



Calculating complexity of large randomized libraries

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ABSTRACT

Randomized libraries are increasingly popular in protein engineering and other biomedical research fields. Statistics of the libraries are useful to guide and evaluate randomized library construction. Previous works only give the mean of the number of unique sequences in the library, and they can only handle equal molar ratio of the four nucleotides at a small number of mutation sites. We derive formulas to calculate the mean and variance of the number of unique sequences in libraries generated by cassette mutagenesis with mixtures of arbitrary nucleotide ratios. Computer program was developed which utilizes arbitrary numerical precision software package to calculate the statistics of large libraries. The statistics of library with mutations in more than 20 amino acids can be calculated easily. Results show that the nucleotide ratios have significant effects on these statistics. The more skewed the ratio, the larger the library size is needed to obtain the same expected number of unique sequences. The program is freely available at http://graphics.med.yale.edu/cgi-bin/lib_comp.pl.

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1. Introduction

Randomized libraries are widely used to select novel proteins with specific biological and physiochemical properties. There are many protocols to create randomized libraries (Neylon, 2004), such as cassette mutagenesis and error-prone PCR (epPCR). In the cassette mutagenesis, random mutagenesis is generated in a particular region or regions of the target gene through incorporation of degenerate synthetic DNA sequence. Usually equal molar nucleotides are used (1 : 1 : 1 : 1 ratio of nucleotides), but on other occasions, different molar ratios of nucleotides in the mixtures are used to create predetermined physiochemical properties in the targeted region(s). For example, in the study of selection and characterization of small transmembrane proteins that bind and activate the platelet-derived growth factor β (PDGF β) receptor, 15 transmembrane amino acids of E5 protein of bovine papilloma-virus (BPV) were replaced with random sequences (Freeman-Cook et al., 2004). The following library design was used to mimic the hydrophobic transmembrane region of BPV E5 protein:

$$\dots (\text{NXR})_3 \text{CAA} (\text{NXR})_{12} \dots, \quad (1)$$

where the three NXR codons are followed by a CAA codon encoding glutamine, which in turn is followed by 12 more NXR codons. For the NXR codons, N stands for an equal mix of A, C, G and T, X is a 5 : 0.1 mixture of T : C, and R is an equal mix of A and G.

In order to guide and evaluate randomized library construction, the statistical properties of the libraries will be useful. One of the most asked questions is the number of unique sequences in the library (the complexity of the library). In the following we are going to give some formulas to calculate the expected number of unique sequences in the library as well as its variance. Our treatment is different from the previous works in several ways (Bosley and Ostermeier, 2005; Firth and Patrick, 2008). Firstly, the previous works deal with only mixtures of equal molar ratio of the four nucleotides, while we can handle mixtures of arbitrary user-defined molar ratios, which is more useful in such situations as described above. As shown in the examples below, the different molar ratios in the nucleotide mixtures make a significant difference in the statistics of the library. Secondly, we present a formula for the variance, and hence the standard deviation, of the number of unique sequences in the library. The standard deviation gives an indication of the spread of the distribution around the expected value. Thirdly, by using a mathematical software library that can handle arbitrary numerical precision, we can calculate the statistics for much larger libraries. The statistics of library with mutations in more than 20 amino acids can be calculated easily. The program can be accessed freely in the web server at http://graphics.med.yale.edu/cgi-bin/lib_comp.pl.

The paper is organized as follows. In the *Theory* section we derive the formulas for the expected number of unique sequences in the randomized library and its variance. Within the assumption of the model, the formulas are exact. For real calculations of randomized libraries, however, these formulas have to be rearranged due to the huge number of possible sequences. In *Software implementation* section we discuss several ways to make the calculation manageable while keeping the numerical accuracy

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of the calculation. In the last section two examples are given for a small library and a relatively bigger library.

