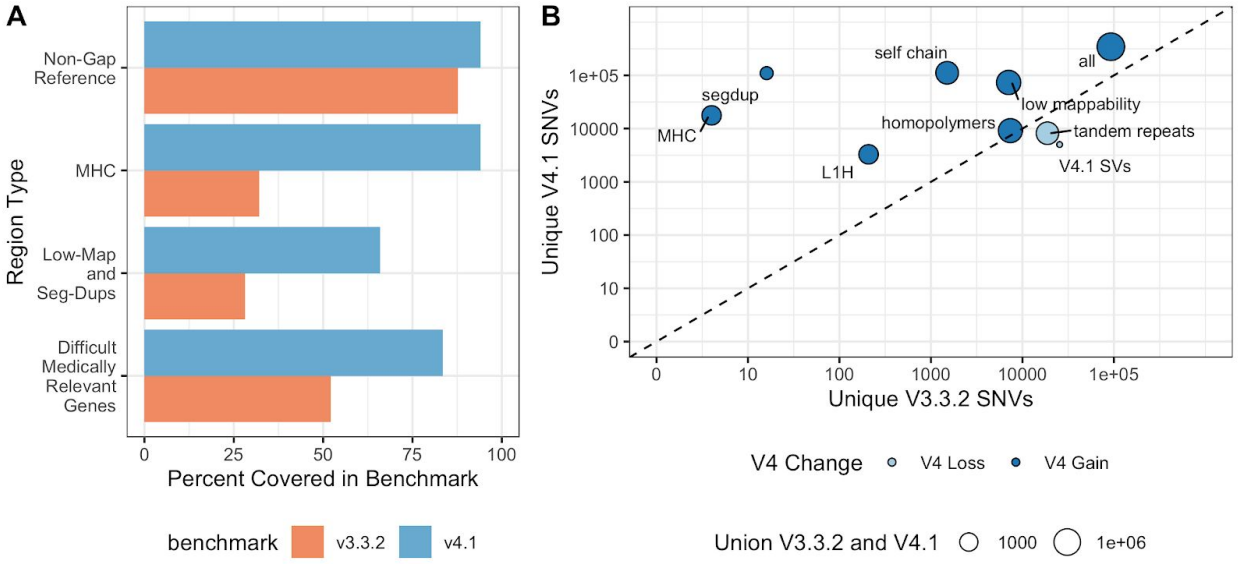
Supplementary Information:
Benchmarking challenging small variants with linked and long reads

Supplementary Table 1: Comparison of v4.1 to v3.3.2 using hap.py with v2.0 genome stratifications are available in SupplementaryTable1_HG002_GRCh37_1_22_truth_v4.1_in_benchmark_query_v3.3.2_in_benchmark_stratification.extended.xlsx



Supplementary Figure 1: New benchmark set for GRCh37 covers more of the reference genome and includes more SNVs and indels**.**

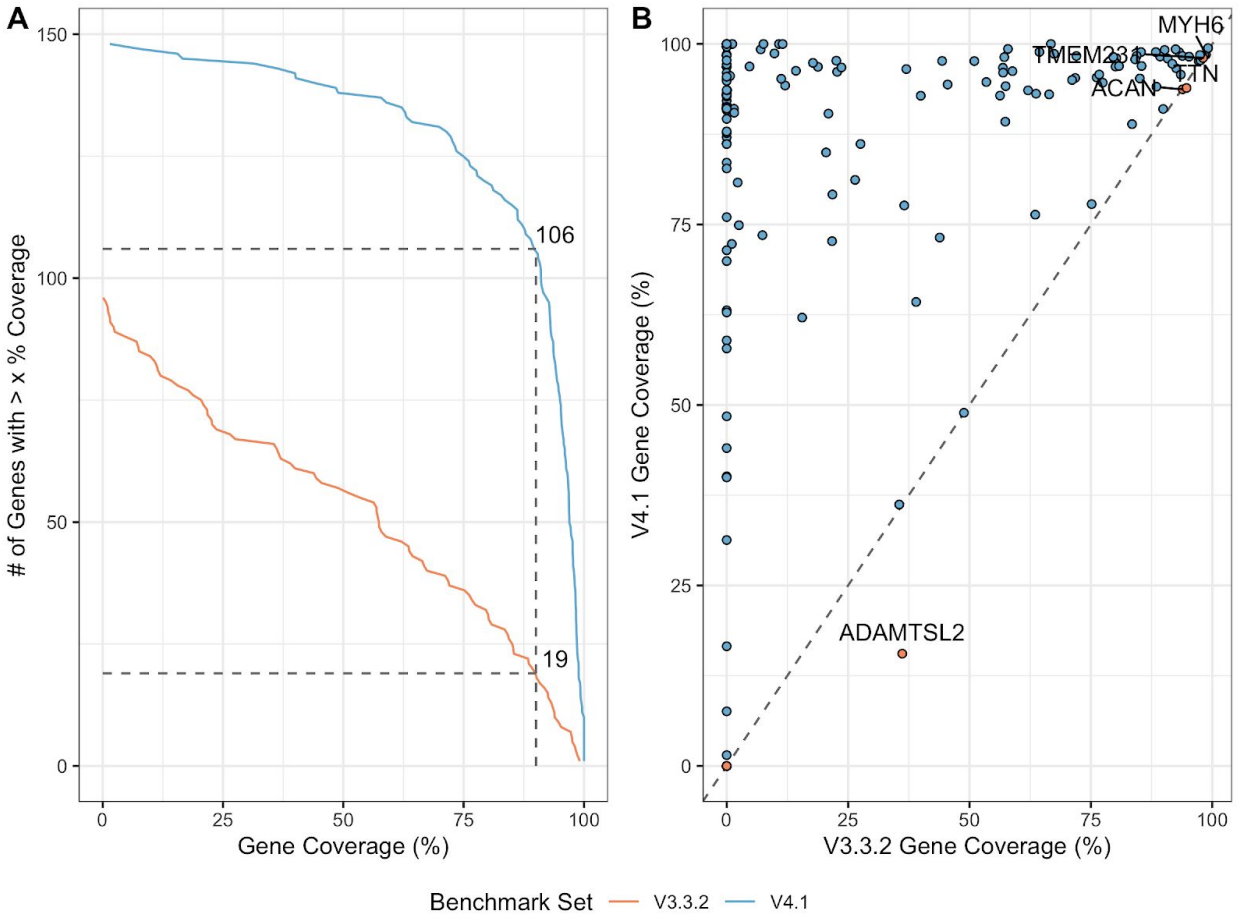
Supplementary Table 2: Errors in v3.3.2 identified in (Wenger et al. 2019) that are updated now matching PacBio HiFi callset or removed from benchmark regions.

