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(54) Title: PEPTIDE RATCHET LIBRARIES FOR CTL-INDUCING VACCINES AND THERAPEUTICS**(57) Abstract**

The present invention relates to ratchet libraries composed of related peptides synthesized simultaneously in a single peptide synthesis. Ratchet libraries are derived from a longer template peptide by sequentially "ratcheting" the template sequence into the shorter ratchet length and are used for cytotoxic T lymphocyte (CTL) induction or stimulation if the CTL epitope is known. If the CTL epitope is unknown, then the ratchet library can be used for identification of CTL epitopes. The ratchet libraries can be prepared from any protein sequence to which an immune CTL response is desired and can be formulated for delivery as a vaccine or therapeutic for the treatment or prevention of disease or malignancy. For example, a ratchet library can be used in the prevention and treatment of infectious or malignant diseases including HIV, influenza, malaria, breast, ovarian, lung and colon cancers.

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1 PEPTIDE RATCHET LIBRARIES FOR CTL-INDUCING
2 VACCINES AND THERAPEUTICS
3
4

5 FIELD OF THE INVENTION

6 The present invention relates to ratchet libraries
7 composed of related peptides synthesized simultaneously in
8 a single peptide synthesis. Ratchet libraries are derived
9 from a longer template peptide by sequentially
10 "ratcheting" the template sequence into the shorter
11 ratchet length and are used for cytotoxic T lymphocyte
12 (CTL) induction or stimulation if the CTL epitope is
13 known. If the CTL epitope is unknown, then the ratchet
14 library can be used for identification of CTL epitopes.
15 The ratchet libraries can be prepared from any protein
16 sequence to which an immune CTL response is desired and
17 can be formulated for delivery as a vaccine or therapeutic
18 for the treatment or prevention of disease or malignancy.
19 For example, a ratchet library can be used in the
20 prevention and treatment of infectious or malignant
21 diseases including HIV, influenza, malaria, breast,
22 ovarian, lung and colon cancers.

23 BACKGROUND OF THE INVENTION

24 The development of vaccines and therapeutics
25 specifically designed to stimulate cytotoxic T lymphocytes
26 (CTL) is needed. CTL are a vital component of the natural
27 immune response against infectious organisms and malignant
28 cells. CTL are CD8⁺ thymus derived lymphocytes which
29 appear early in an immune response and help in the
30 elimination of, for example, virus-infected cells or tumor
31 cells by lysis of the target cells and by secretion of
32 chemical immunomodulators termed cytokines, such as
33 interferons.

34 CTL have been detected following many viral
35 infections, including HIV infection, and extensive
36 evidence points to a major role for CTL in control of
37 virus infections [McMichael et al. (1983) New Eng. J. Med.]

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1 301:13; Nixon et al. (1992) Immunology 76:515). For
2 example, adoptive transfer of specific CTL to influenza
3 [Taylor et al. (1986) Immunology 58:417] or paramyxovirus
4 simian virus 5-infected mice, cleared the virus from the
5 lungs [Young et al. (1990) J. Virol. 64:5403]. Tumor
6 specific CTL have also been shown to clear tumors caused
7 by mouse retroviruses [Cerundolo et al. (1987) Eur. J.
8 Immunol. 17:173] and are also probably critical in the
9 control of certain human malignancies. It has long been
10 the aim of scientists to develop vaccines or therapeutics
11 designed to specifically stimulate CTL immunity.

12 An essential step in the design of a CTL-inducing
13 vaccine is in the identification of the antigenic sites to
14 which CTL react. CTL recognize infected or malignant
15 cells through the interaction of their specific T-cell
16 receptor with a complex displayed on the surface of the
17 target cell. The complex consists of an antigenic peptide
18 specific to the virus or tumor, for example, and a major
19 histocompatibility complex (MHC) class I molecule encoded
20 by the Class I MHC genes of the host [Townsend et al.
21 (1986) Cell 44:959]. Clusters of closely linked MHC
22 alleles are characteristically inherited as a genetic unit
23 termed the "haplotype". In general, individual MHC
24 molecules associate with and present different antigenic
25 protein fragments, so that one fragment of an antigenic
26 protein is recognized by CTL of a specific MHC haplotype,
27 while a different MHC haplotype requires another fragment
28 of the antigen for recognition, i.e., recognition of
29 individual antigenic fragments is MHC-restricted. As the
30 MHC alleles are highly polymorphic between diverse genetic
31 groups, a large number of distinct peptides may be needed
32 to insure CTL stimulation across diverse human
33 populations.

34 The exact fragment(s) of a virus or tumor antigen or
35 other potential antigenic site (i.e., CTL epitope)
36 recognized by a specific CTL was thought to be between 7

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1 and 25 amino acids, but recent characterization of viral
2 peptides naturally processed in virus-infected cells and
3 displayed by Class I MHC molecules have identified the CTL
4 epitopes as peptides of between 7 to 11 amino acids in
5 length [Rötzschke et al. (1991) Immunology Today 12:447]
6 with the majority of these peptides being of 9 amino acids
7 (nonomers).

8 Identification of CTL epitopes in a protein sequence
9 has been achieved by using synthetic peptides to map
10 immunogenic sites. For example, several human CTL epitopes
11 have been defined from HIV through an *in vitro* testing
12 process of the human immune response to HIV infection
13 [Nixon et al. (1988) Nature 336:484-487; Nixon et al. U.K.
14 Patents GB 2,255,093, 2,273,709, 2,273,710]. While many
15 HIV CTL epitopes have been identified in animals, few have
16 been identified in humans. However, because CTL epitopes
17 are simultaneously recognized by a T-cell receptor that is
18 specific for both the virally-encoded peptide and the
19 host-encoded MHC for clearance of an infected cell to
20 occur, CTL-epitopes are species specific. Hence, human
21 CTL epitopes may not be reliably predicted from animal
22 studies.

23 While vaccine development has led to successful
24 vaccine against many infectious diseases, (e.g. polio,
25 measles), there are several important pathogens for which
26 vaccines are either ineffective or simply non-existent,
27 for example HIV, hepatitis C virus (HCV) and herpes
28 simplex virus (HSV). Moreover, there are no vaccines for
29 treatment of malignancies.

30 The identification of CTL epitopes makes it feasible
31 to design CTL-stimulating vaccines and other
32 immunotherapeutics for prevention or treatment of disease
33 by the clearance of virally-infected cells or malignant
34 cancer cells. However, there remain at least four major
35 problems associated with developing CTL-inducing vaccines
36 and immunotherapeutics, namely, (1) the identification of

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1 CTL epitopes or regions of proteins containing such
2 epitopes, (2) the induction of specific CTL responses by
3 peptides, (3) the need to accomodate MHC diversity with a
4 large multiplicity of peptides and (4) the ability to
5 provide for antigenic variation and escape mutations
6 within the CTL epitopic regions.

7 With respect to the identification of CTL epitopes,
8 progress has been made in identifying CTL epitopes from a
9 number of target antigenic proteins,; however, there
10 remain many proteins which contain potential CTL antigenic
11 sites for which epitopes have not been identified. For
12 example, the EBNA 1 protein of Epstein-Barr virus (EBV).
13 The present invention provides a solution to this problem
14 because the ratchet libraries can encompass extensive CTL
15 antigenic regions and eliminate the need to precisely map
16 CTL epitopes, or even to map the CTL epitopes at all. In
17 addition, the ratchet libraries can be used to map
18 antigenic sites.

19 Until recently, it was assumed that the induction of
20 specific CTL responses by peptides could only be
21 stimulated by endogenously-produced peptide fragments of
22 endogenous proteins assembled into HLA Class I-antigenic
23 peptide complexes on the cell surface. However, recent
24 studies have demonstrated that CTL responses can be primed
25 by administration of lipid-derivatized peptides [Deres et
26 al. (1989) Nature 342:561], peptides in liposomes [Friede
27 et al. (1994) Vaccine 12:791-797], or peptides admixed or
28 conjugated to other biologically active substances [Shirai
29 et al. (1994) J. Immunol. 152:549]. Hence, ratchet
30 library peptides can be formulated into an appropriate
31 vehicle to elicit CTL responses in vivo.

32 To accomodate genetic diversity and MHC restriction,
33 the ratchet library peptides provide a major advance since
34 several epitopes can be incorporated into the ratchet
35 libraries rather than relying upon mixtures individually
36 synthesized immunogenic peptides.

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1 The ability to provide for antigenic variation and
2 escape mutations within the CTL epitopic regions is
3 another significant problem. Antigenic variation is a
4 recurrent problem among certain pathogens contributing to
5 unsuccessful or limited success of vaccines. Extensive
6 antigenic variation, for example, is a hallmark of HIV
7 (AIDS), rhinovirus (the common cold), influenza virus
8 (flu), plasmodium falciparum (malaria). In addition, some
9 tumors and infectious agents use utilized escape mutation
10 to avoid immune surveillance. Ratchet libraries can be
11 constructed to embody known antigenic variation and escape
12 mutations to pre-empt these problems.

13 Hence, the ratchet library method of CTL induction
14 provides a solution to obstacles in the development of
15 vaccines and therapeutics for such pathogens or cancers.

17 SUMMARY OF THE INVENTION

18 This invention is directed to a ratchet library of
19 peptides comprising at least one immunostimulatory
20 cytotoxic T lymphocyte (CTL) epitope. The peptides are of
21 length l . The sequences of the peptides in the library
22 are determined from a template peptide of length from $l+1$
23 to n amino acids such that each position x in the library
24 has all the amino acids present in the template peptide at
25 positions x to $n-(l-x)$, inclusive and the ratio of amino
26 acids at each position x is determined by the relative
27 prevalence of amino acids at that position x . In
28 accordance herewith l is from about 7 to about 25 amino
29 acids, preferably 8-10 and more preferably 9; n is from
30 $l+1$ to about 100, preferably from $l+1$ to about 75 and more
31 preferably from $l+1$ to about 50; and x is from 1 to l .

32 Moreover, if a position x is identified as part of an
33 MHC-binding motif of a CTL epitope, then that position x
34 in the ratchet library is fixed as one or more amino acids
35 of the MHC-binding motif in an equimolar ratio.

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1 The CTL epitopes of this invention are from a virus,
2 bacterium, parasite, tumor antigen, allergen or other
3 protein antigen. The ratchet library can be constructed
4 from the template peptides of any one of SEQ ID NOS: 1-11.
5 the peptides can have a covalently attached N-terminal
6 tripalmitoyl-5-glycerol-cysteine moiety or be linked to a
7 branched core sequence, polymerized or conjugated to a
8 carrier molecule.

9 Another aspect of this invention provides a
10 pharmaceutical or vaccine composition comprising the
11 subject ratchet libraries including emulsion or a
12 microparticle formulation with or without the addition of
13 free Pam₃Cys or a derivative thereof. These compositions
14 are useful in a method of treating or preventing a disease
15 or a malignancy which comprises administering an amount of
16 the ratchet library as a vaccine or pharmaceutical
17 composition to a mammal effective to stimulate a CTL
18 response against the disease or the malignancy associated
19 with the CTL epitope present in the library.

20 Still another aspect of the invention is directed to
21 a method of constructing a library of related peptides to
22 provide a ratchet library which comprises identifying a
23 template peptide; calculating a distribution of amino
24 acids at each position x having those amino acids present
25 in the template peptide at positions x to $n-(l-x)$,
26 inclusive, wherein l is from about 7 to about 25, n is
27 from $l+1$ to about 100, and x is from 1 to l ; and
28 synthesizing said ratchet library.

29 BRIEF DESCRIPTION OF THE DRAWINGS

30 Fig. 1 depicts malaria ratchet libraries 1 and 2 from
31 *Plasmodium berghei* circumsporozoite (CS) protein and their
32 construction. Fig 1A shows the template peptide with the
33 known CTL epitope (CS 252-260) indicated by a box. Below
34 the template peptide is the corresponding set of
35 overlapping nonmer peptides. Fig. 1B provides an example
36

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1 of a sequence alignment for malaria ratchet library 1 (top
2 panel), its corresponding amino acid (AA) distribution by
3 position (middle panel) and the percent of each amino acid
4 at each position in the library (bottom panel).

5 Fig. 2 is a graphic illustration of malarial-specific
6 CTL activity against CS 252-260. The graph shows the
7 percent specific cell lysis as a function of the effector
8 to target cell (E:T) ratio in mice immunized with 100 μ g
9 doses of malaria ratchet library 1 in microparticles.

10 Fig. 3 is a graphic illustration of malarial-specific
11 CTL activity against CS 252-260. The graph shows the
12 percent specific cell lysis as a function of E:T ratio in
13 mice immunized with 1 mg doses of malaria ratchet library
14 2 in microparticles.

15 Fig. 4 is a graphic illustration of malarial-specific
16 CTL activity against CS 247-266. The graph shows the
17 percent specific cell lysis as a function of E:T ratio in
18 mice immunized with 1 mg doses of malaria ratchet library
19 2 in microparticles.

20 Fig. 5 is a graphic illustration of malarial-specific
21 CTL activity against CS 252-260. The graph shows the
22 percent specific cell lysis as a function of E:T ratio in
23 mice immunized with 10 μ g doses of malaria ratchet library
24 1 as lipopeptides.