2. Theory

Assume that the size of the library (the number of transformants) is L and the total number of all possible sequences is n . Usually n is a huge number for a large randomized library. For example, if 60 nucleotide bases are mutated, the number of potential sequences is $n = 4^{60} \approx 1.3 \times 10^{36}$. We denote the probability of sequence s_i as p_i . For each sequence s_i among these possible sequences, we can associate a random variable $\mathbf{X}_i$, which is either 1 or 0, according as the sequence s_i is or is not in the library. The respective probabilities of $\mathbf{X}_i$ to take these two values are

$$\Pr\{\mathbf{X}_i = 0\} = (1 - p_i)^L = v_i, \quad (2a)$$

$$\Pr\{\mathbf{X}_i = 1\} = 1 - (1 - p_i)^L = u_i. \quad (2b)$$

The number of unique sequences in the library is given by the random variable $\mathbf{U}_n$:

$$\mathbf{U}_n = \mathbf{X}_1 + \mathbf{X}_2 + \cdots + \mathbf{X}_n = \sum_{i=1}^n \mathbf{X}_i.$$

The properties that we are interested in are the average (expectation) of $\mathbf{U}_n$ and its variance. The expectation gives the average of the number of distinct sequences in the library, and the variance shows the tightness of the distribution of the number of distinct sequences around its average. The average is given by the expectation of $\mathbf{U}_n$ as

$$\mathbf{E}(\mathbf{U}_n) = \sum_{i=1}^n \mathbf{E}(\mathbf{X}_i) \quad (3)$$

and its variance is given by

$$\sigma^2(\mathbf{U}_n) = \sum_{i=1}^n \sigma^2(\mathbf{X}_i) + 2 \sum_{i>j} \text{Cov}(\mathbf{X}_i, \mathbf{X}_j), \quad (4)$$

where $\text{Cov}(\mathbf{X}_i, \mathbf{X}_j)$ is the covariance of $\mathbf{X}_i$ and $\mathbf{X}_j$:

$$\begin{aligned} \text{Cov}(\mathbf{X}_i, \mathbf{X}_j) &= \mathbf{E}((\mathbf{X}_i - \mathbf{E}(\mathbf{X}_i))(\mathbf{X}_j - \mathbf{E}(\mathbf{X}_j))) \\ &= \mathbf{E}(\mathbf{X}_i \mathbf{X}_j) - \mathbf{E}(\mathbf{X}_i) \mathbf{E}(\mathbf{X}_j). \end{aligned}$$

From Eq. (2) we know that both $\mathbf{E}(\mathbf{X}_i)$ and $\mathbf{E}(\mathbf{X}_i^2)$ equal to u_i : $\mathbf{E}(\mathbf{X}_i) = 0 \cdot v_i + 1 \cdot u_i = u_i$, $\mathbf{E}(\mathbf{X}_i^2) = 0^2 \cdot v_i + 1^2 \cdot u_i = u_i$. Hence the variance of $\mathbf{X}_i$ is

$$\sigma^2(\mathbf{X}_i) = \mathbf{E}(\mathbf{X}_i^2) - \mathbf{E}(\mathbf{X}_i)^2 = u_i v_i.$$

To calculate $\sigma^2(\mathbf{U}_n)$ we need $\text{Cov}(\mathbf{X}_i, \mathbf{X}_j)$, which in turn depends on $\mathbf{E}(\mathbf{X}_i \mathbf{X}_j)$. The joint probability distribution of $\mathbf{X}_i$ and $\mathbf{X}_j$ are given by

$$\Pr\{\mathbf{X}_i = 0, \mathbf{X}_j = 0\} = (1 - p_i - p_j)^L = w_{ij},$$

$$\Pr\{\mathbf{X}_i = 0, \mathbf{X}_j = 1\} = (1 - p_i)^L - (1 - p_i - p_j)^L = v_i - w_{ij},$$

$$\Pr\{\mathbf{X}_i = 1, \mathbf{X}_j = 0\} = (1 - p_j)^L - (1 - p_i - p_j)^L = v_j - w_{ij},$$

$$\begin{aligned} \Pr\{\mathbf{X}_i = 1, \mathbf{X}_j = 1\} &= 1 - (1 - p_i)^L - (1 - p_j)^L \\ &\quad + (1 - p_i - p_j)^L = 1 - v_i - v_j + w_{ij}. \end{aligned}$$

From the joint probability the expectation of $\mathbf{X}_i \mathbf{X}_j$ can be obtained as

$$\begin{aligned} \mathbf{E}(\mathbf{X}_i \mathbf{X}_j) &= 1 - (1 - p_i)^L - (1 - p_j)^L + (1 - p_i - p_j)^L \\ &= 1 - v_i - v_j + w_{ij} \end{aligned}$$

from which we obtain the covariance of $\mathbf{X}_i$ and $\mathbf{X}_j$ as

$$\text{Cov}(\mathbf{X}_i, \mathbf{X}_j) = w_{ij} - v_i v_j.$$

Putting all the pieces together we have the average and variance of $\mathbf{U}_n$ as

