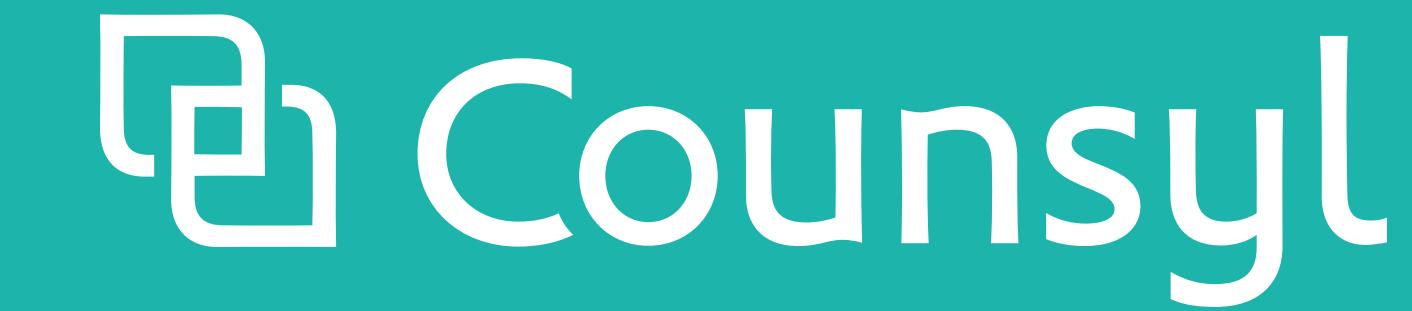


# Expert review of NGS results removes need for routine Sanger sequencing confirmation

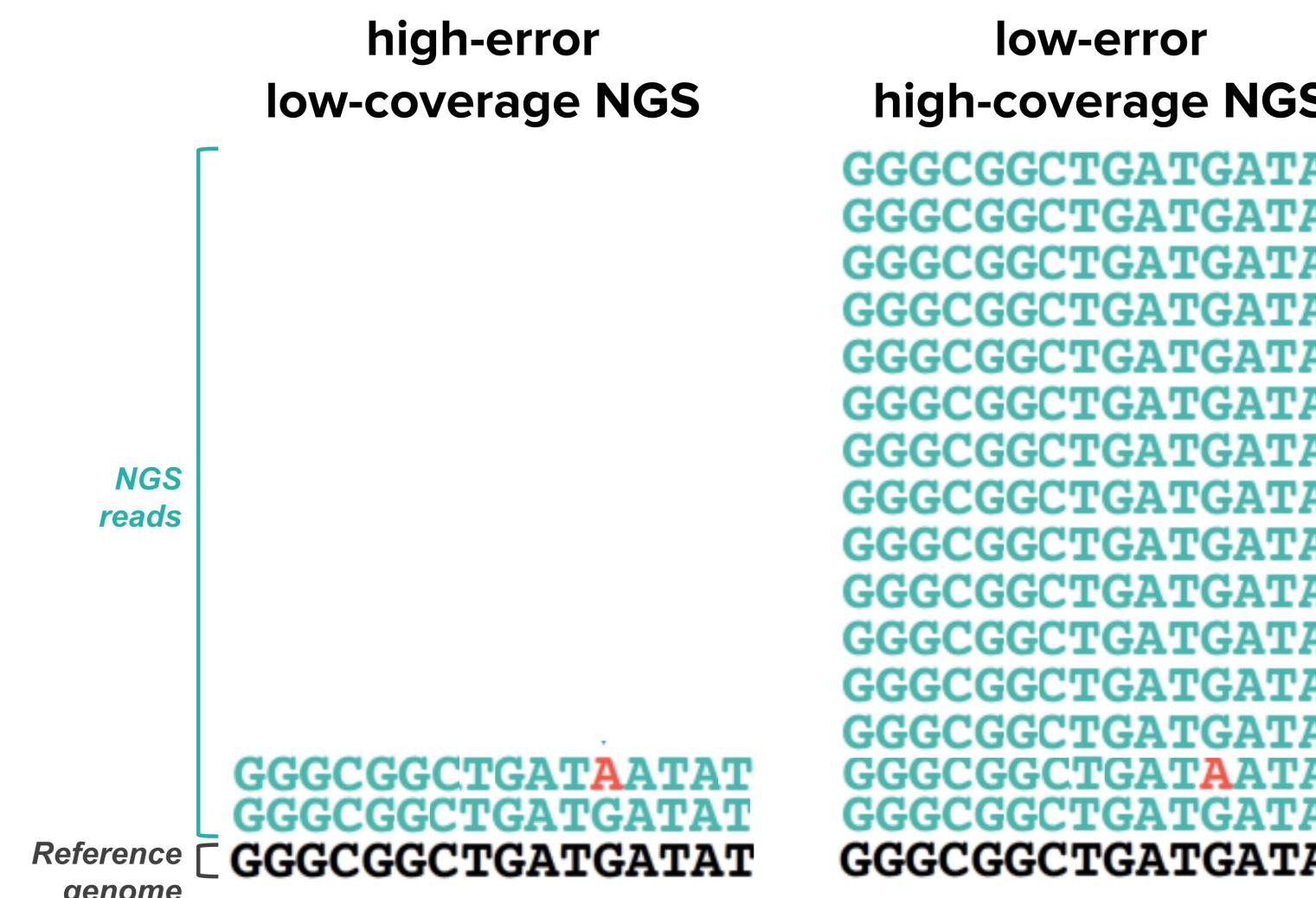


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## Introduction and background

Variant calling with next-generation sequencing (NGS) incurs rare but systematic mistakes (**Figure 1**). In hereditary cancer testing, such mistakes have enormous consequences as they could lead to incorrect screening or surgical management decisions. It has been suggested that routine orthogonal confirmation with Sanger sequencing is required to verify NGS results, especially for sites where the allele balance is between 10% and 30% (Mu et al., 2016). We sought to demonstrate that manual review of NGS data can also identify and eliminate false calls otherwise permitted by automated call-quality thresholds.



**Figure 1: Origins of argument for routine orthogonal confirmation.** The relatively high error and expense associated with the early days of NGS meant that a clinical test with low coverage could not disambiguate a SNP from an error (left). As sequencers and targeting technologies matured, however, the depth and accuracy per base rose, enabling conspicuous distinction of a SNP from a sequencer error (right).

