A Comparison of Variant Calling Pipelines Using Genome in a Bottle as a Reference

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High-throughput sequencing, especially of exomes, is a popular diagnostic tool, but it is difficult to determine which tools are the best at analyzing this data. In this study, we use the NIST Genome in a Bottle results as a novel resource for validation of our exome analysis pipeline. We use six different aligners and five different variant callers to determine which pipeline, of the 30 total, performs the best on a human exome that was used to help generate the list of variants detected by the Genome in a Bottle Consortium. Of these 30 pipelines, we found that Novoalign in conjunction with GATK UnifiedGenotyper exhibited the highest sensitivity while maintaining a low number of false positives for SNVs. However, it is apparent that indels are still difficult for any pipeline to handle with none of the tools achieving an average sensitivity higher than 33% or a Positive Predictive Value (PPV) higher than 53%. Lastly, as expected, it was found that aligners can play as vital a role in variant detection as variant callers themselves.

1. Background

In the past few years there have been many advances made to high-throughput sequencing technologies. Due to these advances, it is now possible to detect a great number of potential disease-causing variants [1], and, in a few cases, next generation sequencing (NGS) data has even been used for diagnostic purposes [2–4]. This is partially due to the developments in sequencing technologies over the past few years but also due to the number of improvements made to the various bioinformatic tools used to analyze the mountains of data produced by NGS instruments [5].

When searching for mutations in a patient, a typical workflow is to sequence their exome with an Illumina sequencer, align the raw data to the human reference genome, and then identify single nucleotide variants (SNVs) or short insertions and deletions (indels) that could possibly cause or influence the phenotype of interest [6]. While this is fairly straightforward, deciding on the best tools to use at each stage of the analysis pipeline is not. There are a large number of tools that are used in various intermediate steps, but the two most important steps in the entire process are aligning the raw reads to the genome and then searching for variants (i.e., SNVs and indels) [7]. In this study, we aim to help today’s bioinformatician by elucidating the correct combination of short read alignment tool and variant calling tool for processing exome sequencing data produced by NGS instruments.

A number of these studies have been performed in the past, but they all had drawbacks of some form or another. Ideally one should have a list of every known variant contained in a sample so that when a pipeline of analysis tools is run, you can test it to know with certainty that it is performing correctly. However, in the past no such list existed, so validation had to be performed by less complete methods. In some instances, validation was performed by
generating simulated data so as to create a set of known true positives (TP) and true negatives (TN) [8–10]. While this conveniently provides a list of every TP and TN in the dataset, it does a poor job of accurately representing biology. Other methods of validating variant calling pipelines include using genotyping arrays or Sanger sequencing to obtain a list of TPs and false positives (FP) [11]. These have the upside of providing biologically validated results, but they also have the downside of not being comprehensive due to the limited number of spots on genotyping arrays and the prohibitive cost of Sanger validation when performed thousands of times. Lastly, none of these studies aimed at looking at the effect of the short read aligner had on variant calling. Consequently, the upstream effect of aligner performance could not be assessed independently.

In this study, we have the advantage of a list of variants for an anonymous female from Utah (subject ID: NA12878, originally sequenced for the 1000 Genomes project [12]) that was experimentally validated by the NIST-led Genome in a Bottle (GiaB) Consortium. This list of variants was created by integrating 14 different datasets from five different sequencers, and it allows us to validate any list of variants generated by our exome analysis pipelines [7]. The novelty of this work is to validate the right combination of aligners and variant callers against a comprehensive and experimentally determined variant dataset: NIST-GiaB.

To perform our analysis we will be using one of the exome datasets originally used to create the NIST-GiaB list. We chose only one of the original Illumina TruSeq-generated exomes because we wanted to provide a standard use case scenario for someone who wishes to perform NGS analysis, and while whole genome sequencing is continuing to drop in price, exome sequencing is still a popular and use case scenario for someone who wishes to perform NGS generated exomes because we wanted to provide a standard list. We chose only one of the original Illumina TruSeq-exome datasets originally used to create the NIST-GiaB list. Then, the novelty of this work is to validate the right combination of aligners and variant callers against a comprehensive and experimentally determined variant dataset: NIST-GiaB.

Table 1 shows the workflow used in this study, which is similar to the one outlined in the Best Practices guide produced by The Broad Institute [30]. This involves a number of steps to ensure that the alignment files produced are of the highest quality as well as several more to guarantee the variants are called correctly. First, raw reads were aligned to hg19, and then PCR duplicates were removed from the alignment. Next, to help with indel identification later in the pipeline, read realignment was performed around indels. The last step of alignment processing was to perform a base quality score recalibration step, which helps to ameliorate the inherent bias and inaccuracies of scores issued by sequencers. Unfortunately, despite these steps, the alignment rate of each aligner was significantly lower than expected, so to offset this, the fastx toolkit was used to filter out low quality reads (Table 1). Low quality reads were defined as those reads that had at least half of their quality scores below 30. Following alignment processing, variant calling and variant filtering were performed.

