The Sensitivity of HIV Deep Sequencing

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Abstract

Background: Deep sequencing, primarily the 454/pyrosequencing platform, is increasingly being used to examine HIV viral populations, including for the detection of minor variants. We have examined how different variables impact the introduction of sequencing errors, thus affecting the sensitivity for detection of rare variants and accuracy of description of HIV quasispecies.

Methods: We performed pyrosequencing on multiple HIV plasmid control sequences, using a variety of PCR conditions and DNA polymerases to determine what factors affected error rates. We also sequenced a variety of plasmid mixtures at known ratios to examine sensitivity and reproducibility of detection of minor variants.

Results: The polymerase used for PCR had the largest impact on substitution errors. PCR conditions, including the input number of templates and multiplexing, also had an effect.

Discussion: We found that the conditions of the PCR carried out prior to sequencing is the largest source of substitution errors following sequencing. Optimization of PCR enzymes and conditions can reduce background error rate and thus increase the depth of analysis of HIV quasispecies by deep sequencing methodologies.

Background

454/pyrosequencing has emerged as a powerful new technology to obtain vast amounts of sequencing data, allowing study of HIV variation and population dynamics on a greater scale than was previously practical. For example, pyrosequencing has been used to examine HIV dual (Campbell et al, 2011) and superinfection (Redd et al. 2011), drug resistance (Lataillade et al. 2012, Gianella et al. 2011), viral tropism (Bunnik et al. 2011), and viral escape from immune pressure (Fischer et al. 2010).

These studies estimated variant frequencies and reported these estimates as representative of the true HIV population. However, as Swanstrom and colleagues (2011) have noted, a possible bias in variant frequencies may occur due to PCR amplification. We examined this bias on a mixture of 16 HIV plasmids at different input levels that have undergone PCR using different polymerase combinations to determine the effect that PCR has on substitution errors and on thresholds for determining variant frequencies.

Although pyrosequencing is often called "deep sequencing," it is unknown how deep into a intrahost population this technology can actually probe. A 1% cutoff is often drawn on variants in order to classify them as real or not. Often, the two critical determinants for the depth of this sequencing, *the number of input templates that are initially amplified prior to sequencing*, and the *background error rate* are not determined carefully. We observed that PCR is the cause of the majority of the background substitution errors, and sought PCR conditions to lower the background error rate, and therefore increase the reliable depth of deep sequencing.

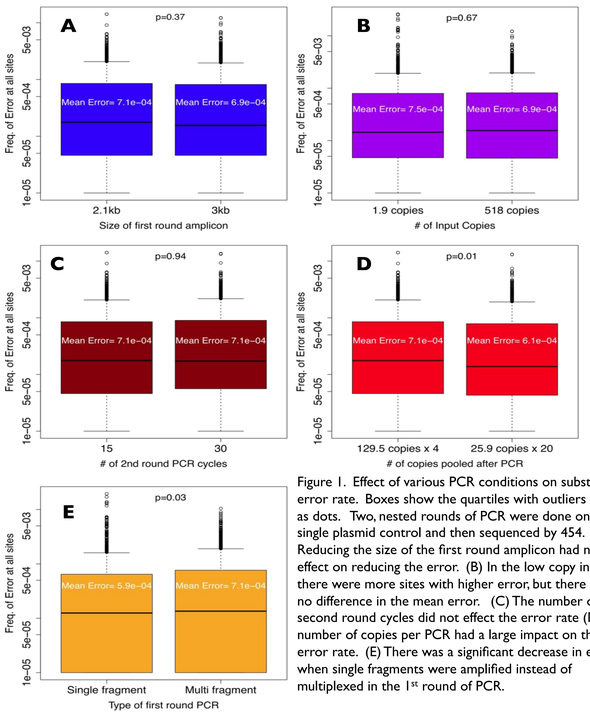

Figure 1. Effect of various PCR conditions on substitution error rate. Boxes show the quartiles with outliers shown as dots. Two, nested rounds of PCR were done on a single plasmid control and then sequenced by 454. (A) Reducing the size of the first round amplicon had no effect on reducing the error. (B) In the low copy input, there were more sites with higher error, but there was no difference in the mean error. (C) The number of second round cycles did not effect the error rate (D) The number of copies per PCR had a large impact on the error rate. (E) There was a significant decrease in error when single fragments were amplified instead of multiplexed in the 1st round of PCR.