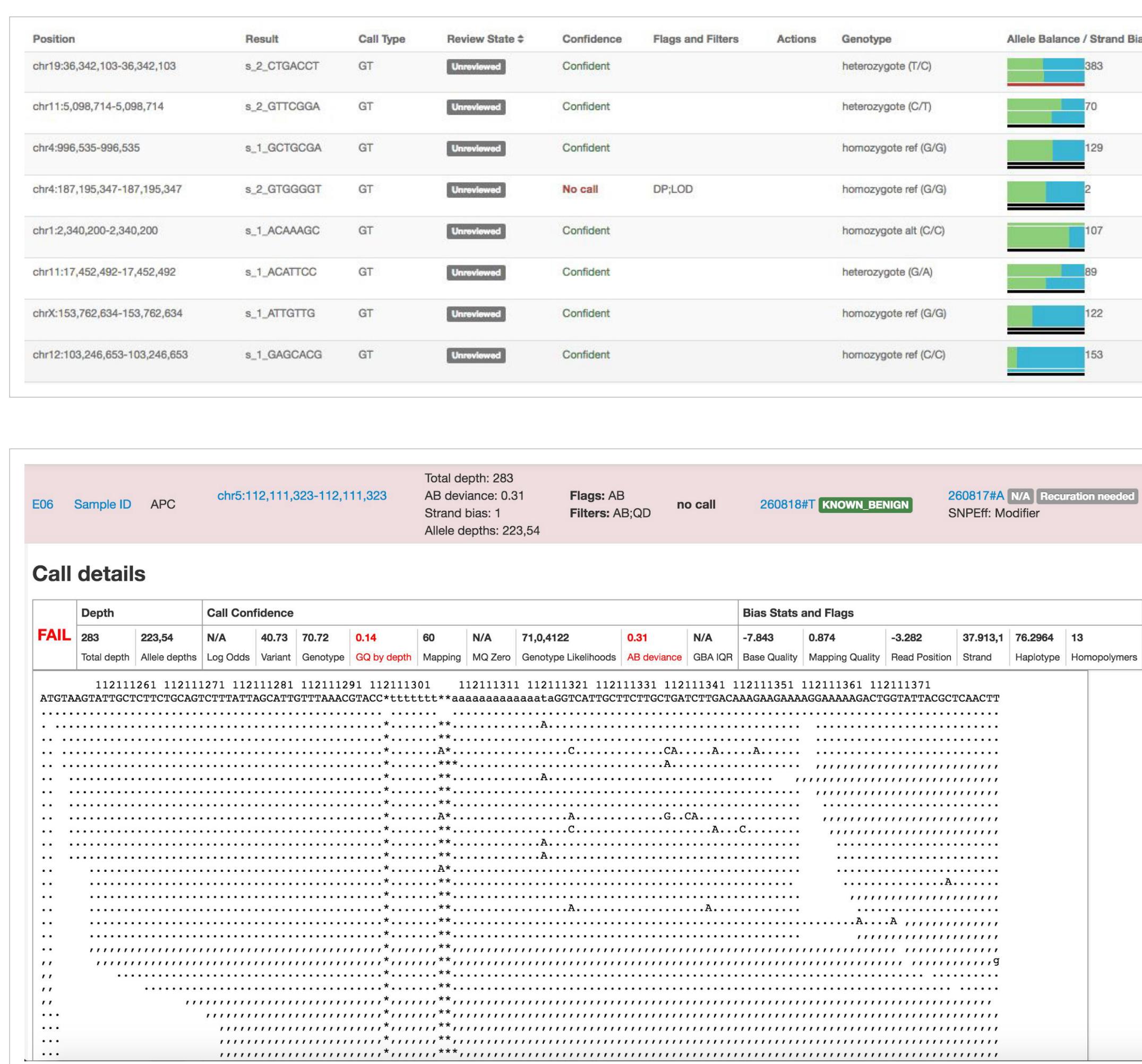
## Materials and methods

### Call Review

For over 20,000 patient samples tested with the Counsyl Reliant™ Cancer Screen, we evaluated our custom variant-review software, which enables licensed reviewers to perform manual inspection of detailed quality-control criteria for batch-, sample-, and variant-level QC metrics, including evaluation of NGS read pileups (**Figures 2–6**).

