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Technical Note

Optimized determination of T cell epitope responses

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Abstract

Pools of overlapping peptides corresponding to specific antigens are frequently used to identify T cell immune responses to vaccines or pathogens. While the response to the entire pool of peptides provides important information, it is often desirable to also know to which individual peptides within the pool the immune responses are directed. In this report, we analyzed various ways of deconvoluting an immune response to a pool of peptides to determine the number of different peptides to which the T cells are responding. We used a Monte Carlo simulation to optimize the construction of peptide pools that could identify responses to individual peptides using the fewest numbers of assays and patient material. We find that the number of assays required to deconvolute a pool increases by the logarithm of the number of peptides within the pool; however, the optimum configuration of pools changes dramatically according to the number of responses to individual peptides that are expected to be in the sample. Our simulation will help in the design of clinical trials in which the breadth of the response is being measured, by allowing a calculation for the minimum amount of blood that needs to be collected. In addition, our results guide the design and implementation of the experiments to deconvolute the responses to individual peptide epitopes.
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1. Introduction

Recently, there have been great advances in the ability to detect and characterize antigen-specific T cells. Small overlapping peptides that span an entire protein(s) of interest are being routinely used to determine the number of antigen-reactive T cells within clinical samples by intracellular flow cytometry or ELISpot (Kern et al., 1998, 1999, 2002; Addo et al., 2001; Altfeld et al., 2001; Betts et al., 2001; Maecker et al., 2001; Yu et al., 2002). Typically, such pools contain peptides that are between 9 and 20 amino acids in length, and overlap to a degree that ensures that every T cell epitope is represented. We have adopted the approach of using peptides that are 15 amino acids long, and overlap by 11 (i.e., starting every 4th amino acid through a protein) (Betts et al., 2001; Maecker et al., 2001). For protein such as HIV gag, which is approximately 500 amino acids long, this translates into a set of 120–125 peptides (depending on the strain of HIV from which the gag was derived).

As an alternative, some investigators have used pools of peptides that represent predicted or known HLA class-I-restricted T cell epitopes (Dalod et al.,

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1999; Betts et al., 2000). However, we feel that there are advantages to using the overlapping peptides approach: (1) the HLA type of the patient, and the corresponding dominant epitopes relevant to the patient's haplotype, do not need to be determined in advance; and (2) both CD4+ and CD8+ T cell responses can be assessed (Betts et al., 2001; Maecker et al., 2001). Irrespective of these considerations, the methods described here are relevant to deconvoluting peptide responses from pools of overlapping peptides or optimized class I epitopes.

Enumeration of T cell responses to peptide pools by flow cytometric assays usually requires between 0.5 and 1 million cells; ELISpot assays use a few hundred thousand (but are usually done in duplicate or triplicate). Further testing of every individual peptide within a pool to determine to which peptides the T cells are responding would be prohibitive, from both a sample requirement and a reagent requirement. For example, for the HIV gag, one would have to perform 120 separate assays, requiring at least 50 ml of blood and 120 tests. Instead, peptides may be pooled together in sets to determine responses. This has previously been accomplished by creating multiple smaller pools in a "matrix" format, and has been relatively successful for determining individual peptide responses for pools of 100–120 peptides (Kern et al., 1999; Betts et al., 2000).

However, there is a need to deconvolute responses from larger pools of peptides. As an example, we are currently planning a clinical trial where we will immunize with DNA vectors that express the HIV envelope protein from each of three clades as well as an HIV gag–pol–nef polyprotein. The combined immunogen expresses four proteins, and the total number of peptides we will use to determine immunogenicity is approximately 800. For some of the vaccinees we will wish to determine the breadth of the response. However, the composition of the "matrix" pools of peptides that would best accomplish this is not self-evident.

