

Ensuring high accuracy of clinically relevant results from NGS data using high-throughput, custom software that enables expert human review



South San Francisco, California

Kevin R. Haas PhD, Dale Muzey PhD, Greg Hogan PhD, Shera Kash PhD, Jillian Johnson MS, Thi Tran BS, Eric Olson BS, Aaron Packer PhD, Peter Krenesky BS, Sanjay Siddhanti MS, Imran S. Haque PhD, H. Peter Kang MD

Introduction

Variant calling with next-generation sequencing (NGS)—now used extensively in clinical genetic testing—incurs rare but systematic mistakes, particularly in regions of low sequence complexity or regions with homology within the genome. Because application of one-dimensional quality control (QC) metrics is not sufficient to filter out all false results, it has been suggested that orthogonal confirmation is required to verify NGS results. However, manual review of NGS data can also identify and correct false calls otherwise permitted by automated call quality thresholds.

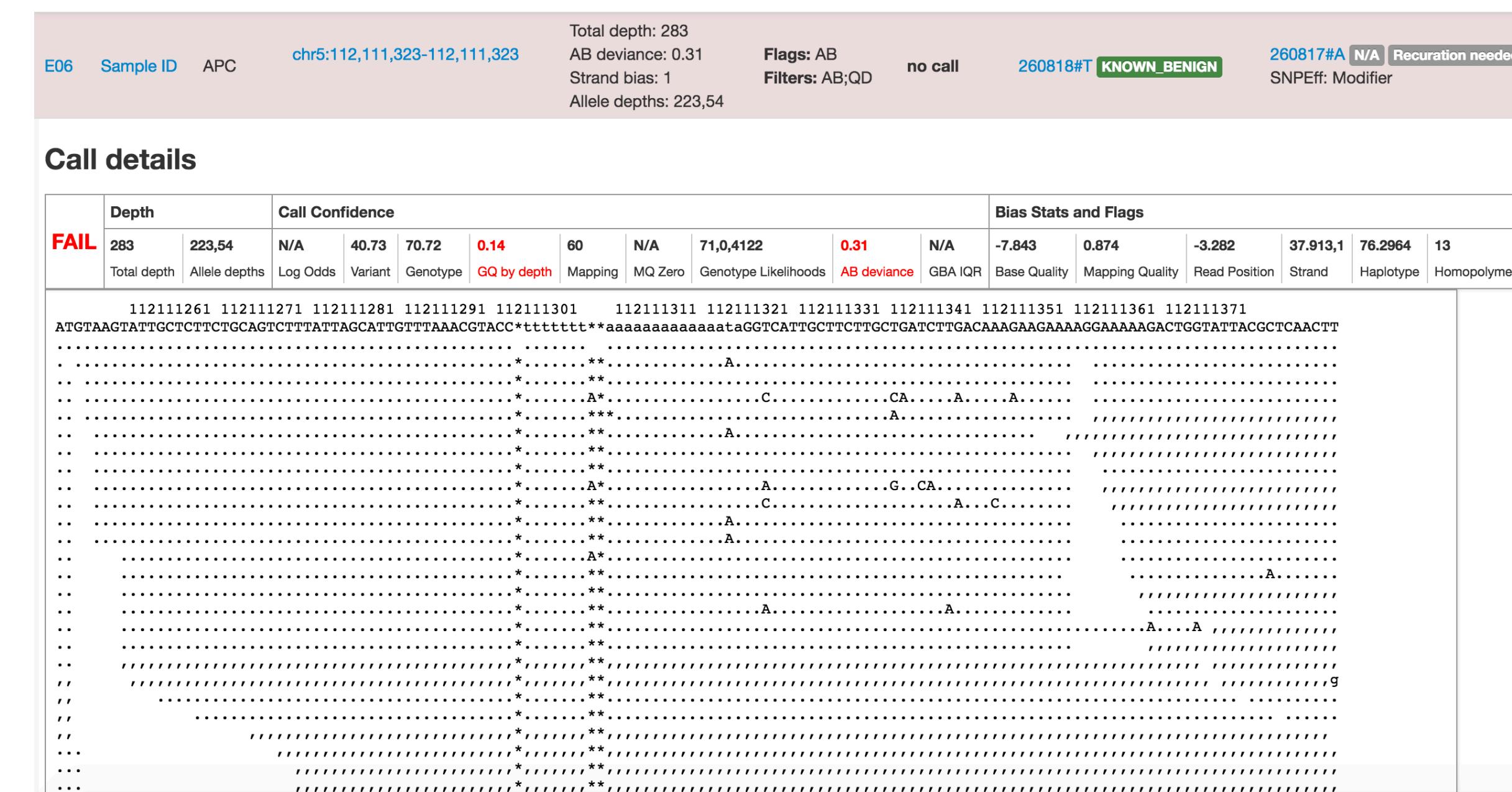
Comprehensive software system for detailed call review

