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Integration of wet and dry bench processes optimizes targeted next-generation sequencing of low-quality and low-quantity tumor biopsies

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Abstract:	All next-generation sequencing (NGS) procedures include assays performed at the laboratory bench ("wet bench") and data analyses conducted using bioinformatics pipelines ("dry bench"). Both elements are essential to produce accurate and reliable results, which are particularly critical for clinical laboratories. Targeted NGS technologies have increasingly found favor in oncology applications to help advance precision medicine objectives, yet the methods often involve disconnected and variable wet and dry bench workflows and uncoordinated reagent sets. In this report, we describe a comprehensive system for targeted NGS of challenging cancer specimens. This system integrates functional DNA quantification and qualification, single-tube multiplexed PCR enrichment, and library purification and normalization using analytically verified single-source reagents with a push-button, standalone bioinformatics suite that enables accurate variant calls from low-quality and low-quantity FFPE and FNA tumor biopsies. The method can routinely assess cancer-associated variants from an input of 400 amplifiable DNA copies, and is modular in design to accommodate new gene content. Two different types of analytically-defined controls provide quality assurance and help safeguard accuracy with clinically-relevant samples. A flexible "tag" PCR step embeds platform-specific adaptors and index codes to allow sample barcoding and compatibility with common benchtop NGS instruments. Importantly, the protocol is streamlined to be responsive to the needs of

	clinical laboratories, and can produce 24 sequence-ready libraries in a single day. Finally, the approach links wet and dry bench processes by incorporating pre-analytical sample quality control results directly into the variant calling algorithms to improve mutation detection accuracy and differentiate false-negative and indeterminate calls. This targeted NGS method advances both wetware and software to achieve high-depth, multiplexed sequencing and sensitive analysis of heterogeneous cancer samples for diagnostic applications.
Author Comments:	<p>This manuscript is submitted in response to an invitation by Dr. Nandita Singh, Senior Science Editor at JoVE. The content and approach were discussed with her prior to its preparation.</p> <p>Sincerely, Gary Latham</p>
Additional Information:	
Question	Response
If this article needs to be "in-press" by a certain date to satisfy grant requirements, please indicate the date below and explain in your cover letter.	



13 June 2015

Dear Dr. Singh,

We are pleased to submit the attached manuscript "Integration of wet and dry bench processes optimizes targeted next-generation sequencing of low-quality and low-quantity tumor biopsies" for consideration of publication in JoVE. We recognize the value of the unique format that JoVE offers for sharing complex information and protocols, and believe that that the dynamic scientific video medium, combined with detailed text instructions and other supporting information, will enhance adoption, mastery and reproducibility of the procedure we describe for targeted next-generation sequencing (NGS). Targeted NGS procedures are rapidly emerging for molecular diagnostic applications, but the lack of cohesion across wetware, hardware, and software requirements challenges their validation and implementation. Our cross-platform method addresses these gaps by enabling a comprehensive systems approach that links pre-analytical, analytical, and post-analytical steps. We believe that the benefits of this consolidated approach—particularly the workflow advantages—are an excellent fit with the visual format that JoVE offers through its multimedia interface.

The attached manuscript is responsive to your previous invitation for a submission to JoVE, and consistent with our discussions in April. The method described addresses a compelling need for more streamlined and integrated targeted NGS methods that can advance molecular diagnostic testing needs for clinical oncology applications using FFPE and FNA tumor biopsies. This work significantly extends our 2013 publication in the Journal of Molecular Diagnostics (Hadd et al.) by incorporating multi-variant controls, a functional DNA quantification and qualification assay, a single-tube multiplex PCR enrichment step, a unified library purification method, a novel calibration-free library quantification assay, and, critically, an innovative, personal computer-compatible bioinformatics pipeline that is informed by the results of the pre-analytical QC analyses and tuned to reliably call clinically-actionable DNA variants from challenging cancer specimens. As a result, this method addresses a number of limitations of existing methods by improving both the workflow complexity (both hands-on time and total time-to-result) and the accuracy of variant calling, particularly using low-quantity and low-quality tumor DNA. In addition, the integrated dry bench software eases the requirements for cryptic bioinformatics expertise and a high performance computing infrastructure.

In our submission, we have provided contacts for 6 potential reviewers, including several that have worked directly with the protocol described. As previously discussed, we are highly motivated to achieve publication by the end of October should the manuscript meet the journal's standards.

Finally, author contributions for this work are as follows:



- JH helped develop the methodology, performed experiments, and helped write and edit the manuscript.
- AGH helped develop the methodology, designed and directed studies, and performed data analysis.
- RZ helped develop the methodology and performed data analysis.
- BCH helped develop the methodology, performed data analysis, and helped write and edit the manuscript.
- GJL helped develop the methodology, conceived, designed, and directed the studies, performed data analysis, and wrote the manuscript.

We look forward to the next steps in the review process.

Sincerely,

A handwritten signature in black ink, appearing to read "Gary J. Latham".

Gary Latham, PhD
Vice President, Research
Asuragen, Inc.

TITLE:

Integration of wet and dry bench processes optimizes targeted next-generation sequencing of low-quality and low-quantity tumor biopsies

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SHORT ABSTRACT:

An integrated system for targeted next-generation sequencing of oncology specimens is described. This cross-platform system is optimized for low-quality and low-quantity tumor biopsies, accommodates low DNA inputs, includes well-characterized multi-variant controls, and features a novel variant caller that is informed by quantitative pre-analytical quality control measures.

LONG ABSTRACT:

All next-generation sequencing (NGS) procedures include assays performed at the laboratory bench (“wet bench”) and data analyses conducted using bioinformatics pipelines (“dry bench”). Both elements are essential to produce accurate and reliable results which are particularly critical for clinical laboratories. Targeted NGS technologies have increasingly found favor in oncology applications to help advance precision medicine objectives, yet the methods often involve disconnected and variable wet and dry bench workflows and uncoordinated reagent sets. In this report, we describe a method for sequencing challenging cancer specimens with a 21-gene panel as an example of a comprehensive targeted NGS system. The system integrates functional DNA quantification and qualification, single-tube multiplexed PCR enrichment, and library purification and normalization using analytically-verified, single-source reagents with a standalone bioinformatics suite. As a result, accurate variant calls from low-quality and low-quantity formalin-fixed, paraffin-embedded (FFPE) and fine-needle aspiration (FNA) tumor biopsies can be achieved. The method can routinely assess cancer-associated variants from an input of 400 amplifiable DNA copies, and is modular in design to accommodate new gene content. Two different types of analytically-defined controls provide quality assurance and help safeguard call accuracy with clinically-relevant samples. A flexible “tag” PCR step embeds platform-specific adaptors and index codes to allow sample barcoding and compatibility with common benchtop NGS instruments. Importantly, the protocol is streamlined and can produce 24 sequence-ready libraries in a single day. Finally, the approach links wet and dry bench processes by incorporating pre-analytical sample quality control results directly into the variant calling algorithms to improve mutation detection accuracy and differentiate false-negative and indeterminate calls. This targeted NGS method uses advances in both wetware and software to achieve high-depth, multiplexed sequencing and sensitive analysis of heterogeneous cancer samples for diagnostic applications.

INTRODUCTION:

Precision medicine relies on the individualization of diagnostic and therapeutic options for patients. The promise of tailored treatments is a direct consequence of an improved understanding of disease pathways that can inform the linkage of molecular diagnostics and targeted therapeutics. For example, the use of molecularly-targeted therapies increased from 11% to 46% from 2003 to 2013¹, and anti-cancer drugs such as vemurafenib and crizotinib are FDA-cleared with companion diagnostic tests. With its ability to accurately recover low-abundance sequence targets across highly multiplexed sample sets, next-generation sequencing (NGS) has emerged as a method of choice for evaluating genetic aberrations associated with cancer and identifying molecular targets for precision medicine.

The most common solid tumor biopsies for molecular testing include formalin-fixed, paraffin-embedded (FFPE) and fine-needle aspiration (FNA) specimens. These samples are fraught with low-quantity and/or low-quality nucleic acids that challenge accurate NGS assessments²⁻⁵. Current commercial NGS methods for the analysis of these specimens are based on a patchwork of different reagents, protocols, and informatics tools that represent moving targets of continual improvements. For example, changes in assay chemistries and/or software occurred every 1-2 months for the most commonly-used targeted NGS kits⁶. This instability

reflects a lack of coherence in constructing and verifying a unified NGS system for challenging specimen types, particularly for cancer testing, and puts an undue burden on laboratories to develop cohesive protocols that are optimized from sample-to-results. Indeed, one recent survey of NGS users highlighted the difficulties of these “rapidly changing” technologies, along with the requirements for established, medically-actionable content, entrenched bioinformatics expertise, a solidified and integrated procedure that can be rapidly implemented, and streamlined workflows and simplified protocols that facilitate on-the-job training⁷. In this article, a comprehensive system for targeted NGS is described that addresses these gaps.

The presented methodology integrates all procedural steps from pre-analytical to post-analytical—both wet and dry bench—to improve the accuracy, sensitivity, and reliability of target quantification and detection for NGS of clinically-relevant cancer gene loci. This approach begins with the quantification of “functional” DNA⁴ to assess DNA quality, guide input into the PCR enrichment step, and guard against false-positive calls that can arise from the interrogation of very low template copies. A single-tube multiplex PCR then enriches for 46 loci in 21 cancer genes using only 400 amplifiable DNA copies, followed by incorporation of platform-specific sequences for NGS using common desktop sequencing instruments. Libraries are purified using a simple magnetic bead procedure and quantified with a novel, calibration-free qPCR assay. A standalone bioinformatics suite, informed by sample DNA QC results to improve call performance, provides sequence analysis following NGS. We present data using this systems approach for targeted NGS to reveal base-substitution mutations, insertion/deletions (indels), and copy number variants (CNVs) in low-quality and low-quantity tumor biopsies such as FFPE and FNA samples, and run controls.

