Published in final edited form as:

J Virol Methods. 2013 December ; 194(0): . doi:10.1016/j.jviromet.2013.07.062.

Factors Affecting Relative Fitness Measurements in Pairwise Competition Assays of Human Immunodeficiency Viruses

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Abstract

Cell culture growth competition assays of human immunodeficiency virus type 1 (HIV-1) are used to estimate viral fitness and quantify the impact of mutations conferring drug resistance and immunological escape. A comprehensive study of growth competition assays was conducted and identified experimental parameters that can impact measurements of relative fitness including multiplicity of infection, viral input ratio, number, timing and interval of time points used to evaluate selective outgrowth, and the algorithm for calculating fitness values. An optimized protocol is developed here that is a multi-point growth competition assay that resolves reproducibly small differences in viral fitness. The optimized protocol uses an MOI of 0.005, a consistent ratio of mutant: parental viruses (70:30), and a multipoint [1+s 4,7] algorithm that uses data points within the logarithmic phase of viral growth for assessing fitness differences

Keywords

HIV-1; Viral Fitness; Replication Capacity; Growth Competition

1. Introduction

Adaptation and escape from selective forces such as antiretroviral drug therapy and host immunity are fueled by mutations in the HIV genome, but these mutations often impair the ability of the virus to survive and replicate (*i.e.*, they diminish viral “fitness”). This loss in fitness is balanced by the positive effect of affording escape from the suppressive pressure. To determine the fitness cost of mutations in viral sequences, investigators typically use one of two multi-cycle assays: a monoinfection assay, in which the replication kinetics of individual isolates are evaluated in parallel over the course of several days (Brockman et al., 2010; Quinones-Mateu and Arts, 2002; Quinones-Mateu and Arts, 2006), or pairwise growth competition assays (Miao et al., 2008; Miao et al., 2009; Quinones-Mateu et al., 2000; Song et al., 2012). In the absence of an internal control, the outcomes of monoinfections are likely to vary between experiments and may not reliably reveal subtle differences in viral fitness. Pairwise growth competition assays are more labor-intensive, but are more reliable because they use an internal reference strain to quantify the relative

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replication rate of the virus. In addition, competition assays provide a more realistic measure of the ability of a virus to contribute to the next generation in situations where multiple variants are competing for host cell targets, suggesting that they may be more relevant biologically (although no cell culture system can fully mimic the target cell populations or selective forces found *in vivo*).

Although several experimental protocols have been used for growth competition studies, a consensus regarding a protocol standardized for measuring viral fitness has not been reached. Inconsistencies between protocols include differences in the methods for measuring infectious titer, the multiplicity of infection (MOI) used to initiate the assay, the initial ratio of the strain to be tested relative to the reference strain, and variations in the number and interval of time points sampled to evaluate mutant frequency. In the present study, an analysis of these assay parameters was performed in an effort to improve the reliability of pairwise growth competition assays. Several factors were identified as necessary for robust measurements of HIV-1 fitness in cell culture.

2. Materials and Methods

2.1 Generation of HIV-1 chimeric viruses

Mutations of interest were introduced into a variant of NL4-3 that contains an HIV-1 group M subtype B Center-of-Tree (COTB) sequence in the p24-encoding region of *gag* (HIV-1_{NL4-3(COTB)}) (Rolland et al., 2007; Rolland et al., 2013), here referred to as VifA. The same plasmid except for six synonymous mutations in the NL4-3 *vif* gene (VifB) (Troyer et al., 2009) was used as a control in competition assays. Plasmid DNAs were prepared using the QIAprep Spin Miniprep Kit or HiSpeed Plasmid Midi Kit (Qiagen, Valencia, US), with an additional endotoxin removal step for use in mammalian cell transfection experiments. Infectious viruses were generated by transfecting HEK 293T-17 cells with 1 µg of plasmid DNA using the XtremeGENE 9 DNA transfection reagent (Roche, San Francisco, US) (Figure 1). Cell-free supernatants were harvested after 48 hours and filtered through a 0.22 µm filter. Viral stock aliquots of 250 µl were stored at -80 °C until use. The viral titer for each stock was calculated by determining endpoints in a serial dilution assay (Reed and Muench, 1938). Positive wells were quantified using a p24 antigen capture assay (McClure et al., 2007).

