# Inference with Viral Quasispecies

Methods for Individual Samples Comparative Analysis

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## Abstract

The study of viral quasispecies structure and diversity presents unique challenges in comparing samples, particularly when dealing with single observations of single biosamples, taken from different time points or conditions. Traditional statistical methods are often inapplicable in these scenarios, where a biosample observation is represented by a vector of haplotypes and associated frequencies, necessitating the use of resampling techniques to empirically generate haplotype distributions from which estimate estimate expected values and corresponding variability. The authors propose a random delete-d jackknife to estimate standard errors followed by a z-test for comparing single quasispecies observations. The research highlights several key challenges in quasispecies analysis, including the need for high sequencing depth, limited clinical samples, technical inconsistencies leading to coverage disparities, and the sensitivity of diversity indices to sample size differences. To address these issues, the authors suggest using a combination of metrics with varying susceptibilities to large sample sizes, ranging from observed differences and ratios to multitest adjusted p-values. The paper emphasizes the importance of not relying solely on p-values, as the high statistical power resulting from large sample sizes can lead to very low p-values for small, potentially biologically insignificant differences.

## 1 Introduction

A quasispecies is the complex and dynamic population of closely related but genetically diverse viral variants that arises during RNA virus infection. This population structure arises due to the high mutation rates of RNA viruses, which can introduce multiple errors during genome replication. The quasispecies consists of a mutant spectrum or cloud of viral genomes that are continuously generated and subject to selection within the host [1–3].

A quasispecies observation consists in sequencing to high depth a quasispecies sample taken from a biosample infected with a RNA virus, and it is represented by a set of genomes (haplotypes) and observed frequencies, in the form of NGS sequencing read counts.

In the study of quasispecies structure and diversity, we are often confronted with a situation where we need to compare single quasispecies observations from single biosamples, possibly taken at two different infection or treatment time points. This situation presents statistic challenges, because traditional inferencial tests are not applicable when comparing diversity indices or structure indicators of single quasispecies observations. A useful test must be based on estimates of expected diversity and its variability for each of the quasispecies to be compared. Analytical approximations are available for some diversity indices [4–6], like Shannon entropy or

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Simpson index, but for others they are complex and depend on assumptions about the underlying haplotypes abundance distribution. Furthermore, we know that some diversity indices, which may be of interest, are sensitive to sample size differences, which means that unbalanced samples will require normalization. We focus on diversity indices which may be calculated directly from the vector of haplotype NGS read counts.

In this situation we rely on tools based on resampling to generate empirical distributions from which estimate standard errors, and possibly confidence intervals and p-values. A resampling method commonly used to generate such distributions to estimate variances is the bootstrap [7–9], where at each resampling cycle, we sample with replacement a number of reads equal to the sample size. At each bootstrap cycle diversity indices are computed, and at the end the estimated values and corresponding variances result from the mean and variance of the observed values in the B bootstrap cycles. However we know that the bootstrap suffers from the 0.632 limitation, which implies that in each resampling cycle with replacement at the full sample size, at most 63.2% of the reads in the sample are observed, meaning that 36.8% of the reads are replicates of already observed reads, and likewise that 36.8% of the reads in the quasispecies sample remain unobserved [10]. This limitation results in biases in the estimation of diversity indices sensitive to low and very low haplotype frequencies. Particularly affected are the fraction of singletons and rare haplotypes, the total number of haplotypes and indices such as Shannon entropy or the Hill number at q=1 [10]. Note that any approach based on parametric multinomial resampling is submitted to the same limitations; resampling from a multinomial given its parameters and a sample size, is equivalent to resampling with replacement.

In this context, a valid alternative to the bootstrap is the jackknife [11]. While similar to the bootstrap in that it involves resampling, the key difference is that the jackknife samples without replacement, which aligns with our requirements. However, it is well known that the traditional delete-1 jackknife estimator performs poorly with non-smooth estimators—those that are sensitive to one or a few values in the dataset, such as the median or other empirical quantiles. This makes it particularly unsuitable for computing confidence intervals. It has been shown that the delete-d Jackknife [7,12], where $\sqrt{n} < d < (n-1)$, is a consistent estimator [7,13] and overcomes most of the limitations in the delete-1 jackknife. This method has a similar form to the delete-1 estimator but incorporates a different normalizing constant, accounting for the number of distinct sets with $d$ items deleted from the full set of $n$, $\binom{n}{d}$. However, as the complexity of the computations increases rapidly with larger $d$,-especially for large n-the estimator may be evaluated for a subset of all possible $\binom{n}{d}$ sets [7,14,15]. In this work, we employ a random delete-d jackknife, in which a fixed fraction of reads is randomly removed across a large number of resampling cycles. This scheme is closely related to repeated rarefaction to a lower sample size, subsampling without replacement. Note that in our context $n$ is in the order of 1.e+5, $d$ is chosen to be a 10%, 1.e+4, and the number of jackknife resamplings 1.e+3.

We show in the results section the implementation of this method in a study of in-vitro HCV quasispecies evolution, with samples at baseline and at different passages up to 200, representing the quasispecies evolution in a controlled non-coevolving cellular environment free of external pressures, for 700 days [16,17].

The R code implementing the proposed method is included as an appendix, along with the session info giving platform requirements, and software and library versions.

# 2 Methods

## 2.1 Random delete-d jackknife

Given a quasispecies represented by a vector of haplotype read counts, with a total of $n$ reads, we remove a fraction $f$ of reads, $d = n \times f$, at each resampling cycle. The reads have been

previously shuffled to prevent correlations among repeated samplings. A fraction $f$ of 10% has been selected as a good balance between perturbation and stability for a consistent estimation of variance across resampling cycles.

At each cycle, $j$, we estimate a quasispecies indicator, $\hat{\theta}_j$, and after $R$ delete-d jackknife cycles we estimate the indicator's standard error, $SE$ [13–15], as:

$$SE_{\text{jack}} = \sqrt{\frac{n-d}{d\,R}\sum_{j=1}^{R}\left(\hat{\theta}_j - \bar{\theta}_{(\cdot)}\right)^2}$$

where

$$\bar{\theta}_{(\cdot)} = \sum_{j=1}^{R}\hat{\theta}_j$$

The standard deviation, $S$, which will be used in the computation of effect sizes, is obtained from the standard error in the usual way:

$$S\text{jack} = SE\text{jack} \times \sqrt{R}$$

## 2.2 Normality assessment

The normality of the jackknife values may be assessed by the Kolmogorov-Smirnov, and the Shapiro-Wilks test [18]. The later is a correlation test that may detect even trivial departures from the null hypothesis [19], it is the most stringent test of normality. It was originally restricted to sample size less than 50. It is able to detect departures from normality due to either skewness or kurtosis, or both. However, significant results may be caused by effects too small to be of any practical significance. The Kolmogorov-Smirnov test [19] quantifies a distance between the empirical distribution function of the sample and the cumulative distribution function of the reference distributionis, and is less sensitive to deviations than the Shapiro-Wilks. To put in context the p-values of both tests, the p-value obtained from a set of 1000 randomly generated normal values is provided besides the p-values for the distribution of 1000 jackknife values of each quasispecies indicator. Additionally q-q plots, and the back-of-the-envelop test giving the sample coverage between SD units, are used.

## 2.3 Very large sample sizes and p-values

With sample sizes (N) so high, even a tiny difference in diversity measures between two quasispecies can appear statistically significant due to the extremely small standard errors. However, this statistical significance may not translate to biological or practical significance. In these cases, 99% confidence intervals are more appropriate. They assess the precision of the diversity estimates and the range of plausible values for the true difference between quasispecies. However, the main focus should be on the magnitude of the difference (effect size) rather than just statistical significance. Effect size measures, like Cohen's d, can provide more meaningful information about the practical importance of observed differences, and are independent of sample sizes [20–23].

## 2.4 Effect size

Cohen's $d$ is a standardized measure of effect size, independent of the sample size, that expresses the observed difference between two populations in units of the pooled standard deviation [20–23].

$$d = \frac{\bar{T}_a - \bar{T}_b}{S_p}$$

where, $T_a$ and $T_b$ are the observed indicator values at a normalized sample size, and $S_p$ is the pooled standard deviation:

$$SD_p = \sqrt{\frac{1}{2}(S_a^2 + S_b^2)}$$

By contrast, the z-statistic is highly affected by the sample size, providing information about the statistical power of the test.

$$z = \frac{\bar{T}_a - \bar{T}_b}{\sqrt{\frac{S_a^2}{n_a} + \frac{S_b^2}{n_a}}} = \frac{\bar{T}_a - \bar{T}_b}{\sqrt{SE_a^2 + SE_b^2}}$$

When the two populations being compared show diversity values that are normally distributed, or when the sample size is large enough that the difference is approximately normally distributed, Cohen's $d$ may be interpreted in probabilistic terms. Table 1 provides a guide for interpreting $d$ values [20,23,24]:

Table 1: Cohen's $d$ values interpretation. Effect magnitude and corresponding probabilistic interpretations, non-overlap (NOV) and probability of superiority (PS).

| d | Magnitude | NOV (1) | PS (2) |
|---|---|---|---|
| 0.0 | Null | 0 | 50.0 |
| 0.2 | Small | 8.0 | 55.6 |
| 0.5 | Medium | 19.7 | 63.8 |
| 0.8 | Large | 31.1 | 71.4 |
| 1.2 | Very large | 45.1 | 80.2 |

(1) NOV: Non-overlap between the two distributions.

$$NOV = \left[1 - 2\Phi\left(\frac{-\text{abs}(d)}{2}\right)\right] \times 100 \tag{1}$$

(2) PS: Probability of superiority. Probability that a randomly sampled individual from one group will have a higher score than a randomly sampled individual from the other group. It is a metric similar to the AUC.

$$PS = \Phi\left(\text{abs}(d)/\sqrt{2}\right) \times 100 \tag{2}$$

Where $\Phi$ represents the cumulative distribution function of the standard normal distribution. Note that in taking the absolute value of $d$ we ensure a minimum $PS$ of 50%. The sign of $d$ will dictate which is the condition of superiority.

### 2.5 Quasispecies maturity indicators

Table 2 shows the set of selected quasispecies structure indicators to account for the evolutionary state of a quasispecies in its path from a founding quasispecies dominated by a highly abundant master genome and a peaked fitness landscape, towards its hypothetical end-point in the form of a flat fitness landscape, with a high number of genomes at low and equivalent abundances, with no dominance [25,26].