PROTOCOL:

Note: This protocol describes the simultaneous processing of samples using a MiSeq NGS System but can be adapted for the Personal Genome Machine (PGM) instrument. For the recommended minimum DNA input of 400 amplifiable template copies, the assay is capable of producing at least 3000x median coverage for each of 96 samples per NGS run, and equivalent coverage depth for 24 samples using the PGM on a 318 chip. The method also requires the use of a real-time PCR instrument.

1. DNA Functional Quantification and Quality Control (QC)

1.1) Thaw reagents: 2x Master Mix, Primer Probe Mix, Inhibition Primer Probe Mix, 6-carboxy-X-rhodamine (ROX), Diluent, and the four human genomic DNA calibration curve standards (DNA Standard (50 ng/μL), DNA Standard (10 ng/μL), DNA Standard (2 ng/μL), and DNA Standard (0.4 ng/μL)) (**Table 1**). Vortex all reagents for 10 sec and centrifuge at maximum speed for 10 sec to collect contents. Keep the 2x master mix on ice.

1.2) Prepare a sufficient amount of master mix for the total number of samples to be tested and include 10% more volume to avoid shortages due to pipetting. Prepare the master mix in a microcentrifuge tube using the following volumes per sample: 5 μL 2x Master Mix, 0.5 μL

Primer Probe Mix, 0.5 μ L Inhibition Primer Probe Mix, 0.05 μ L ROX and 2.95 μ L Diluent. Vortex for 10 sec and centrifuge at maximum speed for 10 sec to collect contents.

1.3) Add 9 μ L master mix into wells of a 96-well plate.

1.4) Add 1 μ L of the DNA Standards in duplicate to generate a calibration curve. Mix by pipetting up-and-down 5 times.

1.5) Ensure the nucleic acid sample is well mixed before use. Add 1 μ L sample to the master mix and mix by pipetting up-and-down 5 times.

1.6) Seal the plate, vortex for 10 sec and centrifuge at maximum speed for 10 sec to collect contents.

1.7) Place the plate into the PCR System. Assign both FAM (functional quantification) and VIC (functional inhibition) detectors for each sample according to the manufacturer's instructions. Perform PCR cycles of 10 min at 95 $^{\circ}$ C, and 40 cycles (15 sec at 95 $^{\circ}$ C, 1 min at 60 $^{\circ}$ C).

1.8) Analyze the qPCR data by generating a linear regression plot for each of the duplicate DNA standards using software protocols.

1.9) Plot the Log₁₀ of the copy number for each DNA standard on the x-axis and the corresponding FAM C_q value on the y-axis.

1.10) Confirm that the results from the nucleic acid sample falls within the dynamic range of the DNA Standards calibration curve, and then calculate the concentration of the unknown DNA in "functional" or amplifiable copy number per μ L from its corresponding position on the reference standard curve. Figure 1 shows examples of calibration curves that passed and failed.

1.11) Determine if amplification occurred in each reaction by checking for the presence of the non-human target amplicon in the VIC channel.

Note: As a positive control, the Inhibition Primer Probe Mix contains primers specific for a non-human exogenous target and the non-human exogenous target. The Inhibition Primer Probe Mix is a component of the master mix which is added to each reaction, including the no-template control (NTC). In absence of an inhibitor, the PCR product for the non-human target should always be detected in the VIC channel. An "undetected" C_q for a sample in the VIC channel indicates the presence of PCR inhibitors that may benefit from subsequent clean-up of the sample prior to further processing.

2. Library Preparation – Gene-specific (GS) PCR

2.1) Prepare a sufficient amount of master mix for the total number of samples to be tested and include 10% more volume to avoid shortages due to pipetting. Prepare the GS PCR master

mix in a microcentrifuge tube using the following volumes per sample: 5 μ L 2X Amplification Master Mix (**Table 1**), and 1 μ L Pan Cancer Primer Panel (**Table 1**). Mix by pipetting up-and-down, vortex for 10 sec and centrifuge at maximum speed for 10 sec to collect contents.

2.2) Aliquot the 6 μ L GS PCR master mix into wells of a 96-well plate. Add 4 μ L of each nucleic acid sample into individual wells. To other wells, add 4 μ L of the FFPE Control (**Table 1**), 4 μ L of the Multi-Variant control (**Table 1**), and 4 μ L of nuclease-free water for a procedural NTC. For each addition, mix by pipetting up-and-down 5 times.

2.3) Seal the plate, vortex for 10 sec and centrifuge at maximum speed for 10 sec to collect contents.

2.4) Place the plate in the thermocycler for the following PCR cycles: 5 min at 95 °C, 2 cycles (15 sec at 95 °C, 4 min at 60 °C), 23 cycles (15 sec at 95 °C, 4 min at 72 °C), and a final extension of 10 min at 72 °C. Hold at 4 °C.

Note: After completion of step 2.4, the plate will be referred to as the GS PCR plate.

3. Library Preparation – Tag PCR

3.1) Thaw reagents: 2X Index Master Mix (**Table 1**), Index Codes (**Table 1**), vortex for 10 sec and centrifuge at maximum speed for 10 sec to collect contents.

Note: Index Codes are premixed to provide a unique set of pairwise indices (barcodes) for each sample.

3.2) In a 96-well plate, add 7.5 μ L of the 2X Index Master Mix and 5.5 μ L of an Index Code to a specified well and mix by pipetting up-and-down 5 times.

3.3) Carefully open the GS PCR plate, and add 2 μ L GS PCR product to the new plate with the master mix. Mix by pipetting up-and-down 5 times. For each sample, record the Sample ID and the corresponding pairwise Index Codes. Seal the plate, vortex for 10 sec and centrifuge at maximum speed for 10 sec to collect contents.

3.4) Place the plate in the thermocycler and PCR for 5 min at 95 °C, 10 cycles (30 sec at 95 °C, 30 sec at 55 °C, 1 min at 72 °C), and a final extension of 10 min at 72 °C. Hold at 4 °C.

Note: After completion of step 3.4, the plate will be referred to as the Tag PCR plate.

4. Library Purification and Size Selection

4.1) Remove the Library Pure Prep magnetic beads (**Table 1**) and Elution Buffer (**Table 1**) from 2 - 8 °C and allow to equilibrate to room temperature for 30 minutes. Add 9.6 mL 100%

ethanol to the Wash Buffer (**Table 1**) container, cap and mix by inverting the bottle several times.

4.2) Vortex the magnetic beads for 10 sec and add 11 μ L into separate wells of a 96-well plate.

4.3) Open the Tag PCR plate and add 10 μ L of Tag PCR product to the beads and pipet mix 5 times. Incubate the mixture for 4 min at room temperature.

4.4) Place the 96-well plate on the magnetic stand (**Table 1**) for 4 min. With the 96-well plate still on the stand, remove and discard the supernatant with a pipette.

4.5) Remove the 96-well plate from the magnetic stand and add 100 μ L ethanol-containing Wash Buffer to each well and mix by pipetting up-and-down 5 times. Incubate for 2 min.

4.6) Place the 96-well plate on the magnetic stand for 2 min, then remove and discard the supernatant with a pipette.

4.7) Repeat step 4.5, for a total of 2 ethanol washes, removing as much wash solution possible after the second wash.

4.8) With the 96-well plate on the magnetic stand, dry the beads for 2 minutes at room temperature, then remove the plate from the stand.

4.9) Resuspend the beads by adding 20 μ L Elution Buffer to each well and pipet up-and-down 5 times.

4.10) Incubate for 2 min at room temperature.

4.11) Place the 96-well plate back on the magnetic stand for 4 min, and carefully remove and transfer 18 μ L of the clear supernatant to a new well.

Note: The procedure may be safely stopped at this step and samples stored at -15 to -30°C. To restart, thaw frozen samples on ice before proceeding.

5. Library Quantification

5.1) Thaw Library Quant (LQ) reagents: 2x LQ Master Mix, LQ Primer / Probe Mix, LQ Standard, LQ Positive Control, LQ Diluent, and LQ ROX (**Table 1**). Vortex for 10 sec and centrifuge at maximum speed for 10 sec to collect contents.

5.2) Using purified library products, perform a serial dilution of each individual sample in LQ Diluent.

5.2.1) Add 2 µL (library purified product) to 198 µL LQ Diluent and mix up-and-down with a pipet 10 times.

5.2.2) Add 2 µL (1:100 dilution) to 198 µL LQ Diluent and mix up-and-down with a pipet 10 times.

5.3) Prepare a sufficient amount of LQ master mix for the total number of samples to be tested and include 10% more volume to avoid shortages due to pipetting. Prepare the LQ Master Mix in a microcentrifuge tube using the following volumes per sample: 5 µL 2X LQ Master Mix, 2 µL LQ Primer / Probe Mix, 0.5 µL LQ Standard and 0.5 µL LQ ROX. Mix by pipetting up-and-down, vortex for 10 sec and centrifuge at maximum speed for 10 sec to collect contents.

5.4) Add 8 µL LQ master mix to a well of an optical 96-well plate.

5.5) In separate wells, add 2 µL diluted library, 2 µL LQ Positive Control and 2 µL LQ Diluent (NTC) and mix by pipetting up-and-down 5 times. Seal the plate with optical adhesive film, vortex for 10 sec and centrifuge at 400 x g for 10 sec to collect contents.

