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TITLE:

Oncogenic Gene Fusion Detection Using Anchored Multiplex Polymerase Chain Reaction Followed by Next Generation Sequencing

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SUMMARY:

This article details the use of an anchored multiplex polymerase chain reaction-based library preparation kit followed by next-generation sequencing to assess for oncogenic gene fusions in clinical solid tumor samples. Both wet-bench and data analysis steps are described.

ABSTRACT:

Gene fusions frequently contribute to the oncogenic phenotype of many different types of cancer. Additionally, the presence of certain fusions in samples from cancer patients often directly influences diagnosis, prognosis, and/or therapy selection. As a result, the accurate detection of gene fusions has become a critical component of clinical management for many disease types. Until recently, clinical gene fusion detection was predominantly accomplished through the use of single-gene assays. However, the ever-growing list of gene fusions with clinical significance has created a need for assessing fusion status of multiple genes simultaneously. Next generation sequencing (NGS)-based testing has met this demand through the ability to sequence nucleic acid in massively parallel fashion. Multiple NGS-based approaches that employ different strategies for gene target enrichment are now available for use in clinical molecular diagnostics, each with its own strengths and weaknesses. This article describes the use of anchored multiplex PCR (AMP)-based target enrichment and library preparation followed by NGS to assess for gene fusions in clinical solid tumor specimens. AMP is unique among amplicon-based enrichment approaches in that it identifies gene fusions regardless of the identity of the fusion partner. Detailed here are both the wet-bench and data analysis steps that ensure accurate gene fusion detection from clinical samples.

INTRODUCTION:

The fusion of two or more genes into a single transcriptional entity can occur as the result of large scale chromosomal variations including deletions, duplications, insertions, inversions, and translocations. Through altered transcriptional control and/or altered functional properties of the expressed gene product, these fusion genes can confer oncogenic properties to cancer cells[1]. In many cases, fusion genes are known to act as primary oncogenic drivers by directly activating cellular proliferation and survival pathways.

The clinical relevance of gene fusions for cancer patients first became apparent with the discovery of the Philadelphia chromosome and the corresponding *BCR-ABL1* fusion gene in chronic myelogenous leukemia (CML)[2]. The small molecule inhibitor imatinib mesylate was developed to specifically target this fusion gene and demonstrated remarkable efficacy in *BCR-ABL1*-positive CML patients[3]. Therapeutic targeting of oncogenic gene fusions has also been successful in solid tumors, with inhibition of *ALK* and *ROS1* fusion genes in non-small cell lung cancer serving as primary examples[4,5]. Recently, the NTRK inhibitor larotrectinib was FDA approved for *NTRK1/2/3* fusion-positive solid tumors, regardless of disease site[6]. Beyond therapy selection, gene fusion detection also has roles in disease diagnosis and prognosis. This is particularly prevalent in various sarcoma and hematologic malignancy subtypes that are diagnostically defined by the presence of specific fusions and/or for which presence of a fusion directly informs prognosis[7-11]. These are but a few of the examples of the clinical application of gene fusion detection for cancer patients.

Due to the critical role in clinical decision making, accurate gene fusion detection from clinical samples is of vital importance. Numerous techniques have been applied in clinical laboratories for fusion or chromosomal rearrangement analysis including: cytogenetic techniques, reverse transcription polymerase chain reaction (RT-PCR), fluorescence in situ hybridization (FISH), immunohistochemistry (IHC), and 5'/3' expression imbalance analysis (among others)[12-15]. Presently, the rapidly expanding list of actionable gene fusions in cancer has resulted in the need to assess fusion status of multiple genes simultaneously. Consequently, some traditional techniques that can only query one or a few genes at a time are becoming inefficient approaches, especially when considering that clinical tumor samples are often very finite and not amenable to being divided among several assays. Next generation sequencing (NGS), however, is an analysis platform that is well suited for multi-gene testing, and NGS-based assays have become common in clinical molecular diagnostic laboratories.

Currently used NGS assays for fusion/rearrangement detection vary in regard to the input material used, the chemistries employed for library preparation and target enrichment, and the number of genes queried within an assay. NGS assays can be based on RNA or DNA (or both) extracted from the sample. Although RNA-based analysis is hampered by the tendency of clinical samples to contain highly degraded RNA, it circumvents the need to sequence large and often repetitive introns that are the targets of DNA-based fusion testing but have proven to be difficult for NGS data analysis[16]. Target enrichment strategies for RNA-based NGS assays can be largely divided into hybrid capture or amplicon-mediated approaches. While both strategies have been successfully utilized for fusion detection, each contains advantages over the other[17,18]. Hybrid capture assays generally result in more complex libraries and reduced allelic

dropout, whereas amplicon-based assays generally require lower input and result in less off-target sequencing[19]. However, perhaps the primary limitation of traditional amplicon-based enrichment is the need for primers to all known fusion partners. This is problematic since many clinically important genes are known to fuse with dozens of different partners, and even if primer design allowed for detection of all known partners, novel fusion events would remain undetected. A recently described technique termed anchored multiplex PCR (or AMP for short) addresses this limitation[20]. In AMP, a 'half-functional' NGS adapter is ligated to cDNA fragments that are derived from input RNA. Target enrichment is achieved by amplification between gene specific primers and a primer to the adapter. As a result, all fusions to genes of interest, even if a novel fusion partner is involved, should be detected (see **Figure 1**). This article describes the use of the ArcherDx FusionPlex Solid Tumor kit, an NGS-based assay that employs AMP for target enrichment, for the detection of oncogenic gene fusions in solid tumor samples (see **Supplementary Table 1** for complete gene list). The wet-bench protocol and data analysis steps have been rigorously validated in a Clinical Laboratory Improvement Amendments (CLIA)-certified laboratory.

PROTOCOL:

1. Library preparation and sequencing

1.1. General assay considerations and pre-assay steps

1.1.1. Assay runs typically consist of seven clinical samples and one positive control (although the number of samples per library preparation run can be adjusted as necessary). Use a positive control that contains at least several gene fusions (that the assay targets) that have been confirmed by a manufacturer and/or have been confirmed by an orthogonal methodology. A non-template control (NTC) must be included as an additional sample in every assay run, but is only carried through second strand cDNA synthesis and pre-sequencing quality control (Pre-Seq QC; to ensure no cDNA synthesis). Use sample diluent (10 mM Tris HCl pH 8.0) for the NTC.

1.1.2. Perform total nucleic acid (TNA) extraction of formalin-fixed paraffin-embedded (FFPE) tissue.

1.1.3. Quantify the RNA component of the TNA using a fluorometric assay.

NOTE: Prevent RNA degradation in the samples by limiting freeze-thaw cycles, keeping thawed nucleic acid at low temperature (chilled block, ice, or alternative), and preventing RNase contamination (wearing gloves, using an RNase decontaminator spray).

1.2. Random priming

NOTE: The desired input for the assay is 200 ng RNA (based on fluorometric quantitation of the TNA). Lower inputs can be used if 200 ng cannot be achieved. If the sample fails post-sequencing QC, repeating with higher input may result in acceptable QC metrics.

1.2.1. Dilute TNA in 10 mM Tris HCl pH 8.0 to achieve desired RNA concentration. For each sample, transfer 20 µL of the dilution into the random priming reagent strip tubes (placed in pre-chilled aluminum block) and mix by pipetting up and down 6−8 times. Briefly spin down and transfer the entire volume to a 96 well PCR plate and seal with RT film.

1.2.2. Insert plate in thermocycler block, cover with compression pad, and close lid. Incubate at 65 °C for 5 min (heated lid).

1.2.3. Remove plate from thermocycler and place on ice for 2 min.

1.3. First strand cDNA synthesis

1.3.1. Transfer the entire volume of the random priming product into the first strand reagent strip tubes (placed in pre-chilled aluminum block). Mix by pipetting up and down 6−8 times. Briefly spin down, transfer entire volume to a 96 well PCR plate and seal with RT film.

1.3.2. Insert plate in thermocycler block, cover with compression pad, and close lid. Run thermocycler program: 25 °C 10 min, 42 °C 30 min, 80 °C 20 min, 4 °C hold (heated lid).

1.4. Second strand cDNA synthesis

1.4.1. In a new set of PCR strip tubes, create a 1:10 dilution of the first strand product by adding 1 µL of first strand product to 9 µL nuclease free water. Set the dilution aside to be used in the Pre-Seq QC assay.

1.4.2. Add 21 µL of nuclease free water to the remaining first strand product. Transfer 40 µL of the first strand product and nuclease free water to the second strand reagent strip tube (placed in pre-chilled aluminum block). Mix by pipetting up and down 6−8 times. Spin down, transfer entire volume to a 96 well PCR plate and seal with RT film.