25 Fig. 6 is a graphic illustration of the lack of
26 malarial-specific CTL activity against a self peptide.
27 The graph shows the percent specific cell lysis as a
28 function of E:T ratio in mice immunized with 100 μ g doses
29 of malaria ratchet library 1 as lipopeptides.

30 Fig. 7 depicts an MHC-restricted malaria ratchet
31 library constructed from malarial ratchet library 1 (top
32 panel). The middle panel shows the known anchor residues
33 for four MHC haplotypes, K^d, D^b, K^b and L^d. These anchor
34 residues are at positions 2 for K^d and L^d, 5 for D^b, K^b and
35 L^d, 8 for K^b and 9 for K^d, D^b and L^d. The bottom panel

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1 shows the resulting MHC-restricted malaria ratchet
2 library.

3 Fig. 8 depicts an HIV ratchet library from a 35 amino
4 acid sequence of the HIV-1 gp120 V3 loop region (residues
5 305-339). Fig. 8A provides the sequence of 15 HIV-1
6 variants from this region (top panel) and the
7 corresponding SSAL from those sequences. The D^d
8 restricted CTL epitope at gp120 amino acid positions 318-
9 326 is indicated by a box. Fig. 8B provides the amino
10 acid (AA) distribution by position (middle panel) and the
11 percent of each amino acid at each position in the library
12 (bottom panel) in a ratchet library constructed from the
13 SSAL. Amino acids divergent from the consensus B sequence
14 are shown as upper case letters and conserved amino acids
15 in the consensus sequences are shown as lower case
16 letters.

17 Fig. 9 depicts an HIV-1 gag peptide linear ratchet
18 library containing a mouse HIV CTL epitope prepared from a
19 100 amino acid template (top panel). The amino acid (AA)
20 distribution by position (middle panel) and the percent of
21 each amino acid at each position in the library (bottom
22 panel) is shown. The D^b restricted epitope (at gag
23 residues 390-398) is indicated by the box.

24 Fig. 10 depicts an HIV-1 gag peptide linear ratchet
25 library containing a mouse HIV CTL epitope prepared from a
26 40 amino acid template (top panel). The amino acid (AA)
27 distribution by position (middle panel) and the percent of
28 each amino acid at each position in the library (bottom
29 panel) is shown. The D^b restricted epitope (at gag
30 residues 390-398) is indicated by the box.

31 Fig. 11 depicts an HIV-1 gag peptide linear ratchet
32 library containing a mouse HIV CTL epitope prepared from a
33 20 amino acid template (top panel). The amino acid (AA)
34 distribution by position (middle panel) and the percent of
35 each amino acid at each position in the library (bottom

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1 panel) is shown. The D^b restricted epitope (at gag
2 residues 390-398) is indicated by the box.

3 Fig. 12 is a graphic illustration of HIV-specific CTL
4 activity against HIV-1 gag residues 390-398. The graph
5 shows the percent specific cell lysis as a function of E:T
6 ratio in mice immunized with 100 µg doses of the HIV
7 ratchet library from the 40-mer template in an emulsion.

8 Fig. 13 depicts a mucin ratchet library from a 20-mer
9 repeating sequence (top line). The next two lines
10 illustrate two alternate template peptides for
11 construction of this mucin ratchet library. The boxed
12 residues are the additional sequences added at the termini
13 to allow representation of all possible nonomers of the 20
14 amino acid repeat sequence. The amino acid (AA)
15 distribution by position (middle panel) and the percent of
16 each amino acid at each position in the library (bottom
17 panel) is shown.

18 Fig. 14 depicts a mutant p53 ratchet library
19 constructed from a template peptide of amino acids 124-151
20 (top panel). There are additional amino acids, which
21 represent known p53 mutants incorporated at positions 9-
22 12. The boxed residues are 10 amino acid CTL epitope
23 identified in Balb/C mice. The amino acid (AA)
24 distribution by position (middle panel) and the percent of
25 each amino acid at each position in the library (bottom
26 panel) is shown.

27 Fig. 15 depicts influenza ratchet library 1
28 constructed from a 25-mer template sequence of residues
29 139-163 of influenza A A/34/PR8 nucleoprotein (top panel).
30 The known K^d-restricted epitope of residues 147-155 is
31 indicated by a box. The amino acid (AA) distribution by
32 position (middle panel) and the percent of each amino acid
33 at each position in the library (bottom panel) is shown.

34 Fig. 16 depicts influenza ratchet library 2 from a
35 template peptide which is a linkage of 3 CTL epitopes in
36 order from N to C terminus of residues 50-58, 147-155 and

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366-374 of the nucleoprotein. Each CTL epitope is indicated by a box. The amino acid (AA) distribution by position (middle panel) and the percent of each amino acid at each position in the library (bottom panel) is shown.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to a ratchet library of peptides comprising at least one immunostimulatory cytotoxic T lymphocyte (CTL) epitope. The peptides of the ratchet library are of length l and the sequences of the peptides in the library are determined from a template peptide having a length from $l+1$ to n amino acids. Each position x in the library peptides has those amino acids which are present in the template peptide at positions x to $n-(l-x)$, inclusive. Accordingly, the ratio of the individual amino acids at each position x is determined from the relative numbers (or prevalence) of the amino acids at that position x . In accordance with this invention, l is from about 7 to about 25, n is from $l+1$ to about 100, and x is from 1 to l .

For example, in a ratchet library of the above formula, position 1 contains all the amino acids of the template peptide at positions 1 to $n-(x-1)$, position 2 contains all the amino acids of the template peptide at positions 2 to $n-(x-2)$, position 3 contains all the amino acids of the template peptide at positions 3 to $n-(x-3)$, ... , position $x-1$ contains all the amino acids of the template peptide at positions $x-1$ to $n-1$, and position x contains all the amino acids of the template peptide at positions x to n .

Fig. 1 provides an example of a malaria ratchet library, showing the relative ratio of amino acids at each position in the ratchet library derived from a longer malaria template peptide as well as the percentage of amino acids required for synthesis at each position of the ratchet library. More specifically, the malaria template

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1 peptide is divided up into sequential 9-mers which are
2 aligned at the amino terminus of the template peptide to
3 create a set of overlapping peptides from which to
4 calculate the ratchet library composition. After
5 calculation of its composition, the ratchet library is
6 prepared in a single synthesis based on the calculated
7 amino acid distributions at each position.

8 The size of the ratchet l, or ratchet length, can be
9 determined from the actual or approximate size of the
10 target CTL epitope. CTL epitopes have been identified
11 which vary in length from 7 to 25 residues. However, the
12 majority of CTL peptides are from 8-10 amino acids, and
13 many are 9 amino acids. While the actual size of the CTL
14 inducing peptide is preferred to determine the length l of
15 the ratchet library, e.g. 9 amino acids, the ratchet
16 length can be determined by other means, including an
17 arbitrary selection of size within the range of 7 to 25
18 amino acids.

19 The size of the template peptide ranges from l+1 to
20 about 100 amino acids, and preferably from l+1 to about 75
21 amino acids, and more preferably from l+1 to about 50.
22 Selection of the template peptide length is an important
23 factor in determining the overall complexity of the
24 ratchet library, so that shorter template peptides tend to
25 yield less complex ratchet libraries, i.e., have fewer
26 peptides in the library.

27 If the CTL epitope is known, then the template
28 peptide should be of a length n such that the CTL epitope
29 is flanked by sufficient adjoining sequences, preferably
30 at least l-1, to insure that the CTL epitope is
31 represented in the ratchet library. If the CTL epitope is
32 not known, then the template peptide can have a length
33 which covers a significant region of the protein being
34 tested. Typically such a length can range from about 20
35 to about 100 residues, and preferably ranges up to 50 or
36 75 residues. The template peptide can also be selected on

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1 the basis of clustering of epitopes, of hydrophobicity, of
2 stretches containing basic amino acids or of another
3 biological characteristic. Selection of a longer template
4 peptide is useful in identifying unknown CTL epitopes.

5 MHC binding motifs have identified particular amino
6 acids residues within CTL epitopes which are important in
7 peptide binding to the MHC receptor. When the MHC binding
8 motif is known for a particular CTL epitope, then the
9 ratchet library can be simplified by replacing the
10 calculated distribution of amino acids at a particular
11 site with the ratio of known amino acids from the MHC
12 binding motif at that site. An example of this is shown
13 in Example 2. In another example, the proportion of amino
14 acids within a ratchet library can be altered to reflect
15 human HLA binding motifs. For example, human HLA binding
16 motifs for 9-mer or 10-mer peptides typically have the
17 designated amino acids at the indicated positions: for
18 HLA-A2, leucine at position 2, valine or leucine at the C
19 terminus; for HLA-B35, proline at position 2, tyrosine at
20 the C terminus; for HLA-B53, proline at position 2,
21 phenylalanine or tryptophan at the C terminus; for HLA-B8,
22 lysine at position 3, lysine at position 5, isoleucine at
23 the C terminus; for HLA-B27, arginine at position 2,
24 lysine or arginine at the C terminus; for HLA-B7, alanine
25 at position 1, proline at position 2, arginine at position
26 3, leucine or valine at the C terminus; for HLA-A68,
27 threonine or valine at position 2, arginine at the C
28 terminus; for HLA-A3.1, isoleucine or leucine at position
29 2, phenylalanine at position 3, lysine or tyrosine at the
30 C terminus; and for HLA-A11, isoleucine or leucine at
31 position 2, lysine at the C terminus.

32 When the CTL epitope is contained in a multiple
33 tandemly repeated sequence, then the template peptide
34 length n can be equal to the length of the CTL epitope
35 plus 1-1 residues where the 1-1 residues carboxy-terminal
36 amino acids of the epitope are placed at its amino

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1 terminus, or alternatively, the 1-1 amino-terminal
2 residues of the epitope are placed at its carboxyl
3 terminus, for example as shown in Fig. 5 for the mucin
4 ratchet library.

5 To accomodate antigenic variation, the ratchet
6 library can be constructed from a template peptide which
7 is itself a "structured synthetic antigen library" or
8 SSAL. SSALs are defined and exemplified in U.S. Serial
9 No. 08/143,412, filed October 26, 1993, which is
10 incorporated herein by reference. Briefly, the sequence
11 of an SSAL is determined by aligning the primary amino
12 acid sequences of a related family of CTL epitopes and
13 identifying the invariant and variant loci within the
14 alignment. The invariant loci generally represent the
15 structural framework of the SSAL. The degeneracy within
16 the SSAL is determined by the loci within the alignment
17 that harbor different amino acid residue types relative to
18 an arbitrary prototype sequence. After determining which
19 amino acids are to be at each position, the degree of
20 degeneracy for the multiresidue position in the SSAL
21 library is determined from the number of variants each
22 individual amino acid represents by one of three methods.
23 Thus in a simple manner, the specific amino acids and
24 their frequency of appearance at each position within the
25 SSAL is defined by the primary sequences of the different
26 CTL antigens or molecules in the alignment of multiple
27 primary sequences.

28 The degeneracies for the variant amino acid positions
29 used for an SSAL can be determined in one of three ways.
30 In one method, the identity and ratio of residues is
31 determined by the relative prevalence of the amino acids
32 in a compilation of known sequences for the epitope. In
33 another method the identity of the amino acids at the
34 variant position is determined from the compilation of
35 known sequences for the epitope but the ratio of amino
36 acids is set to be equimolar. Finally, the identity and

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1 ratio of amino acids at a variant position can be
2 determined by a modification of the first method to
3 provide a simplified SSAL. When some of the residue types
4 are present at a low frequency (i.e., less than 5-10%
5 representation), the complex SSALs are modified to ensure
6 adequate representation of all variants. The process
7 follows three rules: 1) any amino acid present in the
8 primary sequence list at a proportion less than 10% is set
9 at 5% to allow for adequate representation of all variable
10 positions; 2) amino acids occurring at frequencies greater
11 than or equal to 10% are rounded to the nearest 10% of
12 prevalence. If the sum of percent prevalence exceeds
13 100%, the percent of the amino acid with the highest
14 prevalence is correspondingly reduced so that the amino
15 acids occurring at a given position are each represented in
16 the SSAL but the total representation does not exceed
17 100%.

18 Once an SSAL is calculated, then it can be the
19 "template peptide" for construction of a ratchet library
20 in accordance with the formula such that each position x
21 in the library peptides has those amino acids which are
22 present in the template SSAL at positions x to $n-(1-x)$,
23 inclusive. In other words, each full composition and
24 ratios of amino acids at each position x in the SSAL is
25 "ratcheted" and used to calculate the final distribution
26 of amino acids in the ratchet library.

27 The ratchet libraries can be prepared as a ensemble
28 of linear peptides. Similarly, it can be attached to a
29 branched core sequences, conjugated to a carrier or
30 polymerized.

