Comparison of error correction algorithms for Ion Torrent PGM data: application to hepatitis B virus

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Ion Torrent Personal Genome Machine (PGM) technology is a mid-length read, low-cost and high-speed next-generation sequencing platform with a relatively high insertion and deletion (indel) error rate. A full systematic assessment of the effectiveness of various error correction algorithms in PGM viral datasets (e.g., hepatitis B virus (HBV)) has not been performed. We examined 19 quality-trimmed PGM datasets for the HBV reverse transcriptase (RT) region and found a total error rate of 0.48% ± 0.12%. Deletion errors were clearly present at the ends of homopolymer runs. Tests using both real and simulated data showed that the algorithms differed in their abilities to detect and correct errors and that the error rate and sequencing depth significantly affected the performance. Of the algorithms tested, Pollux showed a better overall performance but tended to over-correct ‘genuine’ substitution variants, whereas Fiona proved to be better at distinguishing these variants from sequencing errors. We found that the combined use of Pollux and Fiona gave the best results when error-correcting Ion Torrent PGM viral data.

Next-generation sequencing (NGS) has been widely used in the study of viruses and has opened new avenues for research and diagnostic applications (e.g., viral mutant spectra\textsuperscript{1,2}, virus quasispecies theory and dynamics\textsuperscript{3-7}, fitness landscape\textsuperscript{8,9} and discovery of novel viruses\textsuperscript{5}). Ion Torrent Personal Genome Machine (PGM) technology is a mid-length read, low-cost and high-speed NGS platform\textsuperscript{10} with special applications in microbial sequencing\textsuperscript{11}. However, PGM has a relatively high insertion and deletion (indel) error rate of 1.5% (range from 0.46% to 2.4%)\textsuperscript{12-14}.

Several algorithms have been proposed to correct sequencing errors for PGM data (Table 1). These algorithms differ with respect to error models, statistical techniques, data features, the determined parameters, and performances. These methods are classified into the following three categories: (1) suffix array/tree-based methods that use a suffix tree to detect and correct substitution and indel errors (e.g., Fiona\textsuperscript{15}); (2) \textit{k}-spectrum-based methods that divide reads into \textit{k}-mer lengths and generate a \textit{k}-mer depth profile to correct the \textit{k}-mer profile (e.g., Blue\textsuperscript{16} and Pollux\textsuperscript{17}); and (3) multiple sequence alignment (MSA)-based methods that use \textit{k}-mers as seeds and construct a consensus sequence from the multiple alignments to correct errors (e.g., Coral\textsuperscript{18} and Karect\textsuperscript{19}). Two review articles\textsuperscript{12,20} have systematically surveyed these methods for PGM data and provided guidance concerning which tools to consider for benchmarking based on the data properties. Sequencing data generated in NGS platforms were analyzed in four microbial genomes to assess the coverage distribution, bias, GC distribution, variant detection and accuracy\textsuperscript{13}. However, these algorithms have not been fully assessed and applied to viral sequencing data (e.g., hepatitis B virus, HBV).

HBV has a partially double-stranded DNA genome, and its replication depends on reverse transcription of an RNA intermediate by reverse transcriptase (RT). Since the RT lacks proofreading, errors in HBV DNA replication occur at a relatively higher rate than other DNA viruses, with an estimated nucleotide substitution rate of

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Estimation of the error rate in the empirical PGM data. We excluded the defined ‘genuine’ mutations (i.e., a variant with a frequency ≥ hl) to ensure that indel errors, which were common on this platform, were truly reflected by the error rate. To estimate the substitution error rate, we used the following equation:

$$R_{\text{error}} = \frac{\sum_{i=1}^{n} r_i}{\sum_{i=1}^{n} n_{\text{base}}}$$

where $r_i$ was the number of errors in each read ($i$), $n_{\text{base}}$ was the total number of sequenced bases, and $n$ was the total number of reads. For example, the deletion error rate in the homopolymers was calculated by dividing the total number of deletion errors by the total number of sequenced bases in the homopolymer region. A homopolymer region was defined as a homopolymer repeat with a length $\geq hl$, where $2 \leq hl \leq 5$. This definition was established to ensure that indel errors, which were common on this platform, were truly reflected by the error rate. To estimate the substitution error rate, we excluded the defined ‘genuine’ mutations (i.e., a variant with a frequency...
≥0.5% based on the TEF file from the pre-corrected alignments), because Ion Torrent PGM could detect substitutions occurring at frequencies ≥0.1%26.