We developed a custom software interface that enables CGMBS-licensed and expertly trained reviewers to perform manual review of detailed quality-control criteria (Fig. 1, 2). In addition to assessing sample- and batch-level holistic QC metrics, the reviewers are guided through the interface to perform human review of each called variant and its associated call metrics that may be implicated in reporting. Reviewers assess both standard and custom QC metrics, along with the sequencing read pileups to detect technical anomalies possible with NGS.

Figure 1 (A)

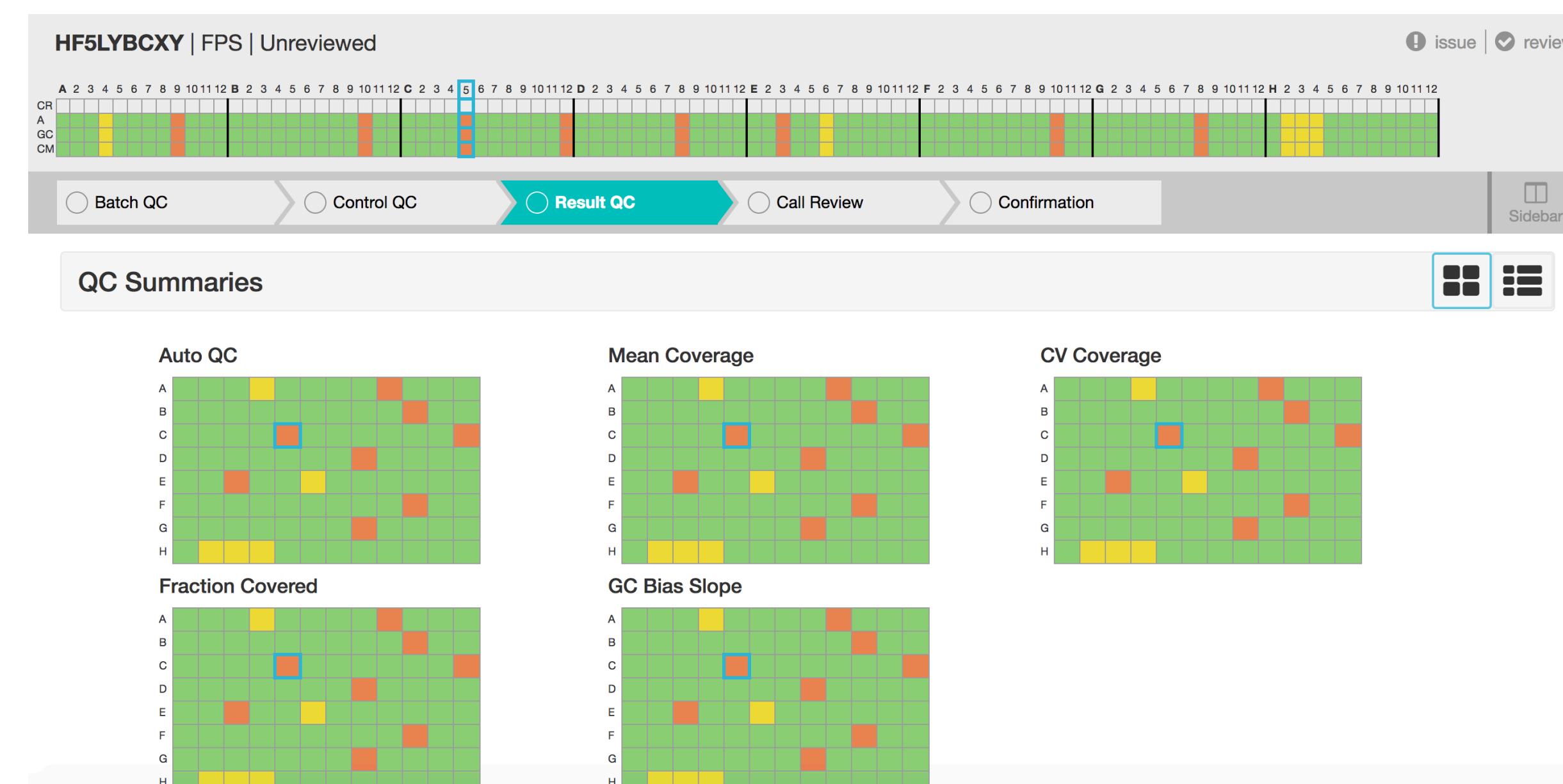
Position	Result	Call Type	Review State	Confidence	Flags and Filters	Actions	Genotype	Allele Balance / Strand Bias	CN
chr1:43,054,553-43,054,553	s_1_CGACCGG	GT	Unreviewed	No call	DP<50		homozygote alt (C/C)		>
chr1:45,709,656-45,709,656	s_1_GCCAACC	GT	Unreviewed	Confident			homozygote ref (C/C)		>
chr4:123,665,428-123,665,428	s_2_CGGCATC	GT	Unreviewed	Confident			heterozygote (G/A)		>
chr5:34,532,860-34,532,861	s_2_CGGCATC	GT	Unreviewed	Confident			homozygote ref (AC/AC)		>
chr4:100,495,488-100,495,488	s_1_CAGAGTT	GT	Unreviewed	Confident			heterozygote (G/T)		>
chr7:117,176,588-117,176,572	s_2_CGGCATC	GT	Unreviewed	Confident			heterozygote (AGATT/A)		>