1.4.3. Insert plate in thermocycler block, cover with compression pad, and close lid. Run thermocycler program: 16 °C 60 min, 75 °C 20 min, 4 °C hold (heated lid).

NOTE: This is an acceptable stopping point. The plate can be stored at -20 °C.

1.5. Pre-Seq QC

NOTE: This quality control (QC) assay is used primarily for verification of no cDNA synthesis from the NTC. However, the data may also be informative in assay troubleshooting. For example, if a sample has a good Pre-Seq QC value but poor assay metrics (see below) then it might be an indication of a problem in the assay run, necessitating a repeat.

1.5.1. Run each sample and the NTC in duplicate. Also include in the Pre-Seq QC run a reaction

NTC, which is nuclease free water (also run in duplicate). To each applicable well of an optical 96 well plate add 5 µL of the assay master mix, 1 µL of 10x VCP primer, and 4 µL of the 1:10 dilution of the first strand product that was created in step 1.4.1.

1.5.2. Seal the plate with RT film, spin down, and load into a quantitative PCR (qPCR) instrument. Run the program: pre-amp: 1 cycle 95 °C 20 s, amp: 35 cycles 95 °C 3 s (4.4 °C/s ramp rate) − 60 °C 30 s (2.2 °C/s ramp rate).

1.5.3. Confirm lack of a cycle threshold (Ct) value for both the assay NTC and reaction NTC.

NOTE: If there is no Ct observed in the assay NTC it will no longer be carried forward in the assay. Observation of a Ct value in the reaction NTC necessitates a repeat of the Pre-Seq QC assay. Observation of a Ct in the assay NTC suggests sample contamination at some point prior in the assay, thus requiring that the entire assay (all samples) be started over. The Pre-Seq QC Ct value for an adequate quality sample should be below 30 (lower Ct values correlate with more product and thus higher quality RNA). Values above 30 are not absolute indicators of failure and these sample should be continued in the assay. However, all data derived from such samples should be reviewed carefully, particularly in cases for which no fusion is called.

1.6. End repair and bead purification

1.6.1. Transfer 40 µL of the second strand product into the end repair reagent strip tube (placed in pre-chilled aluminum block). Mix by pipetting up and down 6−8 times, spin down and place into thermocycler block (keeping the heated lid open) and run the thermocycler program: 25 °C 30 min, 4 °C hold.

1.6.2. Remove purification beads from 4 °C and allow to equilibrate to room temperature. Make up enough 70% ethanol to last throughout the entire library preparation. Vortex beads well before use and pipet 100 µL into the appropriate number of wells of a U-bottom plate.

NOTE: For every 8 samples that are run, 20 mL of 70% ethanol will be needed.

1.6.3. Add entire volume of the end repair product to the beads. Pipet up and down 6−8 times to mix and incubate at room temperature for 5 min, followed by a 5-min incubation on the magnet.

1.6.4. Remove and discard the supernatant and perform two 200 µL 70% ethanol washes with 30-s incubations. After the final wash remove all 70% ethanol and let air dry for 5 min.

1.6.5. Remove from magnet and re-suspend beads in 22 µL of 10 mM Tris HCl pH 8.0. Incubate off the magnet for 3 min followed by a 2-min incubation on the magnet. Proceed immediately to ligation step 1.

1.7. Ligation step 1 and bead purification

1.7.1. Transfer 20 µL from the end repair bead purification plate (taking care not to disturb bead pellet) into the ligation step 1 strip tubes (placed in pre-chilled aluminum block). Mix by pipetting up and down 6−8 times, spin down and transfer the entire volume into a 96 well PCR plate.

1.7.2. Insert plate in thermocycler block, cover with compression pad and close lid. Run thermocycler program: 37 °C 15 min, 4 °C hold (heated lid).

1.7.3. Remove purification beads from 4 °C and allow to equilibrate to room temperature. Vortex beads well before use and pipet 50 µL into the appropriate number of wells of a U-bottom plate.

1.7.4. Add entire volume of ligation step 1 product to the beads. Pipet up and down 6−8 times to mix and incubate at room temperature for 5 min, followed by a 5-min incubation on the magnet.

1.7.5. Remove and discard the supernatant and perform two 200 µL 70% ethanol washes with 30-s incubations. After the final wash remove all 70% ethanol and let air dry for 5 min.

1.7.6. Remove from magnet and re-suspend beads in 42 µL of 10 mM Tris HCl pH 8.0. Incubate off the magnet for 3 min followed by a 2-min incubation on the magnet. Proceed immediately to ligation step 2.

1.8. Ligation step 2 and bead purification

1.8.1. Remove molecular barcode (MBC) adapter strip tube reagents from 4 °C storage. Proper numbering of the MBC adapter strip is critically important as sample-specific indexes are added at this point. Position the tubes horizontally with the hinges to the back and use a permanent marker to label the tubes 1, 2, 3… from left to right. It is also critical to record the sample-specific indexes for sequencing purposes (they must be entered in the sequencer worksheet).

1.8.2. Transfer 40 µL from the ligation step 1 bead purification plate (taking care not to disturb bead pellet) to the MBC adapter strip tubes (placed in pre-chilled aluminum block). Mix by pipetting up and down 6−8 times.

1.8.3. Spin down and transfer the entire volume to the ligation step 2 reagent strip tubes (also placed in pre-chilled aluminum block). Mix by pipetting up and down 6−8 times, spin down and place in thermocycler block. With the heated lid off run the thermocycler program: 22 °C 5 min, 4 °C hold.

NOTE: This is a safe stopping point and samples can be stored at -20 °C.

1.8.4. Remove ligation cleanup beads from 4 °C storage and allow to equilibrate to room

temperature. Prepare 1 mL of fresh 5 mM NaOH.

1.8.5. Vortex the ligation cleanup beads and add 50 µL to a new set of PCR strip tubes. Incubate on the magnet for 1 min. After the 1-min incubation remove and discard supernatant. Remove strip tubes from magnet and re-suspend in 50 µL of ligation cleanup buffer by pipetting up and down 6−8 times.

1.8.6. Transfer entire volume of the ligation step 2 product into the ligation cleanup bead strip tubes. Mix the samples by vortexing and incubate at room temperature for 5 min. Again, mix samples by vortexing and incubate at room temperature for another 5 min. After the second 5-min incubation, mix the samples by vortexing, spin down briefly and incubate on the magnet for 1 min.

1.8.7. Remove and discard supernatant. Add 200 µL of ligation cleanup buffer and vortex to re-suspend. Perform a quick spin and place on the magnet for 1 min. Perform a second wash using the ligation cleanup buffer again.

1.8.8. After the two washes with the ligation cleanup buffer perform an identical wash using ultrapure water. Following the ultrapure water wash re-suspend the beads in 20 µL of 5 mM NaOH and transfer to a 96 well PCR plate. Place the plate into thermocycler block with a compression pad and run the thermocycler program: 75 °C 10 min, 4 °C hold (heated lid).

1.8.9. Spin down the PCR plate once the samples have cooled to 4 °C. Place the plate on magnet for at least 3 min and proceed immediately to first PCR.

1.9. First PCR and bead purification

1.9.1. Remove the first PCR reagent strip tubes from 4 °C Storage and place in a pre-chilled aluminum block. Also, remove the GSP1 primers from -20 °C and allow to equilibrate to room temperature.

1.9.2. Add 2 µL of the GSP1 primers to each well of the first PCR reagent strip tubes. Transfer 18 µL of the ligation 2 cleanup product to the first PCR reagent strip tubes and mix by pipetting up and down 6−8 times.

1.9.3. Spin down and transfer to a 96 well PCR plate. Place the plate into thermocycler block with a compression pad and run the thermocycler program: 95 °C 3 min, 15 cycles 95 °C 30 s – 65 °C 5 min (100% ramp rate), 72 °C 3 min, 4 °C hold (heated lid).

NOTE: This is a safe stopping point and samples can be stored at 4 °C overnight or at -20 °C for long term storage.

1.9.4. Remove purification beads from 4 °C and allow to equilibrate to room temperature. Vortex beads well before use and pipet 24 µL into the appropriate number of wells of a U-

bottom plate.

1.9.5. Transfer 20 µL of the first PCR product to the 24 µL of beads. Pipet up and down 6−8 times to mix and incubate at room temperature for 5 min, followed by a 2-min incubation on the magnet.

1.9.6. Remove and discard the supernatant and perform two 200 µL 70% ethanol washes with 30-s incubations. After the final wash remove all 70% ethanol and let air dry for 2 min.

1.9.7. Remove from magnet and re-suspend the beads in 24 µL of 10 mM Tris HCl pH 8.0. Incubate off the magnet for 3 min followed by a 2-min incubation on the magnet. Proceed immediately to second PCR.

