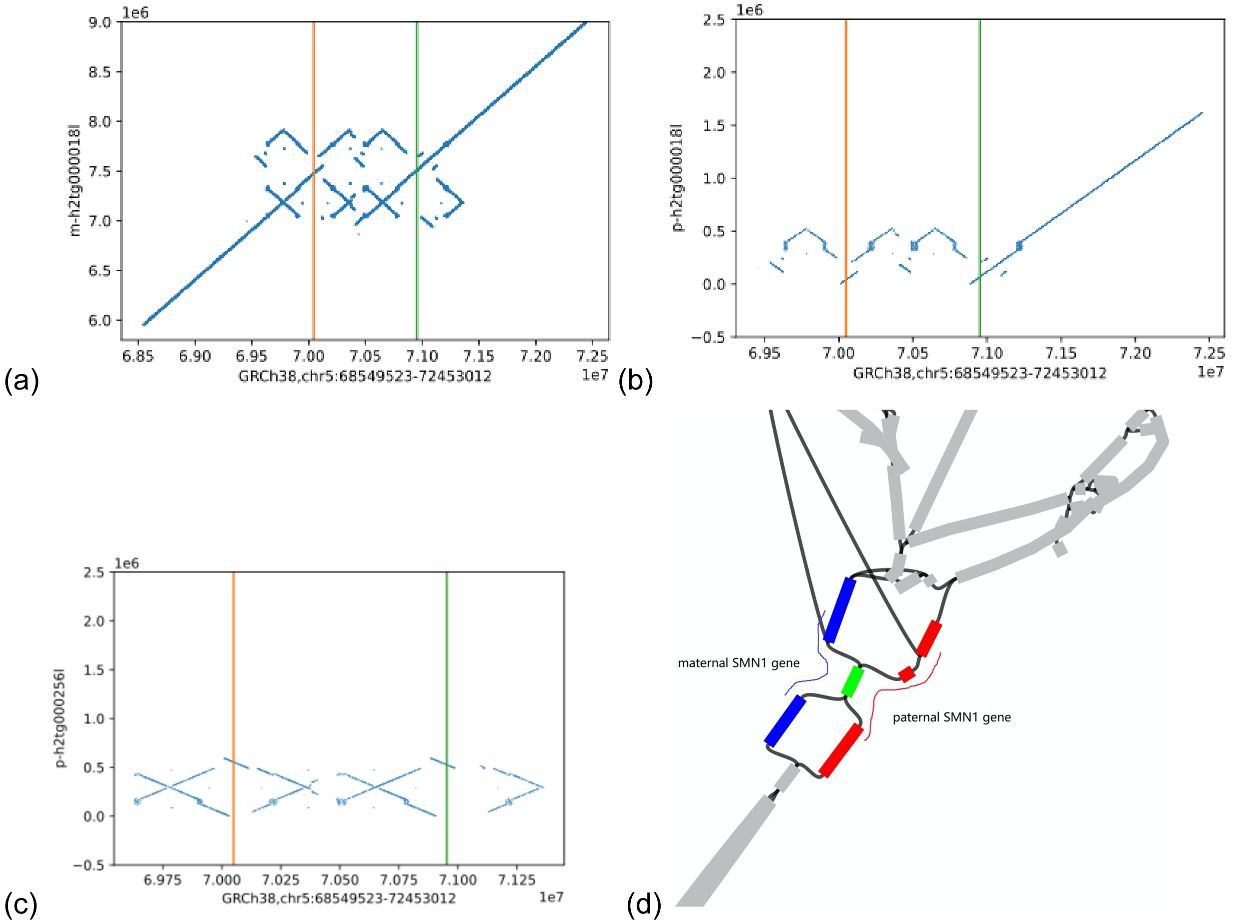
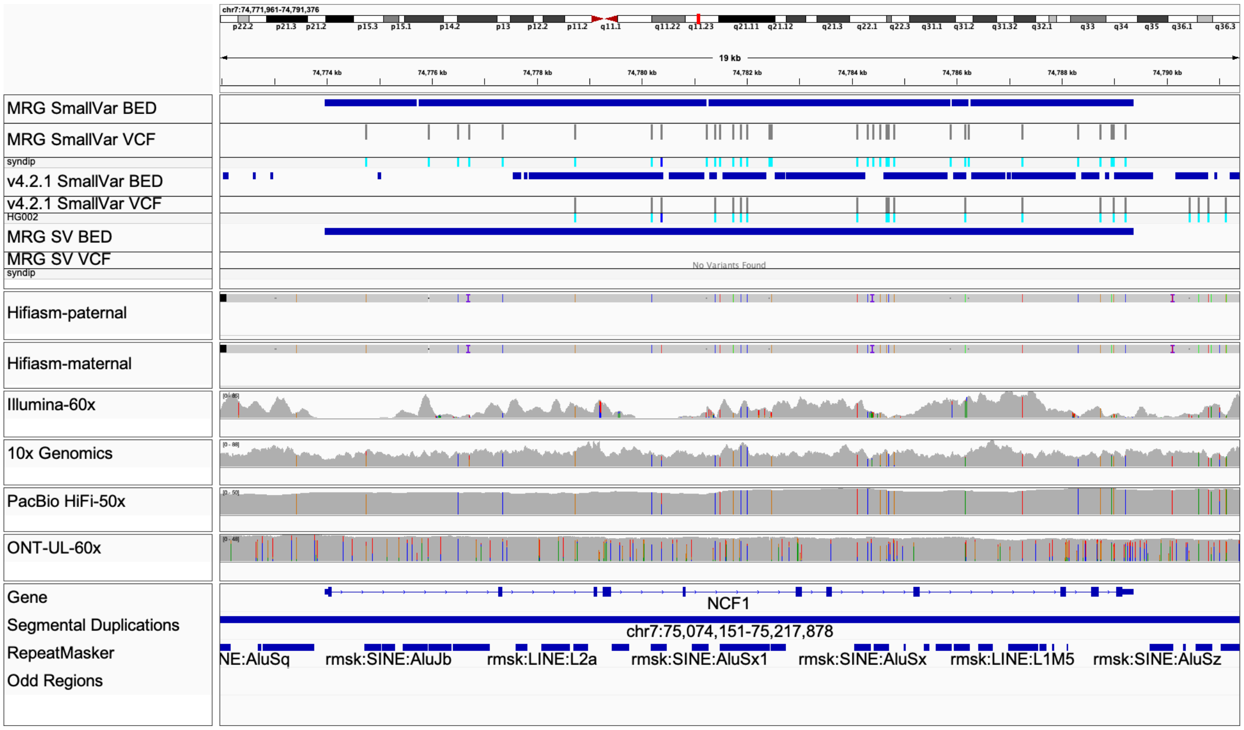
Supplementary Information for:

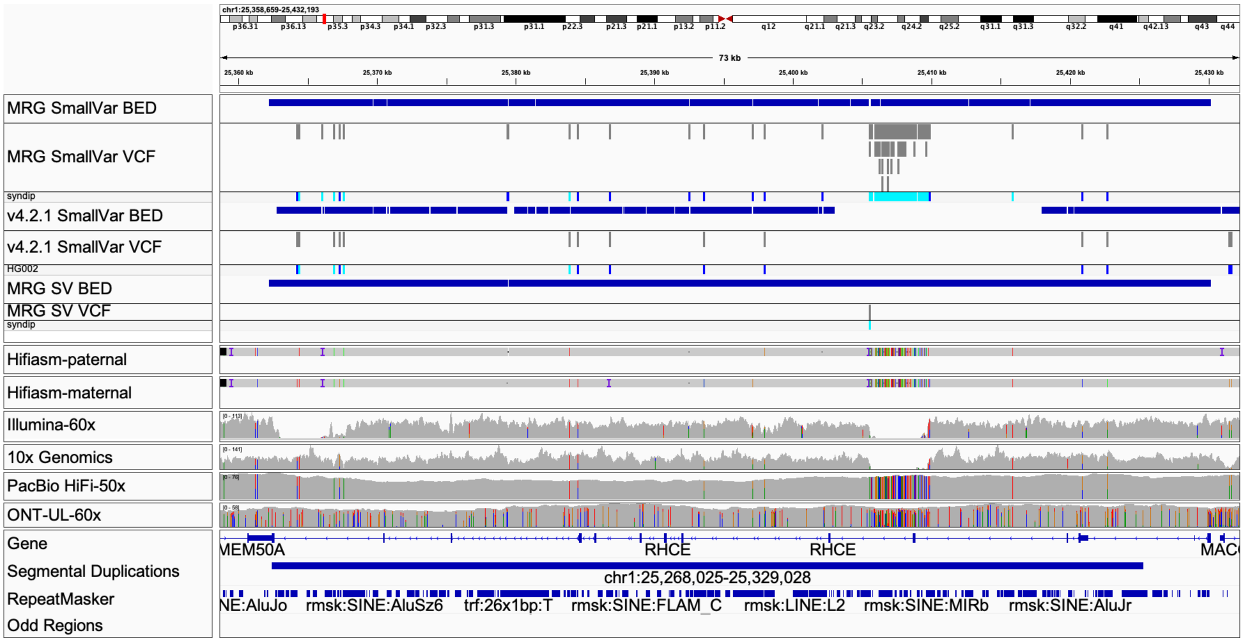
Curated variation benchmarks for challenging medically-relevant autosomal genes