Figure 1 (B)



Custom interface provides easy access to relevant data, ranging from distilled metrics (A, colored boxes at right) to the raw sequencing data itself (B). Every instance of high- or low-confidence deviation from reference—e.g., SNP/indel/CNV “calls” or “no-calls”—is represented as a distinct, expandable row in the call-review interface (A). Among several confidently called sites is a site with potentially insufficient depth. The questionable site is flagged in red to indicate that it requires closer scrutiny of more data, which can be performed by expanding the call’s row in the interface (B) to reveal far more data, including representation of raw sequencing reads.

Figure 2

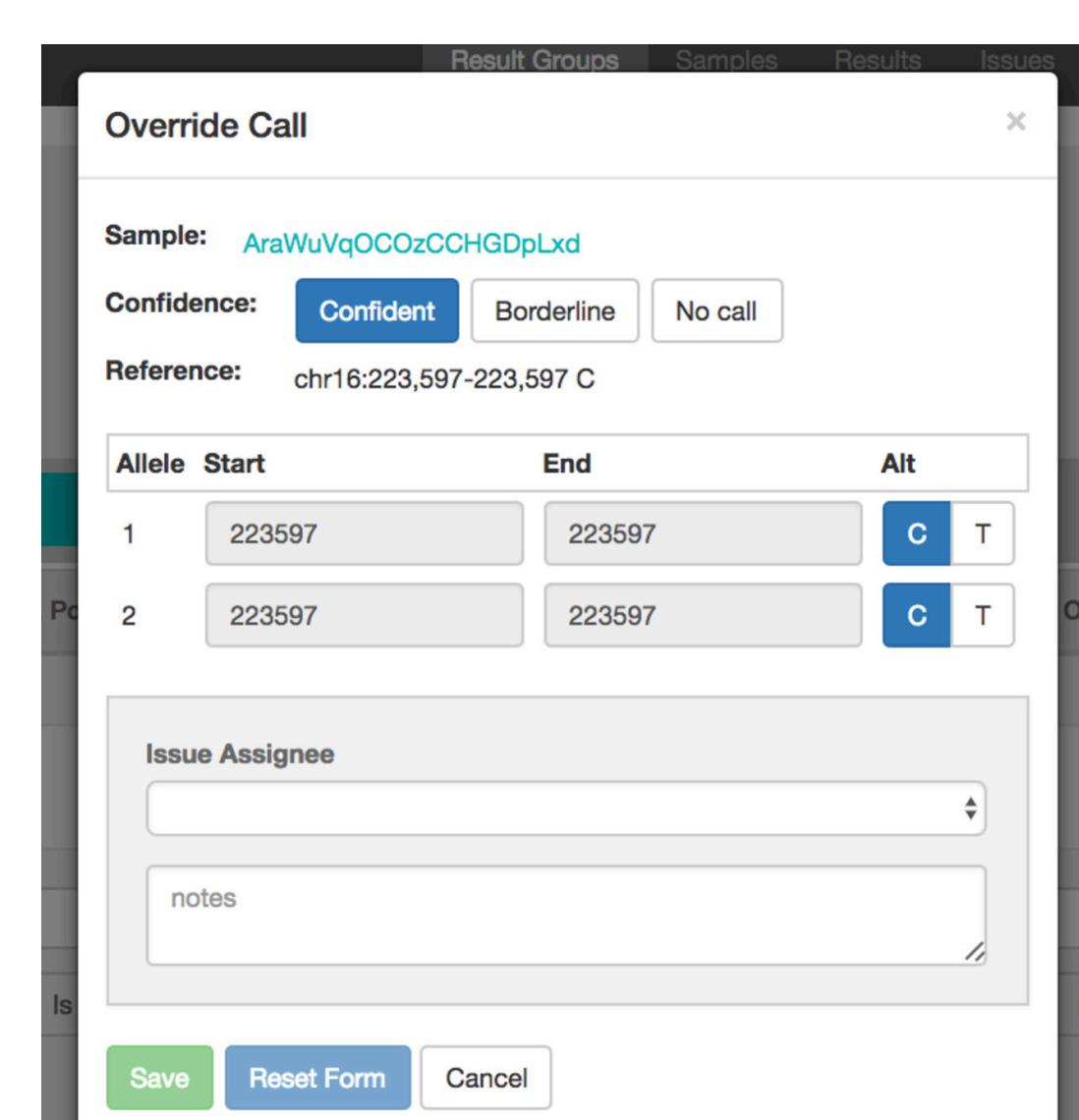