2.2 Pairwise growth competition assay

Target cells for all experiments were peripheral blood mononuclear cells (PBMCs), from a single uninfected donor, that had been stimulated for 72 hours with 1.5 µg/mL PHA. Post-stimulation, cells were cultured in Iscove's Modified Dulbecco's Medium (IMDM) supplemented with 20 U/ml of human interleukin 2 (hIL-2; Roche, San Francisco, US), 10% fetal bovine serum (Sigma-Aldrich, St. Louis, US) and 1% penicillin/streptomycin. Experiments were done in triplicate at 3×10^5 PBMCs/well, in 0.5 ml of complete medium in 48-well plates. Concurrently, PHA-stimulated PBMCs were seeded at 2×10^4 cells/well in a 96-well flat-bottom microtiter plate to titer the experimental inoculum and back-calculate the MOI of each experiment. Cultures were incubated at 37 °C with a 5% CO₂ atmosphere.

A virus inoculum master mix was prepared by dilution of virus in fresh media. PHA-stimulated PBMCs were inoculated with 0.5 ml of the master mix for a final culture volume of 1.0 ml. After 16 hours, 0.75 ml of the culture supernatant was removed and replaced with warm complete IMDM. Cultures were centrifuged for 5 minutes at $228 \times g$, and then 0.75 ml of the culture supernatant was replaced for a total of two washes (final inoculum dilution of ~94%). Starting at day 0, and on two or three day intervals, 0.5 ml of culture supernatant was removed and transferred to a 1.8-ml microcentrifuge tube, centrifuged for 5 minutes at $228 \times g$, and the cell free supernatant was removed and stored at -80 °C in 200-µl aliquots.

2.3 Nucleic acid preparation and amplification

Viral RNA was extracted from 200 µl of cell-free supernatant using an ion exchange membrane technology on a QIAextractor robot (Qiagen) according to the manufacturer's protocol. RNA samples were stored at -80 °C until cDNA synthesis.

Each 25-µl cDNA synthesis reaction consisted of master mix 1 [final concentration: 0.5 mM dNTPs, 0.5 µM of primer RT-2 (5'-GCTGGACTGTCAATGACATAC-3'; HXB2 positions 3301–3321) or VifExt (5'-CTTCCACTCCTGCCCAAGTATCCC-3'; HXB2 positions 5733–5710)], master mix 2 [final concentration: 1X first strand buffer (Life Technologies, Grand Island, US), 5 mM dithiothreitol, 10 U/µl SuperScript III (Life Technologies), 2 U/µl RNase Inhibitor (Roche)], and 10 µl RNA. Four µl of master mix 1 was transferred to each reaction well in a 96-well PCR plate. Ten microliters of RNA was added to each reaction well and incubated at 60 °C for 5 minutes. The reaction was held at 4 °C before 10 µl of master mix 2 was added and incubated for 90 minutes at 50 °C. Reverse transcriptase was inactivated by incubation for 15 minutes at 70 °C and held at 4 °C until 2U/µl of RNase H was added and incubated for 20 minutes at 37 °C. The reaction was held at 4 °C until PCR amplification.

