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Short Communication

Criteria for Clinical Reporting of Variants from a Broad Target Capture NGS Assay without Sanger Verification

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Abstract

Increasing clinical interest and decreasing sequencing costs are driving the wider implementation of clinical next generation sequencing assays for the diagnosis of inherited disease, including among a growing number of small to medium sized clinical laboratories. Therefore, an optimal combination of cost-effectiveness and clinical specificity is required to continue this broad adoption of genomic technology for clinical diagnosis. Sanger confirmation of all NGS variants is a common practice that increases both cost and turnaround time for clinical reporting. We reviewed 300 cases of Sanger verified NGS results as well as 60 suspected (and subsequently confirmed) artifacts, and developed a set of multiple criteria to report NGS variants without Sanger verification with 100% accuracy. Using these criteria, we project greater than 80% of clinically reported variants could be confidently released without Sanger confirmation.

ABBREVIATIONS

NGS: Next Generation Sequencing; WES: Whole Exome Sequencing; GATK: Genome Analysis Tool Kit; SNV: Single Nucleotide Variant; Indel: Insertion Deletion Variant; IGV: Integrative Genome Viewer; STR: Short Tandem Repeat; GQ: Genome Quality; VAF: Variant Allele Frequency; VCF: Variant Call File; CI: Confidence Interval

INTRODUCTION

The clinical implementation of massively parallel, high-throughput "next generation" sequencing (NGS) has enabled improved diagnostic success for patients with genetic disease [1,2]. Prior to clinical utilization of NGS, molecular confirmation of genetic disease was often performed through narrowly targeted Sanger sequencing guided by clinical phenotype and family history. Testing by this method for diseases with stereotypical phenotypes and a small number of causative genes yields reasonably high diagnostic rates due to the high pretest probability [3,4]. However, inherited diseases with subtle, non-specific phenotypes and incompletely defined genetics are not amenable to this narrowly targeted approach. For these types of cases, NGS testing has led to a reasonable improvement in the diagnostic rate [5-8]. Increasing clinical interest and rapidly decreasing sequencing costs have both driven the utilization of more broadly targeted NGS gene panels ranging up to whole exome sequencing (WES). However, large gene panels generate logistic difficulties for clinical laboratories that routinely use Sanger sequencing to fill low coverage areas and confirm all reported variants. Previously, we have reported on clinical validation of a 568 gene inherited disease NGS panel at our institution [9,10]. Here in, we describe the performance verification of an expanded 2400 gene testing menu including a number of large gene panels for congenital deafness, retinal disease, ataxia, and others. We experienced an immediate increase in test volume and total number of genes tested after the release of this assay, which prompted a quality improvement review of our need to continue Sanger confirmation of all reported variants. In order to improve turnaround time and maintain specificity, we propose

a set of criteria for reporting of sequence variants based on our NGS results without Sanger confirmation.

MATERIALS AND METHODS

Test Design, Ordering, Library Preparation and Sequencing

We perform transposase-based library creation and target enrichment using the Illumina Tru Sight One Sequencing Panel followed by NGS using the HiSeq 2500 (Illumina, Inc. San Diego, CA). This kit enriches for 62,000 exons from 4,813 genes, producing a cumulative target region size of 12 Mb. The TruSight gene list was curated by a genetic counselor (MB) to identify approximately 2,462 genes associated with Mendelian disease phenotypes which were verified for clinical testing (referred to as the 2400 gene panel, Supplemental Table 1). Clinical testing can be ordered either as pre-defined gene panels, single genes, or multiple clinician-selected genes. DNA is extracted from peripheral blood (Qiagen, Valencia, CA) and 50 ng is used for Nextera-based library preparation following manufacturer's protocols (Illumina). Nine clinical samples and 1 HapMap control are pooled and run across two lanes on a HiSeq 2500 for an average of 5 samples per lane, which produces approximately 25-30 million paired end reads per sample. For targeted clinical panels (<50 genes), areas with <15X coverage are routinely Sanger sequenced to achieve adequate analytic sensitivity.

