High-resolution Inference of Multiplexed Anti-HIV Gene Editing using Single-Cell Targeted DNA Sequencing

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Abstract

Gene therapy-based HIV cure strategies typically aim to excise the HIV provirus directly, or target host dependency factors (HDFs) that support viral persistence. Cure approaches will likely require simultaneous co-targeting of multiple sites within the HIV genome to prevent evolution of resistance, and/or co-targeting of multiple HDFs to fully render host cells refractory to HIV infection. Bulk cell-based methods do not enable inference of co-editing within individual viral or target cell genomes, and do not discriminate between monoallelic and biallelic gene disruption. Here, we describe a targeted single-cell DNA sequencing (scDNA-seq) platform characterizing the near full-length HIV genome and 50 established HDF genes, designed to evaluate anti-HIV gene therapy strategies. We implemented the platform to investigate the capacity of multiplexed CRISPR-Cas9 ribonucleoprotein complexes (Cas9-RNPs) to simultaneously 1) inactivate the HIV provirus, and 2) knockout the *CCR5* and *CXCR4* HDF (entry co-receptor) genes in microglia and primary monocyte-derived macrophages (MDMs). Our scDNA-seq pipeline revealed that antiviral gene editing is rarely observed at multiple loci (or both alleles of a locus) within an individual cell, and editing probabilities across sites are linked. Our results demonstrate that single-cell sequencing is critical to evaluate the true efficacy and therapeutic potential of HIV gene therapy.

Introduction

Despite the advent of antiretroviral therapy (ART), the human immunodeficiency virus (HIV) was responsible for the deaths of 650,000 people worldwide in 2021, while 38.4 million individuals are currently living with HIV infection.[1] Moreover, ART needs to be taken on a lifelong basis due to the persistence of HIV latently-infected cells during treatment, which typically reinitiate spreading infection when ART is stopped. [2,3] As access to lifelong ART is extremely challenging in resource-limited settings where HIV predominates, there is a desperate need for effective prophylactic and curative strategies. [4,5]

Several HIV cure strategies are currently in development, including “shock-and-kill”,[6] “block-and-lock”[7], and oncology-derived immunomodulatory [8] and bone marrow transplantation approaches targeting the latent HIV reservoir. [9–11] The discovery of the CRISPR (Clustered Regularly Interspaced Short Palindromic Repeats)-Cas9 gene editing system has raised new hopes for gene therapy-based HIV cure strategies.[12–14] The Cas9 enzyme interacts with the scaffold sequence of a guide RNA (gRNA) complementary to a target sequence, enabling knockout of a targeted gene in a highly specific manner.[15] HIV gene therapy-based cure strategies in development typically aim to excise the HIV provirus directly,[16–22] or target host dependency factors (HDFs) including viral entry receptors that support viral persistence.[23–25] In recent years, a number of preclinical HIV cure studies have showed promising results using this technology. [17,19,26–29] An important caveat, however, arises from the high mutation and recombination rate of HIV-1,[30,31] which allows the virus to navigate broad expanses of sequence space in short periods of time. Studies of CRISPR-based HIV excision strategies targeting the viral genome at a single site demonstrate rapid evolution of viral resistance to the gene editing modality, mirroring the evolution of antiviral drug resistance in the setting of monotherapy.[32–36] Therefore,

the HIV genome will need to be targeted at multiple sites simultaneously. Drawing from lessons learned during the development of combination ART, a minimum of three distinct genome sites may need to be targeted to prevent evolution of resistance.[16] Similarly, the neutralization of a single HDF supporting HIV replication is unlikely to completely protect cells from infection, suggesting that multiple HDFs will likely need to be targeted simultaneously to achieve a functional or sterilizing cure for HIV infection.[16] Even when considering a single HDF locus, genes exhibiting codominance between alleles will likely require biallelic knockout to fully eliminate generation of the encoded protein and, in this manner, block viral infection. Illustration of this concept is provided in nature; CCR5-Δ32 heterozygotes typically produce enough CCR5 protein at the target cell surface to support HIV replication, while Δ32 homozygotes are fully protected from HIV infection (by the highly prevalent CCR5-tropic HIV strains).[37–39]

Based on the need for multitargeting to achieve an HIV cure, molecular diagnostics enabling detection of multiple edits within individual cells will be critical in assessing the true, real-world potency of anti-HIV gene therapy approaches. Currently-implemented technologies, including Sanger sequencing of bulk cell populations and gel-based imaging of excision products, do not enable inference of co-editing within single cells.[40] Furthermore, standard deep sequencing approaches will not suffice, due to read length limitations that prevent inference of genetic linkage between distal sites.[40] To address this need, we report here a novel, targeted single-cell DNA sequencing (scDNA-seq) pipeline featuring a hybrid experimental and computational structure to characterize anti-HIV gene editing at viral and host genome sites with extreme resolution. Our platform characterizes the near full-length HIV genome and 50 established HDF genes (identified in a primary cell-based CRISPR screen) at the single-cell level. We implemented the platform to investigate the capacity of multiplexed CRISPR-Cas9 ribonucleoprotein complexes (Cas9-RNPs) to simultaneously 1) inactivate the HIV provirus, and

2) knockout the *CCR5* and *CXCR4* HDF (entry co-receptor) genes in microglia and in primary monocyte-derived macrophages (MDMs), leveraging our previously published work[29]. Our findings demonstrate the utility and critical importance of scDNA-seq analysis to accurately and comprehensively evaluate the efficacy of antiviral gene editing strategies.

Results

Generation and validation of a customized panel allowing detection of CRISPR-Cas9 edits in the near-full length HIV provirus and HIV HDFs at the single-cell level

To develop our single-cell sequencing strategy to detect CRISPR-Cas9 editing, we first designed a sequencing primer panel. To evaluate gene editing within both the HIV provirus and HDFs supporting viral replication, we designed a panel targeting tiled amplicons spanning the HIV genome, and 53 HDF genes that were previously identified using a genome-wide CRISPR screen in primary CD4+ T cells (e.g. *CXCR4*, *CCR5* and *CD4*, which also facilitate HIV entry into myeloid cells).[41] All targeted genes, chromosomal positions, primer pair names, sequences, and genome coordinates are described in the supplementary materials (Supp. Table 1). Thirty-six primer pairs cover the HIV genome from position 30 to position 9516 based on the HIV HXB2 reference genome (Figure 1). This includes the 5' long terminal repeat (LTR) region, which has been targeted specifically in several HIV gene therapy strategies,[16,17,19,26–28,42] allowing detection of edits in highly conserved regions such as the Primer Binding Site (PBS) and packaging signals (PSI sequences).

Bulk cell and single-cell sequencing analyses provide concordant estimates of antiviral gene editing efficiencies.

In order to test our single-cell sequencing approach, we first targeted the HIV 5'LTR along with the host HIV co-receptor genes *CXCR4* and *CCR5* using CRISPR-Cas9 RNP complexes. We first tested our approach in the HC69.5 cell line, a clone derived from the C20 immortalized microglial cell that has been infected with an HIV-derived construct.[43] The presence of a d2eGFP sequence under the control of the viral promoter permits the detection of viral expression through flow cytometry measurement of GFP signal. Moreover, no latency reversal agent treatment is required to detect the GFP signal as the HC69.5 cell line expresses HIV constitutively.[44] We nucleofected HC69.5 cells with RNPs targeting the HIV 5'LTR, *CXCR4* and *CCR5* and evaluated editing efficiency using our scDNA-seq platform, Sanger sequencing and Tracking of Indels by Decomposition (TIDE) analysis, and flow cytometry (Figure 2A).

In regards to coverage across the HIV genome (Supplementary Figure 1A), we observed a gap in coverage between amplicons 6 and amplicon 21. This reflects the fact that the provirus in the HC69.5 clone lacks the corresponding region of the HIV genome (consisting of a part of *gag* and the whole *pol* gene). We independently evaluated and validated the performance of our panel by applying our scDNA-seq pipeline to the J-Lat cell line (Jurkat cells harboring a near-complete, latent HIV proviral genome). The read depths observed across amplicons indicate robust coverage across the HIV provirus (Supplementary Figure 1B). Similar metrics were generated for the host genes indicating robust coverage (Supplementary Figure 1B and C).

We next evaluated gene editing efficiencies at viral and host loci using both Sanger (bulk) and single-cell NGS approaches. Three measurements were generated for each targeted site: 1) TIDE analysis of Sanger bulk sequencing data, 2) Pseudo-bulk analysis of single-cell NGS data using CRISPResso2 software (ignoring single-cell barcodes), and true single-cell analysis using CRISPResso2 (interpreting single-cell barcodes). Overall, the three distinct analytical methods

(leveraging two distinct sequencing approaches) provided highly concordant estimates of gene editing efficiencies at all three loci (Figure 3). We observed high editing efficiencies in the HIV LTR and *CXCR4* (mean efficiency of 74.75 and 60.7%, respectively), while editing of *CCR5* as measured by all three methods was less efficient (21.11% mean editing efficiency) (Figure 3A).

The observed highly potent editing of the HIV LTR was correlated with a 88% reduction of GFP signal in edited cells as compared to cells nucleofected with control guides (48.7% to 6.31%) (Figure 3C), demonstrating the accuracy of our sequence-based inference of gene editing.

In addition to immortalized microglia, we applied our scDNA-seq pipeline to infer antiviral gene editing in primary monocyte-derived macrophages (MDMs), which are key constituents of the latent HIV reservoir *in vivo* that will likely need to be targeted by HIV cure strategies.[45] We isolated monocytes from freshly-collected blood, and nucleofected them with *CXCR4*- and *CCR5*-targeting RNPs. After a 7-day M-CSF treatment, MDMs were harvested and characterized by genetic and immunophenotypic analyses (Figure 2B). As it is known that CRISPR editing can impact cell function and metabolism, we first sought to evaluate the impact of editing on macrophage immune function. We incubated edited and unedited cells with fluorescent beads and measured phagocytic uptake by macrophages using flow cytometry (Supplementary Figure 2). We observed a similar level of phagocytosis in edited and unedited cells, demonstrating that a key macrophage immune function was not impacted by nucleofection or Cas9 activity.

Similar to our observations in immortalized microglia, bulk-cell and single-cell analyses provided highly concordant estimates of gene editing efficiencies in primary MDMs. We observed high

editing efficiency in *CXCR4* (mean efficiency of 73.52%), while editing of *CCR5* as measured by all three methods was once again less efficient (17.54% mean editing efficiency) (Figure 3B).

Single-cell sequencing enables highly-precise characterization of CRISPR-induced indel spectra.

Precisely characterizing the indel spectra induced by CRISPR gene editing is critical to evaluating therapeutic efficacy and safety. It is now known that the CRISPR-induced indel spectrum is correlated with editing efficiency at a specific site; the less diverse the indel spectrum, the higher the editing efficiency.[46] We therefore sought to determine if our scDNA-seq pipeline enabled higher-resolution characterization of indel spectra as compared to standard Sanger sequencing and TIDE analysis. Our analyses unequivocally demonstrate that scDNA-seq identifies CRISPR-induced indel spectra with significantly enhanced sensitivity ($p=0.025$, paired t test), typically revealing a broad range of indels and minority variants (Figure 4). In contrast, Sanger sequencing only enables detection of dominant, majority indel variants (Figure 4).

Single-cell sequencing enables inference of antiviral gene editing at multiple loci within individual cells

HIV enters target cells such as T cells and myeloid cells using the CD4 receptor and the CXCR4 or CCR5 co-receptors. It has been described that a switch occurs during the course of HIV infection, in favor of one or the other co-receptor.[47] It is therefore necessary to knock-out both co-receptors to avoid a switch, preventing viral rebound. Leveraging our single cell sequencing approach, we sought to evaluate co-editing efficiency at a single-cell level in HC69.5 and primary MDMs. We processed our single-cell sequencing data through CRISPRessoV2 software (as described in the Materials and Methods), and summarized the data in Figure 5. Each line represents one cell, characterized by its editing frequency at each targeted site. The

shade of green indicates the editing frequency (dark green for high editing frequency to white for low editing frequency) (Figure 5A&C) Full co-editing (editing of all targets) was observed in 37.4% of HC69.5 cells (Figure 5B) and in 22.38% of primary macrophages (Figure 5D). The single-cell resolution of our analysis demonstrates the extreme heterogeneity of antiviral gene editing patterns across cells (Figure 5), informing therapeutic efficacy while demonstrating that cell-specific factors likely modulate the intake and activity of gene editing machinery.

Single-cell sequencing enables allele-specific evaluation of antiviral gene editing performance

This single-cell approach also allows us to survey editing in each of the two alleles of each targeted host gene by using specific editing efficiency thresholds.[48] This is of particular importance as editing on one chromosome may not be enough to fully shut down the expression of a HDF and prevent HIV infection. For this purpose, we categorized allele-specific editing based on observed editing efficiencies (defined as the frequency of NGS reads harboring indels); genes with an editing efficiency above 0.8 were considered to be edited at both alleles, genes with an editing efficiency value between 0.2 and 0.8 were considered to be edited at only one allele, and genes with an editing efficiency below 0.2 were categorized as non-edited (Figure 5A&C). We observed that 43% of the HC69.5 cells were $CXCR4^{mut}/CXCR4^{mut}$ and 42% $CXCR4^{wt}/CXC4^{mut}$. For CCR5, we observed that 10% of the cells $CCR5^{mut}/CCR5^{mut}$ and 36% of the cells were $CCR5^{wt}/CCR5^{mut}$ (Figure 5B). In primary macrophages, approximately 57% $CXCR4^{mut}/CXCR4^{mut}$ and 28% of the cells were $CXCR4^{wt}/CXCR4^{mut}$. For *CCR5*, we observed that 4% $CCR5^{mut}/CCR5^{mut}$ and 21% of cells were $CCR5^{wt}/CCR5^{mut}$ (Figure 5D).

Gene editing events exhibit interdependence

Finally, we leveraged the power of our single-cell inference approach to determine whether antiviral gene editing events occur independently of one another. Briefly, we built contingency tables for each gene pair across both tested cell types (HIV-LTR, *CXCR4* and *CCR5* for HC69.5 and *CXCR4* and *CCR5* for MDMs) and determined if the co-occurrence of editing at both considered sites was observed more or less than expected by random chance. The expected values were determined by multiplying single cell editing efficiencies as measured in Fig. 4 from each considered pair (assuming that there are independent probabilities of editing at each targeted site). We observed that in both HC69.5 and MDMs, mono-editing (editing at a single targeted locus) was lower compared to the expected value, while co-editing and co editing frequency was higher than expected (Figure 6A, Fisher's exact t-test p-values ranging from 0.003 to <0.0001). Similarly, we sought to determine if editing at two alleles could be explained by independent probabilities. Again, we obsesrved higher than expected frequencies of co-editing, suggesting that antiviral gene editing events are not independent (Figure 6B&C) , p-value ≤ 0.001).