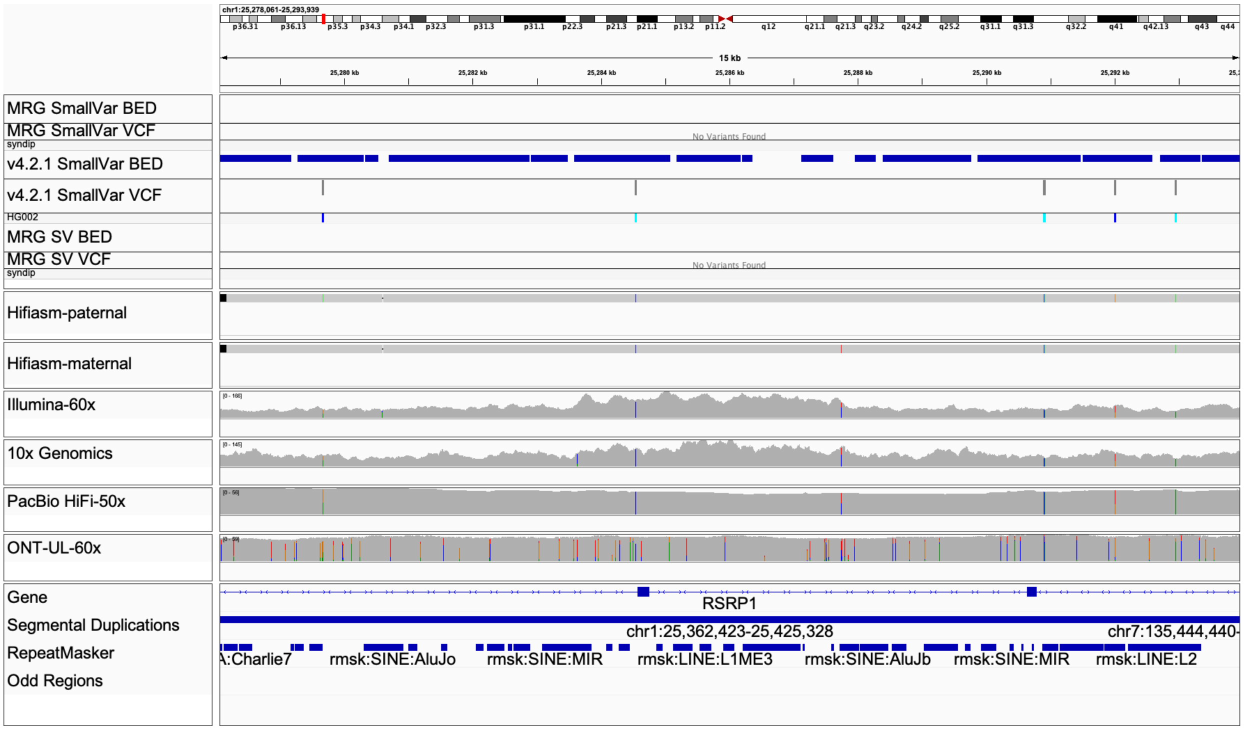
Supplementary Figure 1: Dot plots and assembly graph for HG002 assemblies in SMA region. (a) Maternal contig containing *SMN1* vs. GRCh38 in SMA region. (b) Paternal contig containing *SMN1* vs. GRCh38 in SMA region. (c) Paternal contig containing *SMN2* vs. GRCh38 in SMA region (*SMN2* was not assembled in the maternal contig). (d) Assembly graph for *SMN1* region, with maternal *SMN1* gene in blue and paternal *SMN1* gene in red.



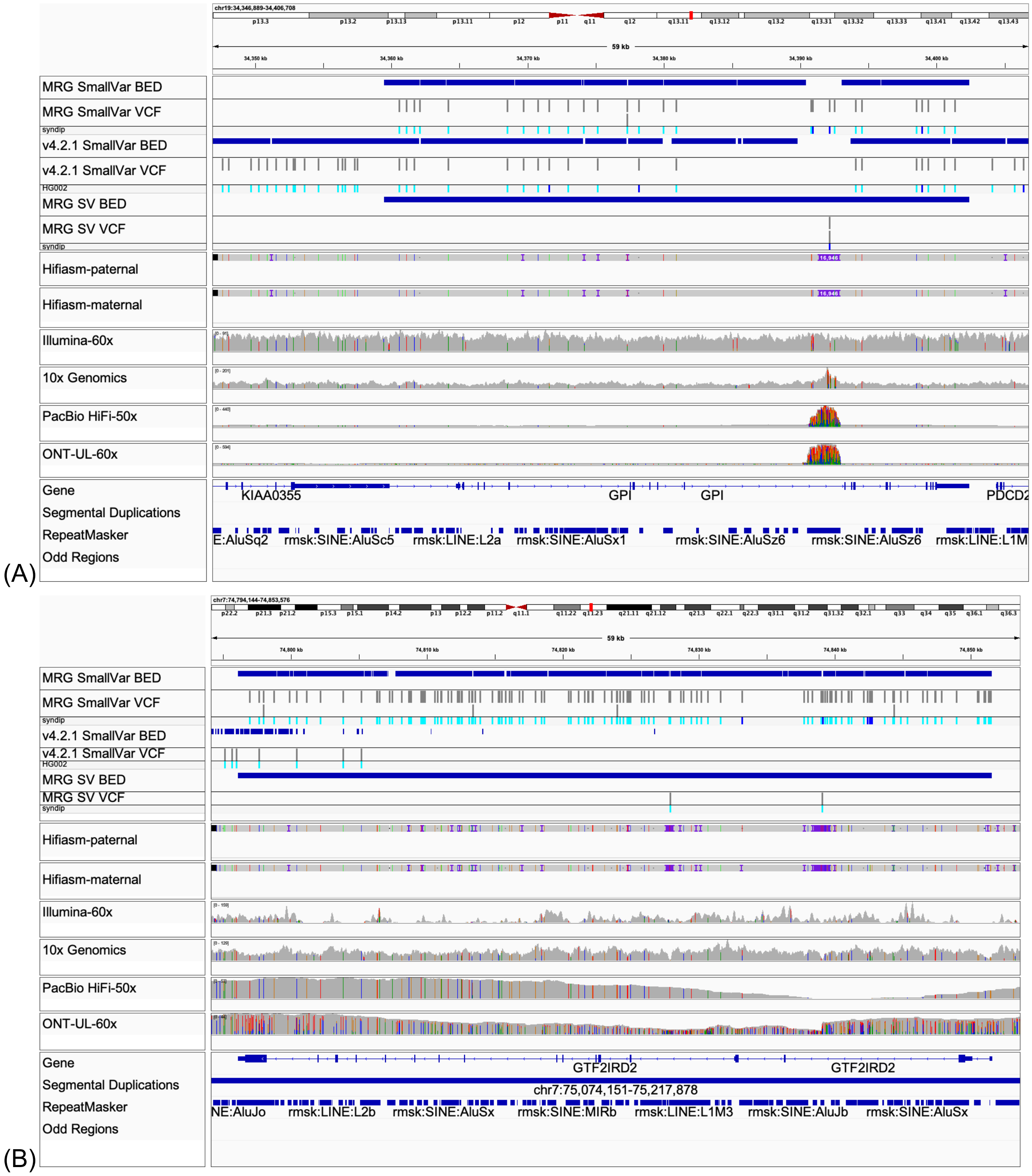
Supplementary Figure 2: Our new assembly-based benchmark almost completely resolves the gene *NCF1*. *NCF1* is in a 140 kb segmental duplication, resulting in some regions missing coverage by short reads, and the first 2 exons and some other variants missing from the v4.2.1 benchmark in GRCh38.



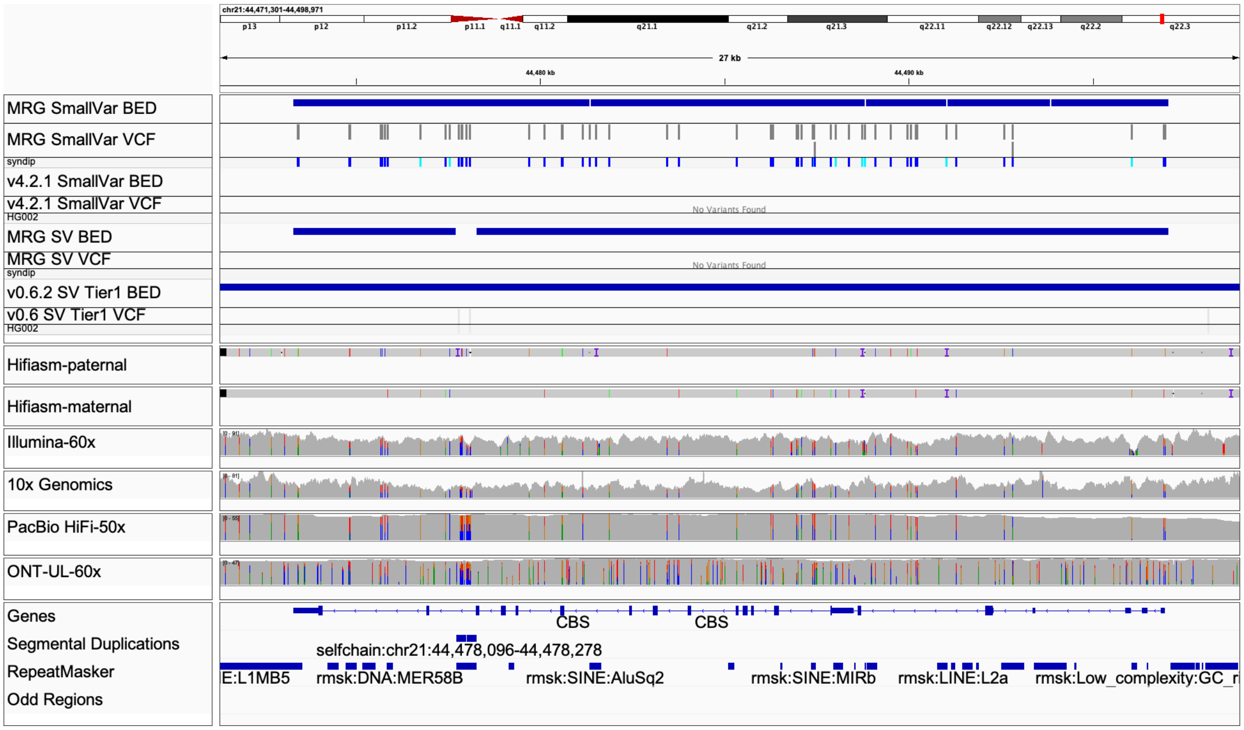
Supplementary Figure 3: HG002 has an approximately 4.5 kb region with an SV and many homozygous small variants surrounding exon 2 of *RHCE*, a gene that is part of the Rh blood group antigens. *RHCE* and *RHD* are part of an inverted duplication on chromosome 1. This complex set of variants results from a 4.5 kb region of *RHCE* in both HG002 haplotypes that have a sequence very similar to a 4.5 kb region of *RHD* in GRCh37 and GRCh38, which may be related to past gene conversion events. Short reads and linked reads from this region incorrectly map to *RHD* (GRCh38 shown).