### Sanger Sequencing

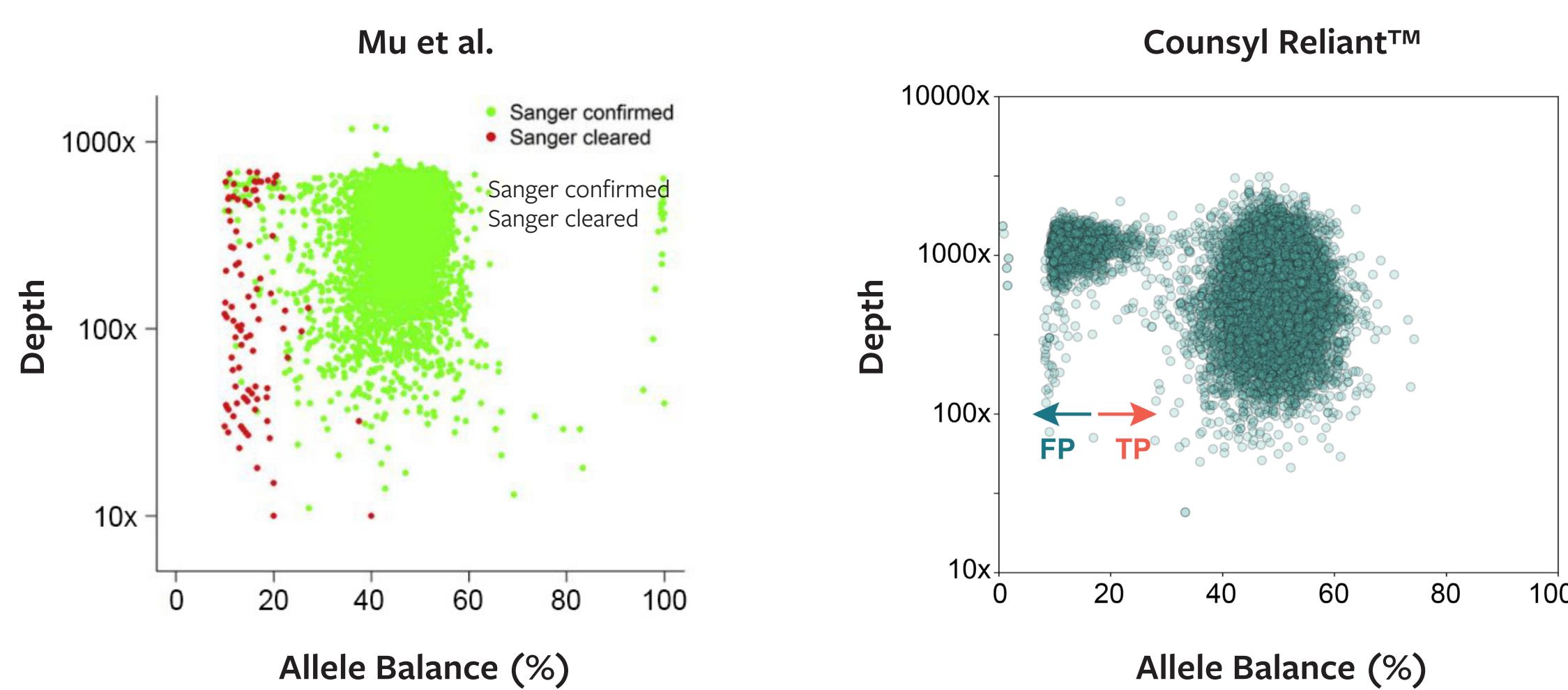
Samples with putative heterozygous variant calls with allele balances below 30% were Sanger sequenced, and the respective outcomes of Sanger sequencing and manual NGS review were compared (**Figure 7**).



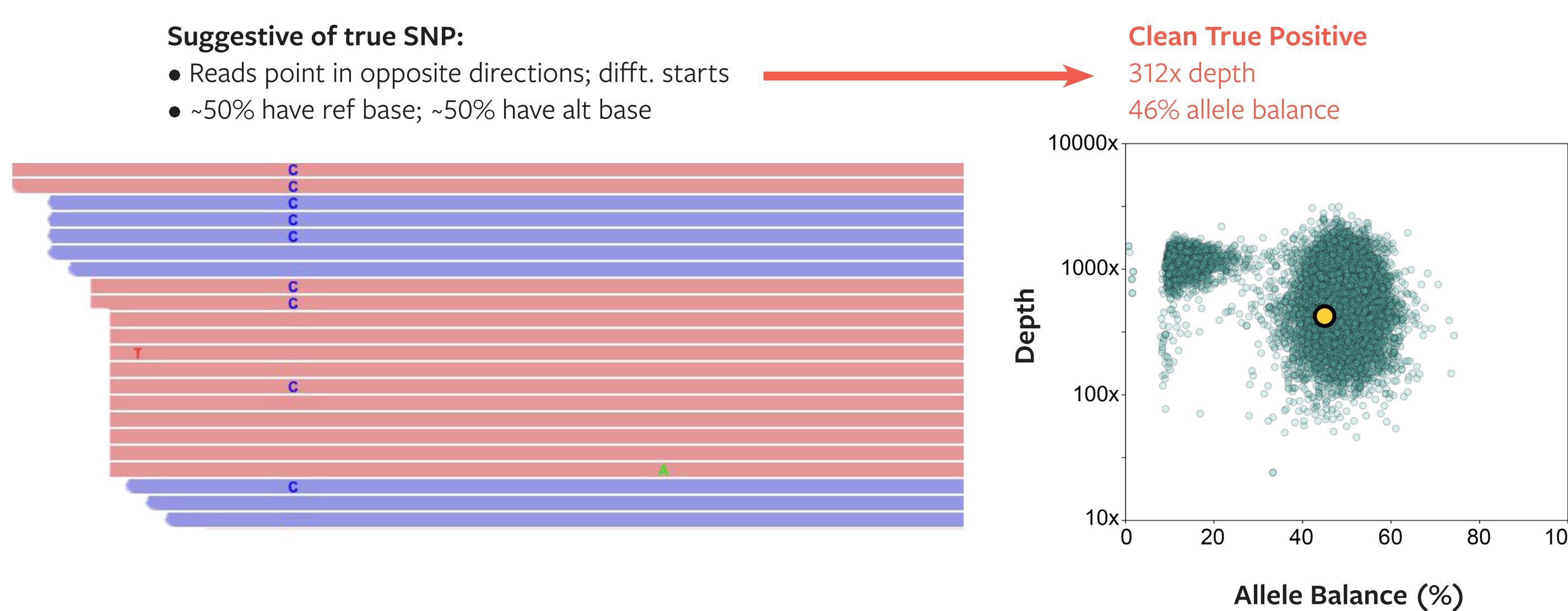
**Figure 2: Manual review interface.** Every potentially reportable variant (e.g., pathogenic variants and VUSs that have high or low confidence) undergoes software-assisted manual review. The interface presents cascading levels of complexity to the reviewer, with high level metrics in the first view (top). Clicking a particular variant's row in the interface opens a second panel (bottom) presenting detailed metrics, as well as the NGS data itself plus a link to a genome browser (IGV).