Therefore, we wrote a program to model the determination of peptide responses. The software does a complete test of all possible peptide pool configurations against a varying number of potential positive responses from within the immunogen. We were able to determine the optimal configuration of peptide pools for deconvoluting peptide responses, with the goal of minimizing the number of assays and the amount of clinical sample required. Here, we show this optimization for either 120 or 800 peptide libraries; additional results for 64, 480, or 1200 peptide pool libraries are available. The software can also be used to output the optimal peptide pool configuration, and, given the results of a series of assays, can identify which peptides from those pools comprise the response.

2. Materials and methods

The software used in this analysis, "DeconvoluteThis" version 1.0, was written in C++ using the Metrowerks Codewarrior Pro for Macintosh framework. The application and source code are available from the author by request. The software is implemented for Mac OSX, but may be run on earlier versions of the operating system. The software is capable of both simulating a series of deconvolutions to identify the optimal peptide configuration, as well as aiding in such an experiment by outputting the peptide pool configuration and reading in a set of positive responses to identify which peptides must be re-tested in a second round of assays.

3. Results and discussion

The simplest way to deconvolute the individual responses within a peptide pool is to simply test each of the peptides from the pool separately. From a pool of 100 peptides, however, this would require 100 assays to find the few that are responding: this is highly inefficient. For the purpose of explaining how we approached this problem, we will consider a peptide pool comprising 100 distinct peptides. Typically, the number of peptides that will generate a response from such a pool will be less than six.

A more efficient process is to break the large pool into a number of smaller pools. For example, 10 pools of 10 peptides each could be tested. If there were only one positive peptide, only one of those pools would be positive—then, each of those 10 peptides could be individually tested. Thus, a total of 20 assays is performed in two rounds to identify the peptide (i.e., deconvolute the response). However, if there are three

peptides that are positive, this approach will likely require a total of 20–40 tests: 10 pools of 10, followed by testing one to three positive pools individually, depending upon whether the responding peptides fall into one, two, or three pools.

One way to improve the efficiency is to implement additional pools of peptides that contain different mixtures of peptides. For example, the standard matrix approach would use 20 pools of 10 peptides, where each peptide is contained within two different pools (Fig. 1). Now a single peptide response can be determined with 20 assays, and a three-peptide response can be determined with 29 or fewer assays. We use the term "coverage" to mean the number of times each peptide is represented within the pools. In this case, the coverage is 2; in the previous example, the coverage is 1.

Fig. 1 shows an extension, to this process, where the coverage is now 3 (i.e., each peptide is represented in three different pools). In addition, the pool size was increased from 10 to 20 peptides; this reduces the number of pools that need to be tested in the first round. It appears from this example that 15 pools of 20 peptides each (coverage = 3) may be more efficient for determining peptide responses than 20 pools of 10 peptides (coverage = 2), which had been our standard method for deconvoluting responses.

In considering our upcoming requirement to deconvolute from 800 peptides, we realized that it would be impossible to guess at the optimal peptide pool construction. Therefore, we used a software program to analyze pool configuration and potential responses, testing all possible combinations, to determine the optimal configuration.