Chromosome	Position	Result	Region Type
4	11,468,804	Outside v4.1 benchmark regions	
5	42,740,225	Outside v.41 benchmark regions	LINE:L1PA2
2	5,143,996	Call matches in benchmark region	

13	48,291,499	Outside v4.1 benchmark regions	LINE:L1PA3
8	5,930,728	Outside v4.1 benchmark regions	
15	41,943,823	Outside v4.1 benchmark regions	
6	9,737,425	Outside v4.1 benchmark regions	
7	157,385,671	Reference call in benchmark regions	
17	32,064,214	Outside v4.1 benchmark regions	
1	94,256,825	Call matches in benchmark region	LINE:L1PA2
2	153,864,971	Call matches in benchmark region	LINE:L1HS
4	112,819,087	Call matches in benchmark region	LINE:L1HS
4	165,026,074	Call matches in benchmark region	LINE:L1PA2
11	23,338,682	Call matches in benchmark region	LINE:L1P1
1	35,034,071	Call matches in benchmark region	LINE:L1HS
3	79,181,734	Call matches in benchmark region	LINE:L1HS
4	94,532,444	Call matches in benchmark region	LINE:L1HS
8	46,873,565	Outside v4.1 benchmark regions	
9	22,350,168	Call matches in benchmark region	LINE:L1PA2
21	42,288,851	Call matches in benchmark region	LINE:L1PA2

Supplementary Table 3: Benchmark set overlap of 163 difficult, medically-relevant genes in GRCh37. There are 10,152,047 bps in GRCh37 for medically-relevant genes that are difficult to sequence for short reads genes on the primary assembly for chromosomes 1-22.

Benchmark Set	Coverage	SNVs	INDELS
v3.3.2	5,283,743 (52.0%)	6,364	997
v4.1	8,513,217 (83.9%)	10,957	1,483



Supplementary Figure 2: GRCh37 v4.1 coverage of difficult, medically-relevant genes.

Supplementary Table 4: Long PCR and Sanger sequencing results for confirmation of challenging variants. We confirmed all new variants covered cleanly by Sanger sequencing in 10 challenging genes and 4 challenging LINEs , affirming the accuracy of v4.1 in these new difficult regions.