1.10. Second PCR and bead purification

1.10.1. Remove the Second PCR reagent strip tubes from 4 °C and place in a pre-chilled aluminum block. Proper numbering of the second PCR strip tubes is critically important as sample-specific indexes are added at this point. Position the tubes horizontally with the hinges to the back and use a permanent marker to label the tubes 1, 2, 3… from left to right. Also, remove the GSP2 primers from -20 °C and allow to equilibrate to room temperature.

NOTE: It is also critical to record the sample-specific indexes for sequencing purposes (they must be entered in the sequencer worksheet).

1.10.2. Add 2 µL of the GSP2 primers to each well of the second PCR reagent strip tubes. Transfer 18 µL of the first PCR cleanup product to the second PCR reagent strip tubes and mix by pipetting up and down 6−8 times.

1.10.3. Spin down and transfer to a 96 well PCR plate. Place plate into thermocycler block with a compression pad and run the thermocycler program: 95 °C 3 min, 18 cycles 95 °C 30 s − 65 °C 5 min (100% ramp rate), 72 °C 3 min, 4 °C hold (heated lid).

NOTE: This is a safe stopping point and samples can be stored at 4 °C overnight or at -20 °C for long term storage.

1.10.4. Remove purification beads from 4 °C and allow to equilibrate to room temperature. Vortex beads well before use and pipet 24 µL into the appropriate number of wells of a U-bottom plate.

1.10.5. Transfer 20 µL of second PCR product to the beads. Pipet up and down 6−8 times to mix and incubate at room temperature for 5 min, followed by a 2-min incubation on the magnet.

1.10.6. Remove and discard the supernatant and perform two 200 µL 70% ethanol washes with 30-s incubations. After the final wash remove all 70% ethanol and let air dry for 2 min.

1.10.7. Remove from magnet and re-suspend the beads in 24 µL of 10 mM Tris HCl pH 8.0. Incubate off the magnet for 3 min followed by a 2-min incubation on the magnet. Transfer 20 µL of the second PCR cleanup product to a new 96 well PCR plate.

NOTE: This is a safe stopping point and libraries can be stored at -20 °C for long term storage or proceed immediately to library quantification.

1.11. Library quantitation

1.11.1. Remove the library quantitation master mix and standards from -20 °C storage and allow to equilibrate to room temperature.

1.11.2. Perform 1:5 dilution of all libraries (second PCR product) using 10 mM Tris HCl pH 8.0 followed by a serial dilution of 1:199, 1:199, and 20:80 using 10 mM Tris HCl pH 8.0 0.05% polysorbate. The final two dilutions of 1:200,000 and 1:1,000,000 respectively will be run in triplicate.

1.11.3. Add 6 µL of master mix to each well of an optical 96 well plate followed by 4 µL of appropriate dilution or standard. Spin down the plate and load it onto the qPCR instrument. Use the following cycling conditions: 1 cycle 95 °C 5 min, 35 cycles 95 °C 30 s (4.4 °C/s ramp rate) – 60 °C 45 s (2.2 °C/s ramp rate).

1.11.4. After library quantification is complete and library concentrations are determined (through averaging both tested dilutions), normalize all libraries to 2 nM using 10 mM Tris HCl pH 8.0. Make the library pool by combining 10 µL of each normalized library into one 1.5 mL micro centrifuge tube.

1.12. Sequencing of libraries

1.12.1. Remove sequencer reagent cartridge from -20 °C storage and place in deionized (DI) water up to the fill line for at least 1 h. Also, remove sequencer reagent kit from 4 °C and allow to equilibrate to room temperature for at least 1 h. The maximum number of libraries that can be sequenced on this sequencing platform is 8 (one of which must be the positive control).

1.12.2. Make the denatured amplicon library (DAL) pool by combining 10 µL of the library pool with 10 µL of 0.2 N NaOH and incubate for 5 min at room temperature. After the 5-min incubation add 10 µL of 200 mM Tris HCl pH 7.0, followed by 970 µL of HT1 hybridization buffer.

1.12.3. Make the final load tube by combining 300 µL of HT1, 25 µL of 20 pM PhiX and 675 µL of the DAL pool. Vortex and spin down the load tube. Add entire volume of the load tube to the sample well of the sequencer reagent cartridge and load cartridge into the sequencer running 2 x 151 bp reads with 2 x 8 index reads.

2. Data analysis

2.1. Sequencing data handling and analysis

2.1.1. Download sequencing metrics from the NGS instrument.

2.1.2. Ensure that sequencing data falls into the below ranges (specific for the kit used in this example). Cluster density (density [K/mm^2]): 945–1800 K; % of reads passing filter (clusters PF [%]): >90%; quality scores (% ≥Q30): >85%; PhiX alignment (aligned %): 1.5−6.0%; PhiX error rate (error rate [%]): <1.0%; reads passing filter (reads PF [M]): >22 million.

NOTE: Sequencing runs not meeting these metrics are subject to repeat.

2.1.3. Review the % of reads attributed to each sample (i.e., each sample-specific index). For a typical run in which the PhiX spike-in was ~5% and 8 samples were sequenced, each sample should ideally account for approximately 11.9% of the reads. The acceptable range for each sample is 5−25%. If any samples do not fit within this range, repeat library quantitation and sequencing.

2.2. Submission of raw sequence data to the analysis algorithm

NOTE: Version 4.1.1.7 of ArcherDx Analysis is described here. In this example, the analysis software is deployed as a 'virtual machine' run on an internal server. One central processing unit (CPU) and 12 GB of random access memory (RAM) are required for each sample to be analyzed concurrently (typically 8 samples are processed concurrently requiring 8 CPU and 96 GB RAM). Processing typically takes 3−8 h.

2.2.1. Use default analysis settings within the analysis system, with the exception of MIN_AVERAGE_UNIQUE_RNA_START_SITES_PER_GSP2_CONTROLS (this is changed from 10 to 20) and READ_DEPTH_NORMALIZATION (this is set to 0 to prevent down-sampling).

2.2.2. To start an analysis job, click on **Perform Analysis** in the user interface, submit a name for the job, and then select the necessary FASTQ files (ensuring that both reads [R1 and R2] are selected for each sample).

2.2.3. Select **RNA Fusion** for RNA analysis types, **Illumina (paired)** for platform, and **FusionPlex Solid Tumor Panel** for target region. Then click on **Submit Analysis**.

2.3. Assay data interpretation

2.3.1. Open the user interface of the analysis system and select the desired job. Select the positive control sample. Ensure that all expected fusions and oncogenic isoforms have been detected and are listed in the **Strong Evidence** tab.

NOTE: If any of the tracked fusions in the positive control are not detected for a given run of the assay, it may be an indication of a problem in that run, which necessitates a repeat (from beginning of the assay).

2.3.2. Examine the read statistics for each clinical sample in the run by clicking on the **Read Statistics** tab. Each sample should be represented by at least 1 million total fragments. If less than 1 million, re-sequence the library with considerations for re-quantitation to ensure the initial quantitation was not aberrantly high.

NOTE: Good quality samples will generally display **On Target %** >85%, and the RNA **Molecular Bins** should be >20,000 and comprise >30% of the reads. However, failure to meet these metrics does not automatically trigger sample failure.

2.3.3. Inspect the **Average Unique Start Sites per GSP2 Control** value.

NOTE: This value is a measure of sequencing complexity from primers to four housekeeping genes. This metric is a critical determinant of RNA quality in the sample (if sequencing of genes expected to be expressed in the sample is poor, then confidence in the ability to detect an expressed gene fusion is low). The cutoff for this metric is 20. If any sample displays a value less than 20, then results for the sample must be reported as **uninformative** if no high confidence fusion is found. The status of this metric can also be determined in the summary page of the run (status will be listed as **Fusion QC: Pass** or **Fusion QC: Number of Unique RNA GSP2 Control Start Sites Low** depending on whether the value is greater than or less than 20). A high confidence fusion in a sample that did not pass this QC metric can still be reported if it is determined to be real (see below). Generally, higher QC scores using this metric correlate with lower PreSeq Ct scores, as expected (**Figure 2**).

2.4. Fusion call interpretation

2.4.1. Carefully scrutinize every called fusion under the **Strong Evidence** tab (the majority of strong evidence fusions called by the system are artifactual). Also scan the weak evidence fusion bin, although the presence of a non-artifactual fusion in this bin is an extremely rare event. To assess validity of a fusion, first note the **Reads (#/%)** and **Start Sites** metrics.

NOTE: Generally, confidence in a called fusion increases if these metrics are higher. Fusions that are supported by less than 5 start sites and by less than 10% of the reads from the supporting primer should be considered as highly suspicious as being artifactual.