## Results

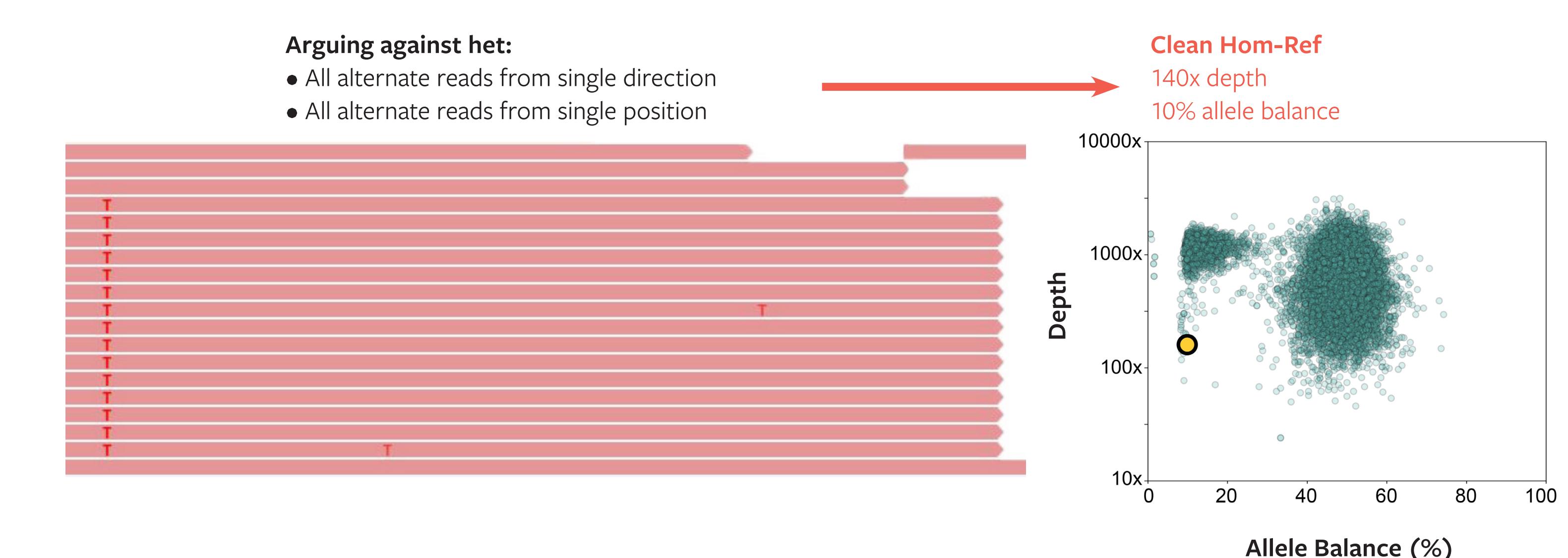
There were 3,937 putative heterozygous sites where at least one patient had a pathogenic variant or variant of uncertain significance (**Figure 3**), and calls at all such sites underwent manual review, which focused on the position, strand, and capture origin of the reads in the NGS pileup. The large majority of sites (98.4%) had multiple pieces of evidence supporting a heterozygous call (**Figure 4**). Forty-seven (1.6%) sites had samples with variant calls at <30% allele balance (2003 calls in total) and call review revealed the source of the unexpected allele balance, allowing >99% of calls (1991) at these sites to be classified as true negatives (often conspicuous NGS artifacts; **Figure 5**) or true positives (enriched for long indels and homopolymers; **Figure 6**). The remaining <1% of calls (12) appear to be mosaic. Sanger sequencing on all of the low allele-balance sites verified that expert manual review correctly classified all such variants as positive, negative, or mosaic (**Figure 7**).