The six tools used to generate alignments were Bowtie2, BWA mem, BWA sampe, CUSHAW3, MOSAIK, and Novoalign, and the five tools used to generate variants were FreeBayes, GATK HaplotypeCaller, GATK UnifiedGenotyper, SAMtools mpileup, and SNPSVM, as can be seen in Table 2.

### Table 1: Alignment percentages for filtered reads and unfiltered reads. The average depth of coverage is for the alignment files created with the filtered reads.

| Aligner      | % reads aligned (unfiltered) | % reads aligned (filtered) | Average depth of coverage |
|--------------|------------------------------|---------------------------|--------------------------|
| Bowtie2      | 89.73                        | 98.73                     | 47.97                    |
| BWA mem      | 92.91                        | 99.85                     | 46.89                    |
| BWA sampe    | 85.95                        | 97.49                     | 46.67                    |
| CUSHAW3      | 85.00                        | 99.81                     | 47.69                    |
| MOSAIK       | 85.68                        | 96.22                     | 45.14                    |
| Novoalign    | 82.21                        | 94.20                     | 45.62                    |

### 2. Methods

#### 2.1. Datasets

Human reference genome hg19 was downloaded from the UCSC browser (http://hgdownload.cse.ucsc.edu/goldenPath/hg19/chromosomes/) and was used to perform the alignments. The human exome, SRR098401, was downloaded from the Sequence Read Archive (SRA) (http://www.ncbi.nlm.nih.gov/sra). For annotation and calibration purposes, dbSNP137 without sites after version 129, HapMap 3.3, Human Omni 2.5 BeadChip, and Mills and 1000 G gold standard indel set lists were used (all from ftp://ftp.broadinstitute.org/distribution/gsa/gatk_resources.tgz).

#### 2.2. The Pipeline

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### 2.3. Filtering

Raw data was acquired from the SRA (SRR098401), split with fastq-dump, and filtered using the fastx toolkit. Specifically, fastq-dump used the --split_files and --split_spot flags, and
Filter raw reads

Align reads to hg19 using six aligners

Remove PCR duplicates from alignments

Realign reads around indels

Recalibrate base quality scores

Perform variant calling using five variant callers

Filter variants using tool-specific filters

Compare variant to Genome in a Bottle results

Figure 1: Schematic of the data analysis pipeline used. To ensure that the highest quality alignments are created, reads are first filtered and then aligned to the human reference genome, hg19. Next, PCR duplicates are removed, reads are aligned around putative indels, and base quality scores are recalibrated. Finally, variants are called and validated against the NIST-GiaB list of variants.

Table 2: These are the 11 different tools used that made up the 30 (six aligners * five variant callers) different pipelines. Software versions are also included to ensure reproducibility.

| Tool          | Type        | Version | Reference |
|---------------|-------------|---------|-----------|
| Bowtie2       | Aligner     | 2.1.0   | [14]      |
| BWA sampe     | Aligner     | 0.7.5a  | [15]      |
| BWA mem       | Aligner     | 0.7.5a  | [16]      |
| CUSHAW3       | Aligner     | 3.0.3   | [17]      |
| MOSAIK        | Aligner     | 2.2.3   | [18]      |
| Novoalign     | Aligner     | 3.02.07 | N/A       |
| FreeBayes     | Genotyper   | v9.9.2-19-g011561f | [22] |
| GATK          | Genotyper   | 2.7-2   | N/A       |
| GATK UnifiedGenotyper | Genotyper | 2.7-2 | [23] |
| SAMtools mpileup | Genotyper | 0.1.19 | [24] |
| SNPSVM        | Genotyper   | 0.01    | [25]      |

fastq_quality_filter was run with the following arguments: -Q 33 -q 30 -p 50. Then reads were properly paired with a custom script.

2.4. Aligning. Aligners used default arguments except when a threads argument was used where available. The commands used are as follows.