Table 2: Selected maturity indicators.

| Indicator | Description | Regular Qs | Flat-like Qs |
|---|---|---|---|
| Top25 | Fraction of reads for top 25 hpl. | High | Low |
| Master | Dominant haplotype frequency | High | Low |
| Rare1 | Fraction of reads for hpl $\leq 1\%$ | Low | High |
| Rare2 | Fraction of reads for hpl $\leq 0.1\%$ | Low | High |
| $RLE_1$ | Relative logarithmic evennes at $q = 1$ | Low | High |
| $RLE_2$ | Relative logarithmic evennes at $q = 2$ | Low | High |
| $RLE_\infty$ | Relative logarithmic evennes at $q = \infty$ | Low | High |
| R5 | Evenness among top 5 haplotypes | Low | High |
| R10 | Evenness among top 10 haplotypes | Low | High |

### 2.6 Two limiting quasispecies states

Table 3 shows the characterization of the two limiting quasispecies states, as ideal models A and Z.

Table 3: Quasispecies maturity indicator values characterizing the two limiting quasispecies models A and Z.

| Model | Top25 | Master | Rare2 | Rare1 | RLE1 | RLE2 | RLEinf | R5 | R10 |
|---|---|---|---|---|---|---|---|---|---|
| A | 1.000 | 1.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 | 0.000 |
| Z | 0.000 | 0.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 | 1.000 |

A global quasispecies maturity score for a given quasispecies, Qs, may be computed as the distance from this quasispecies to Model A, $d_A$, in the multidimensional space represented by this set of indicators [26].

$$d_A(Qs) = \sqrt{\sum_i [(f_i(Qs) - f_i(A))^2]}$$

where $f_i$ is the set of maturity indicators. This score may be normalized to the range 0-1 as $d_N(Qs) = d_A(Qs)/d_A(Z)$. Whereas each indicator refers to a given aspect of quasispecies structure, $d_N$ represents the combined effect of all changes in pushing the quasispecies towards its origin, A, or its fate, Z [26].

### 2.7 Samples, cells and viruses

HCV RNA expressed from plasmid Jc1FLAG2(p7-nsGluc2A) (genotype 2a) [27] was transfected into Huh-7 Lunet cells and amplified in Huh-7.5 cells to produce the initial virus population HCV p0 [28]. HCV p0 was subjected to 200 serial passages in Huh-7.5 reporter cells in the absence of any drug. Biosamples were taken at different passages: p0, p3, p100, p110 and p200. The

populations at passage 100 (HCV p100) and at passage 200 (HCV p200) displayed increased replication in Huh-7.5 reported cells [16,29]. HCV p100 and p200 increased their fitness 2.2-fold relative to HCV p0 [29,30]. The 200 passages are equivalent to about 700 days of continuous evolution in a non-coevolving environment [16,17] free of external pressures. A single NS5A amplicon spanning positions 7523:7864 is deeply sequenced with a Illumina® MySeq instrument for each biosample.

# 3 Results

## 3.1 Samples and NGS coverage

Table 4 lists the quasispecies considered in the tests, and the coverage in number of reads for each strand of the sequenced amplicon.

Table 4: Reads per amplicon sample and strand

| ID | FW | RV |
|---|---|---|
| p0 | 124253 | 120939 |
| p3 | 186398 | 182387 |
| p100 | 179401 | 176168 |
| p110 | 136432 | 133209 |
| p200 | 123807 | 123946 |

In what follows only the fasta files for the forward strands of the amplicon are considered.

## 3.2 Quasispecies maturity indicators, raw values

Values of these indicators are first computed on the quasispecies as they are observed, without size normalization, see Table 5.

Table 5: (A) - Quasispecies maturity indicators. Raw values.

| ID | nHpl | Master | Top25 | Rare1 | Rare2 | dN |
|---|---|---|---|---|---|---|
| p0 | 5858 | 0.72884 | 0.80131 | 0.24107 | 0.19969 | 0.18117 |
| p3 | 8325 | 0.73095 | 0.78525 | 0.26905 | 0.18898 | 0.18507 |
| p100 | 11948 | 0.49214 | 0.74294 | 0.33202 | 0.23125 | 0.27241 |
| p110 | 9744 | 0.39303 | 0.75431 | 0.28186 | 0.24154 | 0.29475 |
| p200 | 11490 | 0.43506 | 0.70577 | 0.37739 | 0.25735 | 0.31201 |

Table 5: (B) - Quasispecies maturity indicators. Raw values.

| ID | R5 | R10 | RLE1 | RLE2 | RLEinf | dN |
|---|---|---|---|---|---|---|
| p0 | 0.02398 | 0.02458 | 0.27677 | 0.07278 | 0.03646 | 0.18117 |
| p3 | 0.02070 | 0.02845 | 0.27276 | 0.06939 | 0.03472 | 0.18507 |
| p100 | 0.06210 | 0.06696 | 0.38164 | 0.14308 | 0.07552 | 0.27241 |
| p110 | 0.10277 | 0.03230 | 0.40176 | 0.17882 | 0.10168 | 0.29475 |
| p200 | 0.16119 | 0.08438 | 0.43750 | 0.17121 | 0.08902 | 0.31201 |

## 3.3 Maturity indicator values on rarefied quasispecies

## 3.4 Observed effects

Observed differences between consecutive time points, in rarefied maturity indicators. These values provide a reference scale for the magnitude of changes experienced by the quasispecies along the passages.

Note that differences observed between p0 and p3, or between p100 and p110 are caused by stochastic variations in the short term, and that the differences between p100 and p0, or between p200 and p100 are caused by increased quasispecies maturity, the evolution expected in the long run, with decreased Master and Top25, and increased rare fractions and evenness.

Table 6: (A) - Quasispecies maturity indicators. Rarefied values to 120,000 reads.

| ID | nHpl | Master | Top25 | Rare1 | Rare2 | dN |
|---|---|---|---|---|---|---|
| p0 | 5701.0 | 0.72882 | 0.80132 | 0.24108 | 0.19871 | 0.18119 |
| p3 | 5820.5 | 0.73085 | 0.78539 | 0.26915 | 0.18985 | 0.18686 |
| p100 | 8810.0 | 0.49220 | 0.74294 | 0.33206 | 0.23073 | 0.27427 |
| p110 | 8905.0 | 0.39302 | 0.75434 | 0.28183 | 0.24104 | 0.29533 |
| p200 | 11223.0 | 0.43506 | 0.70578 | 0.37738 | 0.25743 | 0.31217 |

Table 6: (B) - Quasispecies maturity indicators. Rarefied values to 120,000 reads.

| ID | R5 | R10 | RLE1 | RLE2 | RLEinf | dN |
|---|---|---|---|---|---|---|
| p0 | 0.02398 | 0.02460 | 0.27747 | 0.07301 | 0.03657 | 0.18119 |
| p3 | 0.02096 | 0.02843 | 0.28194 | 0.07229 | 0.03617 | 0.18686 |
| p100 | 0.06222 | 0.06592 | 0.39169 | 0.14784 | 0.07803 | 0.27427 |
| p110 | 0.10286 | 0.03238 | 0.40484 | 0.18059 | 0.10269 | 0.29533 |
| p200 | 0.16116 | 0.08442 | 0.43832 | 0.17164 | 0.08924 | 0.31217 |

Table 7: (A) - Observed differences in quasispecies structure indicators. Quasispecies rarefied to 120,000 reads.

| | nHpl | Master | Top25 | Rare1 | Rare2 | dN |
|---|---|---|---|---|---|---|
| p3-p0 | 119.5 | 0.0020 | -0.0159 | 0.0281 | -0.0089 | 0.0057 |
| p100-p0 | 3109.0 | -0.2366 | -0.0584 | 0.0910 | 0.0320 | 0.0931 |
| p110-p100 | 95.0 | -0.0992 | 0.0114 | -0.0502 | 0.0103 | 0.0211 |
| p200-p100 | 2413.0 | -0.0571 | -0.0372 | 0.0453 | 0.0267 | 0.0379 |

Table 7: (B) - Observed differences in quasispecies structure indicators. Quasispecies rarefied to 120,000 reads.

| | R5 | R10 | RLE1 | RLE2 | RLEinf | dN |
|---|---|---|---|---|---|---|
| p3-p0 | -0.0030 | 0.0038 | 0.0045 | -0.0007 | -0.0004 | 0.0057 |
| p100-p0 | 0.0382 | 0.0413 | 0.1142 | 0.0748 | 0.0415 | 0.0931 |
| p110-p100 | 0.0406 | -0.0335 | 0.0132 | 0.0327 | 0.0247 | 0.0211 |
| p200-p100 | 0.0989 | 0.0185 | 0.0466 | 0.0238 | 0.0112 | 0.0379 |

Table 8: (A) - Observed ratios in quasispecies structure indicators. Quasispecies rarefied to 120,000 reads.

| | nHpl | Master | Top25 | Rare1 | Rare2 | dN |
|---|---|---|---|---|---|---|
| p3/p0 | 1.021 | 1.003 | 0.980 | 1.116 | 0.955 | 1.031 |
| p100/p0 | 1.545 | 0.675 | 0.927 | 1.377 | 1.161 | 1.514 |
| p110/p100 | 1.011 | 0.798 | 1.015 | 0.849 | 1.045 | 1.077 |
| p200/p100 | 1.274 | 0.884 | 0.950 | 1.136 | 1.116 | 1.138 |

Despite very large NGS sample sizes, only a single experimental sample from each quasispecies is analyzed here. It's important to note that these experimental samples are susceptible to stochastic fluctuations, methodological discrepancies, and procedural inconsistencies When comparing single experimental samples, the inherent experimental variability may lead to unexpected or misleading

Table 8: (B) - Observed ratios in quasispecies structure indicators. Quasispecies rarefied to 120,000 reads.

| | R5 | R10 | RLE1 | RLE2 | RLEinf | dN |
|---|---|---|---|---|---|---|
| p3/p0 | 0.874 | 1.156 | 1.016 | 0.990 | 0.989 | 1.031 |
| p100/p0 | 2.595 | 2.680 | 1.412 | 2.025 | 2.134 | 1.514 |
| p110/p100 | 1.653 | 0.491 | 1.034 | 1.222 | 1.316 | 1.077 |
| p200/p100 | 2.590 | 1.281 | 1.119 | 1.161 | 1.144 | 1.138 |

results, particularly when the two quasispecies are very similar in structure.