31 These core sequences include dendritically branched
32 cores, linear array type branched cores or randomly
33 branched cores (e.g. poly-L-lysine). The branched cores
34 can be composed of an amino acid or an amino acid analog
35 having two amino groups and one carboxyl group, each group
36 capable of forming a peptide bond linkage. Preferably

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1 such amino acids are lysine or a lysine analog such as
2 ornithine. The amino acid analog can be an α -amino acid,
3 a β -amino acid, or any other either natural or non-natural
4 amino acid with two amino groups and one carboxyl group
5 available for forming peptide bonds. Preferred branched
6 peptides of the invention are dimers, tetramers and
7 octamers, especially those having a branching core
8 structure composed of lysine such as a heptalysine core.
9 Similarly, the branched cores can contain other residues
10 interspersed among the branching residues as depicted, for
11 example, in Fig. 12 of U.S. Serial No. 143,412.

12 When branched ratchet libraries are made, the library
13 can have a C-terminal methionine as the residue that is
14 attached to the branched core. The methionine provides a
15 cleavable site to facilitate analysis of the ratchet
16 library.

17 In addition, the ratchet can have one or more lysine
18 residues (added at the amino terminus) to increase peptide
19 solubility, cysteine and haloacylated residues can be
20 added to facilitate directed coupling to carrier
21 molecules, and methionine can be added for cyanogen
22 bromide cleavage if necessary. Pam₃Cys, or a similar
23 lipid tail, can be added to create a lipopeptide.

24 The subject ratchet libraries can also be used to
25 form conjugates, i.e., the ratchet library, either in
26 branched or linear form, can be coupled directly or
27 indirectly, by methods known in the art, to carriers such
28 as bovine serum albumin (BSA), human serum albumin (HSA),
29 or other proteins, red blood cells or latex particles. In
30 another embodiment, a ratchet library can be polymerized
31 to homo- or hetero-dimers or higher oligomers by cysteine
32 oxidation, by induced disulfide cross-linking, or by use
33 of homo- or hetero-functional multivalent cross-linking
34 reagents.

35 As used herein, a CTL epitope is a fragment of an
36 antigen which binds to the peptide-binding cleft of an MHC

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1 molecule such that the fragment-MHC complex is recognized
2 by a T cell antigen-specific receptor (TCR) and thereby
3 stimulates a CTL response. CTL and T cell epitopes are
4 reviewed in Encyclopedia of Immunology (Roitt et al.,
5 eds.), 1992, Academic Press Ltd, London, in Vol. I at
6 pages 447-450 and pages 515-517, respectively.

7 The protein sequence selected for a ratchet library
8 can be from a protein with known immunogenic CTL epitopes,
9 or from a protein whose CTL-stimulating ability has not
10 been determined, in which case the ratchet library method
11 can be used to identify CTL epitopes. Ratchet libraries
12 can be constructed from CTL epitopes (or putative CTL
13 epitopes) of viruses, bacteria, parasites, tumor
14 antigens, allergens, amino acid sequences deduced from an
15 intron or exon/intron mixtures, or from aberrant proteins
16 often associated with malignancy and generated by
17 frameshift mutations (i.e. frameshift sequences), or any
18 other proteins known to stimulate a CTL response. More
19 specifically, ratchet libraries can be prepared from the
20 following proteins or proteins from the listed organisms
21 or diseases (with the cited references indicating known
22 CTL response to those proteins): melanoma proteins
23 [Bakker et al. (1994) J. Exp. Med. 179:1005] including
24 MAGE-1, -2, and -3 [Gaugler et al. (1994) J. Exp. Med.
25 179:921]; proteins associated with renal cell carcinoma;
26 proteins associated with colon carcinoma [Townsend et al.
27 (1994) Nature: 371:662]; proteins associated with prostate
28 cancer (malignant or benign) including PSA; tyrosinase
29 [Brichard et al. (1993) J. Exp. med. 178:489]; oncogenes
30 such as the HER-2/neu proto-oncogene; ras [Gedde-Dahl et
31 al. (1994) Eur. J. Immunol. 24:410]; MUC1 [Barnd et al.
32 (1989) Proc. Natl. Acad. Sci. USA 86:7159]; p53 [Mijman et
33 al. (1994) Immunol. Lett. 40:171]; p16; TL [Morita et al.
34 (1994) J. Exp. Med. 179:777]; proteins from HIV-1 or -2
35 including envelope, gag, pol, nef, tat, rev, vpx, vpu
36 [Nixon et al. (1992)]; HTLV-I or -II including envelope,

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gag, pol, pX and TAX [Jacobson et al. (1990) Nature 348:245; lymphocytic choriomeningitis virus of mice (LCMV) [Aebischer et al. (1991) Proc. Natl. Acad. Sci. USA 88:11047]; influenza A, B or C including PB1, PB2, PA, NS1, M1, NP, HA [McMichael et al. (1978) Eur. J. Immunol. 8:705]; Epstein-Barr virus (EBV) including TETA, EENL, EBNA3, EBNA1 and LMP [Brooks et al. (1993) J. Exp. Med. 178:879]; respiratory syncytia virus (RSV) [Bangham et al. (1985) J. Virol. 56:55]; hepatitis B virus (HBV) [Bertoletti et al. (1993) J. Virol. 67:2367]; hepatitis C virus (HCV) [Koziel et al. (1992) J. Immunol. 149:3339]; herpes simplex virus (HSV) [Bonneau (1993) Virology 195:62]; cytomegalovirus (CMV) [Borysiewicz et al. (1988) Eur. J. Immunol. 18:269]; parainfluenza virus 1 including hemagglutinin, neuraminidase, phosphoprotein and nucleoprotein [Dave et al. (1994) Virology 199:376]; intracisternal A particle gag [de Bergeyck et al. (1994) Eur. J. Immunol. 24:2203]; bovine leukemia virus [Gatei et al. (1993) J. Virol. 67:1796]; papilloma viruses [Feltkamp et al. (1993) Eur. J. Immunol. 23:2242]; malaria including *P. falciparum*, *P. berghei*, *P. ovale*, *P. vivax*, *P. malaria* [Aggarwal et al. (1990) J. Exp. Med. 172:1083]; *Histoplasma capsulatum* [Deepe (1994) J. Immunol. 152:3491]; *Listeria* [Harty et al. (1992) J. Exp. Med. 175:1531]; Toxoplasmosis [Khan et al. (1994) J. Immunol. 152:1856]; *Trypanosoma cruzi*; *Yersinia*; *M. tuberculosis*; *M. leproi*; *Pneumocystis carinii*; Kaposi's sarcoma or frameshift sequences [Townsend (1994)].

The preferred ratchet libraries of this invention are those libraries provided in the Examples and the Figures.

CTL responses can be measured by conventional techniques known to ordinarily skilled artisans, including, for example, the dye exclusion test and the Cr-release assay described in Encyclopedia of Immunology, supra at page 451. Another method to assay CTL is

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1 described by McDonald et al. (1980) Immunol. Rev. 51:93-
2 123.

3 The ratchet libraries are prepared by chemical
4 synthesis using standard techniques well known in the art
5 such as the solid-phase synthetic route pioneered by
6 Merrifield. The coupling of multiple amino acids at a
7 given position is accomplished by providing a mixture of
8 the desired amino acids at the ratios determined by the
9 ratchet process. If necessary the ratio of amino acids in
10 the mixture can be varied to account for different
11 coupling efficiency of those amino acids.

12 Based on CTL induction of the ratchet libraries, they
13 are useful in a vaccine composition to treat or prevent
14 disease or malignancy in accordance with the source of the
15 CTL epitope in the ratchet library. In other words an HIV
16 ratchet library can be used as an HIV CTL vaccine (either
17 as a vaccine component or as a therapeutic in the
18 treatment of AIDS), an HCV ratchet library as an HCV CTL
19 vaccine, an influenza ratchet library as a flu CTL
20 vaccine, a mutant p53 ratchet library as a cancer CTL
21 vaccine and the like.

22 For example, efforts to develop a malaria vaccine
23 have been hampered by the complexity of the parasite life
24 cycle and the inability for an antibody-inducing vaccine
25 alone to provide sufficient efficacy. A CTL response to
26 liver stage antigens of the malaria parasite *Plasmodium*
27 *falciparum* has been recently reported [Hill et al. (1992)
28 Nature 360:434]. Stimulation of the CTL response against
29 the parasite appears necessary for an effective vaccine,
30 since CTL can eliminate parasite-infected cells at the
31 liver stage when the parasite load is low.

32 Vaccine compositions containing one or more distinct
33 ratchet libraries can be introduced into normal subjects
34 to stimulate production of CTL by immunization protocols
35 known in the art. Similarly the subject ratchet libraries
36 (one or more libraries) can be formulated in a vaccine

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1 composition using adjuvants, pharmaceutically-acceptable
2 carriers or other ingredients routinely provided in
3 vaccine compositions. Adjuvants for use in this invention
4 include incomplete Freund's adjuvant (IFA), alum, lipidic
5 amino acids and Pam₃Cys (see description in the Examples).
6 These latter two adjuvants can be either covalently
7 attached to the ratchet to produce a lipopeptide ratchet
8 library or formulated together with the ratchet library
9 for co-administration.

10 Vaccine formulations are readily determined by one of
11 ordinary skill in the art and include formulations for
12 immediate release and for sustained release. Formulations
13 contemplated by this invention include microparticles,
14 microcapsules, emulsions, liposomes, DMSO-glycerol and the
15 like.

16 The present vaccines can be administered by any
17 convenient route including subcutaneous, oral,
18 intramuscular, intravenous, intra-dermal, intraocular,
19 vaginal, trans-dermal or other parenteral or enteral
20 route. Similarly the vaccines can be administered as a
21 single dose or divided into multiple doses for
22 administration.

23 The vaccine compositions of the instant invention
24 contain an immunoeffective amount of a ratchet library to
25 treat or prevent disease or malignancy associated with the
26 source of the CTL epitope in that ratchet library.
27 Preferred vaccine compositions are effective for CTL
28 induction with respect to malaria, HIV, HCV, mucin, p53
29 and influenza and their associated pathogenic conditions.
30 Such compositions in dosage unit form can contain about 10
31 ng to about 2 mg of the peptide (or mixture of peptides)
32 per kg body weight. When delivered in multiple doses, the
33 dosage unit form is conveniently divided into the
34 appropriate amounts per dosage.

35 Accordingly, another aspect of this invention
36 provides a method of treating or preventing a disease or a

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1 malignancy which comprises administering an amount of the
2 library of Claim 1 to a mammal effective to stimulate a
3 CTL response against the disease or malignancy associated
4 with a CTL epitope present in said library. Based on the
5 source of the ratchet library (i.e., the virus, bacterium
6 or other organism from which the template peptide was
7 derived or a protein associated with malignancy), then one
8 skilled in the art can readily determine the amount needed
9 for delivery to obtain the desired therapeutic result,
10 that is, the amount of library to induce a CTL response of
11 therapeutic benefit for the disease or condition under
12 treatment. Typically these dosages ranges are as
13 indicated above for the vaccine formulation. Likewise,
14 one of ordinary skill in the art can readily determine an
15 efficacious formulation for delivery of the ratchet
16 library.

17 In another embodiment of this invention, a ratchet
18 peptide can be used to identify CTL epitopes within a
19 protein sequence. Hence, this invention is directed to a
20 method of constructing a library of related peptides to
21 provide a ratchet library which comprises identifying a
22 template peptide; calculating a distribution of amino
23 acids at each position x having those amino acids present
24 in the template peptide at positions x to $n-(1-x)$,
25 inclusive, wherein 1 is from about 7 to about 25, n is
26 from $1+1$ to about 100, and x is from 1 to 1; synthesizing
27 said ratchet library; and assaying said ratchet library
28 for the ability to stimulate CTL activity. For example,
29 the ratchet peptide is constructed and used to immunize
30 animals, typically though not necessarily mice. The
31 immunized animals are sacrificed and splenocytes removed
32 and cultured in vitro with a pool of overlapping
33 individual peptides that span the ratcheted template. The
34 activate splenocytes are then tested on target cells
35 pulsed with 50 μ M (for example) pooled peptides, and if
36 any CTL activity is present, the splenocytes are tested on

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1 the individual peptides derived from the region. If a
2 single peptide derived from the pool of overlapping
3 peptides is recognized, a new CTL epitope has been
4 identified.

5 The examples serve to illustrate the present
6 invention and are not to be used to limit the scope of the
7 invention.

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EXAMPLE 1

Malaria Ratchet Libraries Generate Malaria-Specific CTL

A. General Methods

Ratchet libraries were synthesized by standard F-moc chemistry using solid phase peptide synthesis with an F-moc RINK MBHA resin [4-(2',4'-dimethoxyphenyl-Fmoc-aminomethyl)-phenoxyacetamido-norleucyl-MBHA resin; MBHA is methylbenzhydrylamine] according to manufacturer's instructions on an ABI Model 433 peptide synthesizer or similar model. The ratchet libraries were synthesized as linear peptides or as branched peptides using a heptalysyl core.

Ratchet libraries were formulated at the indicated concentrations and then used for immunization as microparticles, emulsions or lipopeptides.

Microparticles were prepared according to the water-in-oil-water solvent evaporation method described in U.S. Serial No. 08/263,841, filed June 22, 1994, which is incorporated herein by reference, using polylactide-co-glycolide polymer Resomer RG 505 (Boehringer Ingelheim). Microparticles containing 100 µg of ratchet library were suspended in 0.5 ml phosphate-buffered saline (PBS) for intraperitoneal immunization of mice on days 0, 10 and 20 followed by sacrifice of the animals 7 to 10 days later.