2.4.1. Bowtie2

(l) bowtie2 -p 10 -x $INDEX -1 raw_data/read1_filtered.fastq -2 raw_data/read2_filtered.fastq -S alignments/NA12878.bt2.sam

2.4.2. BWA Sampe

(l) bwa aln -t 10 genome/hg19.fa raw_data/read1_filtered.fastq > alignments/NA12878.R1.sai

(2) bwa aln -t 10 genome/hg19.fa raw_data/read2_filtered.fastq > alignments/NA12878.R2.sai

(3) bwa sampe genome/hg19.fa alignments/NA12878.R1.sai alignments/NA12878.R2.sai raw_data/read1_filtered.fastq raw_data/read2_filtered.fastq > alignments/NA12878.bwa-sampe.sam

2.4.3. BWA Mem

(l) bwa mem -t 10 genome/hg19.fa raw_data/read1_filtered.fastq raw_data/read2_filtered.fastq > alignments/NA12878.bwa-mem.sam

2.4.4. CUSHAW3

(l) cushaw3 align -r $INDEX -t 10 -o alignments/NA12878.CUSHAW3.sam -q raw_data/read1_filtered.fastq raw_data/read2_filtered.fastq

2.4.5. MOSAIK

(l) MosaikBuild -q raw_data/read1_filtered.fastq -q2 raw_data/read2_filtered.fastq -st illumina -out alignments/NA12878.MOSAIK.mkb
(2) MosaikAligner -in alignments/NA12878.MOSAIK.mkb -out alignments/NA12878.MOSAIK -p 10 -ia genome/hg19.dat -j genome/hg19_15 -annpe tools/MOSAIK/src/networkFile/2.1.78.pe.ann -annse tools/MOSAIK/src/networkFile/2.1.78.se.ann

2.4.6. Novoalign

(1) novoalign -d $INDEX -f raw_data/
read_1_filtered.fastq raw_data/
read_2_filtered.fastq -o SAM -c 10 > alignments/NA12878.novoalign.sam

2.5. Alignment Depth of Coverage Calculation. To ensure proper depth of coverage calculation, the Picard Tools module CalculateHsMetrics was used with the following arguments:

(1) java -jar CalculateHsMetrics.jar
I=NA12878.ALN.BQSR.bam O=ALN.O.log
R=genome/hg19.fa TI=genome/
truseq_exome.bed BI=genome/
truseq_exome.bed VALIDATION_STRINGENCY=SILENT PER_TARGET_COVERAGE=ALN.ptc.bed

It is important to note that the TruSeq exome bed file must have the header from the SAM alignment file prepended to it for this module to function. Further, column 6 must be moved to column 4, and column 5 needs to be removed from the TruSeq bed file.

2.6. Alignment File Processing. Processing the alignment files (SAM/BAM files) required the following steps for all aligners:

(1) SAM to BAM conversion with SAMTools view:

(a) samtools view -bS alignments/
NA12878.ALN.sam -o alignments/
NA12878.ALN.bam

(2) BAM file sorting using the Picard Tools module, SortSam:

(a) java -jar bin/SortSam.jar
VALIDATION_STRINGENCY=SILENT
I=alignments/NA12878.ALN.bam
OUTPUT=alignments/NA12878.ALN.sorted
.bam SORT_ORDER=coordinate

(3) PCR duplicate removal using the Picard Tools module, MarkDuplicates:

(a) java -jar bin/MarkDuplicates.jar
VALIDATION_STRINGENCY=SILENT
I=alignments/NA12878.ALN.sorted.bam
O=alignments/NA12878.ALN.dups_removed.bam REMOVE_DUPLICATES=true M=alignments/metrics

(4) Read Group added to alignment files using the Picard Tools module, AddOrReplaceReadGroups:

(a) java -jar bin/AddOrReplaceReadGroups.
.jar VALIDATION_STRINGENCY=SILENT
I=alignments/NA12878.ALN.dups_removed.bam O=alignments/NA12878.ALN.RG.bam S=coordinate
RGID=NA12878 RGLB=NA12878
RGPL=illumina RGPU=NA12878
RGSM=NA12878 CREATE_INDEX=true

(5) Realignment around indels using the GATK modules RealignerTargetCreator and IndelRealigner:

(a) java -XX:-DoEscapeAnalysis -jar
bin/GenomeAnalysisTK.jar -T
RealignerTargetCreator -R
genome/hg19.fa -I alignments/
NA12878.ALN.RG.bam -known
genome/mills.vcf -o tmp/ALN.
intervals

(b) java -XX:-DoEscapeAnalysis -jar
bin/GenomeAnalysisTK.jar -T
IndelRealigner -R genome/hg19.fa
-I alignments/NA12878.ALN.RG.bam
-known genome/mills.vcf -o
alignments/NA12878.ALN.indels.bam
--maxReadsForRealignment 100000 --maxReadsInMemory 1000000
-targetIntervals tmp/ALN.
.intervals

(6) Base recalibration using the GATK modules BaseRecalibrator and PrintReads:

(a) java -XX:-DoEscapeAnalysis -jar
bin/GenomeAnalysisTK.jar -T
BaseRecalibrator -R genome/hg19.fa
-I alignments/NA12878.ALN.indels.bam
-knownSitesgenome/dbsnp
137.excluding
sites after
129.only standard
chroms.vcf -o
tmp/NA12878.ALN.grp