Manual overrides with secondary approval

If required, reviewers are capable of overriding the calls within the results system to match the findings of manual review. Only in the rare cases in which a call cannot be adjudicated by manual review is it necessary to perform testing using an orthogonal method, such as Sanger sequencing, multiplex ligation-dependent probe amplification (MLPA), or array comparative genomic hybridization (array CGH).

Figure 3

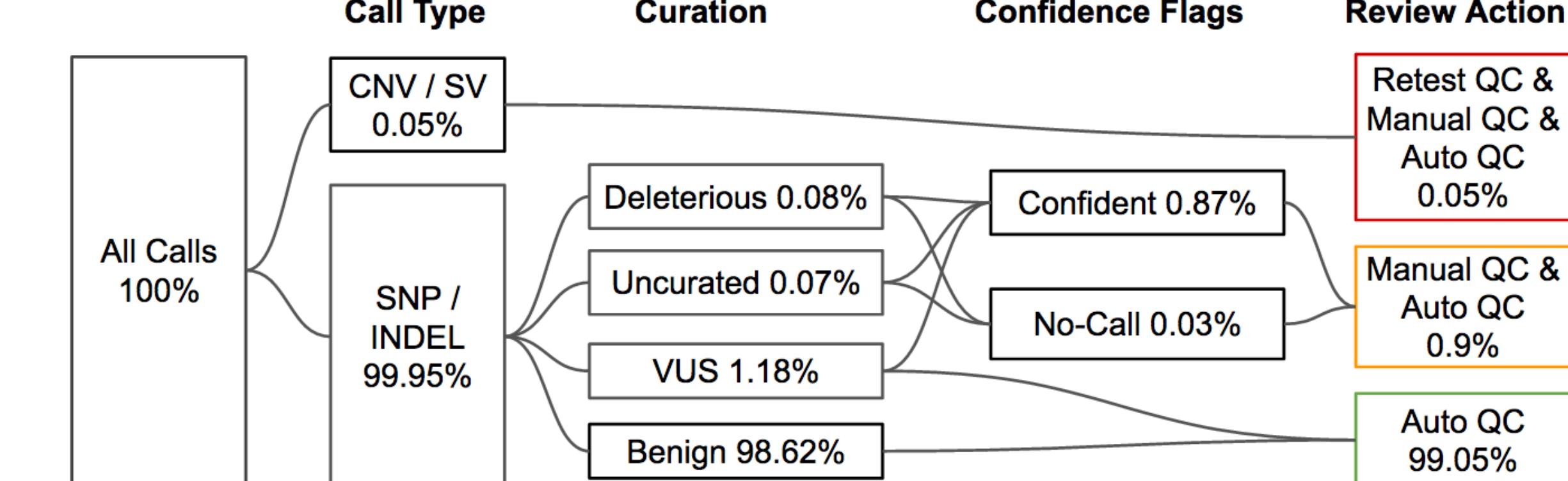
Example of call-override interface. In addition to specifying a genotype, the override framework requires a statement of confidence (top), free text to explain the reason for override, and the specification of a secondary reviewer to audit and confirm the override.