Discussion

Anti-HIV gene therapy will likely require editing of multiple host genes and/or sites in the proviral genome. According to mathematical models, more than 99% of total provirus in people living with HIV needs to be inactivated to achieve a functional cure.[16] Provirus inactivation requires editing on several sites due to viral genetic diversity and plasticity, which may result in the generation of escape mutants considering the exceptional adaptability of HIV.[35] Moreover, it is unlikely that 99% of infected cells will be targeted by the CRISPR-Cas9 system: some macrophages for example can infiltrate deep into tissues,[49] where delivery of the CRISPR payload can be very challenging.[50] One way to overcome this issue is to target HDFs such as

HIV co-receptors *CXCR4* and *CCR5* to prevent viral spread from cells harboring unedited provirus. [51–54] In this case, mathematical models predict that inactivating provirus in 60% of the infected cells and reducing the pool of susceptible cells by 68% is enough to achieve a functional cure.[16]

So far, no method has been proposed to rigorously assess the frequencies of such events *in vitro* or *in vivo*. Here, we demonstrate that single-cell encapsulation and targeted next generation sequencing, in combination with a specific bioinformatic pipeline, is a reliable and powerful method to characterize antiviral gene editing in both cell lines and primary cells. The editing efficiencies measured with our single-cell method are close but not identical to the results obtained with Sanger sequencing and TIDE analysis. The difference observed between the two methods can likely be explained by the reduced sensitivity of Sanger sequencing in comparison to NGS approaches,[55] resulting in minority genetic variants being excluded from the final results. Interestingly, the editing efficiency in cells targeted with only one guide at a time is similar to editing obtained in our multiplexed approach, likely due to saturation effects (data not shown).

Highly precise CRISPR editing at a targeted site is key to avoid lethal editing or production of aberrant cells. It has been shown that indel spectra directly correlate with editing precision and efficiency; the less diverse the indels, the more precise and potent the editing.[46] However, Sanger sequencing and TIDE analysis do not capture indel spectra with sufficient sensitivity, as revealed by our analyses. We demonstrated that indel spectra characterized with our single-cell approach were significantly more detailed and reliable than spectra obtained through TIDE, revealing several indel variants that were unseen in population sequencing data. This result is of particular importance when evaluating a therapeutic approach to predict gene editing efficiency and overall editing precision.

Single-cell sequencing is the only method that enables inference of co-editing events within the same targeted cell. Our results demonstrate that the frequency of co-editing events cannot simply be predicted by considering the product of editing efficiencies across individual loci due to linkage effects. Single cell sequencing also allows us to determine if gene editing is bi-allelic or mono-allelic; this is critical as neutralization of both alleles is often required to completely abrogate target protein expression. In this study, we were able to achieve 42% mono-allelic editing and 43% bi allelic-editing on the *CXCR4* gene in HC69.5 cells. For the *CCR5* gene, 36% of the cells showed mono-allelic editing and 10% bi-allelic editing. The results for *CCR5* reveal a considerable bias towards mono-allelic editing for this locus. Similar results were observed for primary MDMs, where bi-allelic editing of *CXCR4* was observed in 57% versus 28% for mono-allelic editing. For *CCR5*, 21% of the cells were edited in one allele and 3.5% of the cells in both alleles. Interestingly, flow cytometry data for the latter case showed a decrease of 11.3% in the frequency of CCR5-expressing cells compared to the control, and a 13% decrease in mean fluorescence intensity (MFI) (data not shown), without any impact on cell function (as represented by phagocytic activity). This demonstrates that the relationship between gene editing and protein expression is complex, and in some cases, the intact allele may compensate for the knockout allele resulting in a lower-than-expected impact on protein expression. These results highlight the need for a comprehensive method to evaluate gene editing activity such as the single cell approach used here.

The fact that our observed gene editing frequencies across loci and alleles on a cellular level were not independent suggests that some cells may be more permissive than others to editing. The permissiveness of a cell to Cas9-RNPs could be due to several factors: the efficiency of

nuclear transport machinery, epigenetic factors such as chromatin state or methylation, or DNA repair machinery.

In conclusion, our study demonstrates that our single-cell pipeline is a powerful approach to evaluate the potency of multi-targeting antiviral gene editing strategies. It yields critical insights into co-editing events across targeted loci and alleles, of key relevance to anti-HIV gene therapy. Our method and panel design can be further expanded to survey a broad range of bioinformatically-predicted off-target sites, to determine the specificity and safety of gene therapy approaches. In addition, our panel can be expanded to reliably characterize non-subtype B proviruses, which will be an essential developmental step when evaluating therapeutic efficacy in diverse global settings. In combination with rational guide design and continuing innovation in the engineering of gene editing machinery, our single-cell sequencing approach provides another key tool to facilitate the clinical development of cure-focused HIV gene therapy approaches.

Materials and Methods

Primary Human Monocyte Isolation

Primary monocytes were isolated from blood obtained from healthy donors (Vitalant, San Francisco, CA). A total of 15 mL of blood from TRIMA residual was diluted at a 1:1 ratio with a 3mM EDTA PBS 2% FBS buffer. The resulting mixture was layered onto a Ficoll-Paque density gradient medium and centrifuged at 1200 g for 30 minutes at room temperature. The peripheral blood mononuclear cell (PBMC) layer was carefully collected and subsequently resuspended in 500 µL of ACK lysis buffer (Gibco), followed by a 3-minute incubation at room temperature. To this, 40 mL of 3mM EDTA PBS 2% FBS buffer was added, and the cells were centrifuged for 2

minutes at 1200g. The PBMCs were then subjected to two washes with 50 mL of 3mM EDTA in PBS with 2% FBS, each followed by a 2-minute centrifugation at 1200g. After cell counting, 5 x 10^7 PBMCs were resuspended in 8.5 mL of 3mM EDTA in PBS with 2% FBS. Monocytes were subsequently isolated using the EasySep™ Human Monocyte Isolation Kit (StemCell Technologies) in accordance with the manufacturer's protocol.

Cell culture and Macrophage differentiation:

The HC69.5 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) (ThermoFisher Scientific, Cat. # 11965-118) supplemented with 10% fetal bovine serum (FBS) (Corning, Inc., Cat. #35-010-CV) and 1% Penicillin/Streptomycin (Fisher Scientific, Cat. #11548876) at 37°C with 5% CO2.

Freshly isolated monocytes were seeded in 12-well plates with 800 µL of differentiation medium comprising ImmunoCult™-SF Macrophage Medium (StemCell Technologies) supplemented with 50 µg/mL human M-CSF (PeproTech) for macrophage differentiation. After 7 days, fully differentiated cells were harvested using StemPro Accutase (Gibco) for downstream analysis.

RNP preparation and cell nucleofection

CRISPR RNA (crRNA) were designed *in silico* using the IDT Design Tool (Integrated DNA Technologies) based on their on-target and off-target score (highest score and lowest score, respectively). The sequences are for *CXCR4* : 5'-GAAGCGTGATGACAAAGAGG-3', CCR5: 5'-CATCATCTATGCCTTTGTCG-3' and HIV LTR: 5'-CTACAAGGGACTTTCCGCTG-3'. A mixture of 160 µM tracrRNA (Integrated DNA Technologies) and 160 µM crRNA (Integrated DNA Technologies) was incubated at 37°C for 30 minutes to generate guide RNA (gRNA) at a concentration of 80 µM. Subsequently, 4 µL of gRNA was combined with 4 µL of purified

SpCas9-NLS protein (QB3 core, Berkeley) at 40 µM and incubated at 37°C for 15 minutes to form Cas9-Ribonucleoproteins (Cas9-RNPs). For nucleofection, 8 µL of Cas9-RNPs were nucleofected per 1 x 10^6 HC69.5 cells or 1 x 10^6 monocytes, following the manufacturer's recommendations for a Lonza Nucleofector. The DN-100 program with SF Buffer was employed for HC69.5 cells, while the DS-150 program with P3 buffer was utilized for monocytes.

DNA Extraction, Sanger Sequencing and TIDE analysis.

Cells were harvested, and genomic DNA was extracted from approximately 0.5 x 10^6 cells using Qiagen Blood and Tissue Kits following the manufacturer's recommendations. The CRISPR-targeted sequences were subsequently amplified using Phusion® High-Fidelity DNA Polymerase (NEB). The primers used for the PCR were as follows: for *CXCR4*, 5'-AGAGGAGTTAGCCAAGATGTGACTTTGAAACC-3' and 5'-GGACAGGATGACAATACCAGGCAGGATAAGGCC-3'; for *CCR5*, 5'-CTCACTATGCTGCCGCCCAGTG-3' and 5'-TCACCAGCCCACTTGAGTCCGT-3'; and for HIV LTR, 5'-GCAAGTAGAAGAGGCCAATGAAGG-3' and 5'-TGCTAGAGATTTTCCACACTGACTA-3'. The PCR products were purified using NucleoSpin Gel and PCR Clean-Up kits (Macherey-Nagel) and subsequently sent for Sanger sequencing (ElimBio, California). AB1 sequencing files were then analyzed using TIDE software [56].

Flow Cytometry.

For GFP measurement, HC69.5 cells were harvested and washed three times in PBS (Gibco). Cells were resuspended at a concentration of 1 x 10^6 cells per mL and analyzed using an LSRII flow cytometer.

For CCR5 flow cytometry, approximately 1 x 10^6 cells were washed three times with PBS and resuspended in 100 µl of cell staining buffer. Cells were incubated with 5 µl of Human TruStain FcX™ to block Fc receptors for 15 minutes, followed by APC anti-human CD195 (CCR5) (clone J418F1, BioLegend) or 5 µl of the corresponding isotype control staining for 30 minutes. Afterward, cells were washed three times with cell staining buffer and resuspended in 500 µl of staining buffer. Samples were run on a BD LSR II flow cytometer (BD Biosciences).

For the phagocytosis assay, macrophages were washed and resuspended in serum-free IMDM at a concentration of 1 x 10^6 cells/mL. A mixture of 100 µl of macrophages and 100 µl of pHrodo™ Green E. coli BioParticles (Invitrogen, P35366) at 1 mg/mL was incubated for 45 minutes at 37°C. Phagocytosis was stopped by adding 900 µl of cold FACS buffer (PBS+2% PBS). Cells were centrifuged at 500g for 5 minutes and washed with 1 mL of FACS buffer. Subsequently, cells were resuspended in 100 µl of FACS Buffer and incubated with 5 µl of APC anti-human CD14 for 30 minutes. After three washes with FACS Buffer, cells were resuspended in 110 µl of FACS Buffer supplemented with 1 µM Propidium Iodide and analyzed on a FACS Calibur Flow Cytometer (BD Biosciences).

Flow cytometry data were analyzed, and plots were generated using FlowJo (v10.9.0).

Single-cell library preparation and sequencing

Libraries were prepared following the manufacturer's recommendations for custom panels (Mission Bio). Briefly, 300,000 HC69.5 or MDMs were encapsulated and barcoded using the Mission Bio Tapestri single-cell sequencing platform. Specific sequences, including HIV LTR, *CXCR4* and *CCR5*, were amplified by a multiplexed targeted PCR using primers described in Supplementary Table 1. The conditions were as follows: incubation at 95°C for 10 minutes,

followed by 20 cycles consisting of 30 seconds at 95°C, 10 seconds at 72°C, 6 minutes at 61°C, and 30 seconds at 72°C. Samples were then incubated at 72°C for 2 minutes and kept at 4°C until the next step. All samples exhibited DNA quantities within the expected yield range after PCR clean-up steps. Following library PCR and clean-up, samples were pooled using the Tapestri Sample Quantification Tool, and libraries were sequenced on an Illumina MiSeq using MiSeq Reagent Kit v2 (300-cycles) and a 2x150bp reaction, following the manufacturer's recommendations.

Bioinformatic analysis

Sequences were analyzed using a custom pipeline to determine genome editing rates for both the human and HIV genomes at each targeted amplicon in each cell. Briefly, the two 8bp cell barcodes were read from individual sequencing reads and compared to the list of 768 possible barcodes (v1 chemistry) with a custom Python script. Barcodes that were an exact match passed the filter, and those with a Levenshtein distance of 1 away from a unique barcode in the allow list were corrected to that barcode and also passed the filter. Reads that did not have both 8bp barcodes passing the filter were discarded (overall barcode fail rate was 5.3-6.5%). All reads with the same 16bp barcode were collected into individual files. Only barcodes with a minimum of 100 reads were processed using CRISPResso2 in batch mode.[57] The average number of aligned reads per cell for each amplicon was then plotted. The percentage of reads modified, and the modification state and frequency of *CXCR4*, *CCR5*, and HIV LTR for each cell and each amplicon were analyzed using Python (v3.x) and R Studio (v2023.0.2+561). Briefly, each mutation (indel) was characterized by its size. Cells with fewer than 5 reads for each CRISPR-targeted amplicon were filtered out, and the overall frequency for each indel size and total editing efficiency were plotted.

For the heterozygosity analysis, amplicons corresponding to targeted sites in CXCR4 and CCR5 genes and HIV-LTR were labeled with their mutational state (Wild Type or Mutated), and the frequency of each event was recorded. For each amplicon, each cell was classified into one of three classes: WT/WT, WT/Mut, or Mut/Mut based on the overall percentage of reads with indels in a given cell. Cells with < 20% modified reads were considered WT/WT. Cells with ≥ 20% and < 80% modified reads were considered heterozygous WT/Mut. Cells with ≥ 80% mutated reads were classified as homozygous Mut/Mut. A heatmap has been plotted summarizing these results where each line represents one cell. Custom source code used for the analysis can be found at this address: https://github.com/Pillai-Lab/sc-allelic_antiviral_gene_editing .

Statistical analysis

The results presented were analyzed with GraphPad Prism v10 (GraphPad Software, SanDiego, CA, USA). Information on specific tests that were used are described in the Results section.

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Author Contributions

M.S.B., L.R.C. and S.K.P. initiated the project and designed the experiments. M.S.B., K.A.R. and S.D. performed the bulk sequencing and M.S.B. analyzed the data. M.S.B., K.A.R. L.R.C. performed the single cell sequencing. Z.Y.D. and M.S.B. analyzed the scDNA-seq data. C.D.B. performed the phagocytosis assay. M.S.B. and S.K.P. prepared the manuscript and M.S.B., P.B. and S.K.P. jointly supervised the work.

Declaration of interests

There is no conflict of interest regarding the publication of this article.

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Figures and Tables

Figure 1. Map of primer binding sites across the HIV genome. Open reading frames across the entire HIV genome are represented. Blue and red bars represent forward and reverse sequencing primers, respectively. A total of 36 primer pairs were employed to achieve near-complete coverage of the HIV genome. Nucleotide coordinates corresponding to each primer pair are provided below the bars.

Figure 2. Schematic of anti-HIV gene editing workflow. A) RNP complexes were generated using guides targeting the LTR, CXCR4, or CCR5 loci. The resulting complexes were pooled at equimolar concentrations and delivered to HC69.5 cells utilizing a Lonza 4D-Nucleofector. After 48 hours of culture, cells were harvested for flow cytometry, DNA isolation, PCR and TIDE analysis and Tapestri single cell analysis. B) PBMCs were isolated from whole blood obtained from healthy donors. Monocytes were then isolated using a negative selection antibody cocktail. Simultaneously, CXCR4 and CCR5 targeting guides were generated and complexed with purified Cas9 protein to form CXCR4 and CCR5 targeting Cas9-RNPs. These RNPs were pooled at equimolar concentrations and delivered to freshly isolated monocytes using a Lonza 4-D Nucleofector. Following stimulation with M-CSF for 7 days, MDMs were polarized. Harvested cells underwent DNA extraction for PCR amplification and Sanger sequencing, scDNA-seq library preparation, flow cytometry, and phagocytosis assays.

Figure 3. Comparison of inferred gene editing efficiencies between single-cell and bulk Sanger sequencing approaches. Inferred editing efficiency comparison obtained using bulk TIDE analysis (blue), bulk CRISPResso2 (red) and single cell CRISPResso2 (green) methods in HC69.5 (A) or primary MDMs (B). Editing efficiencies are indicated above the bars for each

method and each target, expressed as percentages. (C) Flow cytometry on HC69.5 cells nucleofected with scrambled guide RNPs (control) or CXCR4, CCR5 and HIV LTR-targeting RNPs. GFP signal indicates HIV LTR activity.