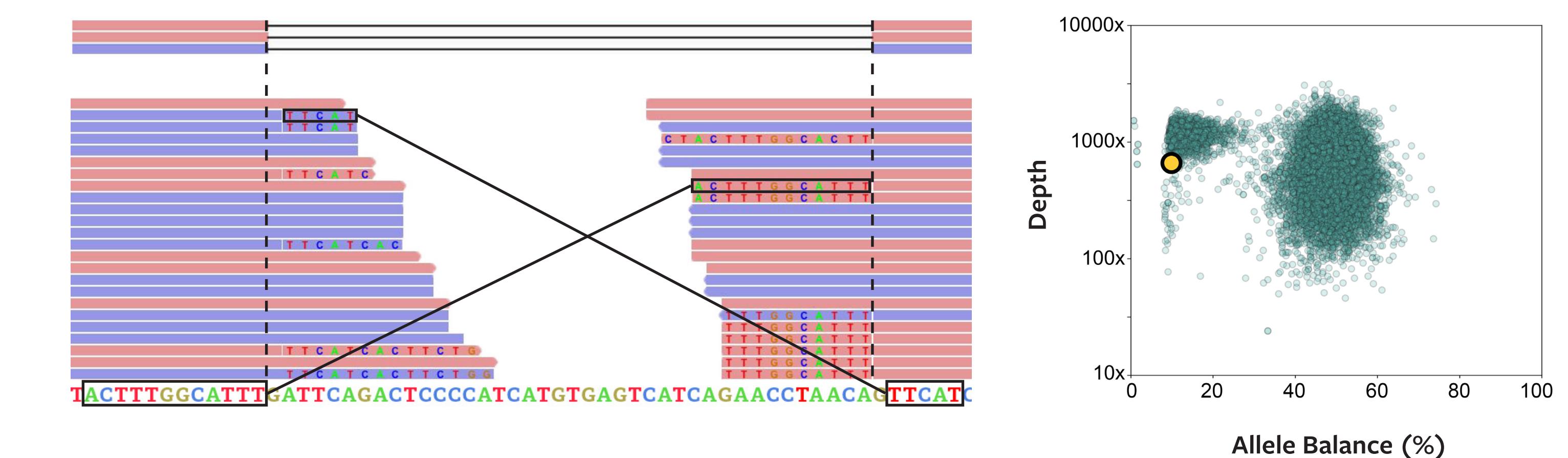
**Figure 3: Distribution of sites as function of depth and allele balance.** Mu et al. (left) suggest that putative heterozygous sites with depressed allele balance (i.e., <30%) should undergo routine confirmation with Sanger sequencing, as depth and allele balance alone cannot resolve the genotype. A similar plot from Counsyl's Reliant™ Cancer Screen also reveals two populations, one centered near 50% allele balance that contains clear heterozygous calls (note that all such calls still undergo manual review), and another at depressed allele balance (right). The low allele-balance calls emitted by the algorithm—and subsequently addressed via call review—are either false positives with enriched allele balance or true positives with depressed allele balance.