Data Processing, Variant Analysis, Clinical Reporting, and Statistical Analysis

A full description of our bioinformatics pipeline has been previously reported [9,10]. Briefly, data is processed on a cloud-based Galaxy instance utilizing Genome Analysis Tool Kit's (GATK) (version 1.6) best practices pipeline [11,12] and mapping to the human reference genome (hg 19 version) using BWA [13]. The Unified Genotyper was used for SNV and indel discovery and genotyping. SNV recalibration and indel filtration were both done following guidelines from GATK's best practices recommendations (http://www.broadinstitute.org/gatk/guide/topic?name=best-practices). Following these recommendations, the variant recalibrator determination is based on a variety of GATK metrics that can signal inaccurate calls, such as Fisher Strand, homopolymer run, MQ Rank Sum, quality by depth and Read Pos Rank Sum [11,12,14]. The Fisher strand metric measures the presence of sequencing strand bias, i.e. whether reads supporting the variant allele are predominantly only in either the forward or reverse direction, which can signal a false positive. The homopolymer run metric reports, if present, the length of a homopolymer run of the variant allele at the site; homopolymer runs are difficult to sequence accurately and can signal false positive calls. MQ Rank Sum (mapping quality rank sum test) is an assessment of the mapping qualities of reads with the reference allele vs. those with the alternate allele, since a large difference in the mapping qualities between the two alleles may identify an incorrect call of the allele with lower quality. Quality by depth is the variant quality score normalized by depth; since variant call quality increases with increasing numbers of reads supporting the variant allele, this normalization accounts for the default – but perhaps incorrect – higher confidence in variant call quality awarded to variants with deeper coverage. Read Pos Rank Sum (read position rank sum test) is an assessment of the difference in the distribution of the position of the reference and alternate alleles within the reads supporting each. An allele found only at the end of reads is more likely to be a false positive. Please see the GATK documentation for details on the statistical calculations underlying each of these metrics (https://www.broadinstitute.org/gatk/gatkdocs/index).

The variant call file (VCF) produced by the analysis pipeline contains all variants detected regardless of whether a specific variant was flagged by one of the quality filters or not. A Hap Map sample (NA12877) is processed with every clinical run for longitudinal quality control. Concordance comparisons are made against established consensus calls (publically available) as well as between a rotating set of 50 genes compared between consecutive runs of the control. For clinical samples, all potentially pathogenic variant calls are reviewed manually via assessment of quality scores and visual inspection of the data in the Integrative Genome Viewer (IGV) (Broad Institute, Boston, MA) and then confirmed by Sanger sequencing prior to clinical reporting. Analytic sensitivity and specificity were calculated as described [15] and confidence intervals calculated using the Confidence Interval Calculator (Accessed on 1/2/2015) [16].

RESULTS AND DISCUSSION

Cross-platform Verification of the 2400 Gene Assay

We have previously described the methodology and validation of a NGS assay covering 568 genes using 18 samples orthogonally characterized by Sanger sequencing [10]. This 568 gene assay was performed with a custom designed Sure Select (Agilent Technologies Inc., Santa Clara, CA) hybrid capture library preparation and sequenced on an Illumina Hi Seq 2000. To implement the larger 2400 gene panel, we utilized a different target capture/library preparation kit (Tru Sight One) with sequencing on a different instrument (Hi Seq 2500). The bioinformatics analysis pipeline remained the same for both assays, with the exception that sequencing data was mapped to the full human genome rather than using the reduced genome constructed for the 568 gene assay. In order to verify equivalent performance of the new capture procedure and the new sequencing instrument, experiments were carried out with 12 of the samples previously used for the 568 gene assay validation.

Generation of coverage metrics for the 2400 gene panel was performed to ensure adequate baseline performance of the assay. A minimum of 20X coverage was previously established for clinical reporting with a high degree of sensitivity and specificity. In the 2400 gene panel, 94.6%of targeted exons (across the entire Tru Sight One capture) achieved at least 20X coverage in all samples. The remaining exons either captured poorly in the assay, demonstrated incomplete coverage of extreme 5' and 3' ends, or did not have baits in the design. Exons without baits or that routinely drop below 15X coverage were identified for reflex Sanger assessment as part of the standard workflow based on the clinically ordered gene set. Exons with coverage between 15-20X are noted in the report to have slightly reduced sensitivity for detection of heterozygous variants (98.8-99.9%).