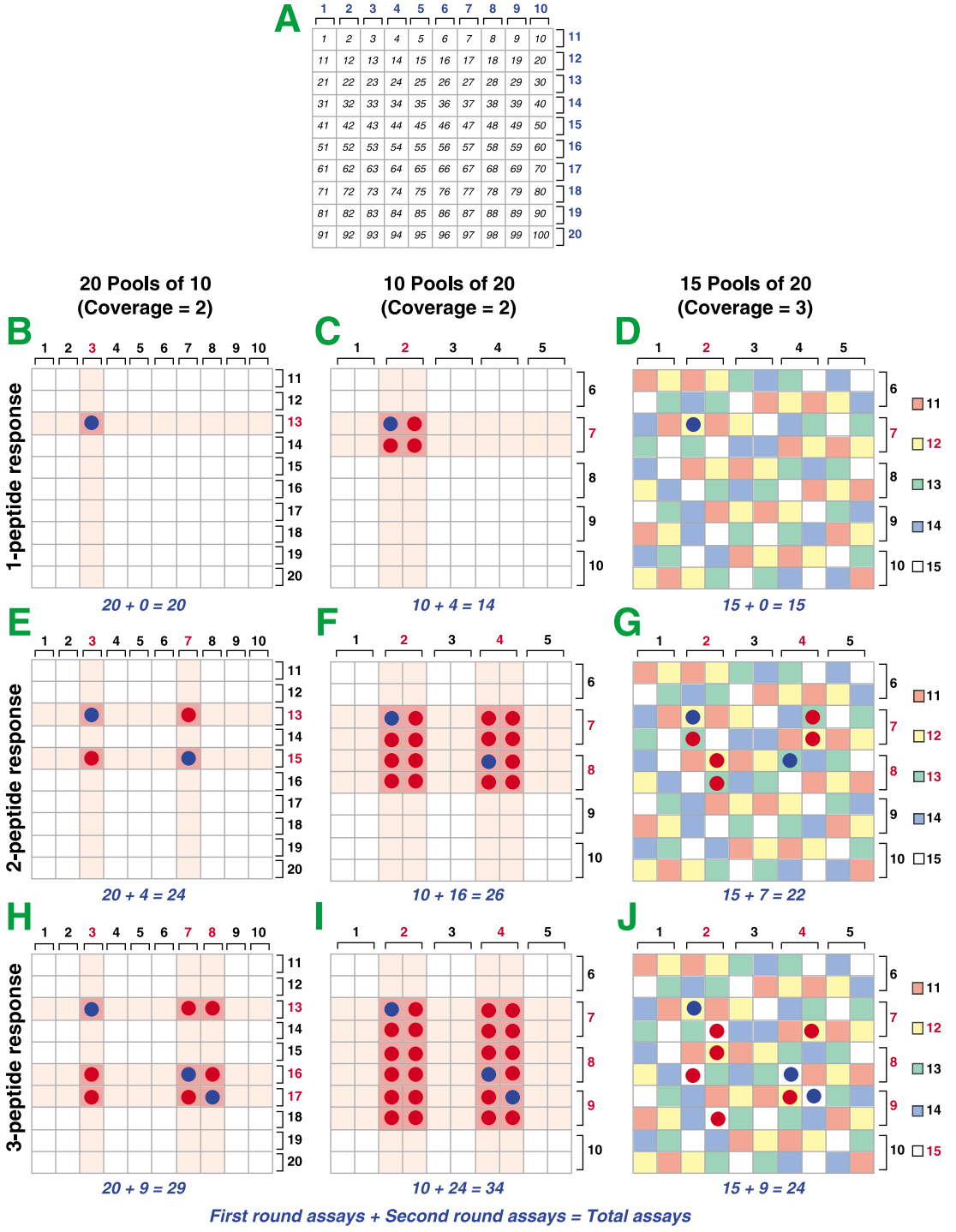
There were three variables that were modeled in the software, once the total number of peptides were entered: (1) The size of each peptide pool used in the first round of assays; (2) The coverage; and (3) The number of positive peptides in a simulated response. The total number of pools is determined by the first two variables. For each unique configuration of pools (from variables #1 and #2), we ran 25,000 simulations generating a random set of positive peptides (defined by variable #3). For each simulation, the number of second-round assays required to finish the deconvolution was determined. The optimal pool configuration was defined as that which required the minimum total number of assays (round one plus round two). The results of some of these simulations are shown in Table 1.

The first conclusion from these simulations is that the optimal peptide configuration depends on the expected number of positive peptides in the pool. For example, the optimal configuration to detect one peptide from 800 is to use 20 pools of 160 peptides. With this configuration, on average only 26 tests (maximum of 41) need to be performed to identify the peptide uniquely. However, if there are 5 positive peptides in the pool, this particular configuration is highly inefficient, requiring on average 188 assays. Contrast this with the optimal configuration designed with the expectation of five positive peptides (48 pools of 100 peptides), in which the deconvolution will require only 62 assays. On the other hand, deconvoluting a single peptide with that configuration requires 50 assays.