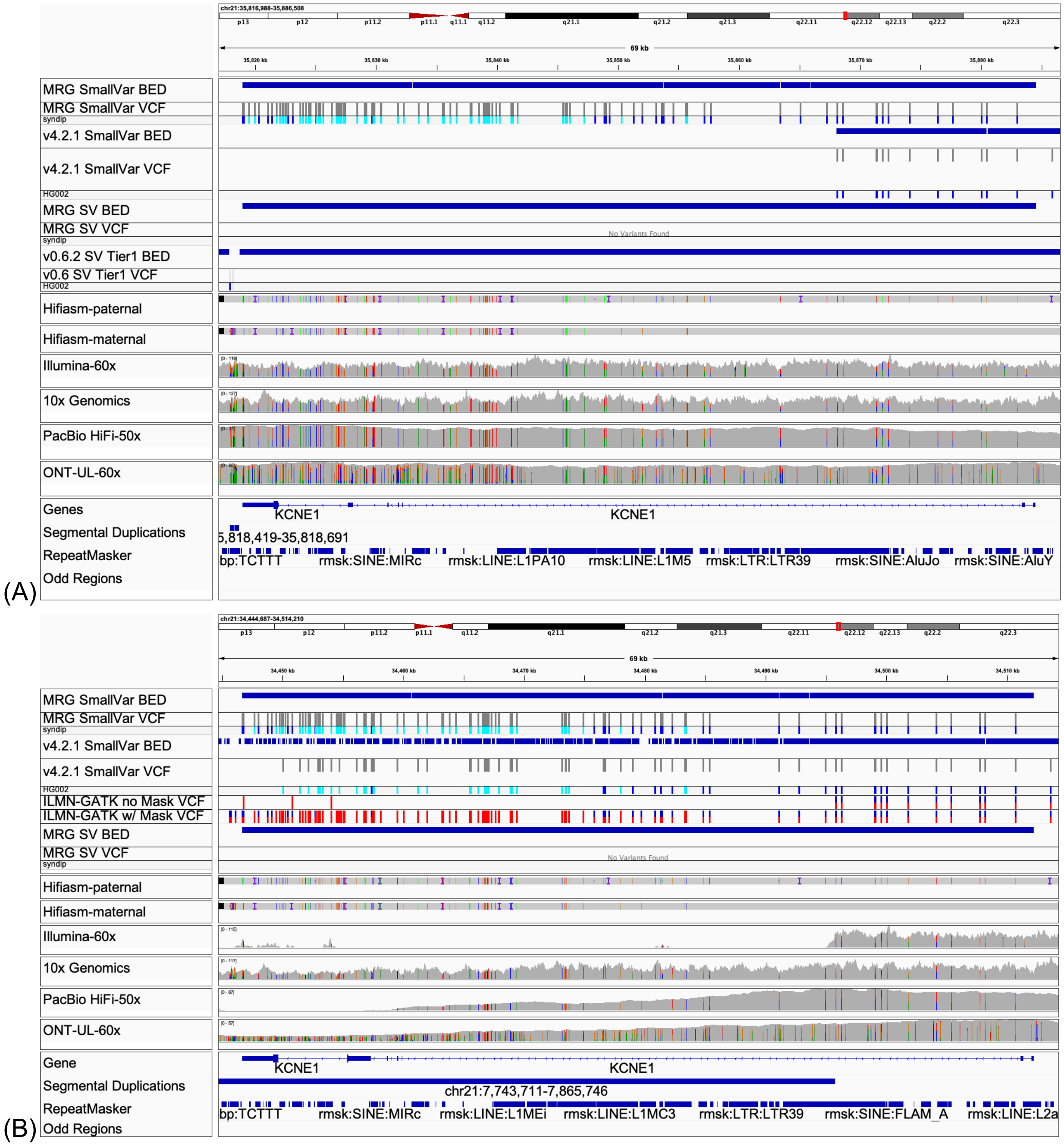
Supplementary Figure 4: An approximately 4.5 kb region (chr1:25,283,400-25,287,900 on GRCh38) in the segmentally duplicated gene *RHD* has about twice the normal coverage of short- and linked-reads due to reads mis-mapping from a gene conversion event in the homologous gene *RHCE*. Note that the gene *RSRP1* overlaps with *RHD*, so it is in the label in the IGV session above.



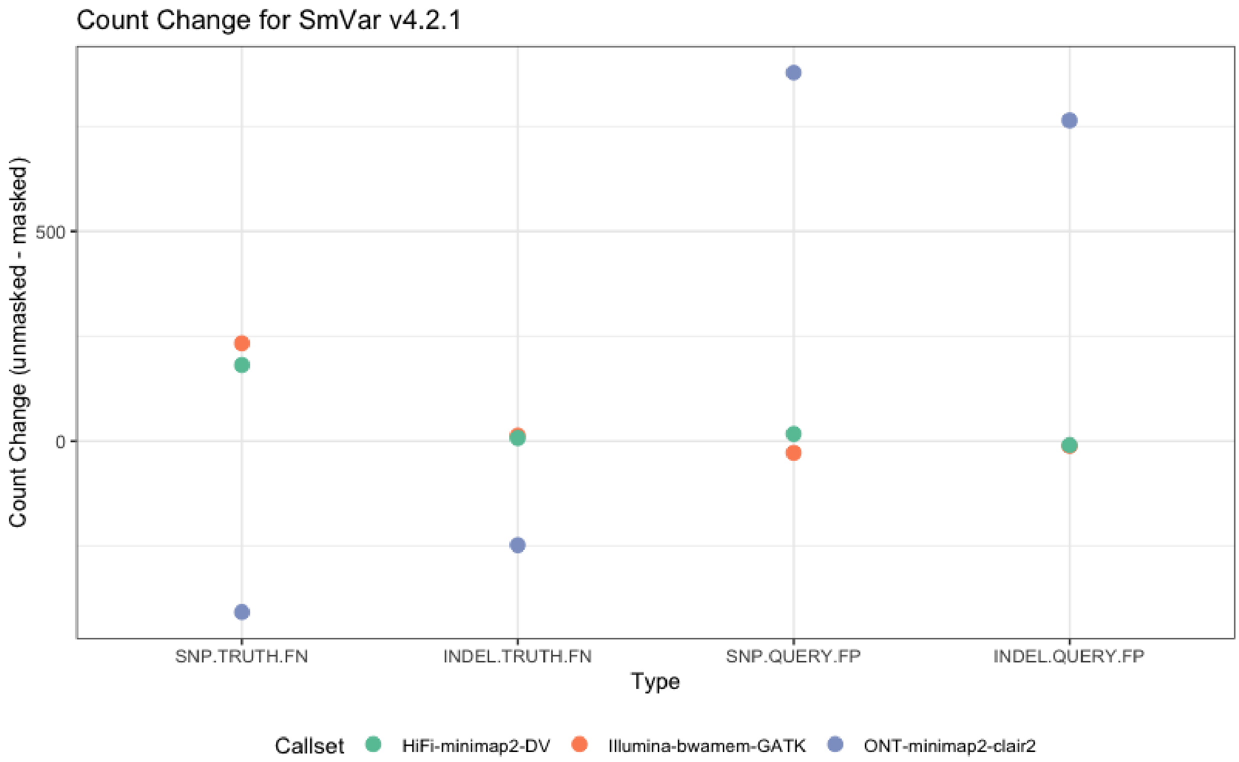
Supplementary Figure 5: The new CMRG benchmark contains challenging SVs excluded from previous benchmarks. (A) The new benchmark includes a 16,946 bp insertion in an intronic VNTR in the gene *GPI*. Even with long reads, this variant is challenging to call with current mapping-based SV callers. (B) The new benchmark includes two homozygous insertions in the segmentally duplicated gene *GTF2IRD2*. Many long reads containing the insertions align to the wrong copy of the segmental duplication, making it challenging to call SVs with mapping-based methods.