Emulsions were prepared so that the final preparations contained 100 µg of ratchet library and 50 µg Pam₃Cys-seryl--lysyl-lysyl-lysyl-lysyl (Pam₃Cys-SKKKK) (SEQ ID NO:12) in a volume of 0.5 mL unless indicated otherwise. To prepare the emulsion, 4 mg of ratchet library was dissolved in 16 mL H₂O and 240 mg of egg lecithin was dispersed therein by homogenization (Model STD 1 fitted with a 0.25" tubular head, Silverson Machines, East Longmeadow, MA) at 10,000 rpm for 5 min. Pam₃Cys-SKKK (2 mg) was mixed with 4 g soya oil and then added to the library mixture by homogenization at 10,000

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rpm for 5 min. Further mixing was conducted by ultrasonic pulsation of the emulsion with an ultra sonic probe (Vibra cell, Sonics and Materials, Inc., Danbury, CT). The emulsions (0.5 mL) were injected intraperitoneally into mice on days 0 and 10 followed by sacrifice of the animals 7 to 10 days later.

For lipopeptide ratchet libraries, the peptides of the ratchet library were covalently coupled to Pam₃Cys (tripalmitoyl-5-glycerol-cysteine) as generally described (Deres et al.) to produce the corresponding lipopeptide ratchet library. The lipopeptide ratchet libraries (100 µg) were suspended in a 0.5 mL of 1% DMSO in glycerol and injected intraperitoneally into mice at day 0. The animals were sacrificed 7-9 days later.

Control mice were injected with 0.5 mL phosphate-buffered saline (PBS) using the corresponding injection schedule as that of the formulated ratchet library.

Upon sacrifice, the spleens were removed and splenocytes were pooled and cultured in vitro with the indicated peptide at a concentration of 1 µg/mL for one week to produce activated splenocytes.

CTL assays were then conducted according to the method of McDonald et al. (1980) as briefly described below.

B. Malarial Ratchet Libraries

A ratchet library was designed from a 20 amino acid sequence of residues 247-266 from the circumsporozoite protein of *Plasmodium berghei* (CS 247-266). This sequence contains the nonamer CTL epitope designated as CS 252-260 [Eberl et al. (1993) Int. Immunol. 5:1489-1492].

Malarial ratchet library 1 from the template peptide of SEQ ID NO:1 (Fig.1B, bottom panel) was prepared in linear form and formulated in microparticles at a final concentration of 200 µg/mL. Three BALB/C mice were immunized with 0.5 mL per injection as described above.

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1 Pooled splenocytes were cultured with *Plasmodium berghei*
2 CS peptide 252-260.

3 CS 252-260-specific CTL activity was then assayed.
4 Briefly, H-2^d target cells (mouse mastocytoma cell line
5 P815 or A20.1) were ⁵¹Cr labeled for one h, washed and then
6 incubated for one h in the presence of CS 252-260 peptide
7 at 50 μM, in the presence of an unrelated control peptide,
8 influenza nucleoprotein peptide NP 147-155 at 50 μM or in
9 media (i.e., in the absence of a peptide antigen). CTL
10 activity was determined by incubating these target cells
11 with activated splenocytes (effector cells) for 4 hours in
12 round-bottomed 96-well plates at a range of
13 effector:target (E:T) ratios of 100:1, 50:1, 25:1 and
14 12.5:1 and measuring the release of ⁵¹Cr. Cell lysis was
15 calculated as per cent target cell lysis from the formula
16 $(E-M/T-M) \times 100$, where E = experimental ⁵¹Cr release (cpm);
17 M = ⁵¹Cr release in presence of culture medium; and T =
18 total ⁵¹Cr released by 10% Triton X-100®.

19 The results are shown in Fig. 2 and indicate that
20 significant CTL lysis was elicited in an MHC-restricted
21 manner, since the K^d-restricted CS 252-260 peptide epitope
22 sensitized target cells incubated with that peptide and
23 not with any of the controls. Significant CTL lysis
24 occurs if there is greater than 10% lysis above the
25 control level of lysis at the highest E:T ratio.
26 Splenocytes from control mice did not elicit specific CTL
27 activity in any experiment.

28 Malaria ratchet library 2 (Fig. 1B, bottom panel) was
29 synthesized as branched octameric peptides on a heptalysyl
30 core and formulated in microparticles at a final
31 concentration of 2 mg/mL and injected into mice as
32 described above. Splenocytes were incubated and CTL
33 activity was assayed as described above using E:T ratios
34 of 100:1, 50:1 25:1 and 12.5:1. The results are shown in
35 Fig. 3.

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1 Malaria ratchet library 2 was formulated and injected
2 into mice as described in the preceding paragraph to
3 determine CTL activity against the 20 residue CS 247-266
4 peptide. Splenocytes were incubated with CS 252-260
5 peptide and CTL activity was determined as above, except
6 that the target cells were incubated in the presence of 50
7 μ M of CS 252-260 peptide, 50 μ M of CS 247-266 peptide or
8 in media using E:T ratios of 100:1, 50:1 25:1 and 12.5:1.
9 The results are shown in Fig. 4.

10 Malaria ratchet library 1 was formulated and injected
11 as a lipopeptide except that the lipopeptide ratchet
12 library was formulated at a concentration of 20 μ g/mL.
13 Splenocytes were incubated CS 252-160 and CTL activity was
14 assayed as described above using E:T ratios of 100:1, 50:1
15 25:1 and 12.5:1. The results are shown in Fig. 5.

16 Malaria ratchet library 1 was formulated and injected
17 as a lipopeptide ratchet library at a concentration of 200
18 μ g/mL. Splenocytes were incubated with CS 252-260 and CTL
19 activity was as described above to determine CTL activity
20 against a self peptide. For the CTL activity
21 determination, the target cells were incubated in the
22 presence of 50 μ M of CS 252-260 peptide, 50 μ M of a K^d-
23 restricted self peptide (SYFPEITHI; SEQ ID NO:13) or in
24 media using E:T ratios of 100:1, 50:1 25:1 and 12.5:1.
25 The results are shown in Fig. 6.

26 These malaria ratchet libraries elicit malaria-
27 specific CTL at immunogen doses ranging from 10 μ g to 1 mg
28 (Figs. 2-5). The CTL activity is MHC restricted and is
29 elicited when presented with the processed epitope as a
30 longer peptide (Fig. 4). CTL activity was absent when
31 target cells were pulsed with a K^d restricted self-peptide
32 to test for any auto-immune reaction. No significant
33 lysis was seen on the targets pulsed with the self
34 peptide.

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EXAMPLE 2

MHC-Binding Motif Restricted Ratchet Library

Fig. 7, bottom panel, illustrates an MHC-restricted malaria ratchet library from the template peptide of SEQ ID NO:1. This library is constructed from malaria ratchet library 1 of Fig. 1B (also top panel of Fig. 7) by replacing those positions which contain anchor residues for the K^b, D^b, K^d, and L^d molecules (see below) with an equal proportion of the anchor residues at the position in question. The anchor residues are those amino acids which have been identified as necessary for binding to the MHC class 1 molecule for the given haplotype. The anchor residues for the indicated haplotypes are shown in the middle panel of Fig. 7 and the MHC-restricted malaria ratchet library is shown in the bottom panel of Fig. 7.

At each of these positions (i.e., positions 2, 5, 8 and 9), the ratchet incorporates only those anchor amino acids shown in the middle panel. Thus, position 2 contains 50% tyrosine Y and 50 % proline; position 5 contains 33% asparagine, 33% tyrosine and 33% phenylalanine; position 8 contains 100% leucine and position 9 contains 25% isoleucine, 25% leucine, 25% phenylalanine and 25% methionine. Thus, this ratchet has the anchor residues involved in binding MHC class I molecules and stimulating CTL.

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EXAMPLE 3Ratchet Libraries Can Accomodate
Antigenic Diversity

An HIV ratchet library was constructed from the SSAL (Fig. 8A, bottom panel) of template peptide SEQ ID NO:2 and the additional 14 HIV-1 sequences (Fig. 8A) from a 35 amino acid sequence of the HIV-1 gp120 V3 loop region, the principle neutralizing domain known to have extensive sequence variability. This region contains a D^d restricted CTL epitope at amino acid positions 318-326. The antigenic diversity of this region is accommodated by taking 15 HIV-1 consensus sequences including the sequence HIV-MVP5188 and constructing an SSAL library where the identity and ratio of amino acids at each position is determined by the relative prevalence of amino acids in those 16 sequences. Next, it is the SSAL library which is "ratcheted" to yield the HIV-1 ratchet library shown in the bottom panel of Fig. 8.

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EXAMPLE 4Peptide Ratchet Libraries from
Different Length Template Peptides

To examine the effect of template peptide length on the efficacy of CTL induction via ratchet libraries, three HIV-1 gag peptide linear ratchet libraries (Figs. 9-11, bottom panel) containing a mouse HIV CTL epitope were synthesized using template peptides of lengths 100, 40 or 20 amino acids of the gag sequence as shown in Figs. 9-11, respectively, and designated by SEQ ID NOS:3-5, respectively.

These libraries were formulated with 100 μ g in 0.5 mL as microparticles, emulsions or lipopeptides and injected as described in Example 1 with the following modifications: The immunized mice were C57BL/6 mice. Activated splenocytes were prepared by culturing with 1 μ g/mL HIV gag peptide 390-398 [Elvin et al. (1993) J. Immunol. Methods 158:161-171]. The target cells were EL4 and were incubated with 50 μ M HIV gag peptide 390-398. The results are presented in Table 1. Fig. 12 show the specific cell lysis results for the emulsion formulation of the ratchet library from the 40 amino acid template.

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Table 1

CTL Response from HIV-1 Ratchet Libraries

Formulation ^a	Pam ₃ Cys ^b	Template Peptide Length ^c		
		20	40	100
Lipopeptide	+ ^d	+	±	++
Emulsion	+	±	+++	-
Microparticle	-	-	-	-

^a The formulations are described in Example 1 and the experimental protocol in Example 4.

^b Pam₃Cys is covalently bound to the ratchet library for the lipopeptide preparation but not in the emulsion preparation.

^c The template peptide of the indicated amino acid length was ratcheted to 9-mers as described in Example 4.

^d The symbols are as follows: +++, ++ and + mean significant specific target cell lysis in the indicated relative amounts with +++ as the most lysis; ±, inconclusive amount of specific cell lysis; -, no significant specific cell lysis.

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EXAMPLE 5

Mucin Ratchet Libraries

Fig 13. (bottom panel) provides a mucin ratchet library constructed from the template peptide of SEQ ID NO:7 or SEQ ID NO:8. Mucin is a large, heavily glycosylated molecule expressed and secreted by ductal epithelial cells and tumors. Mucin consists of multiple copies of a 20 amino acid tandem repeat (SEQ ID NO:6) which appears to elicit non-MHC restricted CTL responses.

Because the 20-mer repeat will not contain every possible nonomer when used as a template, due to end effects in the ratchet, an alternative approach was used to generate all possible nonomers of the repeating 20-mer peptide. In this case the last eight carboxy-terminal amino acids of the 20-mer repeat peptide were placed at its amino terminus to yield a template peptide of 28 amino acids before calculation of the ratchet (Fig. 13, middle panel). Alternatively, the first eight amino-terminal amino acids were placed at its carboxyl terminus to yield a template peptide of 28 amino acids before calculation of the ratchet (Fig. 13, middle panel). With either method the calculated mucin ratchet library is the same.

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EXAMPLE 6

Mutant p53 Ratchet Libraries

The protein p53 is a tumor supressor which fails to function effectively when mutated. More than 50% of human tumors contain cells which express a mutant form of p53, due to one or more point mutations in the protein. Class I mutations in p53 affect residues that directly contact DNA and include the residues lysine at postion 120, serine at position 241, arginine at position 248, arginine at position 273, alanine at position 276, cysteine at position 277 and arginine at positon 283. In this group, the mutations of arginine at positions 248 and 273 appear most frequently. Class II mutants affect residues that do not contact DNA but rather appear to have a role in stabilizing protein structure and include mutations of arginine at positions 175 and 249.

This example provides four peptide ratchet libraries containing four hot spots of mutation of p53.

The four ratchets are designed around mutation hot spots in the protein: (1) template peptide of amino acids 124-151 (SEQ ID NO:9) as a 10-mer ratchet library (Fig. 14, bottom panel); (2) template peptide of amino acids 166-187 as a 9-mer ratchet library; (3) template peptide of amino acids 228-256 as a 9-mer ratchet library; and (4) template peptide of amino acids 264-289 as a 9-mer ratchet library.

The ratchet library from the 166-187 template is especially useful for treating colon cancer since this mutation is frquently encountered with this malignancy.