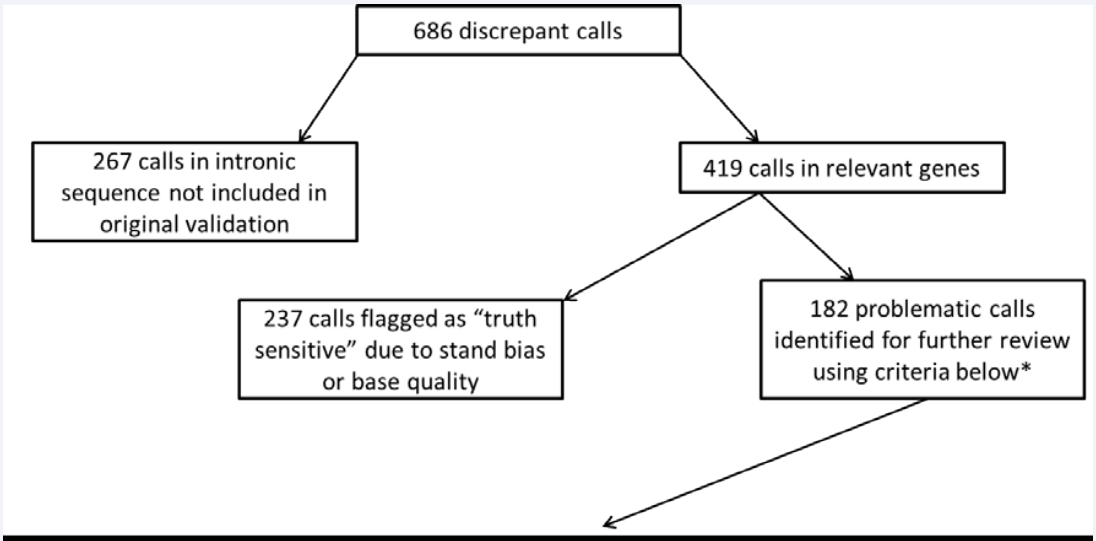
Twelve samples previously characterized on the 568 gene assay were processed on the 2400 gene assay. The VCF files

were compared across platforms, limited to only the 568 genes present on the initial clinical assay. A variant call was considered concordant if it was present in both VCF files and received a "PASS" from the variant recalibrator. Based on this definition, the average raw concordance between the 24 paired replicates was 92.1% (range 89.0%-93.8%) with an average of 697 calls per sample. Across the 24 replicates, there were 686 discrepant calls (out of 16,716 total calls), 419 of which were within exonic regions of the relevant genes (Figure 1). Of these, 237 calls were discrepant only due to a truth-sensitive label in one replicate due to either strand bias or base quality. The remaining 182 calls were manually reviewed in IGV to determine the underlying cause of the discrepancy. Known causes of artifactual calls comprised 97% of these 182 discrepancies, including known highly homologous sequence (37%), improperly sized trinucleotide repeats (13%), mismappedindels (22%), and high GC content regions (25%). Of the six "unexplained" discrepancies that did not clearly fit into one of these categories, two were likely due to artifactual G/C variant calls in areas of repetitive G/C content, two were due to skewed ref/alt ratios (0.87/0.13 and 0.75/0.25) in regions with no discernable homology by BLAT search, one was due to a skewed ref/altratio (0.87/0.13) at a location with a possible homology/pseudogene region by BLAT search, and one was due to a mapping quality issue that prevented the call from being made on the 568 gene assay. Therefore, the final cross-assay reproducibility between the 568 and 2400 gene assays (corrected for known artifacts) was 99.1 %.