Figure 4. Comparison of inferred indel spectra between single-cell and bulk Sanger sequencing approaches. The sum of the number of peaks inferred using TIDE (green) or single-cell analysis (blue) were plotted for each target in each cell type. The target names and cell types are labeled along the x-axis, with the number of detected indel sizes indicated on the y-axis. Statistical significance was determined using a paired t-test (*$p<0.05$)

Figure 5. Heatmap visualization of multi-site gene editing within individual cells. Each row in the heatmap represents a single cell, with the shade of green indicating the editing frequency for each target. (A) Detailed view of the editing per cell and per loci analyzed in HC69.5 cells (editing efficiency at each site is represented as a continuous variable). (B) Discretized editing heatmaps for HC69.5 cells. Editing values above 0.8 indicate bi-allelic editing, between 0.2 and 0.8 indicate mono-allelic editing, and below 0.2 indicate no editing. Biallelic editing is represented in dark green, monoallelic editing in light green and unedited in pale green. The number of cells in each group is listed (when counts exceed 100). (C) Detailed view of the editing per cell and per loci analyzed in MDMs. (D) Discretized editing heatmaps for MDMs.

Figure 6. Interdependency of gene editing events. Expected values were determined as the coefficient between the single-cell editing efficiency frequency for one target (as measured in Fig. 4) to the single-cell editing efficiency frequency of the other target (assuming that the probabilities of editing at each targeted site are independent). Expected values of cells harboring no editing, mono editing, or co-editing between CXCR4 and CCR5 loci (A) or between

CXCR4 and CCR5 alleles (B & C) have been calculated and plotted (green) and compared to observed values (blue). Statistical significance was determined using Chi-square tests, comparing observed values to expected values (***P < 0.001, ****P < 0.0001).

Supplementary Figure 1. Amplicon coverage across host and viral genome targets. (A) Read coverage for each amplicon in the HC69.5 cell line obtained through the CRISPResso2 pipeline are plotted. The amplicon names are labeled along the x-axis, with the average reads per cell plotted on the y-axis. A gap in reads observed between AMP6 and AMP21 corresponds to the deletion identified in the HC69.5 HIV provirus. (B) Coverage across the HIV genome and host genes in the J-Lat 11.1 cell line. (C) Host gene coverage in HC69.5 cells. The amplicon name is indicated on the x-axis and the average reads per cell is indicated in the y-axis.

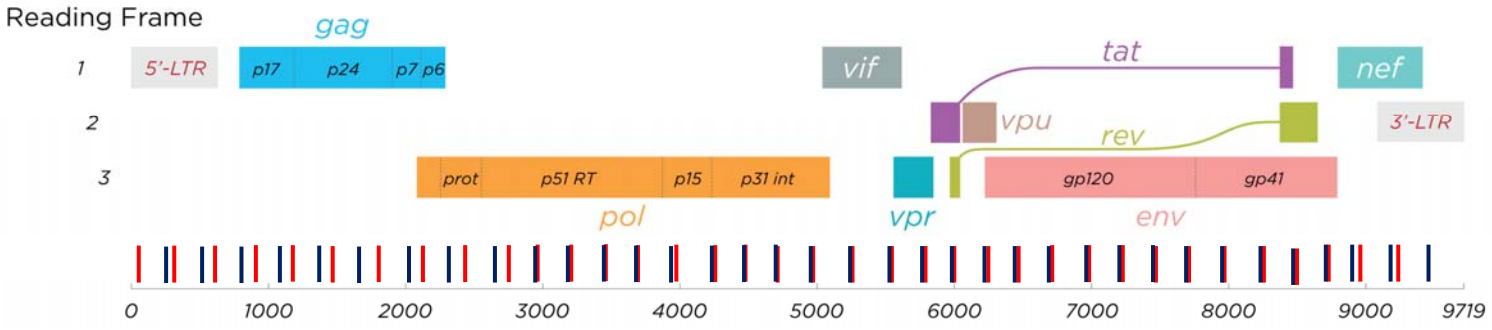
Supplementary Figure 2. Gene editing workflow does not impede the functional activity of primary MDMs. Cells were incubated for 45 minutes with fluorescent bioparticles to measure phagocytosis and analyzed by flow cytometry. The gating strategy is indicated on the left and the frequency of CD14+ Bioparticles+ cells compared to the total of CD14+ cells and the mean fluorescent intensity (MFI) for control cells and knocked out cells is indicated in the table on the right.

Supplementary Figure 3. Indel spectra in HC69.5 and MDMs infered by TIDE analysis or single cell analysis. A) Indel spectra determined using Sanger sequencing and TIDE software. Red bars indicate indel variants occuring at statistically significant frequencies as determined by TIDE analysis (Pearson's chi-squared p<0.001), and black bars represent indel variants that did not meet the statistical cutoff. Indels in size from -25 (25 nucleotide deletion) to +25 (25 nucleotides insertions) were plotted. Percentage contribution for each indel size is indicated

above each bar (when percentages exceed 10%). B) Indel spectra characterized using our custom bioinformatic pipeline for targeted single-cell sequencing.

Supplementary Table 1. Complete list of targets in scDNA-seq panel. For each amplicon in the panel, the following information is indicated: amplicon ID, chromosome location (Genbank accession number K03455.1 pertaining to the HIV-1 HXB2 reference genome is listed for HIV amplicons), primer coordinates, forward and reverse primer sequences, amplicon sequence, and targeted gene ID are provided.

Figure 1

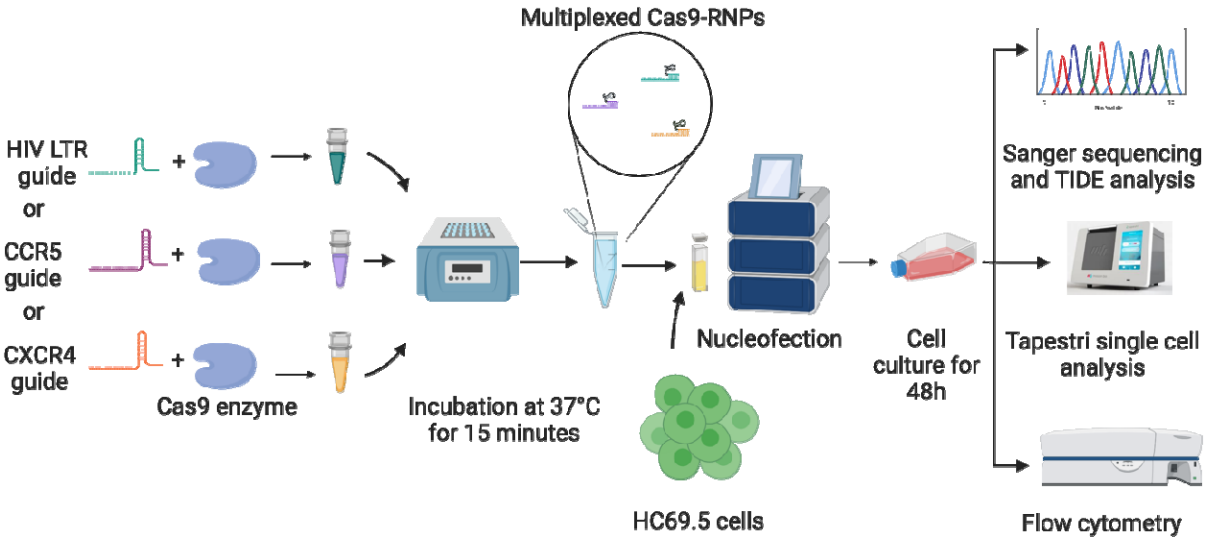


| : 5' primers

| : 3' primers

Figure 2

A.


Multiplexed Cas9-RNPs
HIV LTR guide
or
CCR5 guide
or
CXCR4 guide
Cas9 enzyme
Incubation at 37°C for 15 minutes
Nucleofection
HC69.5 cells
Cell culture for 48h
Sanger sequencing and TIDE analysis
Tapestri single cell analysis
Flow cytometry

B.

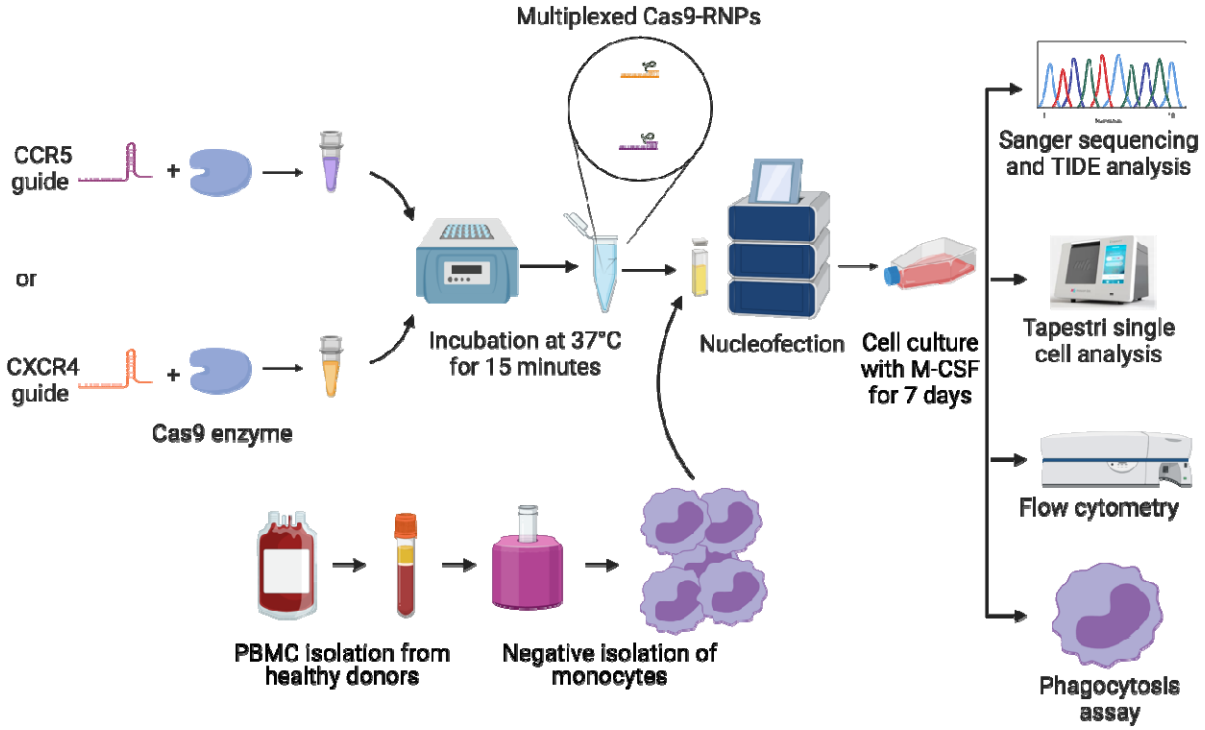

Multiplexed Cas9-RNPs
CCR5 guide
or
CXCR4 guide
Cas9 enzyme
Incubation at 37°C for 15 minutes
Nucleofection
Cell culture with M-CSF for 7 days
Sanger sequencing and TIDE analysis
Tapestri single cell analysis
Flow cytometry
Phagocytosis assay
PBMC isolation from healthy donors
Negative isolation of monocytes

Figure 3

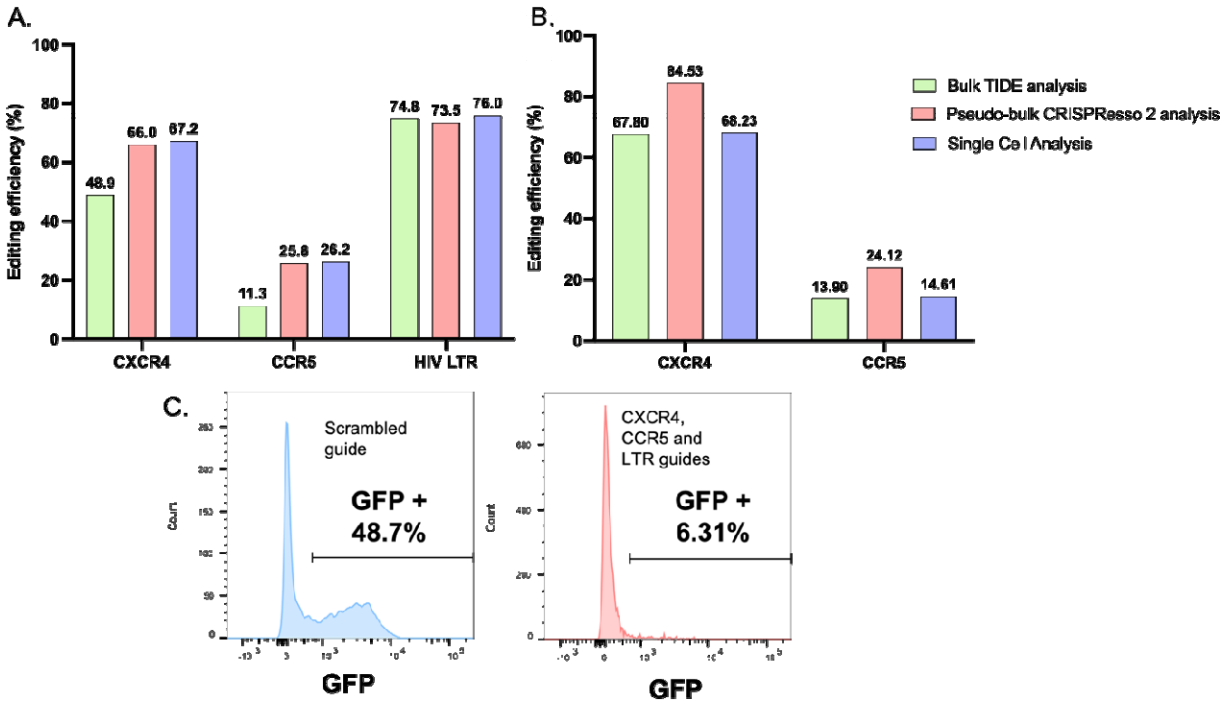

A.
Editing efficiency (%)
100
80
60
40
20
0
48.9
66.0
67.2
11.3
25.6
26.2
74.8
73.5
76.0
CXCR4
CCR5
HIV LTR
B.
Editing efficiency (%)
67.80
84.53
68.23
13.90
24.12
14.61
CXCR4
CCR5
Bulk TIDE analysis
Pseudo-bulk CRISPResso 2 analysis
Single Cell Analysis
C.
Scrambled guide
GFP +
48.7%
Count
GFP
CXCR4, CCR5 and LTR guides
GFP +
6.31%
Count
GFP

Figure 4.