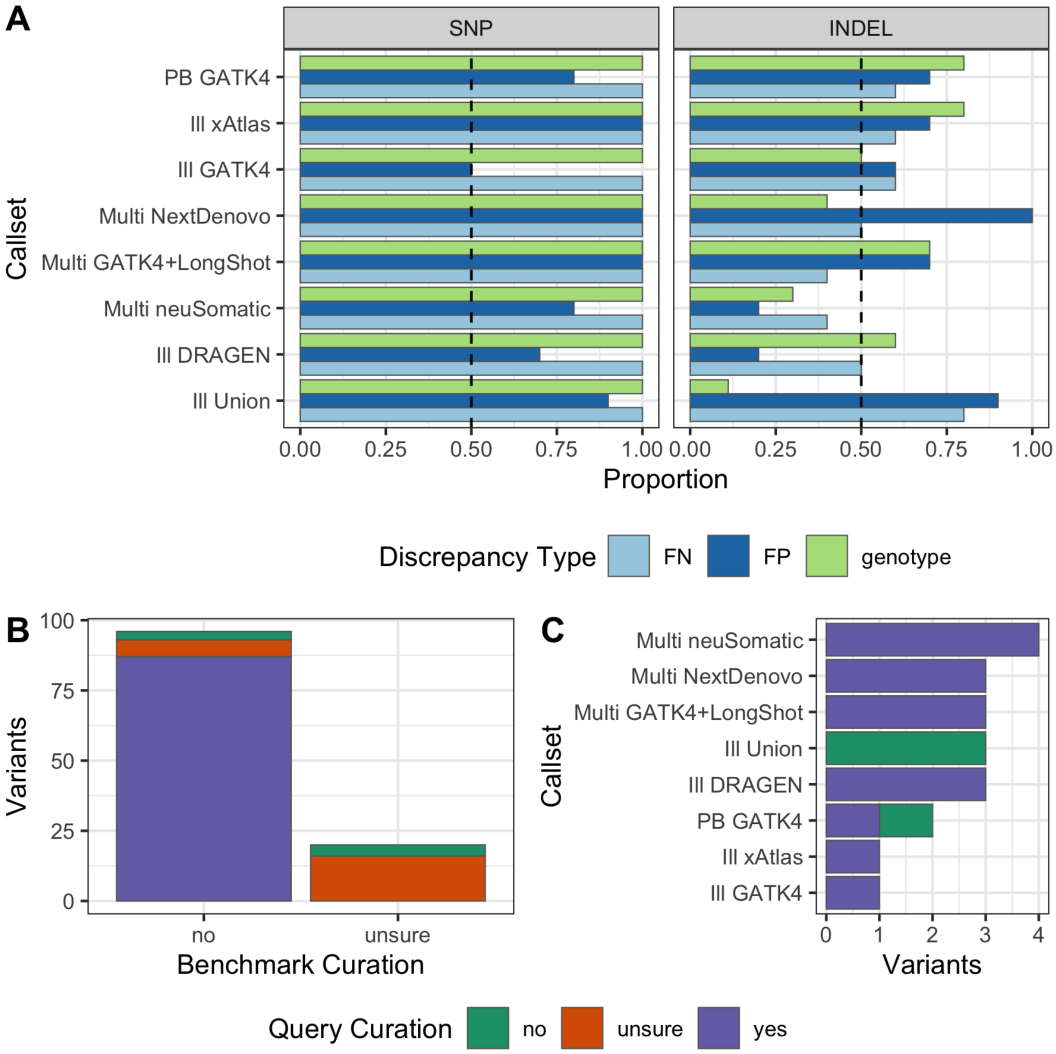
Supplementary Figure 6: Unlike GRCh38, variant calls across technologies are consistent with the benchmark on GRCh37 because GRCh37 does not contain the false duplication.



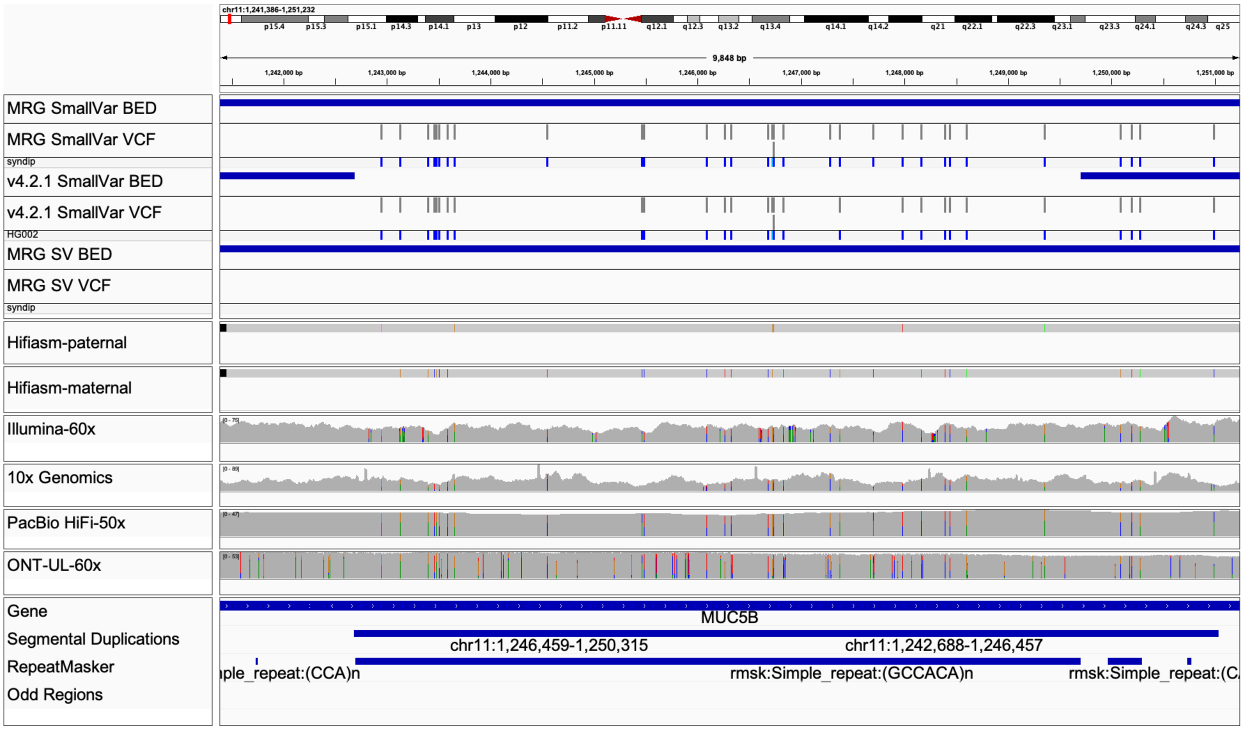
Supplementary Figure 7: KCNE false duplication in GRCh38. (A) Variant calls across technologies are consistent with the benchmark on GRCh37, because GRCh37 does not contain a false duplication. (B) GRCh38 contains a false duplication of part of the gene, so many reads mis-map to the false copy of the gene, *KCNE1B*



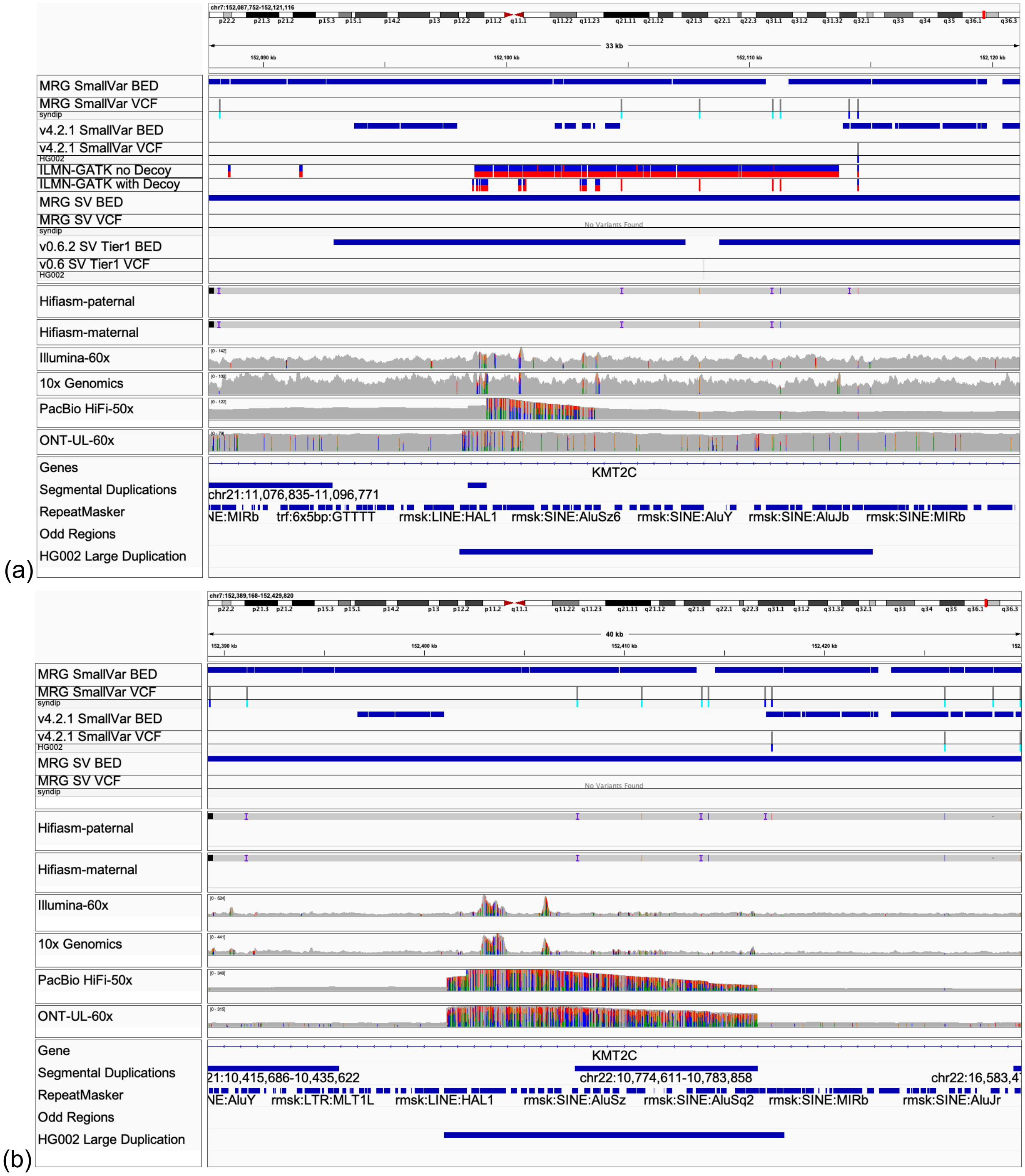
Supplementary Figure 8: Comparison of variant accuracy for GRCh38 before and after masking false duplications on chromosome 21. The new benchmark demonstrates only small changes to false positive and false negative counts for the whole genome benchmark v4.2.1 when mapping to the masked GRCh38, since v4.2.1 excludes most of the falsely duplicated regions.



