Confirmation for clinical genetic testing

CURRENT CLINICAL APPROACH

For decades, Sanger sequencing has been accepted by clinicians as a gold-standard in genetic testing. However, its low throughput is not ideal for assessing the growing number of genes in a modern clinical gene panel. When clinical testing began to employ next-generation sequencing (NGS), a two-step approach was adopted whereby many genes were assayed by NGS sequencing and reportable variants were verified by Sanger sequencing. This continues to be the standard as set out by the ACMG guidelines: "...it is recommended that all disease-focused and/or diagnostic testing include confirmation of the final result using a companion technology."[1]

It has since been reported that Sanger sequencing may introduce more errors than it actually prevents, and may be unnecessary for high quality variants.[2,3,4] Here we examine the results of this two-step confirmatory approach as it has been implemented in our laboratory.

QUALITY IN CONFIRMATION

For reasons both of efficiency and accuracy, Invitae this year validated Pacific Biosciences (PacBio) sequencing technology for clinical use. It is an alternate confirmatory sequencing method that, in our hands, provides an accurate and higher throughput method that is orthogonal to NGS sequencing. PacBio provides a more quantitative measurement of variants and additional QC metrics compared to Sanger sequencing. We continue to use both PacBio and Sanger to confirm variants.

CONFIDENT NGS VARIANT CALLS ARE HIGHLY SPECIFIC

Using this hybrid method of NGS followed by either Sanger or PacBio to deliver tens of thousands of clinical reports, Invitae has built a large dataset of variants detected by all three technologies. This has allowed us to complete a direct comparison of NGS with the two orthogonal technologies (see table on next page). Standard orthogonal confirmation assays rely on PCR-based targeting of the variant loci, which is susceptible to allele dropout and amplification failure. To guard against a false negative result due to these types of failures, we run multiple overlapping assays to redundantly target each variant.

In a cohort of approximately 70,000 individuals who have undergone genetic testing at Invitae, we identified nearly 6,800 high quality variant calls that because of our reporting policies required orthogonal confirmation. Our data show that among 6,788 variants (57% SNVs and 43% indels) detected with high confidence according to our QC parameters, 6,752 (99.5%) were confirmed by Sanger or PacBio sequencing. We have **never** observed a discordant result during the orthogonal confirmation process of high confidence variant calls. For the remaining 36 (0.5%) high confidence variants detected by NGS, the orthogonal assay generated no useable data after several attempts and the variant was reported with a corresponding limitation regarding the failure to confirm according to ACMG guidelines. These events tend to be in regions known to be difficult for these assays, such as areas of high GC content or within repetitive sequences.

[1] Rehm *et al*. ACMG clinical laboratory standards for next-generation sequencing. *Genet. Med*. 2013
[2] Beck *et al*. Systematic evaluation of Sanger validation of next-generation sequencing variants. *Clin. Chem*. 2016
[3] Baudhuin *et al*. Confirming variants in next-generation sequencing panel testing by Sanger sequencing. *JMD* 2015
[4] Mu *et al*. Sanger confirmation is required to achieve optimal sensitivity and specificity in next-generation sequencing panel testing. *JMD* 2016

Table: Next-generation sequencing and orthogonal methods show perfect concordance for high quality variant calls

Confirmation result	Technology	Number of events	Total
True positive	Sanger	4964	6752
	PacBio	1788	
False positive	Sanger	0	0
	PacBio	0	
Orthogonal assay failed	Sanger	26	36
	PacBio	10	

FALSE POSITIVE RATE AND SENSITIVITY

In order to maintain high sensitivity, a wide net must be cast when initially calling variants. Our validated, custom computational pipeline identifies variants and assesses their quality based on a large number of sequencing attributes (including strand bias, read depth, Phred-scaled p-value, and many others). These quality attributes are used to separate variants into those that are high-quality and others that may be false positives and are "flagged" for further evaluation and mandatory confirmatory testing.

In the same testing period in which the 6,788 high quality variants were found, 508 low-quality, flagged variants were also detected and, among these, 411 (81%) confirmed as true positives and 97 (19%) were found to be false positives by an orthogonal method. Thus, out of 7,296 total variants, 1.3% (97 of 7,296) were NGS false positives that were correctly identified during confirmatory testing.

IN CONCLUSION

Our data show that confirmatory sequencing has not improved the accuracy of the final report for high quality NGS variant calls. It does however continue to be important when the quality metrics for the variant do not exceed empirically determined thresholds set in our lab.

N.B.: Although it is outside the scope of this white paper, note that Invitae confirms any reported CNV event and has performed more than 1,000 such confirmations.

Next-generation sequencing, as implemented at Invitae, is a high-quality, clinical grade technology. Our team understands that the stakes for clinical genetic testing are high. Results can lead to irreversible action and emotional distress for both the patient and their family. We are committed to maintaining the highest quality, while continually improving our processes in a responsible and data driven manner.

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