### 3.5 Z-tests with random delete-d jackknife SE estimates

A random delete-d reads jackknife resampling scheme is carried out, removing 10% reads, selected randomly, in each of $R = 1000$ resampling cycles. Besides jackknife values, these values may be considered as observed quasispecies indicator values in $R$ rarefactions from $N$ reads to $0.9 \cdot N$ reads. Jackknife estimated standard errors of each indicator will be used in a z-test contrasting the indicator values of two quasispecies. A random delete-d reads jackknife is carried out for each sample to be compared. When the samples are unbalanced, a rarefaction of the read counts of the larger sample to the size of the smaller is performed previous to the jackknife.

#### 3.5.1 Test p0 vs p3

The coverages as reads number of the quasispecies p0 and p3 are shown in 9.

Table 9: Quasispecies coverage

| ID | Reads |
|---|---|
| p3 | 186398 |
| p0 | 124253 |

**3.5.1.1 Normality of jackknife values** The normality of the jackknife values should be assessed to provide validity to the test. The Q-Q plot of selected indicators for the jackknife values of the p0 quasispecies is shown in Figure 1. The Shapiro-Wilk and Kolmogorov-Smirnov test p-values, and the jackknife values coverage within SD units are given in Table 10 for p0 and in Table 11 for p3. The 'Normality' row gives the expected values under normality, where the Kolmogorov-Smirnov and the Shapiro-Wilk test p-values are obtained from a test on a set of 1000 random $\mathcal{N}(0, 1)$ values generated for each indicator's test.

Table 10: Normality assessment. Within n-SD data fraction for the jackknife values of p0. Fraction of data within 1, 2, 2.5, and 3 SD, Kolmogorov-Smirnov, and Shapiro-Wilk normality test p-values

| | w1SD | w2SD | w2.5SD | w3SD | KS.pval | SW.pval |
|---|---|---|---|---|---|---|
| Normality | 0.683 | 0.954 | 0.988 | 0.997 | 0.897 | 0.330 |
| nHpl | 0.669 | 0.958 | 0.991 | 0.999 | 0.922 | 0.440 |
| Top25 | 0.689 | 0.948 | 0.985 | 0.995 | 0.625 | 0.717 |
| Master | 0.688 | 0.957 | 0.983 | 0.997 | 0.896 | 0.542 |
| Rare1 | 0.697 | 0.957 | 0.984 | 0.996 | 0.870 | 0.812 |
| Rare2 | 0.686 | 0.954 | 0.987 | 0.994 | 0.904 | 0.121 |
| R5 | 0.688 | 0.954 | 0.988 | 0.996 | 0.913 | 0.062 |
| R10 | 0.701 | 0.966 | 0.987 | 0.994 | 0.003 | 0.000 |
| RLE1 | 0.692 | 0.951 | 0.985 | 0.998 | 0.867 | 0.856 |
| RLE2 | 0.689 | 0.957 | 0.984 | 0.997 | 0.831 | 0.383 |
| RLEinf | 0.688 | 0.957 | 0.985 | 0.996 | 0.890 | 0.394 |
| dN | 0.673 | 0.950 | 0.986 | 0.998 | 0.768 | 0.332 |

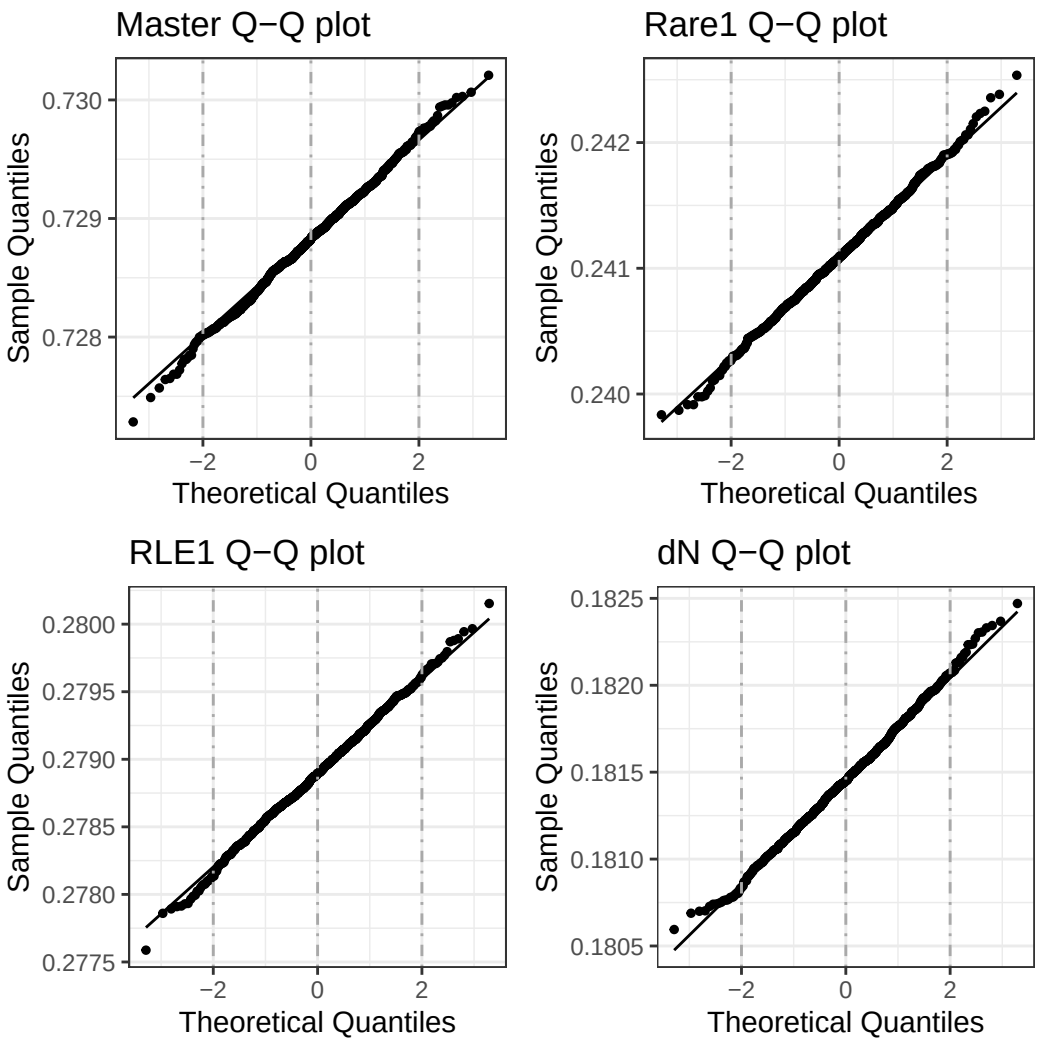


Figure 1: Q-Q plots to assess normality in the jackknife values of selected quasispecies indicators of p0.

Table 11: Normality assessment. Within n-SD data fraction for the jackknife values of p3. Fraction of data within 1, 2, 2.5, and 3 SD, Kolmogorov-Smirnov, and Shapiro-Wilk normality test p-values

| | w1SD | w2SD | w2.5SD | w3SD | KS.pval | SW.pval |
|---|---|---|---|---|---|---|
| Normality | 0.683 | 0.954 | 0.988 | 0.997 | 0.878 | 0.532 |
| nHpl | 0.669 | 0.958 | 0.991 | 0.999 | 0.922 | 0.440 |
| Top25 | 0.689 | 0.948 | 0.985 | 0.995 | 0.625 | 0.717 |
| Master | 0.688 | 0.957 | 0.983 | 0.997 | 0.896 | 0.542 |
| Rare1 | 0.697 | 0.957 | 0.984 | 0.996 | 0.870 | 0.812 |
| Rare2 | 0.686 | 0.954 | 0.987 | 0.994 | 0.904 | 0.121 |
| R5 | 0.688 | 0.954 | 0.988 | 0.996 | 0.913 | 0.062 |
| R10 | 0.701 | 0.966 | 0.987 | 0.994 | 0.003 | 0.000 |
| RLE1 | 0.692 | 0.951 | 0.985 | 0.998 | 0.867 | 0.856 |
| RLE2 | 0.689 | 0.957 | 0.984 | 0.997 | 0.831 | 0.383 |
| RLEinf | 0.688 | 0.957 | 0.985 | 0.996 | 0.890 | 0.394 |
| dN | 0.673 | 0.950 | 0.986 | 0.998 | 0.768 | 0.332 |

**3.5.1.2 Z-test of p0 versus p3** Table 12 shows the results of the z-test contrasting quasispecies indicator values of quasispecies p0 to quasispecies p3, with indicator observed values, their difference and ratio, the 95% confidence interval of the difference, the z statistic, the multitest Benjamini-Hochberg adjusted p-value, the Cohen's d effect size, and the probability of superiority (PS) in percentage, where 50 implies no effect difference. Figure 2 represents the 95% confidence intervals, and the Cohen's effect sizes.

Table 12: Z-test results for p3-p0

| Feat | p0 | p3 | Ratio | Dif | CI.low | CI.upp | z | adj.p.val | d | PS |
|---|---|---|---|---|---|---|---|---|---|---|
| Top25 | 0.801 | 0.786 | 0.981 | -0.015 | -0.019 | -0.012 | -8.2 | 1.0e-15 | -0.37 | 60.3 |
| Master | 0.729 | 0.731 | 1.004 | 0.003 | -0.001 | 0.007 | 1.3 | 2.2e-01 | 0.06 | 51.6 |
| Rare1 | 0.241 | 0.269 | 1.114 | 0.027 | 0.024 | 0.031 | 13.9 | 7.1e-43 | 0.62 | 67.0 |
| Rare2 | 0.199 | 0.189 | 0.951 | -0.010 | -0.020 | 0.000 | -2.0 | 7.7e-02 | -0.09 | 52.5 |
| R5 | 0.024 | 0.021 | 0.861 | -0.003 | -0.007 | 0.000 | -2.0 | 7.7e-02 | -0.09 | 52.5 |
| R10 | 0.025 | 0.030 | 1.203 | 0.005 | 0.001 | 0.009 | 2.3 | 4.2e-02 | 0.10 | 53.0 |
| RLE1 | 0.279 | 0.283 | 1.015 | 0.004 | 0.001 | 0.007 | 2.4 | 4.2e-02 | 0.11 | 53.0 |
| RLE2 | 0.073 | 0.073 | 0.988 | -0.001 | -0.002 | 0.000 | -1.4 | 1.9e-01 | -0.06 | 51.8 |
| RLEinf | 0.037 | 0.036 | 0.987 | 0.000 | -0.001 | 0.000 | -1.6 | 1.6e-01 | -0.07 | 52.0 |
| dN | 0.181 | 0.187 | 1.029 | 0.005 | 0.002 | 0.008 | 3.7 | 8.8e-04 | 0.16 | 54.6 |