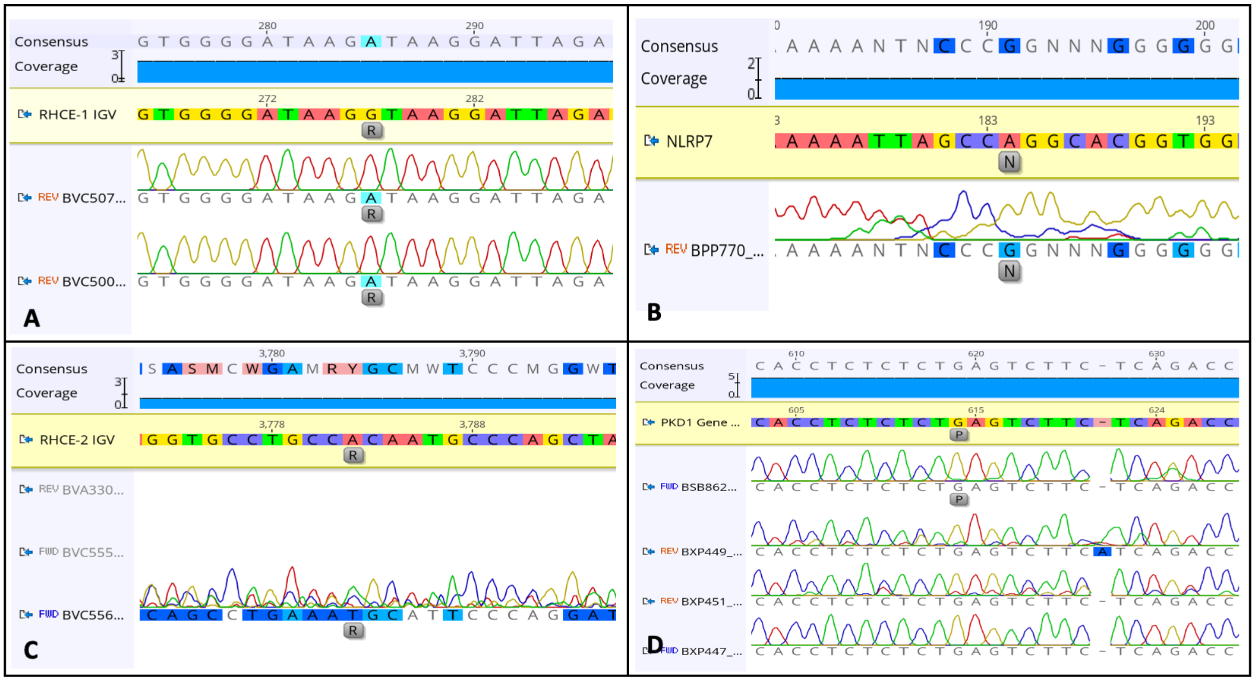
Supplementary Figure 9: The v0.02.03 small variant CMRG benchmark reliably identified most types of errors, but some putative INDEL FPs and genotype errors needed to be excluded from v1.00. We used hap.py with the vcfeval engine to compare each of the evaluation callsets against the v0.02.03 small variant CMRG benchmark. We later excluded all sites manually curated as unsure or error in the benchmark from the v1.00 small variant CMRG benchmark. A) The proportion of curated FP and FN variants by callset where the benchmark set was correct and the query callset was incorrect. The dashed black line indicates the majority threshold, 50%, because GIAB's goal for benchmarks is to exceed this threshold. Curated variants from both GRCh37 and GRCh38 (20 total) were used to calculate proportions. (B) Breakdown of the total number of variants by manual curation category, excluding variants from panel A where the benchmark was deemed correct and query incorrect, showing some sites were difficult to curate with current technologies. (C) Benchmark unsure variants by callset. 44/50 and 59/63 of the errors identified by the evaluation on GRCh37 and GRCh38, respectively, were excluded by curation of the common false positives and false negatives, so the accuracy of the v1.00 benchmark is higher. Technology abbreviations are: ONT=Oxford Nanopore, PB=PacBio HiFi, Ill=Illumina PCR-free, Multi= Combined ONT, PB, and Ill.



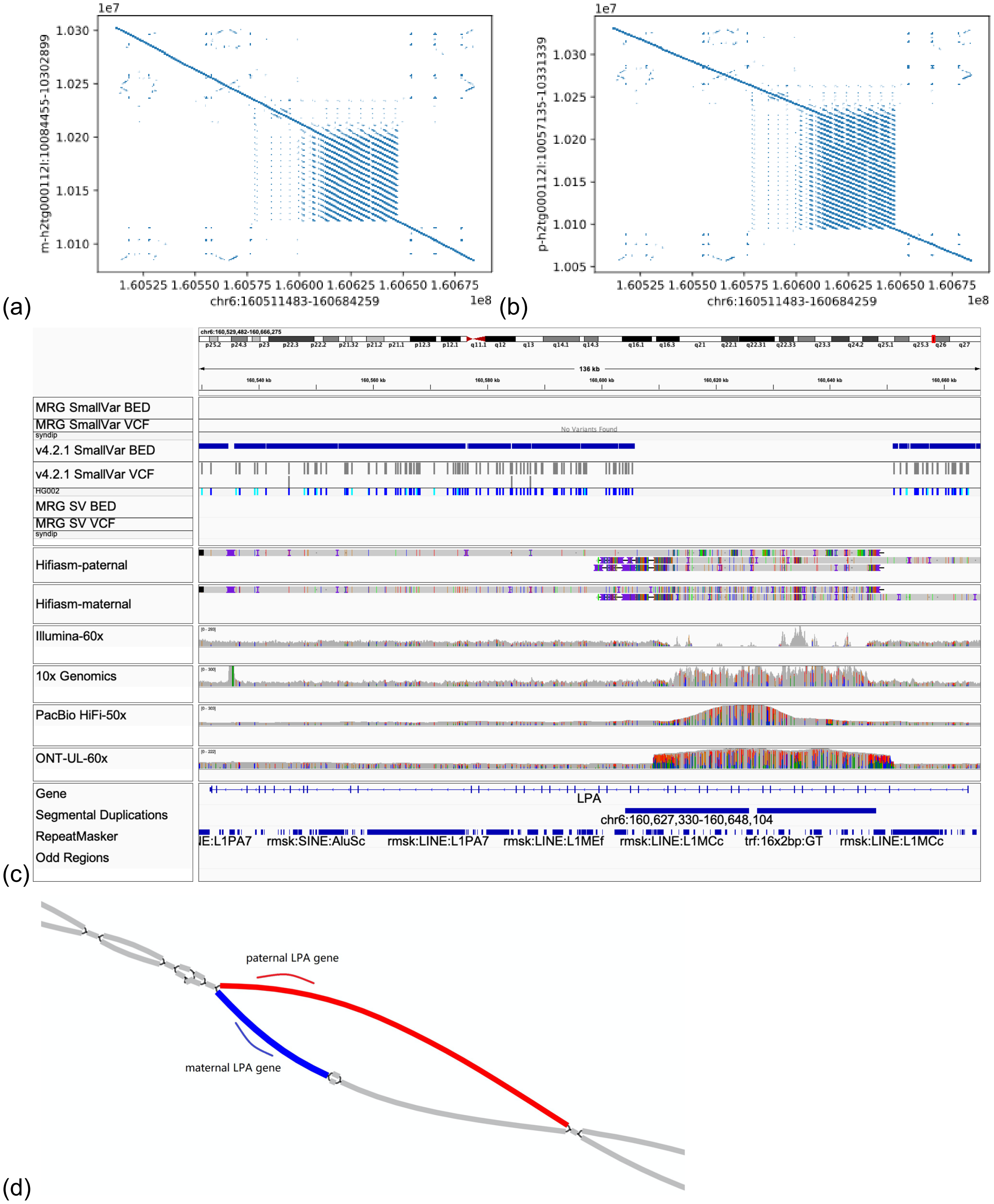
Supplementary Figure 10: The new benchmark contains a series of small variants in this long exonic tandem repeat in *MUC5B*. When there are many variants within a tandem repeat, they can often be called in a variety of ways. They may not be counted as true positives when benchmark if they are represented differently from the benchmark and partially called incorrectly or filtered. We created a new stratification to highlight false positives and false negatives in these regions in HG002.



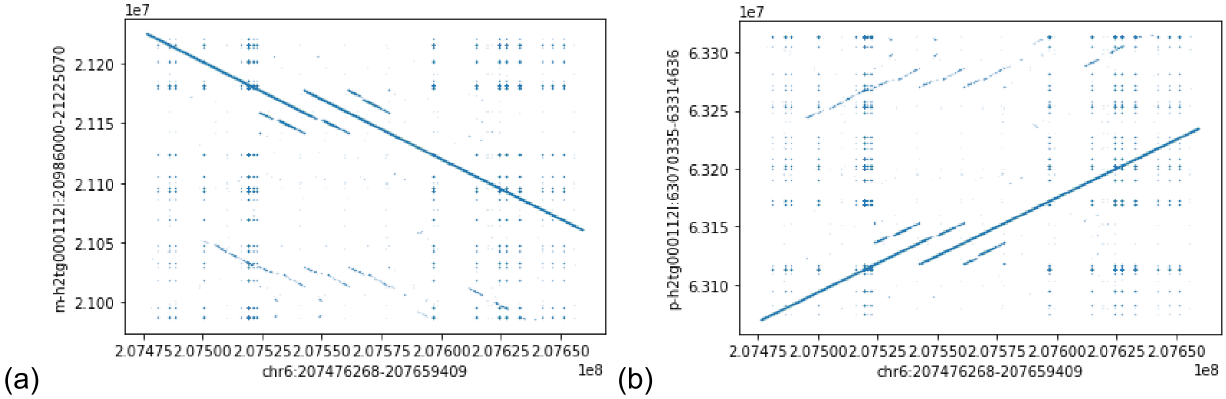
Supplementary Figure 11: We created a new stratification for a large, divergent duplication of part of the gene *KMT2C* in HG002 missing from GRCh38 (b) and only included in the hs37d5 decoy for GRCh37 (a). Mapping-based methods can call many false positives in this region in GRCh38 or in GRCh37 without the decoy, because reads from the duplication incorrectly map to the gene, as shown in the very high coverage and high density of variants in the alignments across technologies on GRCh38.