In experimental situations, of course, one cannot know in advance how many peptides will be positive for any given sample. Therefore, the goal is to choose a peptide configuration that is likely to minimize the number of assays for the average sample.

For all configurations of pools, the number of assays required to deconvolute increases by the cube of the number of peptides that are positive. Furthermore, the number of extra assays required when the number of positive peptides is less than that for which the configuration was optimized is relatively smaller. Therefore, one should in general choose a configuration that is optimized for the upper end of the range of expected positive peptides. For example, if the expected number of positive responses within a pool of 800 peptides is, on average, 6, then one would probably choose to use 64 pools of 50 peptides (coverage = 4), the optimum for nine positive responses. Using this configuration, the number of assays required for deconvolution will be, on average, between 64 (one positive peptide) and 110 (10 positive peptides).

We considered other possible methods to increase the efficiency. For example, if there are 10 positive peptides (out of 800), and the above configuration is tested, after the first round of assays we would have to test 46 peptides to determine which ones are the positive ones. One could consider making another matrix of these 46 (for example, 14 pools of 6 or 7, in a 7×7 matrix) as an intermediate step. However, it is


A
1 2 3 4 5 6 7 8 9 10
1 2 3 4 5 6 7 8 9 10 11
11 12 13 14 15 16 17 18 19 20 12
21 22 23 24 25 26 27 28 29 30 13
31 32 33 34 35 36 37 38 39 40 14
41 42 43 44 45 46 47 48 49 50 15
51 52 53 54 55 56 57 58 59 60 16
61 62 63 64 65 66 67 68 69 70 17
71 72 73 74 75 76 77 78 79 80 18
81 82 83 84 85 86 87 88 89 90 19
91 92 93 94 95 96 97 98 99 100 20
20 Pools of 10 (Coverage = 2)
10 Pools of 20 (Coverage = 2)
15 Pools of 20 (Coverage = 3)
B
C
D
1-peptide response
20 + 0 = 20
10 + 4 = 14
15 + 0 = 15
E
F
G
2-peptide response
20 + 4 = 24
10 + 16 = 26
15 + 7 = 22
H
I
J
3-peptide response
20 + 9 = 29
10 + 24 = 34
15 + 9 = 24
First round assays + Second round assays = Total assays

impossible to construct a configuration of peptide pools (out of 46) that will identify the 10 positives in less than 46 assays (the number required to test each individually). When the fraction of peptides that are positive is significantly greater than a few percent of the total peptide pool, then the most efficient deconvolution is to test them individually.

It must also be remembered that the creation of these peptide pools is not only labor-intensive, but also reagent-intensive. The smallest pools that can be made will have enough peptides for testing hundreds of samples (because of pipetting limitations). Therefore, it will be unlikely that a laboratory would construct different configurations of peptide pools for deconvolution purposes. Hence, the pools for the first round will always be the same, and the optimal configuration will need to be determined in advance.

In deconvoluting by the matrix approach, it can be helpful to consider the absolute value of the positive response. In other words, a positive response is not just a ''yes'' or ''no'', but is quantitative in nature. Consider a two-peptide response in a matrix approach such as that shown in Fig. 1. If one of the peptides gave a response of 1% of CD8 T cells, and the other gave a response of 0.1% of CD8 T cells, then there would be no ambiguity: the two pools containing the first peptide would show 1% and the two pools containing the second peptide would show 0.1% as well. The peptide at the intersection of one of the first pools with one of the second pools could be eliminated since the pool values are different. However, this additional information can only help with a very limited number of positive responses, and only if the responses are sufficiently different in magnitude that they can be distinguished with a high degree of confidence.