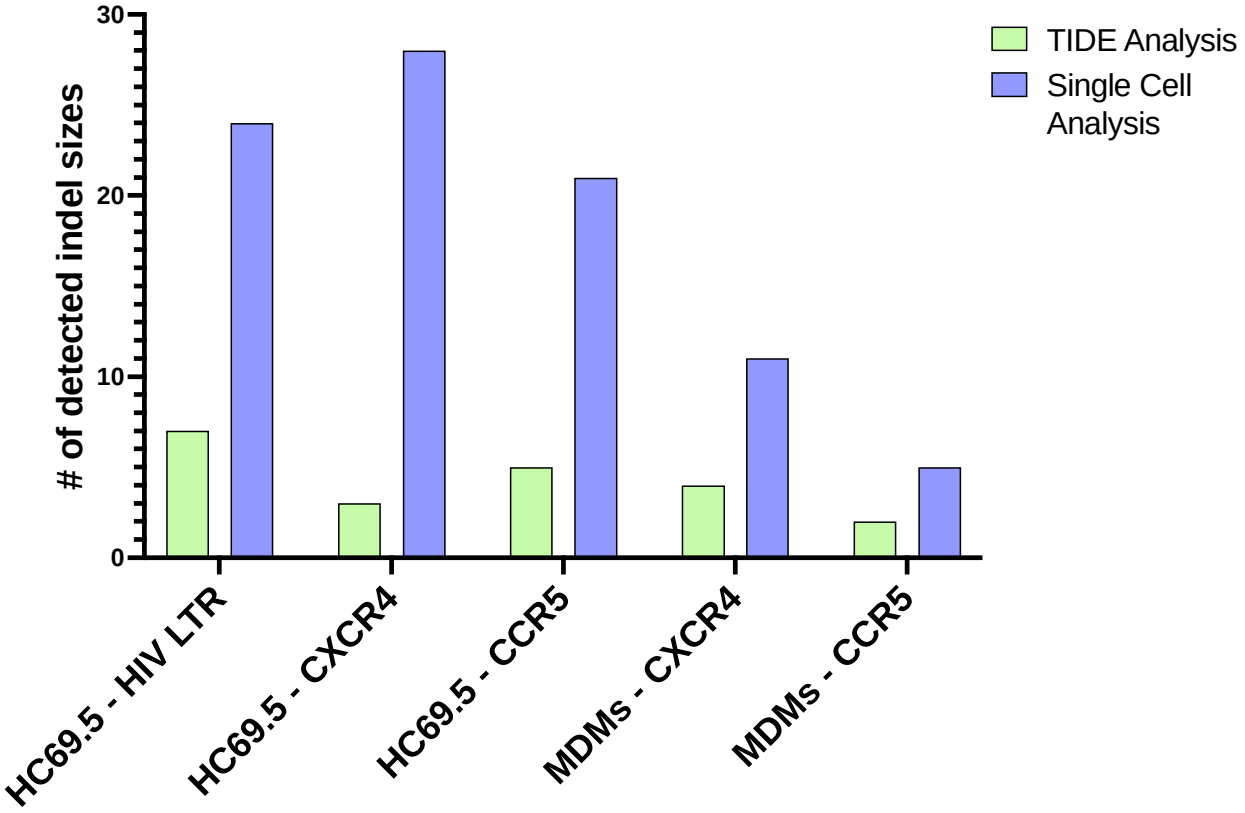

30
20
10
0
# of detected indel sizes
TIDE Analysis
Single Cell Analysis
HC69.5 - HIV LTR
HC69.5 - CXCR4
HC69.5 - CCR5
MDMs - CXCR4
MDMs - CCR5

Figure 5

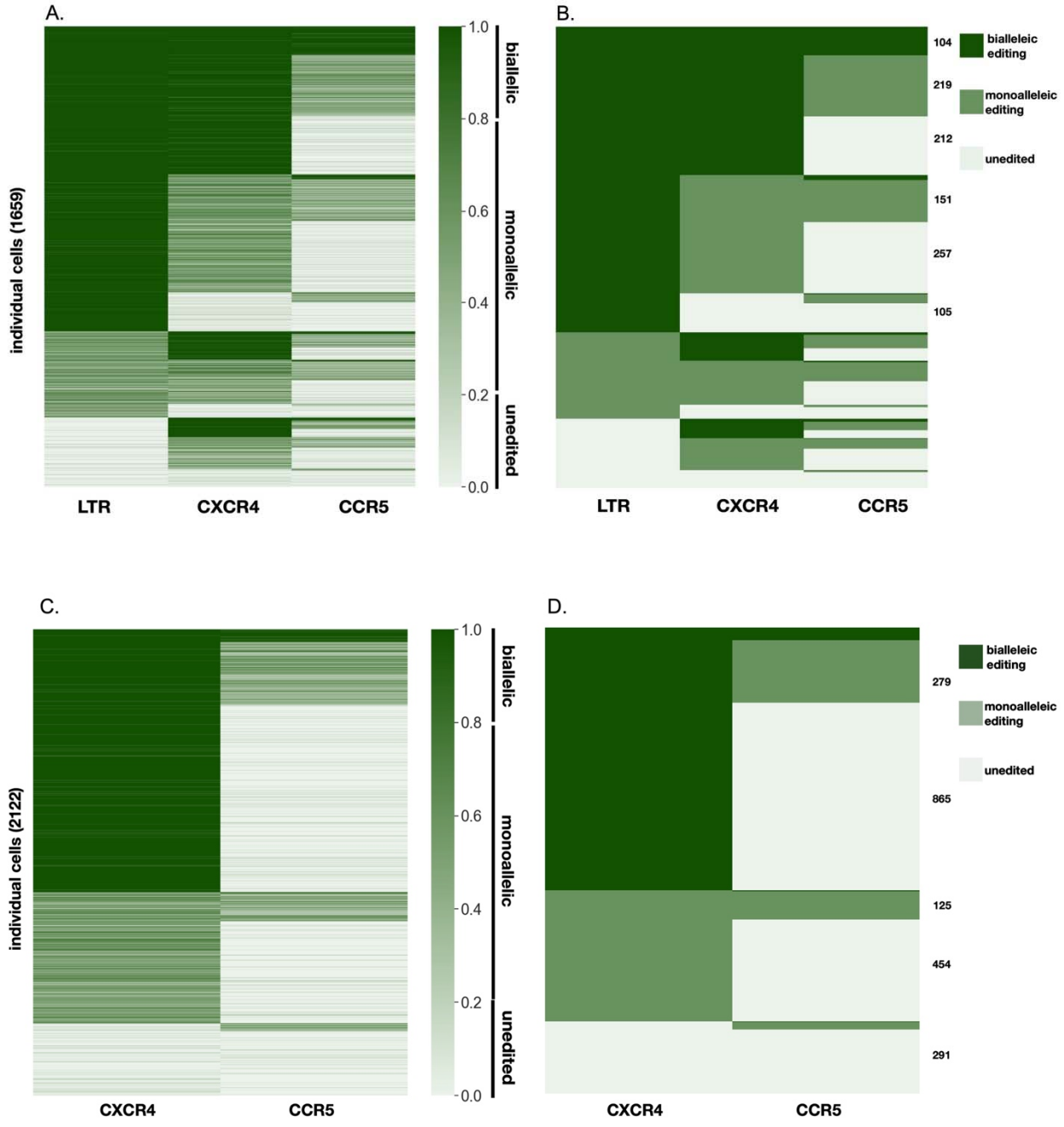
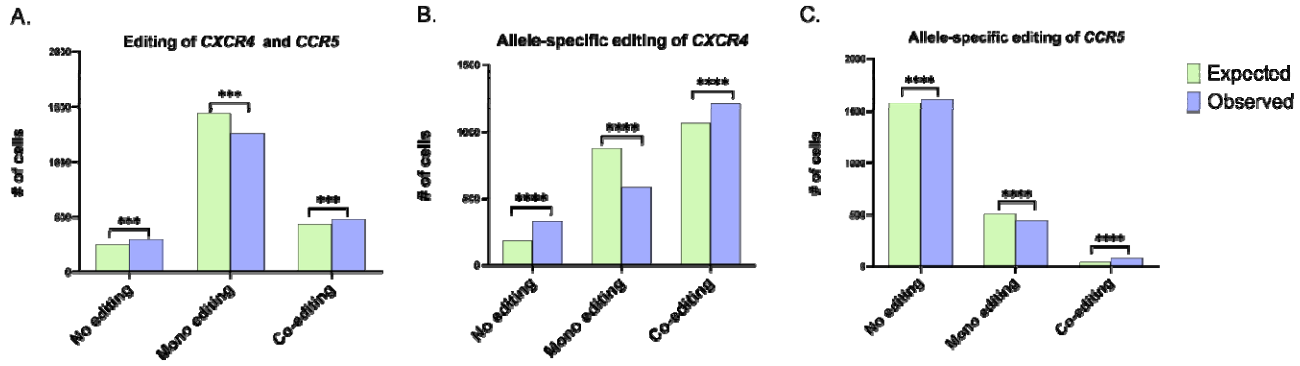

A.
individual cells (1659)
LTR
CXCR4
CCR5
1.0
0.8
0.6
0.4
0.2
0.0
biallelic
monoallelic
unedited
B.
104
219
212
151
257
105
bialleleic editing
monoalleleic editing
unedited
C.
individual cells (2122)
D.
279
865
125
454
291

Figure 6


A.
Editing of CXCR4 and CCR5
# of cells
No editing
Mono editing
Co-editing
B.
Allele-specific editing of CXCR4
# of cells
No editing
Mono editing
Co-editing
C.
Allele-specific editing of CCR5
# of cells
No editing
Mono editing
Co-editing
Expected
Observed