$$\mathbf{E}(\mathbf{U}_n) = \sum_{i=1}^n u_i = \sum_{i=1}^n [1 - (1 - p_i)^L] \quad (5)$$

and

$$\begin{aligned} \sigma^2(\mathbf{U}_n) &= \sum_{i=1}^n u_i v_i + 2 \sum_{i>j} [w_{ij} - v_i v_j] \\ &= \sum_{i=1}^n v_i - \left[\sum_{i=1}^n v_i \right]^2 + 2 \sum_{i>j} w_{ij} \\ &= \sum_{i=1}^n (1 - p_i)^L - \left[\sum_{i=1}^n (1 - p_i)^L \right]^2 + 2 \sum_{i>j} (1 - p_i - p_j)^L. \end{aligned} \quad (6)$$

3. Software implementation

When the number of possible sequences n is small, the average and variance of the unique sequences in the library can be calculated directly using Eqs. (5) and (6). When n becomes large, however, a direct calculation is not feasible. If in one position along the sequence we have m possibilities (for nucleotide m usually is 4), and we have mutation in b such positions, a direct calculation would have $n = m^b$ possible values of p_i , which, as stated earlier, is too big to tackle directly. Not all these p_i , however, are unique. For a particular mixture ratio of x , we can calculate the probability q_i of each nucleotide in that position from the nucleotide ratio, which is just the fraction of each nucleotide at positions with mixture ratio x . For example, if the ratio is 1 : 2 : 3 : 4 for A, C, G and T, then q_i will take values of 1/10, 2/10, 3/10 and 4/10. All possible p_i are given in the following multinomial expansion

$$(q_1 + q_2 + \cdots + q_m)^b = \sum_{i_1, i_2, \dots, i_m} \binom{b}{i_1, i_2, \dots, i_m} q_1^{i_1} q_2^{i_2} \cdots q_m^{i_m}. \quad (7)$$

For each possible $p_i = q_1^{i_1} q_2^{i_2} \cdots q_m^{i_m}$, the number of such p_i is given by the multinomial coefficient,

$$c_i = c(i_1, i_2, \dots, i_m) = \binom{b}{i_1, i_2, \dots, i_m} = \frac{b!}{i_1! i_2! \cdots i_m!}.$$

The number of such unique probabilities, which equals the number of unique terms in the expansion of Eq. (7), is given by

$$h = \binom{b+m-1}{m-1} = \frac{(b+m-1)!}{b!(m-1)!}, \quad (8)$$

which is much smaller than m^b .

Hence the mean in Eq. (5) can be rewritten as

$$\mathbf{E}(\mathbf{U}_n) = \sum_{j=1}^h c_j u_j \quad (9)$$

and the variance in Eq. (6) as

$$\begin{aligned} \sigma^2(\mathbf{U}_n) &= \sum_{i=1}^h c_i v_i - \left[\sum_{i=1}^h c_i v_i \right]^2 \\ &\quad + 2 \left[\sum_{i=1}^h \frac{c_i(c_i-1)}{2} w_{ii} + \sum_{i>j} c_i c_j w_{ij} \right]. \end{aligned} \quad (10)$$

Furthermore, the number of the terms in Eqs. (9) and (10), h , can be reduced if there is degeneracy among q_i :

$$(q_1 + q_2 + \cdots + q_m)^b = (\alpha_1 q'_1 + \alpha_2 q'_2 + \cdots + \alpha_m q'_m)^b,$$

where we combine terms of q_i of the same value together (there are α_i of them): q'_i are just unique items among q_i . In such cases

the number of unique probabilities of p_i is given by

$$h' = \binom{b+m'-1}{m'-1} = \frac{(b+m'-1)!}{b!(m'-1)!}. \quad (11)$$

For example, if we want to mutate nucleotide acids in 100 positions with nucleotide ratio 1 : 2 : 1 : 2, then $b = 100$, $m = 4$ and $m' = 2$. The brute force calculation would have to add up $m^b = 1.6 \times 10^{60}$ terms. Eq. (8) gives $h = 176851$, while Eq. (11) gives $h' = 101$, a significant reduction.