Consensus
Coverage
280
290
G T G G G G A T A A G A T A A G G A T T A G A
3
0
RHCE-1 IGV
272
282
G T G G G G A T A A G G T A A G G A T T A G A
R
REV BVC507...
G T G G G G A T A A G A T A A G G A T T A G A
R
REV BVC500...
G T G G G G A T A A G A T A A G G A T T A G A
R
A
Consensus
Coverage
0
190
200
A A A A N T N C C C G G N N N G G G G G G
2
0
3
183
193
NLRP7
A A A A T T A G C C A G G C A C G G T G G
N
REV BPP770_...
A A A A N T N C C C G G N N N G G G G G G
N
B
Consensus
Coverage
3,780
3,790
S A S M C W G A M R Y G C M W T C C C M G G W T
3
0
RHCE-2 IGV
3,778
3,788
G G T G C C T G C C A C A A T G C C C A G C T A
R
REV BVA330...
FWD BVC555...
FWD BVC556...
C A G C C T G A A A T G C A T T C C C A G G A T
R
C
Consensus
Coverage
610
620
630
C A C C T C T C T C T G A G T C T T C – T C A G A C C
5
0
PKD1 Gene ...
605
615
624
C A C C T C T C T C T G A G T C T T C – T C A G A C C
P
FWD BSB862...
C A C C T C T C T C T G A G T C T T C – T C A G A C C
P
REV BXP449_...
C A C C T C T C T C T G A G T C T T C A T C A G A C C
REV BXP451_...
C A C C T C T C T C T G A G T C T T C – T C A G A C C
FWD BXP447_...
C A C C T C T C T C T G A G T C T T C – T C A G A C C
D

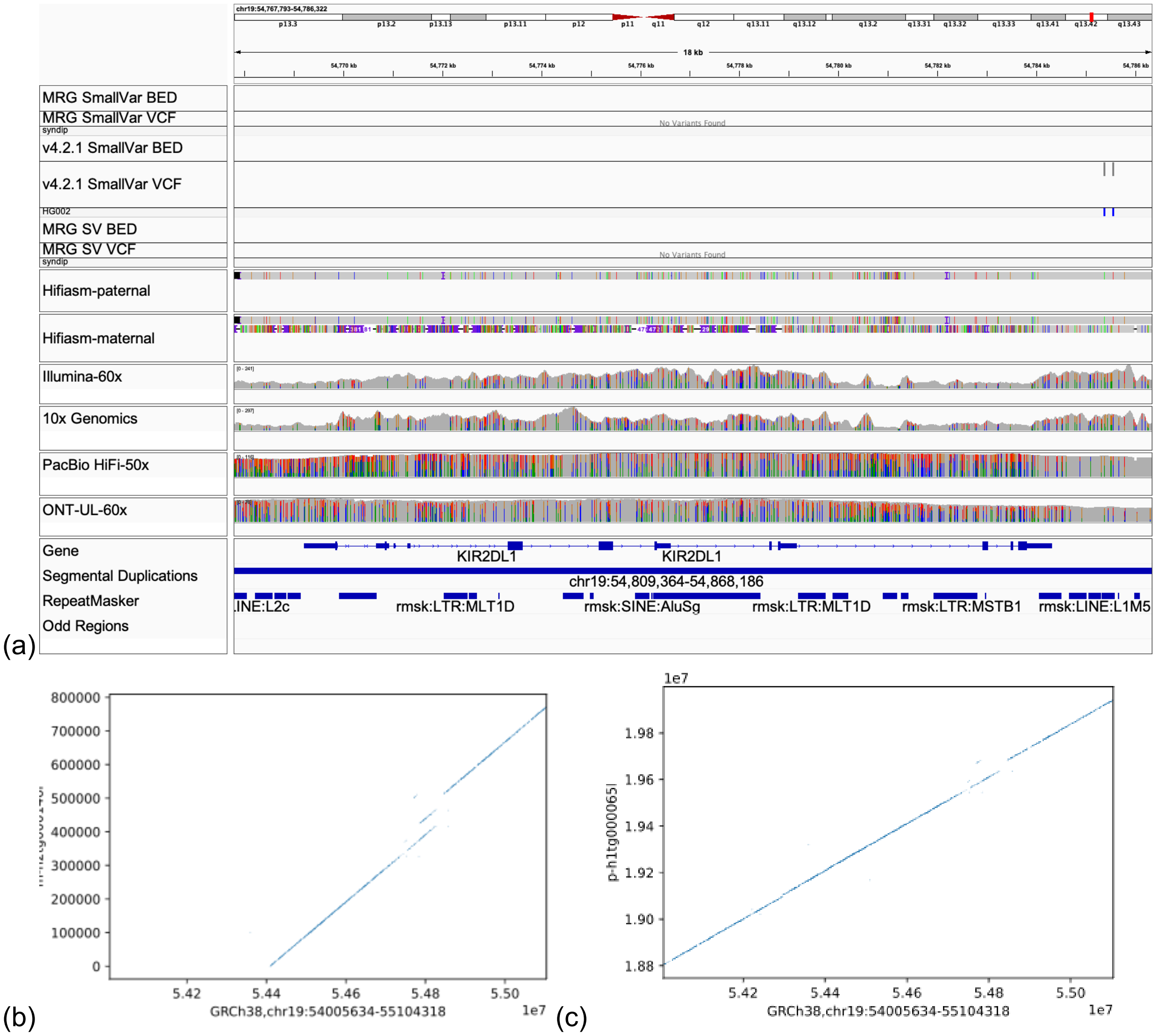
Supplementary Figure 12: A. *Variant Confirmed*. These variants have clean coverage by at least 1 primer, the base call is clearly variant at the expected base location, and all other base calls in the surrounding region clearly match the reference; **B**. *Variant Supported Not Confirmed*. These variants show support for a variant at the given base location, but other, unsupported variants are shown in the surrounding region as a result of noise in the chromatogram; **C**. *Variant Covered but not Confirmed*. These variants have coverage by at least 1 primer, but the sequence cannot be discerned by the Sanger trace. Usually this is a result of messy sequencing with multiple nucleotide peaks at all bases around the variant; **D**. *PKD1*. This variant does not show support for the G-->C variant identified by NGS. Several forward and reverse primers show a homozygous reference base call at this location.



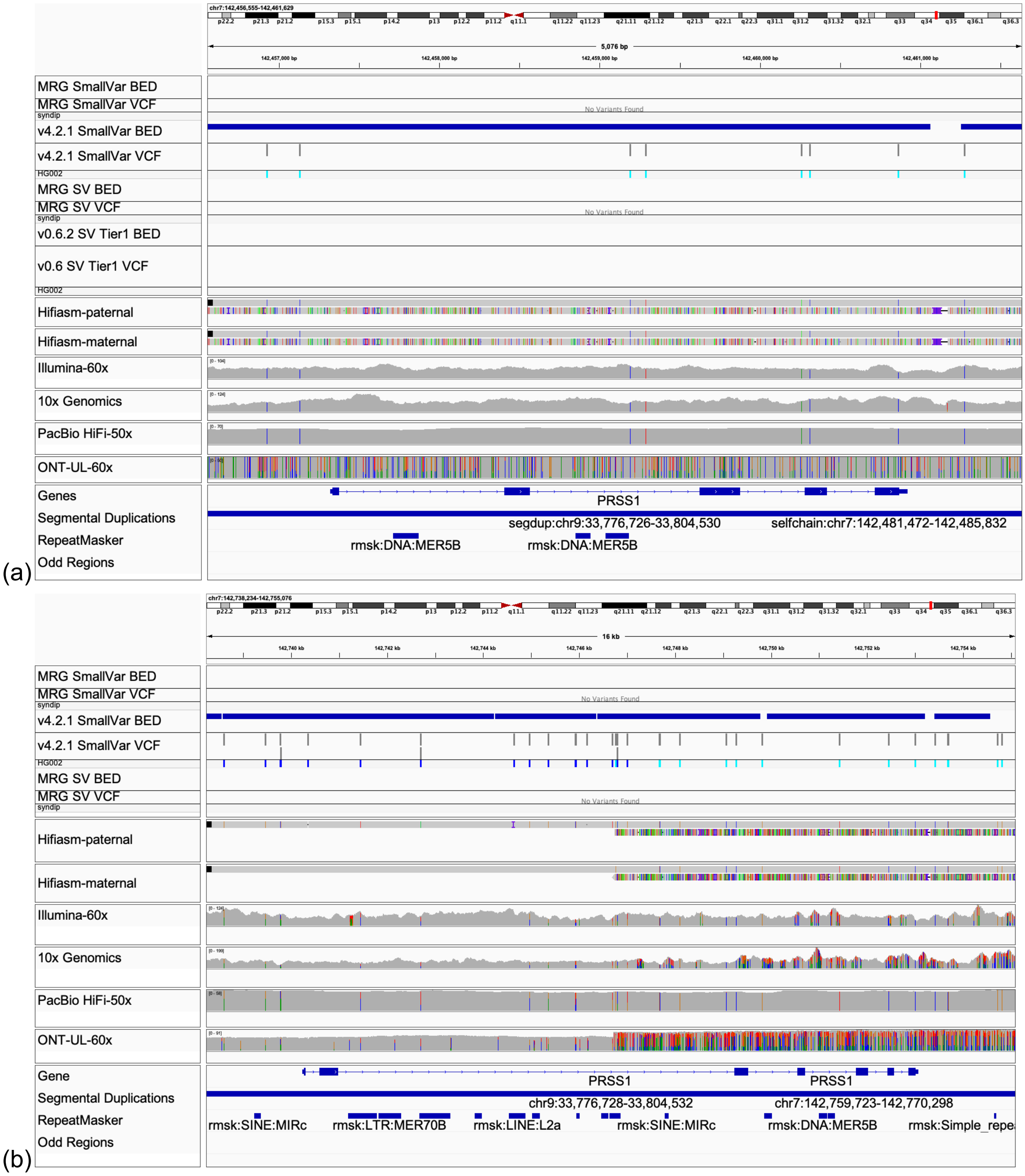
Supplementary Figure 13: Dot plots, IGV, and assembly graph for HG002 assemblies in the medically important gene *LPA*, which contains 45 kb and 100 kb expansions of the tandemly duplicated kringle IV repeats relative to GRCh38. (a) Maternal contig containing 45 kb expansion vs. GRCh38. (b) Paternal contig containing 45 kb expansion vs. GRCh38. (c) IGV showing complex assembly and read alignments in repeat expansion. (d) Assembly graph for LPA region, with maternal LPA gene in blue and paternal LPA gene in red.