Supplementary Table 1

Amplicon ID	Chromosome	Forward Primer Amplicon start coordinates	Amplicon end coordinates	Reverse Primer coordinates	Forward Primer Sequence	Reverse Primer Sequence	Amplicon Sequence	Target
B19_HIV_AMP1	KIDAS1x1	7	30	180	201	GGCCAATTCGAGCGCGGTGGTCGTCGTTT	GGCCAATTCGACCCGAACCGAACGAGATCCTTHIV_LHK82	HK82
B19_HIV_AMP2	KIDAS1x1	206	225	402	420	ACGCCGTCGAACTGCGAGGGCCGCCACT	ACGCCGTCGAGCGCTCGATGGAATGGATGACCGCHIV_LHK82	HK82
B19_HIV_AMP3	KIDAS1x1	441	462	678	696	TTTTGCCGTGTACTCCGGTCGAGAGAGCT	TTTTGCCGTGACTCCGGTCGTCGTCGGTTAGACCGACTHIV_LHK82	HK82
B19_HIV_AMP4	KIDAS1x1	738	756	989	1012	CGACTGGTGAGTCTGTCGACGAAATTTGACTACGGCHIV_LHK82	CGACTGGTGAGTCGGCGAAAATTTGACTACGGCHIV_LHK82	HK82
B19_HIV_AMP5	KIDAS1x1	1075	1099	1288	1315	GACACCAAGGAAGCTTAGAGACGATAGAGCHIV_LHK82	GACACCAAGGAAGCTTAGAGACGATAGAGCHIV_LHK82	HK82
B19_HIV_AMP6	KIDAS1x1	1364	1383	1615	1638	GGGGGACGTGACGAGCGTGGTAGGGGCATACA	GGGGGACGTGACGAGCGCGATGGAAATGTTAAAAGCHIV_LHK82	HK82
B19_HIV_AMP7	KIDAS1x1	1765	1794	1989	2009	GCGAACCGCGAGCCGCCGAATTTCGGGCGT	GCGAACCGCGATCTGAAGACTATTTTAAAAGGATHIV_LHK82	HK82
B19_HIV_AMP8	KIDAS1x1	2153	2173	2394	2419	GAACGCGCCCACGAAGAGAGCTCGGTCGGCHIV_LHK82	GAACGCGCCCACGAAGAGAGCTCGGTCGGCHIV_LHK82	HK82
B19_HIV_AMP9	KIDAS1x1	2420	2452	2668	2694	AAGACGATTTGCAGCTACTCATAGAAATCTGT	AAGACGATTGATCAGCTACTCATAGAAATCTGTHIV_LHK82	HK82
B19_HIV_AMP10	KIDAS1x1	2685	2719	2913	2939	AAAATTCGGCGCGAAAATCCATCAGAATCGTT	AAATTCGGCGCGAAAATCCATCAGAATCGTTHIV_LHK82	HK82
B19_HIV_AMP11	KIDAS1x1	2940	2968	3160	3183	ACCATACCTACTGAGCTGAGCGTGTTGCTCA	ACCATACCTACTAAAGAATGAGCACCGGGAHIV_LHK82	HK82
B19_HIV_AMP12	KIDAS1x1	3184	3204	3399	3429	GGGGGACTTACGCTATTCATCGTTATGCGT	GGGGGACTTACGACCGAAGAAAAAGATGAAHIV_LHK82	HK82
B19_HIV_AMP13	KIDAS1x1	3400	3434	3674	3699	CACTAACGAGAGAGGCAATTATCGCTTCGT	CACTAACGAGAGAGGCGAGATCGGAGATCGGAHIV_LHK82	HK82
B19_HIV_AMP14	KIDAS1x1	3702	3727	3949	3973	AAGACTCCTAAATTTAAACTGCCATAGAAAGGA	AAGACTCCTAAATTTAAACTGCCATAGAAAGGAHIV_LHK82	HK82
B19_HIV_AMP15	KIDAS1x1	3978	4005	4206	4232	ACTGAGTTAGAAGCAATTTATTCAGCTTGCG	ACTGAGTTAGAAGCAATTTATTCAGCTTGCGHIV_LHK82	HK82
B19_HIV_AMP16	KIDAS1x1	4223	4239	4454	4478	TTAGATGGAATAGAGGCGGATGAGATGCT	TTAGATGGAATAGAGGCGGATGAGATGCTHIV_LHK82	HK82
B19_HIV_AMP17	KIDAS1x1	4479	4504	4706	4734	ATAGAGCCGCAATTATTCGCGAGAAGAGGG	ATAGAGCCGCAATTATTCGCGAGAAGAGGGHIV_LHK82	HK82
B19_HIV_AMP18	KIDAS1x1	4735	4760	4963	4990	CTGAAGTCTTAACAGCGCGATGCAAATGCG	CTGAAGTCTTAACAGCGCGATGCAAATGCGHIV_LHK82	HK82
B19_HIV_AMP19	KIDAS1x1	4991	5018	5213	5237	TAGTGAGTAAATATGTGCGAAGAAAGAGGH	TAGTGAGTAAAGATATGTGCGAAGAAAGAGGHIV_LHK82	HK82
B19_HIV_AMP20	KIDAS1x1	5238	5263	5451	5481	AAGAAGTATTGCGGCGTGGATACGCAGAAGA	AAGAAGTATTGCGGCGTGGATACGCAGAAGAHIV_LHK82	HK82
B19_HIV_AMP21	KIDAS1x1	5485	5509	5709	5729	GCACTAGCAGCCGACTCCTGCCCAGCATCCC	GCACTAGCAGCGCTTAATAACGCAAAAAAGATAAHIV_LHK82	HK82
B19_HIV_AMP22	KIDAS1x1	5710	5756	5958	5977	GAACCCATAATCCTGCGATACGGACGATCCGT	GAACCCATAATAGAATTTGCGAACGTGCCTTHIV_LHK82	HK82
B19_HIV_AMP23	KIDAS1x1	5978	5996	6198	6222	AAGAAGCGGCGGAGAGGCGTCTATGAGTTC	AAGAAGCGGCGGAGAGGCGTCTATGAGTTCHIV_LHK82	HK82
B19_HIV_AMP24	KIDAS1x1	6223	6252	6447	6467	GAATGAGAGTTTGGGTTGGGGTCTGTGGCTA	GAATGAGAGTTTGGGTTGGGGTCTGTGGCTAHIV_LHK82	HK82
B19_HIV_AMP25	KIDAS1x1	6468	6496	6689	6713	GAACATCATATTGCTAAATGTGATGAGAAAT	GAACATCATATTGCTAAATGTGATGAGAAATHIV_LHK82	HK82
B19_HIV_AMP26	KIDAS1x1	6714	6737	6934	6957	AGCATAAGACCTCTGCTGACTTTGTATGAT	ACGTAAGACCTCTGACCTGCTGACTTTGTATGAHIV_LHK82	HK82
B19_HIV_AMP27	KIDAS1x1	6958	6983	7188	7216	TAGAATTGAGCGATGCTGTCTGTCATATTTC	TAGAATTGAGCGATGCTGTCTGTCATATTTCHIV_LHK82	HK82
B19_HIV_AMP28	KIDAS1x1	7217	7247	7434	7461	TAAGATTATGCAGGAAATCGAATAACACTTA	TAAGATTATGCAGGAAATCGAATAACACTTAHIV_LHK82	HK82
B19_HIV_AMP29	KIDAS1x1	7462	7488	7693	7717	CAATCGCCTCGTGGATAAAACAAATTATAHH	CAATCGCCTCGTGGATAAAACAAATTATAHH_LHK82	HK82
B19_HIV_AMP30	KIDAS1x1	7718	7740	7941	7960	CACCAACGGCAACTGCCTGCGGACGTCCTT	CACCAACGGCAAACAGACGACGTGCTGACGGHIV_LHK82	HK82
B19_HIV_AMP31	KIDAS1x1	7961	7994	8176	8205	AATCCTGCCTCGAAACATACCAAAAGGTGAA	AATCCTGCCTCGAAACATACCAAAAGGTGAAHIV_LHK82	HK82
B19_HIV_AMP32	KIDAS1x1	8206	8238	8430	8452	TCGAATTAGATAAGTCGGGCAGTTGTGGAT	TCGAATTAGATAAGTCGGGCAGTTGTGGATHIV_LHK82	HK82
B19_HIV_AMP33	KIDAS1x1	8453	8478	8675	8696	GAGATCCATCCGATTAGTGAACGCATCCTT	GAGATCCATCCGATTAGTGAACGCATCCTTGGCHIV_LHK82	HK82
B19_HIV_AMP34	KIDAS1x1	8697	8726	8919	8942	GGGAGCAGATAGGGTTATAGAAGTACAGGH	GGGAGCAGATAGGGTTATAGAAGTACAGGAHIV_LHK82	HK82
B19_HIV_AMP35	KIDAS1x1	8972	8992	9223	9245	AAGGACGAGGAGGAGCGCGGTCATCGTTGT	AAGGACGAGGAGGAGCGCGGTTCATCGTTGTHIV_LHK82	HK82
B19_HIV_AMP36	KIDAS1x1	9250	9277	9494	9516	AAGATGAAGAGCTGCCATAAAGGAGAGAACCDH	AAGATGAAGAGCGCCATAAAGGAGAGAACCDHHK82	HK82
B19_HIV_AMP37	chr1	19565711	19565737	19565927	19565949	TTCTGAATACTTCCCATCGCTGATTAAATGCCTG	TTCTGAATACTTCCCATCGCTGATTAAATGCCTG	HK82
B19_HIV_AMP38	chr1	19567484	19567510	19567630	19567656	TCATCGATGATCTTGTTCTTCCTCCTTACTTA	TCATCGATGATCTTGTTCTTCCTCCTTACTTAHMC1-02	HK82
B19_HIV_AMP39	chr1	19570370	19570392	19570549	19570574	TGTAGGAATGATATTTCTTATGTCTTCGGTTGCCA	TGTAGGAATGAAGGACCCAAGGAATCGGACGCGEMC1-03	HK82
B19_HIV_AMP40	chr1	19977199	19977396	19977214	19977231	ATCTTACGGCAGAAGGGGTTAGGGGACCCAA	ATCTTACGGCAGAAGGGGTTAGGGGACCCAA1A RS-02	HK82
B19_HIV_AMP41	chr1	19977857	19977882	19977976	19977976	TTAGCGTCCCAAAACCAGCCCCGTTGATGAA	TTAGCGTCCCAAAACCAGCCCCGTTGATGAA RS-03	HK82
B19_HIV_AMP42	chr1	19979821	19979842	19978041	19978065	ATGAGAGTTGAGGGGACGTCCCTCTGGGACG	ATGAGAGTTGAGGGGACGTCCCTCTGGGACG1A RS-01	HK82
B19_HIV_AMP43	chr1	15267786	15267813	15267990	15268016	GATCGTTATCTCCCGCTGTTAGTGTTGATCG	GATCGTTATCTCCCGCTGTTAAAGGCGAGCGCGAT	CDE1-02
B19_HIV_AMP44	chr1	15275202	15275394	15275498	15275525	TGCTCGCCATAATTGTGATGTTGCTGTCGTC	TGCTCGCCATAATTGTGATGTTGCTGTCGTCGAAACCDE1-03	HK82
B19_HIV_AMP45	chr1	15282308	15282336	15282492	15282519	ATTACCTCTATAGTCTTGGAGGTTGCCATTC	ATTACCTCTATAGTCTTGGAGGTTGCCATTCCDE1-01	HK82
B19_HIV_AMP46	chr2	27355010	27355051	27355214	27355237	CGCGGAGGGACCTTTCCAACCCCCTCACGCA	CGCGGAGGGACCTTTCCAACCCCCTCACGCPE8-03	HK82
B19_HIV_AMP47	chr2	27355657	27355688	27355835	27355856	GATAACGACCTCGTCCTTCCTCCTCCCTCCT	GATAACGACCTCGTCCTTCCTCCTCCCTCCTPE8-01	HK82
B19_HIV_AMP48	chr2	27356319	27356342	27356516	27356338	AAGCCAGCTCCACTCCCATGAGCAGCAGACC	AAGCCAGCTCCTCTAGAGACCAAGACTTGACPE8-02	HK82
B19_HIV_AMP49	chr2	47693660	47693692	47693906	47693934	CTGGTAGATAAACATTGTGAGTATCGTGAGT	CTGGTAGATAATAATTGTGAGTATCGTGAGTHH3-03	HK82
B19_HIV_AMP50	chr2	47703335	47703358	47703757	47703784	ATTCGACGAAACTCGGGTCATAGTATCTTT	ATTCGACGAAACTCGGGTCATAGTATCGTGCDM1HH3-02	HK82
B19_HIV_AMP51	chr2	47705395	47705420	47705616	47705644	TAATTATGTGCTAAGCGATAGTTAAGGTCT	TAATTATGTGCTAAGCGATAGTTAAGGTCTHH3-01	HK82
B19_HIV_AMP52	chr2	74689449	74689471	74689665	74689686	TATCTGCTCCCCCAATCCAGCGATCTGCGGGT	TATCTGCTCCCCCAATCCAGCGATCTGCGGGTHGB1-02MMG1-03	HK82
B19_HIV_AMP53	chr2	74691678	74691708	74691867	74691889	CTCTGACGAAGTCCGTGCTCTTTGACTACGC	CTCTTGACGAAGTCCGTGCTCTTTGACTACGCCDM1-01	HK82
B19_HIV_AMP54	chr2	136872764	136872787	136872982	136873003	ATGAGGAATGACCGTTGCAGCGTCGACATT	ATGAGGAATGACCGTTGCAGCGTCGCCCACPH4	HK82
B19_HIV_AMP55	chr2	136873185	136873206	136873368	136873393	GAAAGTACCAGTTTGCCAGTGGCAGCCGCC	GAAAGTACCAGTTTGCCAGTGGCAGCCGCCACPH4	HK82
B19_HIV_AMP56	chr2	237998976	237998997	237998776	237998801	TACGACCGATCGATGAGAAGGGTGATTAGGG	TACGACCGATCGACGTCCGTGGTCGAGAGGCCGCP18-01CDP1 8-02	HK82
B19_HIV_AMP57	chr3	238012735	238012736	238012924	238012949	ACCCGACCACTCGCGACTCCGAACGGATATT	ACCCGACCACTCGCGACTCCGAACGGACGCCGP18-03	HK82
B19_HIV_AMP58	chr3	46415146	46415170	46415361	46415385	GAACGATTCCTTCCTCGTGGAAAATAAGACG	GAACGATTCCTTCCTCGTGGAAATCATCGCCP5	HK82
B19_HIV_AMP59	chr3	127778890	127778914	127779111	127779135	TCTTTTGTTCGTCGGTGTGCTTGAATTCGTTA	TCTTTTGTTCGTCGTGGTGCCCGCTTGAAAACGUCEGEA1A-02	HK82
B19_HIV_AMP60	chr3	127779409	127779431	127779603	127779631	GCCTTGGCCTCTCGAGGATAAACCTAAACGT	GCCTTGGCCTCTCGATTCTTCGTCTGATTGGACDA1EGEA1A-03	HK82
B19_HIV_AMP61	chr3	127785943	127785967	127786042	127786068	AGTACAACACCTGATGAGGAAGGACTGAGAA	AGTACAACACCTATCCGATGAAGTCTCATACGT	EGEA1A-01	HK82
B19_HIV_AMP62	chr4	73957688	73957714	73957904	73957930	GGATATCCTACAAAGAATCGTTTGAAAGCCT	GGATATCCTACATCGAGAGAAGTGGAAAACGAHNKRD17-01	HK82
B19_HIV_AMP63	chr4	74800740	74800767	74800966	74800988	GGAGTCTTTGAGAACATGAGTGAGTGGAGAG	GGAGTCTTTGAGAACATGAGTGAGTGGAGAGCHNKRD17-02A NKRD17-02	HK82
B19_HIV_AMP64	chr4	83970944	83970973	83971129	83971162	GAATTGCGATTTAGAATAACTTTCCATTTATT	GAATTGCGATTTAGAATAACTTTCCATTTATTTTCDP14-03CDP14-01	CDP14-02	HK82
B19_HIV_AMP65	chr4	87969467	87969495	87969693	87969705	CGATCATAAAAGATCGGGAGGGCATTTGGAGT	CGATCATAAAAGAGACCCCCGAGGACTTTGGTGAAFF1-02AFF1-01	HK82
B19_HIV_AMP66	chr4	88055741	88055767	88055936	88055963	GTGACGATCGAGAATAAGAGCTACGATTCCGT	GTGACGATCGAGAATAAGAGCTACGATTCCGTFF1-03	HK82
B19_HIV_AMP67	chr4	177241149	177241167	177242361	177241382	GATCGCAGGGGGGCTCACTCAGCGATGATC	GATCGCAGGGGGCCGGTCCGCCGGCCGGAACCGGGGTPC13-01	PC13-03	HK82
B19_HIV_AMP68	chr4	177249261	177249281	177249447	177249477	AAGTATTGAATCATCTTCGTTATTTCATATGTATCTGGA	AAGTATTTGAGATCTACGTGTTTGTCTTAAATTTTTPC13-02	HK82
B19_HIV_AMP69	chr5	133311501	133311525	133311704	133311725	GGATTCAACAATGGGTTTTGGAGGAATGACG	GGATTCAACAATGCAGGCATTGGCCTGGCGTGV1A-01	HK82
B19_HIV_AMP70	chr5	133316477	133316497	133316729	133316751	GTGACTGGGATTTTGGAGTCTGAAATTCATCGW	GTGACTGGGATTTTGGAGTCTGAAATTCATCGWDA1-01/W DA1-02	HK82
B19_HIV_AMP71	chr5	138651324	138651348	138651518	138651548	AGAAGGCTGTAAAGTCGTTTTGTAAGAAGGA	AGAAGGCTGTAAAGTCGTTTTGTAATTTATGGA	HK82
B19_HIV_AMP72	chr5	138652661	138652679	138652871	138652895	TTGTGTTTGCCAGTCTGAGGATGGTTTTTCG	TTGTGTTTGCCAGTCTGAGGATGGTTTTTCGATAAATTMAT8A-02	HK82
B19_HIV_AMP73	chr5	138654481	138654506	138654658	138654680	GCCGCTTTTGTTTTGTATTTTGATTTCGATT	GCCGCTTTTGTTTTGTATTTTGATTTCGATTCGAAGATMAT8A-01	HK82
B19_HIV_AMP74	chr5	177819268	177819298	177819460	177819483	TGGGTAGAGTATCGTCGAGAGGTGACTGTCT	TGGGTAGAGTGATGGTGACGTCCTGGTGGTGCTMED8-03	HK82
B19_HIV_AMP75	chr5	177026868	177026894	177026947	177026939	ATCCCTACGTACTATCCGCAAGGTGAAGGGC	ATCCCTACGTACTATCCGCAAGGTGTATCCTMED8-02	HK82
B19_HIV_AMP76	chr5	177026999	177027022	177027202	177027224	CATGGGGCTAAGTCGAACGAAGGGCCGCGTCT	CATGGGGCTAAGACGTCAAGGGCCGCGTCTMED8-01	HK82
B19_HIV_AMP77	chr6	27839710	27839738	27839979	27839997	CGCAAGGTGGATCTTCTTAGGCATAATAGTGA	CGCAAGGTGGATCTTCTTAGGCATAATAGTGAHT1HB4-01;HT1HB4-02;HT1HB4-03	HK82
B19_HIV_AMP78	chr6	43492369	43492392	43492712	43492733	CCCAACGAGCAGTGGCCAAAGGAGCGTTGT	CCCAACGAGCAGTGGCGAGTGATGAAGGKPO5-01	HK82
B19_HIV_AMP79	chr6	43498310	43498338	43498505	43498529	ATAATCTATCTATCGGAGGACTTTCGGTGT	ATAATCTATCTATCTTCCTTCGAGCTTTCCKPO5-02	HK82
B19_HIV_AMP80	chr6	43516067	43516092	43516267	43516294	CGTCCTGAGTTGGACTGCTTTGGTTTTCTTT	CGTCCTGAGTTCTAAATTGGTTGCTAATGAGAACPO5-03	HK82
B19_HIV_AMP81	chr8	74898819	74898836	74898945	74898975	ATAATTTTTTTCAACCTGAAACAGGGATTAA	ATAATTTTTTTCAACCTGAAACAGGGATTAAAATACTCEE1-03	HK82
B19_HIV_AMP82	chr8	74867987	74868014	74868173	74868199	GAGACCTGTCACGAAGAGAGGAATCGATAA	GAGACCTGTCACGTCAAGAAGAAGAAAACTCEE1-01	HK82
B19_HIV_AMP83	chr8	74868200	74868227	74868436	74868459	TTACGAATAATTTAGTACGAGACGGGCTTGT	TTACGAATAAATTCATGGCGATCAGATGATATCEE1-02	HK82
B19_HIV_AMP84	chr8	109241239	109241265	109241411	109241438	TTTTCGCATGAGAATTTAAAGAGCTCGAGGA	TTTTCGCATGAGAGAGCAATTTAAAGAGCTCGAGGAHF3A6-01	HK82
B19_HIV_AMP85	chr8	109247209	109247237	109247382	109247413	AGCATGCTATATACTACCAGGCATCAAAAAA	AGCATGCTATATACTACCAGGCATCAAAAAATAGHF3A6-02	HK82
B19_HIV_AMP86	chr8	109254025	109254055	109254271	109254288	TACCATGAGGAATATGATGCTGCGATTATTA	TACCATGAGGAATATGATGCTGCGATTATTAGHF3A6-03	HK82
B19_HIV_AMP87	chr8	117658619	117658644	117658832	117658859	AAATTTCGCTTCGTTCGAATTTATATGAGGA	AAATTTCGCTTCGTTCGAATTTATATGAGGAGCHF3A6-04	HK82
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B19_HIV_AMP89	chr9	154795311	154795339	154795727	154795754	AAATGGACTGTGCTTGCGATCGAATTAAACTT	AAATGGACTGGAATATTAATGAGAGCAGCPJP1	PJ-01	HK82
B19_HIV_AMP90	chr9	155365441	155365468	155367669	155367649	GCTAAGGAGCTGACGTTGTCGATGAGTTTCG	GCTAAGGAGCTGACGTTGTCGATGAAAAPJP1	PJ-02	HK82
B19_HIV_AMP91	chr9	155101077	155101127	155101352	155101371	TGACTACCGAGGTGGCGAACTGGCGATCAT	TGACTACCGAGGTGGCGAACTGGCGATCATTCEDGP1	PJ-03	HK82
B19_HIV_AMP92	chr9	102776983	102776606	102776754	102776782	TGGAGGATGAGAATAGAATATACTTTGCCAG	TGGAGGATGAAAGCCATACCTCCATTTTGAAAERP4-02	HK82
B19_HIV_AMP93	chr9	102784319	102784364	102784556	102784579	TTCTGCTAGCAAACTTTCTGAATTGGGAAACAG	TTCTGCTAGCAAACTTTCTGAATTGGGGTCACERP4A-01	ERP4A-03	HK82
B19_HIV_AMP94	chr10	70897755	70897780	70897761	70897788	GGAATGTTTGAGCAATTTAACGCGATCCCGC	GGAATGTTTGAGCGTTTAACGCTTGTCCCGTGATGHNRNPH5-02;HNRNPH5-01	HK82
B19_HIV_AMP95	chr10	72610832	72610855	72611057	72611081	TGGTTGCATAGGAGGATAAGAGACGAAATCG	TGGTTGCATATCCAGGATGATATCGATGGAATTTGPL1-02	HK82
B19_HIV_AMP96	chr10	72628022	72628045	72628219	72628243	CAAACGTTGTGAAGATCGGGGCATGAAGGCG	CAAACGTTGTGAAGATCGGGGCATGAAGGCGGCGPL1-01	HK82
B19_HIV_AMP97	chr10	72631121	72631146	72633341	72633364	CAAAAGCGTCATGGTCTCCGAGGATACTCTG	CAAAAGCGTCATGGTCTCCGAGGATACTCTGGPL1-03	HK82
B19_HIV_AMP98	chr11	145351051	145351118	145352624	145352594	AAAAGCAAGCGCGTCATCACCATCAAATTCAUP1	AAAAGCAAGCGCGTCATCACCATCAAATTCAUP1MA1-03	HK82
B19_HIV_AMP99	chr11	145399184	145399123	145399284	145399311	AGAGAACTCAAACACCTATCGGACTCTTTTP1	AGAGAACTCAAACACCTATCGGACTCTTTTP1MA1-02P1MA1-01	HK82
B19_HIV_AMP100	chr11	326055 2	326105 6	326106 4	326107 6	TGGTTGGTGATTTCATCTATACGACAGAGA	TGGTTGGTGATTTCATCTATTGTTGTTTTHF3A6-01	HK82
B19_HIV_AMP101	chr11	326153807	326153832	326155519	326155541	GTGACCCTTATCTCATGATCGGTGTGAACCCT	GTGACCCTTATAATGTGATGAGCATGATCGCCGHF3A6-02;HF3A6-01	HK82
B19_HIV_AMP102	chr11	118920203	118920224	118920429	118920450	CTTCGCGGCCACTCCTGTGATCGTGAAGGTT	CTTCGCGGCCACTCCCTGTCGTCGTCGAHFOU1-01	HK82
B19_HIV_AMP103	chr11	118922265	118922289	118922489	118922510	CGCGCCGCCGCCGTCCTTTCACC	CGCGCCGCCGCCGTCCTTTCACCGAGCGHFOU1-03	HK82
B19_HIV_AMP104	chr11	118926775	118926797	118926998	118927021	TAGATACGCTGACGTGCCGACCAGGTCGAT	TAGATACGCTGACGTGCCGACCAGGTCGATCGHFOU1-02	HK82
B19_HIV_AMP105	chr11	134946188	134946116	134946301	134946324	CGAAACGCTGACTGCAGTGATCGCAGCCTGA	CGAAACGCTGACTGCAGTGATCGCAGCCTGAAACPO5-02	HK82
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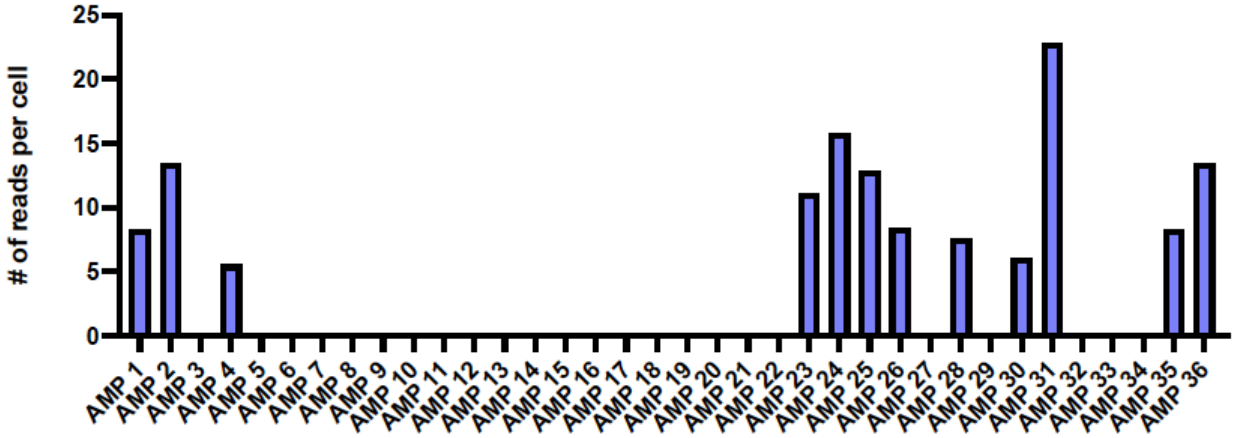
B19_HIV_AMP112	chr12	7079476	7079497	7079704	7079724	CGCTGCTGAGGAGAATGAGAGTCGATGGAT	CGCTGCTGAGGAGAATGAGAGTCGATGGATPHB2-02	HK82
B19_HIV_AMP113	chr12	16035647	16035667	16035898	16035921	AATGAGAGCGCCTGACCTCCTCCTGGCGACGT	AATGAGAGCGCCTGACCTCCTCCTGGCGACGTRAF4-01;TRAF4-03	HK82
B19_HIV_AMP114	chr12	16048160	16048186	16048409	16048434	TTGTGACTTGGAATGATTGTTTTTCTGTATT	TTGTGACTTGGAATGATTGTTTTTCTGTATTTRAF4-02	HK82
B19_HIV_AMP115	chr12	49899640	49899648	49899671	49899697	GGATTGAAATAGATCAAGATCGACGGATCGT	GGATTGAAATAGATCAAGATCGACGGATCGTGGCCDNT1-03CDNT1-02	HK82
B19_HIV_AMP116	chr12	49110352	49110376	49110552	49110573	GAAAGTTCTTGCTATGCGGAAGTTCGTCAAG	GAAAGTTCTTATCTGGTCGACGGCAAACGGACCCCNT1-01	HK82
B19_HIV_AMP117	chr13	25027674	25027697	25027873	25027896	AAAGCATGGTACGCAAAAGACCCAGCGAGTA	AAAGCATGGTACGCAAAAGACCCAGCGAGTARPA-01	HK82
B19_HIV_AMP118	chr13	25052349	25052371	25052595	25052622	ACGTCAAAATGAGCGAGGAGTCTGGTGGATA	ACGTCAAAATGAGCGAGGAGTCTGGTGGATARPA-02	HK82
B19_HIV_AMP119	chr13	25075721	25075746	25075937	25075959	GTGTATCATCGACCGATCATCAATAAATGCT	GTGTATCATCGACCGATCATCAATAAATGCTGPA RPA-03	HK82
B19_HIV_AMP120	chr14	78174373	78174396	78174560	78174585	GAACGAGATTAAAATTCGTACTATCCCGACT	GAACGAGATTACGTTCGTTCGTCGGGAATTTGRR RD1;RR RD1	HK82
B19_HIV_AMP121	chr14	78183756	78183784	78183989	78184005	CTGAGTCCCTAATAACATGATTTCAGTTTCAA	CTGAGTCCCTAATAACATGATTTCAGTTTCAATGRR RD3	HK82
B19_HIV_AMP122	chr5	41535856	41535882	41536063	41536083	GCTGTTTAGTTTCGAATGATGCGAAGACGTA	GCTGTTTAGTTTCGAATGATGCGAAGACGTACHPS-01	HK82
B19_HIV_AMP123	chr5	41554963	41554938	41550085	41550115	GTGATTCACATGCGTTCTGTTTGATATTTAA	GTGATTCACATGCGTTCTGTTTGATATTTAACHPS-02	HK82
B19_HIV_AMP124	chr5	41562742	41562767	41562964	41562984	CTTTCCCTTACGGTTGGTTTTCGACTATGCAT	CTTTCCCTTACGGTTGGTTTTCGACTATGCATTHPS-03	HK82
B19_HIV_AMP125	chr6	2825397	2825358	2825561	2825583	CGAGGAAGACGTCCCGTCGATCGTCCACC	CGAGGAAGACGTCCCGTCGATCGTCCACCCBS2-03	HK82
B19_HIV_AMP126	chr6	2826903	2826924	2827108	2827129	GCCCCTACTGTGGACGTGTCCATCGATCGT	GCCCCTACTGTTAGATCCCGGACGGATGGCGTCBS2-01;TCBS2-02	HK82
B19_HIV_AMP127	chr6	30356107	30356128	30356310	30356332	TTGGTGCCCACCTTTTCATGGGGGGAGAGGT	TTGGTGCCCACCTTTTCATGGGGGGAGAGGTGAL-03	HK82
B19_HIV_AMP128	chr6	30307646	30307667	30307873	30307895	ATTCTGCGTCCCCAGGGCGGCGGACGTCGA	ATTCTGCGTCCCCAGGGCGGCGGACGTCGAGAL-01	HK82
B19_HIV_AMP129	chr6	30529128	30529149	30529222	30529244	TATGGGCGTCGAGCTGAGGACGCGTGGGCGT	TATGGGCGTCGAGCTGAGGACGCGTGGGCGTGAL-02	HK82
B19_HIV_AMP130	chr6	67063554	67063556	67063710	67063731	GCCTCGCTGGCGATCATGCCATGCATCGCTA	GCCTCGCTGGCCGTAATGGGGGCGCTGGGACCBFB-03;CBFB-01	HK82
B19_HIV_AMP131	chr6	67132725	67132753	67132950	67132974	GTCTGTGGCCATCTTCTCTCTGATTTTGTATA	GTCTGTGGCATCTTCTCTCTGATTTTGTATACATCBFB-02	HK82
B19_HIV_AMP132	chr6	74384906	74384934	74385129	74385155	GTTTCATCGAGGACCCAAACTAACTAAATTCG	GTTTCATCGAGGACCCAAACTAACTAAATTCGPS MD7-03	HK82
B19_HIV_AMP133	chr6	74386083	74386107	74386282	74386309	ACTTCCAAGAAAACACACCTCTTTTGTACGGA	ACTTCCAAGAATTCTGTTTCTGCGAGGATTCGTPS MD7-01	HK82
B19_HIV_AMP134	chr6	74389343	74389367	74389492	74389512	AATGGTTGAAATCGTGAACGTCGGCTTCGCC	ATGGTTGAAATCGTGAACGTCGGCTTCGCCPS MD7-02	HK82
B19_HIV_AMP135	chr6	74508428	74508451	74508654	74508677	GCTGATCACCATCTACCTCGATTCAAGTGAG	GCTGATCACCATCTACCTCGATTCAAGTGAGGLGS-03	HK82
B19_HIV_AMP136	chr6	74519675	74519702	74519890	74519914	GATGGAGAGATAACTTGCGAGAGAGTTCGA	GATGGAGAGATAACTTGCGAGAGAGTTCGAGLGS-01	HK82
B19_HIV_AMP137	chr6	74537429	74537452	74537620	74537646	CTTTCTCCAACGGCAAACTGAAGGAGTCGA	CTTTCTCCAACGGCAAACTGAAGGAGTCGAGLGS-02	H