2.4 PCR amplification

A 1.67 kb fragment was amplified in a 50-µl PCR reaction consisting of 1 µl cDNA template and master mix [final concentration: 1X NH₄ buffer (Bioline, Seattle, WA), 1.5 mM MgCl₂, 0.2 mM dNTP, 2.5 U Taq Polymerase (Bioline, Seattle, US), and the gene specific primers Gag-2 (5'-GTGCGAGAGCGTCGGTATTAAGCG-3'; HXB2 positions 794–817) and RSP15R (5'-GGAAACCAAAAATGATAGGGGAATTG-3'; HXB2 positions 2377–2403) at 0.45 µM each]. PCR cycling conditions were 3 cycles of 94 °C for 1 minute, 55 °C for 1 minute, and 70 °C for 1 minute followed by 34 cycles of 94 °C for 15 seconds, 58 °C for 30 seconds, and 70 °C for 1 minute, and then hold at 4 °C. Amplicons were confirmed by automated electrophoresis using a QiaXcel (Qiagen), and PCR products were purified and concentrated using the QIAquick PCR Purification kit (Qiagen). Purified PCR products were subjected to dye terminator sequencing with 3.2 µM of primer NL43_F_1087 (5'-GCCTTAGATAAGATAGAGGAAGAGC-3'; HXB2 positions 1087–1111) and 8 µl of PCR product.

2.5 Quantitative PCR (qPCR)

Viral RNA from parallel monoinfection cultures was quantified to determine the growth kinetics of each virus using an ABI 7300 Real-Time PCR System (ABI, Foster City, US). Each 25-µl PCR reaction contained 12.5 µl TaqMan® Gene Expression Master Mix (ABI), 5 µM probe (5'-AAAGAGATATACCACACAAGTAGACCCTGACC-3'; HXB2 positions 5313–5344), 20 µM of the forward (5'-GAAAGAGACTGGCATTGTTGGGTCAGGG-3'; HXB2 positions 5266–5291) and reverse primers (5'-GTCTTCTGGGGCTTGTTCATCTGTCC-3'; HXB2 positions 5579–5553), and 1 µl of cDNA. PCR cycling parameters were 50 °C for 2 min, 95 °C for 10 min, and 40 cycles at 95 °C for 15 sec and 60 °C for 1 min. A standard curve was generated using 1 µl of pNL4-3 serially diluted 10-fold from 3×10⁷ to 3 copies/µl, with each dilution run in triplicate. The copy number of cDNA and corresponding Ct values were used to extrapolate cDNA copy from each experimental sample. Each experimental sample was analyzed in duplicate PCR reactions.

2.6 Allele quantitation

Raw sequence files were imported into Sequencher v4.7 (Gene Codes, Ann Arbor, US) and assessed for quality by alignment to the HIV-1_{NL4-3}(COTB) reference sequence. Raw (*.ab1)

sequence files with an average read quality score below 85% were not used for analysis and in these cases PCR products were resynthesized and sequenced. Sequence trace files were analyzed by measuring the absolute peak intensity at the nucleotide position of interest. The mutant frequency was calculated as the relative proportion of the mutant peak intensity divided by the total peak height intensity ($f_m/(f_w+f_m)$) for the bases corresponding to the mutant and wild type virus sequences (Figure 1) using an in-house web-based front end (<http://indra.mullins.microbiol.washington.edu/cgi-bin/chromatquant.cgi>) to the Staden trace dump utility (Bragg et al., 2012).

2.7 Calculations for estimating relative fitness

Relative fitness was estimated using two methods: a web-based linear regression algorithm (<http://bis.urmc.rochester.edu/vFitness/FitnessMulti.aspx>) for assessing the rate of change in mutant frequency across multiple time points (1+s) (Wu et al., 2006), and a relative fitness (w) (Quinones-Mateu and Arts, 2006) equation f_o/i_o , where f_o is the final relative amount of virus produced, and i_o is the initial amount of virus in the inoculum. A linear regression analysis was performed on mutant frequency values for each virus at the MOI examined. An analysis of variance (ANOVA) was performed to determine significance ($p < 0.05$) between groups for all conditions tested. The Pearson product moment correlation coefficient (PMCC) was used to determine the extent of the linear relationship between mutant frequency and days of infection at each MOI tested. All statistical analyses were performed using Prism version 5.0d (GraphPad Software, La Jolla, US).

