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Biomathematical Description of Peptide Library Properties

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ABSTRACT

Motivation: Libraries of randomised peptides displayed on phages or viral particles are essential tools in a wide spectrum of applications. However, there is only limited understanding of a library's fundamental dynamics and the influences of encoding schemes and sizes on their quality measured as peptide diversity.

Results: We are presenting a mathematical framework to derive the expected number of different peptides expressed in specified libraries. We also define coverage and relative efficiency, which allows researchers to describe libraries in enough detail to plan new experiments in a more informed manner. In particular, these values allow an answer to "What are the chances that a library contains one of the 'best' possible peptides?"

Availability: The framework is implemented in two freely available packages to the statistical software environment `R`: `discreteRV` and `peptider`. A graphical user interface implementing all aspects is made available at the PeLiCa website `http://www.pelica.org/`.

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Supplementary Information: At Bioinformatics online

1 INTRODUCTION

Since the year 2000, almost 500 publications per year have been published in which peptide libraries have been used (PubMed query November 2013 on *"peptide library"*), reflecting the importance of such libraries as tools for a wide spectrum of biological applications ranging from the identification of protein interaction sites (e.g. Rodi *et al.*, 1999) and the development of enzyme inhibitors (e.g. Lu *et al.*, 2012) to identification of peptides that mediate cell type specific gene delivery by viral vector systems (e.g. Müller *et al.*, 2003). For such studies, randomised oligonucleotides are introduced into plasmids encoding structural proteins of bacteriophages (Binder *et al.*, 2011) or viruses, such as adeno-associated viruses (Müller *et al.*, 2003), adenoviruses (Nishimoto *et al.*, 2012) or retroviruses (Bupp and Roth, 2003). These plasmids are ligated and transformed into bacteria to generate a plasmid library, which then is used to produce virus or phage libraries. These can be utilised in a variety of selection procedures, aiming to isolate peptide bearing viruses and phages with desired properties or scaffold independent, functional peptides (e.g. peptide inhibitors; Lu *et al.*, 2012). The success of this method is highly dependent on the diversity of the initial peptide pool, as the chance to identify the "best possible" sequence, or even a suitable sequence, directly correlates with the number and diversity of the peptides contained in the library used for the screening procedure.

However, determining the diversity of a library is problematic, as the number of distinct peptides cannot be measured easily. Even with the advent of widely accessible next-generation sequencing, the size of current libraries (e.g. 2×10^{10} clones; Deshayes *et al.*, 2002) makes the use of this technique generally impracticable due to time and financial effort necessary to reach the very high sequencing depth necessary to gain sufficient sequencing coverage. Therefore, several attempts to describe diversity of peptide libraries by other means have been made. DeGraaf *et al.* (1993) show the diversity of their phage decapeptide display library by estimating the distribution of single amino acids and dipeptides in a sample. While this is a useful way to show that a population is diverse, it does not quantify the diversity nor does this method give any information about the actual number of distinct peptides in the library.

Rodi *et al.* (2002) present another approach by defining *functional diversity* as a measure to describe the quality of a peptide library. The functional diversity reflects the distribution of the different peptides encoded in the library. If every peptide has the same frequency, the functional diversity is at its maximum (set to 1). With increasingly skewed distributions, this value drops towards zero. Functional diversity does not reflect the actual number of sequences in a library but describes diversity at a theoretical level based on specific peptide length and encoding scheme. *Clonal diversity* is another approach to describe diversity at the level of the plasmid library by counting successfully transformed bacterial colonies (e.g. Noren and Noren, 2001; Michelfelder *et al.*, 2009). This number is easily assessable, and represents the maximally achievable diversity for the phage/virus library, as the diversity cannot be increased after the cloning and transformation process. Particular precautions must be taken to avoid – or at least, to minimise – losses to diversity in subsequent steps of the library production to make the clonal diversity a valid qualifier for the peptide library. Clonal diversity on its own is of limited value, as the relevant metric is the diversity of the presented peptides (*peptide diversity*). However, both values are connected and clonal diversity can be used to determine peptide diversity if certain considerations are taken into account: peptide diversity of the library is always lower than clonal

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diversity, due to the possibility that different bacterial clones encode identical peptides. This is caused either by several clones containing identical peptide encoding DNA, or by clones harboring distinct DNA sequences that encode the same peptide. The reason for the latter possibility is the degenerate nature of the genetic code: amino acids can be encoded by up to six distinct codons, and therefore the same peptide can be described by multiple DNA sequences. This has the effect that, for instance, a pool of randomised seven codon DNA sequences has a nominal diversity of 64^7 (64 codons; 4.4×10^{12}) while it encodes only 23^7 (20 amino acids and three stop codons; 3.4×10^9) distinct amino acid sequences. Further, stop codons in the random nucleotide sequence prematurely terminate the peptide and can cause dysfunctional proteins in display systems (Lindner *et al.*, 2011; Michelfelder and Trepel, 2009).