Supplementary Figure 1

A.



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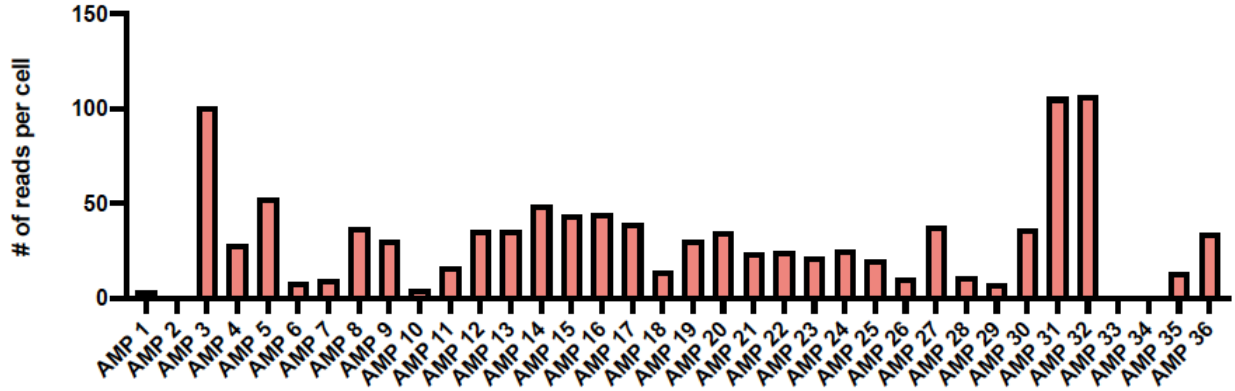