5.6) Assign both FAM and VIC detectors for each sample. Perform PCR amplification using cycling conditions of 5 min at 95 °C, and 40 cycles (15 sec at 95 °C, 1 min at 60 °C).

5.7) Determine the concentration of each sample (nM) using the comparative C_q method. Calculate the difference of the known LQ Standard (C_q VIC) to the unknown library (C_q FAM). The concentration of the Diluted Sample (pM) is calculated using the following equation:

$$[Conc_{lib}]_{nM} = 12.5 \times 2^{\Delta C_q}$$

If using a dilution factor other than 10,000, calculate the ratio of the target dilution factor to 10,000, and multiply this factor by the result of the equation in 5.7.

6. Library Normalization and Sample Pooling

6.1) Determine the median concentration (nM) across all samples (each containing a unique pairwise index) to be pooled.

6.2) Determine the individual sample volume (µL) to pool by multiplying the median concentration across all samples by 5, then dividing by its individual concentration (nM). Round the resulting value to the nearest integer. Round volumes with values of <2 µL to 2 µL, and volumes >15 µL to 15 µL.

6.3) Add the normalized volume (µL) for each sample to a single microcentrifuge tube to create the sample pool.

6.4) Calculate the new concentration for each sample using rounded integer values and record the results.

6.5) To determine the concentration of the sample pool, calculate the sum of all individual concentrations and record the resulting value (nM).

6.6) Dilute the sample pool to 1.25 nM using Sequencing Diluent (**Table 1**).

Note: The procedure may be safely stopped at this step and samples stored at -15 to -30°C. To restart, thaw frozen samples on ice before proceeding.

7. Sequencing

7.1) Denature the sample pool in the presence of PhiX Control v3 (**Table 1**) by adding the following volumes: 15 µL of 1.25 nM Sample Pool, 3 µL of 0.5 nM PhiX and 2 µL of 1 N NaOH. Vortex briefly followed by a brief centrifugation and incubate for 5 min at room temperature.

7.2) Place the denatured sample pool on ice.

7.3) Add 8 µL of denatured library to 992 µL of pre-chilled HT1-Hyb buffer to a microcentrifuge tube. Vortex briefly to mix, followed by a brief centrifugation to collect contents. Keep on ice.

7.4) Add 600 µL of the denatured and diluted library to position #17 of the reagent cartridge.

7.5) Thaw Read 1 Sequencing Primers (**Table 1**), Index Read Sequencing Primers (**Table 1**), and Read 2 Sequencing Primers (**Table 1**). In microcentrifuge tubes, separately dilute 4 µL Sequencing Primers with 636 µL HT1-Hyb buffer. Note: HT1-Hyb buffer is provided with the sequencing reagent kit (**Table 1**).

7.6) Mix by vortexing for 10 sec and centrifuge at maximum speed for 10 sec to collect contents. Add 600 µL of diluted Read 1 Sequencing Primers to position #18 of the sequencing reagent cartridge, 600 µL of diluted Index Read Primers to position #19, and 600 µL of diluted Read 2 Sequencing Primers to position #20.

7.7) Load the reagents on the NGS instrument (**Table 1**) and sequence according to the manufacturer's instructions. Perform a paired-end 2 x 150 cycle sequencing run.

8. Data Analysis

Note: The NGS instrument software converts cluster images to base calls and quality scores, and demultiplexes pairwise indices to generate individual gzip-compressed FASTQ (*.fastq.gz) files for each sample. Prior to analyzing the demultiplexed files, the reader must download and install the associated bioinformatics software (**Table 1**). The software can be installed on a

consumer-grade Windows PC and does not require specialized computing hardware or an internet connection to perform the data analysis.

- 8.1) Double-click the software desktop icon.
- 8.2) Login to the system using the username and password provided in the software manual.
- 8.3) Open the project dashboard, and click “New Project”.

8.3.1) Name the project and provide an optional project description. Select the targeted NGS panel type and NGS instrument type. Click “Save and continue”.

8.3.2) Upload the compressed FASTQ files for the forward and reverse reads. Do not upload the “unassigned” FASTQs, which contain reads that failed to demultiplex. Click “Save and continue”.

8.3.3) Input the number of functional input copies used to prepare each library as determined by the DNA functional quantification assay (see Step 1 above). Manually add values or copy and paste values from a spreadsheet into the annotation table. Click “Save and continue.”

8.3.4) Review the annotated libraries uploaded for analysis and click “Submit analysis” to initiate the analysis.

- 8.4) Monitor the progress of the analysis displayed through the project dashboard.

Note: An analysis complete status indication is presented when the results are ready for review.

8.5) Review the analyzed results for sample QC metrics including total coverage per library, percentage of reads passing filters, amplicon coverage depth and uniformity. Review the variant calls for each sequenced library with dbSNP, COSMIC, 1000 genomes and other sources of functional and population level annotation.

8.6) Export the raw results as summary spreadsheet tables, *.bam files and *.vcf files for long-term storage or downstream analysis with complementary informatics tools.

REPRESENTATIVE RESULTS:

A total of 90 samples (74 unique) representing positive and negative controls, previously characterized cell lines, and residual clinical FFPE tumor biopsies were assessed for amplifiable DNA, input into multiplex PCR enrichment, tagged with sequence adapters, barcoded, and analyzed in a single benchtop NGS instrument run (**Figure 2**) that produced 19.1M reads passing filter. Equimolar sample pooling resulted in high depth sequencing (3692x reads) and uniform coverage (97.8% of amplicons covered within 5-fold of the median read depth). Outliers were comprised of no-template controls, one cell-line DNA with a large copy number amplification, and one FFPE DNA that was flagged for PCR inhibition by the pre-analytical QC assay (**Figure 3**). Coverage uniformity across the 46 amplicons was maintained using three different operators

(**Figure 4A**), and for different low-quality FFPE DNA samples (**Figure 4B**). An FFPE tumor DNA control, formulated from a mixture of residual clinical specimens to achieve 5% BRAF V600E (quantified by droplet digital PCR), was reported to have the target BRAF mutation at abundances of 3.9, 5.3, and 6.5% by three operators using an input of 400 amplifiable copies (and thus only 20 mutant copies) (**Figure 4B** and “FFPE3” of inset table). Further, a mixture of 12 synthetic DNA templates, each representing a known “driver” base-substitution mutation, revealed the expected mutations at the intended range of 9-17% mean allele frequency (**Table 2**). Dilution of cell-line and FFPE DNA samples with copy number amplifications demonstrated dose-dependence for variants in EGFR and KRAS, respectively (**Figure 5**). Importantly, FFPE DNA input could be reduced to as few 100 amplifiable copies or 1.2 ng of bulk DNA while preserving the detection of known mutations without false-positive calls (**Figure 6**). DNA inputs were accommodated over a 100-fold range up to at least 50,000 amplifiable copies (**Table 3**). In this and related experiments, variant calls in 22 FFPE and 20 FNA specimens were reported in agreement with independent methods with shared mutation coverage (**Table 4**).

The sensitivity and positive predictive value for the assay was determined from an analysis of 97 samples, including FFPE, FNA, fresh-frozen, and cell-line DNA, and a total of 195 sequencing results. The results revealed 365 true positive variant calls, 4 false negative calls, and 1 false positive call for a sensitivity of 98.9% (95% CI: 97.1-99.7%) and a positive predictive value (PPV) of 99.7% (95% CI: 98.2-99.99). Analyses of indels were performed for two common EGFR variants (p.E746_A750delELREA and p.V769_D770insASV) in 33 sample-runs, demonstrating a sensitivity of 93.9% (95% CI: 78.4%-98.9%) and a PPV of 100% (95% CI: 86.3%-100%) with variants detected over a range of 2.4-84.8%.

Figure 1: An example of DNA quantification calibration curves that pass and fail QC criteria. A) A passing standard curve. B) A failing standard curve. In this case, the failure was caused by duplicate pipetting of the lowest input DNA standard.

Figure 2: Overview of a comprehensive targeted NGS system for oncology applications that integrates pre-analytical, analytical, and post-analytical workflows.

Figure 3: Read coverage and uniformity for targeted NGS of cancer genes in low-quality FFPE DNA compared to intact cell-line DNA and controls. A total of 90 samples that included residual clinical FFPE (closed circles), cell-line (open circles), and synthetic template DNA (plus symbols) were processed using single-tube, 21-gene multiplex PCR enrichment. Each amplicon library was tagged with adapter sequences for the NGS instrument, barcoded with a distinct dual-index code, purified, quantified, and normalized to a concentration of 2.5 nM. The DNA library was sequenced and analyzed by the companion bioinformatics software. Sample deviations included a dilution series of MDA-MB-468 cell-line DNA bearing a large EGFR copy number amplification (top, open rectangles within dotted circle) that distorted coverage uniformity and one melanoma FFPE sample that failed to generate an appreciable number of reads due to carryover of PCR inhibitors from the DNA extraction. The melanoma sample failure (bottom, dotted circle) was predicted by the pre-analytical qPCR DNA QC assay. NTC, no-template control (x symbols).

Figure 4: Amplicon-by-amplicon read coverage, uniformity and variant detection in residual clinical FFPE tumor DNA. A) Read coverage across all enriched loci in a representative FFPE DNA measured across three different operators. Operator 1, Op1 (blue bars); Operator 2, Op2 (green bars); Operator 3, Op3 (black bars). B) Coverage uniformity and variant calls evaluated using three FFPE tumor samples, including a control mixture (FFPE3, gray bars and text) comprised of a known 5% BRAF c.1799T>A mutation. FFPE1 (blue bars and text), FFPE2 (orange bars and text).