Libraries can also be encoded by limited subsets of the standard 64 codons to at least partially counteract both effects (as also discussed in Patrick and Firth, 2005). Instead of the NNN scheme, where "N" represents any of the four bases, encoding schemes like NNB, NNK or NNS (B: C/G/T; K: G/T; S: G/C) can be used. These schemes encode all twenty amino acids and one stop codon each, while the total number of codons is reduced to 48 (NNB) and 32 (NNK and NNS), respectively. Another approach are trimer libraries (Kayushin *et al.*, 1996). Here, oligonucleotides are synthesised by assembling prefabricated trinucleotide phosphoramidites or trimers. This allows a ratio of one codon per amino acid for all amino acids and a complete avoidance of stop codons. It has been shown that such libraries possess increased functional diversity in phage display (Krumpe *et al.*, 2007).

Another important consideration regarding peptide diversity are cysteines. Pairs of cysteines flanking randomised sequences are often used in phage display as they form controlled disulfide bridges that enhance half-lives and binding characteristics of the library peptides (McConnell *et al.*, 1994). However, random integration of odd numbers of cysteines has repeatedly been shown to inhibit the generation of peptide bearing phages (reviewed by Fukunaga and Taki, 2012). Further, even though the situation is less well understood for other display systems, a strong underrepresentation of cysteine-containing peptides was observed in peptide libraries on different AAV vectors (Waterkamp *et al.*, 2006; Naumer *et al.*, 2012; Perabo *et al.*, 2006; Varadi *et al.*, 2012). This again suggests unfavorable effects of cysteine incorporation on basic functions of the display system. In line with this is the notable lack of capsid surface-exposed cysteine residues on wild type AAV2 (Xie *et al.*, 2002). Also, the surface of human Adenovirus type 5 is naturally devoid of cysteines. If they are artificially integrated, the particles were shown to be prone to aggregation due to the formation of interparticle disulfide bridges (Kreppel *et al.*, 2005).

With regard to the aforementioned factors, we will determine peptide diversity by using clonal diversity, but consider effects of encoding schemes and stop codons. For the purpose of discussing diversity, we will consider cysteine-containing peptides as non-functional. Diversity discussions considering cysteines are available at our website PeLiCa. Other biological restraints that negatively affect peptide diversity do exist, but are not taken into account here, as they are largely unknown and highly dependent on the individual system and its specific characteristics, such as the differences between distinct incorporation sites (Naumer *et al.*, 2012; Girod *et al.*, 1999). However, depending on the system and its intended use (e.g. generation of a functional viral vector with peptide mediated tropism), compatibility with such restrictions might be considered as a first step in the selection process.

Due to the sheer number of peptides possible in a library, determining the peptide diversity is a mathematically taxing problem that becomes ever more challenging with increasing peptide length. In this publication, we introduce a mathematical framework capable of facilitating this task. As the quality of a peptide library is not only defined by the peptide diversity, we further use the concepts of *coverage* and *efficiency* to allow a detailed evaluation of libraries. Further, we discuss effects of insert length, different encoding schemes (NNN, NNB, NNK, NNS, and trimer), and answer one of the important questions for researchers working with peptide libraries: "What are the chances that my library contains one of the 'best' possible peptides?"

In contrast to other methods describing library diversity, our framework allows a systematic analysis of the behavior of large peptide libraries, which in turn facilitates a deeper understanding and allows for a more informed planning of new, optimised libraries. To make the framework easily accessible, we generated a user-friendly web-interface called PeLiCa (available at `http://www.pelica.org`), which allows the user to determine all of these factors for libraries of sizes 10^6 to 10^{15} bacterial clones, using different encoding schemes (including custom-designed schemes and those that consider cysteine viable) and peptide lengths (6 to 10 aa).

2 MEASURING DIVERSITY

While not studied in detail for peptide libraries, studies on diversity at the amino acid level have been performed in the related field of site saturation mutagenesis generated protein libraries. Here, proteins are mutated at a limited number of positions to detect variants with improved properties. The program "GLUE-IT" (available at `http://guinevere.otago.ac.nz/stats.html`) generates values for diversity and coverage for protein libraries with up to six modified codons per protein (Firth and Patrick, 2008). Though this program was designed for another purpose and does not allow evaluation of cysteines as disruptive, it can also be used to gain some information for peptide libraries with short peptides. However, it is insufficient to describe most libraries currently used, which are generally longer and range from five up to twenty or more amino acids in length (e.g. Naumer *et al.*, 2012; Fukunaga and Taki, 2012; Scholle *et al.*, 2005).