The cumulative distribution of errors in the sequencing reads after quality trimming indicated that 99.48% of the sequencing reads had ≤9 errors (Fig. 1a). We did not find any ‘true’ indels using Sanger sequencing; therefore,
Previously, PGM data were sensitive to homopolymers, and the indel error rate increased as the sequence coverage increased. Notably, deletion errors were more likely for homopolymers and were correlated with the homopolymer lengths. When the homopolymer length was greater than 4, the mean deletion error rate in the homopolymers was 0.59%, although the insertion error rate (0.27%) was more likely to be greater than the deletion error rate (0.13%) in the total sequenced regions. As noted previously, PGM data were sensitive to homopolymers, and the indel error rate increased as the homopolymer length increased.

Comparison of error correction algorithms using empirical PGM data. Several measures have been proposed to evaluate the quality of error correction, including the measure of gain, sensitivity, and specificity. The gain

\[ \text{gain} = \frac{(TP - FP)}{(TP + FN)} \]  

is a widely used measure that is equivalent to the number of true errors corrected (TP) minus the number of introduced errors (FP) divided by the total number of errors initially present in the data. This measure penalizes failing to detect an erroneous base, correctly detecting but wrongly correcting an erroneous base, and characterizing a correct base as an erroneous base. Generally, real sequencing errors were obtained by mapping the sequencing reads to the reference genome and recording the differences. When both substitution and indel errors were targeted for correction, the TP, FP, and FN were inferred as follows. The algorithm defines r as an original read and rc as the read post-correction. The set of real sequencing errors (En) is derived by mapping r to the reference and recording the differences, and the set of errors remaining in rc (Er) is measured by applying a global alignment between rc and the genomic region to which r was mapped and recording the differences in the alignment. Accordingly, TP, FP, and FN are calculated as: TP = |Er \cap En|, FP = |Er \setminus En|, and FN = |En \setminus Er|.

On average, 0.48% and 8.21% of the reads were discarded by Pollux and Blue, respectively (Table S1). The error correction performance in the 19 PGM data sets differed significantly among the algorithms (Fig. 2). The measures of gain obtained by Pollux (mean of 0.74) and Blue (0.60) were significantly greater than those of gain obtained by Fiona, Coral, and Karect (ANOVA, p = 3.41 (3.45) × 10^{-14}, p = 1.24 (1.24) × 10^{-14}, and p = 1.15 (1.15) × 10^{-14}, respectively). The sensitivity of the five algorithms appeared to be similar to the measure of gain (Fig. 2b), and the specificity was similar (Table S2). A negative correlation was found between the measure of gain and the residual error rate of the post-corrected reads (r = −0.8) (Table S2).

We manually investigated the behaviors of these algorithms in correcting for insertion (blue arrow), deletion (red arrow) and substitution (green arrow) errors (Fig. 3). We found that Pollux and Blue had a greater power for indel error correction but were unable to distinguish ‘genuine’ substitutions from errors. For example, at position 651 (a G → A Sanger-confirmed mutation), most of the mutated ‘A’ alleles (959 out of 7427) were falsely corrected by Pollux (956/959) and Blue (788/959) but not by Fiona, Coral, and Karect. For the insertion errors between positions 762 and 763 (1,070 out of 7,208 sequencing reads), Pollux and Blue corrected 98.2% and 100% of the erroneous insertions, followed by Coral (25.3%), Fiona (1.3%) and Karect (0%). We noted similar behaviors of these algorithms for deletion error (e.g., at position 525) corrections.