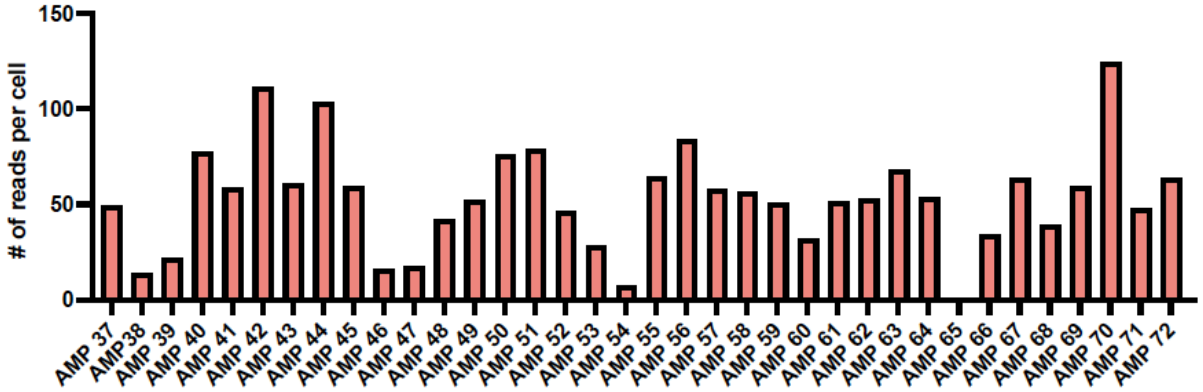
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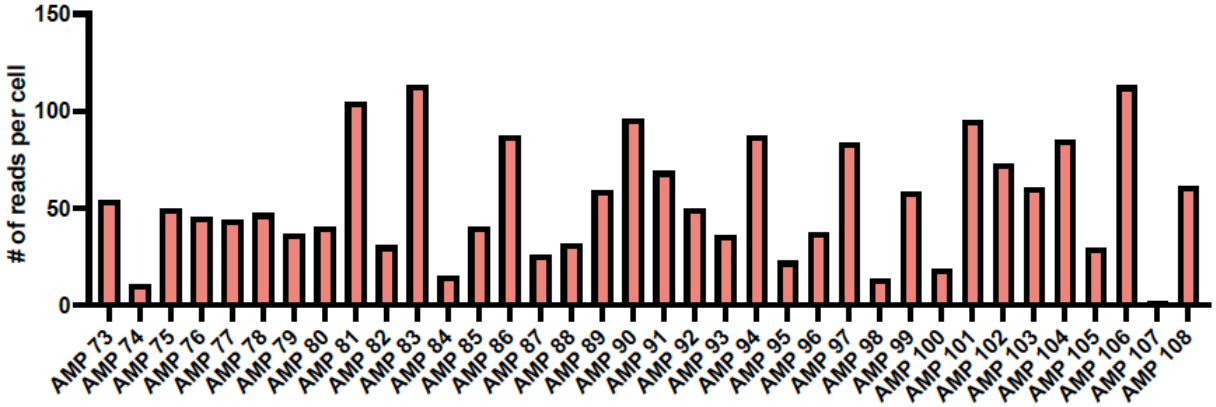
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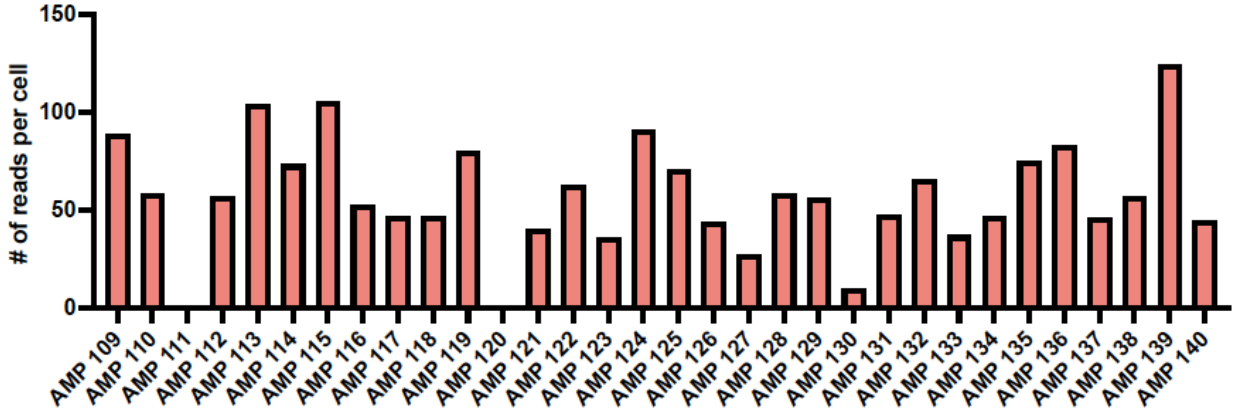
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100
50
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AMP 109
AMP 110
AMP 111
AMP 112
AMP 113
AMP 114
AMP 115
AMP 116
AMP 117
AMP 118
AMP 119
AMP 120
AMP 121
AMP 122
AMP 123
AMP 124
AMP 125
AMP 126
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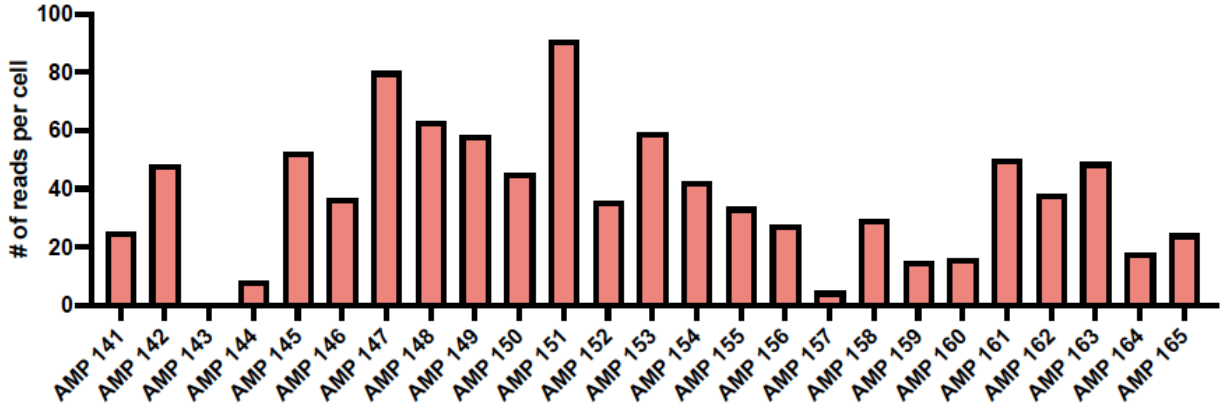
AMP 133
AMP 134
AMP 135
AMP 136
AMP 137
AMP 138
AMP 139
AMP 140


# of reads per cell
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60
40
20
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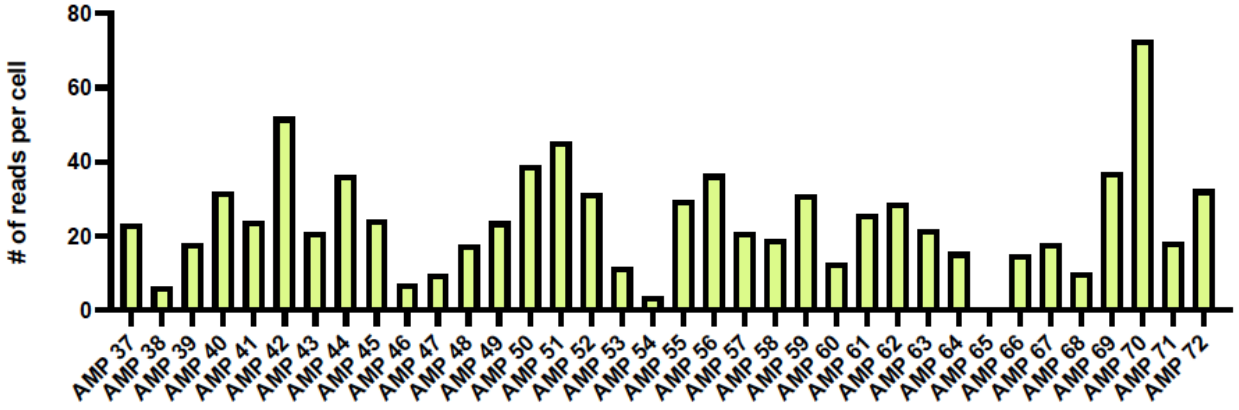
AMP 148
AMP 149
AMP 150
AMP 151
AMP 152
AMP 153
AMP 154
AMP 155
AMP 156
AMP 157
AMP 158
AMP 159
AMP 160
AMP 161
AMP 162
AMP 163
AMP 164
AMP 165

C.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40
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AMP 56
AMP 57
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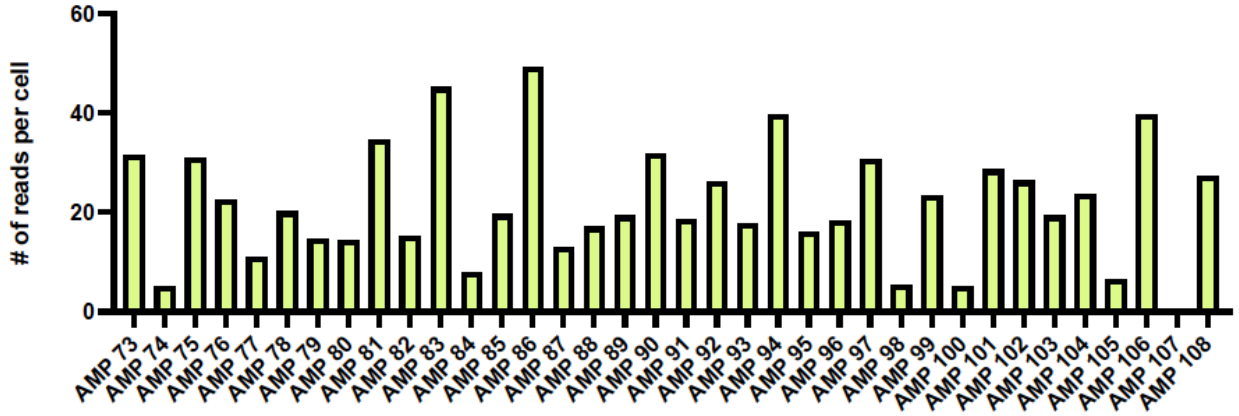
AMP 69
AMP 70
AMP 71
AMP 72


# of reads per cell
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20
40
60
AMP 73
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AMP 81
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AMP 83
AMP 84
AMP 85
AMP 86
AMP 87
AMP 88
AMP 89
AMP 90
AMP 91
AMP 92
AMP 93
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AMP 101
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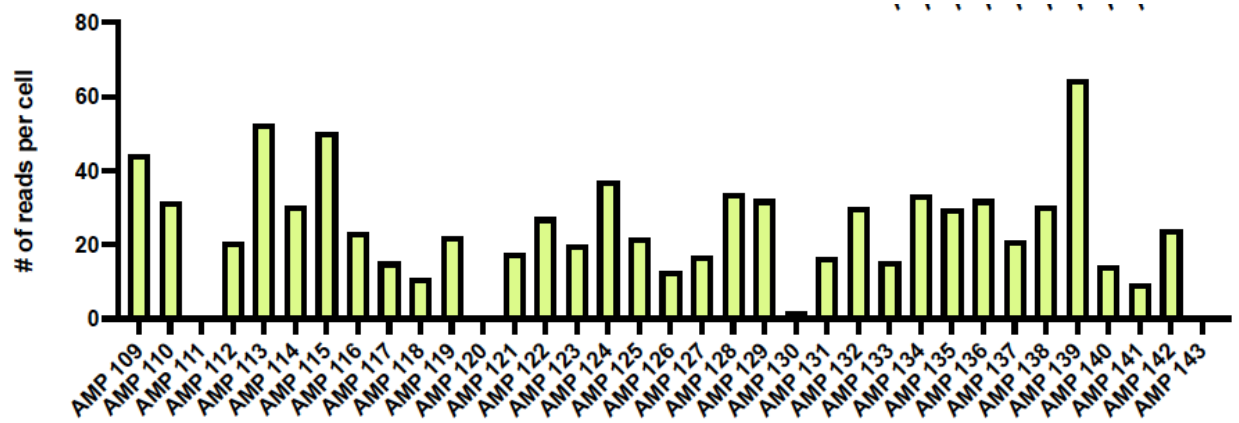
AMP 103
AMP 104
AMP 105
AMP 106
AMP 107
AMP 108


# of reads per cell
0
20
40
60
80
AMP 109
AMP 110
AMP 111
AMP 112
AMP 113
AMP 114
AMP 115
AMP 116
AMP 117
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AMP 120
AMP 121
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AMP 123
AMP 124
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AMP 128
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AMP 138
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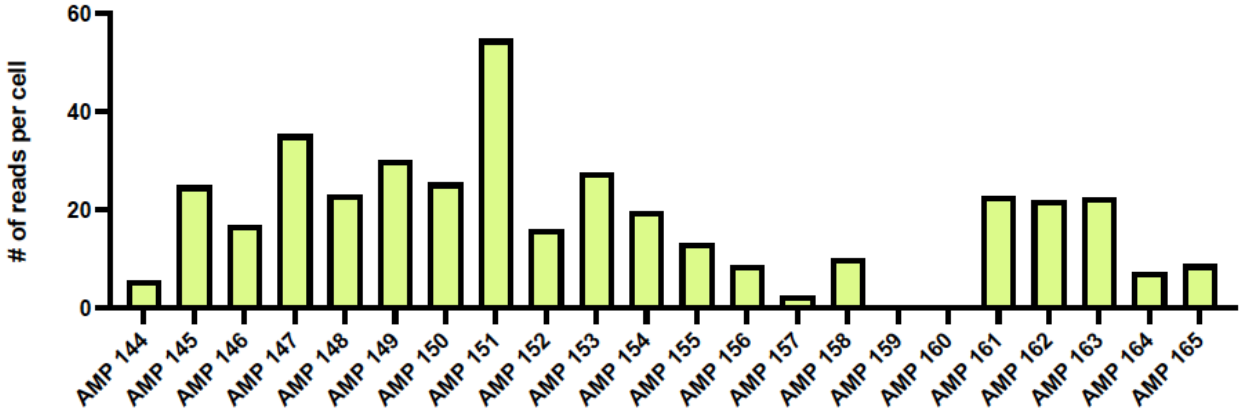
AMP 140
AMP 141
AMP 142
AMP 143


# of reads per cell
0
20
40
60
AMP 144
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AMP 160
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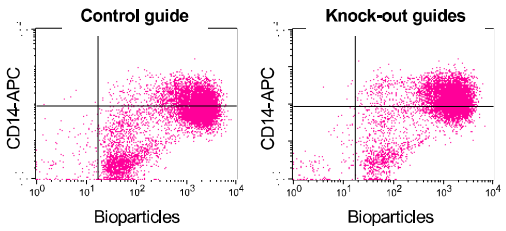
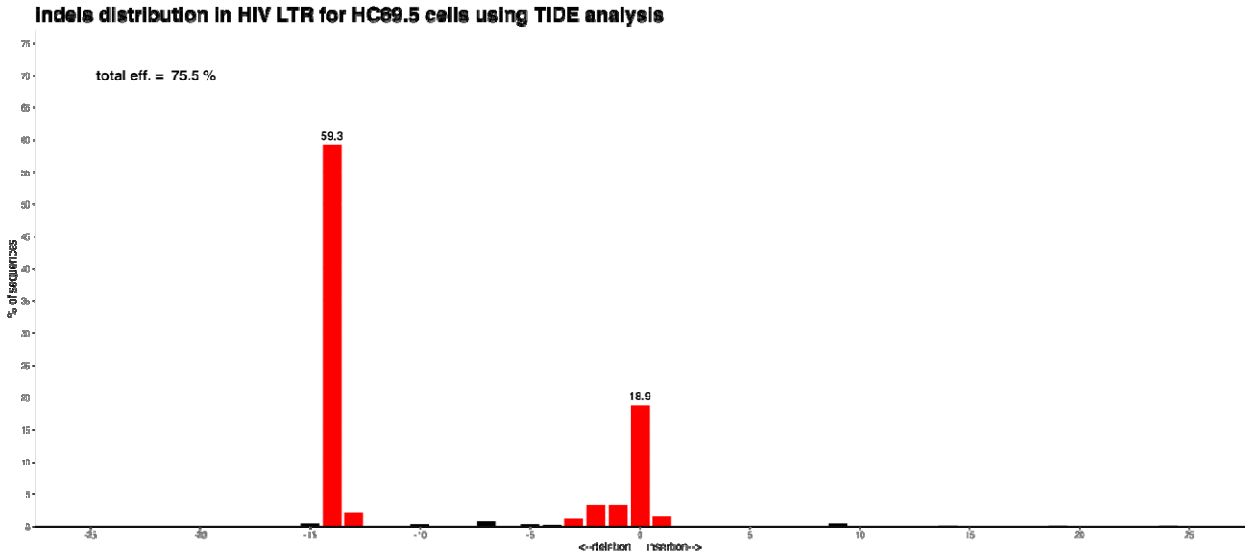
AMP 164
AMP 165