3. Results

3.1 Effect of MOI on HIV-1 replication kinetics in monoinfections

To evaluate assay parameters that influenced relative viral fitness in competition assays, three variants of HIV-1_{NL4-3(COTB)}, each differing from the parental strain by single amino acid replacements in Gag p24 (T186M, T242N, or I256V) were constructed. Infectious titers produced by the mutant clones following transfection of HEK 293T-17 cells (CD4-negative) were comparable to those of the parental virus (Supplementary Figure 1). The replication capacities of the parental and mutant viruses in parallel monoinfections were evaluated in PHA-stimulated PBMCs that were initiated at four different MOI: 0.005, 0.001, 0.0005, and 0.0001. An analysis of the time-dependent accumulation of HIV-1 RNA over an 11-day period showed that the kinetics of viral replication varied depending on the MOI used to initiate the infections (Figure 2). In most cases, the largest increases in viral RNA occurred between days 3 and 7 after infection; supernatants harvested on days 9 and 10 showed relatively small increases in viral RNA or were unchanged relative to the day 7 samples. However, infections initiated with parental or T186M virus at an MOI of 0.0001 showed linear increases in viral RNA from days 3–11, whereas cultures of I256V and T242N that were initiated at an MOI of 0.005 reached maximal RNA levels at day 5 post-infection. Thus, lower input multiplicities tended to result in a longer growth phase of virus production, with MOI 0.001 consistently resulting in logarithmic growth between days 3–7 of culture.

3.2 Variation between biological replicates is influenced by MOI

To evaluate the effect of MOI on the outcome and reproducibility of mixed-virus competition experiments, mutants T186M, T242N, I256V and a variant of the parental virus clone that contained six synonymous nucleotide changes in the *vif* gene (VifB) were competed against a wild-type reference strain (VifA). The VifB allele was previously shown to have no effect on viral fitness in culture (Troyer et al., 2009), enabling us to use mixed infections between parental VifA and VifB viruses as a negative control (i.e., competitions between these viruses should not lead to selective outgrowth of either strain). As in the

monoinfection experiments, the competition assays were performed in PHA stimulated PBMCs at MOI of 0.005, 0.001, 0.0005, and 0.0001. Samples were collected from the cultures at two to three day intervals, and the frequency of mutant virus in each of the populations (relative to the reference strain) was determined by sequencing PCR products amplified from viral RNA (collected on days 0, 3, 5, 7, 9, and 11).

Experiments performed at MOI = 0.0001 generally resulted in substantial inter-replicate variation, with large confidence intervals for the linear regressions. For example, as expected, competitions between parental VifA and VifB viruses showed no fitness differences ($p > 0.05$, ANOVA with Bonferroni correction, Figure 3) except at an MOI of 0.0001, where there was a trend towards higher fitness for the VifB construct (Figure 3, top right panel). Furthermore, variants T186M, T242N and I256V were all less fit than the parental virus with the exception of T186M at an input MOI of 0.0001 ($p = 0.13$; Figure 3). Consistent results were achieved in competitions initiated at an MOI of 0.005, which yielded minimal variation between replicates and narrow confidence intervals. As a result, all subsequent competition experiments in this study were performed at an MOI of 0.005.

3.3 Relative Fitness Assessed Using Different Algorithms

To determine the reproducibility of the assay and compare different methods for calculating relative fitness, input frequency values from a total of 95 independent competition experiments were used in two different algorithms: $1+s$ (Wu et al., 2006) or w (Quinones-Mateu et al., 2000). Time points 0–10 (0,4,7 and 10) and 4,7 were used in the $1+s$ algorithm ($1+s$) to assess fitness differences during the entirety and log growth phase of the experiment, respectively. The w method uses only two time points to calculate fitness (Quinones-Mateu et al., 2000); relative fitness was calculated using days 4 and 7 ($w_{d4,7}$) to capture the log growth phase, as well as days 0 and 10 ($w_{d0,10}$) (Troyer et al., 2009). Mutant viruses T186M, T242N, I256V, and parental VifB were competed against the isogenic parental VifA reference strain at an MOI of 0.005, and cultures were sampled for viral RNA at day 0, and on two or three day intervals until 10 days post infection.