Figure 5: Dose-dependent detection of copy number variants in cell-line and FFPE DNA. MDA-MB-468 cell-line DNA with a well-characterized EGFR copy number amplification was progressively diluted in a background of a reference non-mutated cell-line DNA to illustrate the decrease in copy number change as a function of the dilution. The percentage of each cell-line DNA sample is shown with a distinct line (0, 12.5, 25, 50, and 100%). Dilution of an ovarian FFPE tumor sample with a known KRAS amplification revealed a similar profile using the same titration series but with replicates of 100% FFPE DNA for the two top lines.

Figure 6: Accurate mutation detection and quantification to 50 amplifiable FFPE DNA copies, or 1.2 ng bulk DNA. The amplifiable copy number of a colon cancer FFPE DNA was determined by the qPCR-based QC assay, and diluted from 400 to 25 copies as an input into multiplex PCR enrichment prior to sequencing. The bioinformatics pipeline correctly called both of the known variants down to 50 copies, or the equivalent of ~10 mutant templates.

Table 1: Reagents and kits. Upon first use of the ROX, store the vial at 2 to 8°C. Do not refreeze. Software can be downloaded at www.asuragen.com

Table 2: A pooled synthetic control is comprised of 12 “driver” cancer gene variants that are quantified at 9-17% abundance. A mixture of 12 different double-stranded synthetic templates bearing 12 distinct mutations was evaluated following sequencing. All variants were correctly called with no false positives.

Table 3: Coverage and variant calling are preserved over a >100-fold range of DNA input. Amplifiable DNA from a BCPAP cell line was input into multiplex PCR enrichment at 400 to 50,000 copies and sequenced. Read depth, coverage uniformity, mutation detection, and mutation accuracy were preserved across the input range.

Table 4: Variant calls in 22 FFPE and 20 FNA tumor biopsies agree with results from independent mutation assays. A set of 22 FFPE tumor DNA with mutation status previously determined by orthogonal targeted NGS assays was input at 400 to 2928 amplifiable copies into the PCR enrichment step and sequenced using the 21-gene Pan Cancer panel. In addition, a cohort of 20 FNA DNA samples previously characterized using a liquid bead array mutation assay⁸ was PCR amplified using 156 to 36080 input amplifiable copies and sequenced. All overlapping calls between the Pan Cancer NGS panel and the reference methods were in agreement.

DISCUSSION:

NGS technologies have redefined expectations for interrogating the molecular profiles of tumor biopsies in clinical settings⁹. A number of targeted NGS panels have been developed as research technologies, laboratory developed tests, and commercially available products and custom panels for assessing multiple types of clinical specimens^{3,5,10-15}. Reports from multiple studies have demonstrated the value of NGS as a sensitive and specific clinical tool for the detection of genomic alterations^{3,10-12,14}. Yet studies have also demonstrated the risk of artifacts which can cause false-positive results from challenging cancer biopsies such as FFPE specimens^{2-5,12,14}. Additionally, recent publications have highlighted high failure rates for NGS using oncology specimens¹⁶ and false-positive calls with commercial targeted NGS panels that are aggravated by the use of low-input DNA¹². As a result, some laboratories have either modified or added additional checks and balances to commercially available targeted NGS technologies to improve performance, often to ensure accuracy or confirm the findings from the bioinformatic analyses^{5,10,11}.

The 21-gene panel (Figure 1) was developed as targeted content for a comprehensive NGS system to interrogate evidence-based, actionable mutations in challenging specimen types such as FFPE and FNA tumor biopsies. The workflow has a number of benefits: 1) consistency by providing uniform amplicon coverage (Figures 2 and 3, Table 3); 2) ease-of-use by providing pre-formulated, optimized reagent sets, and simplifying the bioinformatics requirements; 3) efficiency by streamlining the workflow and reducing the number of pipetting steps as compared to other commercial NGS methods; and 4) accuracy by incorporating a DNA QC assay for assessing the amplifiable DNA copy number to ensure acceptable template diversity and avoid stochastic fluctuations in variant detection⁴. The integration of the pre-analytical QC data with the bioinformatics analysis allows for low inputs of FFPE DNA. This was achieved by training a decision-tree algorithm using different functional copies of DNA input across 400 FFPE samples with independent measures of truth, and incorporating this algorithm into the bioinformatic software. As a result, the recommended input of 400 amplifiable copies, typically equivalent to ~5-20 ng of FFPE DNA, compares favorably to other methods¹⁰⁻¹², including hybridization-based enrichment where ~250 ng of FFPE DNA is recommended^{17,18}. Although the technique is described for use on a MiSeq platform, it can be modified by using Tag PCR primers with instrument-specific adaptors to enable sequence analysis on other NGS platforms.

Several steps are critical to ensure success of the procedure. The pre-analytical QC assay determines the amplifiable copy number of DNA and reports functional inhibition. However, if less than 400 amplifiable DNA copies are used in the PCR enrichment step, there is an increased risk of a false-negative call from samples with low-abundance mutations (Figure 5). Additionally, care must be taken during library purification to prevent over-drying of the magnetic beads during the washing or elution steps. Furthermore, successful library quantification is highly dependent on the accurate dilution of library DNA. For the best outcome, the difference of the qPCR results for the sample library (Cq FAM) compared to the LQ Standard (Cq VIC) should be ≤ 3.3 Cq. If the difference is greater than 3.3 Cq, re-dilution and testing of the sample is recommended. Although an excellent correlation has been observed between this competitive qPCR method and commercial kits that offer absolute quantification

using a standard curve, an offset of the library input into the clonal amplification step relative to other methods may be necessary to achieve optimal seeding density.

Some cancer specimens are particularly challenging to sequence because of inhibitors that persist after DNA isolation. To identify these samples prior to library preparation, the qPCR QC assay also detects amplification inhibition by including an exogenous template that serves as both an internal control and a sentinel for functional inhibition. An example is presented in Figure 3 in which a melanoma DNA sample failed to pass the QC inhibition metric prior to sequencing and then failed to generate a library that could be sequenced. The failure was likely a consequence of melanin contamination, a known PCR inhibitor, carried over from the FFPE DNA isolation step. Samples that are identified by the QC assay to be at risk for amplification failure may be salvaged through an extra clean-up step to remove potential inhibitors.

The targeted 21-gene panel focuses on evidence-based gene hotspots and provides a complete system with optimized reagents and controls for DNA QC, NGS and bioinformatics software that is informed by pre-analytical “functional” DNA quantification results. The method accurately detects base-substitution mutations and indels from low-input DNA, and provides an example of an NGS system with the option to expand panel content, to detect additional variants such as CNVs and be adapted for targeted RNA sequencing.

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DISCLOSURES:

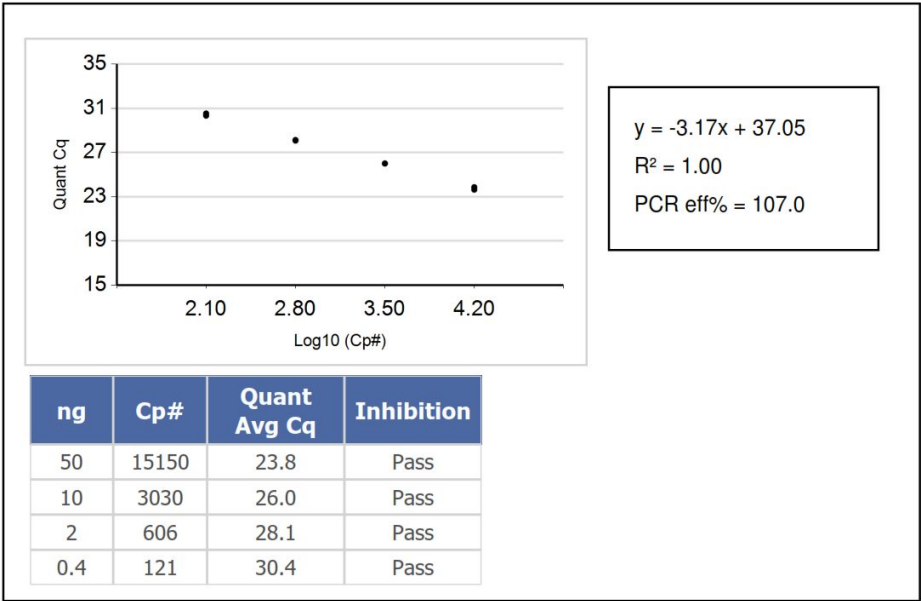
JH, AH, RZ, BCH, and GJL are employees and have stock ownership in Asuragen, Inc. RZ, BCH, and GJL are co-inventors on a patent application for improving variant calling using amplifiable copy number information determined for each sample.