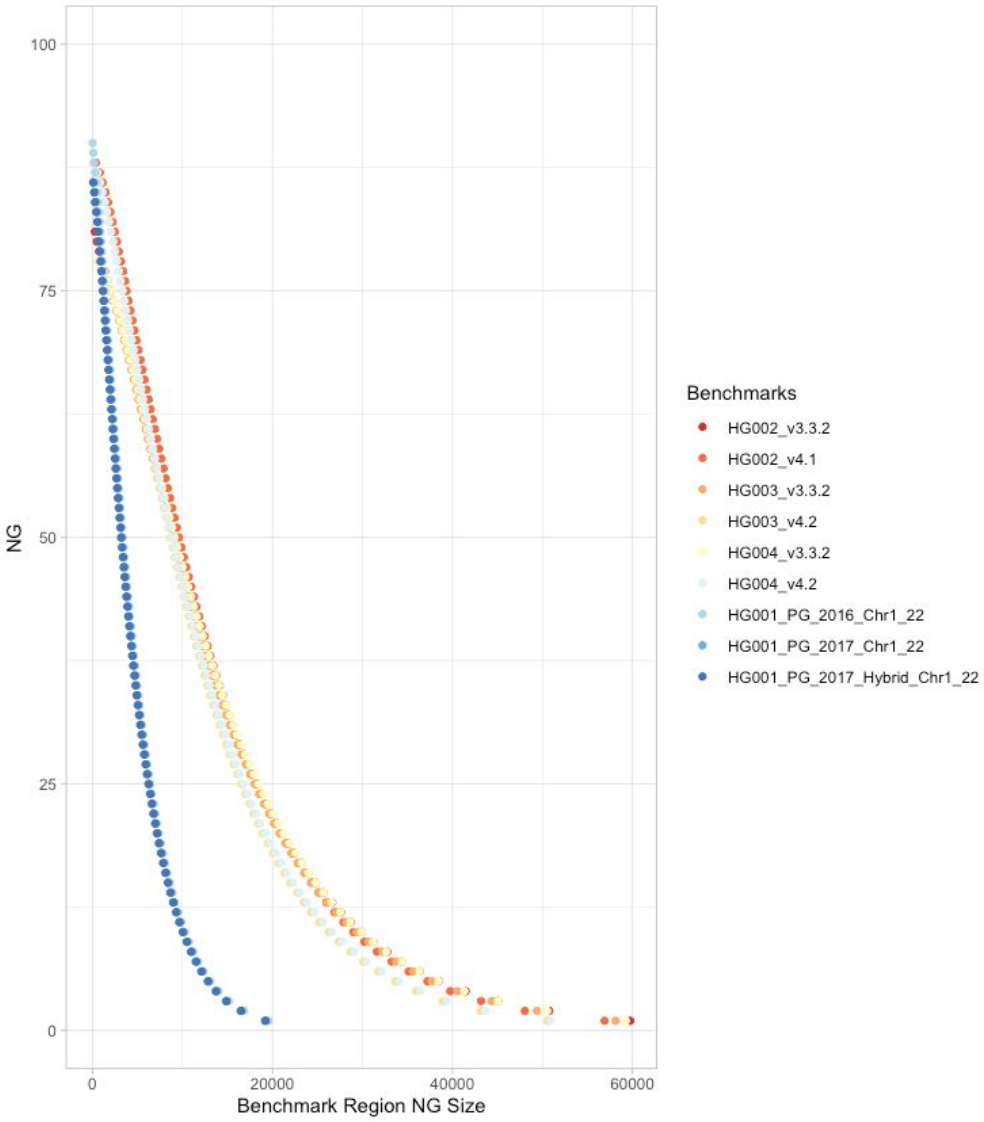
	Total Variants Covered by Sanger	Total Variants Covered by LR PCR	Variants NOT Covered by Sanger	Variants Covered but Not Confirmed by Sanger	Notes
TnxA	4	7	0	3	Messy sequencing around variant sites
TnxB	14	14	0	0	
C4A	1	1	0	0	
C4B	2	2	0	0	

DMBT1	4	4	0	0	
STRC	6	6	0	0	
HSPG2	21	22	0	1	Variant very close to beginning of LR PCR product
Cyp2D6	18	18	0	0	
Cyp21A	12	16	0	4	Messy sequencing around variant sites
LINE chr1	8	8	0	0	
LINE chr3	5	5	0	0	
LINE chr9	17	17	0	0	
LINE chr21	20	20	0	0	
PMS2	5	5	0	0	

Supplementary Table 5: Manual curation results of 10 random sites in HG002 v4.1 that match Category 1 SNVs in Platinum Genomes.