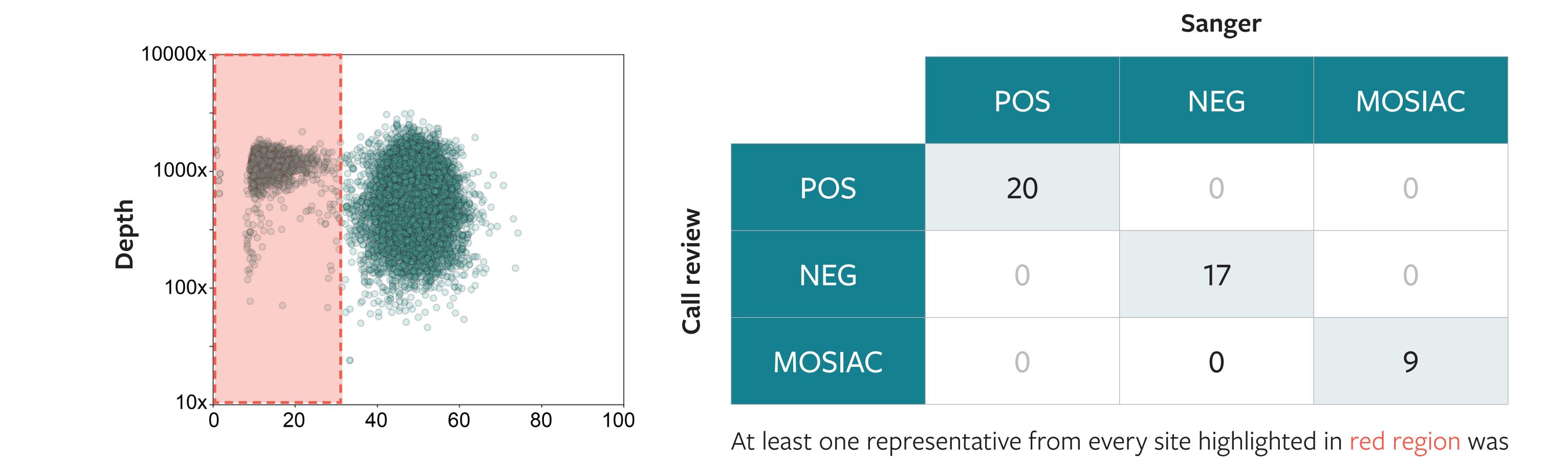
**Figure 4: NGS pileup snippet for confident heterozygous site.** With the technology used in the Counsyl Reliant™ Cancer Screen, a true-positive heterozygous site will (1) have allele balance near 50%, (2) have reads from both strands, (3) have a diverse set of read-start positions, and (4) originate from different probes (probe origin not shown above).



**Figure 5: NGS false positive detected in call review.** Manual inspection of NGS pileups for some sites with unexpected allele balance (10% in this case, which issued a low-confidence "no-call" from the analysis software to signal reviewer attention) revealed that all reads harboring the alternate base originated from a single strand and a single start position. These data are consistent with a PCR artifact and strongly argue against the site being heterozygous.



**Figure 6: Heterozygous large indel with depressed allele balance.** For a large putative 40nt deletion that has too few "alt" reads to yield a confident heterozygous call, some of the "alt" reads that harbor the deletion are miscounted as "ref" reads. These misaligned reads have insufficient sequence spanning one of the flanking regions for the aligner to register them as "alt". If such sites were removed from the "ref" count and populated the "alt" count, the allele balance would be more compellingly heterozygous.