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EXAMPLE 7

Influenza Ratchet Libraries

Existing influenza vaccines are complex to design and manufacture as the prevalent strain of influenza can rapidly change and a vaccine designed to stimulate antibodies against influenza A of strain one may not be effective in eliciting cross-reactive immunity against strain two. The CTL response has been shown to be effective against influenza in animal models and in humans, and the addition of an influenza specific CTL component to existing vaccines, or a CTL inducing vaccine alone, would dramatically broaden protection against many strains of influenza.

To provide a vaccine capable of stimulating a CTL response against influenza, known CTL epitopes can be ratcheted. Fig. 15 (bottom panel) shows influenza ratchet library 1 from a 25-mer template peptide of residues 139-163 (SEQ ID NO:10) of influenza A A/34/PR8 nucleoprotein. This library encompasses the known K^d-restricted epitope of residues 147-155. Fig. 16 (bottom panel) shows influenza ratchet library 2 constructed from the template peptide of SEQ ID NO:11 which is a linkage of 3 CTL epitopes in order from N to C terminus of residues 50-58, 147-155 and 366-374 of the nucleoprotein.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

- (i) APPLICANT: Kuebler, Peter J.
Nixon, Douglas F.
- (ii) TITLE OF INVENTION: Peptide Ratchets for Vaccines
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- (v) COMPUTER READABLE FORM:
 - (A) MEDIUM TYPE: Floppy disk
 - (B) COMPUTER: IBM PC compatible
 - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
 - (D) SOFTWARE: PatentIn Release #1.0, Version
#1.25
- (vi) CURRENT APPLICATION DATA:
 - (A) APPLICATION NUMBER:
 - (B) FILING DATE:
 - (C) CLASSIFICATION:
- (viii) ATTORNEY/AGENT INFORMATION:
 - (A) NAME: Wilson, M. Lisa
 - (B) REGISTRATION NUMBER: 34,045
 - (C) REFERENCE/DOCKET NUMBER: 2012
- (ix) TELECOMMUNICATION INFORMATION:
 - (A) TELEPHONE: (516)273-2828
 - (B) TELEFAX: (516)273-1717

(2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 20 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: peptide

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```

Asn Asn Asn Asp Asp Ser Tyr Ile Pro Ser Ala Glu
 1           5           10
Lys Ile Leu Glu Phe Val Lys Gln
      15           20

```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 35 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```

Cys Thr Arg Pro Asn Asn Asn Thr Arg Lys Ser Ile
 1           5           10
His Ile Gly Pro Gly Arg Ala Phe Tyr Thr Thr Gly
      15           20
Glu Ile Ile Gly Asp Ile Arg Gln Ala His Cys
 25           30           35

```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 100 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```

Ile Arg Gln Gly Pro Lys Glu Pro Phe Arg Asp Tyr
 1           5           10
Val Asp Arg Phe Tyr Lys Thr Leu Arg Ala Glu Gln
      15           20
Ala Ser Gln Glu Val Lys Asn Trp Met Thr Glu Thr
 25           30           35
Leu Leu Val Gln Asn Ala Asn Pro Asp Cys Lys Thr
      40           45
Ile Leu Lys Ala Leu Gly Pro Ala Ala Thr Leu Glu
      50           55           60
Glu Met Met Thr Ala Cys Gln Gly Val Gly Gly Pro
      65           70
Gly His Lys Ala Arg Val Leu Ala Glu Ala Met Ser
      75           80
Gln Val Thr Asn Ser Ala Thr Ile Met Met Gln Arg
      85           90           95

```

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Gly Asn Phe Leu
100

(2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 40 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Glu	Met	Met	Thr	Ala	Cys	Gln	Gly	Val	Gly	Gly	Pro
1				5			10				
Gly	His	Lys	Ala	Arg	Val	Leu	Ala	Glu	Ala	Met	Ser
	15					20					
Gln	Val	Thr	Asn	Ser	Ala	Thr	Ile	Met	Met	Gln	Arg
25					30					35	
Gly	Asn	Phe	Leu								
			40								

(2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

Glu	Ala	Met	Ser	Gln	Val	Thr	Asn	Ser	Ala	Thr	Ile
1				5					10		
Met	Met	Gln	Arg	Gly	Asn	Phe	Leu				
	15					20					

(2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 20 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

Pro	Asp	Thr	Arg	Pro	Ala	Pro	Gly	Ser	Thr	Ala	Pro
1				5					10		

(2) INFORMATION FOR SEQ ID NO:7:

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

```

Pro  Ala  His  Gly  Val  Thr  Ser  Ala  Pro  Asp  Thr  Arg
 1          5          10
Pro  Ala  Pro  Gly  Ser  Thr  Ala  Pro  Pro  Ala  His  Gly
          15          20
Val  Thr  Ser  Ala
 25

```

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