In addition, 3 of the 12 samples were run in duplicate on two separate runs of the 2400 gene assay to analyze intra-run and inter-run reproducibility. Comparison of intra-run and inter-run replicates on the 2400 gene assay demonstrated high (>99%) analytic precision and similar distribution of causality for the discrepant calls (data not shown). To address analytic sensitivity of the 2400 gene assay, 15 known mutations previously verified by Sanger sequencing (including 6 SNVs and 9indels) were successfully detected across the twelve samples. To address analytic specificity, eleven exons (encompassing approximately 4kb) that were negative for variation by Sanger xsequencing were confirmed to be negative for variation by NGS. Therefore, in a limited sample size, the 2400 gene assay demonstrated 100% analytic sensitivity (95% CI= 0.80-1.0) and 100% specificity (95% CI=0.74-1.0). In addition, a HapMap control sample with publically available whole genome sequence data is included on all clinical sequencing runs for comparison with externally available data and for comparisons across different analytical runs as a part of ongoing quality assurance to monitor assay performance [15].

Clinical Implementation and Review of Sanger Confirmation for Reported Variants

After the clinical launch of the 2400 gene assay, average test volume per month increased 133% and the average size of gene panels ordered also increased, leading to a seven-fold expansion of the number of genes tested per month (Figure 2A,2B). These factors created logistical challenges in our standard practice of verifying all potentially pathogenic variants identified using NGS with confirmatory Sanger sequencing. Therefore, we evaluated



Pseudogene/ Homologous Sequence	Trinucleotide repeat sequences	Mismapped indels	GC rich sequence	Tri- or tetra-allelic SNPs	Unexplained
68	23	40	45	0	6

Figure 1 Analysis of discrepancies between the 568 gene panel and the 2400 gene panel assays. There were 686 total discrepant calls among 24 duplicates run (12 unique samples run once on each assay). Calls outside of reportable regions (target exons plus intronic pad) were ignored (n=267). 237 calls were flagged as Truth Sensitive in one replicate due to strand bias or base quality, but were otherwise reproducible. 182 discrepant calls were flagged for further review based on the following 4 criteria (*): 1) heterozygous ref/alt ratio greater than 70/30 or less than 30/70, 2) insertion/deletion variant, 3) variant completely absent in one duplicate, or 4) variant filtered in one duplicate due to mapping quality (indicative of a potential pseudogene/homology region). These 182 discrepant calls were categorized by likely causative issue as indicated.