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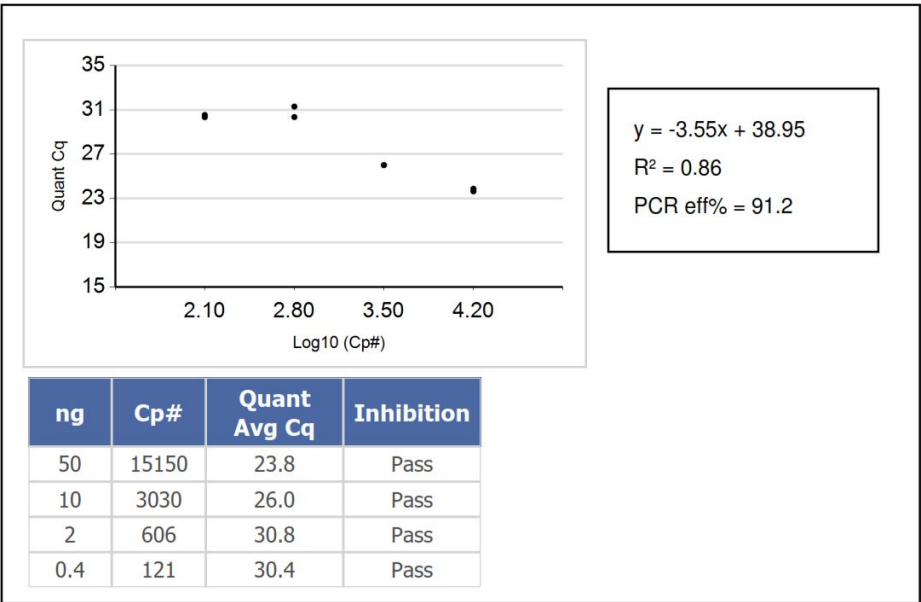
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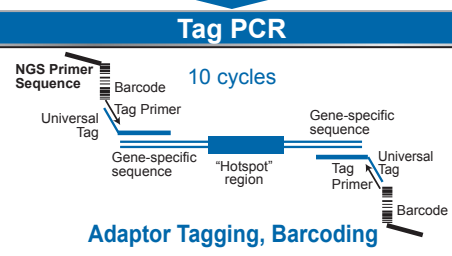
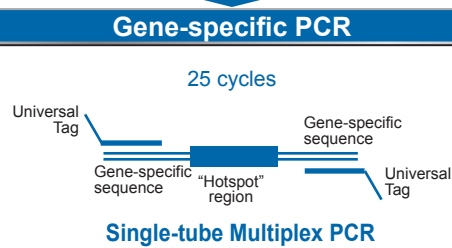
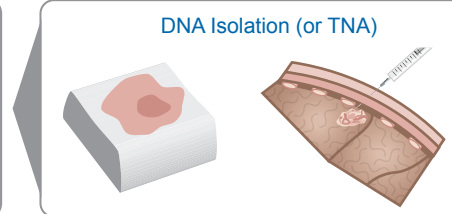
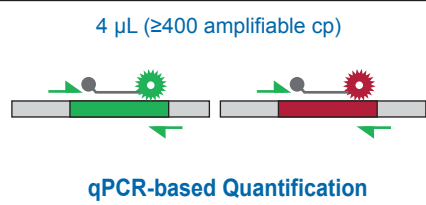
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A

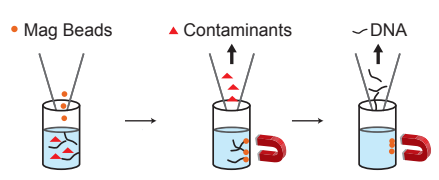


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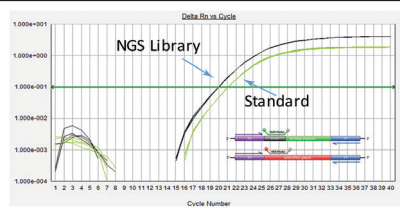




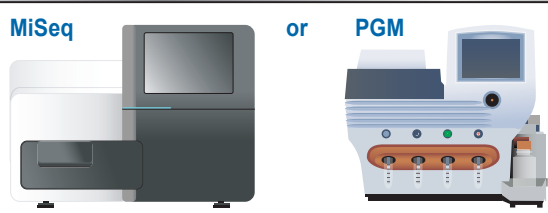
Library Purification & Size Selection



Library Quantification



Normalization & Pooling, Dilution, Loading



Cancer Gene Panel

Pan Cancer DNA Panel			
Gene	Codon Range	Gene	Codon Range
ABL1	249-258	HRAS	9-20
	303-319		59-76
AKT1	16-27		113-121
AKT2	16-26	IDH1	122-134
	1174-1196		138-145
ALK1	1274-1278	IDH2	163-174
	465-474		607-620
BRAF	591-612	JAK2	557-579
	486-493		815-826
	709-722		9-20
	737-761		55-65
	767-798		104-118
	849-861		137-148
	755-769	MET	1245-1256
	774-788		9-20
ERBB2	839-847		55-67
	877-883		110-119
	123-136		144-150
FGFR1	250-262		560-572
	247-260		840-852
	363-374		540-551
FGFR3	638-653		1038-1049
	829-840		916-926