The ECE toolkit takes all bases differing from the reference as errors and counts all corrections changed to the reference as a TP, resulting in a bias in the calculation of these measures. We set different frequency thresholds (0.1%, 0.5%, and 1%) to distinguish ‘genuine’ substitutions and errors, because Ion Torrent PGM can detect substitutions occurring at frequencies ≥0.1% (i.e., a variant was considered to be ‘true’ if its frequency was greater than the cutoff). Based on the pre- and post-corrected TEF files, we counted the proportion of the identified ‘genuine’ mutations and the corrected errors under different algorithms (Fig. 4). We calculated the proportion of the identified ‘genuine’ mutations by dividing the number of mutated alleles in the corrected reads by the total number of reads with mutations.

**Figure 2.** Comparison of the error correction algorithms using 19 empirical data sets sequenced in the HBV RT region with respect to the measure of gain (a), sensitivity (b), and specificity (c).
number in the original reads. We found that Pollux and Blue over-corrected for ‘genuine’ substitutions with a higher frequency, whereas Karect and Coral had a lower power for error correction. Fiona corrected most of the substitution errors with frequencies <1% and preserved the variants with relatively higher frequencies. However, this algorithm had limited power for correcting indels, which are the main type of errors in the Ion PGM data. The greater gain of Pollux may be due to its power for indel error correction as well as its effect on falsely correcting ‘genuine’ substitutions. Therefore, we suggest the combination of Pollux (for indel error correction only) and Fiona (for substitution error correction) for Ion Torrent PGM data (Pollux_Fiona). The measures of gain ($p = 0.79$), sensitivity ($p = 0.52$), and specificity ($p = 0.35$) obtained by Pollux_Fiona did not differ significantly from the measures obtained with Pollux (Fig. 2).

We also changed the $k$-mer parameter to optimize the $k$-spectrum-based algorithms (Blue and Pollux) and the MSA-based method using $k$-mer (Coral) for error correction. The measure of gain did not differ significantly under different $k$-mer values (ANOVA, $p = 0.45$ (Pollux) and 0.20 (Coral)) but was marginal in Blue ($p = 0.04$) (Fig. S1). The average time costs for Pollux, Blue, Fiona, Coral, and Karect were 5.2, 2.2, 36.1, 18.6 and 1.2 minutes, respectively, showing that Fiona was the most time-consuming algorithm.

**Performance of error correction algorithms using simulated data.** We studied the performance of the different algorithms under different simulation scenarios. First, a model of indel errors (Fig. 5a) showed that the measures of gain differed significantly among these algorithms (ANOVA, $p < 2.2 \times 10^{-16}$) and the indel error rates ($p = 2.1 \times 10^{-16}$). In concordance with the empirical data, Pollux had a better performance in measure of gain (~1) and remained similar with an increased indel error rate; Blue exhibited similar behavior, but its performance decreased when the insertion error rate was $\geq 0.06$ or the deletion rate was $\geq 0.09$. Fiona showed a relatively higher measure of gain in the setting of an error rate $\leq 0.02$, since most of the substitution errors (the major errors under this setting) were removed. The introduction of a large number of insertion errors (rate of 0.01) at the homopolymer regions ($hl \geq 5$) resulted in a negative gain for Coral and a relatively lower specificity. Both Fiona and Coral obtained moderate measures of gain and sensitivity with an increased error rate. Karect had a lower
performance for correcting indel errors regardless of the rate. As expected, the combined use of Pollux and Fiona had a similar performance with Pollux.