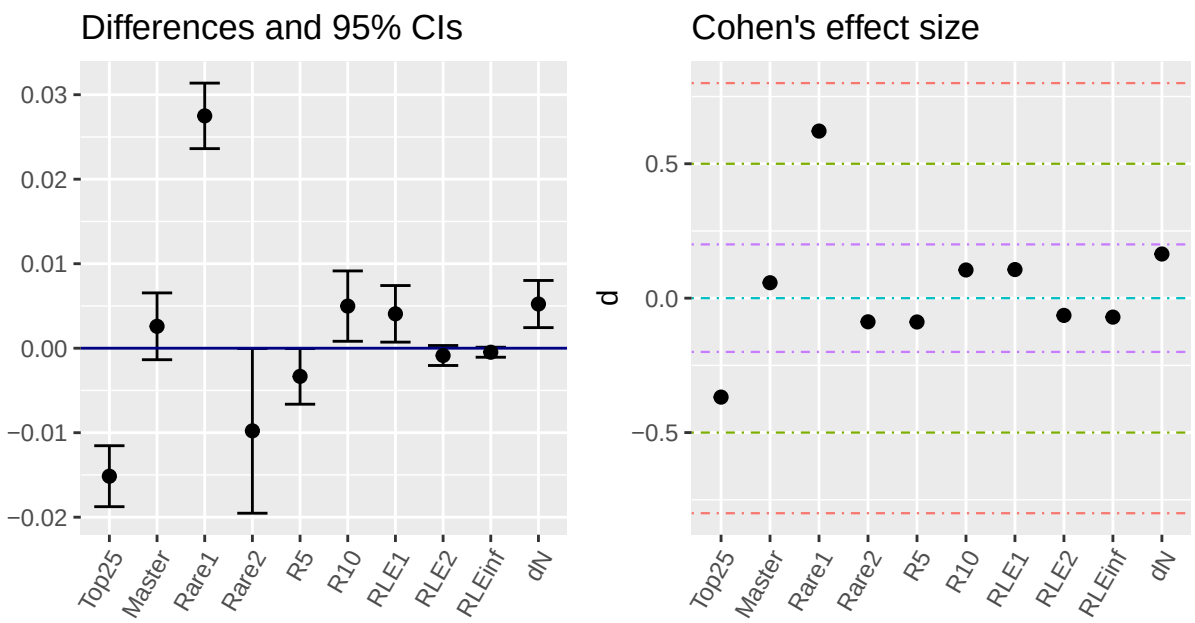


Figure 2: Z-test results for p3-p0. Left: 95% CI of the difference. Right: Effect size as Cohen's d, with 0.2, 0.5 and 0.8 borders.

The jackknife values show acceptable normality distributions for all indicators except for R10, which has low p-values in the Shapiro-Wilk and Kolmogorov-Smirnov tests. However, the coverage within SD units for this indicator, beyond 2SD, shows limited deviations. Statistically significant differences (adjusted p-value $< 0.05$) are observed for Top25, Rare1, R10, RLE1, and dN. The effect after 3 passages is null ($0 \leq |d| < 0.2$) for all indicators, except for Top25, which is small ($0.2 \leq |d| < 0.5$), and Rare1, which is medium ($0.5 \leq |d| < 0.8$). These results highlight the stochastic variations in quasispecies structure caused by quasispecies dynamics in the short term, with dN remaining just below the threshold for a small effect [26].

### 3.5.2 Test p0 vs p100

The coverages as reads number of the quasispecies p0 and p100 are shown in 13.

Table 13: Quasispecies coverage

| ID | Reads |
|---|---|
| p100 | 179401 |
| p0 | 124253 |

**3.5.2.1 Normality of jackknife values** The Shapiro-Wilk and Kolmogorov-Smirnov test p-values, and the jackknife values coverage within SD units are given in Table 14 for p100. The 'Normality' row gives the expected values under normality.

Table 14: Normality for the jackknife values of p100

| | w1SD | w2SD | w2.5SD | w3SD | KS.pval | SW.pval |
|---|---|---|---|---|---|---|
| Normality | 0.683 | 0.954 | 0.988 | 0.997 | 0.908 | 0.141 |
| nHpl | 0.669 | 0.958 | 0.991 | 0.999 | 0.922 | 0.440 |
| Top25 | 0.689 | 0.948 | 0.985 | 0.995 | 0.625 | 0.717 |
| Master | 0.688 | 0.957 | 0.983 | 0.997 | 0.896 | 0.542 |
| Rare1 | 0.697 | 0.957 | 0.984 | 0.996 | 0.870 | 0.812 |
| Rare2 | 0.686 | 0.954 | 0.987 | 0.994 | 0.904 | 0.121 |
| R5 | 0.688 | 0.954 | 0.988 | 0.996 | 0.913 | 0.062 |
| R10 | 0.701 | 0.966 | 0.987 | 0.994 | 0.003 | 0.000 |
| RLE1 | 0.692 | 0.951 | 0.985 | 0.998 | 0.867 | 0.856 |
| RLE2 | 0.689 | 0.957 | 0.984 | 0.997 | 0.831 | 0.383 |
| RLEinf | 0.688 | 0.957 | 0.985 | 0.996 | 0.890 | 0.394 |
| dN | 0.673 | 0.950 | 0.986 | 0.998 | 0.768 | 0.332 |

**3.5.2.2 Z-test of p0 versus p100** Table 15 shows the results of the z-test contrasting quasispecies indicator values of quasispecies p0 to quasispecies p100, with indicator observed values, their difference and ratio, the 95% confidence interval of the difference, the z statistic, the multitest Benjamini-Hochberg adjusted p-value, the Cohen's d effect size, and the probability of superiority (PS) in percentage, where 50 implies no effect difference. Figure 3 represents the 95% confidence intervals, and the Cohen's effect sizes.

The jackknife values for p0.p100, show acceptable normality distributions for all indicators except for R10, as seen previously. Statistically significant differences (adjusted p-value $< 0.05$) are found for all indicators. The effect after 100 passages is small ($0.2 \leq |d| < 0.5$) for Rare2, medium ($0.5 \leq |d| < 0.8$) for R5 and R10, and very large ($|d| \geq 1.2$) for all other indicators. These results indicate a long-term deterministic evolution toward flatter fitness landscapes, with a very large effect on dN, which increased by 50% [26].

Table 15: Z-test results for p100-p0

| Feat | p0 | p100 | Ratio | Dif | CI.low | CI.upp | z | adj.p.val | d | PS |
|---|---|---|---|---|---|---|---|---|---|---|
| Top25 | 0.801 | 0.744 | 0.928 | -0.058 | -0.061 | -0.054 | -31.0 | 2.6e-211 | -1.39 | 83.7 |
| Master | 0.729 | 0.493 | 0.677 | -0.236 | -0.240 | -0.232 | -109.5 | 0.0e+00 | -4.90 | 100.0 |
| Rare1 | 0.241 | 0.331 | 1.375 | 0.090 | 0.086 | 0.094 | 44.7 | 0.0e+00 | 2.00 | 92.1 |
| Rare2 | 0.199 | 0.229 | 1.151 | 0.030 | 0.021 | 0.039 | 6.6 | 3.7e-11 | 0.30 | 58.3 |
| R5 | 0.024 | 0.063 | 2.638 | 0.039 | 0.034 | 0.045 | 14.6 | 1.9e-48 | 0.65 | 67.8 |
| R10 | 0.025 | 0.066 | 2.694 | 0.042 | 0.036 | 0.048 | 13.6 | 6.2e-42 | 0.61 | 66.6 |
| RLE1 | 0.279 | 0.393 | 1.409 | 0.114 | 0.111 | 0.117 | 69.7 | 0.0e+00 | 3.12 | 98.6 |
| RLE2 | 0.073 | 0.148 | 2.020 | 0.075 | 0.074 | 0.076 | 99.9 | 0.0e+00 | 4.47 | 99.9 |
| RLEinf | 0.037 | 0.078 | 2.129 | 0.042 | 0.041 | 0.042 | 98.9 | 0.0e+00 | 4.42 | 99.9 |
| dN | 0.181 | 0.274 | 1.510 | 0.093 | 0.090 | 0.095 | 67.4 | 0.0e+00 | 3.01 | 98.3 |

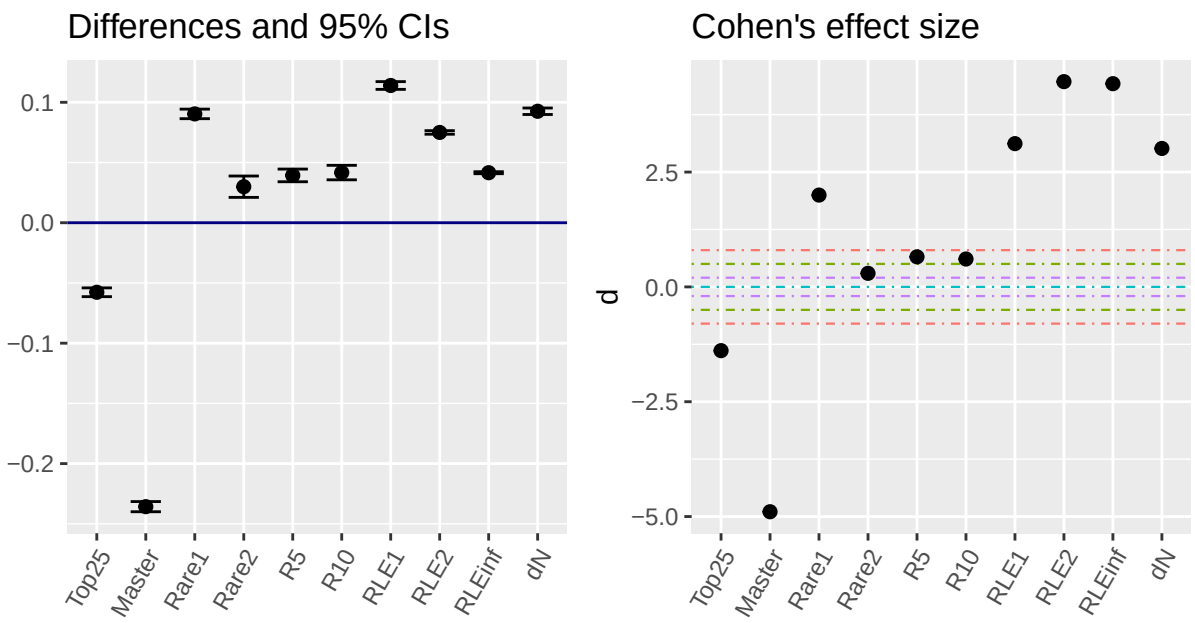


Figure 3: Z-test results for p100-p0. Left: 95% CI of the difference. Right: Effect size as Cohen's d, with magnitude borders.