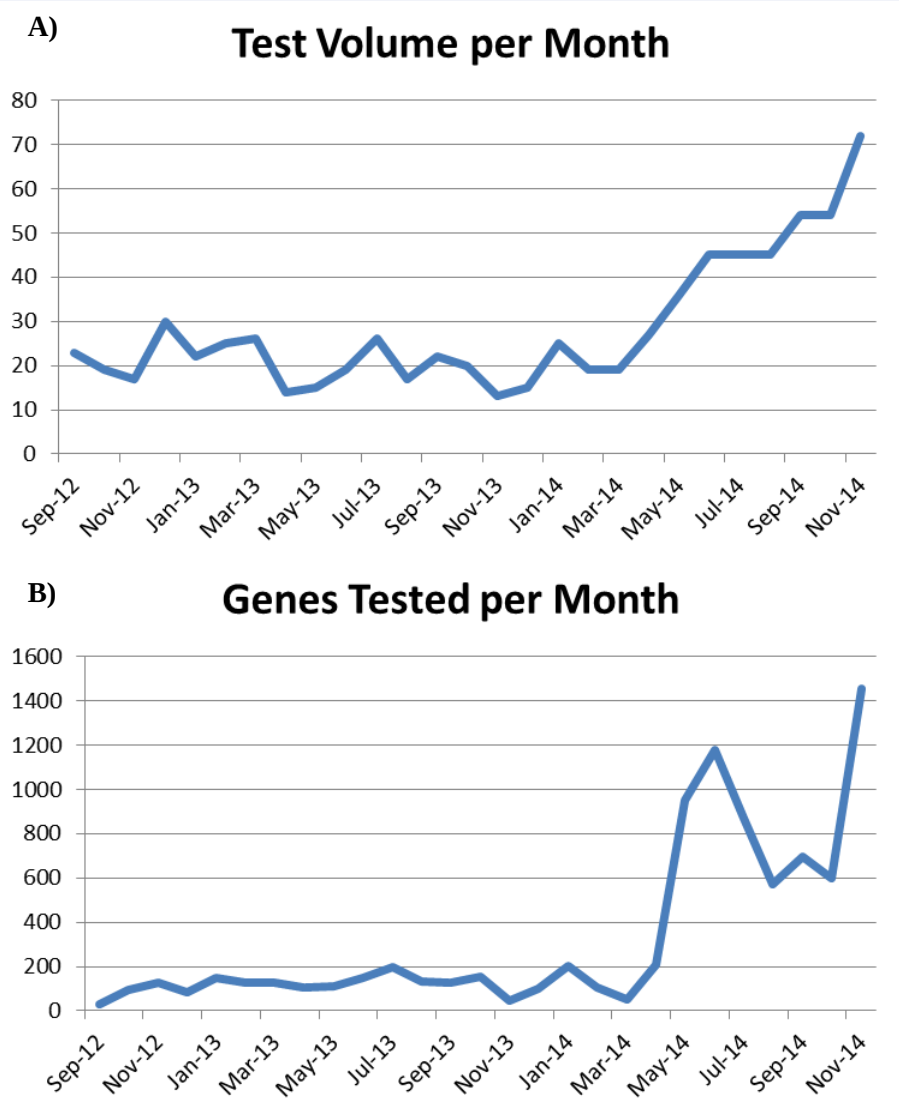


Figure 2 Test and gene volume per month before and after release of Phase 2 (2400 gene) assay. Clinical release of the phase 2 assay occurred in April 2014. 2A) Number of NGS test requisitions received per month. 2B) Total number of genes processed per month.

whether high quality NGS calls could accurately be reported without Sanger confirmation.

We reviewed 300clinically reported variants with Sanger verification data from cases processed on both platforms (200 on the 568 gene panel and 100 on the 2400 gene panel). Four cases were excluded from further analysis for the following reasons: one variant was detected in a low-coverage (4X) exon by supplemental Sanger (and had not been called in the NGS data); one variant showed an unbalanced allele fraction in one strand and complete failure of the other strand in Sanger sequencing (despite multiple primer designs); and two variants were discrepant due to one instance of sample mix-up and one instance of data file mix-up. Of the remaining 296 cases, 294 (99.3%, 95% CI = 0.976-0.998) were concordant between NGS and Sanger results. Two cases with indels were identified by NGS but imprecisely mapped, while 39 indels were precisely concordant between Sanger and NGS. Table 1 demonstrates the distribution of variant types within the 296 cases, which included

Table 1: Distribution of variant types among the 296 Sanger verified, clinically reported NGS variants reviewed. SNV: single nucleotide variant. The "splice" category refers to variants affecting the conserved donor and splice acceptor sequences. The "non-coding" category refers to all other intronic or 5' UTR variants reported, including variants implicated in splicing that lie outside of the highly conserved acceptor/donor sites.

Confirmed Variants	n = 296 (%)
Nonsynonymous SNV	179 (60%)
Stopgain SNV	29 (10%)
Synonymous SNV	8 (3%)
Frameshift deletion	26 (9%)
Nonframeshift deletion	3 (1%)
Frameshift insertion	11 (4%)
Nonframeshift insertion	1 (<1%)
Splice	18 (6%)
Non-coding	21 (7%)

149 unique genes (for full listing, see Supplemental Table 2).

The variant recalibrator passed 93.6% (n = 277) of verified cases. The GQ score (median 2878.73, range 96.25-9907.11) was > 300 in 97.9% of cases (n = 290). The variant allele frequency (VAF) fell between 0.3-0.6 (heterozygous) or >0.9 (homozygous/hemizygous) for 97.6% (n = 289) of cases. In contrast, we also reviewed 60 suspected NGS artifacts subsequently confirmed as negative by Sanger sequencing. 93.3% of these verified artifacts were called Truth Sensitive by the variant recalibrator (n = 56), 68.3% had a GQ < 300 (n = 41), and 66.7% fell outside of the VAF ranges listed above (n = 40).