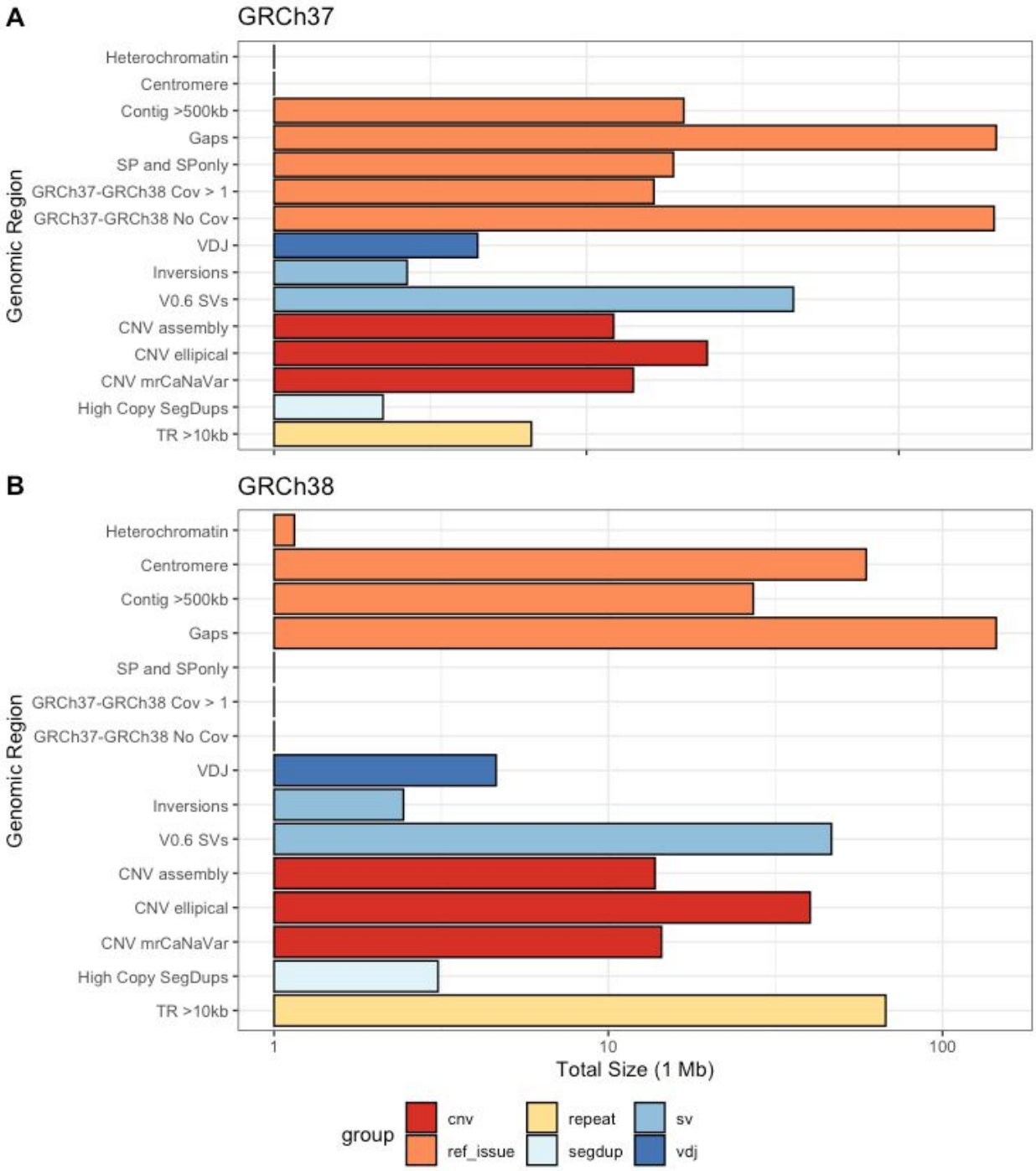
Chromosome	Position	Curation	Notes
16	18288432	Selfchain/segdup	Many variants on one HP CCS, in selfchain/segdup
19	54726776	Selfchain/segdup	Cluster of variants in 10x/Illumina nearby and CCS has more variants on one HP than the other. In high depth selfchain/segdup that is smaller than 10kb
19	41379908	Selfchain/segdup	Cluster of variants, in LINE:L1MA3. In depth 2 segdup and depth 2 selfchain
8	11872949	Selfchain/segdup	Potential SV in segdup, since CCS and ONT have clipped reads nearby. Cluster of CAT1 variants
15	20360478	Possible CNV	Likely CNV given CCS data and high coverage in ONT. Several CAT1 variants in the region.
8	7223157	Selfchain/segdup	Cluster of CAT1 variants, in segdup and normal coverage but in a cluster of variants on one HP CCS and ONT, so may be more complex
15	20453992	Possible CNV	Cluster of CAT1 variants, cluster of variants on one HP CCS, and large change in coverage in CCS and

			nearby SV
7	149749666	Possible CNV	Cluster of CAT1 variants. Large changes in coverage in region in CCS data but overall looks reasonable.
15	20454464	Possible CNV	Many CAT1 variants in region, large change in CCS coverage in region, near what appears to be SV that is excluded from v4.1
12	74899879	Possible CNV	Somewhat elevated CCS coverage. In LINE:L1PA3.



Supplementary Figure 5: The Benchmark Region NG_X is the benchmark region that contains at least X% of the total bases covered by the benchmark region. This metric is the same as

Assembly NG50 except that benchmark region is used in place of contig length. The contiguity of the benchmark improves in v4 compared to v3.3.2 and Platinum Genomes.



Supplementary Figure 6: **Base pairs in genomic regions excluded for all input variant call sets.**

Supplementary Table 6: Errors in v3.3.2 GRCh38 corrected hap.py results are available in SupplementaryTable6_GRCh38_v3.3.2_corrections.xlsx

Supplementary Table 7: Problematic regions in GRCh38 v3.3.2 that were near or in the centromere.

GIAB Sample	Chromosome	Start	End	Region Type
HG002	chr8	43637994	43672749	Centromere
HG002	chr8	43603010	43637285	Centromere
HG002	chr8	43831369	43864819	Centromere
HG002	chr7	62742402	62800702	q11.21 (near centromere)
HG002	chr7	57925899	57969199	p11.1 (centromere)
HG002	chr7	54317738	54350806	p11.2
HG002	chr7	62821943	62851720	q11.21
HG002	chr5	46337535	46371375	Centromere
HG002	chr5	46009909	46041150	Centromere
HG002	chr3	90613721	90676762	Centromere
HG002	chr3	90268364	90303792	Centromere
HG002	chr3	90445745	90478995	Centromere
HG002	chr19	27523978	27570562	Centromere
HG002	chr12	37624574	37664823	Centromere
HG002	chr12	34536432	34575253	Centromere
HG002	chr12	34483102	34520344	Centromere
HG002	chr12	37342974	37379851	Centromere
HG002	chr11	50785848	50821348	p11.12 (near centromere)
HG002	chr10	39052350	39083950	Centromere
HG002	chr10	39116363	39147923	Centromere
HG003	chr8	43601909	43637285	Centromere
HG003	chr8	43637994	43672749	Centromere
HG003	chr7	62742945	62800702	q11.21 (near centromere)
HG003	chr5	50193424	50229094	Centromere
HG003	chr5	46337535	46371375	Centromere
HG003	chr4	8843663	8892454	p16.1
HG003	chr3	90598264	90676761	Centromere
HG003	chr3	90268364	90303792	Centromere