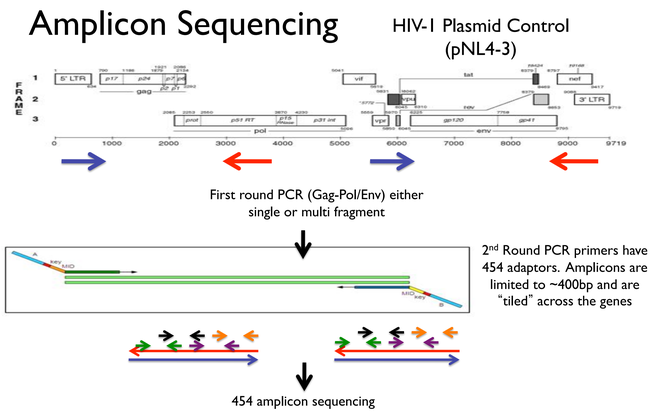

Figure 2. Pipeline of 454 amplicon sequencing

Methods

We first investigated the contribution to error rates of different DNA polymerases and PCR conditions. A known plasmid control was processed through our pyrosequencing amplicon sequencing pipeline (Fig. 1). All PCR conditions were evaluated on the same pyrosequencing plate to remove concerns about run-to-run variability.

Briefly, 1st round single fragment or multiplex PCR was done on linearized pNL4-3 DNA to amplify ~3kb fragments encompassing *gag-Δpol* and *env* (Fig. 2). These products were used for multiple 2nd round PCRs using primers that spanned 300-500 bp segments encompassing the 1st round amplicons. Each 2nd round reaction amplified 2 fragments, one from the *gag-pol* region and one from the *env* gene. These primers contained 454 adaptors so that sequencing could be performed without a shearing or adaptor ligation step.

For the enzyme error tests, 35 cycles of 1st Round and 35 cycles of 2nd round PCR were done on linearized pNL4-3 plasmid DNA. All enzyme comparisons were run on the same pyrosequencing plate. Advantage 2 was the DNA polymerase used in several of the 2nd round PCRs because of its increased sensitivity compared to other enzymes (data not shown). All errors were calculated using in-house Perl scripts. For these experiments, homopolymer errors were not tabulated and only the frequencies of substitutions calculated. A Wilcoxon test was used to determine if there was a significant difference in the error rates of the different PCR conditions.

For the plasmid mixtures, quantification of DNA was performed by Nanodrop to estimate the number of copies for each individual plasmid. They were then mixed in different proportions (Figure 5). The plasmid mixture underwent a limiting dilution endpoint PCR in order to quantify the number of amplifiable templates. This estimate was used for three different inputs into independent PCRs to demonstrate the relationship between input molecules and sensitivity.

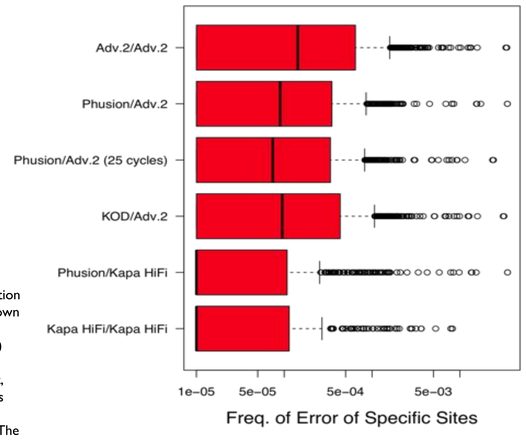

Figure 3. Site specific substitution error varies between different DNA polymerases. Two rounds of PCR were done with 35 cycles 1st round and 35 cycles 2nd round unless noted. The name of the polymerase on the left is the enzyme that was used in the 1st round of PCR, the enzyme on the right is the enzyme that was used in the 2nd round of PCR.

Results

Figure 1 shows the difference between substitution error rates resulting from changes in PCR conditions. Of five different comparisons, only two showed any statistically significant difference. Multiplexing the first round PCR increased the error rate (p=0.003) compared to when the first rounds amplified single fragments (which were then then pooled together for the second rounds). We did not test the difference in error between single fragment 2nd round PCR and multiplexed 2nd round PCR.

The other PCR condition that led to a difference in substitution error was the copies of template present at the start of each PCR. ~125 copies were put into 4 PCR reactions and then pooled afterwards for a total of 520 copies. ~26 copies were put into 20 reactions for the same total number of copies. Interestingly, pooling 20 reactions resulted a lower error rate than when 4 reactions were pooled (p=0.01).

For the enzyme comparisons, Kapa HiFi used for both rounds of PCR showed a lower frequency of substitution error than any other combination of enzymes (Fig. 3 and Table 2). The enzyme choice for both the first and second rounds had an impact on substitution error.

The upper limit of background substitution error determines the cutoff for which variants can be accurately called. Using our in-house correction pipeline (see Poster A140) we corrected homopolymer errors for the Kapa HiFi sample. This is shown in Figure 4. Correction of the Kapa HiFi plasmid control sequence leads to a maximum site-specific error of 0.05% (95% C.I), vs. 0.12% for Advantage 2.