```

Pro Asp Thr Arg Pro Ala Pro Gly Ser Thr Ala Pro
 1          5          10
Pro Ala His Gly Val Thr Ser Ala Pro Asp Thr Arg
      15          20
Pro Ala Pro Gly
 25

```

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 28 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

Cys Thr Tyr Ser Pro Ala Leu Asn Lys Met Phe Cys
1 5 10

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Gln Leu Ala Lys Thr Cys Pro Val Gln Leu Trp Val
 15 20
 Asp Ser Thr Pro
 25

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 25 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Trp His Ser Asn Leu Asn Asp Ala Thr Tyr Gln Arg
 1 5 10
 Thr Arg Ala Leu Val Arg Thr Gly Met Asp Pro Arg
 15 20
 Met
 25

(2) INFORMATION FOR SEQ ID NO:11:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 26 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

Ser Asp Tyr Glu Gly Arg Leu Ile Thr Tyr Gln Arg
 1 5 10
 Thr Arg Ala Leu Val Ala Ser Asn Glu Asn Met Glu
 15 20
 Thr Met
 25

(2) INFORMATION FOR SEQ ID NO:12:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 6 amino acids
 - (B) TYPE: amino acid
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

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(ix) FEATURE:

- (A) NAME/KEY: Modified-site
- (B) LOCATION: 1
- (D) OTHER INFORMATION: /note= "N-terminal
tripalmitoyl-5-glycerol"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

Cys Ser Lys Lys Lys Lys
1 5

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 9 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

Ser Tyr Phe Pro Glu Ile Thr His Ile
1 5

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We Claim:

1. A ratchet library of peptides comprising at least one immunostimulatory cytotoxic T lymphocyte (CTL) epitope wherein said peptides are of length l ; the sequences of said peptides in said library being determined from a template peptide of length from $l+1$ to n amino acids such that each position x in the library has all the amino acids present in said template peptide at positions x to $n-(l-x)$, inclusive; the ratio of amino acids at each position x being determined by the relative prevalence of amino acids at that position x ; and wherein l is from about 7 to about 25 amino acids, n is from $l+1$ to about 100, and x is from 1 to l .

2. The library of Claim 1 wherein n is from $l+1$ to about 75.

3. The library of Claim 1 wherein n is from $l+1$ to about 50.

4. The library of Claim 1 wherein l is from 8 to 10.

5. The library of Claim 1 wherein l is 9.

6. The library of Claim 1 wherein if a position x is identified as part of an MHC-binding motif of a CTL epitope, then that position x is fixed as one or more amino acids of said MHC-binding motif in an equimolar ratio.

7. The library of any one of Claims 1 to 6 wherein said CTL epitope is from a virus, bacterium, parasite, tumor antigen, allergen or other protein antigen.

8. The library of Claim 1 to 6 wherein said CTL epitope is from a melanoma protein including MAGE-1, -2, and -3; a renal cell carcinoma protein; a colon carcinoma protein; a prostate cancer protein (malignant or benign) including PSA; tyrosinase; an oncogene such as HER-2/neu proto-oncogene; ras; MUC1; p53; p16; TL; an HIV-1 or HIV-2 protein including envelope, gag, pol, nef, tat, rev, vpx or vpu; an HTLV I or II protein including envelope, gag, pol, pX or TAX; lymphocytic choriomeningitis virus of

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mice; influenza A, B or C including PB1, PB2, PA, NS1, M1, NP or HA; an Epstein-Barr virus protein including TETA, EENL, EBNA3, EBNA1 or LMP; respiratory syncytia virus; hepatitis B virus; hepatitis C virus; herpes simplex virus; cytomegalovirus; a parainfluenza virus 1 protein including hemagglutinin, neuraminidase, phosphoprotein or nucleoprotein; intracisternal A particle gag; bovine leukemia virus; papilloma viruses; a malaria protein including proteins from *P. falciparum*, *P. berghei*, *P. ovale*, *P. vivax*, *P. malaria*; *Histoplasma capsulatum*; *Listeria*; *Toxoplasmosis*; *Trypanosoma cruzi*; *Yersinia*; *M. tuberculi*; *M. lepri*; *Pneumocystis carinii*; Kaposi's sarcoma or frameshift sequences.

9. The library of Claim 1 wherein said template peptide is any one of SEQ ID NOS: 1-11.

10. The library of any one of Claims 1-6 or 9 wherein said peptides of said library have a covalently attached N-terminal tripalmitoyl-5-glycerol-cysteine moiety.

11. The library of any one of Claims 1-6 or 9 wherein said peptides are linked to a branched core sequence, are polymerized or are conjugated to a carrier molecule.

12. A pharmaceutical or vaccine composition comprising the library of any one of Claims 1-6 or 9, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

13. The composition of Claim 12 wherein said composition is an emulsion or a microparticle formulation.

14. The composition of Claim 12 wherein said formulation also comprises tripalmitoyl-5-glycerolcysteine or a derivative thereof.

15. A pharmaceutical or vaccine composition comprising the library of Claim 7, or a pharmaceutically acceptable salt thereof, and a pharmaceutically acceptable carrier.

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16. The composition of Claim 15 wherein said composition is an emulsion or a microparticle formulation.