Second, we investigated the effects of the substitution errors for the performance (i.e., a model of substitution errors) (Fig. 5b). Similarly, Pollux out-performed the remaining algorithms under different rates. However, Karect obtained a higher measure of gain when the substitution rate was 0.1% partly due to its effects in correcting for low-frequency substitution errors. Obviously, Blue, Fiona and Coral had better performances at higher substitution rates, but the performances of Blue and Fiona decreased as the errors continued to accumulate. Blue had an especially good performance when the substitution error rate was \( \leq 0.4\% \), but its power for error correction decreased significantly when the rate was \( \geq 0.4\% \). We speculated that the enrichment of errors in reads might have a significant effect on the \( k \)-mer count profile and error inference. We also simulated a set of data by randomly introducing known variants into the reads, including five rare mutations (with frequencies of 0.1–0.5%) and three low-frequency variants (approximately 5%). The proportion of the remaining mutated alleles and sequencing errors after error correction (Table S3) indicated that Pollux and Blue could not distinguish rare and low-frequency variants from sequencing errors, whereas Fiona could identify low-frequency variants but not rare mutations. Although Coral and Karect could identify the rare and low-frequency variants, these algorithms had little power for sequencing error correction. These results were consistent with our analyses of the empirical data (Fig. 4).

Finally, we explored how the sequencing depth affected the performance (i.e., a model of the sequencing depth). The sequencing depth had little effect on Blue and Pollux (Fig. 5c), whereas Fiona and Karect exhibited a better performance with a lower depth. However, Coral obtained a negative measure of gain under a lower depth (e.g., 6,000 reads), probably resulting from a higher FP introduced by insertion errors. The combined use of Pollux and Fiona had a similar performance as Pollux.

Discussion

Relatively higher mutation and replication rates in viruses lead to an increased number of mutations, including a large number of rare variants. Ultra-deep sequencing has been widely used for analyses of viral populations\(^{32,33}\) and enables the examination of the diversity of the whole viral population and the identification of important variants present within the viral population at low frequencies (i.e., mutations that increase pathogenicity or convey drug resistance\(^{33}\)). Therefore, the characteristics of viral sequencing data include a higher sequencing depth and a broad frequency spectrum of mutations compared with sequencing data for macro-organisms. Therefore, effectively distinguishing low-frequency variants from sequencing errors remains a great challenge.

Bragg et al.\(^{26}\) described the biases and errors introduced by PGM across a combination of factors in two bacterial species. The average GC content of Bacillus amyloliquefaciens (46%)\(^{26}\) is similar to the empirical (49.9%) and simulated data (46.4%) in our study. The authors found indel errors at a rate of 1.38% after quality clipping, which accounted for most of the errors due to inaccurate flow calls. In our PGM data, the deletion errors in the

Figure 4. The proportion of the identified ‘genuine’ mutations and the corrected errors under different algorithms based on the pre- and post-corrected TEF files in the empirical PGM data.
homopolymers (i.e., a polymer consisting of ≥4 identical nucleotides) were significantly greater than those in the non-homopolymers, but the insertion error rate was 1.5 times the insertion error rate. (b) A model of substitution errors. We assumed a fixed insertion (0.04%) and deletion (0.06%) error rate and read number (60,000) with varied substitution error rates; and (c). A model of sequencing depth. We assumed fixed insertion (0.04%) and deletion (0.06%) error rates and substitution error rates (0.17%) with different sequencing depths.

Of these correction algorithms, we noted different performances in both the empirical and simulated PGM data (Figs 2 and 5). Generally, Pollux and Blue had similar performances, and their measures of gain were significantly greater compared to the remaining algorithms, which was consistent with previous studies. There are several explanations for their ‘outperformances’. First, Pollux and Blue filter and discard reads that appear to still be faulty after correction (averages of 0.48% and 8.21%, respectively, in our 19 PGM data sets). Second, Pollux performs homopolymer corrections independently after exhausting all other correction possibilities. Third, both algorithms over-corrected for the ‘genuine’ substitutions (Figs 3, 4 and Table S3) (e.g., more than 97% of the...
HBV DNA extraction and RT region amplification. Consent. All of the experiments were performed in accordance with the relevant guidelines and regulations. Patients with chronic HBV infection were recruited from the Department of Infectious Diseases, the Second Affiliated Hospital of Chongqing Medical University, Chongqing, China. None of the patients were receiving oral nucleoside/nucleotide analogues (NAs) or interferon-alpha (IFN-α) antiviral therapy. The study was approved by the Institutional Review Board (IRB) of the Second Affiliated Hospital of Chongqing Medical University, and the patients provided written informed consent. All of the experiments were performed in accordance with the relevant guidelines and regulations.