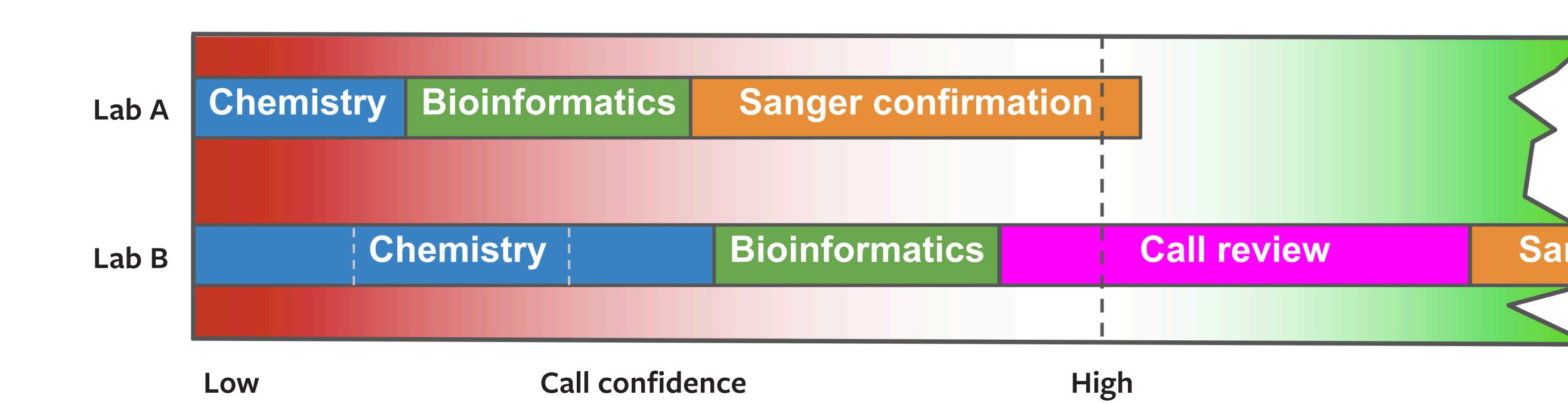


At least one representative from every site highlighted in red region was tested via Sanger.

**Figure 7: Manual call review on Counsyl Reliant™ Cancer Screen performs the same level of confirmation as Sanger sequencing.** Sanger sequencing was retrospectively performed on at least one representative sample for all sites identified to have allele balance <30% (left; red-shaded region). The retrospective analysis confirmed all genotypes identified via a combination of NGS and manual call review (right).

## Conclusions

Clinical NGS variant calling requires great care to avoid false reporting of systematic artifacts, yet this care can manifest in different ways (**Figure 8**). If a lab chooses to forgo careful review of NGS data, routine orthogonal methods such as Sanger sequencing should indeed be used. Alternatively, we have demonstrated that a highly trained NGS expert operating within an optimized and platform-specific software review interface achieves comparable quality to routine Sanger confirmation.



**Figure 8: Labs differ in the ways that they achieve confidence in variant calling, and Sanger sequencing need not be part of the routine workflow.** The primary goal of any lab performing genetic testing should be the achievement of high confidence in genotype calls, but the methods employed to achieve confidence may differ. Though most clinical labs use Illumina sequencers, their library-preparation and capture methods differ ("chemistry"). Some capture methods (e.g., in Lab B) provide more information about variant-call fidelity by giving strand- and capture-probe-specific information. Chemistry plus bioinformatics alone may be insufficient to achieve high confidence, as shown herein for sites with ambiguous allele balance. In such cases, a lab may choose to perform routine Sanger confirmation (as argued in Mu et al.). However, we have shown that in >20,000 samples, manual call review provided adequate confidence, as confirmed retrospectively with Sanger. We note that in future cases where call review fails to yield sufficient confidence, Sanger sequencing may be performed, but this would no longer be considered routine use.

REFERENCES 1. Mu W, Lu HM, Chen J, Li S, Elliott AM. Sanger Confirmation Is Required to Achieve Optimal Sensitivity and Specificity in Next-Generation Sequencing Panel Testing. *J. Mol. Diagn.* Nov 2016; (18:6) 923-932.