17. The composition of Claim 15 wherein said formulation also comprises tripalmitoyl-5-glycerolcyteine or a derivative thereof.

18. A method of treating or preventing a disease or a malignancy which comprises administering an amount of said composition of Claim 12 to a mammal effective to stimulate a CTL response against said disease or said malignancy associated with said CTL epitope present in said library.

19. A method of treating or preventing a disease or a malignancy which comprises administering an amount of said composition of Claim 15 to a mammal effective to stimulate a CTL response against said disease or said malignancy associated with said CTL epitope present in said library.

20. A method of constructing a library of related peptides to provide a ratchet library which comprises identifying a template peptide; calculating a distribution of amino acids at each position x having those amino acids present in the template peptide at positions x to $n-(1-x)$, inclusive, wherein 1 is from about 7 to about 25, n is from $1+1$ to about 100, and x is from 1 to l ; and synthesizing said ratchet library.

[illegible]

Figure 1A

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Sequence Alignment by Position

1	2	3	4	5	6	7	8	9
N	N	N	D	D	S	Y	I	P
N	N	D	D	S	Y	I	P	S
N	D	D	S	Y	I	P	S	A
D	S	Y	I	P	S	A	E	K
S	Y	I	P	S	A	E	K	I
Y	I	P	S	A	E	K	I	L
I	P	S	A	E	K	I	L	E
P	S	A	E	K	I	L	E	F
S	A	E	K	I	L	E	F	V
A	E	K	I	L	E	F	V	K
E	K	I	L	E	F	V	K	Q

AA Distribution by Position

	1	2	3	4	5	6	7	8	9
AA residue									
N	3	2	1						
D	2	2	2	2	1				
S	2	2	2	2	2	2	1	1	1
Y	1	1	1	1	1	1	1		
I	1	1	2	2	2	2	2	2	1
P	1	1	1	1	1	1	1	1	1
A	1	1	1	1	1	1	1	1	1
E	1	1	1	1	2	2	2	2	2
K		1	1	1	1	1	1	2	2
L				1	1	1	1	1	1
F						1	1	1	1
V							1	1	1
Q									1

AA Percentage by Position

	1	2	3	4	5	6	7	8	9
AA residue									
N	25	17	8						
D	17	17	17	17	8				
S	17	17	17	17	17	17	8	8	8
Y	8	8	8	8	8	8	8		
I	8	8	17	17	17	17	17	17	8
P	8	8	8	8	8	8	8	8	8
A	8	8	8	8	8	8	8	8	8
E	8	8	8	8	17	17	17	17	17
K		8	8	8	8	8	8	17	17
L				8	8	8	8	8	8
F						8	8	8	8
V							8	8	8
Q									8

Figure 1B

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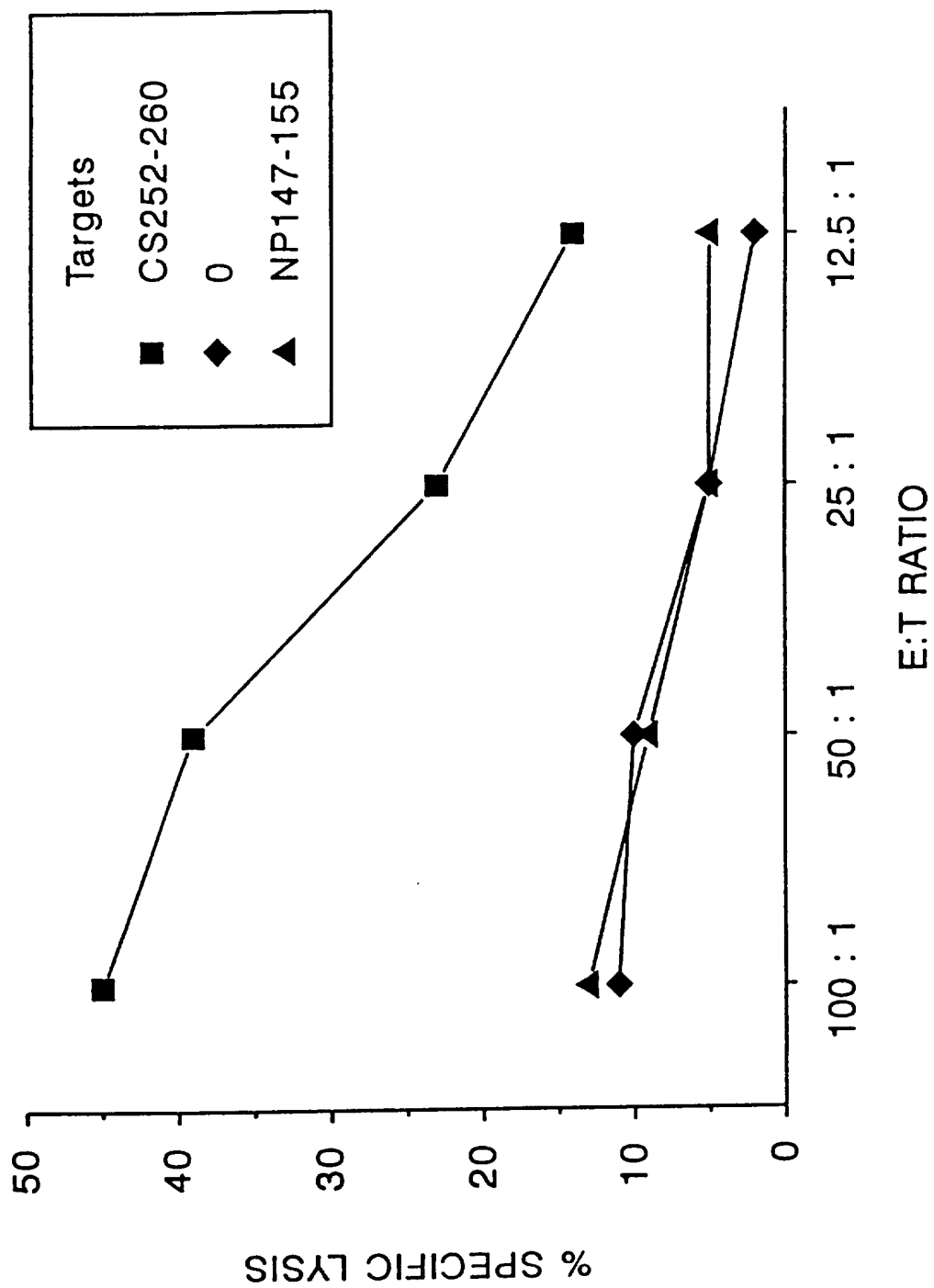


Figure 2

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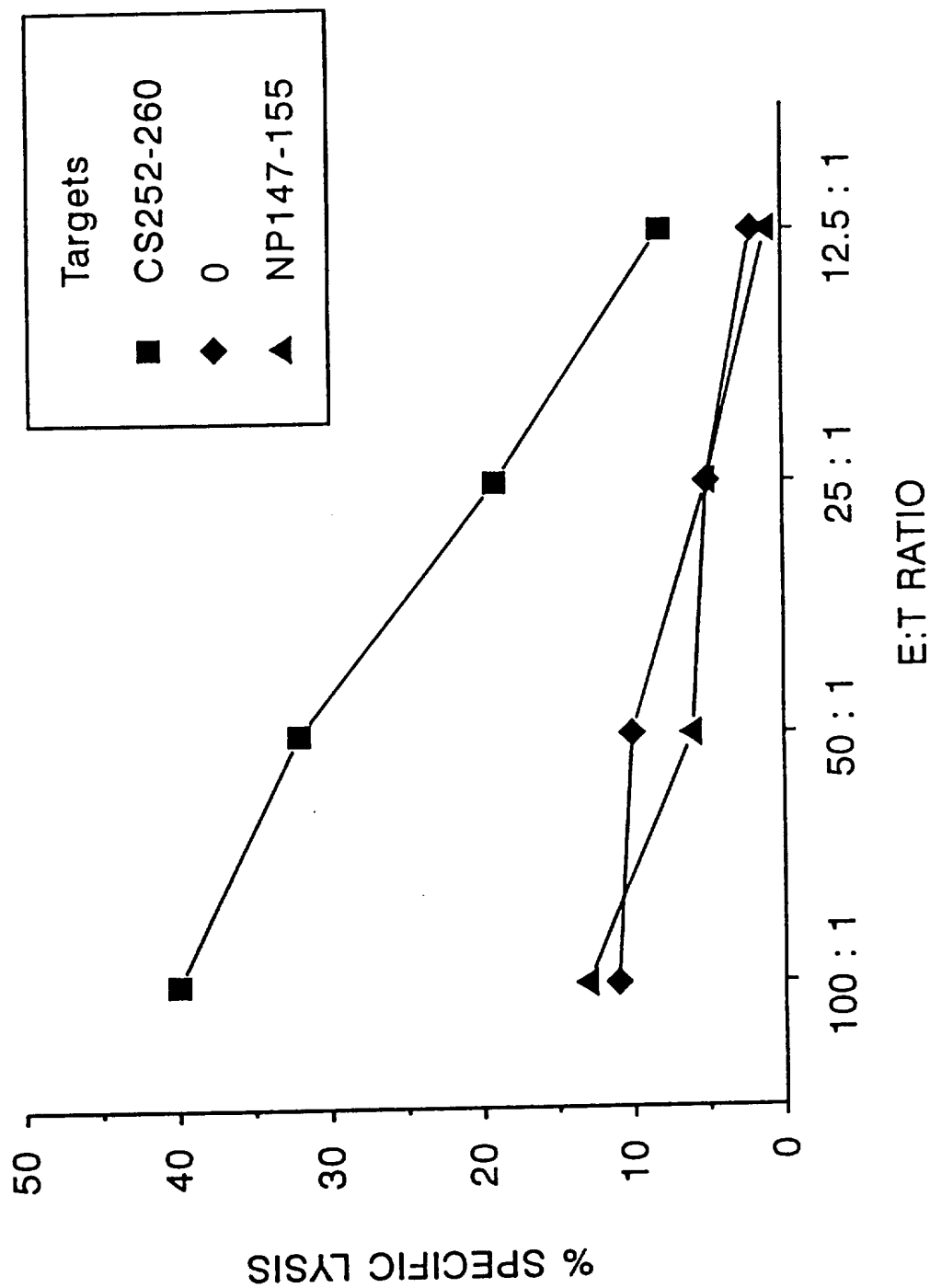


Figure 3

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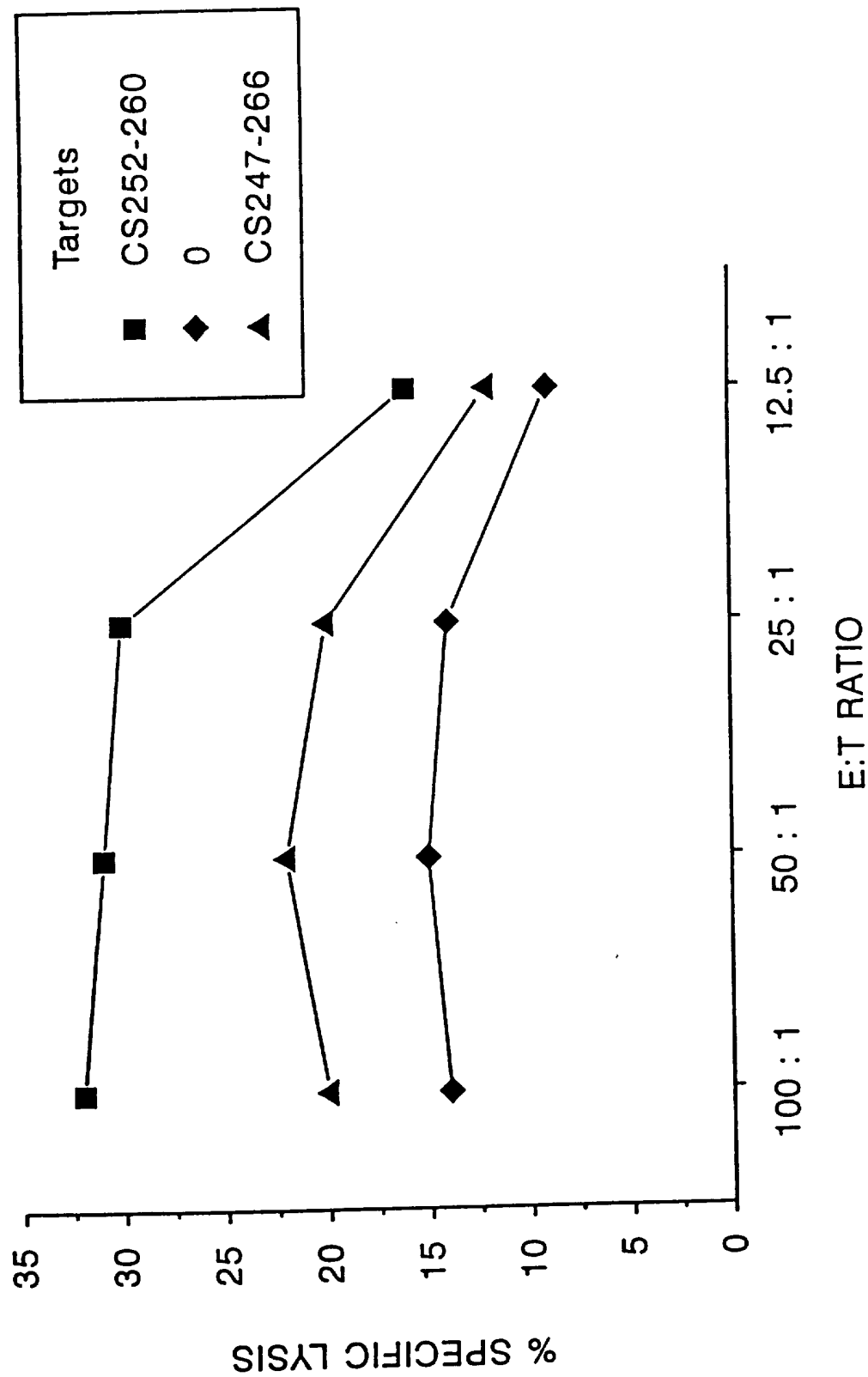


Figure 4

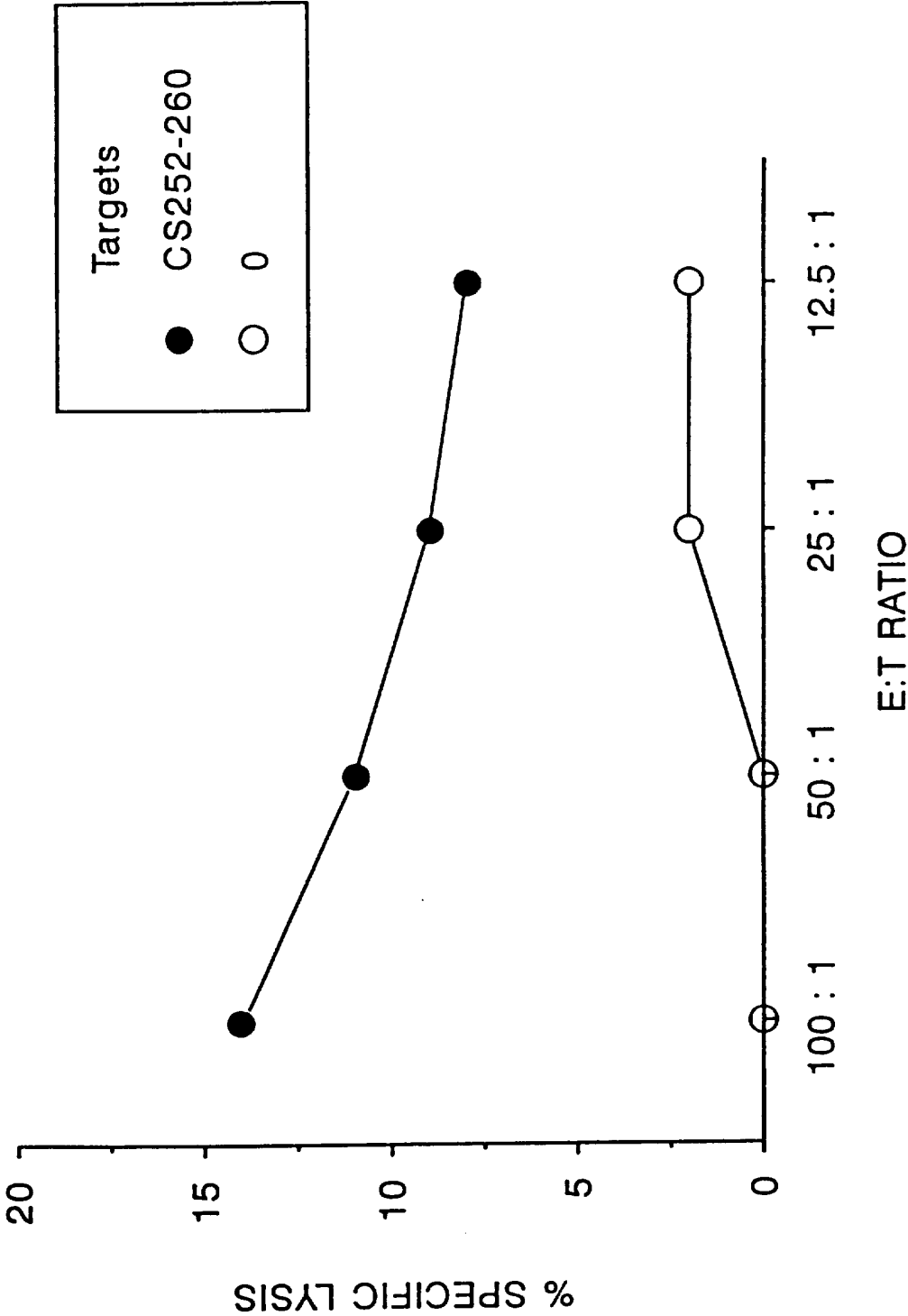


Figure 5

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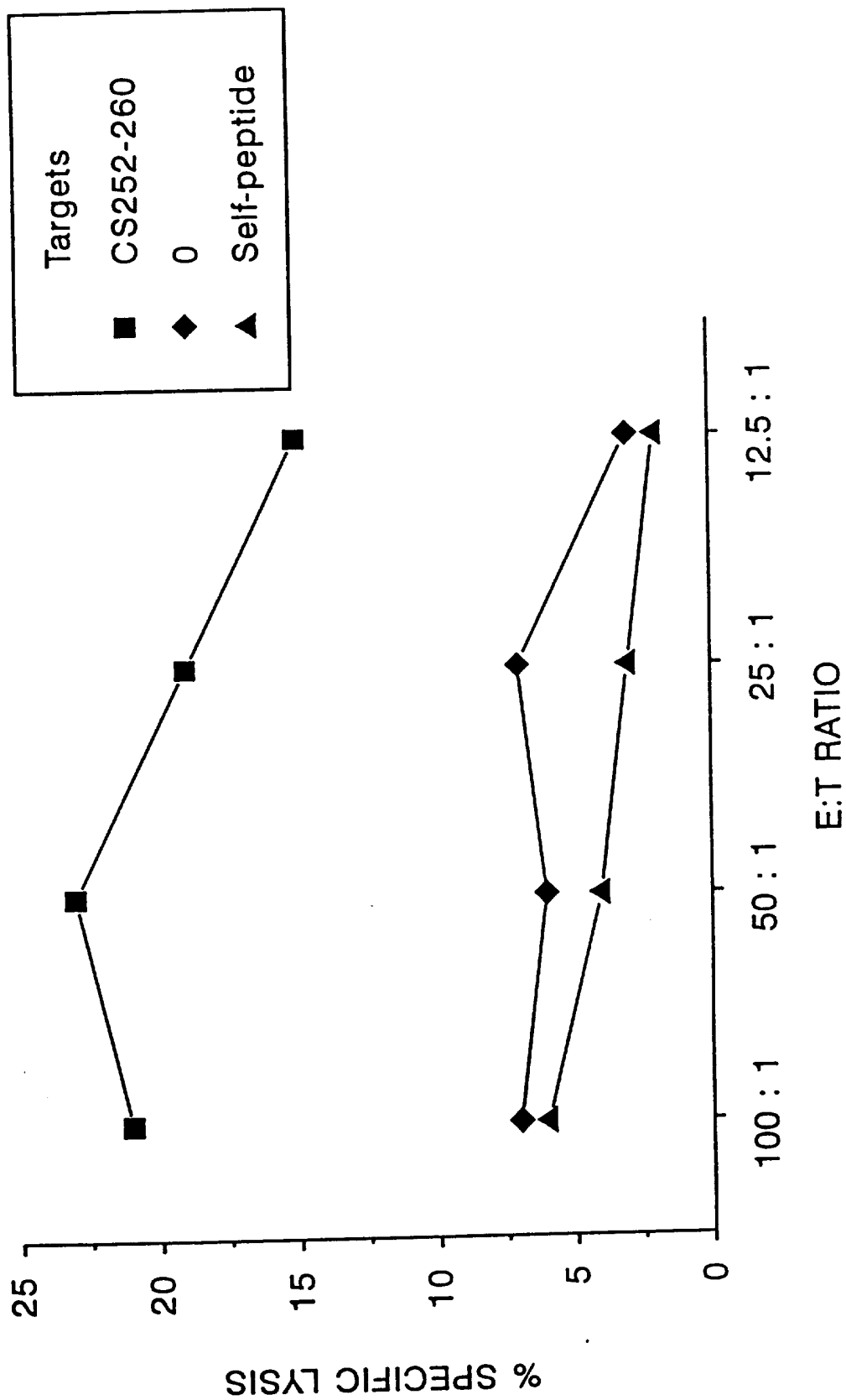


Figure 6

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		AA Percentage by Position								
AA residue		1	2	3	4	5	6	7	8	9
N		25	17	8						
D		17	17	17	17	8				
S		17	17	17	17	17	17	8	8	8
Y		8	8	8	8	8	8	8		
I		8	8	17	17	17	17	17	17	8
P		8	8	8	8	8	8	8	8	8
A		8	8	8	8	8	8	8	8	8
E		8	8	8	8	17	17	17	17	17
K			8	8	8	8	8	8	17	17
L					8	8	8	8	8	8
F							8	8	8	8
V								8	8	8
Q										8

		AA Restrictions by Position								
Haplotype										
K ^d		Y								I
										L
D ^b										M
K ^b										
L ^d		P								M
										L
										F

		Restricted AA Percentage by Position								
AA residue		1	2	3	4	5	6	7	8	9
N		25		8		33				
D		17		17	17					
S		17		17	17		17	8		
Y		8	50	8	8	33	8	8		
I		8		17	17		17	17		25
P		8	50	8	8		8	8		
A		8		8	8		8	8		
E		8		8	8		17	17		
K				8	8		8	8		
L					8		8	8	100	25
F						33	8	8		25
V								8		
Q										
M										25

Figure 7

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CONSENSUS																																				
B	C	T	R	P	N	N	N	T	R	K	S	I	H	I	G	P	G	R	A	F	Y	T	T	G	E	I	I	G	D	I	R	Q	A	H	C	
1	c	t	r	p	n	n	n	t	t	k	s	i	h	i	g	p	g	r	a	f	y	t	t	g	d	i	i	g	d	i	r	q	a	h	c	
2	c	t	r	p	n	n	n	t	t	k	s	i	h	i	g	p	g	r	a	f	y	A	A	t	g	e	i	i	g	d	i	r	q	a	h	c
3	c	t	r	p	n	n	n	t	t	k	s	i	S	i	g	p	g	r	a	f	y	A	A	t	g	k	i	i	g	d	i	r	q	a	h	c
4	c	t	r	p	n	n	n	t	t	k	s	i	R	i	g	p	g	Q	a	f	y	A	A	t	g	d	i	i	g	d	i	r	q	a	h	c
5	c	I	r	p	n	n	n	t	t	k	s	i	h	i	g	p	g	r	a	f	y	A	A	t	g	d	i	i	g	d	i	r	q	a	h	c
6	c	t	r	p	n	S	k	n	t	t	s	i	h	i	g	p	g	Q	V	f	y	t	t	t	K	Q	i	i	g	d	i	r	q	a	h	c
7	c	t	r	p	p	S	k	n	t	t	s	i	T	i	g	p	g	Q	V	f	y	R	R	t	g	d	i	i	g	d	i	r	q	a	h	c
8	c	t	r	p	p	F	k	n	t	t	s	i	T	i	g	p	g	r	V	f	y	K	K	t	g	s	i	T	g	d	i	r	K	a	h	c
9	c	t	r	p	p	Y	n	n	t	t	s	i	h	h	i	g	p	Q	a	l	y	K	K	t	g	e	i	i	g	d	i	r	q	a	h	c
10	c	t	r	p	p	Y	n	n	t	t	s	i	h	h	i	g	p	Q	a	l	y	t	t	t	n	i	i	g	D	I	R	Q	A	H	C	
11	c	t	r	p	p	Y	n	n	t	t	s	i	h	h	i	g	p	Q	a	l	y	t	t	t	n	i	i	g	D	I	R	Q	A	H	C	
12	c	t	r	p	p	Y	n	n	t	t	s	i	h	h	i	g	p	Q	a	l	y	t	t	t	n	i	i	g	D	I	R	Q	A	H	C	
13	c	t	r	p	p	Y	n	n	t	t	s	i	h	h	i	g	p	Q	a	l	y	t	t	t	n	i	i	g	D	I	R	Q	A	H	C	
14	c	t	r	p	p	Y	n	n	t	t	s	i	h	h	i	g	p	Q	a	l	y	t	t	t	n	i	i	g	D	I	R	Q	A	H	C	
HIVMVP5180	c	I	r	E	G	I	A	E	V	Q	D	i	Y	t	g	p	M	M	T	f	y	N	N	t	l	k	r	s	n	t	s	p	r	s	r	

C T R P N N N T R K S I H														I G P G R A F Y T														T G E I I G D I R Q A H C													
C15 T13 R15 P14 I2	E1	S2	K2	K1	M1	S1	T2	R3	T5	P2	P1	H6 I13	G15 P12 G14 R9	A10 F11	Y14	T7	Q1	M1	Q6	V2	L3	L1	A4	T15	G8	E2	I10	I8	G9	D9	I9	P9	Q7	A9	H7	C9					
F1	D1	A1	I1	K1	Q6	A1	F1	S2	T1	L2	M1	Q1	Y1	K2	N1	K2	T1	G4	Y1	N1	G2	K1	A5	R1	S1	E1	Q1	K1	S2	I1	T1	S1	P1	C5							
Y6	Q1	G1	I1	E1	Q1	G1	D1	P2	Y1	S1	T1	R1	N1	L1	S2	R3	I4																								

Figure 8A

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AA Distribution by Position									
	1	2	3	4	5	6	7	8	9
AMINO ACID									
C	15							5	9
T	59	59	46	47	47	47	47	47	36
R	48	48	48	36	45	45	46	46	45
P	29	29	29	29	16	16	16	16	16