2.4.2. Make note of the icons displayed in the **Filters** column.

NOTE: Several of these icons alert the user of a potential source of an artifactual call. Frequent among these (and primarily found in the weak evidence bin of called fusions) are instances where the partners are known paralogs or show similarity to each other in sequence (indicating likely mis-priming or erroneous alignment). Another common source of artifactual fusion calls

occurs when the reads supporting the fusion contain a high error rate that is indicative of poor mapping to the reference genome, and this is associated with a distinct icon. Transcriptional readthrough events are identified via another icon. These are common and are generally ignored. Several other icons are also used in the user interface, but a full description of all is beyond the scope of this article. Common artifacts that can generally be ignored without further investigation include: *ADCK4-NUMBL, SYN2-PPARG, DGKG-ETV5, ETV6-AXL, ETV1-ERG, ETV4-ETV1, FGFR3-PDGFRB, EGFR-NTRK1, RAF1-RET, ALK-TFEB, NOTCH1-RET, RELA-RET, NTRK3-ALK, BRAF-ADAMTS8,* and *FCGR2C-MAST.*

2.4.3. Visualize the supporting reads for each potential fusion by clicking on the **Visualize** link in the user interface, which takes the user to a web-based JBrowse view of pileups of individual fusion-supporting reads. Confirm that the reads are generally free of mismatch (although some noise is to be expected), that a significant proportion (ideally >30 bases) of the reads align to the fusion partner, and that sequences immediately adjacent to the breakpoint between the genes and the primer binding sites are free of insertions or deletions. Confirm that aligned sequence includes the 3' portion of the primer binding sequences for primers that support the fusion reads (if 3' portions are not included, it can be an indication of mis-priming).

2.4.4. If coding sequences of both genes are involved in the fusion, ensure that the 3' partner remains in-frame. Click on the **Translation** link in the interface (which will give a **True** or **False** status for each reference sequence combination) and also manually confirm that fusion is in frame through inspection of the called breakpoints in a genome browser. The called breakpoints can be determined by hovering the mouse over the red dots in the fusion schematic.

NOTE: Since most (but not all) genomic breakpoints for legitimate fusions occur in intronic regions and result in splicing together of whole exons, fusions in which exon boundaries comprise the breakpoints for both partners are generally viewed with a higher degree of confidence. However, since there are many published examples of mid-exonic breakpoints in fusions, this should not be used as absolute criteria in the interpretation of a fusion.

2.4.5. For each fusion, manually ensure that sequences adjacent to the breakpoint are not homologous to the next expected contiguous bases for each partner. Inspect all possible contiguous exons in a genome browser.

NOTE: A common source of artifactual fusion is misalignment or mis-priming due to homology between two gene partners, and this is not always called by the system via the icons described above.

REPRESENTATIVE RESULTS:

Shown in **Figure 3**, **Figure 4** and **Figure 5** are screenshots from the analysis user interface demonstrating results from a lung adenocarcinoma sample. In **Figure 3**, the sample summary is shown (top) that lists the called strong evidence fusions, as well as the QC status (circled in red). The *ADCK4-NUMBL* fusion (of which 3 isoforms are listed) is immediately ignored because it is a

persistent transcriptional readthrough event (noted by the broken circle icons next to the listings). The bottom of **Figure 3** is a screenshot of the Read Statistics page. Particularly informative metrics are circled in green. The critical QC metric that determines the pass/fail nature of the sample is highlighted in red (this is the metric that dictates pass/fail status in the summary above). If this value is below 20, a negative fusion result is deemed 'uninformative'. **Figure 4** and **Figure 5** are screenshots of the fusion schematics (top) and JBrowse views of fusion supporting reads (bottom) for the two fusions that warranted further investigation. The *KIF5B-RET* fusion (**Figure 4**) demonstrates a high number of supporting reads, a high % of reads from the primer supporting the fusion, and a high number of start sites. Additionally, several contiguous exons of the fusion partner (*KIF5B*) are included in the alignment, the fusion was verified to be in frame, and the reads supporting the fusion are generally free of mismatch. For these reasons, the fusion is deemed real and reportable. The *LOC101927681-PDGFRB* fusion (**Figure 5**) demonstrates lower supporting metrics. Furthermore, the portion of the reads mapping to the partner are relatively short and contain a high error rate, which strongly suggests misalignment and an artifactual fusion call. Finally, intronic sequence of *PDGFRB* is included, further suggesting an artifact (most, but not all, legitimate fusions are comprised solely of sequence that maps to coding regions of both genes). For these reasons, this fusion is deemed an artifact and not reportable.

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic representation of AMP approach. Traditional amplicon-mediated approaches for target enrichment are limited by the fact that primers are needed for all fusion partners. Thus, novel fusion partners will not be detected. In AMP, the opposing primer is specific to the adapter, thus novel partners are detected.

Figure 2: Correlation between PreSeq QC score and post-sequencing QC. The PreSeq Ct and average unique start sites per GSP2 Control values were correlated for a series of 100 samples. Generally, as expected, low PreSeq scores correlate with higher SS/GSP2 values (both are indicators of good quality RNA).

Figure 3: Example sample summary and read statistic views. Top: view of a sample summary in the user interface with strong evidence fusions listed. Bottom: view of the read statistics page in the user interface.

Figure 4: Example of a legitimate fusion call. Top: view of the fusion schematic in the user interface. Bottom: JBrowse view of individual reads supporting the fusion.

Figure 5: Example of an artifactual fusion call. Top: view of the fusion schematic in the user interface. Bottom: JBrowse view of individual reads supporting the fusion.

Supplementary Table 1: List of genes for which gene-specific primers are included in the assay (to various exons and introns).

DISCUSSION:

Anchored multiplex PCR-based target enrichment and library preparation followed by next-generation sequencing is well suited for multiplexed gene fusion assessment in clinical tumor samples. By focusing on RNA input rather than genomic DNA, the need to sequence large and repetitive introns is avoided. Additionally, since this approach amplifies gene fusions regardless of the identity of the fusion partner, novel fusions are detected. This is a critical advantage in the clinical realm, and there have been many examples of actionable novel gene fusions identified through AMP reported in the literature[21-25].

Since the assay is RNA based, it is critical to preserve RNA quality in samples during processing. It is also critical to determine which samples produced RNA sequencing that was too poor to trust negative fusion results. This is achieved by assessment of sequencing data from primers to four housekeeping genes. If sequencing of these genes is poor, then negative fusion results are deemed uninformative. In addition, given the complexity and multitude of wet-bench steps in the assay, it is important to include a fusion-positive control in every assay run. By doing so, compromised assay runs become apparent through analysis of expected fusion events in the control.

As with all amplicon-based approaches, AMP is highly reliant on individual primer performance. When assessing multiple exons of multiple genes, it is inevitable that some primers will not perform as well as others. Therefore, it is critical for users to know where the assay underperforms due to primer inefficiency. Additionally, NGS-based assays require complex bioinformatic data analysis. If the algorithms employed are not thoughtfully designed, false-negative and false-positive results are likely. It is very important that all gene fusions called by analysis algorithms be manually inspected by the user.

With an ever-growing list of actionable gene fusions that should be assessed in clinical tumor specimens, use of multiplexed assays like AMP will continue to increase in clinical laboratories. Future applications of the technique will likely focus on combining fusion and mutation assessment within a single assay. Regardless of the molecular assay approach, users must always be aware of assay limitations and should always establish quality control metrics to guide data interpretation.