### 3.5.3 Test p100 vs p110

The coverages as reads number of the quasispecies p0 and p3 are shown in Table 16.

Table 16: Quasispecies coverage

| ID | Reads |
|---|---|
| p110 | 136432 |
| p100 | 179401 |

**3.5.3.1 Normality of jackknife values** The Shapiro-Wilk and Kolmogorov-Smirnov test p-values, and the jackknife values coverage within SD units are given in Table 17 for p110. The 'Normality' row gives the expected values under normality.

Table 17: Normality tests for the jackknife values of p110

| | w1SD | w2SD | w2.5SD | w3SD | KS.pval | SW.pval |
|---|---|---|---|---|---|---|
| Normality | 0.683 | 0.954 | 0.988 | 0.997 | 0.825 | 0.557 |
| nHpl | 0.677 | 0.958 | 0.986 | 0.997 | 0.919 | 0.440 |
| Top25 | 0.689 | 0.954 | 0.985 | 0.995 | 0.764 | 0.439 |
| Master | 0.687 | 0.955 | 0.988 | 1.000 | 0.602 | 0.414 |
| Rare1 | 0.687 | 0.951 | 0.989 | 0.998 | 0.637 | 0.831 |
| Rare2 | 0.665 | 0.955 | 0.991 | 1.000 | 0.624 | 0.110 |
| R5 | 0.688 | 0.960 | 0.983 | 0.998 | 0.992 | 0.649 |
| R10 | 0.684 | 0.960 | 0.987 | 0.995 | 0.104 | 0.000 |
| RLE1 | 0.699 | 0.950 | 0.988 | 0.998 | 0.817 | 0.700 |
| RLE2 | 0.687 | 0.958 | 0.989 | 1.000 | 0.725 | 0.597 |
| RLEinf | 0.683 | 0.956 | 0.989 | 0.999 | 0.926 | 0.675 |
| dN | 0.690 | 0.953 | 0.987 | 0.997 | 0.566 | 0.495 |

**3.5.3.2 Z-test of p100 versus p110** Table 18 shows the results of the z-test contrasting quasispecies indicator values of quasispecies p100 to quasispecies p110, with indicator observed values, their difference and ratio, the 95% confidence interval of the difference, the z statistic, the multitest Benjamini-Hochberg adjusted p-value, the Cohen's d effect size, and the probability of superiority (PS) in percentage, where 50 implies no effect difference. Figure 4 represents the 95% confidence intervals, and the Cohen's effect sizes.

Table 18: Z-test results for p110-p100

| Feat | p100 | p110 | Ratio | Dif | CI.low | CI.upp | z | adj.p.val | d | PS |
|---|---|---|---|---|---|---|---|---|---|---|
| Top25 | 0.742 | 0.754 | 1.017 | 0.012 | 0.009 | 0.016 | 6.8 | 1.4e-11 | 0.30 | 58.5 |
| Master | 0.492 | 0.393 | 0.799 | -0.099 | -0.103 | -0.095 | -47.7 | 0.0e+00 | -2.13 | 93.4 |
| Rare1 | 0.333 | 0.282 | 0.846 | -0.051 | -0.055 | -0.047 | -26.2 | 2.4e-151 | -1.17 | 79.7 |
| Rare2 | 0.233 | 0.241 | 1.037 | 0.009 | 0.000 | 0.017 | 1.9 | 6.7e-02 | 0.08 | 52.4 |
| R5 | 0.062 | 0.103 | 1.645 | 0.040 | 0.034 | 0.046 | 13.0 | 2.3e-38 | 0.58 | 65.9 |
| R10 | 0.066 | 0.032 | 0.492 | -0.033 | -0.040 | -0.027 | -10.2 | 4.7e-24 | -0.45 | 62.6 |
| RLE1 | 0.392 | 0.404 | 1.032 | 0.013 | 0.010 | 0.015 | 8.3 | 1.4e-16 | 0.37 | 60.4 |
| RLE2 | 0.147 | 0.180 | 1.222 | 0.033 | 0.031 | 0.034 | 41.8 | 0.0e+00 | 1.87 | 90.7 |
| RLEinf | 0.078 | 0.103 | 1.317 | 0.025 | 0.024 | 0.026 | 49.2 | 0.0e+00 | 2.20 | 94.0 |
| dN | 0.275 | 0.295 | 1.075 | 0.020 | 0.018 | 0.023 | 16.5 | 1.8e-60 | 0.74 | 69.9 |

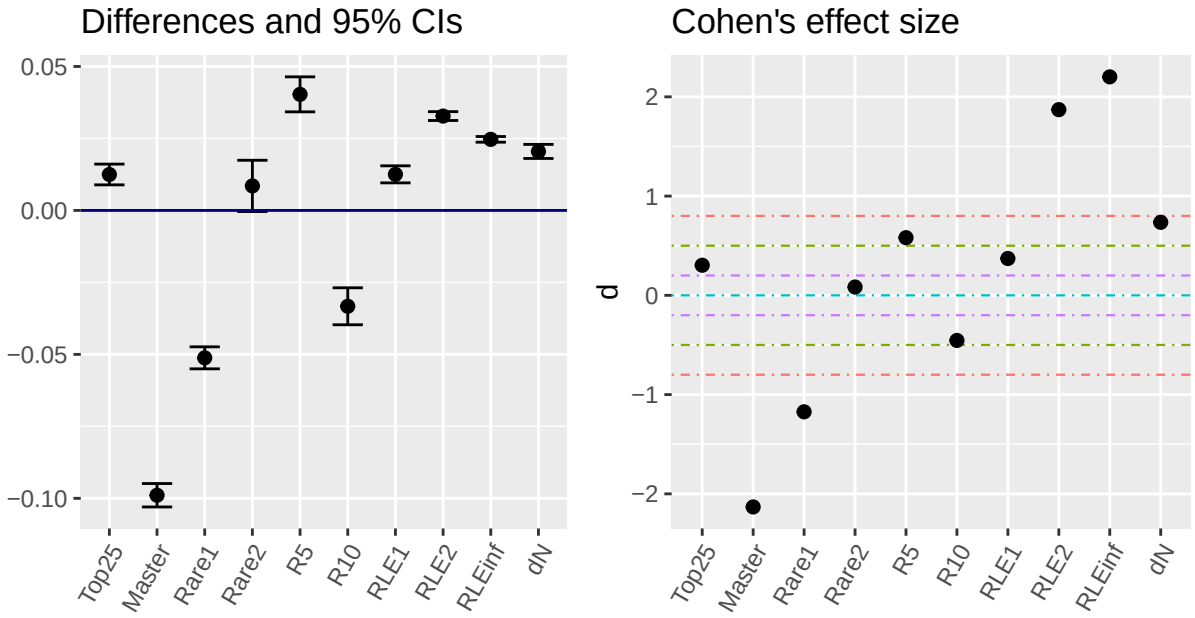


Figure 4: Z-test results for p110-p100. Left: 95% CI of the difference. Right: Effect size as Cohen's d, with magnitude borders.

The jackknife values for p110 show acceptable normality distributions for all indicators except for R10, as seen previously. Statistically significant differences (adjusted p-value $< 0.05$) are found for all indicators, except for Rare2. The effect after 10 passages is null ($|d| < 0.2$) for Rare2, small ($0.2 \leq |d| < 0.5$) for Top25 and RLE1, medium ($0.5 \leq |d| < 0.8$) for R5, large ($0.8 \leq |d| < 1.2$) for dN, and very large ($|d| \geq 1.2$) for all other indicators. These results are more difficult to interpret, as Top25 and Rare1 exhibit changes with signs opposite to those expected in the long run, while evenness increases in all indicators except for R10. The very large effect on the decrease of Master, along with the increase in all evenness indicators—particularly RLE2 and RLEinf—results in a medium effect on dN. This reflects a combination of stochastic effects in the short term and a moderated long-term evolution toward flatter fitness landscapes [26].

### 3.5.4 Test p100 vs p200

The coverages as reads number of the quasispecies p0 and p3 are shown in Table 19.

Table 19: Quasispecies coverage

| ID | Reads |
|---|---|
| p200 | 123807 |
| p100 | 179401 |

**3.5.4.1 Normality of jackknife values** The Shapiro-Wilk and Kolmogorov-Smirnov test p-values, and the jackknife values coverage within SD units are given in Table 20 for p110. The 'Normality' row gives the expected values under normality.

Table 20: Normality tests for the jackknife values of p200

| | w1SD | w2SD | w2.5SD | w3SD | KS.pval | SW.pval |
|---|---|---|---|---|---|---|
| Normality | 0.683 | 0.954 | 0.988 | 0.997 | 0.765 | 0.707 |
| nHpl | 0.673 | 0.965 | 0.990 | 0.999 | 0.924 | 0.224 |
| Top25 | 0.690 | 0.953 | 0.979 | 0.993 | 0.744 | 0.096 |
| Master | 0.667 | 0.959 | 0.991 | 0.998 | 0.782 | 0.355 |
| Rare1 | 0.680 | 0.959 | 0.984 | 0.996 | 0.726 | 0.140 |
| Rare2 | 0.712 | 0.949 | 0.983 | 0.995 | 0.747 | 0.000 |
| R5 | 0.674 | 0.959 | 0.991 | 0.998 | 0.363 | 0.047 |
| R10 | 0.685 | 0.954 | 0.985 | 0.997 | 0.430 | 0.001 |
| RLE1 | 0.686 | 0.956 | 0.982 | 0.996 | 0.568 | 0.180 |
| RLE2 | 0.661 | 0.959 | 0.993 | 0.998 | 0.919 | 0.287 |
| RLEinf | 0.657 | 0.962 | 0.991 | 0.999 | 0.956 | 0.232 |
| dN | 0.672 | 0.956 | 0.987 | 0.997 | 0.782 | 0.263 |

**3.5.4.2 Z-test of p100 versus p200** Table 21 shows the results of the z-test contrasting quasispecies indicator values of quasispecies p100 to quasispecies p110, with indicator observed values, their difference and ratio, the 95% confidence interval of the difference, the z statistic, the multitest Benjamini-Hochberg adjusted p-value, the Cohen's d effect size, and the probability of superiority (PS) in percentage, where 50 implies no effect difference. Figure 5 represents the 95% confidence intervals, and the Cohen's effect sizes.