In our approach to develop a mathematical framework for the characterization of peptide libraries we use three basic assumptions of randomness: (I) *the randomised oligonucleotides are in fact completely random (in a statistical sense).* (II) *the used pool of oligonucleotides can contain the same nucleotide sequence multiple times as its synthesis is completely random and* (III) *the technical processes that limit the number of DNA sequences in the libraries work at random, to the effect that each nucleotide sequence has an equal chance of getting selected into the library.*

We define and discuss three measures of library quality: *peptide diversity*, defined as the number of distinct peptides in a library, *expected coverage*, describing the fraction of all theoretically possible peptide sequences covered by the library, and *relative efficiency* given as the ratio of the expected number of distinct peptides in a library relative to the overall number of encoding

oligonucleotides. We investigate these measures for NNN, NNK, NNS, NNB and trimer encoding schemes. Trimer libraries can be constructed with any number of selected codons. In this publication, we regard them as composed of peptides assembled from 19 distinct codons (one per amino acid; excluding cysteine).

2.1 Libraries with equal codon representations

An easily tractable case for determining diversity is a setting in which all different sequences have the same probability of being included in the library. This can be assumed if diversity is investigated at DNA level or for the special case of trimer libraries in which every amino acid has the same number of codon representations. In that case, calculating expected peptide diversity of a library is relatively simple: the probability that a peptide is present in the library is determined by the maximum number of different peptide sequences and the size of the library. Denote the number of all different possible peptides in the library by b, the size, measured as clonal diversity, of the library by N.

The number of different peptides Z that can actually be achieved in the library is the primary point of interest. In practice, this will differ from library to library, but we can give an expected value $E[Z]$ describing this diversity.

THEOREM 1. *For a library of size n chosen from a scheme with b different peptides, the expected number of different peptides in the library is given as*

$$E[Z] \approx b(1 - e^{-N/b}). \tag{1}$$

The approximation becomes more accurate as values of b and $|\log(N/b)|$ increase. The proof and a more detailed discussion of the approximation error can be found in the supplementary material, parts A.1 and A.2.

In investigating DNA diversity in site saturation mutagenesis libraries, other groups (Patrick *et al.*, 2003; Bosley and Ostermeier, 2005) obtained the same result as Theorem 1 based on a Poisson approximation. While this approach is usable for an analysis at the DNA level or trimer libraries, it cannot be used directly for library schemes in which the number of codons per amino acid varies, because in this case, the probability that a peptide will be included in the library varies in dependence of its sequence. In a standard 64 codon based library there are one to six codons describing individual amino acids (aa). Therefore, some peptide sequences like `SLRLLRS` are encoded by $6^7 = 279936$ distinct codon sequences, as each amino acid in the sequence has six independent possibilities to be encoded. At the other end of the scale, there are peptides that are encoded by a single nucleotide sequence. We will therefore partition the overall library into classes of peptides that all have the same number of encodings (similar conceptual approaches have previously been mentioned, e.g. Firth and Patrick, 2008; Scott and Smith, 1990) and determine overall diversity based on diversity seen within each of these classes. For that, we need to specify the library under observation in more detail.

2.2 Partitioning of Peptide Libraries

To be able to determine the peptide diversity, we have to partition the libraries. In the following, we focus on the 32 codon-based encoding schemes NNK and NNS. All other schemes work similarly, an overview of the class partitioning is given in tables 1 and 2 of the supplementary material part B, According to their multiplicity and functionality NNK and NNS are equivalent, and we can distinguish four classes of aa based on a common NNK/S scheme (see Table 1). Amino acids are given in single letter code. Size s defines the number of different amino acids in an aa class, the number of codons, c, reflects how many codons describe each amino acid in the class. Classes A to C contain all codons for feasible amino acids, while class Z contains corruptive codons. The number of valid classes is therefore $v = 3$.

Table 1. NNK/S Library Scheme

aa class	amino acids	size s	# codons c
A	S, L, R	3	3
B	A, G, P, T, V	5	2
C	D, E, F, H, I, K, M, N, Q, W, Y	11	1
Z	cysteine C, stop `TAG`	2	1

As discussed earlier, stop codons as well as cysteines are treated as nonviable amino acids (aa class 'Z'); sequences containing one or more of these codons will therefore be excluded.