In our clinical trials, we are immunizing with four different immunogens: Clades A, B, and C envelope, and a gag–pol–nef polyprotein. For routine analysis, we will test immunogenicity with seven peptide pools: one for each envelope, one for gag, two for pol, and one for nef. These pools are designed to give us biologically relevant information about the immune response generated in our vaccines. Therefore, when we are preparing to deconvolute, we would know which of these seven pools need to be interrogated for individual responses.

Might we not achieve additional efficiency by testing matrix pools for only those proteins in which there is a response? The answer is a resounding no. Constructing individual configurations means that we would have seven different sets of pools. However, we can construct a single pool set for all seven immunogens. In our simulations, we find that the number of assays required to deconvolute responses increases by the logarithm of the number of total peptides; specifically, a $10\times$ increase in the number of peptides only requires $1.6\times$ increase in the number

Fig. 1. Construction of peptide pool ''matrices'' for deconvolution of peptide responses. For this example, we consider how to identify which of 100 peptides contribute to an immune response. (A) For display purposes, consider the 100 peptides arranged in a 10×10 array. In the standard matrix approach, 20 peptide pools are made of different mixtures of peptides. For example, Pool #1 has peptides 1, 11, 21, . . .91. Pool #11 has peptides 1, 2, 3, . . .10. Note that each peptide is represented in two pools, and no two peptides share membership in more than one pool. (B–J) These panels illustrate the approach to identifying peptide responses using three different ways of constructing pools. The left three panels using 20 pools of 10 peptides (standard matrix approach). The middle panel illustrates 10 pools of 20 peptides. In both of these approaches, each peptide is in two different pools. The right panel illustrates 15 pools of 20 peptides. In addition to the 10 pools shown in the middle panels, an additional five pools (color coded) are constructed to minimize the shared membership of groups of peptides. Each row of Panels illustrates the process of identifying a single (peptide #23), double (peptides #23 and 47), and triple (peptides #23, 57, and 68) response. Blue dots indicate the true positive peptides; red dots illustrate the peptides that are potential positives after the first round—both blue and red peptides must be retested in the second round to identify the true responders. (B) A single response causes pools 3 and 13 to be positive. There is only one possible peptide that belongs to these two pools, so no further assays are necessary. Deconvolution required only the 20 original pools. (C) A single peptide caused pools 2 and 7 to be positive. There are four peptides belonging to these two pools; hence, a second round testing each of these four peptides is necessary to identify which one was positive. 10 assays were performed in the first round, and four in the second, for a total of 14 assays. (D) A single peptide caused pools 2, 7, and 12 to become positive. Only one peptide belongs to all three pools; hence a second round was not necessary. Deconvolution required 15 assays. (E) Two positive responses cause four pools to become positive. After the first round, it is known that the four pools are positive, but it is not know whether the positive response is from two, three, or four of the peptides that lay at the intersections. Hence, four additional assays are necessary to fully deconvolute this response. (F–J) Similar analyses are carried out to illustrate, given the positive peptides which other peptides must be tested. Note that the optimal configuration of peptide pools (as defined by the minimum total number assays) is 10 pools of 20 when there is one positive peptide, and 15 pools of 20 for two or three positive peptides—20 pools of 10 peptides is never the optimum.

Table 1
Optimal configurations for deconvoluting peptides and number of assays required