(b) java -XX:-DoEscapeAnalysis -jar
bin/GenomeAnalysisTK.jar -T
PrintReads -R genome/hg19.fa
-I alignments/NA12878.ALN.indels.bam
-BQSR tmp/NA12878.ALN.grp -o
alignments/NA12878.ALN.BQSR.bam

2.7. Variant calling. Default arguments were used for each variant caller unless it contained a "threads" or "parallel" flag in which case that was used as well. Additionally, indels were called separately from SNVs where possible. Specifically, the commands used are as follows.
2.7.1. FreeBayes

(l) freebayes -f genome/hg19.fa -i -X -u -v vcf_files/NA12878.ALIGNER.freebayes.raw.snv.vcf alignments/NA12878.ALIGNER.BQSR.bam
(2) freebayes -f genome/hg19.fa -I -X -u -v vcf_files/NA12878.ALIGNER.freebayes.raw.indel.vcf alignments/NA12878.ALIGNER.BQSR.bam

2.7.2. GATK HaplotypeCaller

(l) java -XX:-DoEscapeAnalysis -jar bin/GenomeAnalysisTK.jar -T HaplotypeCaller -R genome/hg19.fa -I alignments/NA12878.ALIGNER.BQSR.bam --dbsnp $DBSNP -o vcf_files/NA12878.ALIGNER.HC.UG.raw.vcf -stand_call_conf 50
(2) java -XX:-DoEscapeAnalysis -jar bin/GenomeAnalysisTK.jar -T HaplotypeCaller -R genome/hg19.fa -I alignments/NA12878.ALIGNER.BQSR.bam --dbsnp $MILLS -o vcf_files/NA12878.ALIGNER.HC.UG.raw.vcf

2.7.3. GATK UnifiedGenotyper

(l) java -XX:-DoEscapeAnalysis -jar bin/GenomeAnalysisTK.jar -T UnifiedGenotyper -R genome/hg19.fa -nt 10 -I alignments/NA12878.ALIGNER.BQSR.bam -o vcf_files/NA12878.ALIGNER.HC.UG.raw.vcf -glm SNP -D $DBSNP
(2) java -XX:-DoEscapeAnalysis -jar bin/GenomeAnalysisTK.jar -T UnifiedGenotyper -R genome/hg19.fa -nt 10 -I alignments/NA12878.ALIGNER.BQSR.bam -o vcf_files/NA12878.ALIGNER.HC.UG.raw.vcf -glm INDEL -D $MILLS

2.7.4. SAMtools Mpileup

(l) samtools mpileup -uf genome/hg19.fa alignments/NA12878.ALIGNER.BQSR.bam | bcftools view -bvcg - > vcf_files/NA12878.ALIGNER.mpileup.bcf & & bcftools view vcf_files/NA12878.ALIGNER.mpileup.bcf > vcf_files/NA12878.ALIGNER.mpileup.raw.vcf

2.7.5. SNPSVM

(l) java -XX:ParallelGCThreads=10 -jar tools/SNPSVM/snpsvm.jar predict -R genome/hg19.fa -B alignments/NA12878.ALIGNER.BQSR.bam -M tools/SNPSVM/models/default.model -V vcf_files/NA12878.ALIGNER.SNPSVM.raw.vcf

Due to the nonexistence of requisite CIGAR flags in the alignment file, SNPSVM failed to call variants for CUSHAW3, and SAMtools mpileup could not call variants on MOSAIK alignments for the same reason. Also, due to the fact that SNPSVM only detects SNVs, no indels were reported for this program.

2.8. Variant Filtration. Filtration varied depending on the variant caller being used. In the cases of GATK Haplotype-Caller and GATK UnifiedGenotyper, the GATK modules, VariantRecalibrator and ApplyRecalibration, were used to filter SNVs using HapMap 3.3, the Omni 2.5 SNP BeadChip, and dbSNP 137 without 1000 Genome data as training sets. For SNPSVM, QUAL scores ≥ 4 and DP values ≥ 6 were used. For FreeBayes and SAMtools, QUAL scores ≥ 20 and DP values ≥ 6 were used.

2.9. Variant Comparison. For variant comparison, USeq 8.8.1 was used to compare SNVs shared between all datasets. To compare indels, the vcflib tool vcflib intersect was used. The TruSeq hg19 exome bed file truseq_exome_targeted_regions hg19.bed.chr, obtained in December 11, 2013, was used to restrict comparisons to locations that could be captured by the exome pull down kit used in the sequencing of SRR098401. This file can be obtained from Illumina here: http://support.illumina.com/sequencing/sequencing_kits/truseq_exome_enrichment_kit/downloads.ilmn. To ensure that variants were represented identically between different call sets, the vcflib tool vcfallelicprimitives was used to preprocess vcf files.

2.10. Statistical Calculations

True Positive (TP). It is a mutation that was detected by the pipeline being tested and is one that exists in the NIST-GiaB list.