As observed in the initial experiments (Figure 3), competitions involving the two parental viruses (VifA and VifB) did not result in selective outgrowth of either strain (Figure 4). Similarly, all three mutants tested exhibited decreased relative fitness (Figures 3 and 4). The w method ($d0,10$) yielded larger confidence intervals, and the resultant fitness values were markedly lower than those produced by the $1+s$ approach (Figure 4).

To determine if varying the input ratio of mutant and parental viruses affected measurements of relative fitness, 47 independent growth competition experiments were performed with T186M against the parental VifA strain at ratios of 90:10, 80:20, 70:30, 60:40, 50:50 and 20:80. Relative fitness values were calculated using all four algorithms ($1+s_{4,7}$ and $1+s_{0-10}$; $w_{d4,7}$; and $w_{d0,11}$). A consistent negative correlation between input ratio and relative fitness was observed when using the $1+s_{0-10}$ calculation (Figure 5; $p = >0.0001$), but no consistent trend when using the $1+s_{4,7}$ or either of the w algorithms ($p > 0.05$).

4. Discussion

Growth competition assays have been applied to estimate viral fitness under differing experimental conditions and using different analysis methods. To assess the effect of these assay and analysis parameters, three HIV-1 p24 Gag mutants (T186M, T242N, and I256V) were evaluated against the parental virus, differing also by six synonymous mutations in the HIV-1 *vif* gene to allow the viruses to be easily distinguished by DNA sequencing or other methods. Several factors were found to be necessary for reliable measurements of relative HIV-1 fitness in cell culture, including the multiplicity of infection, the number and interval

of time points sampled to evaluate mutant frequency, and the initial ratio of the two strains being compared.

To optimize sampling intervals, we performed monoinfections with the parental and mutant strains at four different MOI: 0.005, 0.001, 0.0005 and 0.0001. Each virus expanded rapidly between days 3 and 7 of infection, and additional days of culture produced relatively subtle increases (or no change) in viral RNA levels. This effect was dependent on MOI; lower input multiplicities (i.e., 0.001) tended to result in a longer logarithmic growth phase, as well as poorer reproducibility, with the smallest confidence intervals observed in experiments at the highest MOI tested (0.005). Therefore, the optimal MOI for assessing the molecular clones in this study was 0.005. MOI higher than 0.005 were not tested as higher levels of input virus would shorten the logarithmic phase of viral growth, and increase the risk of recombination between the two strains. In addition, the single passage protocol allows minimal time for recombinants to arise to detectable frequency, even in the unlikely event that the recombinants exhibit a substantial gain in fitness relative to the parental virus strains. Lastly, as many viral mutants replicate poorly and to low titers, higher MOIs would often be difficult to achieve.

Two methods for estimating relative fitness were employed to determine optimal sampling time points - the 1+s method (Wu et al., 2006), which employs multiple longitudinal data points within the logarithmic growth phase, and the *w* method (Quinones-Mateu and Arts, 2006; Quinones-Mateu et al., 2000), which uses two data points – from the start and end of the culture period. The mutant frequencies obtained from days 0–10 and 4–7 of each experiment were used in the multi-point fitness calculation, and the mutant frequencies from 0,10 and 4,7 were used in the two-point fitness calculation. It should also be noted that the fixed input ratio 70:30, not the actual measured value, was used as day 0 data point in the *w* method, as done in the previous study (Quinones-Mateu et al., 2000). The two-time point analysis exhibited a larger standard deviation across experiments, and analyses that used the day 0 and 10 time points exhibited a greater spread of fitness values compared to the same analysis done using days 4 and 7, which spanned the logarithmic phase of viral replication. Our study showed that using data from multiple time points within the exponential growth phase is crucial for obtaining consistent and reproducible results, and superior to using two data points obtained at the start and at the end of the culture. It is also important to quantify precisely the input ratio, and use the ‘real/estimated’ value and not use the value that was aimed for.