Methods

HBV DNA extraction and RT region amplification. In total, 19 serum samples collected at baseline were obtained in the present study. HBV genomic DNA was extracted using the QIAamp UltraSens® Virus Kit according to the manufacturer's protocol. A nested PCR was performed to amplify the HBV RT regions35, and the PCR products were purified using the QIAquick PCR Purification Kit (Qiagen®, Hilden, Germany).

We used the DNA fragmentation strategy to construct shotgun fragment libraries for Ion Torrent PGM sequencing to produce shotgun reads with a target fragment size range of 200–400 bp38. Library preparation was conducted using the Ion Xpress Plus Fragment Library Kit (Cat. no. 4471269, Pub. no. MAN0009847, Rev. C) with 100 ng of HBV DNA. Adapter ligation, size selection, nick repair and amplification were performed according to the manufacturer's protocol. Sample emulsion PCR, emulsion breaking and the enrichment steps were performed using the Ion PGM Template OT2 400 Kit and the associated protocol (Cat. no. 4479878, Pub. no. MAN0007218, Rev. A) according to the manufacturer's instructions. Briefly, an input concentration of template-positive Ion Sphere Particles (ISPs) was added to the emulsion PCR master mix to generate the emulsion. After enriching template-positive ISPs, sequencing was undertaken using the Ion 318 Chip v2 in the Ion Torrent PGM System. The Ion PGM Hi-Q Sequencing Kit was used for all sequencing reactions according to the protocol (Cat. no. A2592, Pub. no. MAN009816, Rev. D). All PGM sequencing was conducted by the WuXi AppTec company (Shanghai, China).

PCR chimeras are common in amplicon sequencing where closely related sequences are amplified but are rare with shotgun sequencing. In the setting of next-generation sequencing, the formation of artificial chimeras during PCR can be consistently suppressed to low levels39. We used the DNA fragmentation strategy to construct shotgun fragment libraries for Ion Torrent PGM sequencing. Therefore, the chimeras from PCR may have little or no influence on the HBV PGM data in our study.

Sanger sequencing. Standard Sanger sequencing reactions were electrophoresed using an Applied Biosystems 3730xl DNA Analyzer (Applied Biosystems, Foster City, CA, USA). Direct sequencing of the PCR products of the HBV RT region was performed in both directions. Sample-specific reference sequences for the HBV RT region were assembled using Sanger sequencing reads with manual finishing.

Data simulation. We used the sequence-read simulator program ‘CuReSim’ to generate in silico PGM data (Fig. 6a). First, we supplied an HBV reference sequence (AB033556) for the simulation to generate error-free reads given the mean and standard error of the read length obtained from our empirical PGM data. Second, indels
and substitution errors were introduced based on a specified error rate. The substitution errors follow an exponential distribution depending on the read position (i.e., the substitution probability increases at the end of the read). Additionally, an iterative algorithm introduced indels in the longer homopolymers. In the empirical data, the number of bases in the total region \(N_{\text{all \_ base}}\) was approximately ten times the number of bases in the homopolymer regions \(N_{\text{homopolymer \_ base}}\) (e.g., the total number of indel errors \(N_{\text{all \_ indel}}\) was approximately equal to the indel errors in the homopolymer regions \(N_{\text{homopolymer \_ indel}}\)). The indel error rates \(PR_{\text{indel}}\) can be estimated by:

\[
PR_{\text{indel}} = \frac{N_{\text{all \_ indel}}}{N_{\text{all \_ base}}} = \frac{N_{\text{homopolymer \_ indel}}}{N_{\text{homopolymer \_ base}}/(10 \times N_{\text{homopolymer \_ base}}) \quad (3)
\]

Therefore, for simulation using ‘CuReSim’, the indel error rate \(PR_{\text{indel}}\) was approximately one-tenth of the indel error rate in the homopolymer regions \(R_{\text{homopolymer \_ indel}}/10\). In our 19 quality trimming PGM data sets, the insertion and deletion error rates in the homopolymer regions were approximately 0.4% and 0.6%, respectively. Therefore, we fixed the error rates of 0.04% and 0.06% (in model 2) to explore the effect of substitutions on the performances of the algorithms. The re-estimated indel error rates based on the simulated data were similar to the empirical indel error rates. Finally, the number of introduced errors was corrected using the errors corresponding to the minimal edit distance.