The above statements apply to mutations with a single base composition, as in $XX \dots X = (X)_b$. They can, however, be generalized easily to handle multiple base compositions, as in $(X_1)_{b_1}(X_2)_{b_2} \dots (X_k)_{b_k}$. For example, the mutation in (1) can be

written as

$$N_{15}X_{15}R_{15}$$

for the computation purpose. In this general situation, the unique probabilities p_i and its associated coefficient c_i are given by the expansion of the product

$$\prod_{i=1}^k (q_{i1} + q_{i2} + \dots + q_{im_i})^{b_i} = \prod_{i=1}^k (\alpha_{i1}q'_{i1} + \alpha_{i2}q'_{i2} + \dots + \alpha_{im_i}q'_{im_i})^{b_i}. \quad (12)$$

A C program has been written that uses Eqs. (9) and (10) to calculate the average and variance of the number of unique

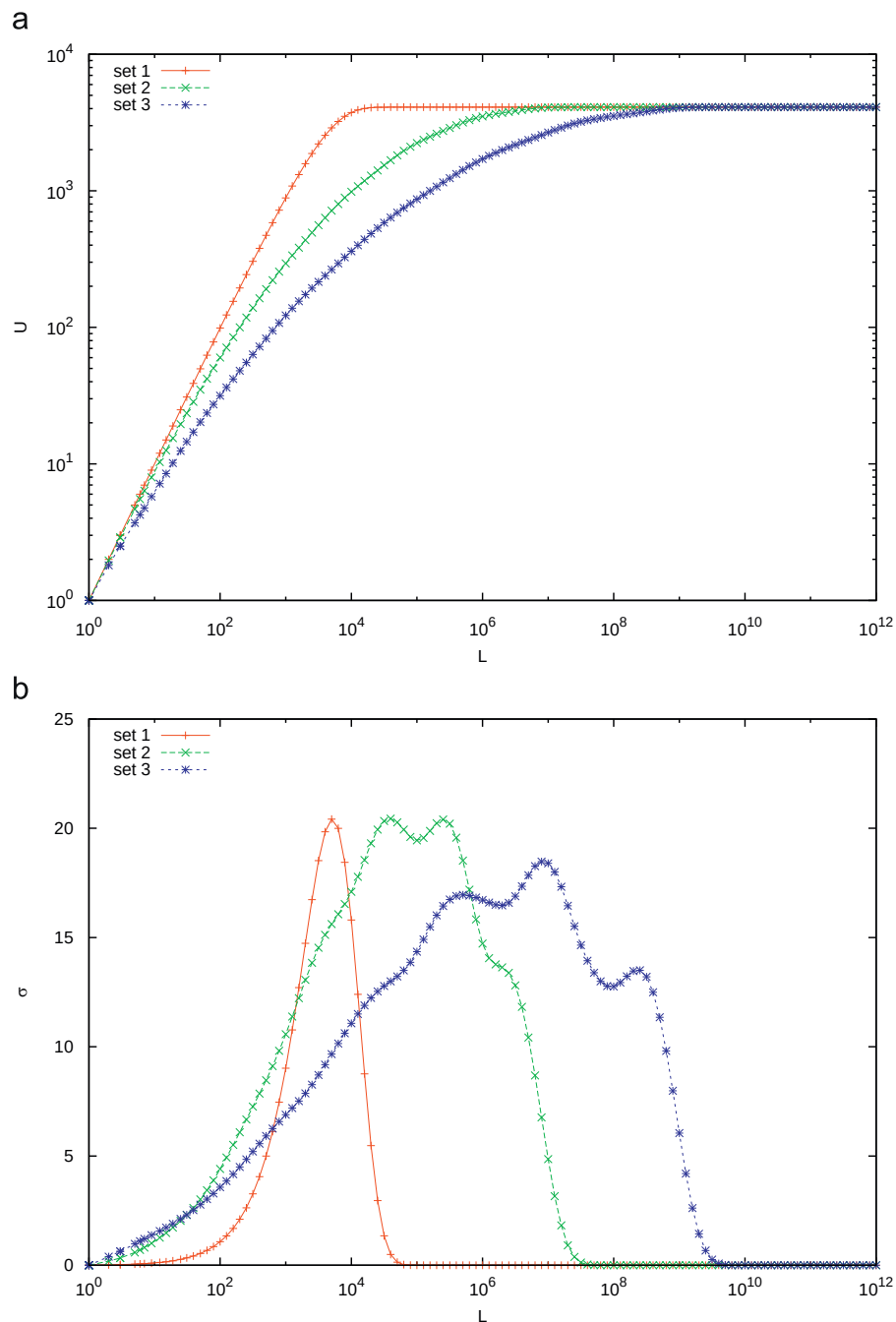


Fig. 1. The expectation and standard deviation (the square root of the variance) of the number of unique sequences in the library with mutations in two amino acids, for three different nucleotide mixture ratios. (a) The expectation of the number of unique sequences in the library. (b) The standard deviation.

sequences in randomized libraries, where c_i and p_i are from the expansion of Eq. (12). For small libraries, numerical accuracy is not a problem: standard programming languages are sufficient to give correct answers. For large libraries, careful attention has to be paid to the numerical stability, since there are many terms involved, and each term of probability p_i is very small, as p_i is the product of many q_j raised to high power. Standard programming languages like C usually cannot handle the situation well.