Pan Cancer Detection Panel



Figure 3

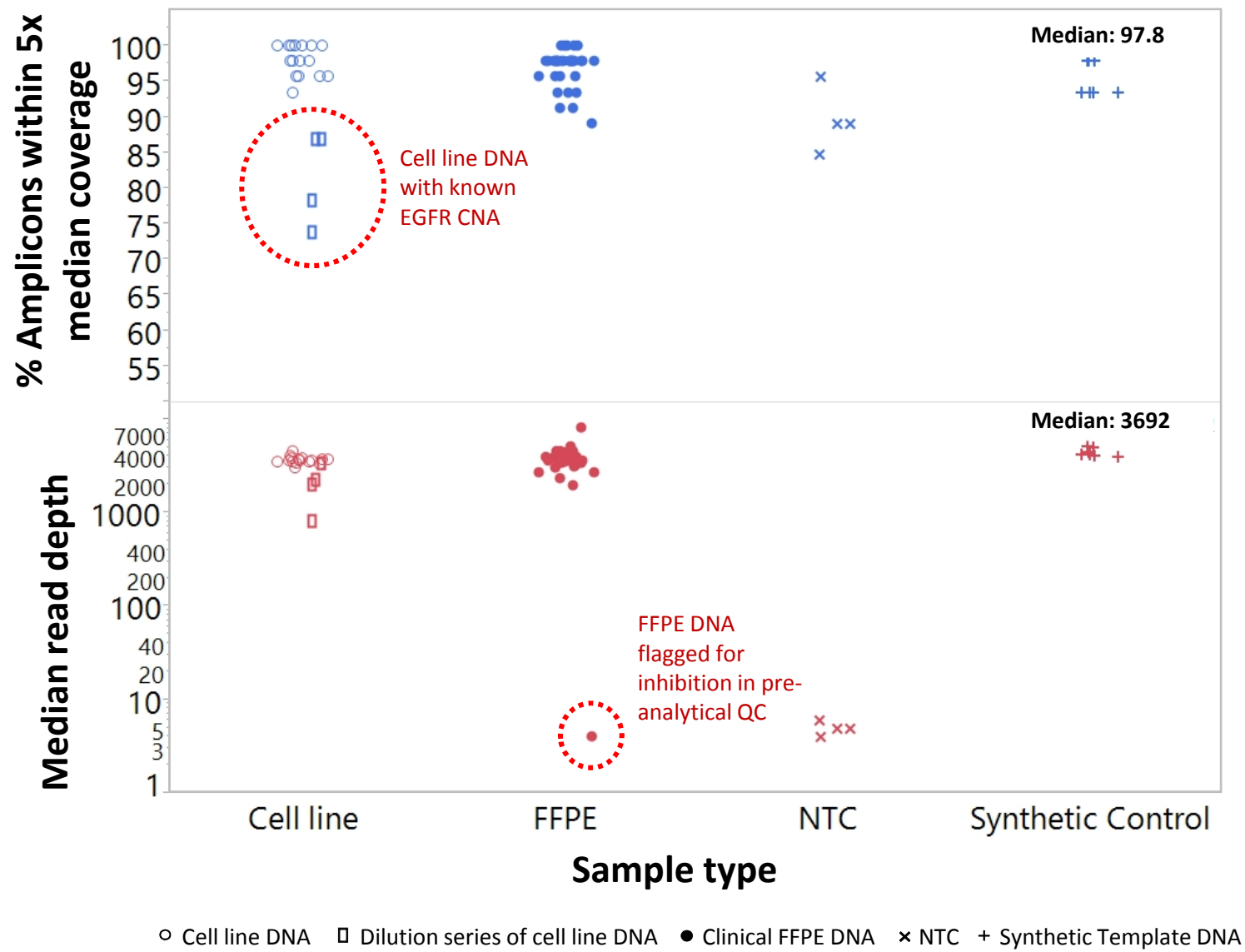


Figure 4

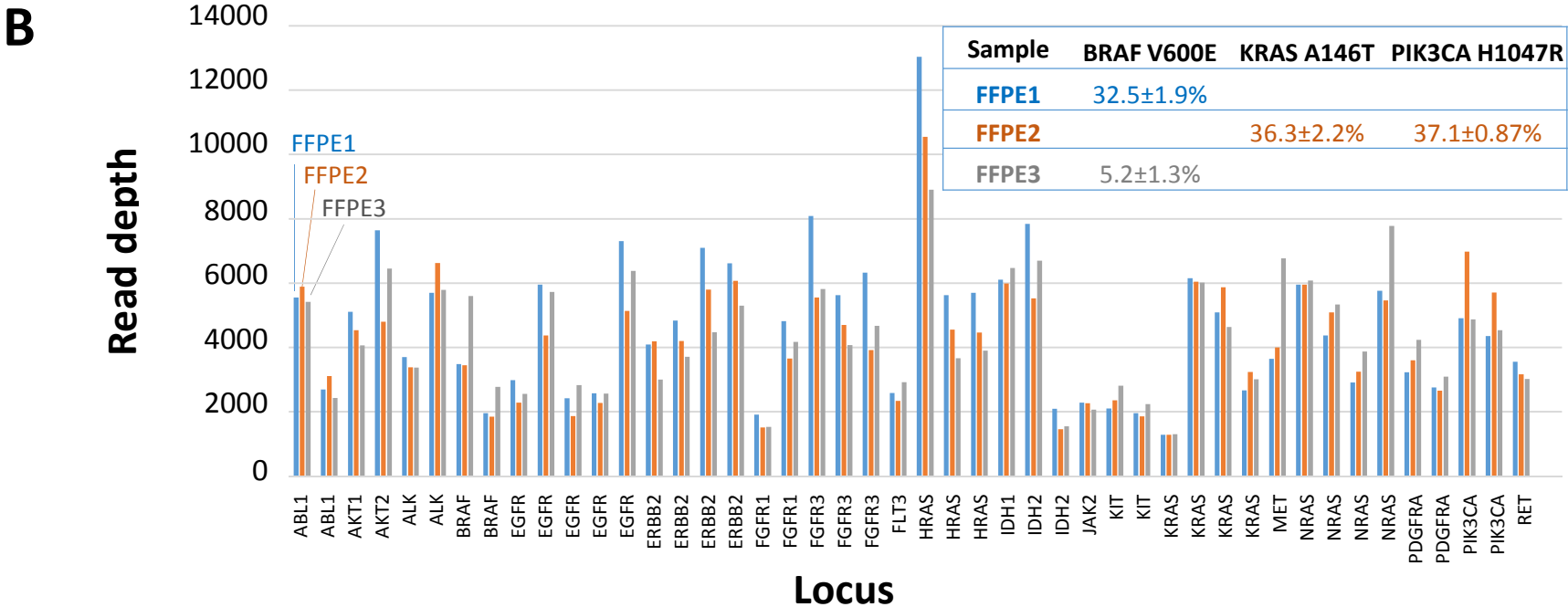
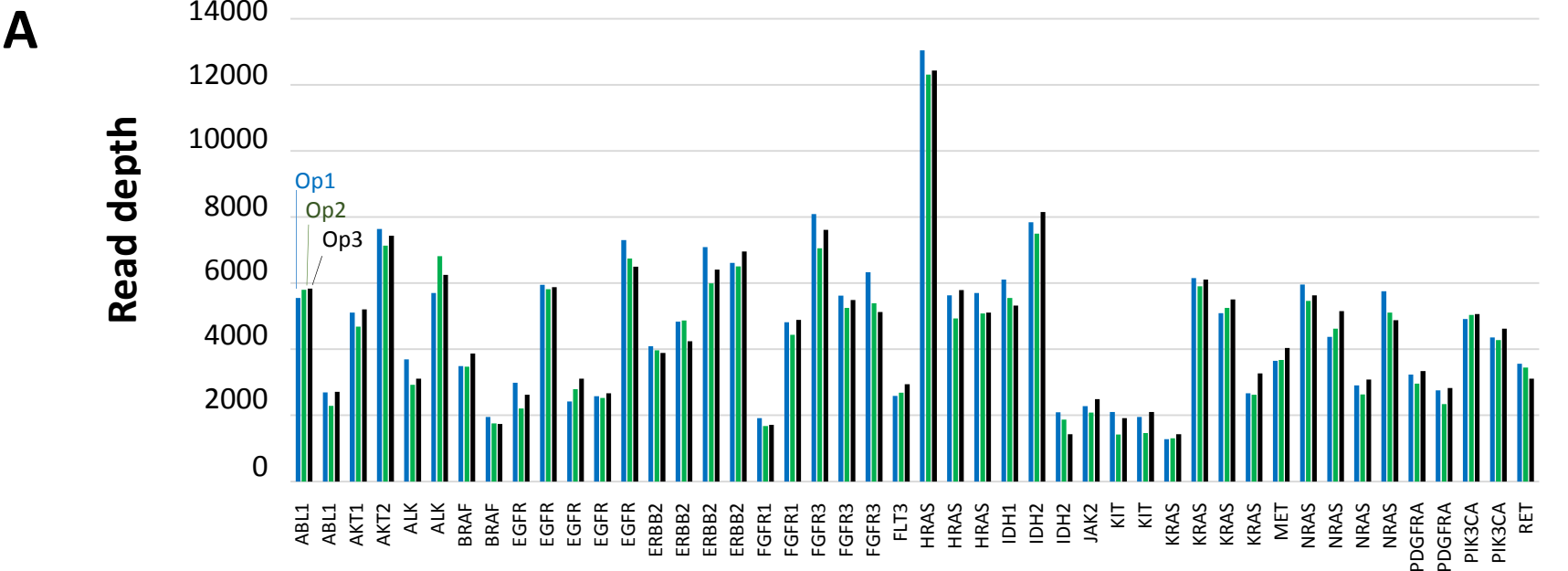


Figure 5

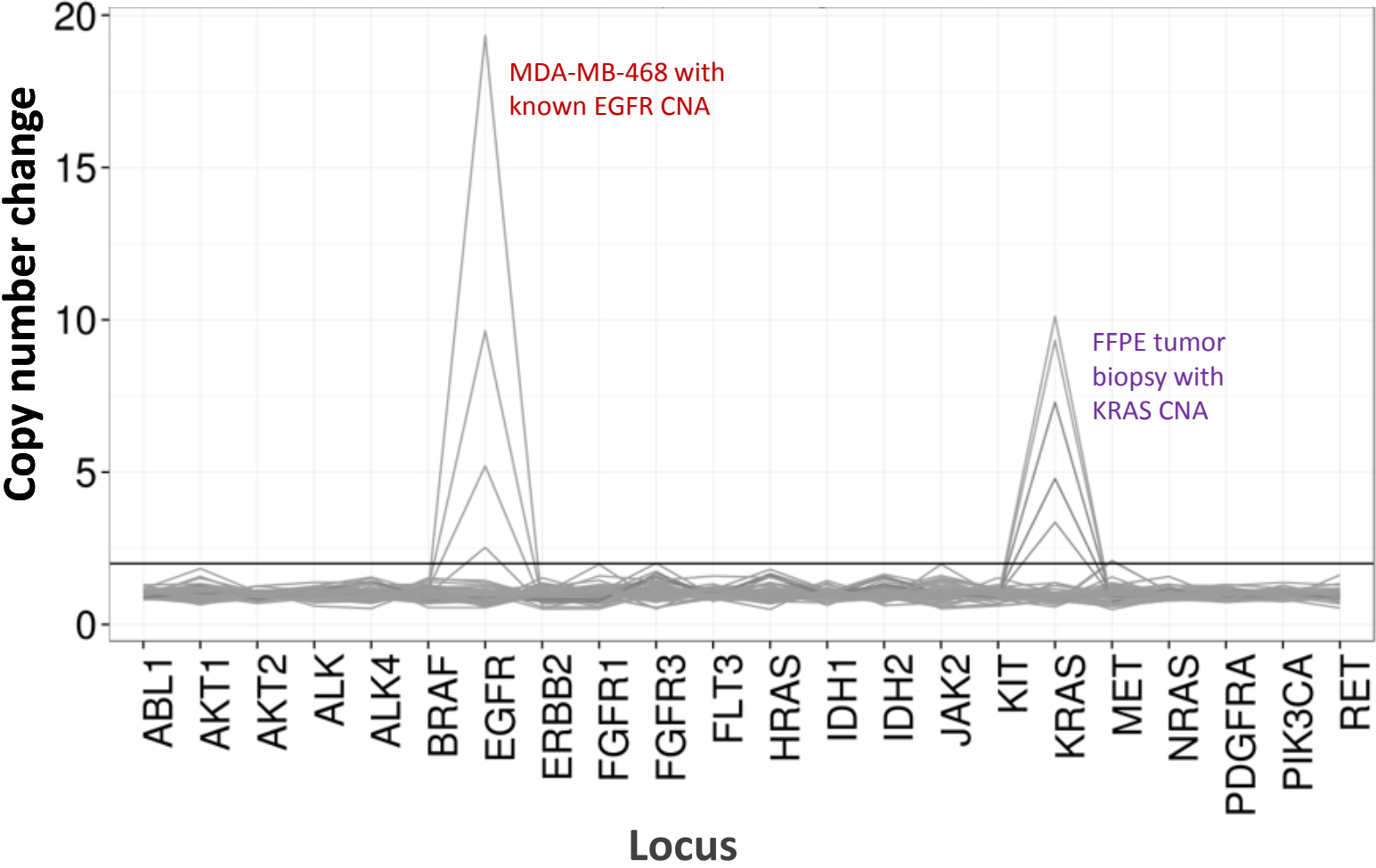
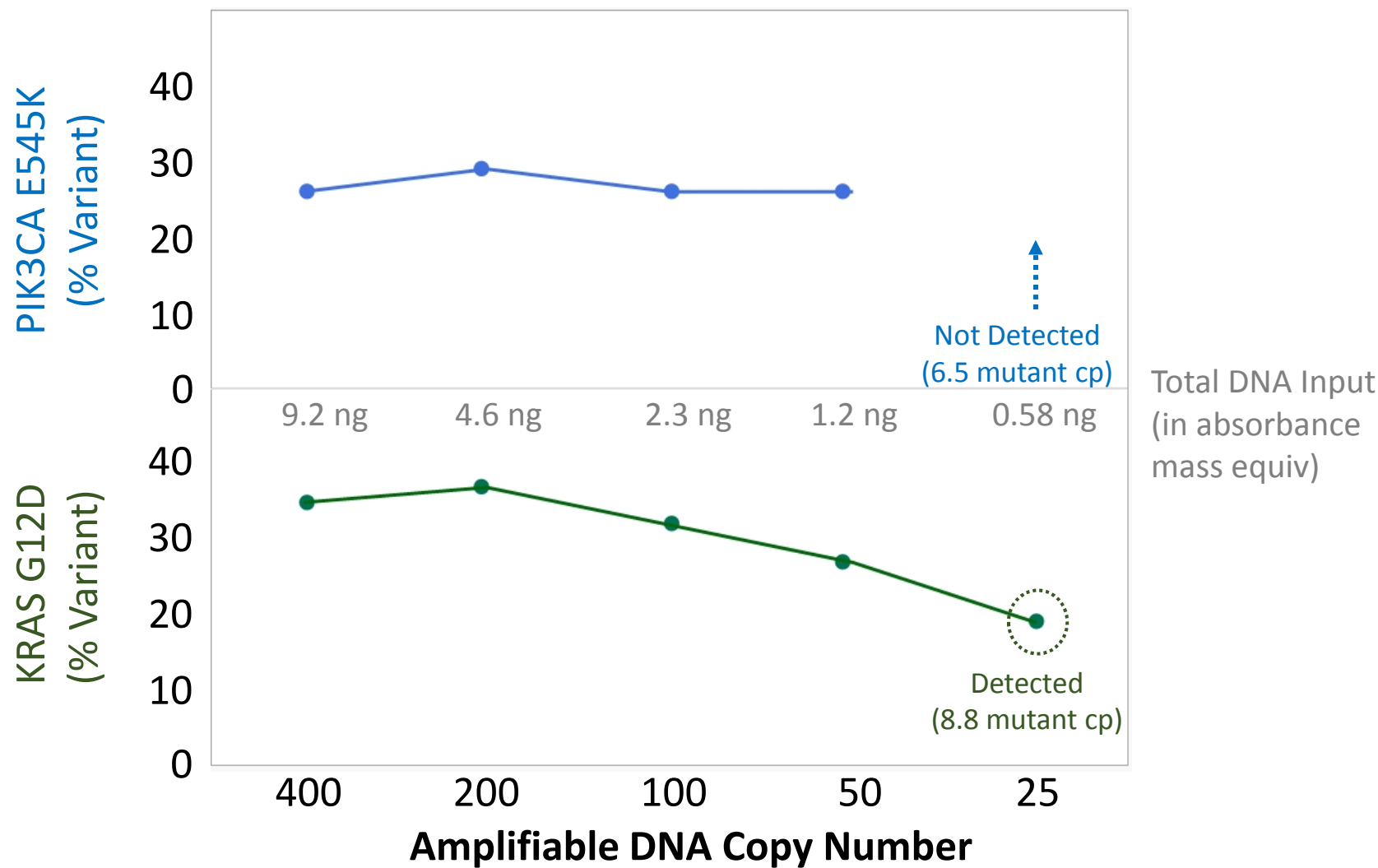