We simulated three models of PGM sequencing reads (Fig. 6b). In the indel error model (Table S4), a set of indel error rates was used given a fixed substitution error rate in the PGM data (0.17%). In the substitution error model (Table S5), we assumed fixed insertion and deletion error rates (0.04% and 0.06%, respectively), and the substitution rate varied from 0 to 0.7%. In the sequencing depth model (Table S6), we simulated a pool of 80,000 reads given a fixed insertion and deletion error rate (0.04% and 0.06%) and a substitution error rate of 0.17%. We down-sampled the pool to generate different numbers of reads from 6,000, 8,000, 10,000, 20,000, 40,000, and 60,000, corresponding to an approximate depth of 1,230 ×, 1,650 ×, 2,050 ×, 4,100 ×, 8,200 ×, and 12,300 ×, respectively. The parameters of the three models are described in detail in the supplementary material (Tables S4–S6).

**Bioinformatics analysis.** We used a pipeline to process the empirical or simulated PGM data, including pre-processing, error correction, alignment, and assessment of error correction (Fig. S2).

**Pre-processing.** The empirical raw fastq data were filtered using the ‘fastq_quality_filter’ in the FASTX-Toolkit (http://hannonlab.cshl.edu/fastx_toolkit/). Low-quality reads were filtered if 20% of the bases had a phred quality score <20.

**Error-correction algorithms.** The five algorithms assessed in the present study (Table 1) could take a ‘fastq’ file as input, and the default setting was used when running each program. The command lines for executing these programs were provided in the supplementary material.

**Alignment.** The pre- and post-corrected PGM sequencing reads were aligned to a sample-specific reference sequence (for the empirical data) and AB033556 (for the simulated data) using the Torrent Mapping Alignment Program (TMAP, https://github.com/iontorrent/TS/tree/master/Analysis/TMAP). TMAP uses a series of algorithms (BWA, BWASW, SSAHA2, the super-maximal exact matching algorithm, and the Smith–Waterman algorithm) to map data to an indexed reference sequence. The alignment was performed in two stages with the option ‘mapall -g 0 stage1 map1 stage2 map2 map3’. This process enabled an alignment using BWA in the first stage (map1) and BWASW (map2) and SSAHA (map3) in the second stage. Since no known indels were previously reported in the HBV RT region, we did not perform a realignment around indels.

**Assessment of error correction.** The measure of gain, TP, FP, and FN were calculated using the ‘compute-stats.py’ script from the Error Correction Evaluation (ECE) Toolkit (http://aluru-sun.ece.iastate.edu/doku.php?id=ecr). The command lines used to execute the assessment are provided in detail in the supplementary material.
All calculations were executed using an IBM server with 4x Intel(R) Xeon(R) CPU E7-8850@2.00 GHz processors and 256 GB of memory.

Data availability. The 19 Ion Torrent PGm sequencing data for the HBV RT region have been uploaded to the NCBI Sequence Read Archive (SRA) (accession number: PRJNA35918). The simulated data were generated using the simulator ‘CuReSim’ and were provided in the Supplementary materials.

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Author Contributions
J.K. recruited the patients and prepared the serum samples. L.S., W.H., and K.D. performed the data analysis. K.D. wrote the manuscript with input from L.S., W.H., and Y.H. J.K., H.R. and K.D. provided funding support, and K.D. conceived and designed the project. All of the authors approved the final manuscript.

Additional Information
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