To overcome the issue of numerical accuracy, the program links the library of PARI/GP package (The PARI Group, 2008), which can do numerical calculations with arbitrary precisions. Furthermore, the package has the ability to do symbolic calcula-

tions, which makes some of the above mentioned calculations easier. A web server has been set up to access the program at http://graphics.med.yale.edu/cgi-bin/lib_comp.pl.

The program is simple to use. The user just type in the library size L and the nucleotide ratios of the mixtures, followed by the number of bases with that nucleotide ratio, separated by a space. Multiple ratios of the mixtures can be handled. If one or more of the nucleotides is not included at a position, simply exclude them in the input (although including them as zeros does not hurt: the program filters them out automatically). For example, to calculate the complexity for the library as described in Eq. (1) in the *Introduction* section, user input for the nucleotide ratios and base numbers is in the

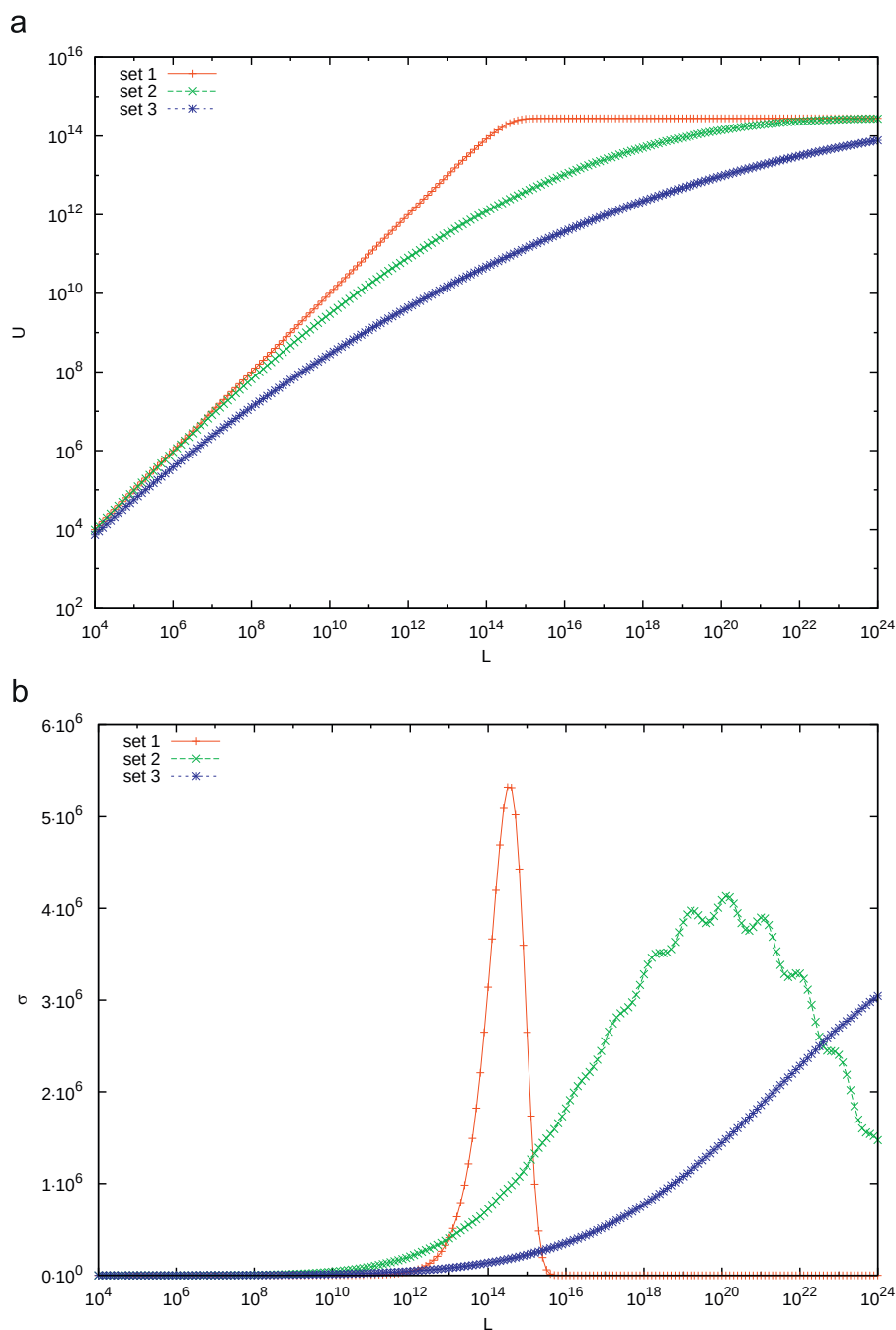


Fig. 2. The expectation and standard deviation (the square root of the variance) of the number of unique sequences in the library with mutations in eight amino acids, for three different nucleotide mixture ratios. (a) The expectation of the number of unique sequences in the library. (b) The standard deviation.

format of

$$1:1:1:1:1:15 \quad 5:0.1:15 \quad 1:1:1:15. \quad (13)$$

4. Examples

In this section two artificial examples are given to show the effects of different molar ratios of the nucleotide mixtures on the statistics of the library. The first example is a small randomized library with mutations on two amino acids, with different nucleotide mixture ratios for the first, second and third codon positions: $(X)_2(Y)_2(Z)_2$. Here we have $k = 3$ and $b_1 = b_2 = b_3 = 2$. The potential number of sequences is $m^b = 4^6 = 4096$. The second library is relatively larger, with mutations in eight amino acids: $(X)_8(Y)_8(Z)_8$. Here we have $k = 3$ and $b_1 = b_2 = b_3 = 8$. The potential number of sequences is $m^b = 4^{24} \approx 2.8 \times 10^{14}$. Three different sets of molar ratios are used for each library:

Set 1: equal ratio.

Set 2: $X = 1:1:1:1:8$, $Y = 1:1:1:1:9$, $Z = 1:1:1:1:10$.

Set 3: $X = 1:1:1:1:10$, $Y = 1:1:1:1:30$, $Z = 1:1:1:1:40$.

Set 3, though not very common in practice, is included to show the effects of nucleotide molar ratio on the library statistics.