To determine if input ratio had an effect on relative fitness, mutant T186M was competed against the parental strain at multiple input ratios. The mutant input ratio did not affect the results except for a small effect on relative fitness when the 1+s 0–10 algorithm was used. This observation suggests that the input viral ratio does not affect the assay outcome when the appropriate data points within the logarithmic growth phase were applied. The viral input ratio of 70:30 (mutant:parental strain) was chosen to allow for the detection of a large fitness deficiency in the mutant viruses. This ratio can also be used if the mutation is expected to have small or insignificant fitness cost. On the other hand, the inverse ratio of 30:70 should be used if the mutants are expected to have substantially higher fitness than the parental strain.

Using the optimized experimental conditions described, all three mutations consistently conferred reduced fitness relative to the parental strain, with T242N having the smallest fitness cost. Recently, our approach was used to study other HIV-1 Gag mutants and we were able to identify mutations which showed, on average, a smaller fitness cost than T242N (Rolland et al., 2013).

All of our experiments were conducted using PBMCs from single donor. We did not address the issue of inter-donor variability, which had been addressed previously by others (Cohen et al., 2012). However, while viral growth may vary widely between cells from different donors, an issue for the comparison of replication capacity in monoinfections, the ratio between two viruses in competition is not likely to be affected by inter-donor variability (Ball et al., 2003; Quinones-Mateu et al., 2000).

5. Conclusions

In the present study, an analysis of various assays parameters was performed in an effort to improve the reliability of pairwise growth competition assays. We identified an optimized set of parameters with which to assess small differences in the relative fitness of HIV-1 variants in pairwise competition assays. The optimized protocol uses an MOI of 0.005, a consistent ratio of mutant: parental viruses (70:30), and the multipoint 1+s algorithm using data points within the logarithmic phase of viral growth for assessing fitness differences. Our procedures make use of routine PCR-based techniques, and Sanger sequencing. Therefore, our method can be conducted in most molecular biology and virology laboratories with standard equipment.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

This work was supported by grants from the NIH to JIM (P01AI057005 and R37AI04773), the University of Washington Center for AIDS Research Computational Biology Core (P30AI27757), and the Bill and Melinda Gates Foundation (43437). Additional funding was provided by grants to RAS from the University of Washington Center for AIDS Research New Investigator Award Program (P30 AI27757) and to MR from the American Foundation for AIDS Research (107005-43-RFN). The above agencies had no role in the study design, collection, analysis or interpretation of the data, nor with the writing of the article or decision to submit the article for publication.