False Positive (FP). It is a mutation that was detected by the pipeline being tested but is one that does not exist in the NIST-GiaB list.

True Negative (TN). It is a mutation that was not detected by the pipeline being tested and is one that does not exist in the NIST-GiaB list.

False Negative (FN). It is a mutation that was not detected by the pipeline being tested but is one that does exist in the NIST-GiaB list:

\[ PPV = \frac{TP}{TP + FP} \]
\[ Sensitivity = \frac{TP}{TP + FN} \]

3. Results and Discussion

3.1. Prefiltering Variants. When performing variant analysis, one of the many pitfalls that must be taken into consideration is the exome sequence space (as defined by the exome capture kit) and how it can affect the analysis results. In this case, we had a single exome (SRR098401) that was extracted using the Illumina TruSeq exome kit and sequenced on a HiSeq 2000.
Table 3: Raw variant statistics for the 30 pipelines, including SNVs and indels.

| Aligner | Genotyper | Raw TP SNVs | Raw FP SNVs | Raw TP indels | Raw FP indels |
|---------|-----------|-------------|-------------|---------------|---------------|
| Bowtie2 | FreeBayes | 23,985      | 73,473      | 806           | 2,482         |
| Bowtie2 | GATK HC   | 21,631      | 273         | 771           | 1,103         |
| Bowtie2 | GATK UG   | 25,136      | 2,276       | 418           | 420           |
| Bowtie2 | mpileup   | 21,930      | 1,030       | 734           | 1,414         |
| Bowtie2 | SNPSVM    | 17,613      | 47          |               |               |
| BWA mem | FreeBayes | 23,857      | 18,256      | 785           | 2,088         |
| BWA mem | GATK HC   | 21,707      | 367         | 779           | 1,348         |
| BWA mem | GATK UG   | 21,925      | 213         | 402           | 408           |
| BWA mem | mpileup   | 25,081      | 2,129       | 761           | 1,772         |
| BWA mem | SNPSVM    | 17,920      | 65          |               |               |
| BWA sampe | FreeBayes | 23,789     | 27,143      | 737           | 1,872         |
| BWA sampe | GATK HC | 21,878      | 263         | 758           | 1,161         |
| BWA sampe | GATK UG | 22,153      | 321         | 394           | 385           |
| BWA sampe | mpileup | 25,206      | 2,205       | 684           | 1,401         |
| BWA sampe | SNPSVM | 18,017      | 78          |               |               |
| CUSHAW3 | FreeBayes | 23,191      | 53,525      | 624           | 3,310         |
| CUSHAW3 | GATK HC | 19,673      | 14,814      | 751           | 4,727         |
| CUSHAW3 | GATK UG | 19,113      | 13,184      | 360           | 1,005         |
| CUSHAW3 | mpileup | 22,171      | 9,694       | 681           | 1,983         |
| CUSHAW3 | SNPSVM | —           | —           |               |               |
| MOSAIK  | FreeBayes | 23,373      | 39,203      | 783           | 3,359         |
| MOSAIK  | GATK HC | 13,528      | 111         | 500           | 458           |
| MOSAIK  | GATK UG | 17,147      | 76          | 392           | 284           |
| MOSAIK  | mpileup | —           | —           |               |               |
| MOSAIK  | SNPSVM | 14,586      | 8           |               |               |
| Novoaalign | FreeBayes | 22,794      | 2,970       | 678           | 1,554         |
| Novoaalign | GATK HC | 21,407      | 473         | 779           | 1,370         |
| Novoaalign | GATK UG | 21,113      | 144         | 387           | 365           |
| Novoaalign | mpileup | 24,512      | 1,861       | 773           | 1,781         |
| Novoaalign | SNPSVM | 17,109      | 164         |               |               |

With this in mind, we wanted to make sure that we were measuring the ability of the bioinformatic tools to do their jobs and not how well the Illumina TruSeq exome capture kit worked. That is, we only want to try to call variants that are supposed to be present in the exons as defined by the pull down kit. For this reason, we use the bed file provided by Illumina, not a generic annotation bed file, for example, RefSeq for hg19. We found that for this particular individual, according to the NIST-GiaB list, there should be a total of 34,886 SNVs and 1,473 indels within the regions defined by the TruSeq bed file.

Once we filtered out variants that were not located in the regions defined by the Illumina TruSeq exome bed file, we went from hundreds of thousands of putative variants (data not shown) to, on average, about 23,000 variants (SNVs and indels) per pipeline (Table 3). This is an important step for researchers to begin with, as it significantly reduces the search space for potentially interesting variants.