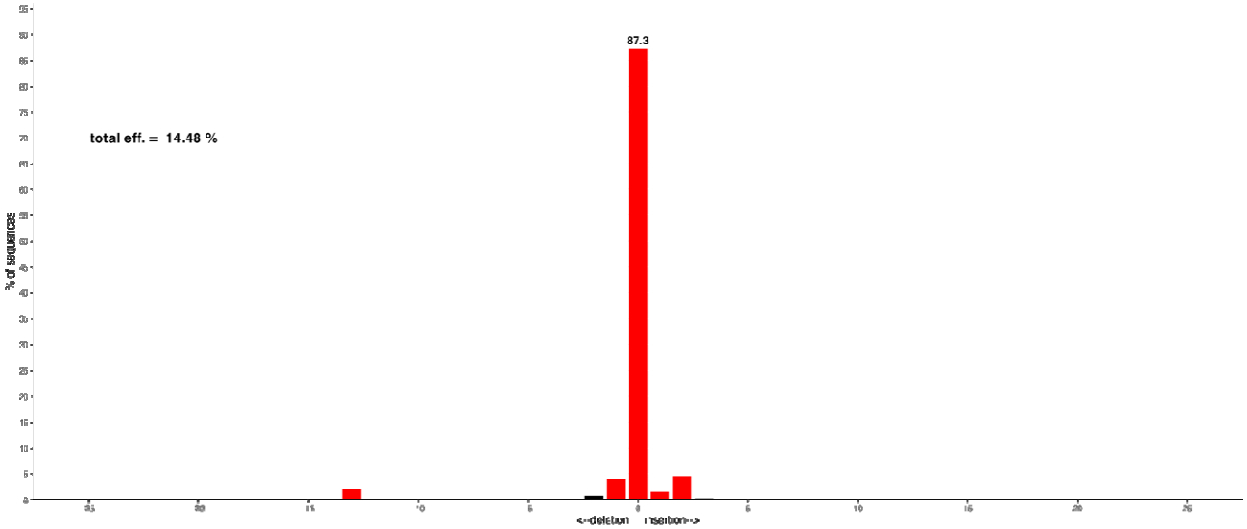
Sample	Phagocytic Index (%)	Green MFI in CD14+ Bioparticle+
Control guide	99.86	1756.6
Knock-out guides	99.90	1595.8

Supplementary Figure 3

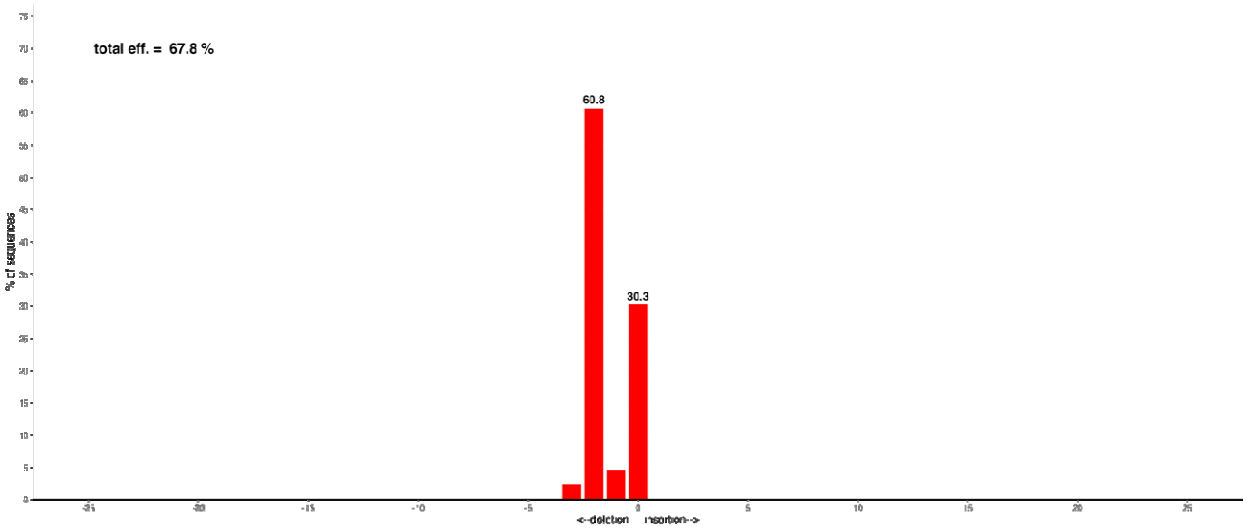
A.



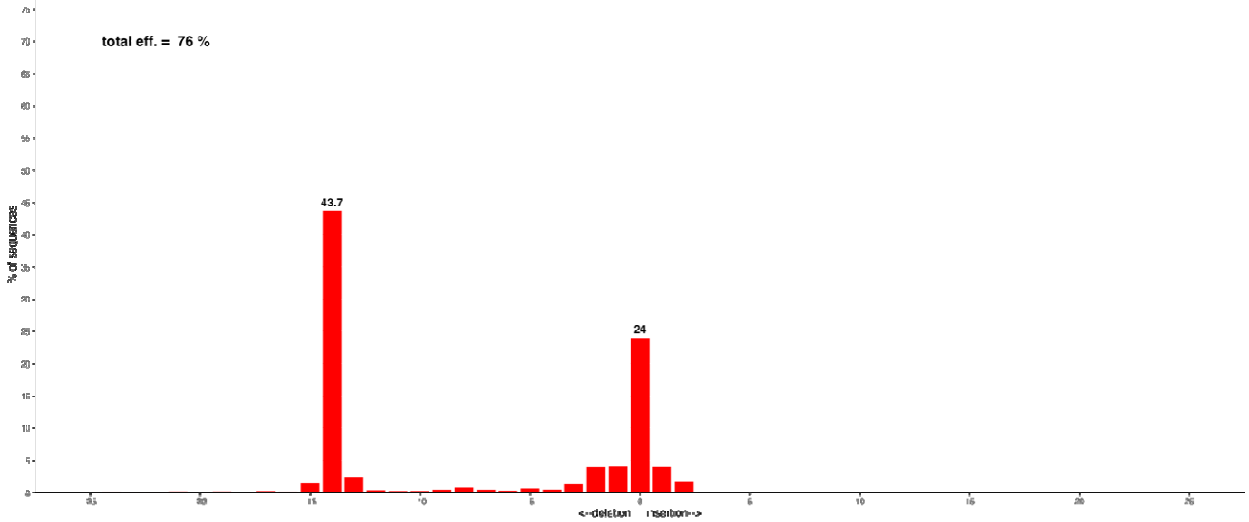
indels distribution in CCR5 for HC69.5 cells using TIDE analysis



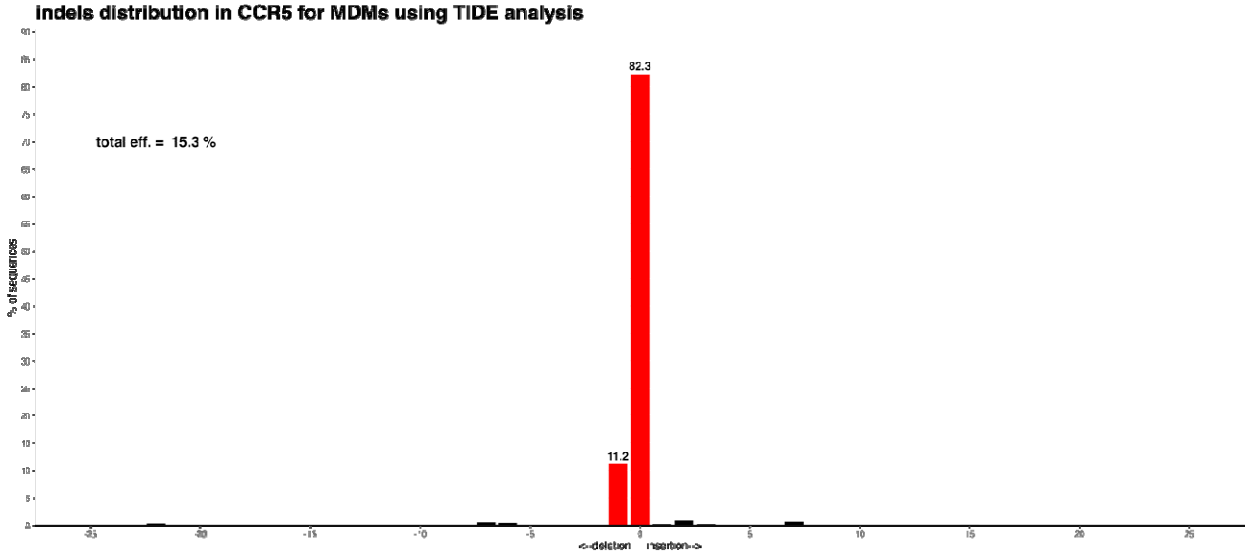
indels distribution in CXCR4 for MDMs using TIDE analysis



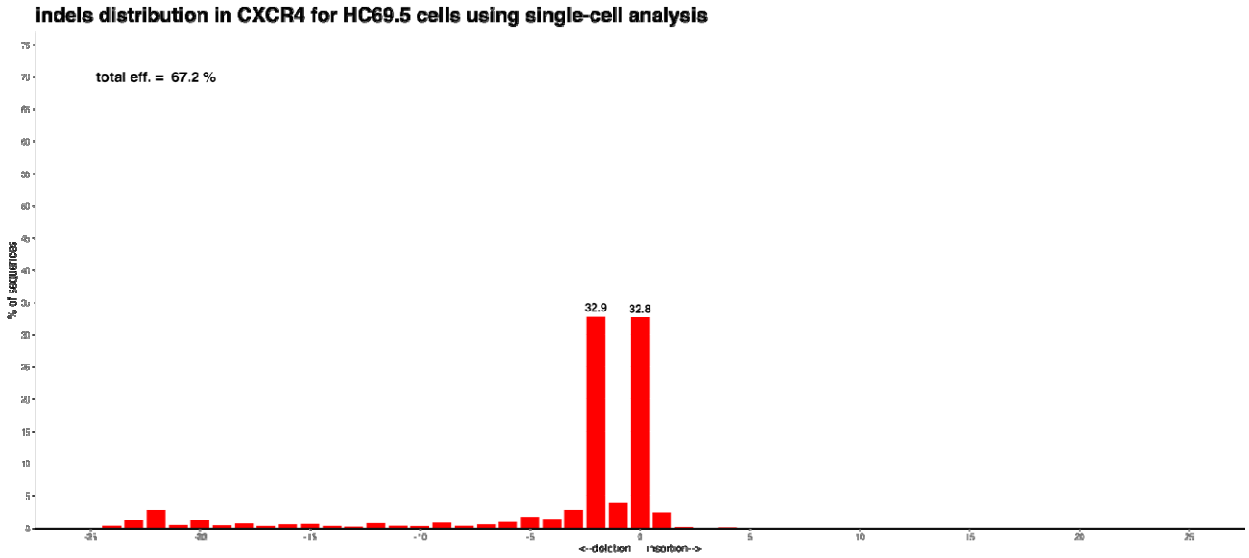
Indels distribution in HIV LTR for HC69.5 cells using single-cell analysis



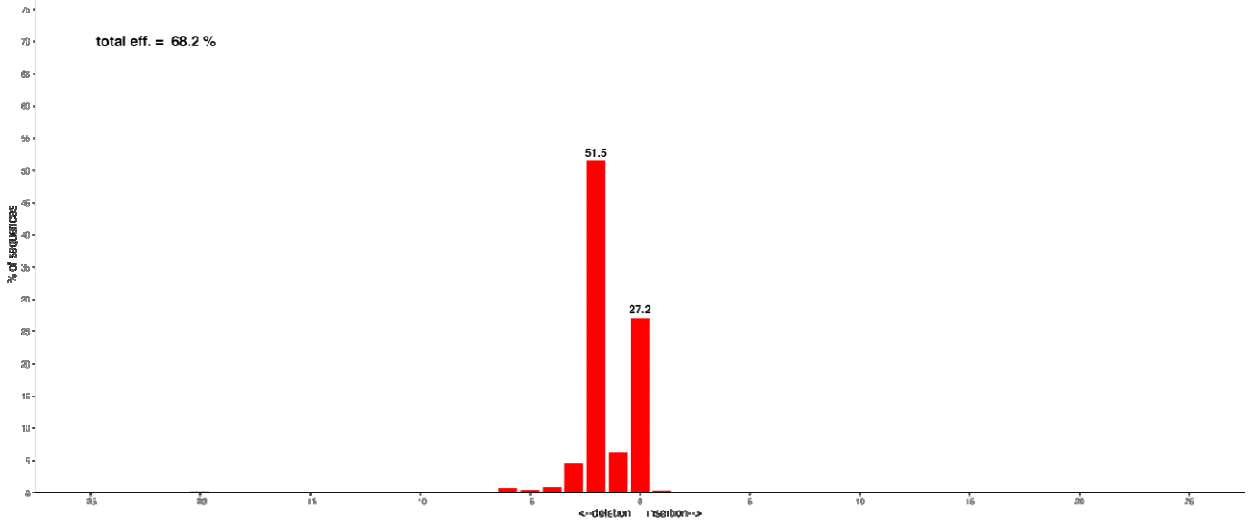
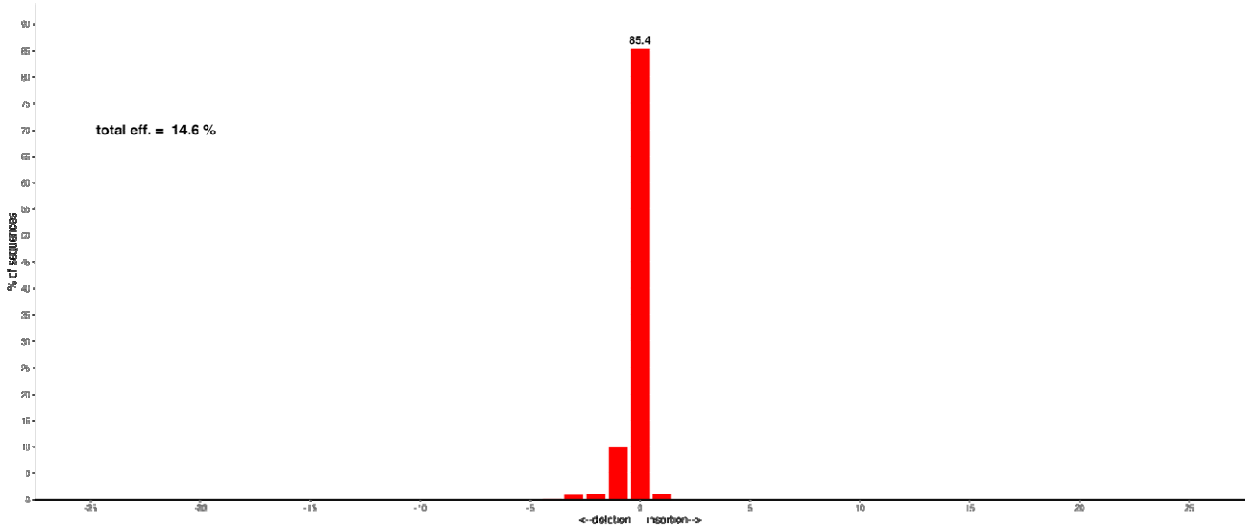
Indels distribution in CCR5 for MDMs using TIDE analysis



B.


Indels distribution in CXCR4 for HC69.5 cells using single-cell analysis
total eff. = 67.2 %
% of sequences
32.9
32.8
<-deletion insertion->
Indels distribution in CCR5 for HC69.5 cells using single-cell analysis
total eff. = 26.6 %
% of sequences
73.4
<-deletion insertion->

indels distribution in CXCR4 for MDMs using single-cell analysis



indels distribution in CCR5 for MDMs using single-cell analysis