The average and standard deviation (square root of the variance) of the number of unique sequences in the libraries are shown in Figs. 1 and 2 as a function of L , the size of the library (or the number of transformants). From these figures we can see that the nucleotide ratios of the mixtures have a significant impact on the statistics. As expected, for both small and large libraries, libraries with equimolar ratios achieve a larger number of unique library members U with the same library size L than libraries with unequal ratios. On average it requires more transformants for the libraries with unequal ratio mixtures to include the same number of unique sequences. For example, for the small library shown in Fig. 1, the library with equal ratio mixtures (Set 1) will have an average number of unique sequences 4096 (the theoretical limit) at $L \approx 10^4$, while the library with unequal ratio mixtures (Set 2) needs a larger library size $L \approx 10^7$ to get the same average number of unique sequences and the library with a more skewed ratio mixtures (Set 3) needs $L \approx 10^9$. However, the ability to calculate precise library statistics allows one to more accurately assess the disadvantages of reduced library complexity against the advantages of selection for particular amino acids (e.g.

hydrophobic amino acids) by using skewed nucleotide ratios. It should be mentioned that the nucleotide complexity considered here is not necessarily the same thing as amino acid complexity, due to the codon degeneracy.

The standard deviation also behaves differently according to the difference in mixture ratios. For equal ratio, the standard deviation has a sharp peak, centered around the point where the mean is about 70% of the saturation. For unequal ratios, however, the distributions of standard deviation is broad and multimodal, and the peaks shift to the higher L . For large libraries, the standard deviation is quite small when compared with the mean.

The number of peaks in the standard deviation depends on the number of distinct sequence probabilities p_i . In fact, if all n probabilities are distinct, there will be $n - 1$ peaks in the standard deviation. If these peaks from the left to the right are labeled from 1 to $n - 1$, then the peak i is associated with the fluctuation of the number of unique sequences in the transition from $U_n = i$ to $U_n = i + 1$ as L increases. When some or all sequence probabilities become degenerate, the peaks in the standard deviation will merge together. In the extreme case of equal ratios, all peaks merge into one peak and the standard deviation becomes single modal.

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