3.2. Raw Variant Results Compared to GiaB. One aspect we wanted to understand when doing this comparison was the importance of filtering variants detected by these tools. The reason for this is that ideally one would like to have as high a level of sensitivity as possible so that the mutations of interest do not get lost in the filtering process. It therefore behooves us to determine whether or not this step is necessary and to what degree it is necessary, since it is clear from the NIST-GiaB results and the Bamshad et al. [13] review that sensitivity could be an issue.

As we can see in Table 3, filtering is needed more for some variant callers than for others when it comes to SNVs, and it is absolutely necessary for indels. In most cases, the number of TP variants is close to or higher than our expected number of about 20,000 [13], but, on the other hand, in some cases the number of FPs is very high.

Clearly there is a lot of variation in the numbers generated by each pipeline. However, one can find some commonalities...
in the numbers that likely stem from the algorithmic origins of each tool. FreeBayes produces both the largest number of unfiltered variants and the highest number of FPs. It is likely that we only see this kind of performance from this tool due to the fact that while it is not the only variant caller based on Bayesian inference it is unique in its interpretation of alignments. That is, it is a haplotype-based caller that identifies variants based on the sequence of the reads themselves instead of the alignment, the latter of which is how GATK’s UnifiedGenotyper operates.

Additionally we see the Burrows-Wheeler based aligners perform very similarly to each other: Bowtie2, BWA mem, and BWA sampe achieve similar results across the board. One might surmise that this is likely due to the fact that all of these tools utilize similar algorithms when performing their designated task. This observation is supported by the fact that MOSAIK (gapped alignments using the Smith-Waterman algorithm) and CUSHAW3 (a hybrid seeding approach) both have very different underlying algorithms and subsequently produce very different results.

This difference in results correlating with different algorithms is seen best in the SNPSVM results. Of the variant callers, it is the only one that utilizes support vector machines and model building to generate SNV calls. It would appear that while it has the disadvantage of not being as sensitive as other methods it does benefit from being extremely accurate regardless of the aligner being used. This suggests that one is able to skip the filtering step altogether when using this variant caller.

With regard to indels, no aligner seems to stand out among the rest as one that handles this type of mutation well. In fact, when looking at the number of FPs, it is clear that it is the variant caller that plays the largest role in the accuracy of indel identification. Additionally, there are data for neither CUSHAW3 plus SNPSVM nor MOSAIK plus SAMtools mpileup pipelines due to the alignment files not containing the necessary CIGAR strings for the variant callers to function downstream. Lastly, the reason there are no indel data for SNPSVM is because this tool is solely used for identification of SNVs.

3.3. Filtered Results Compared to GiaB. As in Table 4, standard filtering practices manage to remove a large number of FP SNVs for each pipeline; however it seems that these filters are a bit too aggressive in most cases for SNV detection, but not strict enough for indels. This is made obvious when looking at the differences in the number of FPs reported in each dataset. For example, Bowtie2 with Freebayes sees a removal of 72,570 FP SNVs (a reduction of 98%) but only a removal of 1,736 FP indels (a reduction of 70%). It should also be noted that the filters used were pipeline-dependent and, for the most part, within each pipeline produced similar reductions in SNV and indel FPs. The one exception here is the number of variants identified from the CUSHAW3 alignments when compared to other alignments: overall the number of TP SNVs is lower, the number of FP SNVs is higher, and it is the only aligner that produces more than 1,000 FP indels after filtering.

Given the fact that filtering significantly reduces the number of TP variants, it might be wise to, with the exception of pipelines using CUSHAW3 and Freebayes, skip this step when searching for rare, high-impact variants. Instead, one might spend more time on a filtering process that is based on biology rather than statistics. For example, it may make more sense to invest time identifying a small list of variants that are likely to be high-impact: splice site mutations, indels that cause frameshifts, truncation mutations, stop-loss mutations, or mutations in genes that are known to be biologically relevant to the phenotype of interest.

3.4. Average TPR and Sensitivity. As can be seen in Table 5, the Positive Predictive Value (PPV) for each tool, with the exception of CUSHAW3, ranges from 91% to 99.9% for SNVs, but the average sensitivity is very low (around 50%). This discrepancy could be due to a number of reasons, but the most likely one is variable depth of coverage across exons. We can see that, in addition to low SNV sensitivity, indel sensitivity is low (around 30%); however the PPV for indels is considerably lower (35.86% to 52.95%). This could be due to any of the following reasons: very short indels are hard to detect by conventional NGS [31], the representation of indels by different variant callers can cause tools to incorrectly claim that two indels are different, or alignment tools produce different representations of the same indel [7].