HG003	chr3	90411794	90445037	Centromere
HG003	chr3	90445745	90478939	Centromere
HG003	chr22	22145576	22178716	q11.22
HG003	chr19	27523978	27577850	Centromere
HG003	chr12	37624574	37664823	Centromere
HG003	chr12	34536383	34575253	Centromere
HG003	chr12	34482540	34520344	Centromere
HG003	chr12	37342974	37379851	Centromere
HG003	chr11	50772422	50821348	p11.12 (near centromere)
HG003	chr10	39013337	39083750	Centromere
HG003	chr10	39116363	39153579	Centromere
HG003	chr10	39183589	39216647	Centromere
HG004	chr8	43637994	43672749	Centromere
HG004	chr8	43831342	43864819	Centromere
HG004	chr7	62742945	62815024	q11.21
HG004	chr7	57925899	57969199	Centromere
HG004	chr5	46009909	46041150	Centromere
HG004	chr5	50193424	50223736	Centromere
HG004	chr4	144161988	144192833	q31.21
HG004	chr3	90445745	90478957	Centromere
HG004	chr3	90507640	90536550	Centromere
HG004	chr2	88861923	88891174	p11.2
HG004	chr19	27523978	27559503	Centromere
HG004	chr12	37263197	37300537	Centromere
HG004	chr12	37342828	37379552	Centromere
HG004	chr12	63767401	63796912	q14.2
HG004	chr12	37815851	37844396	Centromere
HG004	chr12	34492116	34520344	Centromere
HG004	chr11	50772422	50806467	p11.12
HG004	chr10	39120199	39153579	Centromere
HG004	chr10	39055460	39087970	Centromere
HG004	chr10	39183589	39213934	Centromere

Supplementary Table 8: Sequencing technology, mapping or assembler method, and variant caller that was used to generate each evaluation call set. The names used in **Figure 6** are in the fourth column.

Sequencing Technology	**Variant Caller**	**Mapper/Assembler**	**Figure 6 Name**
PacBio HiFi	DeepVariant	mm2	PB DV-mm2
PacBio HiFi	GATK4	mm2	PB GATK4-mm2
PacBio HiFi	Clair	mm2	PB Clair-mm2
PacBio HiFi	DV	Duplomap	PB DV-Duplomap
PacBio HiFi	dipcall	WHDenovo	PB Dipcall-WHDenovo
Illumina PCR-Free TruSeq 2x250bp	Dragen	Dragen	Ill Dragen
Illumina PCR-Free TruSeq 2x250bp	Dragen	VG	Ill Dragen-VG
Illumina PCR-Free HiSeq 2x150bp	SevenBridges	SevenBridges Graph Aligner	Ill SevenBridges GRAF
Illumina PCR-Free HiSeq 2x150bp	xAtlas	NovoAlign	Ill xAtlas
Illumina PCR-Free NovaSeq 2x250bp	GATK	BWA	Ill GATK-BWA
10x Genomics	LongRanger	LongRanger	10x LongRanger
10x Genomics	paftools	Aquila	10x paftools-Aquila
ONT	Clair	mm2	ONT Clair-mm2
ONT	Clair	ngmlr	ONT Clair-ngmlr

Supplementary Note 1: Benchmark Evaluation Call set Generation

Variant callsets used in evaluation

PacBio HiFi reads with GATK Haplotype Caller

HG002 HiFi reads from three publicly available datasets were aligned to the GRCh37 and GRCh38 references using the pbmm2 v0.10.0 with `--preset CCS`. Small variants were called with GATK v4.0.10.1 HaplotypeCaller with `--pcr-indel-model AGGRESSIVE` and `--minimum-mapping-quality 10`. Variants were filtered on the QD (Quality by Depth) value with GATK v4.0.10.1 Variant Filtration, such that:

- SNVs with QD < 2 are filtered
- Indels > 1bp with QD < 2 are filtered
- 1 bp Indels with QD < 5 are filtered

Instrument	Insert Size	SRA	FTP
Sequel I System	10 kb	--	ftp://ftp.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG002_NA24385_son/PacBio_CCS_10kb
Sequel I System	15 kb	SRX5327410	ftp://ftp.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG002_NA24385_son/PacBio_CCS_15kb
Sequel II System	11 kb	SRX5527202	ftp://ftp.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG002_NA24385_son/PacBio_SequelII_CCS_11kb

GRCh37 reference used for alignment:
ftp://ftp-trace.ncbi.nih.gov/1000genomes/ftp/technical/reference/phase2_reference_assembly_sequence/hs37d5.fa.gz
GRCh38 reference used for alignment:
ftp://ftp.ncbi.nlm.nih.gov/genomes/all/GCA/000/001/405/GCA_000001405.15_GRCh38/seqs_for_alignment_pipelines.ucsc_ids/GCA_000001405.15_GRCh38_no_alt_analysis_set.fna.gz
https://github.com/PacificBiosciences/pbmm2
https://github.com/broadinstitute/gatk/releases/tag/4.0.10.1

PacBio Hifi reads using minimap2 with DeepVariant

A set of ~80x coverage PacBio CCS data was mapped to each reference:

minimap2 VN:2.15-r905
minimap2 -ax asm20 -t 32

(Note that the mapping of these files predates some improved recommendations for mapping to use pbmm2)

DeepVariant v0.8 with the PACBIO model was applied to the mapped files. The commands and workflow used are identical to the DeepVariant case-study:

https://github.com/google/deepvariant/blob/r0.8/docs/deepvariant-pacbio-model-case-study.md

No filtering is applied.