Triaging of calls for manual detailed review

The scope of the call review includes any called variant in the assay’s region of interest that has been curated known deleterious, likely deleterious, a variant of uncertain significance (VUS), or for which the curation status has yet to be determined. All calls that are potentially deleterious and fail QC metrics are manually reviewed to resolve if a call should be cleared from reporting or overridden to be included in the report. Any calls that are not resolvable by lab personnel are escalated to dedicated scientists for further investigation. On average, the Counsyl lab scientists invest a business day of sample turnaround time for manual review of results, doing in-depth review of 5 calls per sample, and resolving a low confidence call or revising a call once per every 35 samples.

Table 1

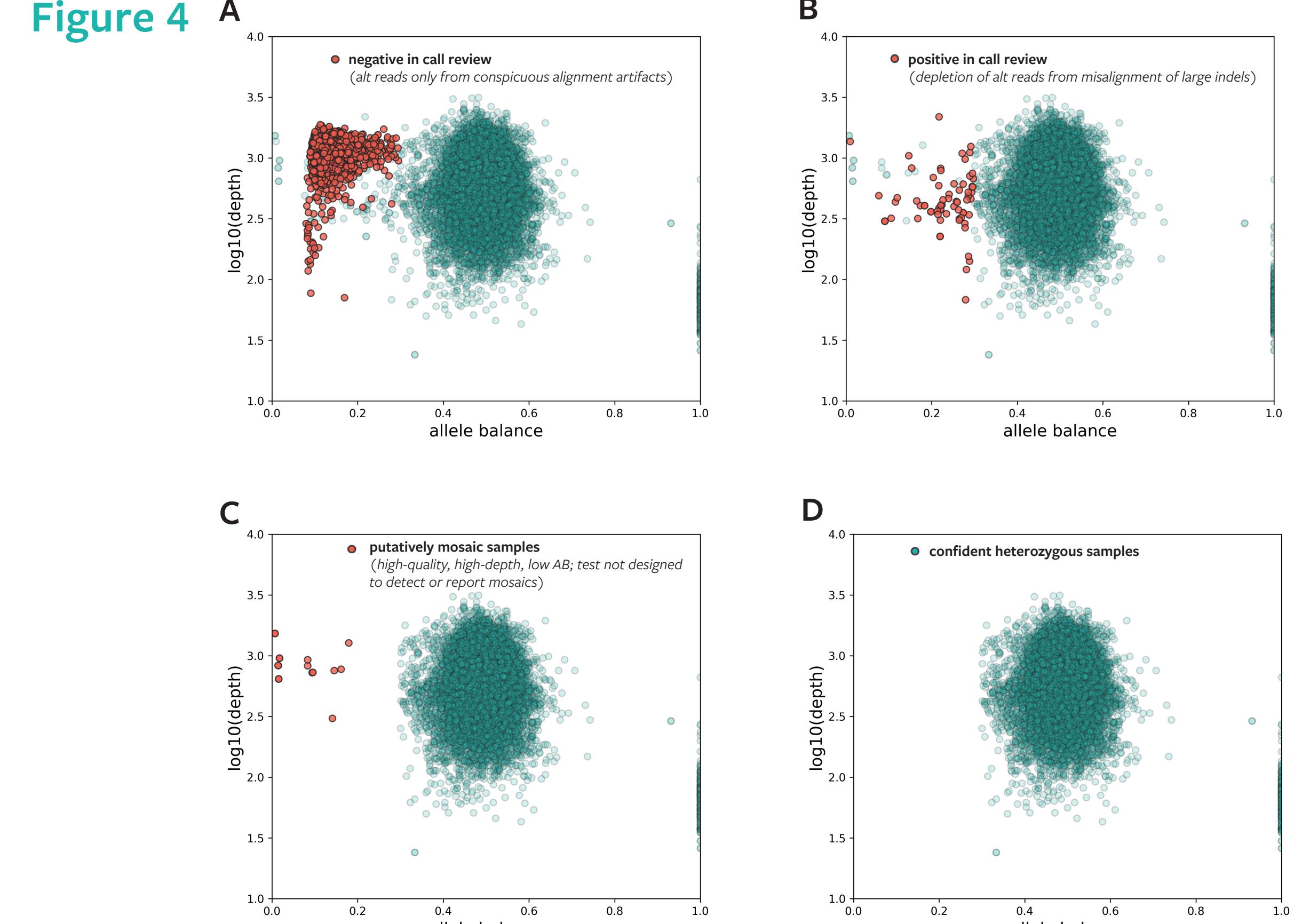


Triage decision tree for calls to trigger focused manual review. Sample calls are bucketed into different categories of review protocol based upon the call type, curation status, and confidence flags. Auto QC protocol includes typical sequencing quality flags like allele balance, strand bias, mapping quality etc. Manual QC includes detailed inspection of call metrics and pileup s by human reviewer. Retest QC includes resequencing of the sample.

Call-review-assisted reconciliation of calls with ambiguous allele balance

To audit the effectiveness of human call review, we retrospectively analyzed results from over 20,000 Counsyl patient samples tested on our expanded hereditary cancer panel. We paid particular attention to sites with allele balances below 30%, which were recently reported by Mu et al. 2016 to be