We are now employing a two-step analysis to retrieve all the relevant probabilistic information to calculate peptide diversity in the resulting library: (I) In a first step we are only interested in whether the outcome is a *valid sequence*, defined to be the case that there is no element of the newly defined aa class Z in the sequence. Valid sequences are therefore those that are expected to be functional in the biological system. (II) In a second step we will investigate the diversity among the remaining peptide sequences.

Any peptide sequence containing a member of aa class Z is by definition not useful for further analysis. In a randomly generated NNK/S library of heptapeptides, these make up 36.35% = $1 - (1 - P(Z))^7$ of the total. We will call this percentage of invalid sequences the *initial loss* and restrict our analysis to valid sequences only.

Analysing peptide sequences directly is too computationally complex of a problem. In order to reduce this complexity, we only differentiate between peptide sequences at the level of the previously introduced classes. If this is performed for an exemplary library of dipeptide sequences, we have a set of nine different peptide classes (peptides composed of aa classes A to C) corresponding to valid peptide sequences based on the classification of amino acids according to Table 1 (as shown in Table 2). Within each of these aa classes, all peptides have an equal number of oligonucleotide sequence representations. The total of all valid peptide sequences adds up to $19^2 = 361$ different peptide sequences of length two.

The peptide class completely determines both the number of unique peptides and the number of nucleotide representations for each of the peptide sequences. For a given sequence, let n_A, n_B, and n_C be the number of codons from A, B, and C (the sum of n_A, n_B, and n_C then adds up to the total length of the sequence). Here, s_A, s_B, and s_C represent the number of different amino acids

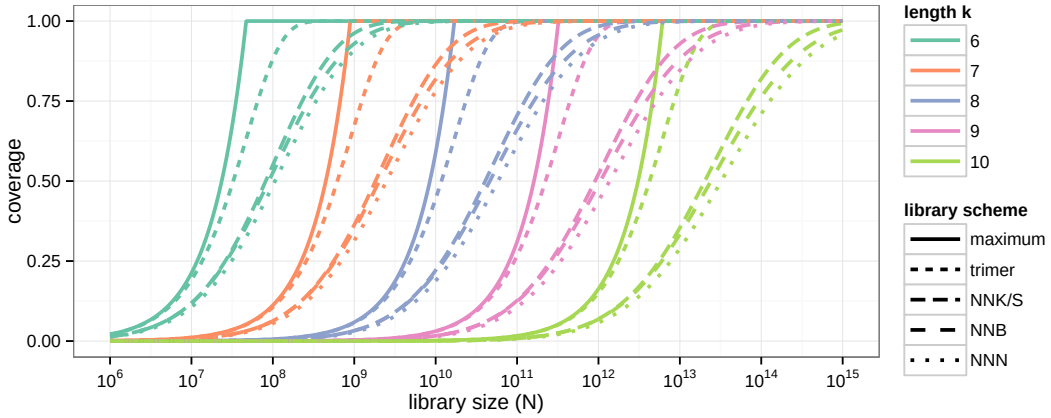

Fig. 1. Overview of expected coverage for k-peptide libraries of different sizes N with the different encoding schemes (NNN, NNB, NNK/S, and trimer). An additional line for maximum possible coverage is shown.

Table 2. Overview of all peptide sequences of length two partitioned according to peptide classes. The peptide class (first line) is defined by the aa class memberships of their codons as defined for NNK/S libraries in Table 1. The number of different unique peptide sequences in each class (second line), and the number of codon representations for each peptide sequence in the class (third line) are given.

peptide class	AA	AB	AC	BA	BB	BC	CA	CB	CC
#peptides	9	15	33	15	25	55	33	55	121
#nucleotides	9	6	3	6	4	2	3	2	1

in aa classes A, B, and C, and c_A, c_B, and c_C stand for the number of codons per amino acid within the corresponding aa class.

The number of peptides (#peptides) and corresponding nucleotide representations for each peptide (#oligonucleotides) is then calculated as

$$\begin{aligned} \text{\#peptides} &= {s_A}^{c_A} \cdot {s_B}^{n_B} \cdot {s_C}^{n_C} \\ \text{\#oligonucleotides} &= {c_A}^{n_A} \cdot {c_B}^{n_B} \cdot {c_C}^{n_C} \end{aligned}$$

The number of oligonucleotide sequences representing a whole peptide class is given as the product of the number of peptides and the number of individual codon representations per peptide. Under the assumption that in a library of peptides with a length of k amino acids all viable codons v (30 codons for NNK/S usage, excluding any class Z codons) are represented with the same probability, this allows us to calculate the probability p for a peptide class to be present in a library as

$$p = \#\text{ peptides} \cdot \#\text{ oligonucleotides}/v^k. \tag{2}$$

2.3 Diversity in general peptide libraries

Combining the information from individual peptide classes we can determine the diversity in the general peptide library.