Table 21: Z-test results for p200-p100

| Feat | p100 | p200 | Ratio | Dif | CI.low | CI.upp | z | adj.p.val | d | PS |
|---|---|---|---|---|---|---|---|---|---|---|
| Top25 | 0.742 | 0.706 | 0.951 | -0.036 | -0.040 | -0.032 | -18.2 | 1.5e-73 | -0.81 | 71.7 |
| Master | 0.492 | 0.435 | 0.884 | -0.057 | -0.061 | -0.053 | -25.6 | 1.4e-143 | -1.14 | 79.0 |
| Rare1 | 0.333 | 0.377 | 1.134 | 0.044 | 0.040 | 0.049 | 20.6 | 2.0e-94 | 0.92 | 74.3 |
| Rare2 | 0.233 | 0.258 | 1.106 | 0.025 | 0.016 | 0.034 | 5.5 | 5.1e-08 | 0.24 | 56.9 |
| R5 | 0.063 | 0.161 | 2.566 | 0.098 | 0.091 | 0.106 | 25.1 | 3.3e-138 | 1.12 | 78.6 |
| R10 | 0.065 | 0.084 | 1.295 | 0.019 | 0.011 | 0.028 | 4.4 | 1.2e-05 | 0.20 | 55.5 |
| RLE1 | 0.394 | 0.440 | 1.117 | 0.046 | 0.043 | 0.049 | 28.4 | 1.9e-176 | 1.27 | 81.5 |
| RLE2 | 0.149 | 0.173 | 1.162 | 0.024 | 0.022 | 0.026 | 27.1 | 7.1e-161 | 1.21 | 80.4 |
| RLEinf | 0.078 | 0.090 | 1.145 | 0.011 | 0.010 | 0.012 | 22.5 | 4.5e-112 | 1.01 | 76.2 |
| dN | 0.275 | 0.313 | 1.136 | 0.037 | 0.035 | 0.040 | 26.8 | 3.5e-157 | 1.20 | 80.1 |

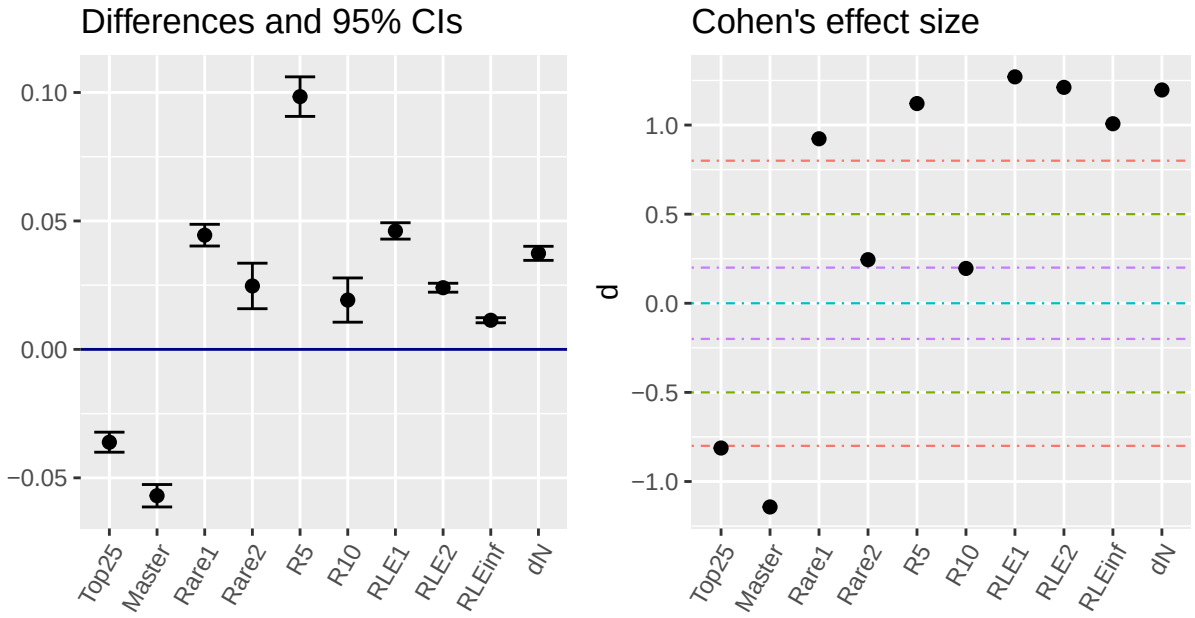


Figure 5: Z-test results for p200-p100. Left: 95% CI of the difference. Right: Effect size as Cohen's d, with magnitude borders.

The p200 jackknife values show acceptable normality distributions for all indicators except for Rare2, R5, and R10 under the Shapiro-Wilk test. However none is statistically significant under the Kolmogorov-Smirnov test, and the coverage profiles within SD units show very limited deviations. Statistically significant differences (adjusted p-value $< 0.05$) are found for all indicators. The effect after extra 100 passages is small ($0.2 \leq |d| < 0.5$) for R10, and Rare2, and very large ($|d| \geq 1.2$) for all other indicators. The trends are as expected, decreased values for Master and Top25, and increased values for the Rare fractions, and the evenness indicators. The effect is very large for dN. These results indicate a long-term deterministic evolution toward flatter fitness landscapes, with a very large effect on dN, which however increased in a limited 14%. The differences and ratios in all indicators are smaller than those observed from p0 to

p0.p100, implying that the evolution within the first 100 passages was faster than in the following 100 passages.

### 3.6 Interpretation of results

The interpretation of results should be based on several complementary metrics:

- Absolute observed difference
- Relative observed difference
- Observed ratio
- Cohen's d
- 95% CIs
- Adjusted p-values

The primary metrics are the observed difference, absolute and relative, and ratio. These are the main results, and often require expert interpretation to determine their biological or clinical significance. The importance of an difference of 1% or 10%, or a ratio of 1.1 or 0.75 depend of the context, and mainly of the level of maturity of the quasispecies being compared. Highly peaked fitness landscape quasispecies are expected to evolve with a sharp decline in Master and Top25, and corresponding increases in Rare1 and Rare2. Highly mature quasispecies in contrast evolve asymptotically in these indicators, with higher variations in the evenness indicators. Cohen's d provides a standardized measure of the magnitude of the effect, as a help to interpret the observed differences in statistical terms. The z-statistic indicates how extreme the observed difference is with respect to the null distribution. It provides information about statistical significance, showing how many standard deviations the observed difference is from the mean of the null distribution. When multiple comparisons are performed, adjusted p-values should be considered to control for the family-wise error rate or false discovery rate.

A comprehensive interpretation of results should consider all these metrics together:

1. Assess the magnitude and direction of the effect using the observed difference and ratio.
2. Evaluate the magnitude of the effect, in statistical terms, using Cohen's d and PS.
3. Consider the statistical significance by means of CIs and adjusted p-values.
4. Contextualize these findings within the biological or clinical framework of the study, and the level of maturity of the involved quasispecies, provided by the dN indicator.

This multi-faceted approach provides a more robust interpretation of the differences between quasispecies, balancing practical significance with statistical considerations.

# 4 Discussion

Inference with viral quasispecies diversity faces several challenges:

- High sequencing depth is required to adequately capture the complexity of these viral populations.
- Due to the scarcity of clinical samples, and the costs associated with the full NGS lab process, typically only a single experimental sample is taken from a given biospecimen. This sample is then amplified and subjected to NGS to obtain a representation of the quasispecies.
- Small technical inconsistencies can lead to dissimilar coverages between the quasispecies to be compared.
- Several diversity indices and maturity indicators are sensitive to sample size differences, necessitating prior normalization of the samples to be compared.

Given these circumstances, the study of clinical quasispecies samples often requires the comparison of two single experimental samples, except when dealing with patient cohorts. In the later case, various traditional statistical methods are available for inferences between two or multiple groups. However, this is not applicable when comparing single samples in a longitudinal study, for instance.

Each quasispecies sample is represented by a set of NGS reads, which in a simplified form consist of a list of haplotypes and their corresponding read counts. With this sample structure, resampling methods are the most appropriated statistic tools for inference.

We proposed a random delete-d jackknife scheme, resampling a high number of times, and normalizing unbalances sampled by rarefaction.

In this study we observed further challenges, including:

1. The required depth leads to very large sample sizes, with very high statistical power, resulting in very low p-values for small differences that may have little biological or clinical significance.

2. Although less affected by sample size than the p-values, effect sizes can also be inflated.

These challenges were mitigated by not relying solely on p-values, but instead using a set of metrics with varying susceptibilities to large sample sizes:

- Observed differences and ratios (Minimally susceptible, clinically relevant)
- Cohen's d effect size (Moderately susceptible, clinically relevant)
- z-test (Highly susceptible, statistically relevant)
- 95% Confidence Intervals (Highly susceptible, statistically relevant)
- Multitest adjusted p-values (Highly susceptible, statistically relevant)

Despite very large NGS sample sizes, typically only a single experimental sample from each quasispecies is analyzed. However, these samples are subject to stochastic variations and procedural inconsistencies, meaning that sample replicates will result in values that differ to some extent. The more complex the quasispecies population, the higher the variability in sampling is expected . For critical analysis, the use of experimental replicates is strongly advised to ensure more reliable and representative results.

Short-term variations are not expected to be of biological, clinical, or practical importance except in the context of emerging variants resistant to current treatments. In these cases the study of reduced susceptibility mutations acquire precedence over quasispecies structural changes.