PacBio HiFi reads re-aligned using Duplomap

HG002 HiFi reads aligned to the GRCh37 reference using Minimap2 were downloaded from ftp://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG002_NA24385_son/PacBio_CCS_15kb_20kb_chemistry2/ and reads overlapping segmental duplications were realigned using a tool Duplomap (https://gitlab.com/tprodanov/duplomap) that used paralogous sequence variants to map reads with multiple possible alignment locations. Small variants were called from the realigned bam file using DeepVariant v.0.8 with default parameters.

10x Genomics using Aquila local assembly

Aquila uses linked-read data for generating a high quality diploid genome assembly, from which it then comprehensively detects and phases personal genetic variation. Here, Aquila merged two link-reads libraries to generate WGS variant calls for NA24385. Assemblies and VCFs for this merged library L5 + L6 can be found at http://mendel.stanford.edu/supplementarydata/zhou_aquila_2019/. The raw linked-reads fastq files can be downloaded in the Sequence Read Archive and its BioProject accession number is PRJNA527321.

Illumina TruSeq DNA PCR-Free reads with Illumina Dragen Bio-IT platform

Illumina PCR-Free reads (2 x 250bp with 350bp insert size) are downloaded from the public file server. Dragen 3.3.7 is used to perform alignment, variant calling, and filtering on GRCh37 and GRCh38 reference assemblies. Variant filtering is based on MQ (Mapping Quality), MQRankSum (Z-score From Wilcoxon rank sum test of Alt vs Ref read MQs), and ReadPosRankSum (Z-score from Wilcoxon rank sum test of Alt vs Ref read position bias) values. For SNVs, MQ < 30.0, MQRankSum < -12.5, or ReadPosRankSum < -8.0 are filtered out. For INDEL, ReadPosRankSum < -20.0 are filtered.

Illumina PCR-Free reads are downloaded from ftp://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG002_NA24385_son/NIST_Illumina_2x250bps/reads/

Illumina TruSeq DNA PCR-Free reads with VG alignment and Illumina Dragen Bio-IT platform

Illumina PCR-Free read pairs (2 x 250bp with 350bp insert size) are downloaded from and extracted from

novoaligned bams that are hosted on the public file server. The process is based on aligning the HG002 to genome graphs that were constructed from HG003 and HG004 parental variants. All alignments are performed using Variation Graph Toolkit (VG) and variant calling is done using Dragen version 3.2. Default variant calling settings in Dragen 3.2 were used during GVCF and VCF variant calling. The methods used to convert graph alignments to linear alignments and parental graph construction are in the workflow defined on the vg_wdl GitHub repository.

The workflow used to process this data can be found here
https://github.com/vgteam/vg_wdl/blob/master/workflows/vg_trio_multi_map_call.wdl

Illumina PCR-Free reads for the trio used in parental graph construction and HG002 alignment are downloaded from
ftp://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG002_NA24385_son/NIST_Illumina_2x250bps/novoalign_bams/
ftp://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG003_NA24149_father/NIST_Illumina_2x250bps/novoalign_bams/
ftp://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG004_NA24143_mother/NIST_Illumina_2x250bps/novoalign_bams/

The population data used for initial graph alignments of the HG002 trio samples are based on the 1000 genomes phase 3 variant dataset and the GRCh37 reference genome.
http://ftp.1000genomes.ebi.ac.uk/vol1/ftp/release/20130502/ALL.wgs.phase3_shapeit2_mvncall_integrated_v5b.20130502.sites.vcf.gz

10x genomics using LongRanger with GATK Haplotype Caller

These callsets, generated independently for each individual in the Ashkenazi trio, used LongRanger[21] (version 2.2, code at https://github.com/10XGenomics/longranger) and GATK v4.0.0.0 as variant caller with default parameters on 10x Genomics linked-reads data for the family trio (84x, 70x, and 69x coverage for HG002 NA24385 son, HG003 NA24149 father, and HG004 NA24143 mother, respectively) against both GRCh37 and GRCh38. The vcf and bam files for each genome are under:
ftp://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/analysis/10XGenomics_ChromiumGenome_LongRanger2.2_Supernova2.0.1_04122018/