Conf	Size	Cov	Pools	NPos	MxPos	Number of positive peptides in the sample														
						1	2	3	4	5	6	7	8	9	10	15	20	25	30	40
800 peptides																				
1	160	4	20	5.2	21	26	40	72	123	188	261	337	408	474	531	717	787	810	817	820
2	160	5	25	8.29	32	27	34	52	87	139	204	276	348	419	484	699	783	812	821	825
3	100	4	32	11.6	35	34	37	44	58	80	109	144	184	226	271	486	640	730	779	820
4	100	5	40	12.3	36	42	43	46	53	65	84	110	141	177	219	434	607	714	774	824
5	100	6	48	13.9	46	50	51	52	56	62	74	92	116	145	181	390	575	700	771	829
6	80	5	50	21.4	66	50	53	54	57	63	72	85	102	123	148	309	476	611	704	799
7	80	6	60	20.6	67	60	63	64	65	68	73	81	92	107	126	267	436	582	687	799
8	50	4	64	25.6	55	64	67	68	70	73	77	83	90	99	110	188	293	402	504	662
9	50	4	64	34.7	80	64	67	68	70	73	77	83	90	99	110	188	293	403	504	661
10	50	5	80	25.6	62	80	83	84	85	86	88	91	95	100	106	161	249	354	459	635
15	32	4	100	44	85	102	103	104	105	106	108	109	112	115	118	145	188	246	312	452
20	32	5	125	55.4	112	127	128	129	130	131	132	133	134	136	138	153	181	224	281	413
25	20	3	120	95.5	165	122	123	124	125	126	128	130	132	134	137	156	182	216	255	344
30	20	4	160	83	150	162	163	164	165	166	167	168	169	170	172	181	196	216	243	314
40	16	4	200	103	183	202	203	204	205	206	207	208	209	210	211	218	228	241	258	303
120 peptides																				
1	24	2	10	4.89	7	15	26	40	53	66	77	87	96	102	108	124	129	130	130	130
2	24	3	15	5.98	12	17	21	30	41	53	66	77	87	96	104	126	133	135	135	135
3	20	3	18	9.95	23	20	23	28	36	46	56	66	75	84	92	120	132	136	138	138
4	15	3	24	10.1	23	26	27	30	35	40	47	54	61	69	76	107	126	136	141	144
5	15	3	24	15.7	35	26	27	30	35	40	47	54	61	69	76	107	126	136	141	144
6	12	3	30	14.8	32	32	33	35	37	41	45	50	56	61	67	96	118	133	141	148
7	12	3	30	19.6	43	32	33	35	37	41	45	50	55	61	67	96	118	133	141	148
8	8	2	30	24.5	49	32	34	36	39	43	47	51	55	59	64	85	104	119	130	142
9	8	2	30	28.9	53	32	34	36	39	43	47	51	55	59	64	85	104	119	130	142
10	8	2	30	33.3	61	32	34	36	39	43	47	51	55	59	64	85	104	119	130	142
15	3	1	40	39.9	45	43	46	49	52	55	58	60	63	66	68	80	91	101	110	125
20	3	1	40	50.9	60	43	46	49	52	55	58	60	63	66	68	80	91	101	110	125
25	3	1	40	60.9	75	43	46	49	52	55	58	60	63	66	68	80	91	101	110	125
30	3	1	40	69.8	87	43	46	49	52	55	58	60	63	66	68	80	91	101	110	125
40	1	1	120	40	40	120	120	120	120	120	120	120	120	120	120	120	120	120	120	120

Conf = Number of positive peptides for which a configuration has been optimized (i.e., blue dots in Fig. 1). Size = Number of peptides in each pool. Cov = Coverage, the number of times a peptide is represented amongst the pools. Pools = total number of pools. Note that Conf × Cov = Size × Pools. NPos = Average number of peptides that would need to be tested in a second round of assays (i.e., blue plus red dots as shown in Fig. 1). MxPos = Maximum number of peptides that would need to be tested in a second round of assays in a worst case scenario. The right-hand columns show the actual average number of assays that would need to be performed if there were a given number of positive peptides, for each configuration. Since each configuration has been optimized for the number of peptides given in the first column, the assays are minimal for that number of peptides. All values were calculated by running a Monte Carlo simulation with 25,000 runs for each configuration. Configurations were calculated for a total of 800 peptides (top) or 120 peptides (bottom) that need deconvolution.