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Figure 1

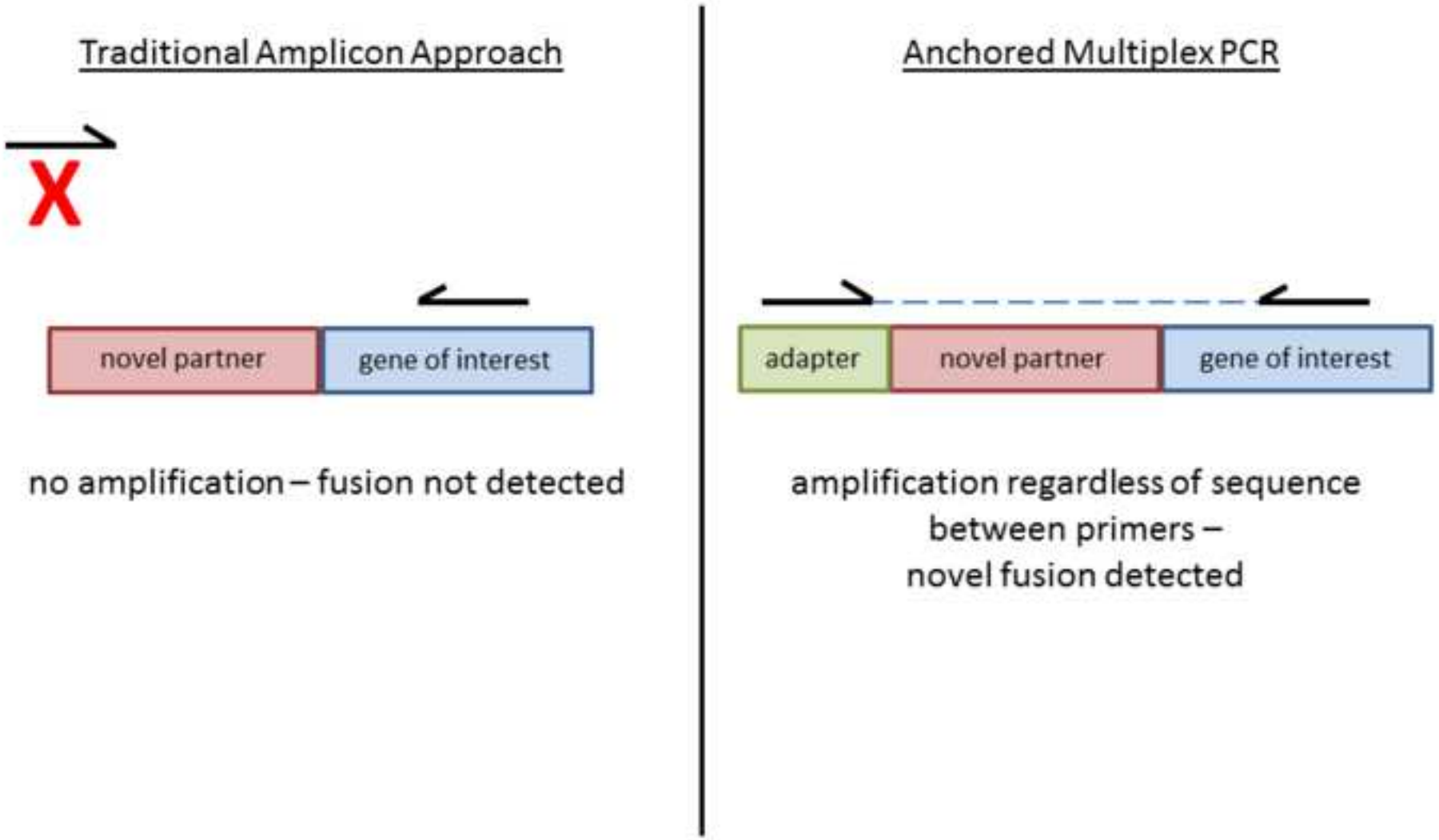

Figure 2

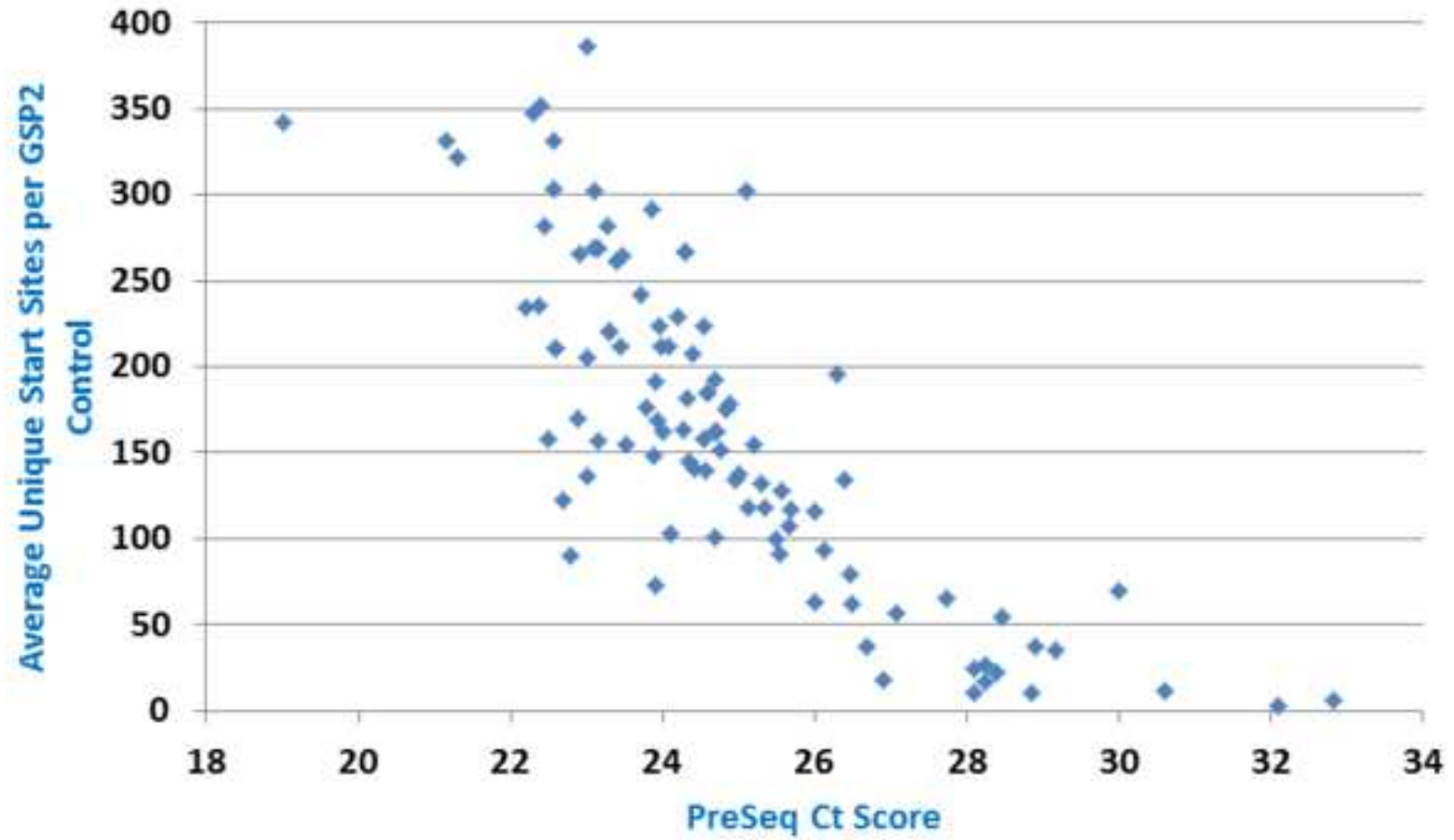

Figure 3

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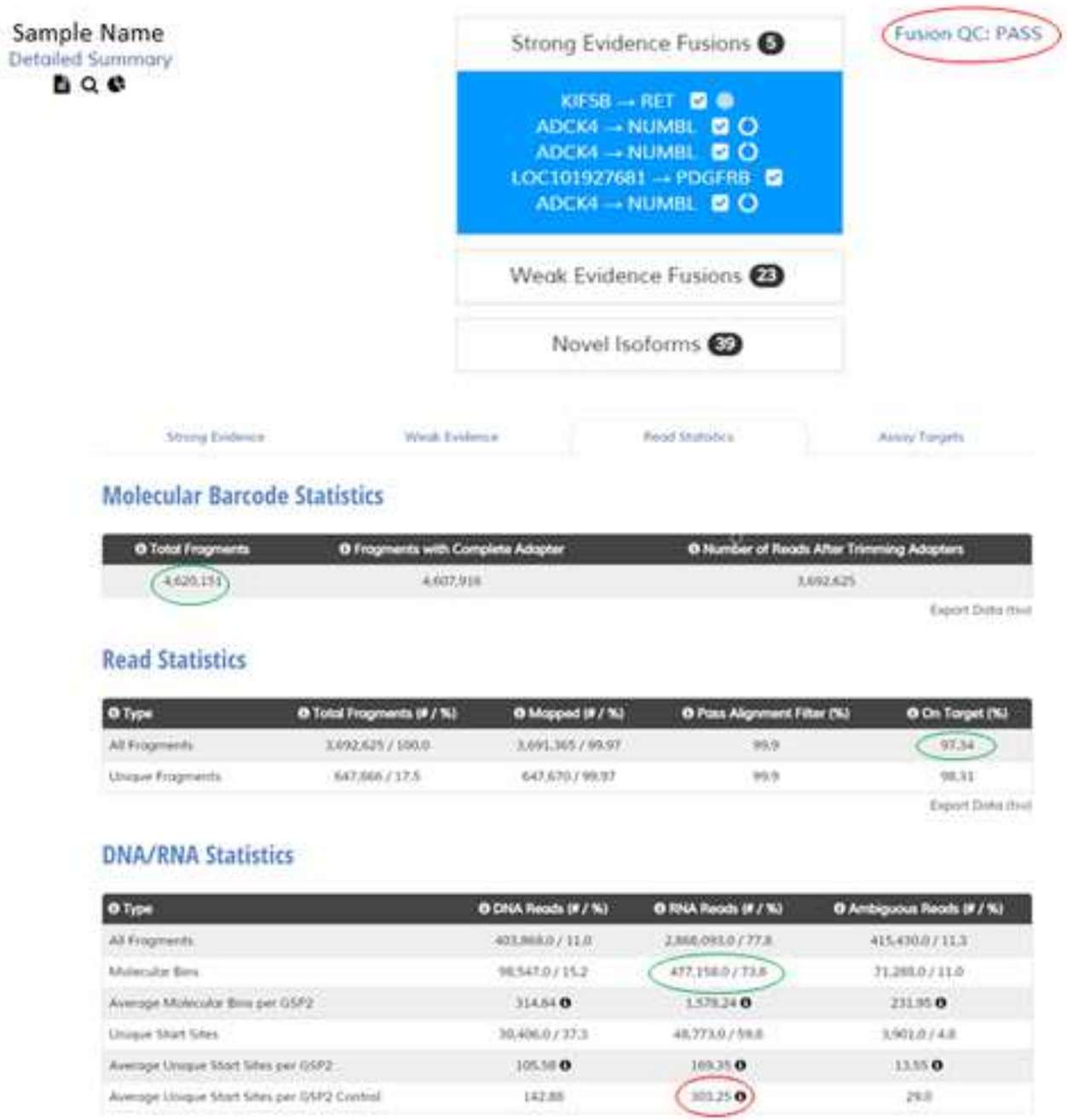