References

- Ball SC, Abraha A, Collins KR, Marozsan AJ, Baird H, Quinones-Mateu ME, Penn-Nicholson A, Murray M, Richard N, Lobritz M, Zimmerman PA, Kawamura T, Blauvelt A, Arts EJ. Comparing the Ex Vivo Fitness of CCR5-Tropic Human Immunodeficiency Virus Type 1 Isolates of Subtypes B and C. *J Virol*. 2003; 77:1021–1038. [PubMed: 12502818]
- Bragg L, Stone G, Imelfort M, Hugenholtz P, Tyson GW. Fast, accurate error-correction of amplicon pyrosequences using Acacia. *Nat Methods*. 2012; 9:425–6. [PubMed: 22543370]
- Brockman MA, Brumme ZL, Brumme CJ, Miura T, Sela J, Rosato PC, Kadie CM, Carlson JM, Markle TJ, Streeck H, Kelleher AD, Markowitz M, Jessen H, Rosenberg E, Altfeld M, Harrigan PR, Heckerman D, Walker BD, Allen TM. Early selection in Gag by protective HLA alleles contributes to reduced HIV-1 replication capacity that may be largely compensated for in chronic infection. *J Virol*. 2010; 84:11937–49. [PubMed: 20810731]
- Cohen CR, Lingappa JR, Baeten JM, Ngayo MO, Spiegel CA, Hong T, Donnell D, Celum C, Kapiga S, Delany S, Bukusi EA. Bacterial vaginosis associated with increased risk of female-to-male HIV-1 transmission: a prospective cohort analysis among African couples. *PLoS Med*. 2012; 9:e1001251. [PubMed: 22745608]
- McClure J, van't Wout AB, Tran T, Mittler JE. Granulocyte-monocyte colony-stimulating factor upregulates HIV-1 replication in monocyte-derived macrophages cultured at low density. *J Acquir Immune Defic Syndr*. 2007; 44:254–61. [PubMed: 17211283]
- Miao H, Dykes C, Demeter LM, Cavanaugh J, Park SY, Perelson AS, Wu H. Modeling and estimation of kinetic parameters and replicative fitness of HIV-1 from flow-cytometry-based growth competition experiments. *Bull Math Biol*. 2008; 70:1749–71. [PubMed: 18648886]

- Miao H, Dykes C, Demeter LM, Wu H. Differential equation modeling of HIV viral fitness experiments: model identification, model selection, and multimodel inference. *Biometrics*. 2009; 65:292–300. [PubMed: 18510656]
- Quinones-Mateu ME, Arts EJ. Fitness of drug resistant HIV-1: methodology and clinical implications. *Drug Resist Updat*. 2002; 5:224–33. [PubMed: 12531179]
- Quinones-Mateu ME, Arts EJ. Virus fitness: concept, quantification, and application to HIV population dynamics. *Curr Top Microbiol Immunol*. 2006; 299:83–140. [PubMed: 16568897]
- Quinones-Mateu ME, Ball SC, Marozsan AJ, Torre VS, Albright JL, Vanham G, van Der Groen G, Colebunders RL, Arts EJ. A dual infection/competition assay shows a correlation between ex vivo human immunodeficiency virus type 1 fitness and disease progression. *J Virol*. 2000; 74:9222–33. [PubMed: 10982369]
- Reed LJ, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg*. 1938; 27:493–497.
- Rolland M, Jensen MA, Nickle DC, Yan J, Learn GH, Heath L, Weiner D, Mullins JI. Reconstruction and function of ancestral center-of-tree human immunodeficiency virus type 1 proteins. *J Virol*. 2007; 81:8507–14. [PubMed: 17537854]
- Rolland M, Manochewa S, Swain JV, Lanxon-Cookson EC, Kim M, Westfall DH, Larsen BB, Gilbert PB, Mullins JI. HIV-1 conserved-element vaccines: relationship between sequence conservation and replicative capacity. *J Virol*. 2013; 87:5461–7. [PubMed: 23468488]
- Song H, Pavlicek JW, Cai F, Bhattacharya T, Li H, Iyer SS, Bar KJ, Decker JM, Goonetilleke N, Liu MK, Berg A, Hora B, Drinker MS, Eudailey J, Pickeral J, Moody MA, Ferrari G, McMichael A, Perelson AS, Shaw GM, Hahn BH, Haynes BF, Gao F. Impact of immune escape mutations on HIV-1 fitness in the context of the cognate transmitted/founder genome. *Retrovirology*. 2012; 9:89. [PubMed: 23110705]
- Troyer RM, McNevin J, Liu Y, Zhang SC, Krizan RW, Abraha A, Tebit DM, Zhao H, Avila S, Lobritz MA, McElrath MJ, Le Gall S, Mullins JI, Arts EJ. Variable fitness impact of HIV-1 escape mutations to cytotoxic T lymphocyte (CTL) response. *PLoS Pathog*. 2009; 5:e1000365. [PubMed: 19343217]
- Wu H, Huang Y, Dykes C, Liu D, Ma J, Perelson AS, Demeter LM. Modeling and estimation of replication fitness of human immunodeficiency virus type 1 in vitro experiments by using a growth competition assay. *J Virol*. 2006; 80:2380–9. [PubMed: 16474144]