The left panels identify the optimum configuration of peptide pools given an expected number of positive peptides in those pools. Thus, the optimal configuration of pools to identify two peptides from 800 is 25 pools of 160 peptides. This configuration results in the minimum total number of assays (on average, 34) when there are two peptides that are positive, but requires 139 assays if there are five peptides. On the other hand, choosing the optimum configuration for five peptides (48 pools of 100 peptides) would require only 52 assays, on average, to deconvolute five responses from a total of 800.

of tests (i.e., blood volume required is proportional to $1.6 \times \text{Log}(\#\text{peptides})$).

Thus, consider a subject who had peptide responses originating from four of the seven pools. For deconvolution, we would need to test configurations for each of the four pools, requiring $4\times$ the amount of blood required to deconvolute just one pool. On the other hand, deconvoluting all peptides simultaneously will

only require about 1.4 × (= 1.6 × Log(7)) the amount of blood.

There are several important considerations in applying this theoretical approach to the experimental situation. First is the issue of false positives. A false positive for any given pool will dramatically increase the number of potential peptides that must be tested. As noted above, the number of assays that must be performed increases by the cube of the number of positives (including false positives). Hence, false positives can have a huge impact on the ability to deconvolute. False positives may result from the combination of sub-optimal responses from multiple peptides within a pool that combine to give a positive response to that pool. For instance, one can imagine a situation where two peptides, each giving a 0.07% response, are present within one pool, thereby resulting in a positive response for that pool (0.14%), but where other pools that contain the peptides individually will not score as positive (assuming a minimum sensitivity of 0.1% of CD8+ T cells). Under this scenario, one must assume that there may be difficulties in deconvoluting very low level responses.

False negatives can also impact, although much less so than false positives. There is a minimum sensitivity of the assay (for example, 0.1% of CD8, depending on the validation data of a given laboratory). If a single peptide response is close to this threshold, then it might have come up positive in the first (pools) round, but negative when individual peptides are tested. Indeed, when the number of peptides in the second round does not fully account for the positives in the first round, it is impossible to know if that was because of false positives in the first round or false negatives in the second round.

Given the need to deconvolute positive responses from a pool of 800 peptides, how much blood will we need to collect from each subject? We estimate that we will have approximately 10 positive responses from the typical subject (in both CD4 and CD8 T cells). Thus, we will probably select a configuration of pools optimized for 15 positive peptides: 100 pools of 32 peptides (coverage = 4). Thus, the first round of assays will always be 100 pools. For this configuration, the second round of assays will be less than 10 for most subjects with 1–7 positive responses, and under 20 for those with 8–10 responses. For subjects with 20 positive responses, the second round will require, on average 88 more assays. The first 100 pools will give us data on both CD4 and CD8 T cells, but since the positive peptides will be different for each subset, the second round must include different sets of peptides. Thus, the average subject (with less than 10 positive peptides) will require a total of 140 assays. Our FACS assays use 0.5 to 1 million cells each, meaning that we will need at least 140 million cells (or 140 ml of blood) for each time point that we wish to deconvolute. If our vaccines generate especially good breadth (for example, 20 positive peptides for CD4 or CD8), then we will need at least twice this much blood.

The ability to deconvolute peptide responses from a pool of overlapping peptides representing an immunogen (or antigen from pathogen) is an extremely powerful technique that will provide much information about the biology and immunology of the system. Our study here determined the optimal methodology for performing such a deconvolution; perhaps the most important conclusion is that the number of assays required increases very slowly with the size of the total pool of peptides. Hence, it makes sense to interrogate as many peptides as possible simultaneously. Our study also helps plan clinical investigations in terms of how much blood needs to be collected in order to have a reasonable chance of success in fully deconvoluting an immunologic response.

References

Addo, M.M., Altfeld, M., Rosenberg, E.S., Eldridge, R.L., Philips, M.N., Habeeb, K., Khatri, A., Brander, C., Robbins, G.K., Mazzara, G.P., Goulder, P.J., Walker, B.D., 2001. The HIV-1 regulatory proteins Tat and Rev are frequently targeted by cytotoxic T lymphocytes derived from HIV-1-infected individuals. Proc. Natl. Acad. Sci. U. S. A. 98, 1781–1786.