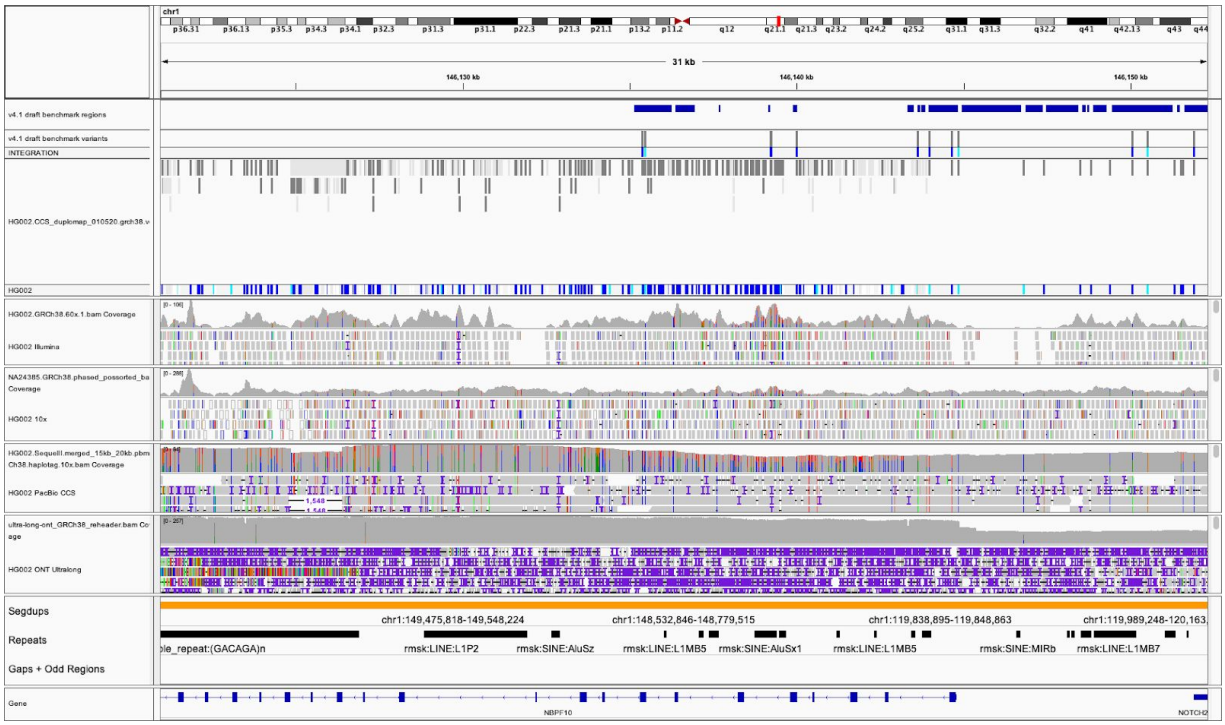
The variant curation used the 10x Genomics VCF from LongRanger 2.2 (SRA accession SRX2225480), which is available at:
ftp://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/analysis/10XGenomics_ChromiumGenome_LongRanger2.2_Supernova2.0.1_04122018/GRCh37/NA24385_300G/NA24385.GRCh37.phased_variants.vcf.gz

All samples were sequenced on the Illumina Xten at 2x150bp. The Ashkenazim trio was done using the v1 of the 10x library prep protocol.

HiFi Clair

This callset was generated using Sequel II 11kbp HiFi reads aligned to the hs37d5 reference with pbmm2, publicly available here: ftp://ftp-trace.ncbi.nlm.nih.gov/ReferenceSamples/giab/data/AshkenazimTrio/HG002_NA24385_son/PacBio_SequelII_CCS_11kb/. The variants were called by using Clair (v1) on these alignments.

Supplementary Table 9: Manual Curation Results are available in SupplementaryTable9_v4.1_ManualCurationResults.xlsx



Supplementary Figure 7: The benchmark and callset both make calls in this region where there is likely a large duplication in HG002 compared to GRCh38 that was not detected by our exclusion criteria. This specific example is a FP SNP in PacBio HiFi Duplomap DV where the benchmark region indicates a reference call at this location.

Supplementary Note 2: Process from v4alpha to v4.1

The Genome in a Bottle Consortium has an iterative evaluation process to ensure new benchmarks are useful for assessing performance across diverse sequencing technologies and variant calling methods. The first version using PacBio HiFi and 10x Genomics data was v4alpha. In particular, GIAB found that some regions contained unreliable calls across technologies, and these regions were excluded from subsequent releases. In addition, the

PacBio HiFi data changed during releases as new data were collected, and the 10x Genomics data remained constant at ~84x coverage.

The v4alpha release used PacBio Sequel I HiFi ~15 kb reads at ~28x coverage, with the difficult regions below (Bold entries changed from v3.3.2):

Difficult Region Description	Method Excluded From
All candidate structural variant regions from the Son-Mother-Father Trio	All methods
All tandem repeats < 51bp in length	All methods except GATK from Illumina PCR-free, Complete Genomics, and **PacBio CCS DeepVariant**
All tandem repeats > 51bp and < 200bp in length	All methods except GATK from Illumina PCR-free and **PacBio CCS DeepVariant**
All tandem repeats > 200bp in length	All methods except **PacBio CCS DeepVariant**
Perfect or imperfect homopolymers > 10bp	All methods except GATK from Illumina PCR-free
Segmental duplications from Eichler *et al*.	All methods except **10X Genomics and PacBio CCS**
Segmental duplications > 10Kbp from self-chain mapping	All methods except **10X Genomics and PacBio CCS**
Regions homologous to contigs in hs37d5 decoy	All methods except **10X Genomics and PacBio CCS**
Difficult to map regions for short reads	All methods except **10X Genomics and PacBio CCS**
Homopolymer > 6bp in length	**All methods except GATK from Illumina PCR-free and Complete Genomics**

The v4beta release used PacBio Sequel I HiFi ~15 kb reads at ~28x coverage. Additionally, v4beta used additional tandem repeat files from UCSC, excluded the entire tandem repeat if any part was not in the benchmark BED, and changed the difficult regions below:

Difficult Region Description	Method Excluded From
v0.6 SV Benchmark	All methods
Regions that are collapsed and expanded from GRCh37/38 Primary Assembly Alignments (corrected)	All methods
Diploid assemblies exhibit more than 2 contigs aligned > 10kb	All methods
Intersected short and long read based copy number > 2.5 (updated)	All Methods
Segmental duplications > 10Kb, Identity > 99%, Count > 5	All methods
mrCaNaVar duplications > 10kb (052119)	All methods except 10X Genomics and PacBio CCS
Outliers from long read coverage	All Methods
LINE:L1Hs > 500	All methods except Illumina MatePair, 10X Genomics, and PacBio CCS
All Tandem Repeats > 10kb in length	All methods

The v4.0 release used PacBio Sequel II HiFi ~11 kb reads at ~32x coverage, updated to DeepVariant v0.8.