Figure 4

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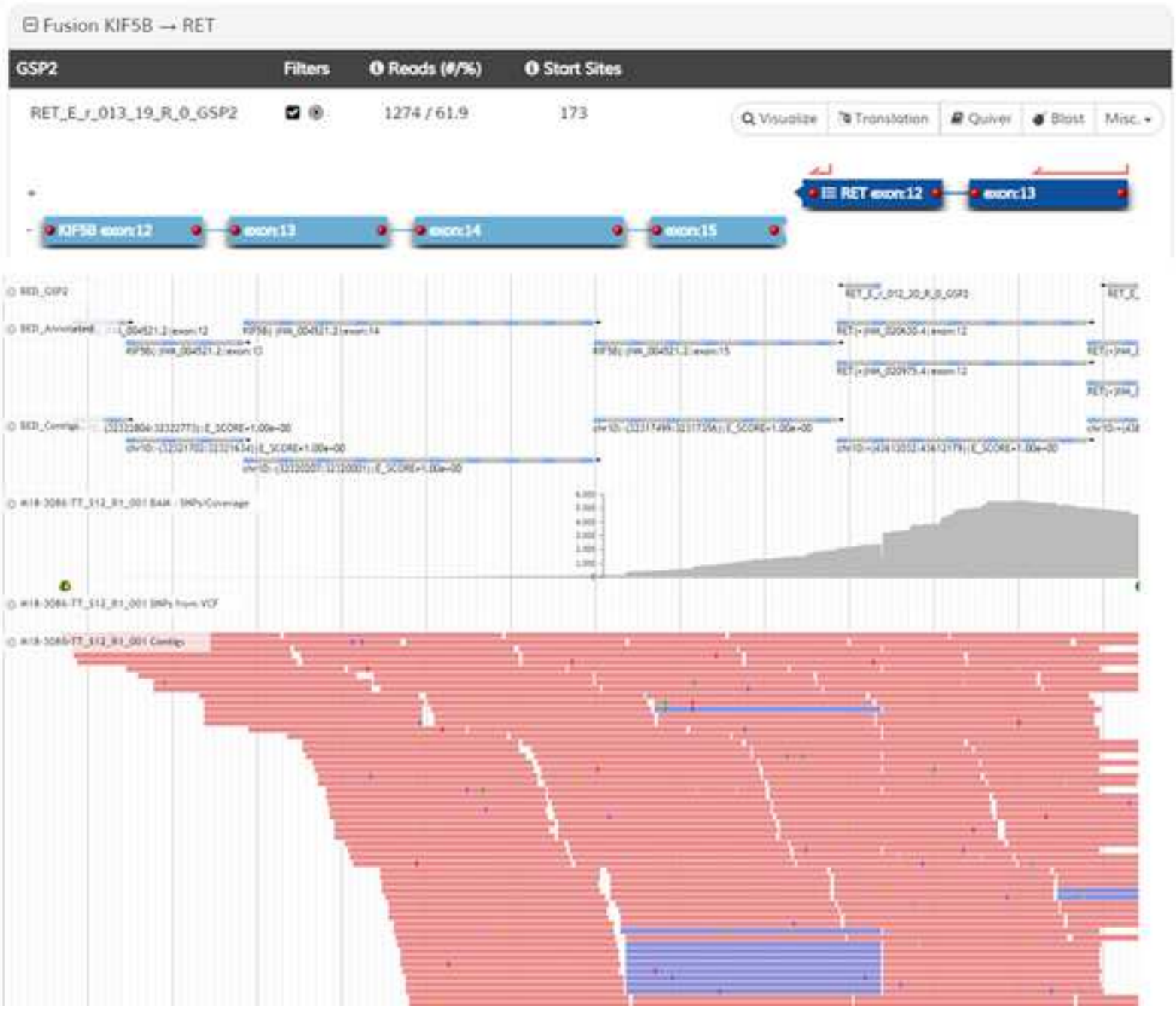

Figure 5

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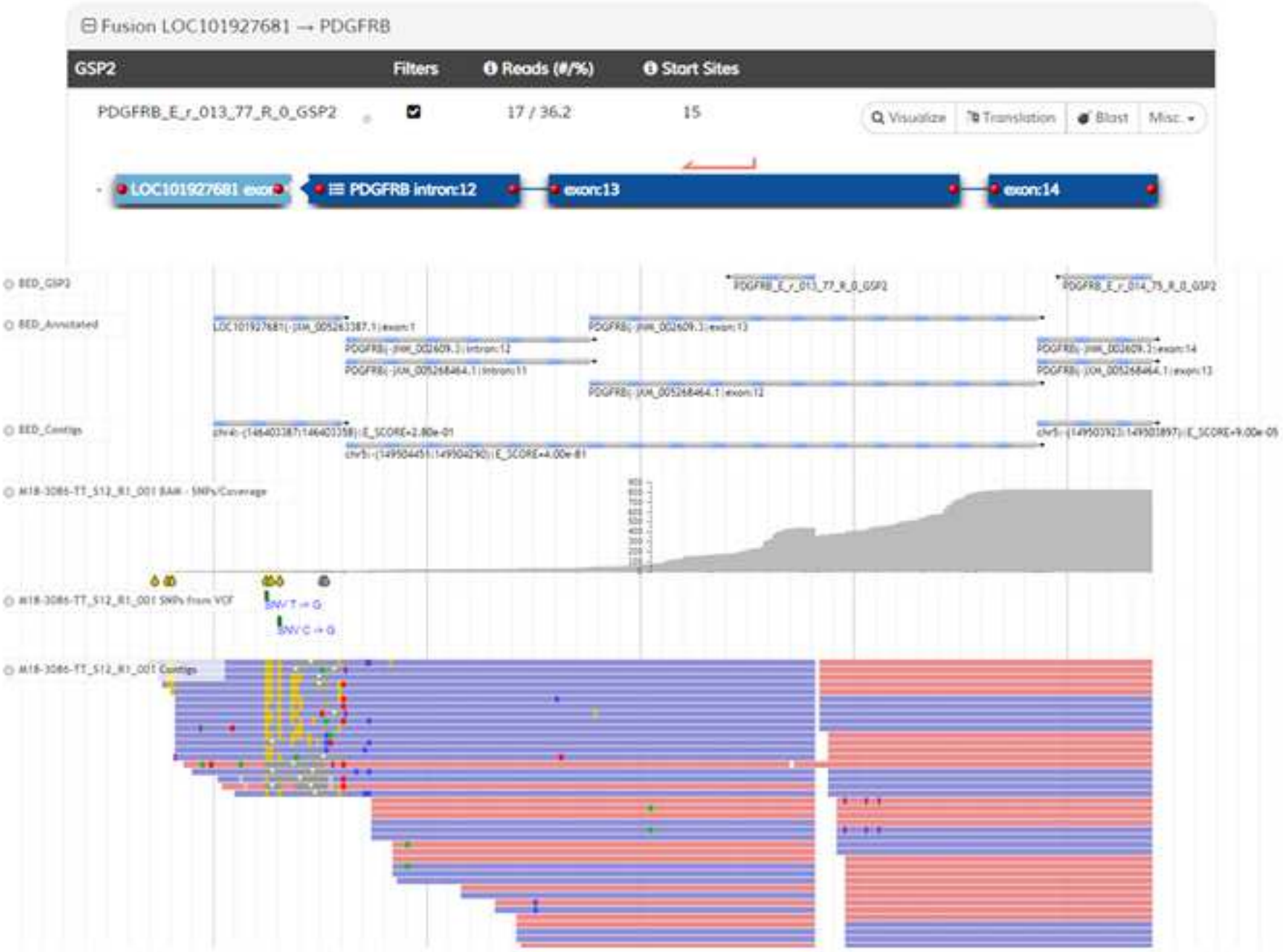