## 5 Acknowledgements

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# 6 Appendix A

## 6.1 R code

```
library(Biostrings)
library(tidyverse)
library(stringr)
library(gridExtra)
library(kableExtra)

# Fasta files
ntDir <- './trim'
flnms <- list.files(path=ntDir,patt='\\.fna')

# Data dictionary
parts <- t(sapply(flnms,function(str) strsplit(str,split='\\.')[[1]]))
parts <- parts[,c(1,3,4,5)]
colnames(parts) <- c('Basal','Pass','Ampl','Str')
dict <- data.frame(parts) %>%
  rownames_to_column(var='FlNm') %>%
  mutate(Str=substr(Str,3,4)) %>%
  mutate(ID=paste('p',as.integer(substring(Basal,2))+
                      as.integer(substring(Pass,2)),sep='')) %>%
  relocate(ID) %>%
     filter(Ampl=='A2' & Pass %in% c('p0','p3','p10')) %>%
  slice(c(1:8,11,12))
snms <- dict$ID


#-----------------------------------------------------------------------#

# Sample coverage
cov.dt <- data.frame(ID=dict$ID,Ampl=dict$Ampl,Str=dict$Str,Reads=0,nHpl=0)
trds <- integer(nrow(dict))
for(i in 1:nrow(dict))
{
  seqs <- readDNAStringSet(file.path(ntDir,dict$FlNm[i]))
  parts <- t(sapply(names(seqs),function(str) strsplit(str,split='\\|')[[1]]))
  rds <- as.integer(parts[,2])
  trds[i] <- sum(rds)
  cov.dt$Reads[i] <- sum(rds)
  cov.dt$nHpl[i] <- length(rds)
}
dict$Reads <- cov.dt$Reads
dict$nHpl <- cov.dt$nHpl

cov.dt %>%
    select(-c(Ampl,nHpl)) %>%
    pivot_wider(names_from=Str,values_from=Reads) %>%
    data.frame() %>%
 mutate(ID=factor(ID,levels=c('p0','p3','p100','p110','p200'))) %>%
```

```r
 arrange(ID) %>%
 kable(caption='Reads per amplicon sample and strand') %>%
 kable_styling(latex_options = "hold_position")

# FW strand only
dict <- dict %>%
  filter(Str=='FW') %>%
  select(-c(Ampl,Str,Basal,Pass))

#-----------------------------------------------------------------------#

###  Function computing selected diversity indices
div.vals <- function(nr)
{
   p <- nr/sum(nr)
   q_0 <- length(p)
   q_1 <- exp(-sum(p*log(p)))
   q_2 <- 1/sum(p^2)
   q_inf <- 1/max(p)
   RLE1 <- log10(q_1)/log10(q_0)
   RLE2 <- log10(q_2)/log10(q_0)
   RLEinf <- log10(q_inf)/log10(q_0)

   p <- sort(p,decreasing=TRUE)
   Master <- p[1]
   Rare1 <- sum(p[p<0.01])
   Rare2 <- sum(p[p<0.001])
   R5 <- 5*p[5]/sum(p[1:5])
   R10 <- 10*p[10]/sum(p[1:10])
   Top25 <- sum(p[1:25])
   return(c(q_0,Master,Top25,Rare1,Rare2,R5,
                                    R10,RLE1,RLE2,RLEinf))
}

cnms <- c('nHpl','Master','Top25','Rare1','Rare2',
          'R5','R10','RLE1','RLE2','RLEinf')

###  Limiting Models A and Z
A <- c(rep(1,2),rep(0,7))
names(A) <- cnms[-1]
Z <- c(rep(0,2),rep(1,7))
names(Z) <- cnms[-1]

### Normalizing constant for maturity indicator
dAZ <- sqrt(sum((Z-A)^2))

### Distance to A
d2A.fn <- function(x)
{ x <- x[,names(A)]
  sqrt(sum((x[1,]-A)^2))
}
```

```r
#-----------------------------------------------------------------------#

### Diversity values, as observed, without sample size normalization
raw.vals <- matrix(nrow=nrow(dict),ncol=length(cnms))
colnames(raw.vals) <- cnms

for(i in 1:nrow(dict))
{
  seqs <- readDNAStringSet(file.path(ntDir,dict$FlNm[i]))
  parts <- t(sapply(names(seqs),function(str) strsplit(str,split='\\|')[[1]]))
  rds <- as.integer(parts[,2])
  raw.vals[i,] <- div.vals(rds)
}
raw.vals <- data.frame(ID=dict$ID,raw.vals)
raw.d2A <- sapply(1:nrow(raw.vals),function(i) d2A.fn(raw.vals[i,]))
raw.dN <- raw.d2A/dAZ

id.lvs <- c("p0","p3","p100","p110","p200")
raw.vals <- raw.vals %>%
     mutate(ID=factor(ID,levels=id.lvs)) %>%
  mutate(d2A=raw.d2A,dN=raw.dN) %>%
  arrange(ID)

#-----------------------------------------------------------------------#

B <- 500  # Rarefaction resampling cycles

### Maturity indicator values on rarefied quasispecies

###  Rarefaction
###  B submostrejos aleatoris a 'size' reads, sense reposició,
###     i amb càlcul de diversitat
###
###     nr: vector amb nombre de reads per haplotip
###   size: mida de la submostra
###      B: nombre de rèpliques
### fn.div: funció de càlcul de diversitat a partir del vector de
###         reads per haplotip d'una submostra
###
###  B subsamples of 'size' reads, without replacement
###    with divrsity computation each time
###  nr: vector of reads per haplotype
subsample.no.rep <- function(nr,size,B,div.fn)
{ # Mapping of reads to haplotypes
  iHpl <- rep(1:length(nr),times=nr)
  # Sample size
  tnr <- sum(nr)

  # Get subsample and compute diversities
  one_time <- function()
```

```
  { # subsample reads
    s <- dqsample.int(tnr,size,replace=FALSE)
    # get haplotype indices
    vh <- iHpl[s]
    # vector of haplotype counts
    bs.rds <- as.integer(table(vh))
    # Compute diversity
    return(div.fn(bs.rds))
  }
  # Replicate B times
  subs.vals <- replicate(B, one_time(), simplify = "array")
  return(subs.vals)
}

if(file.exists('QsTests_Raref_QSstr.RData'))
{ load('QsTests_Raref_QSstr.RData')
} else {
  dqset.seed(120720224)

  rvals <- matrix(nrow=nrow(dict),ncol=length(cnms))
  colnames(rvals) <- cnms

  ###  Raref to 120,000
  ref_size <- 120000

  for(i in 1:nrow(dict))
  {
    seqs <- readDNAStringSet(file.path(ntDir,dict$FlNm[i]))
    parts <- t(sapply(names(seqs),
                      function(str) strsplit(str,split='\\|')[[1]]))
    rds <- as.integer(parts[,2])

    sbv <- subsample.no.rep(rds,ref_size,B,div.vals)
    rvals[i,] <- apply(sbv,1,median)
  }
  suppl.raref.vals <- data.frame(dict[,c('ID','Basal','Pass')],
                                 rvals)
  save(suppl.raref.vals,file='QsTests_Raref_QSstr.RData')
}

raref.d2A <- sapply(1:nrow(suppl.raref.vals),
                    function(i) d2A.fn(suppl.raref.vals[i,]))
raref.dN <- raref.d2A/dAZ

suppl.raref.vals <- suppl.raref.vals %>%
     mutate(ID=factor(ID,levels=id.lvs)) %>%
  mutate(dN=raref.dN) %>%
  arrange(ID)

#-----------------------------------------------------------------------#
```

```r
## Diversity values to be tested
div.nms <- c('nHpl',
             'Top25','Master','Rare1','Rare2',
             'R5','R10',
             'RLE1','RLE2','RLEinf',
             'dN')

###  Function computing selected diversity indices
diversity <- function(nr)
{
   p <- nr/sum(nr)
   q_0 <- nHpl <- length(p)       # Hill number for q=0
   q_1 <- exp(-sum(p*log(p)))     # Hill number for q=1
   q_2 <- 1/sum(p^2)              # Hill number for q=2
   q_inf <- 1/max(p)              # Hill number for q=Infinity
   RLE1 <- log10(q_1)/log10(q_0)      # Rel. log. evennes for q=1
   RLE2 <- log10(q_2)/log10(q_0)      # Rel. log. evennes for q=2
   RLEinf <- log10(q_inf)/log10(q_0)  # Rel. log. evennes for q=Infinity

   p <- sort(p,decreasing=TRUE)  # Frequencies in decreasing order
   Master <- p[1]                # Master frequency
   Top25 <- sum(p[1:25])         # Top 25 haplotypes, reads fraction
   Rare1 <- sum(p[p<0.01])       # Fraction of reads for all Hpl <1%
   Rare2 <- sum(p[p<0.001])      # Fraction of reads for all Hpl <0.1%
   R5 <- p[5]/mean(p[1:5])       # Evenness in top 5 haplotypes
   R10 <- p[10]/mean(p[1:10])    # Evenness in top 10 haplotypes

   vdiv <- c(round(nHpl,1),Top25,Master,Rare1,Rare2,R5,R10,RLE1,RLE2,RLEinf)
   names(vdiv) <- c('nHpl','Top25','Master','Rare1','Rare2','R5','R10',
                    'RLE1','RLE2','RLEinf')
   dN <- sqrt(sum((vdiv[names(A)]-A)^2))/dAZ
   vdiv <- c(vdiv,dN=dN)
   return(vdiv)
}

#-----------------------------------------------------------------------#

## Random delete-d Jackknife
##          to estimate variance of diversity indices

### Fraction to delete
Bfr <- 0.1

# Function to perform counts rarefaction
rarefy_counts <- function(counts, size)
{
  raref <- sample(rep(seq_along(counts), counts),
                  size, replace = FALSE )
  tb <- tabulate(raref)
  return(tb[tb!=0]) # purge hpl with 0 counts
}
```

```r
# Random delete-d jackknife
RandomJackknife <- function(counts, target_size, Bfr=0.1,
                                  R=100, alpha=0.05)
{
  # Rarefy counts to target_size
  n <- total_reads <- sum(counts)
  if(total_reads > target_size*1.1)
  { counts <- rarefy_counts(counts, target_size)
    total_reads <- sum(counts)
  }
  # Number of reads to remove per cycle
  d <- k_size <- ceiling(total_reads * Bfr)

  ### Reads labelled per haplotype index
  qs.rds <- rep(seq_along(counts), counts)

  ### Random d-jackknife cycles
  jack_values <- matrix(0,nrow=R,ncol=length(div.nms))
  colnames(jack_values) <- div.nms
  for (i in 1:R) {
    # Reads to remove
    idx <- sample(1:length(qs.rds),k_size)

    # Counts after removal
    jack_counts <- tabulate(qs.rds[-idx])
    jack_counts <- jack_counts[jack_counts>0]
    jack_values[i,] <- diversity(jack_counts)
  }

  # Compute statistics
  jack_mean <- apply(jack_values,2,mean)
  jack_se <- sapply(1:ncol(jack_values), function(j)
               sqrt((n-d)/(d*R)*sum((jack_values[,j]-jack_mean[j])^2)))

  # 95% confidence interval
  ci_lower <- jack_mean - qnorm(1-alpha/2) * jack_se
  ci_upper <- jack_mean + qnorm(1-alpha/2) * jack_se

  stats<- data.frame(Feat=names(jack_mean),
                     Vals=jack_mean,
                     SE=jack_se,
                     SD=jack_se*sqrt(R),
                     CI_lower=ci_lower,
                     CI_upper=ci_upper)
  return(list(stats = stats, values = jack_values))
}

#-----------------------------------------------------------------------#

# Quasispecies pairs to be contrasted
```

```r
pairs <- matrix(c(2,1,3,1,4,3,5,3),nrow=4,byrow=TRUE)
pairs.ID <- data.frame(B=dict$ID[pairs[,2]],A=dict$ID[pairs[,1]]) %>%
  mutate(Test=paste(A,B,sep=' - '))

#-----------------------------------------------------------------------#

### Test p0 vs p3 (Test 1)

iTest <- 1
tcv <- dict[pairs[iTest,],] %>% select(ID,Reads)
rownames(tcv) <- NULL
tcv %>%
  kable(caption='Quasispecies coverage') %>%
  kable_styling(latex_options = "hold_position")

set.seed(9876)

### Load quasispecies 1
idx1 <- pairs[iTest,2]
ID1 <- dict$ID[idx1]
fasta1 <- dict$FlNm[idx1]
seqs <- readDNAStringSet(file.path(ntDir,fasta1))
parts <- t(sapply(names(seqs),function(str) strsplit(str,split='\\|')[[1]]))
rds1 <- sort(as.integer(parts[,2]),decreasing=TRUE)
n1 <- sum(rds1)

### Load quasispecies 2
idx2 <- pairs[iTest,1]
ID2 <- dict$ID[idx2]
fasta2 <- dict$FlNm[idx2]
seqs <- readDNAStringSet(file.path(ntDir,fasta2))
parts <- t(sapply(names(seqs),function(str) strsplit(str,split='\\|')[[1]]))
rds2 <- sort(as.integer(parts[,2]),decreasing=TRUE)
n2 <- sum(rds2)

### Jackknife quasispecies 1
jack.1 <- RandomJackknife(rds1, min(n1,n2), Bfr=0.1, R=1000,
                          alpha=0.05)

### Jackknife quasispecies 2
jack.2 <- RandomJackknife(rds2, min(n1,n2), Bfr=0.1, R=1000,
                          alpha=0.05)

#-----------------------------------------------------------------------#

### Normality tests

WithInSD <- function(data)
{
  v <- colnames(data)
  nn <- length(v)
```

```r
  P.lst <- vector('list',length(v))
  vlns <- data.frame(x=c(-2,0,2))
  within.sd <- data.frame(w1SD=numeric(nn),w2SD=numeric(nn),
                          w2.5SD=numeric(nn),w3SD=numeric(nn),
                          KS.pval=numeric(nn),
                          SW.pval=numeric(nn))
  for(i in seq_along(v))
  { data <- jack.1$values[,v[i]]
    mean_data <- mean(data)
    sd_data <- sd(data)
    within.sd$w1SD[i] <- sum(abs(data - mean_data) <= sd_data) / length(data)
    within.sd$w2SD[i] <- sum(abs(data - mean_data) <= 2*sd_data) / length(data)
    within.sd$w2.5SD[i] <- sum(abs(data - mean_data) <= 2.5*sd_data) / length(data)
    within.sd$w3SD[i] <- sum(abs(data - mean_data) <= 3*sd_data) / length(data)
     within.sd$SW.pval[i] <- shapiro.test(data)$p.value
     data <- unique(data)
     within.sd$KS.pval[i] <- ks.test(data, "pnorm",mean=mean(data),
                                     sd=sd(data))$p.value
  }
  # p-values for random normal data
  ndt <- rnorm(1000)
  norm.sw.pv <- shapiro.test(ndt)$p.value
  norm.ks.pv <- ks.test(ndt,"pnorm",mean=mean(ndt),
                                     sd=sd(ndt))$p.value
  ndt <- rbind(data.frame(w1SD=0.683,w2SD=0.954,
                          w2.5SD=0.988,w3SD=0.997,
                          KS.pval=norm.ks.pv,
                          SW.pval=norm.sw.pv),
               within.sd)
  rownames(ndt) <- c('Normality',v)
  ndt
}

### Print tests of normality in quasispecies 1 jackknife values
WithInSD(jack.1$values) %>%
  kable(digits=3)

### Print tests of normality in quasispecies 2 jackknife values
WithInSD(jack.2$values) %>%
  kable(digits=3)

#-----------------------------------------------------------------------#

### Z-test after jackknife
jack_test <- function(ID1,jack.1,ID2,jack.2)
{

  ### Difference
  D <- jack.2$stats$Vals - jack.1$stats$Vals
  ### Ratio
  Ratio <- jack.2$stats$Vals / jack.1$stats$Vals
```

```r
  ### Standard error of the difference
  SE.D <- sqrt(jack.1$stats$SE^2 + jack.2$stats$SE^2)
  ### 95% CI
  alpha <- 0.05
  D_CI_lower <- D - qnorm(1-alpha/2) * SE.D
  D_CI_upper <- D + qnorm(1-alpha/2) * SE.D
  ### Z
  Z <- D/SE.D
  ### p-value
  p.vals <- pnorm(-(abs(Z)))*2
  ### BH multitest adjusted p-values
  adj.p.vals <- p.adjust(p.vals, method = "BH")
  ### Sp
  Sp <- sqrt((jack.1$stats$SD^2 + jack.2$stats$SD^2)/2)
  ### Effect size
  d <- D/Sp
  Diff.test <- data.frame(Feat=rownames(jack.1$stats),
              V1=jack.1$stats$Vals,
              V2=jack.2$stats$Vals,
              Ratio=Ratio,
              Dif=D,
              CI.low=D_CI_lower,
              CI.upp=D_CI_upper,
              z=Z,
              adj.p.val = adj.p.vals,
              d=d,
              PS=round(pnorm(abs(d)/sqrt(2))*100,1))
  colnames(Diff.test)[2:3] <- c(ID1,ID2)
  Diff.test
}

Diff.test <- jack_test(ID1,jack.1,ID2,jack.2)

### Print test results

Diff.test %>%
 filter(Feat!='nHpl') %>%
 mutate(adj.p.val=sprintf("%1.1e",adj.p.val),
        z=sprintf("%.1f",z),
        d=sprintf("%.2f",d)) %>%
 kable(digits=3,align='r')

### Plot test results

P <- Diff.test %>%
  filter(Feat!='nHpl') %>%
  select(Feat,Dif,CI.low,CI.upp) %>%
  mutate(Feat=factor(Feat,levels=div.nms[-1])) %>%
  ggplot() +
  geom_segment(aes(x=Feat,xend=Feat,y=CI.low,yend=CI.upp),
               arrow=arrow(angle=90,length=unit(0.2, "cm"),ends='both')) +
```

```
  geom_hline(aes(yintercept=0),col='navy') +
  geom_point(aes(x=Feat,y=Dif),size=2) +
  labs(title='Differences and 95% CIs ',x='',y='') +
  theme(axis.text.x = element_text(angle = 60, vjust = 1, hjust=1))

lm <- max(abs(Diff.test$D))
brdr <- data.frame(d=c(0.8,0.5,0.2,0,-0.2,-0.5,-0.8),
                   Effect=factor(c('High','Medium','Small','Null',
                                   'Small','Medium','High'))) %>%
  filter(abs(d) < lm)

Q <- Diff.test %>%
  filter(Feat!='nHpl') %>%
  mutate(Feat=factor(Feat,levels=div.nms[-1])) %>%
  ggplot() +
  geom_hline(aes(yintercept=d,col=Effect),lty=4,lwd=0.4,data=brdr) +
  geom_point(aes(x=Feat,y=d),size=2) +
  labs(x='',y='d',title="Cohen's effect size") +
  theme(axis.text.x = element_text(angle = 60, vjust = 1, hjust=1)) +
  theme(legend.position = 'none')

grid.arrange(P,Q,ncol=2)

#-----------------------------------------------------------------------#
```