Altfeld, M., Addo, M.M., Eldridge, R.L., Yu, X.G., Thomas, S., Khatri, A., Strick, D., Phillips, M.N., Cohen, G.B., Islam, S.A., Kalams, S.A., Brander, C., Goulder, P.J., Rosenberg, E.S., Walker, B.D., 2001. Vpr is preferentially targeted by CTL during HIV-1 infection. J. Immunol. 167, 2743–2752.

Betts, M.R., Casazza, J.P., Patterson, B.A., Waldrop, S., Trigona, W., Fu, T.M., Kern, F., Picker, L.J., Koup, R.A., 2000. Putative immunodominant human immunodeficiency virus-specific CD8(+) T-cell responses cannot be predicted by major histocompatibility complex class I haplotype. J. Virol. 74, 9144–9151.

Betts, M.R., Ambrozak, D.R., Douek, D.C., Bonhoeffer, S., Brenchley, J.M., Casazza, J.P., Koup, R.A., Picker, L.J., 2001. Analysis of total human immunodeficiency virus (HIV)-specific

CD4(+) and CD8(+) T-cell responses: relationship to viral load in untreated HIV infection. J. Virol. 75, 11983–11991.

Dalod, M., Dupuis, M., Deschemin, J.C., Sicard, D., Salmon, D., Delfraissy, J.F., Venet, A., Sinet, M., Guillet, J.G., 1999. Broad, intense anti-human immunodeficiency virus (HIV) ex vivo CD8(+) responses in HIV type 1-infected patients: comparison with anti-Epstein–Barr virus responses and changes during antiretroviral therapy. J. Virol. 73, 7108–7116.

Kern, F., Surel, I.P., Brock, C., Freistedt, B., Radtke, H., Scheffold, A., Blasczyk, R., Reinke, P., Schneider-Mergener, J., Radbruch, A., Walden, P., Volk, H.D., 1998. T-cell epitope mapping by flow cytometry. Nat. Med. 4, 975–978.

Kern, F., Surel, I.P., Faulhaber, N., Frommel, C., Schneider-Mergener, J., Schonemann, C., Reinke, P., Volk, H.D., 1999. Target structures of the CD8(+)-T-cell response to human cytomegalovirus: the 72-kilodalton major immediate-early protein revisited. J. Virol. 73, 8179–8184.

Kern, F., Bunde, T., Faulhaber, N., Kiecker, F., Khatamzas, E., Rudawski, I.M., Pruss, A., Gratama, J.W., Volkmer-Engert, R., Ewert, R., Reinke, P., Volk, H.D., Picker, L.J., 2002. Cytomegalovirus (CMV) phosphoprotein 65 makes a large contribution to shaping the T cell repertoire in CMV-exposed individuals. J. Infect. Dis. 185, 1709–1716.

Maecker, H.T., Dunn, H.S., Suni, M.A., Khatamzas, E., Pitcher, C.J., Bunde, T., Persaud, N., Trigona, W., Fu, T.M., Sinclair, E., Bredt, B.M., McCune, J.M., Maino, V.C., Kern, F., Picker, L.J., 2001. Use of overlapping peptide mixtures as antigens for cytokine flow cytometry. J. Immunol. Methods 255, 27–40.

Yu, X.G., Addo, M.M., Rosenberg, E.S., Rodriguez, W.R., Lee, P.K., Fitzpatrick, C.A., Johnston, M.N., Strick, D., Goulder, P.J., Walker, B.D., Altfeld, M., 2002. Consistent patterns in the development and immunodominance of human immunodeficiency virus type 1 (HIV-1)-specific CD8(+) T-cell responses following acute HIV-1 infection. J. Virol. 76, 8690–8701.