For a k-peptide library of size N we expect Np_i sequences to be selected from peptide class i, where p_i is the probability (effectively, the size) of peptide class i. Within this class, all peptides have the same number of oligonucleotides. Assuming b_i different peptides in peptide-class i are theoretically possible, we have, according to theorem 1, a diversity given by the number of different peptides as $b_i(1 - e^{-Np_i/b_i})$, resulting in an overall number of different peptides in the NNK/S library of

$$D(N,k) = \sum_{i=1}^{v^k} b_i(1 - e^{-Np_i/b_i}). \tag{3}$$

Based on the overall peptide diversity, we now define two indices measuring different aspects of quality of k-peptide libraries: expected coverage and relative efficiency.

DEFINITION 1 (Expected coverage). *For a k-peptide library of size N the expected coverage is defined as*

$$C(N,k) = D(N,k)/19^k.$$

Expected coverage is an index in $[0,1]$. 0 indicates that no peptide is in the library (which can only happen for a library of size 0), and 1 indicates that every single possible peptide is included in the library; for this, the size of the library has to be at least $N \geq (19$ viable amino acids$)^k$.

Figure 1 shows the expected coverage of k-peptide libraries of sizes between 10^6 and 10^{15} with different encoding schemes. It is obvious that increasing peptide length k has a dramatic negative influence on coverage for a given library size N. Additionally, the used encoding scheme has a profound effect on coverage, with trimer libraries being far superior to the other schemes.

DEFINITION 2 (Relative efficiency). *Relative efficiency is defined as the ratio of expected peptide diversity of a library relative to its overall number of oligonucleotides:*

$$R(N,k) = D(N,k)/N.$$

This makes relative efficiency a number between 0 and 1.

Figure 2 gives an overview of relative efficiency of k-peptide libraries of various sizes. In contrast to ideal (maximum) or trimer libraries, libraries encoded by NNK/S, NNB and NNN schemes suffer from an initial loss due to sequences containing aa class Z codons. This limits their maximal relative efficiency depending on

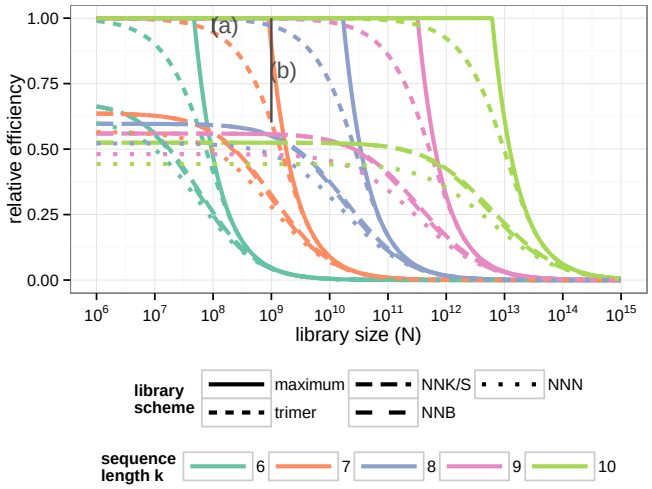

Fig. 2. Overview of relative efficiency for k-peptide libraries (6 to 10) of sizes N from 10^6 to 10^{15}. Relative efficiency decreases with an increased number of oligonucleotides.

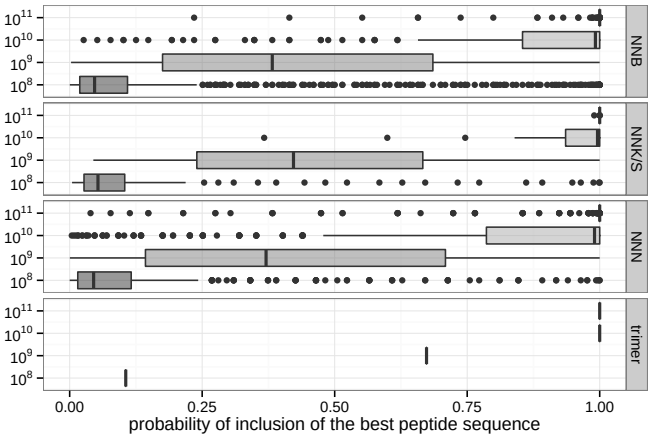

Fig. 3. Overview of the inclusion probabilities for any peptide sequence of length 7 in libraries of sizes $10^8, 10^9$, 10^{10}, and 10^{11} (left) for different encoding schemes (right).

encoding scheme and peptide length k. With increasing library size, relative efficiency decreases due to increasing effects of multiplicity. In an ideal case, this drop only occurs when the library size reaches the maximal possible diversity for the given peptide length k. In practice, however, this loss becomes notable when a library reaches a size of about 1% of the maximal number of possible peptides.