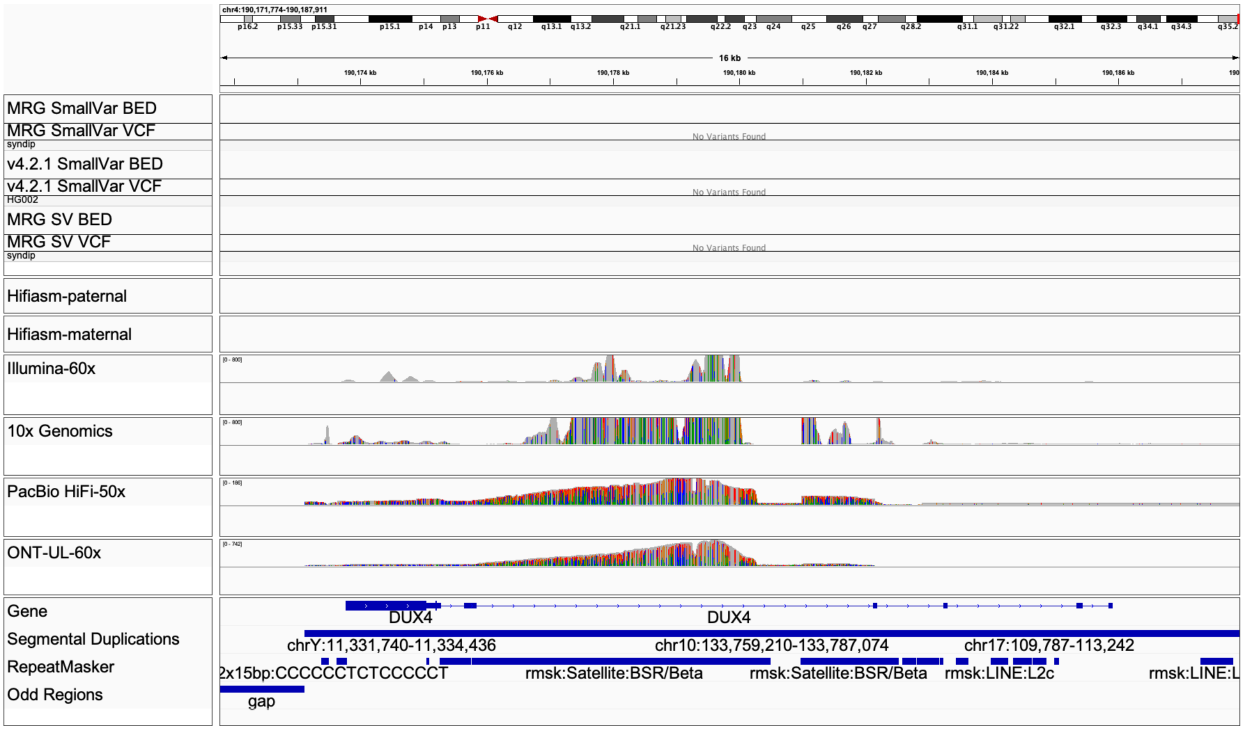
Supplementary Figure 14: Dot plots for HG002 assemblies in the medically important gene *CR1*, which contains 18 kb contractions of the large tandem repeats relative to GRCh38. (a) Maternal contig and (b) Paternal contig dot plots vs. GRCh38. These are excluded in the current benchmark due to breaks in the dipcall alignments.



Supplementary Figure 15: (a) IGV screenshot showing high coverage of the gene *KIR2DL1.* (b) The high coverage results from the duplication of this gene on the maternal haplotype of HG002 relative to GRCh38 shown in the dot plot. (c) The paternal haplotype does not contain the duplication of this region.



Supplementary Figure 16: The gene *PRSS1* has an extra copy in both haplotypes of HG002 relative to GRCh37 (a) and GRCh38 (b). GRCh37 has normal coverage because the extra copy is similar to the decoy sequence hs37d5, whereas GRCh38 only contains this sequence in the alternate locus NT_187562.1, which was not included in our reference for alignments.



Supplementary Figure 17: Very high coverage of the gene *DUX4* due to many gaps and missing sequence in the D4Z4 region in GRCh38, missing extra copies of the gene that are in HG002. This region is not represented at all in GRCh37

Supplementary Note 1:

To identify genes for which a new benchmark was most needed, we next examined the fraction of the 4,697 autosomal medically-relevant gene bodies included in the latest GIAB HG002 v4.2.1 small variant benchmark. We excluded the sex chromosomes, X and Y, because v4.2.1 did not include these haploid chromosomes in males like HG002. The median fraction of each autosomal gene included in v4.2.1 is 97.8%, with 544 (11.6%) genes completely included in v4.2.1, 3,746 included > 95%, and 4,343 (92.5%) included > 90% (**Supplementary Data 2**). Genes were not fully included in v4.2.1 for a variety of reasons, including: (1) putative SVs, specifically regions containing GIAB's HG002 v0.6 Tier 1 or Tier 2 SVs[11], some of which are true SVs and some of which do not contain SVs or only partly contain SVs, (2) regions difficult to characterize with mapping-based methods, including complex variants in segmental duplications and long tandem repeats that were not properly resolved with mapping of Illumina or PacBio HiFi reads, (3) large duplications in HG002 not in GRCh38, in which small variants cannot be represented in standardized ways, and (4) variants (plus 50 bp flanking regions) that were not considered high confidence by the GIAB small variant integration pipeline because differences between methods could not be resolved, or all methods had evidence of bias. We identified 361 and 354 autosomal genes that are included 90% or less by the HG002 v4.2.1 benchmark regions on GRCh37 or GRCh38, respectively, totaling 395 unique genes. We focused this work on genes previously 90% or less included in order to facilitate our manual curation of the genes, thoroughly inspecting variant calls from the diploid assembly and the accuracy of the assembly for these challenging medically relevant genes (CMRG)**.**

Supplementary Note 2:

This curated CMRG benchmark includes new, more challenging classes of SVs not included in the previous GIAB v0.6 benchmark: (1) It includes a sequence-resolved, large 16,946 bp insertion in a VNTR (variant number tandem repeat) in an intron of the gene *GPI*, which is challenging to call with mapping-based methods, even with long reads (**Supplementary Figure 5(a))**. Although VNTRs have been difficult to study, recent evidence points to association of VNTRs with methylation and gene expression[24]. (2) It includes SVs in segmental duplications such as a homozygous 2.3 kb intronic VNTR expansion in *PKD1*, a 5.9 kb homozygous LINE:L1HS deletion in *SMG1*, and two homozygous insertions (588 bp and 1,205 bp) in the gene *GTF2IRD2* on GRCh38 (**Supplementary Figure 5(b))**. The two insertions in *GTF2IRD2* are often missed by mapping-based variant callers because even long reads mismap to the other copy of the segmental duplication, which contains sequences similar to the inserted sequences. GRCh38 corrected a tiling path issue in GRCh37 that mixed haplotypes and resulted in a gap in the region. Interestingly, tThis resulted in a different representation of *GTF2IRD2*, such that instead of the 588 bp and 1,205 bp insertions, HG002 has a homozygous 195 bp deletion relative to GRCh37, but many short and long reads in this region still mismap to the other copy of the segmental duplication. (3) It includes compound heterozygous insertions (i.e., each haplotype has a different large insertion size in a tandem repeat). (4) Other complex SVs like those in *FLG* and *DSPP* are included in the benchmark VCF, but excluded by the benchmark regions because of a lack of tools to do the comparison robustly. Nevertheless, these SVs that cannot be benchmarked with automated tools could be compared manually or with future benchmarking tools. In addition, the SV sizes and estimated insertion and deletion sequences in the new SV benchmark are more accurate than v0.6 because v0.6 did not include accurate HiFi reads or trio-based partitioning of haplotypes for assembly. The small variant benchmark VCF also contains phased SNVs and INDELs near the benchmark SVs, though these are excluded from the small variant benchmark regions. These large SVs, complex SVs, and SVs inside segmental duplications enable benchmarking of more challenging SVs than previously possible.