enriched with false NGS results. We found that manual review aided by our custom NGS-evaluation interface easily revealed the spurious data causing an unexpected allele balance. Omitting the offending reads during call review—e.g., by ensuring that reads with variant bases are not PCR duplicates, and by requiring that reads supporting indels in repeat-rich DNA have unique sequence on each end—effectively pushes non-confident variant calls into either the confidence positive range or confident negative range.

Figure 4



Conclusions

We conclude that clinical use of NGS variant calls requires great care to avoid false reporting of systematic artifacts, yet this care can manifest in different ways. A lab can forgo careful review of NGS data and instead use routine orthogonal test methods; alternatively, we submit and have demonstrated that a highly optimized and platform-specific software interface can achieve comparable quality to routine orthogonal testing, often with faster turnaround time, less susceptibility to sample-handling errors, and lower cost to patients.

REFERENCES: 1. Vysotskai VS, Hogan GJ, Gould GM, Wang X, Robertson AD, Haas KR, Theilmann MR, Spurka L, Grauman PV, Lai HH, Jeon D, Haliburton G, Leggett M, Chu CS, Iori K, Maguire JR, Ready K, Evans EA, Kang HP, Haque IS. (2017) Development and validation of a 36-gene sequencing assay for hereditary cancer risk assessment. PeerJ 5:e3046 | 2. Wenbo Mu, Hsiao-Mei Lu, Jeffrey Chen, Shuwei Li, Aaron M. Elliott. J Mol Diagn. 2016 Nov;18(6):923-932. | 3. Samuel P. Strom PhD, Hane Lee PhD, Kingshuk Das MD, Eric Vilain MD, PhD, Stanley F. Nelson MD, Wayne W. Grody MD, PhD & Joshua L. Deignan PhD. Genetics in Medicine (2014) 16, 510-515