Highlights

Mutations in human immunodeficiency virus type 1 (HIV-1) confer drug resistance and immunological escape

Cell culture growth competition assays are used to estimate viral fitness and quantify the impact of these mutations

We optimized a set of parameters with which to assess small differences in the relative fitness of HIV-1 variants in pairwise competition assays

The optimized protocol uses:

Viral growth determined from monoinfection experiments

MOI of 0.005 in competition assays

Consistent ratio of parental and mutant viruses in competition assays

The multipoint 1+s algorithm for assessing fitness differences within the logarithmic phase of viral growth.

Figure 1. Overview of the methodology used to measure viral fitness

A. Virus production and titration. Plasmids containing full-length proviral DNA, with or without specific point mutations in the HIV-1_{NL4-3(COTB)} genome, were used to transfect HEK 293T-17 cells. After 48 hours, infectious stocks were titrated on PHA-stimulated PBMCs for 14 days, and the TCID₅₀ calculated. **B.** Dual competition assay. Pairwise competition assays were infected at an MOI of 0.005 with inocula comprised of 70% mutant and 30% of the autologous reference HIV-1_{NL4-3(COTB)} VifA virus. Cultures were sampled at days 3, 5, 7, 9 and 11 for viral RNA. The p24 coding sequences were amplified by PCR, and the mutant frequency was estimated at the nucleotide of interest from Sanger consensus sequence chromatograms (see Methods).

Figure 2. Effect of multiplicity of infection on viral replication kinetics

Infections of PHA-stimulated PBMCs were initiated at multiplicities ranging from 0.0001 to 0.005. Viral RNA was extracted from samples of the inocula and from culture supernatants collected at days 3, 5, 7, 9 and 11 post-infection. Viral cDNA was then synthesized from the extracted samples and quantified by real-time qPCR. Each point represents the results from one culture.

Figure 3. Effect of Multiplicity of infection on relative mutant frequency

Mutant viruses COTB VifB, T186M, T242N and I256V were competed against the autologous reference HIV-1_{NL4-3}(COTB) VifA virus at four MOI: 0.005, 0.001, 0.0005 and 0.0001. Supernatants were sampled as described in the legend to figure 2. Linear regression analysis was performed to determine the slope, r^2 and p-value for each data set. The dotted lines represent the 95% confidence interval of the three replicates.

Figure 4. Relative Fitness Assessed Using Different Algorithms

The relative fitness of VifB, T186M, T242N and I256V was assessed in PHA-stimulated PBMCs in competition with COT/NL4-3 VifA at an MOI of 0.005 across multiple experiments. Relative fitness was estimated by 1+s using mutant frequency values derived from two-time points encompassing the log growth phase of the experiment (days 4,7), multiple time points (days 0–10), and by *w* with two time points 4,7 and 0,10.

Figure 5. Effect of Viral Input Ratio on Relative Fitness

Mutant T186M was competed against the autologous reference VifA virus at different input ratios. Relative fitness was estimated by 1+s using mutant frequency values derived from two-time points encompassing the log growth phase of the experiment (days 4,7), multiple time points (days 0–10), and by *w* with two time points 4,7 and 0,10. Values reported are the average of multiple replicates across multiple experiments (for a total of $n=14$ at 90%, 12 at 80%, 9 at 70%, 3 at 60%, 3 at 50% and 6 at 20% mutant virus in the inoculum). Linear regression analysis was performed to determine if the slope of the mutant frequency values over time was significantly ($p>0.05$) different from 0.