Supplementary Note 3: Manually excluded benchmark errors

Curation identified that the initial version of the benchmark contained some errors, which fell in a few categories: (1) regions of high homozygosity, where hifiasm sometimes misses one haplotype, mostly INDELs in homopolymers and dinucleotide tandem repeats (e.g., 1:6496542 on GRCh37/chr1:6436482 on GRCh38), (2) homopolymers and dinucleotide tandem repeats where the benchmark call is off by one or two bps, respectively, likely due to reduced HiFi read accuracy in long homopolymers particularly C/G homopolymers (e.g., 2:1148810 on GRCh37/chr2:1153124 on GRCh38; and 3:136053953 on GRCh37/chr3:136335111 on GRCh38). These are sometimes unclear during curation when PCR-free Illumina is also noisy or biased; (3) large insertions in homopolymers that result in homopolymers >20 bp on one or both haplotypes (e.g., 19:45419394 on GRCh37/chr19:44916137 on GRCh38); (4) a small number of hifiasm consensus errors that cause large INDELs or a series of small variants that are supported by a single read (e.g., 10:135235879 on GRCh37/chr10:133422375 on GRCh38); (5) a small number of adjacent insertion and deletion calls in the assembly alignment, for which a dipcall bug causes only the insertion to be called (e.g., 12:7355777/chr12:7203181 on GRCh37/GRCh38). When curating the homopolymers and dinucleotide tandem repeats, we trusted the PCR-free Illumina reads that traversed the entire repeat region if there was no evidence of mapping errors (e.g., in some segmental duplications) or systematic sequencing errors (e.g., at some G/C homopolymers). In v1.00, we remove all 215 regions with benchmark errors or unclear calls identified by manual curation on GRCh37 or GRCh38.

We evaluated small complex variants in tandem repeats longer than 100 bp in a different way. These variants can be represented in VCF in many ways (e.g. as one line or multiple lines), are often partially filtered by mapping-based methods, and can be very challenging to curate in a genome browser due to different representations in aligned reads from different mappers/technologies. Because assembly-based methods usually perform best in these regions, we compared variants from a trio-hicanu assembly to our hifiasm-based benchmark small variants, because they use different consensus approaches. Variant calls and genotypes agreed between the two methods for 1,343/1,361 SNVs and 944/1,004 INDELs in these complex tandem repeats. Upon curation of the 21 variants unique to the trio-hicanu assembly, we found that most were errors in the benchmark, so we excluded all complex repeats that had any variants unique to the trio-hicanu assembly. Upon curation of the 53 remaining differences on GRCh38, variants with different genotypes or variants unique to the benchmark, we found that 20 were correct in the benchmark (often due to hicanu missing a haplotype), 17 were unclear (often due to C/G homopolymers), and 16 were errors in the benchmark (mostly due to noise in the HiFi reads in homopolymers and dinucleotide tandem repeats).

We evaluated the SV benchmark by comparing four callsets: (1) pbsv from PacBio HiFi alignments, (2) assembly-based calls using ONT and Illumina, (3) a union of 5 Illumina-based callers, and (4) a union of 2 ONT-based callers. We found the benchmark reliably identified false positives and false negatives across all 4 callsets. Upon manual curation only two sites were identified as problematic: a 50 bp net insertion that was represented as two smaller insertions in the tandem repeat in the benchmark, and a 376 bp deletion in the benchmark that was represented in many ONT alignments as two smaller deletions in the tandem repeat. Although these are correct in the benchmark, current SV benchmarking tools fail to compare these different representations, so we exclude these regions from the v1.0 SV benchmark bed. In addition, we found that some callers represent tandem duplications and tandem repeat expansions as duplications with SVTYPE=DUP (and sometimes incorrectly as translocations or inversions), whereas our benchmark calls these as insertions with the annotation REPTYPE=DUP. These can be counted as true positives by ignoring SVTYPE (--type-ignore -p 0 option in truvari) or by changing SVTYPE from DUP to INS in the query VCF. We also compared Bionano optical mapping-based SV calls to the 50 benchmark SVs >=500 in size. Because many of these SVs were near the limit of detection of optical mapping, we curated these calls, and all were supported by the Bionano data.

Supplementary Note 4: New Stratifications

To enable users to better understand performance in new types of challenging regions included in this CMRG benchmark, we created new stratifications for regions where some variant callers may have particularly high error rates relative to the benchmark. The first new stratification is a 16 kb region in an intron of the gene *KMT2C* that has multiple copies in HG002 that are not present in GRCh38 (GRCh37 has similar problems in *KMT2C*, but to a lesser degree when using the hs37d5 decoy sequence). Mis-mapped reads, particularly from long reads, can cause a cluster of many false positives using mapping-based approaches, causing 277 of the 386 false positives in the HiFi-DeepVariant callset, as shown in **Supplementary Figure 11.** Similarly, additional small regions in *KMT2C* have high coverage by short reads, causing false positives. The second new stratification is for complex variants in tandem repeats longer than 100 bp. When more than one variant occurs in a long tandem repeat, the complex variant can often be represented in many different ways. Mapping-based methods sometimes filter part of the complex variant, which can cause the variants in the tandem repeat to be counted as many false positives and false negatives. These contribute to a significant fraction of all false positives and false negatives for most methods (e.g., 28% of errors for HiFi-DeepVariant and 24% of errors for Illumina-GATK). **Supplementary Figure 10** shows an example of a complex variant in a tandem repeat in a coding region of *MUC5B*.

Supplementary Note 5: Medically Relevant Genes not yet benchmarked

The hifiasm assembly resolved both haplotypes of *LPA*, which is an important gene often related to cardiovascular diseases, but it is excluded in the CMRG benchmark due to very large insertions that cause a break in contig alignments (**Supplementary Figure 13)**. The general structure of *LPA* contains multiple tandemly duplicated copies of the same region (ie. kringle IV repeats of ~8 kb). These repeats often range between 10 and 50 repeats that are transcribed and translated[28,29]. The overall repeat number was associated with cardiovascular disease risk and is thus important to resolve correctly. The HG002 hifiasm assembly resolved the entire *LPA* region, including the kringle IV repeats, which have a total length of 7.2 kb in GRCh38. We confirmed that the 44.1 kb and 99.9 kb insertion sizes from hifiasm for the maternal and paternal haplotypes, respectively, were consistent with the insertions predicted by an independent trio-phased Bionano optical mapping assembly (45.0 kb and 101.2 kb). This complex, large expansion of the kringle IV repeats can be represented in many different ways in a VCF with different levels of precision (e.g., as a large insertion, a tandem duplication, or a CNV, and the copies may differ or include small variants). Existing benchmarking tools cannot compare these different representations robustly, partly limited by the VCF format[30]. To benchmark assemblies of this gene in HG002, the sequences could be compared directly to the hifiasm contigs, which we have annotated for *LPA* and other genes using LiftOff[31]. *CR1*, a gene implicated in Alzheimer's disease[8], is similarly resolved by hifiasm but contains a large SV that causes a break in the dipcall/minimap2 alignment (**Supplementary Figure 14)**. For *CR1* in the GRCh38 region chr1:207538089-207573740, the reference allele is 35.6 kb in length. Both paternal and maternal alleles of HG002 are 17.1 kb in length, or a 18.5 kb homozygous deletion, which is consistent with the Bionano deletion prediction of 18.6 kb for both alleles.

Other genes are excluded from the benchmark because they have extra copies in HG002 but not in GRCh38. For example, genes in the KIR region are highly variable and CNVs are observed frequently in the population, with 35 alternate loci and 15 novel patches in GRCh38.p13. Hifiasm resolves the paternal allele in a single contig, but the maternal allele is split into 3 contigs in the KIR region. The maternal allele has an extra copy of the gene *KIR2DL1* that is tandemly duplicated, so that minimap2 aligns both copies to the region, and this duplication is supported by alignments of short-, linked-, and long-reads (**Supplementary Figure 15**). There is no standard way to represent or benchmark small variants in duplicated regions, so we excluded these in our benchmarks. In addition, further work will be needed to fully resolve the maternal haplotype in a single contig in the KIR region. Similarly, the medically important gene *PRSS1* has an extra divergent copy in both HG002 alleles that is similar to an alternate locus in GRCh38 and to the decoy sequence hs37d5 for GRCh37 (**Supplementary Figure 16**). *DUX4* in the D4Z4 locus is also excluded in the CMRG benchmark because it is not well-represented in GRCh38 or GRCh37, with many gaps, and the gene has very high coverage due to multiple copies of the gene in HG002 but not in GRCh38 (**Supplementary Figure 17**).

Supplementary Table 1: Results of Long Range PCR and Sanger Sequencing to confirm variants in genes with segmental duplications in the new HG002 medically relevant gene benchmark.

Gene	Long Range PCR Amplicon Size (bp)	Total Variants	Number of Variants Definitively Confirmed	Number of Variants With No Primer Coverage	Number of Variants Supported (Not Definitively Confirmed)	Number of Variants Covered but Noisy Sanger traces	Number of Variants Contradicted	Reasons for variants not definitively confirmed
CBS	12,216	28	19	5	3	0	0	3 variants fall in a long tandem repeat with a complex variant; the 1 insertion and 1 deletion in the benchmark are likely equivalent to the Sanger and HiFi representation of a series of SNVs
DCLRE1C	12,543	8	5	3	0	0	0	N/A
FCGR2B	14,703	38	34	2	2	0	0	N/A
FLG	7,625	27	24	3	0	0	0	N/A
FXN	3,984	29	23	0	5	1	0	Messy sequencing doesn't allow for call of variant
GTF2IRD2	21,791	51	24	21	2	4	0	Messy sequencing doesn't allow for call of variant
HYDIN	3,392	1	0	0	1	0	0	Long homopolymer shows variant at location but sequencing around it is messy
NCF1	6,466	4	4	0	0	0	0	N/A
NLRP7	12,067	54	24	15	6	9	0	Messy sequencing doesn't allow for call of variant
PKD1 exons 2-7		1	1	0	0	0	0	N/A
PKD1 exons 8-12		2	1	0	0	0	1	1 variant from benchmark is not supported by either of the 2 primers
PKD1 exons 22-26		2	2	0	0	0	0	N/A
PKD1 exons 27-34		5	5	0	0	0	0	N/A
RHCE region 1	6,226	85	41	32	7	5	0	Messy sequencing doesn't allow for call of variant
RHCE region 2	10,000	18	18	0	0	0	0	N/A