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10 mM Tris HCl pH 8.0	IDT
1M Tris pH 7.0	Thermo Fisher
25 mL Reagent Reservoir with divider	USA Scientific
96-well TemPlate Semi-Skirt 0.1mL PCR plate-natural	USA Scientific
Agencourt AMPure XP Beads	Beckman Coulter
Agencourt Formapure Kit	Beckman Coulter
Archer FusionPlex Solid Tumor kit	ArcherDX
Cold block, 96-well	Light Labs
Ethanol	Decon Labs
Library Quantification for Illumina Internal Control Standard	Kapa Biosystems
Library Quantification Primers and ROX Low qPCR mix	Kapa Biosystems
Library Quantification Standards	Kapa Biosystems
Magnet Plate, 96-well (N38 grade)	Alpaqua
MBC Adapters Set B	ArcherDX
Micro Centrifuge	USA Scientific
MicroAmp EnduraPlate Optical 96 well Plate	Thermo-Fisher
Microamp Optical Film Compression Pad	Applied Biosystems
Mini Plate Spinner	Labnet
MiSeq Reagent Kit v3 (600 cycle)	Illumina
MiSeqDx System	Illumina
Model 9700 Thermocycler	Applied Biosystems

nuclease free water	Ambion
Optical ABI 96-well PCR plate covers	Thermo-Fisher
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RNase Away	Fisher
Seraseq FFPE Tumor Fusion RNA Reference Material v2	SeraCare
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SYBR Green Supermix	Bio Rad
TempAssure PCR 8-tube Strips	USA Scientific
Template RT PCR film	USA Scientific
U-Bottom 96-well Microplate	LSP

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BP359-212

172-5120

1402-2700

2921-7800

MP8117-R

Comments/Description

Used for TNA dilution

Used in library pooling

For use with multi-channel pipetters and large reagent volumes

Plate used for thermocycler steps

Used in purification after several assay steps

Used in TNA extraction
This kit contains most of the reagents necessary to perform library preperation for Illumina sequencing (kits for Ion Torrent sequencing are also available)

Used for keeping samples chilled at various steps

Used for bead washes

Used for library quantitation

Used for library quantitation

Used for library quantitation

Used in bead purificiation steps

Adapters that contain sample-specific indexes to enable multiplex sequencing

Used for spinning down PCR tubes

Used for Pre-Seq QClibrary quantitation

Used for library quantitation

Used for collecing liquid at bottom of plate wells

Contains components necessary for a MiSeq sequencing run

NGS Sequencing Instrument

Used for several steps during assay

Used as general diluent

Used for Pre-Seq QClibrary quantitation

Wet-bench assay steps performed in this 'dead air box'

Used in TNA extraction

qPCR instrument used for PreSeq and library quantitation

Use for determing RNA concentration in TNA samples

Used for general RNase decontamination of work areas

Used as the assay positive control

Used in clean-up steps and for sequencing setup

Component of PreSeq QC Assay

Used for reagent and sample mixing etc.

Used for covering 96-well plates

Used during bead purification

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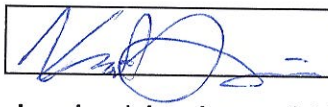
Signature:	
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Assistant Professor

April 2nd, 2019

Dr. Phillip Steindel Ph.D.
Review Editor – Journal of Visualized Experiments

Re: Oncogenic Gene Fusion Detection Using Anchored Multiplex Polymerase Chain Reaction Followed by Next Generation Sequencing

Dear Dr. Steindel:

Thank you very much for the invitation to submit a revised version of our manuscript to the Journal of Visualized Experiments.

We also thank the Editors and Reviewers for their thorough evaluations of the manuscript. We feel that by addressing the concerns they raised the manuscript is much improved. We have included a point-by-point response to all criticisms below (author responses are in blue).

I can confirm that I and all co-authors of this manuscript were original contributors to this work, approve of its submission, and have disclosed all conflicts of interest. This material has not been submitted elsewhere for publication.

Sincerely,

Kurtis D. Davies PhD

Author Responses are below each individual comment in blue

Editorial comments:
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.
The manuscript has been thoroughly proofread and is free of errors.

2. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300dpi.
All figures have been converted to .tif format. The Representative Result figures are screenshots (there is no alternative for this) and current resolution is, from our perspective, the best that can be achieved.

3. Please sort the Materials Table alphabetically by the name of the material.
The Table has been sorted

4. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: ArcherDX FusionPlex Solids Tumor Kit, SeraCare, Qubit, iTaq, MicroAmp, AMPure, Illumina, etc.
All commercial language has been removed from the manuscript body except for 2 instances where it was absolutely critical to name the vendor (in cases where description would not have been coherent without direct reference).

5. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.
The embedded tables have been converted to lines of text.

6. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly.
Several sentences have been changed to reflect an imperative tense.

Please include all safety procedures and use of hoods, etc.
There are no safety procedures beyond standard laboratory practice (personal protective equipment etc.). Hoods are not used for the assay.

7. The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
Description of the wet-bench steps is free of any discussion. Some data analysis steps contain descriptive explanations, but we strongly feel that this is necessary to support the action items that direct decision making. Without these descriptions, the action items will seem largely arbitrary.

8. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
The sections we would like as filmable content have been highlighted (approximately 2.75 pages).

9. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
We have ensured that the highlighted section conforms to the above.

10. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.
The previously included figure legends for the Representative Results have been converted into a single paragraph of text explaining all three figures.

11. Please discuss all figures in the Representative Results. However for figures showing the experimental set-up, please reference them in the Protocol.
These figures are discussed separately in the manuscript.

12. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
The Discussion has been completely re-written to reflect the above guidelines.

13. Please do not abbreviate journal titles.
The reference list has been fixed to include full journal titles.

Reviewers' comments:
Reviewer #1:

Manuscript Summary:

The manuscript by Seager and coworkers describes a method for detecting gene rearrangements or exon skipping events by RNA sequencing. The authors make an excellent case for use of the method, originally described by Zheng et al., and commercialized by ArcherDx. However, it would be nice to see a bit more introduction on the limitations of RNA sequencing and a clear case for the ArcherDx method over competing hybrid capture RNA sequencing methods. Overall the method is described in sufficient detail to allow other laboratories to carry out the procedure. I have several comments and suggestions that would make the procedure more clear. In addition, I believe that some description of the care needed when handling RNA is needed as are the proper precautions needed to prevent PCR amplicon contamination of workspaces.

We thank the Reviewer for the thorough evaluation of the manuscript. As stated below, we do not believe that the AMP/ArcherDx approach is inherently better than hybrid-capture, it just happens to be the assay we use. Therefore, we do not feel comfortable making an argument that it is better. We have, however, stated general advantages of amplicon vs. hybrid capture and vice versa in the revised manuscript. Other concerns are addressed individually below. We feel that prevention of PCR amplicon contamination is more in the realm of general good laboratory practice, and we are not able to fully address it in this manuscript due to word limits.

Major Concerns: None

Minor Concerns:

Introduction

1. The introduction should be better referenced. A large number of statements are not backed-up by literature references. Good examples are lines 64-67, 46-49.

We have added references to support the above referred to statements as well as other statements.

2. The authors point out hybrid capture methods and note that they have pros and cons (lines 81-83). The pros and cons should be listed for the reason that there are quite successful RNA sequencing library prep methods that make use of hybrid capture. In addition, like the AMP method, they are agnostic of the non-oncogenic fusion partner. Is AMP better and why?

We have added commentary (along with references) in the Introduction that compares amplicon vs. hybrid capture target enrichment. We do not think that AMP is necessarily better than hybrid capture and therefore are not going to make this argument (it just happens to be the assay that we use).

3. The description of AMP at the end of the introduction would be greatly aided by a diagram of the method - it's difficult to follow otherwise.

A Figure schematically depicting the primary advantage of the AMP approach has been included in the revised version (Figure 1).

Protocol

1. No mention is made of the precautions required to maintain RNA integrity during handling. One or two sentences outlining the general precautions used would be helpful.

We have added a section on prevention of RNA degradation (1.1.4.)