Current AAV library sizes are in the order of 10^8. Here, the loss due to multiplicity makes up for less than 10% in heptapeptide trimer libraries (see (a) in Figure 2). As peptide libraries increase, the problem grows exponentially. In heptapeptide libraries of size 10^9, the loss due to multiplicity (see (b) in Figure 2) is nearly as large as the initial loss (27.9% compared to 36.3%).

3 APPLICATIONS

Full coverage – especially with longer peptide sequences – might be very difficult to achieve in practice. However, as Yuval Nov describes for saturation mutagenesis in protein evolution (Nov, 2012), it might not always be reasonable to aim for full coverage to ensure that the one 'best' peptide is included in a library (what is 'best' is always defined by the goals of a specific library selection, e.g. to identify the peptide that shows the strongest interaction with a protein). The reasoning behind this is simple: one would expect that there are in fact several peptides which perform similarly well. This assumption is supported by the fact that even in selections using libraries with incomplete coverage, we often observe an enrichment of several sequences that share common sequence motifs (e.g. Naumer *et al.*, 2012; Michelfelder *et al.*, 2007, 2009). With this in mind, it might be more reasonable, instead, to raise the question: "What diversity is necessary to find *at least one of* the best possible peptides?" To answer this, we first estimate the probability that the single best sequence is part of the library (see 3.1), in a next step we assess the probability that any related sequence from an appropriately specified neighborhood around it is included (see 3.2).

3.1 Inclusion probabilities

The probability that a specific peptide sequence is present in a library depends on the overall size of the library and its scheme. Let p_i be the probability that peptide i is in the library, and $\sum_{i=1}^{t} p_i$ be the cumulative probability for the occurrence of any one of a group of t peptide sequences in the library. Define X to be the number of the specified t peptides that occur in a library of size N. The probability that at least one of the t peptides is in the library is then:

$$P(X \geq 1) = 1 - P(X = 0) =$$
$$= 1 - (1 - \sum_i p_i)^N \approx 1 - e^{-N \sum_i p_i}.$$

The approximation is based on the same argument as Theorem 1 and holds for any reasonably large values of N.

The probability p_i of a peptide sequence to occur in a library depends on the number of codons of each of its amino acids. This number varies between library schemes, making an exact *a priori* assessment of the inclusion probability of the 'best' peptide sequence impossible except in the case of trimer libraries, in which each peptide sequence occurs with equal probability. In all other library schemes, the probability of sequences to be included in the library is highly variable. Figure 3 gives an overview of just how much the probability of including the 'best' peptide sequence varies in each encoding scheme with different library sizes. Each of the boxes of the boxplots contain half of all possible 19^7 peptide sequences, with the other quarters on the left and right of the boxes, respectively. For example, in an NNK/S library of size 100 Million, 75% of all peptides have an inclusion probability of below 12.5%.

Compared to trimer libraries, the other library schemes introduce an enormous amount of variability into the inclusion probabilities. This is reflected in more variable results from experiments and makes the probability of peptide inclusion depend strongly on the actual peptide sequence, which *a priori* is unknown to the experimenter.

3.2 Neighborhoods

To determine if at least one of the best possible peptides (or a *top* peptide) is included in a given library, we have to define first what a *top* peptide is. For that we use a rather restrictive definition: a *top* peptide is any peptide that differs from the best possible peptide s in up to one (first degree neighborhood) or up to two (second degree neighborhood) amino acid positions which are conservatively exchanged. To objectively define conservative exchanges we employ the BLOSUM80 matrix (Henikoff and Henikoff, 1992), which provides log-odds scores for the chance to observe a substitution of one amino acid for another. Only exchanges with a positive BLOSUM80 score were considered in determining neighborhoods of top peptides. Further, exchanges to stop codons and cysteines were defined here to lead to invalid sequences. In general, a neighborhood of degree d includes all sequences that differ in at most d amino acids from peptide s. It is obvious, that a d-neighborhood of s includes s itself as well as all sequences of lower degree neighborhoods.