Based on these results and the evaluation of common causes of discrepancy noted in the verification experiments, we propose that Sanger confirmation of NGS variant calls in areas of adequate (>20x) coverage is not required when the variant: 1) is a single nucleotide substitution, 2) receives a "PASS" from the variant recalibrator, 3) has a GQ score of >300, and 4) has a VAF in the accepted range (heterozygous = 0.3-0.6, homozygous/hemizygous >0.9). We propose continued Sanger confirmation to report variants that: 1) are insertion/deletion (due to possible mapping inconsistencies), 2) are flagged as truth sensitive by the variant recalibrator, 3) have a quality score <300 or 4) have a VAF out of the accepted range. Applying these criteria to our cohort of 296 reviewed cases, 80.6% of variants could have been reported without Sanger verification, significantly improving turnaround time and reducing the cost burden in the lab. The majority of cases requiring Sanger would have been indels (70.6%). Of interest, one of the verified SNVs flagged by these criteria (with a ref/alt ratio of 0.84/0.16) represented a case of somatic mosaicism. Finally, 100% of the reviewed NGS artifacts (n =60; 95% CI=0.94-1.0) would have been flagged by one or more of these criteria.

CONCLUSION

Based on our prior clinical validation of a modestly sized NGS assay for inherited disease, we were able to rapidly verify the performance of a larger assay with new library preparation chemistry and four times the gene content. The increased size of this expanded assay brought a rapid increase in the number of genes sequenced per month in our laboratory due to both a wider breadth of clinical offerings and larger gene panels for diseases previously covered by the original assay. Review of our Sanger confirmation procedure for all clinically significant reported results showed that high quality NGS variant calls that met a strict set of criteria had a 100% accuracy rate.

Traditional Sanger sequencing is still considered a gold standard, and the majority of clinical laboratories continue to Sanger verify all reported variants detected by NGS [4]. Several recent reports have proposed that either 30X coverage [17] or GQ > 500 [18] metrics can be utilized to confidently report NGS variants without Sanger verification. However, single metric criteria might be insufficient to ensure accuracy and it should be noted that the correlation between error rate and quality score becomes nonlinear when GQ > 60 [19]. Our data support a model in which multiple interrelated metrics can successfully be used to report the majority of NGS variants confidently without Sanger confirmation and also continue to identify cases with potentially non-typical or incorrect information such as somatic mosaicism or mis-mapped indels.

Processes that minimize potential sample mix-up are a critical component of clinical NGS assay validation [15], and another utility for Sanger confirmation of pathogenic variants is to identify these errors. Highlighting this potential, two examples of sample mix-up were identified in our review of 300 positive cases. In both cases, comparison of Sanger sequencing and NGS data had identified the error prior to report sign-out. However, relying on Sanger confirmation of positive results as the primary mechanism to prevent sample mix-up is insufficient, as it does not apply to the majority of analyzed cases which have negative results. Hence, we have instituted short tandem repeat (STR) analysis of primary extracted samples and working dilutions to ensure proper sample handling and we have also automated data transmission between the sequencing instruments, informatics platform, and the reporting laboratory to minimize the possibility of human error in these downstream processes. Furthermore, we have previously described a synthetic spike-in strategy to detect cross contamination and track specimens through the analysis pipeline with the 568 gene assay [10], and other groups have recently described different spike-in strategies to detect sample mix-up and cross-contamination [20]. Nevertheless, the first step of DNA extraction from the patient's blood (or other tissue) sample is one of the most critical steps prone to sample mix-up, and errors at this stage cannot be identified by downstream methods, including Sanger confirmation of variants. Sample tube barcoding, automated extraction platforms, and attentive laboratory technique are important factors that can help prevent errors at this first, critical step.

The results reviewed in this study were produced entirely on Illumina sequencers using hybrid capture reagents from two companies (Agilent and Illumina), and bioinformatics using BWA mapping with GATK analysis. Therefore, the precise criteria we have proposed may not be generalizable to other popular sequencing platforms. However, our analysis does outline an approach that can be applied to other platforms for validation of appropriate cut-off values within the specific criteria we have identified. Our data demonstrate that high quality NGS variant calls can be confidently reported without Sanger verification, which has the potential to improve clinician satisfaction (via improved turnaround time) and decrease the economic burden on laboratories facing growing demand for NGS testing.

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