Besides errors occurring during PCR, another determinant for the sensitivity of deep sequencing was the number of input template molecules. In the field, these numbers are often, and poorly estimated from plasma viral loads measured on a different and shorter template using different conditions for RNA extraction cDNA production and PCR. We created a mixture of HIV plasmids that were represented in different frequencies. This plasmid mixture underwent PCR and 454 sequencing using 3 different input template numbers (Figure 5). As expected, lower frequency variants dropped out at the lower copy input.

Discussion

Pyrosequencing is often used to detect and quantitate low-frequency HIV variants in a population. An arbitrary 1% cutoff is often used to distinguish background error from real variants. However, this represents lesser depth to detection of variants than would be expected by the use of the term "deep" sequencing, and represents <1 log greater depth than standard, and much lower error-rate Sanger sequencing technology using multiple templates.

In an effort to increase the depth of reliable variant detection using pyrosequencing technology, we evaluated several parameters that we thought might influence error rate. The main causes of substitution error in 454 sequencing was found to be the type of DNA polymerase used, and how much DNA was produced during the PCR reaction. The latter was inferred by the fact that lower error rates were found if fragments were amplified singly rather than in multiplexed reactions. Also in support of this conclusion was the finding that lower error rates were found if the same number of products were amplified in many (n=20) versus fewer (n=4) PCR reactions. Perhaps unexpectedly, however, the number of PCR cycles, at least in the 2nd round (15 vs. 35) did not have a discernable impact on error rates. We did not in these experiments adjust the number of cycles in the first round PCR.

Thus, increasing the number of reactions with fewer copies per reaction and producing single fragments the first round of PCR (vs. multiplexing) would also be helpful in lowering the total error rate. Our results show that Kapa HiFi generated the lowest error rate, and coupled with our homopolymer correction software, had a maximum substitution error of 0.05%(95% C.I).

We also found that sensitivity and detection of minor variants is dependent on the number of HIV templates placed into PCR. Although this may seem trivial, large differences (~1-2 orders of magnitude) are commonly observed between clinical viral load measurements and PCR of other, longer fragments, depending on the length of the fragment and extraction, cDNA and PCR conditions (Mullins et al, unpublished).

Table 1. Polymerases used for the error rate comparison

Enzyme	Manufacturer
Advantage 2	Clontech
Phusion	Finnzyme
KOD	EMD4Biosciences
HiFi	Kapa Biosystems

Table 2. Average error for each enzyme combination

Condition	# of mapped reads	# of positions	% of insertions	% of deletions	% of mismatches	Total % of error
Adv.2/Adv.2	17046	5372632	0.34	0.17	0.20	0.70
Phusion/Adv.2	24115	7539396	0.30	0.17	0.12	0.60
Phusion/Adv.2 (25 cycles)	28481	9202658	0.25	0.15	0.11	0.50
KOD/Adv.2	22697	7147930	0.34	0.16	0.14	0.64
Phusion/Kapa HiFi	20517	6510135	0.34	0.15	0.07	0.56
Kapa HiFi/Kapa HiFi	9412	3302828	0.30	0.09	0.04	0.43

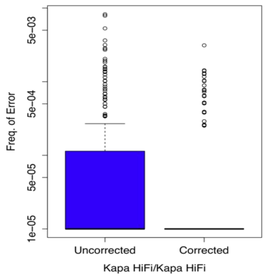

Fig. 4 Difference in error rate after applying our in-house 454 correction pipeline. The correction pipeline only adjusts homopolymer error, it does not change any substitution error. Most high frequency substitution errors fall within homopolymer regions, so the frequency of error decreases when homopolymer correction is applied.

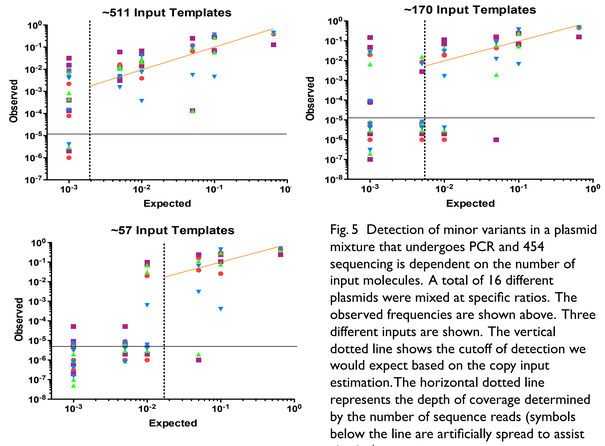

Fig. 5 Detection of minor variants in a plasmid mixture that undergoes PCR and 454 sequencing is dependent on the number of input molecules. A total of 16 different plasmids were mixed at specific ratios. The observed frequencies are shown above. Three different inputs are shown. The vertical dotted line shows the cutoff of detection we would expect based on the copy input estimation. The horizontal dotted line represents the depth of coverage determined by the number of sequence reads (symbols below the line are artificially spread to assist viewing).

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