The v4.1 release used PacBio Sequel II HiFi ~15 kb and ~20 kb reads at ~52x coverage. The diploid assembly-based MHC benchmark was used for the MHC region in v4.1. We also added the difficult regions below:

Difficult Region Description	Method Excluded From
Potential copy number variation including CCS and ONT outlier and CCS, ONT, mrCanavar intersection	**All methods**
VDJ	**All methods**
Inversions	**All methods**

The v4.2 release is the first for HG003 and HG004, and it used hifiasm to perform the assembly of PacBio HiFi reads in the MHC, and used dipcall with this assembly to call variants, including in segmental duplications that were previously not assembled properly. Since it represents complex variants as individual SNVs and indels, dipcall helps improve partial credit in some cases for variants that are only partially called correctly by the query callset. We also excluded entire homopolymers and tandem repeats in the MHC if they were not completely covered by the benchmark bed. Since calls were made for HG003 and HG004 in addition to HG002, we also performed a trio Mendelian analysis and excluded Mendelian violations from the benchmark regions for all individuals (except putative de novo variants in HG002 were not excluded from the benchmark regions).

Supplementary Table 10: Primer Sequences for Long-Range PCR are available in SupplementaryTable10_PrimerSequences.xlsx

Supplementary Table 11: Long Range PCR Components

	LINEs	***C4A***	***C4B***	***Cyp21A2***	***Cyp2D6***	***DMBT1***	***HSPG2***	***PMS2***	***STRC***	***TnxA***	***TnxB***
Buffer (5X)	1X	1X	1X	1X	1X	1X	1X	1X	1X	1X	1X
dNTP (250uM each)	250uM	400uM	400uM	250uM	0.3mM	400uM	200uM	400uM	400uM	250uM	250uM
Forward Primer	0.25uM	0.5uM	0.5uM	10uM	0.5uM	0.4uM	0.3uM	0.2uM	0.4uM	10uM	10uM
Reverse Primer	0.25uM	0.5uM	0.5uM	10uM	0.5uM	0.4uM	0.3uM	0.2uM	0.4uM	10uM	10uM

Polymerase (1.25 units/uL)	1.25 U	1.25 U	1.25 U	1.25 U	1.25 U	2.5 U	0.5 U	1.25 U	2 U	0.5 U	0.5 U
DNA	300ng	100ng	100ng	250ng	1uL	2uL	300ng	100ng	300ng	250ng	250ng
Water	To 50uL	To 50uL	To 50uL	To 30uL	To 25uL	To 50uL	To 50uL	To 25uL	To 50uL	To 30uL	To 30uL

Supplementary Table 12: **Long Range PCR Conditions**

Gene	PCR Conditions
LINEs	30 cycles of 98°C for 10 seconds, 60°C for 15 seconds, and 68°C for 8 minutes.
C4A	98°C for 2 minutes; followed by 40 cycles of 98°C for 45 seconds, 66°C for 60 seconds, and 72°C for 9 minutes, with a final extension step of 72°C for 10 minutes.
C4B	98°C for 2 minutes; followed by 8 cycles of 94°C for 45 seconds, 64°C for 60 seconds, with a decrease of 0.5°C per cycle, and 72°C for 9 minutes; followed by 30 cycles of 94°C for 45 seconds, 59°C for 60 seconds, and 72°C for 9 minutes, with an increase of 10 seconds per cycle, with a final extension step of 72°C for 15 minutes
Cyp21A2 *TnxA* *TnxB*	94°C for 4 minutes; followed by 12 cycles of 94°C for 30 seconds, 62°C for 40 seconds, and 68°C for 5 minutes; followed by 16 cycles of 94°C for 30 seconds, and 68°C for 5 minutes.
Cyp2D6	96°C for 30 seconds; followed by 30 cycles of 94°C for 15 seconds, 68°C for 30 seconds, and 68°C for 7 minutes, with a final extension step of 68°C for 30 minutes.
DMBT1	94°C for 1 minute; followed by 30 cycles of 98°C for 10 seconds, and 68°C for 15 minutes, with a final extension step of 72°C for 10 minutes.
HSPG2	30 cycles of 98°C for 10 seconds, 60°C for 15 seconds, and 68°C for 10 minutes.
PMS2	94°C for 1 minute; followed by 35 cycles of 94°C for 10 seconds, and 65°C for 30 seconds, and 68°C for 15 minutes, with a final extension step of 72°C for 10 minutes.
STRC	93°C for 3 minutes; followed by 38 cycles of 93°C for 15 seconds, 64°C for 30 seconds, and 68°C for 17 minutes, with a final extension step of 68°C for 5 minutes.