Neighborhoods and their sizes depend on the individual peptide sequence. Therefore, we cannot give a single inclusion probability, but we rather have to cite a range of probabilities for including top peptides. To set the boundaries of this range, we consider a best and a worst case scenario under all encoding schemes. In the worst case scenario, the top sequence consists of amino acids with only a single codon each (minimizing the probability to be part of the library) along with the smallest possible number of viable exchanges (minimizing the size of the top peptide neighborhood). Analogously, the top sequence in the best case scenario is one that consists of amino acids with a maximum number of codons in the encoding scheme (maximizing the probability to be found in the library) combined with the largest possible number of viable exchanges (maximizing the size of the top peptide neighborhood).

Figure 4 gives an overview of the minimum and maximum possible probabilities of including one of the sequences in the first and second degree neighborhoods of the best heptapeptide sequence. For an NNK/S library of size 100 Million, we have a minimum chance of about 12% (worst case scenario) that one of the sequences of the first degree neighborhood around the best sequence is included. This chance increases to over 60% to contain at least one sequence for the second degree neighborhood of the sequence.

For individual sequences we can be more specific, and calculate the probability of including any of its d degree neighbors (for $d = 1, 2$) based on the BLOSUM80 matrix, see supplement C.

4 DISCUSSION AND CONCLUSIONS

Peptide libraries are used in a variety of biological systems. For optimum exploitation of this powerful technology, evaluation parameters are needed to allow an in-depth description of the library properties and a more informed library design that can be adjusted to the requirements of the needed peptides and to the technical feasibility in a given experimental setting.

In this publication, we have provided a mathematical framework for an objective assessment of the quality of a wide variety of peptide libraries with different library sizes, encoding schemes (NNN, NNB, NNK, NNS, and trimer) and peptide lengths. In particular, it allows for the first time the mathematical description of peptide libraries of length > 6 aa, and defines peptide diversity, expected coverage, and relative efficiency as evaluation parameters. Further, the framework is suitable to describe properties of even very large current libraries (e.g. AAV libraries of 5×10^8 transformed clones; phage libraries of 2×10^{10} transformed clones; Deshayes *et al.*, 2002; Naumer *et al.*, 2012).

The core of our approach is to classify peptides according to the multiplicity of their encodings first, and then use these peptide classes to regard individual peptide sequences in a second step. This two-step procedure reduces the complexity of the problem sufficiently, making a mathematical assessment of complete libraries analytically feasible. The sheer size of peptide libraries causes alternative approaches to fail. Direct simulation, for instance, is impossible to implement on standard machines due to the limitations of main memory and disk space. Even if these hurdles were taken by more sophisticated simulation strategies, the process would be too slow to be of practical use.

In this publication, we limited our analysis to peptides of 6 to 10 amino acids in length. However, shorter or longer peptides can be investigated as well. Reasonably sized libraries of peptides shorter than 6 aa in length can be expected to show near complete coverage and to contain almost all possible peptides in the investigated encoding schemes. For example, a pentapeptide NNN library of size $N = 10$ Million has an estimated coverage of 98% and a peptide diversity of 3×10^6. When investigating peptide lengths of 11 amino acids or more, it can be assumed even for large current libraries (N up to 2×10^{10}) that the probability that two bacterial clones encode the same peptide is close to zero. Under these conditions, the expected coverage drops towards zero while relative efficiency stays close to the possible maximum defined by peptide length and encoding scheme (Figures 1 and 2). For such libraries, losses in efficiency are strongly dominated by the initial loss and a relative efficiency R, peptide diversity D, and expected coverage C can be approximately described as (defined in dfn 2, eqn 3, and dfn 1, respectively):

$$R(N,k) = \left(\frac{\text{\# of viable codons in the encoding scheme}}{\text{\# of all codons in the encoding scheme}} \right)^k$$

$$D(N,k) = R(N,k) \cdot N$$

$$C(N,k) = D(N,k)/19^k$$

For the experimenter, desirable library features are high peptide diversity as well as good coverage. To improve these factors, the size of the library can be increased. However, this possibility is often limited by technical restraints. Diversity and coverage are, however, also influenced by the used encoding scheme and peptide length. While short peptides (e.g. $k < 6$) reach high coverage with all encoding schemes, their restricted coding capacity limits the number of possible variants and thereby the achievable peptide diversity. If peptides of lengths between 6 and 10 amino acids are considered, the encoding scheme has an important influence. Trimer libraries are superior, as they avoid the initial loss and suffer less from redundancy effects caused by multiple bacterial clones encoding the same peptide. Further, they prevent the bias for preferentially including amino acids with a high number of codons. As trimer libraries are still rather expensive, the majority of currently used libraries are made based on other encoding schemes. When comparing these regarding coverage and relative