Figure 6



Gene	COSMIC variant	COSMIC amino acid	% Variant
NRAS	c.182A>G	p.Q61R	13.3
NRAS	c.35G>A	p.G12D	15.2
HRAS	c.182A>G	p.Q61R	17.8
HRAS	c.35G>A	p.G12D	9.2
KRAS	c.182A>G	p.Q61R	13.5
KRAS	c.35G>A	p.G12D	19.1
PIK3CA	c.1633G>A	p.E545K	9.3
PIK3CA	c.3140A>G	p.H1047R	9.1
KIT	c.2447A>T	p.D816V	14.6
EGFR	c.2369C>T	p.T790M	11.3
EGFR	c.2573T>G	p.L858R	14.9
BRAF	c.1799T>A	p.V600E	17.3

Sample ID	Functional cps	Gene	COSMIC variant	COSMIC amino acid	% Variant	Median read depth	% within 5x of median
BCPAP	400	BRAF	c.1799T>A	p.V600E	99.5	3289	96%
BCPAP	10000	BRAF	c.1799T>A	p.V600E	99.7	4040	98%
BCPAP	25000	BRAF	c.1799T>A	p.V600E	99.4	3687	96%
BCPAP	50000	BRAF	c.1799T>A	p.V600E	99.7	4611	93%

Sample ID	Pan Cancer Panel					Independent Mutation Assay		
	Gene	COSMIC variant	COSMIC amino acid	% Variant	Functional cps/sample	Gene	COSMIC variant	% Variant
FFPE-1	BRAF	c.1799T>A	p.V600E	31.7	2928	BRAF	c.1799T>A	34.3
FFPE-2			None				None	
FFPE-3	IDH1	c.394C>T	p.R132C	42.9	2396		Not Covered	
FFPE-3	NRAS	c.182A>G	p.Q61R	43.4	2396	NRAS	c.182A>G	44.3
FFPE-4	PIK3CA	c.3140A>G	p.H1047R	42.3	2080	PIK3CA	c.3140A>G	43.6
FFPE-5	KRAS	c.35G>T	p.G12V	25.2	2476	KRAS	c.35G>T	29.8
FFPE-6	KRAS	c.35G>T	p.G12V	62.2	2132	KRAS	c.35G>T	60.3
FFPE-7	NRAS	c.34G>A	p.G12S	74.3	1184	NRAS	c.34G>A	71.7
FFPE-8	KRAS	c.35G>A	p.G12D	50.2	880	KRAS	c.35G>A	54.6
FFPE-10	KRAS	c.35G>T	p.G12V	3.8	1712	KRAS	c.35G>T	3.9
FFPE-11	BRAF	c.1397G>T	p.G466V	83.6	400		Not Covered	
FFPE-11	KRAS	c.37G>T	p.G13C	49.3	400	KRAS	c.37G>T	29.0
FFPE-12	ERBB2	c.2301C>G	p.I767M	56.8	400		Not Covered	
FFPE-12	KRAS	c.35G>A	p.G12D	29.0	400	KRAS	c.35G>A	37.8
FFPE-13	BRAF	c.1799T>A	p.V600E	38.2	400	BRAF	c.1799T>A	41.8
FFPE-14	NRAS	c.34G>A	p.G12S	72.6	400	NRAS	c.34G>A	71.7
FFPE-15	PIK3CA	c.3140A>G	p.H1047R	56.4	400	PIK3CA	c.3140A>G	65.1
FFPE-16	KRAS	c.437C>T	p.A146V	46.5	400	KRAS	c.437C>T	45.4
FFPE-17	AKT1	c.49G>A	p.E17K	66.7	400	AKT1	c.49G>A	56.2
FFPE-18	PIK3CA	c.3140A>G	p.H1047R	45.8	628	PIK3CA	c.3140A>G	34.4
FFPE-19	NRAS	c.181C>A	p.Q61K	80.8	400	NRAS	c.181C>A	87.2
FFPE-20	NRAS	c.182A>G	p.Q61R	43.4	400	NRAS	c.182A>G	38.7
FFPE-21	BRAF	c.1799T>A	p.V600E	32.4	400	BRAF	c.1799T>A	32.0
FFPE-22	KRAS	c.436G>A	p.A146T	39.5	400	KRAS	c.436G>A	36.0
FFPE-22	PIK3CA	c.3140A>G	p.H1047R	36.9	400	PIK3CA	c.3140A>G	38.0
FNA-1	BRAF	c.1801A>G	p.K601E	46.9	480		Not Covered	
FNA-2	BRAF	c.1799T>A	p.V600E	14.5	1968	BRAF	c.1799T>A	
FNA-3			None		324		None	
FNA-4	BRAF	c.1799T>A	p.V600E	18.9	1404	BRAF	c.1799T>A	
FNA-5			None		8424		None	
FNA-6	BRAF	c.1799T>A	p.V600E	21.6	5840	BRAF	c.1799T>A	
FNA-7			None		5212		None	
FNA-8	HRAS	c.182A>G	p.Q61R	11.5	1064	HRAS	c.182A>G	
FNA-8	NRAS	c.37G>C	p.G13R	7.0	1064		Not Covered	
FNA-9			None		2220		None	
FNA-10	HRAS	c.182A>G	p.Q61R	25.0	156	HRAS	c.182A>G	
FNA-11			None		4928		None	
FNA-12	KRAS	c.35G>T	p. G12V	2.1	536	KRAS	c.35G>T	
FNA-13			None		36080		None	
FNA-14	NRAS	c.182A>T	p.Q61L	22.9	280	NRAS	c.182A>T	
FNA-15	BRAF	c.1799T>A	p.V600E	4.4	12940	BRAF	c.1799T>A	
FNA-16	NRAS	c.182A>G	p.Q61R	19.9	420	NRAS	c.182A>G	

FNA-17	NRAS	c.182A>G	p.Q61R	25.2	2364	NRAS	c.182A>G
FNA-19			None		5896		None
FNA-20	NRAS	c.182A>G	p.Q61R	9.3	956	NRAS	c.182A>G

Name of Material/Material	Company	Catalog Number
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Quant Primer Probe Mix	Asuragen	145336
Inhibition Primer Probe Mix	Asuragen	145344
ROX	Asuragen	145346
Diluent	Asuragen	145339
DNA Standard (50)	Asuragen	145340
DNA Standard (10)	Asuragen	145341
DNA Standard (2)	Asuragen	145342
DNA Standard (0.4)	Asuragen	145343
2X Amplification Master Mix	Asuragen	145348
Pan Cancer Primer Panel	Asuragen	145347
Pan Cancer FFPE Control	Asuragen	145349
Pan Cancer Multi-Variant Control	Asuragen	145350
Library Pure Prep Beads	Asuragen	145351
Wash Buffer	Asuragen	145352
Elution Buffer	Asuragen	145353
2X LQ Master Mix	Asuragen	145358
LQ Diluent	Asuragen	145354
LQ Positive Control	Asuragen	145355
LQ Standard	Asuragen	145356
LQ Primer / Probe Mix (ILMN)	Asuragen	145357
LQ ROX	Asuragen	145359
Index Codes (ILMN) - Set A AIL001 - AIL048 (48)	Asuragen	150004
Index Codes (ILMN) - Set B AIL049 - AIL096(48)	Asuragen	150005
2X Index Master Mix	Asuragen	145361
Read 1 Sequencing Primers	Asuragen	150001
Index Read Sequencing Primers	Asuragen	150002
Read 2 Sequencing Primers	Asuragen	150003
Sequencing Diluent	Asuragen	145365
Illumina MiSeq	Illumina	
MiSeq Reagent Kit v3 (600-cycle)	Illumina	MS-102-3003
MiSeq Reagent Nano Kit v2 (300-cycle)	Illumina	MS-103-1001
PhiX Control v3	Illumina	FC-110-3001
Magnetic Stand-96 (Or equivalent device)	Ambion	AM10027
Quantidex Reporter Software	Asuragen	