2. Similarly, no mention is made of the unidirectional workflow that is typically employed using sequencing library prep methods that employ PCR steps. This would give the authors an opportunity to describe the use of molecular barcodes in the Archer procedure and why it's important that they are incorporated prior to PCR amplification.
We agree that the molecular barcode incorporation (and corresponding de-duplication process during analysis steps) is an advantage of the ArcherDx assay. However, as this concept is quite complicated (thus requiring significant and "wordy" explanation) and not specifically relevant to instructing users how to perform the assay, we feel it is beyond the scope of the manuscript and would likely put it over word limits.

3. It should be noted where a headed lid is required for thermocycler steps.
We have updated description of steps that require thermocycler incubation with whether or not a heated lid is used.

4. 1.5 Pre-Seq QC. What is the corrective action if the NTC repeatedly has a Ct value? Is the entire batch restarted?
Yes, if the NTC demonstrates a Ct value the entire assay must be restarted. This has now been stated in section 1.5.4.

5. The exact Alpaqua magnet used should be specified, is it the Grade N34 or Grade N48 magnet. The magnet strength can impact the clearing rate of the magnetic beads.
The magnet plate used is N38 grade. The materials table has been updated accordingly.

6. 1.8.1 It should be noted that it's critical to record in the analysis bench sheet the identity of the MBC ligated to each sample. In addition, in Section 1.10 the 2nd PCR tube used for each sample should be recorded in the analysis bench sheet. This critical information in needed to input the correct barcodes into the Illumina MiSeq sample sheet.
Both sections have been updated to state that sample-specific indexes must be tracked for sequencing purposes.

7. 1.12.1 Here the authors indicate that a paired-end 300 bp MiSeq configuration is used to sequence, but later (line 165) they mention 2x151 sequencing. Why not use a MiSeq V2 300 cycle kit (paired-end 150 bp sequencing)? The V2 300 kit has enough cycles to cover the 2x151 reads 1 and 2 plus 2x8 indexes.
The v3 kit (which does not come in a 300 cycle configuration) is necessary to achieve the total fragments per sequencing run necessary for our throughput of up to 8 samples per run. If we used v2, the number of libraries per sequencing run would need to be reduced significantly (likely to 4-5) and result in less efficient sequencing.

Data Analysis
1. 2.1.2 (line 376) 945-1800 K is not what Illumina recommends for this flow cell (https://support.illumina.com/bulletins/2016/10/cluster-density-guidelines-for-illumina-sequencing-platforms-.html). 1400K is the high end of Illumina's recommendations. If the authors run the instrument at 1800K clusters/mm2 I expect that they fall short of the Q30 and cluster Pass filter targets. I recommend that the authors stick with Illumina's recommendations for this flow cell. Their sequencing

metrics objectives are in-line with good practice (lines 377-381), but probably would not be met at 1800K clusters/mm2.
We agree that this is a deviation from Illumina's recommendations, but in our experience we can cluster at up to 1800K and still pass our other metrics (including Q30 and Pass Filter). It is only when cluster density rises above 1800K that we see negative effects on other metrics.

2. 2.1.3 The % for the PhiX spike-in is 5%, not 3.5% (Library pool = 13.5 pM, PhiX = 0.5 pM).
The mixing to achieve an average 5% PhiX alignment is largely empiric and not based on absolute calculated concentration. Section 2.1.3. has been changed to state approximately 5%.

3. The authors need to explain the rationale for running the positive control at such a limiting input amount, 25 ng compared with 200 ng for clinical samples? The low input obviously handicaps the analysis with respect to the exon34 ROS1 fusion and MET exon skipping detection.
This is largely an economical decision (the reference material we use for the positive control is quite expensive). We agree with the reviewer that the ROS1 and MET events would be better detected at higher input, however we are tracking several other fusions that we deem sufficient for this purpose. Another consideration is low-level barcode contamination that the manufacturer has warned us about and that we have seen on one occasion. Because of this, we don't want fusion supporting reads to be too high for the other fusions in the reference material as this may cause barcode contamination-mediated false positive results in clinical samples. By using a lower input, none of the fusions in the positive control reaches a level to be concerning.

Regardless, since the journal prohibits commercial language, we have adjusted positive control description in the revised version to be generic. Thus, description of this specific product (input amount etc.) has been omitted.

Reviewer #2:

Manuscript Summary:
The manuscript by Seager et al. is a thorough and succinct explanation of gene rearrangement detection using anchored multiplex PCR (Archer, Inc) and NGS. All the necessary wet bench steps are appropriately described with detailed parameters for acceptability. Lines 299-301 appear to be a duplication of lines 296-298 and should be deleted or if necessary revised to make clear why the step is repeated. The data analysis steps are explained well. The quality control steps and results interpretations are consistent with the manufacturer guidelines and the general standards accepted by laboratories performing this method for clinical testing.
We thank the reviewer for the thorough evaluation of the manuscript. Yes there was an indeed an erroneous duplication. This has been removed in the revised version.

Major Concerns:
The quality of the three figures is poor in the PDF version. Higher resolution images should be provided as deemed appropriate by the editor.
This has been addressed in the revised version.

Minor Concerns:

It would be helpful to include the list of the gene targets in the Solid Tumor FusionPlex kit.
This has been added as Supplementary Table 1.

Reviewer #3:

Manuscript Summary:
The authors present a detailed protocol of the Archer FusionPlex Solid Tumor Panel, a system based on the Anchored Multiplex Technology published by Zheng et al (Nature medicine, 2014). They discuss critical steps in the protocol and QC steps. Data Analysis and Interpretation using the ArcherDx Analysis Software are discussed and examples of true positive and false positive gene fusions are shown.
We thank the reviewer for the thorough evaluation of the manuscript.

Major Concerns:
No Major concerns

Minor Concerns:

- the PreSeq QC is not only a measure for contamination, but also provides a quality metrics which is useful to determine whether an FFPE-derived RNA is useful for the analysis. It would be helpful for the Reader who wants to estabish the protocol in their own lab, if the authors could provide a table showing samples with different Ct values and the final QC metrics after sequencing.
We agree that the PreSeq QC assay provides a quality metric useful in determining RNA quality. However, since the assay is performed after cDNA synthesis, the user is already 'committed' in that the prescribed input has already been used. Therefore, it is not useful for determining which samples should not be submitted to the assay. In terms of assessing RNA quality for post-sequencing analysis, we believe that the Start Site per GSP2 for Control Genes is the superior metric (and in fact we have seen, albeit rarely, instances where a poor PreSeq value is then followed by an acceptable SS/GSP2 value). However, as is stated in section 1.5.1., the PreSeq score also has value in troubleshooting in cases of poor post-sequencing metrics (i.e. if a sample with good PreSeq score produces poor sequencing metrics, then this may be an indication of a problem in the assay run). We have included a new figure (Fig 2) that demonstrates the correlation between PreSeq QC score and the post-sequencing SS/GSP2 QC metric.

- some of the protocol steps include incubation of the strips in the thermocycler. As some of programs include low temperature and high temperature incubation steps, it not always clear whether or not to use a heated lid option or not. Maybe the authors could include their experience.
We have updated description of steps that require thermocycler incubation with whether or not a heated lid is used.

- The Archer products are not only available for use with Illumina sequencers, but also for IonTorrent Systems. That should be shortly stated and differences in the protocol should be shortly discussed.
Since the journal has prohibited use of commercial language within the text (and thus any specific mention of Illumina or IonTorrent), it is difficult to discuss these differences in the body of the

manuscript. We have, however, updated the Materials table to state that the assay being described is the Illumina-specific version but that Ion Torrent-specific kit versions are also available.

- The ArcherDx Analysis Software comes is deployed as a virtual machine. The authors could help users by explaining their Setup of the system (PC/Server, CPU, available RAM, parallel processing, Analysis time)
This information has been added to the new section 2.2.1.

- The authors could include a short table of common false-positive Fusion detected by the solid tumor Panel.
A list of common artifacts has been added to section 2.4.2.

Supplemental Table 1

Click here to access/download;Supplemental File (Figures, Permissions, etc.);Supplemental Table 1.xlsx

AKT3	EWSR1	NOTCH1	PRKCA
ALK	FGFR1	NOTCH2	PRKCB
ARHGAP26	FGFR2	NRG1	RAF1
AXL	FGFR3	NTRK1	RELA
BRAF	FGR	NTRK2	RET
BRD3	INSR	NTRK3	ROS1
BRD4	MAML2	NUMBL	RSPO2
EGFR	MAST1	NUTM1	RSPO3
ERG	MAST2	PDGFRA	TERT
ESR1	MET	PDGFRB	TFE3
ETV1	MSMB	PIK3CA	TFEB
ETV4	MUSK	PKN1	THADA
ETV5	MYB	PPARG	TMPRSS2
ETV6			

The assay includes gene-specific primers to various exons (and some introns) in the above 53 genes.