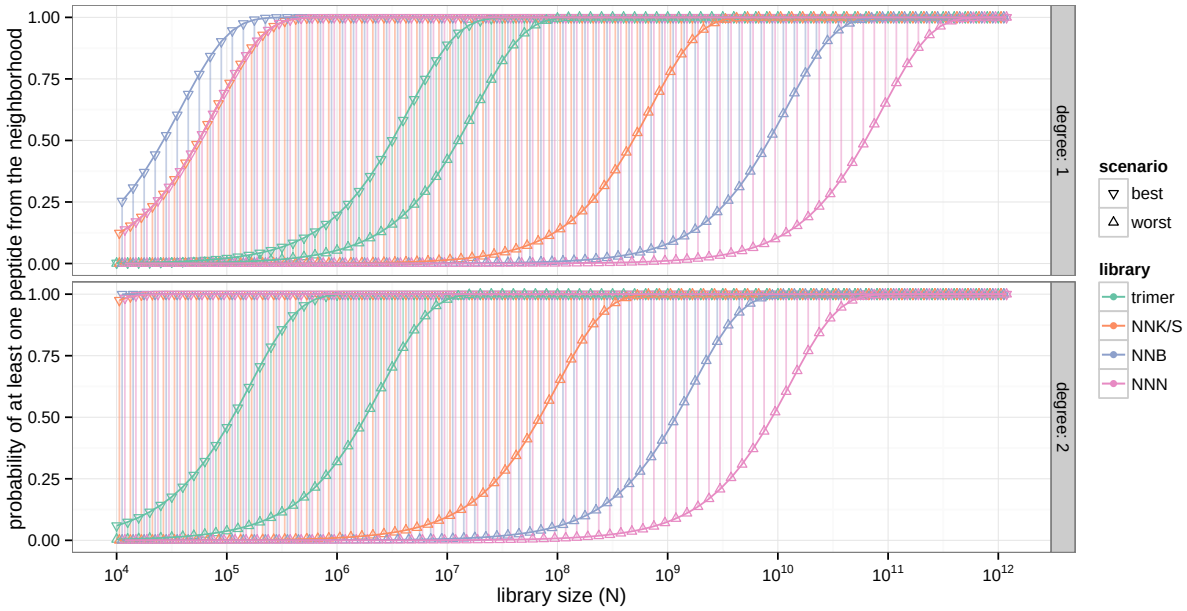

Fig. 4. Overview of the probability to include at least one of the sequences belonging to the first and second degree neighborhoods of the best heptapeptide. Best and worst case probabilities depend on the number of encodings for a sequence and the exchangeability of amino acids. In first degree neighborhoods the best case scenarios for NNK/S and NNN are identical.

efficiency, NNK/S and NNB are very similar and preferable to NNN (see Figures 1 and 2). NNK/S has a slight advantage over NNB in peptide diversity, expected coverage, and relative efficiency. However, if cysteines are considered as viable, NNB encoding has a minor advantage over NNK/S for libraries with a low coverage. At higher coverage, NNK/S encoding again is slightly better than NNB (data not shown). Between NNK and NNS, there is no statistical difference. However, biological considerations like codon preferences of the relevant host organism might cause a difference in a given experimental setting. In *E. coli* and especially in *S. cerevisiae*, codon usage suggests that NNK may generally be the better option (Patrick and Firth, 2005), while in human cells NNS codons are preferred. For all encoding schemes except trimer, there is a peptide length that maximises peptide diversity for a given library size N (see Figure 2). For example, libraries of size 10^8 have a maximum relative efficiency and peptide diversity if k is 8. The peptide length that gives optimum peptide diversity increases with increasing library size. Compared to other library schemes, in trimer libraries longer peptides should in theory always yield higher diversity and efficiency. In practice however, peptides longer than 9 aa are not expected to be notably beneficial in regard to relative efficiency (see Figure 2) at any currently achievable library size. Additionally, increasing peptide length in all encoding schemes will reduce coverage and thereby the chance that top peptides are included in the library.

In conclusion, our framework offers for the first time a set of evaluation parameters that allows for an in-depth analysis of peptide libraries. Thereby, a more informed library design becomes possible, which can be adjusted to the requirements of the needed peptides and to the technical feasibility in a given experimental setting. Further, the chances that a top peptide is included in the library, and thereby indirectly, the probability that the best peptide isolated from a selection is in fact the best possible peptide can now be estimated. This in turn can help to decide if a further optimization of the isolated peptide is potentially worthwhile.

The mathematical framework presented here was implemented in PeLiCa (`http://www.pelica.org`), a web-based tool for scientists to perform *in silico* analyses of various peptide library designs. PeLiCa allows calculations for a wide variety of library sizes, peptide lengths, and encoding schemes.

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