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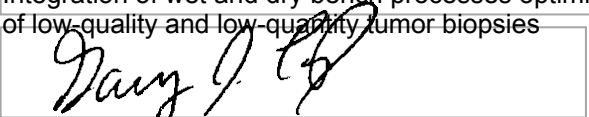
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Oct. 15, 2015

Dear Dr. Nam Nguyen,

We have uploaded a revised version of our manuscript “Integration of wet and dry bench processes optimizes targeted next-generation sequencing of low-quality and low-quantity tumor biopsies” that addresses the requested editorial revisions and reviewer’s comments. These revisions are detailed point-by-point as follows, copied from your original email.

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We have proofread the manuscript.

QC Report 8/17/2015 -

1. This article is essentially a tutorial video providing demonstration of the authors' next generation sequencing system for tumors. While discussion of the merits of the approach is to be expected, it is nevertheless essential to avoid branding and biased language that gives the impression of an advertisement or brochure.

We have revised the manuscript to remove biased language and branding.

2. Academic writing does not typically speak in terms of clinical "needs." This type of language should be avoided throughout the entire document. Please also eliminate the use of the phrase “push-button”.

We have eliminated the term “need” and “push-button” and revised other content that should reflect a more academic style.

3. The Discussion section fails to achieve a balanced or neutral tone. Particularly troublesome phrases and sentences from the discussion are listed below:
-“Further, many of the targeted NGS cancer panels that have been reported are compilations of disaggregated workflows, disparate reagents, and generic data analysis pipelines6” This description of other panels comes from the authors’ own work, and requires a neutral citation.

The sentence was removed.

-“Other targeted NGS methods lack this combination of features, flexibility, capabilities, and workflow integration.”

This sentence has been removed.

-“After sequencing, the “push-button” bioinformatics suite can be run on a desktop computer without the need for an onerous computing infrastructure, niche bioinformatics expertise, or extensive support by information technology specialists.”

The sentence was revised to remove the words “push-button” and the word “onerous”.

-“This limitation, however, is also a strength as the panel only targets evidence-based, actionable content that is of the highest interest to clinical laboratories.”

This sentence has been revised.

-“These opportunities...” “Opportunities” is commercial, not academic, language.

We have removed the word “opportunities”.

-“This proactive approach saves not only the expense of processing the sample through the downstream procedures and expedites troubleshooting, but, more importantly, saves time that would otherwise be lost by repeatedly sequencing a sample that is destined to fail.”

This sentence has been removed.

-“The approach described here is responsive to each of these needs.”

This sentence has been removed.

-Please eliminate references to “needs,” which is not typical academic language.

The word “needs” has been removed.

-Four paragraphs of advantages compared to other technology is excessive. Please condense the first four paragraphs of the discussion into a balanced description of the significance compared to other technologies, with appropriate, neutral citations.

The first four paragraphs have been condensed into a balanced description.

-The last paragraph of the discussion should be deleted, as it does not address any of the required discussion items and merely lists, again, the advantages of the platform.

The last paragraph was deleted.

Reviewers' comments:

Editor's Note: We do not require in depth or novel results for publication in JoVE, only representative results that demonstrate the efficacy of the protocol. However, please ensure that all claims made throughout the manuscript are supported by either results or references to published works.

Claims are supported by the results presented or published works.

Reviewer #1:

Manuscript Summary:

The authors presents a cross-platform system which integrate bench work and data analysis. The system was shown to be sensitive (400 amplifiable copies), flexible (able to accommodate new gene and new platforms), repeatable, and time-saving (one day library prep). A unique aspect of the system is that it incorporates pre-analytical quality control results into the variant calling algorithms, though the authors did not elaborate on this.

Major Concerns:

Some performance characteristics of the system was not discussed, the authors should discuss false positive rate of the assay. Analytic sensitivity for indels and amplifications was also not discussed.

Performance characteristics of the system, including false positive results and sensitivity for indels, are discussed in lines 410-417. Analytic sensitivity (LOD) and results for CNVs are discussed in line 401-403 and shown in Figure 5.

Note: Numbered line references apply to the "Simple Markup" view in Microsoft Word.

Minor Concerns:

The authors should give specific examples on how pre-analytic QC results affects variant calling.

Specific examples are provided in the Discussion, line 537 and Figure 3, and line 393.

Additional Comments to Authors:

N/A

Reviewer #2:*Manuscript Summary:*

Houghton and co-authors, all employees of Asuragen, describe the protocol for a sample-to-genotype experimental and analytical pipeline for generating data on a panel of cancer genes. This approach includes amplification of 46 target loci that may contain potentially actionable genetic mutations. It can be applied to small and difficult FFPE samples. Importantly, the method collects PCR copy number data at the amplification step and then integrates this information at the computational step of calling genotypes.

Major Concerns:

The protocol was reasonably well written and will almost certainly become clearer with an accompanying video. My main concern with this protocol is that it requires proprietary reagents and software to perform. This protocol, as written, will likely be of no value to the community unless they become a customer of Asuragen. It reads like product literature for a cancer panel product they are developing and that is probably how the authors envision this.

In principal, I don't have any objection to this. The product appears to be useful, especially the integration of inferred copy number of target regions with genotype calling from the amplicon sequence data. However, I would recommend, for the sake of full understanding and making the protocol reproducible by the community, that the authors replace Table 4 with the components of each of the products. Also, the authors should describe, at pseudo-code level, how the inferred copy number is used in the genotype calling.

The assay is available for any laboratory to execute the protocol as described and reproduce our results. This includes the provision of specific control samples (both residual clinical FFPE and synthetic DNA) that are detailed in the manuscript. The commercial context for the assay was discussed with Nandita Singh at JOVE after the invitation to submit, and the suitability of the article within this context was agreed upon prior to submission of the manuscript.

The description of how the inferred copy number is used in the genotype calling is described in lines 509-512.

Minor Concerns:

1. Table 4 has many blank rows in the copy of this manuscript I received.

The blank cells in Table 4, now renumbered as Table 1, have been removed.

2. Table 5 (renumbered as Figure 6) provides a useful description of minimum sample input requirements. In this figure or the text, the authors should put this in context with other, similar approaches. What are the input requirements for accurate genotyping using hybridization-based capture, for example?

This has been explained in the text in lines 513-515.

Additional Comments to Authors:

N/A

Reviewer #3:

Manuscript Summary:

This manuscript outlines a new proprietary technology (available as a commercial kit) that enables the assessment of mutation status and copy number alterations in a select panel of 21 genes from difficult samples (either FFPE or FNA specimens, for example). The authors demonstrate robust detection down to nanogram quantities of DNA. This approach should be of interest to those in clinical labs assessing mutation status in the specific genes offered in the kit in difficult samples.

Major Concerns:

My biggest concern is that the methodology in this manuscript is not explicitly described, so that others could not adopt the techniques and technology to other problems. This is somewhat understandable, as this is a commercial kit, but it does limit its broad applicability.

As mentioned above, the assay is available for any laboratory to execute the protocol as described and reproduce our results. This includes the provision of specific control samples (both residual clinical FFPE and synthetic DNA) that are detailed in the manuscript. The commercial context for the assay was discussed with Nandita Singh at JOVE after the invitation to submit, and agreement of the suitability of the article within this context was agreed upon prior to submission of the manuscript.

Minor Concerns:

1. The first table mentioned (line 130) is Table 4. Standard convention is to label the tables consecutively in the order that they are mentioned in the manuscript, so I would recommend renumbering the tables.

As requested, the tables have been renumbered.

2. The authors in step 1.2 make a total of 9 ul of master mix per sample, then aliquot exactly 9 ul of master mix into a 96 well plate. To avoid pipetting errors, they should state that extra master mix should be prepared to avoid shortages due to pipetting. This same issue occurs several additional times in the manuscript.

This is now explained in lines 130-131, 175-176 and 270-271.

3. For step 1 (DNA quantification), no mention is made of the fact that this requires a real



time PCR machine other than one line in step 1.8 stating that it should be analyzed with the qPCR software. This should be stated up front.

This is now stated in lines 119 and 120.

4. For steps 1.8-1.10, it would be helpful to include representative figures demonstrating what an acceptable curve and a failed curve look like.

We have included a new Figure 1 which shows representative examples of a failed and acceptable calibration curve.

5. I realize that the abstract says "targeted NGS", but it would be nice to state that they are looking at a limited oncogene panel here. Targeted can be interpreted in many ways, whereas a "targeted 21-gene panel" much more accurately describes their assay.

We have used the phrase "targeted 21-gene panel" as recommended.

Thank you for the helpful editorial feedback. We look forward to finalizing the manuscript.

Sincerely,

A handwritten signature in black ink, appearing to read "Gary Latham".

Gary Latham, PhD
Vice President, Research
Asuragen, Inc.