Perhaps the most likely explanation for both types of mutations is the issue of depth. As is the case with any variant analysis study, an increase in depth of coverage leads to an increase in sensitivity, but it is impossible to guarantee good depth of coverage due to the inability of exome capture kits to uniformly pull down exons [32–34]. Additionally, no single exome capture kit covers every exon. Indeed, it has been shown that variant analysis of whole genome sequencing at an average depth lower than an exome performs better due to the uniformity of said depth. Thus, it is likely that a large number of variants are missing due to the fact that the NIST-GiaB list was created from a compilation of exomic and genomic sequencing data. Ultimately, to achieve proper sensitivity one will eventually need to perform whole genome sequencing, but that is currently cost-prohibitive for most labs. Fortunately, this cost is continuing to drop, and we will soon see a gradual shift from exome analysis to the more complete whole genome analysis.

3.5. Sensitivity as a Function of Depth. Because sensitivity reflects one of the most important performance metrics of a tool and most of the tools struggle to achieve sensitivity higher than 50%, we would like to further explore how depth affected variant calling sensitivity. We looked at a number of different combinations of tools to determine what the best pipelines, variant callers, and aligners were. For Figure 2, we took the five best combinations of variant callers and aligners as determined by their sensitivity and false positive rate (FPR). That is, we selected those which had the highest number of TP SNVs called in addition to the lowest number of FP SNVs. Upon inspection, the thing that stands out immediately is that the sensitivity is lower than expected.
### Table 4: Filtered variant statistics for the 30 pipelines, including SNVs and indels.

| Aligner     | Genotyper | Filtered TP SNVs | Filtered FP SNVs | Filtered TP indels | Filtered FP indels |
|-------------|-----------|------------------|------------------|--------------------|--------------------|
| Bowtie2     | FreeBayes | 17,504           | 903              | 481                | 746                |
| Bowtie2     | GATK HC   | 17,330           | 29               | 648                | 687                |
| Bowtie2     | GATK UG   | 19,937           | 49               | 395                | 338                |
| Bowtie2     | mpileup   | 17,049           | 153              | 402                | 541                |
| Bowtie2     | SNPSVM    | 13,983           | 8                | —                  | —                  |
| BWA mem     | FreeBayes | 17,376           | 347              | 461                | 739                |
| BWA mem     | GATK HC   | 19,388           | 302              | 689                | 860                |
| BWA mem     | GATK UG   | 20,000           | 48               | 397                | 355                |
| BWA mem     | mpileup   | 17,070           | 57               | 403                | 606                |
| BWA mem     | SNPSVM    | 15,060           | 10               | —                  | —                  |
| BWA sampe   | FreeBayes | 17,435           | 450              | 443                | 647                |
| BWA sampe   | GATK HC   | 19,438           | 214              | 630                | 725                |
| BWA sampe   | GATK UG   | 19,557           | 27               | 384                | 336                |
| BWA sampe   | mpileup   | 17,049           | 111              | 387                | 518                |
| BWA sampe   | SNPSVM    | 15,218           | 10               | —                  | —                  |
| CUSHAW3     | FreeBayes | 16,620           | 7,627            | 362                | 1,294              |
| CUSHAW3     | GATK HC   | 16,590           | 2,195            | 665                | 1,551              |
| CUSHAW3     | GATK UG   | 17,939           | 2,202            | 357                | 545                |
| CUSHAW3     | mpileup   | 15,942           | 4,029            | 368                | 796                |
| CUSHAW3     | SNPSVM    | —                | —                | —                  | —                  |
| MOSAIK      | FreeBayes | 17,177           | 679              | 458                | 645                |
| MOSAIK      | GATK HC   | 11,616           | 33               | 426                | 255                |
| MOSAIK      | GATK UG   | 16,423           | 42               | 381                | 224                |
| MOSAIK      | mpileup   | —                | —                | —                  | —                  |
| MOSAIK      | SNPSVM    | 4,727            | 3                | —                  | —                  |
| Novoalign   | FreeBayes | 16,658           | 219              | 384                | 559                |
| Novoalign   | GATK HC   | 19,406           | 385              | 702                | 872                |
| Novoalign   | GATK UG   | 20,521           | 46               | 386                | 315                |
| Novoalign   | mpileup   | 16,493           | 62               | 396                | 579                |
| Novoalign   | SNPSVM    | 14,451           | 18               | —                  | —                  |