## 6.2 Session info

R version 4.3.3 (2024-02-29 ucrt) Platform: x86_64-w64-mingw32/x64 (64-bit) Running under: Windows 11 x64 (build 22631)

Matrix products: default

locale: [1] LC_COLLATE=Catalan_Spain.utf8 LC_CTYPE=Catalan_Spain.utf8
[3] LC_MONETARY=Catalan_Spain.utf8 LC_NUMERIC=C
[5] LC_TIME=Catalan_Spain.utf8

time zone: Europe/Madrid tzcode source: internal

attached base packages: [1] stats4 stats graphics grDevices utils datasets methods
[8] base

other attached packages: [1] BSDA_1.2.2 lattice_0.22-6 dqrng_0.4.1
[4] kableExtra_1.4.0 gridExtra_2.3 lubridate_1.9.4
[7] forcats_1.0.0 stringr_1.5.1 dplyr_1.1.4
[10] purrr_1.0.2 readr_2.1.5 tidyr_1.3.1
[13] tibble_3.2.1 ggplot2_3.5.1 tidyverse_2.0.0
[16] Biostrings_2.70.3 GenomeInfoDb_1.38.8 XVector_0.42.0
[19] IRanges_2.36.0 S4Vectors_0.40.2 BiocGenerics_0.48.1

loaded via a namespace (and not attached): [1] gtable_0.3.6 xfun_0.49 tzdb_0.4.0
[4] vctrs_0.6.5 tools_4.3.3 bitops_1.0-9
[7] generics_0.1.3 proxy_0.4-27 fansi_1.0.6
[10] pkgconfig_2.0.3 lifecycle_1.0.4 GenomeInfoDbData_1.2.11 [13] compiler_4.3.3 farver_2.1.2 munsell_0.5.1

[16] tinytex_0.54 class_7.3-22 htmltools_0.5.8.1
[19] RCurl_1.98-1.16 yaml_2.3.10 pillar_1.9.0
[22] crayon_1.5.3 tidyselect_1.2.1 digest_0.6.37
[25] stringi_1.8.4 bookdown_0.41 labeling_0.4.3
[28] fastmap_1.2.0 grid_4.3.3 colorspace_2.1-1
[31] cli_3.6.3 magrittr_2.0.3 utf8_1.2.4
[34] e1071_1.7-16 withr_3.0.2 scales_1.3.0
[37] timechange_0.3.0 rmarkdown_2.29 hms_1.1.3
[40] evaluate_1.0.1 knitr_1.49 viridisLite_0.4.2
[43] rlang_1.1.4 Rcpp_1.0.13-1 glue_1.8.0
[46] xml2_1.3.6 svglite_2.1.3 rstudioapi_0.17.1
[49] R6_2.5.1 systemfonts_1.1.0 zlibbioc_1.48.2