### Table 5: Average Positive Predictive Value (PPV) and sensitivity for each tool.

| Tool                    | Average SNV PPV | Average SNV sensitivity | Average indel PPV | Average indel sensitivity |
|-------------------------|-----------------|-------------------------|-------------------|---------------------------|
| Bowtie2                 | 98.69%          | 49.19%                  | 45.45%            | 32.69%                    |
| BWA mem                 | 99.15%          | 50.96%                  | 43.24%            | 33.10%                    |
| BWA sampe               | 99.09%          | 50.85%                  | 45.31%            | 31.30%                    |
| CUSHAW3                 | 80.69%          | 48.08%                  | 29.50%            | 29.74%                    |
| MOSAIK                  | 98.51%          | 35.79%                  | 52.95%            | 28.63%                    |
| Novoalign               | 99.17%          | 50.18%                  | 44.55%            | 31.70%                    |
| FreeBayes               | 90.95%          | 51.00%                  | 35.86%            | 32.79%                    |
| GATK HaplotypeCaller    | 97.05%          | 51.03%                  | 45.17%            | 31.79%                    |
| GATK UnifiedGenotyper   | 99.9%           | 50.77%                  | 52.12%            | 31.57%                    |
| SAMtools mpileup        | 94.99%          | 50.76%                  | 39.15%            | 31.30%                    |
| SNPSVM                  | 99.92%          | 50.85%                  | N/A               | N/A                       |
Figure 2: Sensitivity as a function of depth for the top five pipelines. The top five pipelines are shown here with the depth of every SNV plotted against sensitivity.

All of the pipelines perform at roughly the same level: they identify most of their variants by the time a depth of about 150x has been reached, which indicates that this depth is likely sufficient and that the number of missing variants is probably due to certain exons having lower than average coverage. Note that four out of the five best performing pipelines have GATK UnifiedGenotyper as their variant caller, demonstrating its superior performance irrespective of the aligner used as shown in Figure 3(b).

In addition to looking at the top five pipelines, we determined it would be useful to perform the same analysis on the best aligner coupled with every variant caller (Figure 3(a)), as well as the best variant caller coupled with every aligner (Figure 3(b)). As with the pipelines, the best aligner was identified as that which produced the highest number of TP SNVs and the lowest number of FP SNVs—in this case BWA mem. Despite having the best alignment to work with, we still see a fairly large difference between the variant callers, which is likely attributable to the different algorithms they employ (Figure 3(a)). However, in the case of the best performing variant caller, GATK UnifiedGenotyper, there seems to be less variation among the top four aligners indicating that it performs fairly well in most situations with the exceptions being CUSAHW3 and MOSAIK.

3.6. Shared Variants among the Top Pipelines. Lastly, we wanted to know just how unique the variant call sets were between the different pipelines. To do this, we again focused on the top five variant calling pipelines: Bowtie2 plus UnifiedGenotyper, BWA mem plus UnifiedGenotyper, BWA sampe plus HaplotypeCaller, BWA sampe plus UnifiedGenotyper, and Novoalign plus UnifiedGenotyper. As can be seen in Figure 4, there is a large amount of overlap between

Figure 3: Sensitivity as a function of depth for the top aligner and top variant caller. (a) Results for the depth of every SNV plotted against sensitivity for the top aligner, BWA mem, paired with every variant caller. (b) Results for the depth of every SNV plotted against sensitivity for the top variant caller, GATK UnifiedGenotyper, paired with every aligner.
the five different pipelines in question, with 15,489 SNVs (70%) shared out of a total of 22,324 distinct variants. However, one could also argue that this is largely due to four of the five pipelines using the UnifiedGenotyper as their variant caller. This notion is corroborated by the fact that the largest number of variants unique to a pipeline, 367, belongs to the BWA sampe plus HaplotypeCaller combination. It is also worth noting that the second highest number of unique SNVs also belongs to the BWA sampe aligner, so it is possible that the high number of unique SNVs is better attributed to the aligner than the variant caller.

4. Conclusions

We found that among the thirty different pipelines tested Novoalign plus GATK UnifiedGenotyper exhibited the highest sensitivity while maintaining a low number of FP for SNVs. Of the aligners, BWA mem consistently performed the best, but results still varied greatly depending on the variant caller used. Naturally, it follows that the best variant caller, GATK UnifiedGenotyper, mostly produced similar results regardless of the aligner used. However, it is readily apparent that indels are still difficult for any pipeline to handle with none of the pipelines achieving an average sensitivity higher than 33% or a PPV higher than 53%. In addition to the low overall performance we see in detecting indels, sensitivity, regardless of mutation type, is a problem for every pipeline outlined in this paper. The expected number of SNVs for NA12878's exome is 34,886, but even when using the union of all the variants identified by the top five pipelines, the greatest number identified was very low (22,324). It seems that while still very useful exome analysis has its limitations even when it comes to something as seemingly simple as SNV detection.

Disclosure

Adam Cornish is a graduate student in Chittibabu Guda’s lab with training in computer science and genomics. Chittibabu Guda (Associate Professor) has an interdisciplinary background in molecular and computational biology. He has published a number of computational methods with a variety of applications in biomedical research, since 2001.

Conflict of Interests

The authors are unaware of any competing interests.

Authors’ Contribution

Adam Cornish designed the study, performed all analyses, made the figures, and wrote the paper. Chittibabu Guda provided essential feedback on improvements to the paper and input on the analyses themselves and thoroughly edited the paper. All authors read and approved the final paper.

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