I	48	49	42	51	51	51	50	50	49
K	17	17	17	17	18	18	16	15	14
E	5	5	5	5	4	4	4	4	3
N	30	31	30	30	30	25	15	2	2
F	12	12	12	12	12	11	11	11	11
Y	22	23	23	23	23	17	17	17	17
G	44	53	53	55	55	54	54	54	54
H	6	6	6	6	6	6	11	11	11
L	7	7	7	7	7	7	7	7	7
Q	17	17	17	17	21	21	20	20	20
S	17	17	17	17	18	16	16	16	16
D	6	9	18	18	18	18	17	17	17
A	17	17	17	17	17	17	26	25	25
V	3	3	3	3	3	3	3	3	3
M	3	3	3	3	3	3	3	3	2

AA Percentage by Position									
	1	2	3	4	5	6	7	8	9
AMINO ACID									
C	4							1	3
T	15	15	12	12	12	13	13	13	10
R	12	12	12	9	12	12	12	13	13
P	7	7	7	7	4	4	4	4	5
I	12	12	11	13	13	13	13	13	14
K	4	4	4	4	4	4	4	4	4
E	1	1	1	1	1	1	1	1	1
N	7	7	8	8	8	7	4	1	1
F	3	3	3	3	3	3	3	3	3
Y	5	5	6	6	6	5	5	5	5
G	11	13	13	14	14	14	14	15	15
H	2	2	2	2	2	2	3	3	3
L	2	2	2	2	2	2	2	2	2
Q	4	4	4	4	5	5	5	5	6
S	4	4	4	4	4	4	4	4	5
D	2	2	5	5	5	5	5	5	5
A	5	5	5	5	5	5	7	7	7
V	1	1	1	1	1	1	1	1	1
M	1	1	1	1	1	1	1	1	1

Figure 8B

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
 I R Q G P K E P F R D Y V D R F Y K T L R A E Q A
 26 27 28 29 30 31 32 33 34 35 36 37 38 39 40 41 42 43 44 45 46 47 48 49 50
 S Q E V K N W M T E T L L V Q N A N P D C K T I L
 51 52 53 54 55 56 57 58 59 60 61 62 63 64 65 66 67 68 69 70 71 72 73 74 75
 K A L G P A A T L E E M M T A C Q G V G G P G H K
 76 77 78 79 80 81 82 83 84 85 86 87 88 89 90 91 92 93 94 95 96 97 98 99 100
 A R V L A E A M S Q V T N S A T I M M Q R G N F L

AA Distribution by Position		1	2	3	4	5	6	7	8	9
AMINO ACID										
I		3	2	2	2	2	2	2	2	2
R		5	5	4	4	5	5	5	5	5
Q		6	6	6	6	6	6	6	6	6
G		6	6	6	6	5	6	6	6	6
P		5	5	5	5	5	4	4	4	3
K		6	6	6	6	6	6	5	5	5
E		7	7	7	7	7	7	7	6	6
F		2	2	2	2	2	2	2	3	3
D		3	3	3	3	3	3	3	3	3
Y		2	2	2	2	2	2	2	2	2
V		6	6	6	6	6	6	6	6	6
T		8	8	8	8	8	8	8	8	8
L		7	7	7	7	7	7	7	7	8
A		11	11	11	11	11	11	11	11	11
N		4	4	4	4	4	4	5	5	5
S		3	3	3	3	3	3	3	3	3
W		1	1	1	1	1	1	1	1	1
M		4	5	6	6	6	6	6	6	6
C		2	2	2	2	2	2	2	2	2
H		1	1	1	1	1	1	1	1	1

AA Percentage by Position		1	2	3	4	5	6	7	8	9
AMINO ACID										
I		3	2	2	2	2	2	2	2	2
R		5	5	4	4	5	5	5	5	5
Q		7	7	7	7	7	7	7	7	7
G		7	7	7	7	7	7	7	7	7
P		5	5	5	5	5	4	4	4	3
K		7	7	7	7	7	7	5	5	5
E		8	8	8	8	8	8	8	7	7
F		2	2	2	2	2	2	2	3	3
D		3	3	3	3	3	3	3	3	3
Y		2	2	2	2	2	2	2	2	2
V		7	7	7	7	7	7	7	7	7
T		9	9	9	9	9	9	9	9	9
L		8	8	8	8	8	8	8	8	9
A		12	12	12	12	12	12	12	12	12
N		4	4	4	4	4	4	5	5	5
S		3	3	3	3	3	3	3	3	3
W		1	1	1	1	1	1	1	1	1
M		4	5	7	7	7	7	7	7	7
C		2	2	2	2	2	2	2	2	2
H		1	1	1	1	1	1	1	1	1

Figure 9

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1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E	M	M	T	A	C	Q	G	V	G	G	P	G	H	K	A	R	V	L	A
21	22	23	24	25	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40
E	A	M	S	Q	V	T	N	S	A	T	I	M	M	Q	R	G	N	F	L

AA Distribution by Position										
		1	2	3	4	5	6	7	8	9
AMINO ACID										
I		1	1	1	1	1	1	1	1	1
R		1	1	1	1	2	2	2	2	2
Q		2	2	2	3	3	3	3	2	2
G		4	4	4	4	4	5	5	5	4
P		1	1	1	1	1	1	1	1	1
K		1	1	1	1	1	1	1	1	1
E		2	1	1	1	1	1	1	1	1
V		3	3	3	3	3	3	3	3	3
T		3	3	3	3	2	2	2	2	2
L		1	1	1	1	1	1	1	1	2
A		5	5	5	5	5	4	4	4	4
N		1	1	1	1	1	1	2	2	2
S		2	2	2	2	2	2	2	2	2
M		3	4	4	3	3	3	3	3	3
C		1	1	1	1	1	1			
H		1	1	1	1	1	1	1	1	1
F									1	1

AA Percentage by Position										
		1	2	3	4	5	6	7	8	9
AMINO ACID										
I		3	3	3	3	3	3	3	3	3
R		3	3	3	3	6	6	6	6	6
Q		6	6	6	9	9	9	9	6	6
G		13	13	13	13	13	16	16	16	13
P		3	3	3	3	3	3	3	3	3
K		3	3	3	3	3	3	3	3	3
E		6	3	3	3	3	3	3	3	3
V		9	9	9	9	9	9	9	9	9
T		9	9	9	9	6	6	6	6	6
L		3	3	3	3	3	3	3	3	6
A		16	16	16	16	16	13	13	13	13
N		3	3	3	3	3	3	6	6	6
S		6	6	6	6	6	6	6	6	6
M		9	13	13	9	9	9	9	9	9
C		3	3	3	3	3	3			
H		3	3	3	3	3	3	3	3	3
F									3	3

Figure 10

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1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
E	A	M	S	Q	V	T	N	S	A	T	I	M	M	Q	R	G	N	F	L

		AMINO ACID POSITION#								
		1	2	3	4	5	6	7	8	9
AMINO ACID										
I		1	1	1	1	1	1	1	1	1
R						1	1	1	1	1
Q		1	1	1	2	2	1	1	1	1
G							1	1	1	1
E		1								
V		1	1	1	1	1	1			
T		2	2	2	2	2	2	2	1	1
L										1
A		2	2	2	2	2	2	2	2	2
N		1	1	1	1	1	1	2	2	1
S		2	2	2	2	1	1	1	1	1
M		1	2	2	1	1	1	1	1	1
F									1	1

		AMINO ACID POSITION#								
		1	2	3	4	5	6	7	8	9
AMINO ACID										
I		8	8	8	8	8	8	8	8	8
R						8	8	8	8	8
Q		8	8	8	17	17	8	8	8	8
G							8	8	8	8
E		8								
V		8	8	8	8	8	8			
T		17	17	17	17	17	17	17	8	8
L										8
A		17	17	17	17	17	17	17	17	17
N		8	8	8	8	8	8	17	17	8
S		17	17	17	17	8	8	8	8	8
M		8	17	17	8	8	8	8	8	8
F									8	8

Figure 11

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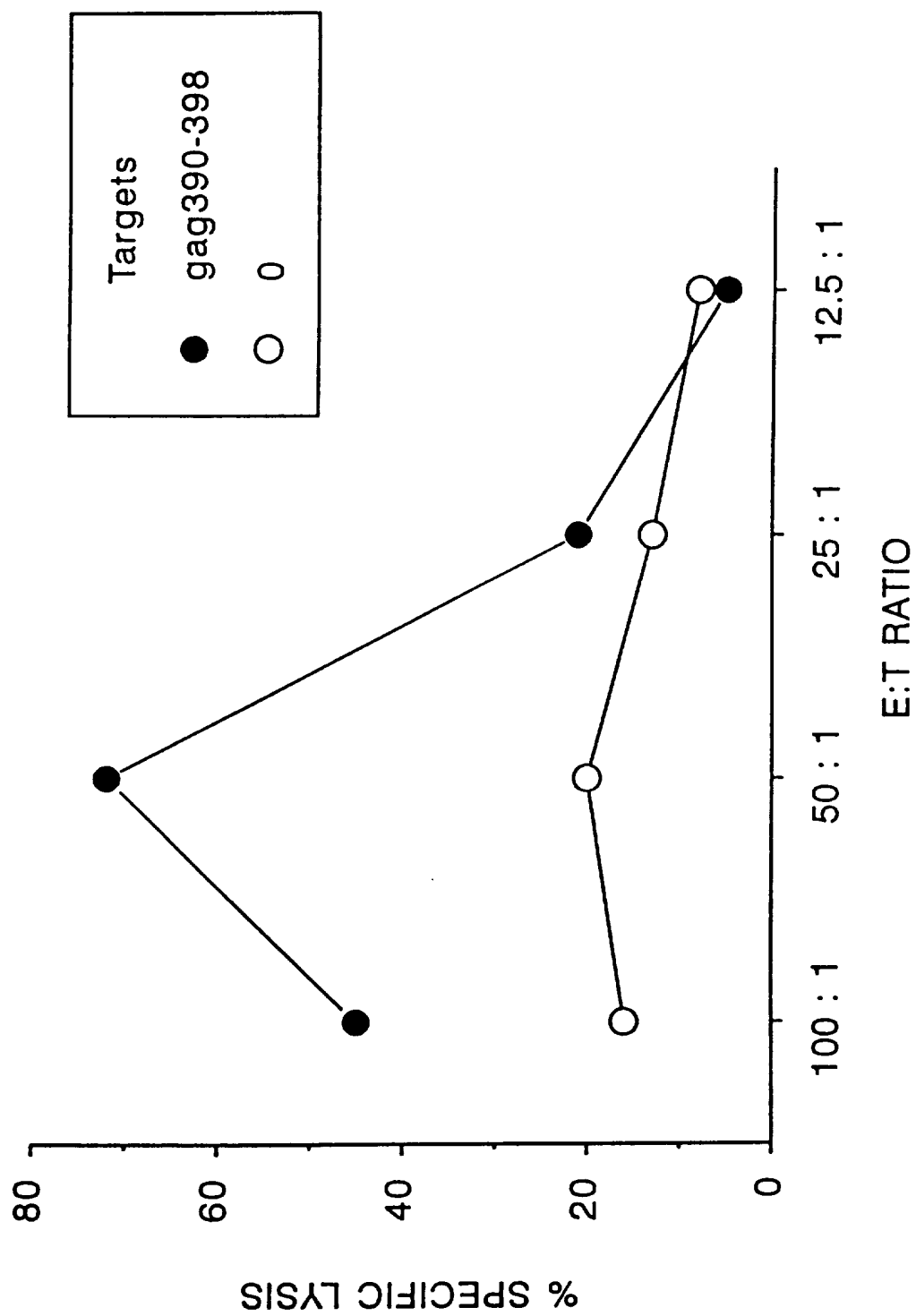
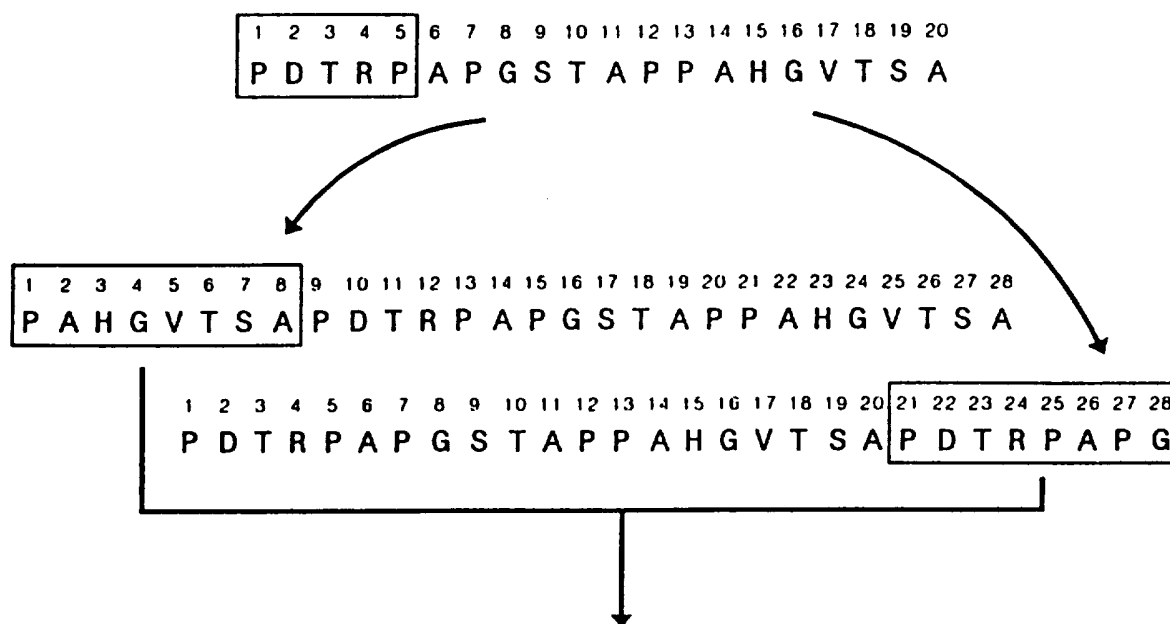


Figure 12



		AA Distribution by Position								
		1	2	3	4	5	6	7	8	9
AMINO ACID	P	5	5	5	5	5	5	5	5	5
	A	4	4	4	4	4	4	4	4	4
	H	1	1	1	1	1	1	1	1	1
	G	2	2	2	2	2	2	2	2	2
	V	1	1	1	1	1	1	1	1	1
	T	3	3	3	3	3	3	3	3	3
	S	2	2	2	2	2	2	2	2	2
	D	1	1	1	1	1	1	1	1	1
	R	1	1	1	1	1	1	1	1	1

		AA Percentage by Position								
		1	2	3	4	5	6	7	8	9
AMINO ACID	P	25	25	25	25	25	25	25	25	25
	A	20	20	20	20	20	20	20	20	20
	H	5	5	5	5	5	5	5	5	5
	G	10	10	10	10	10	10	10	10	10
	V	5	5	5	5	5	5	5	5	5
	T	15	15	15	15	15	15	15	15	15
	S	10	10	10	10	10	10	10	10	10
	D	5	5	5	5	5	5	5	5	5
	R	5	5	5	5	5	5	5	5	5

Figure 13

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25 26 27 28
 C T Y S P A L N K M F C Q L A K T C P V Q L W V D S T P
 M L L Y

AA Distribution by Position

	1	2	3	4	5	6	7	8	9	10
AMINO ACID										
C	3	2	2	2	2	2	2	2	2	2
T	2	2	1	1	1	1	1	1	2	2
Y	2	2	2	1	1	1	1	1	1	1
S	1	1	1	1				1	1	1
P	2	2	2	2	2	1	1	1	1	2
A	2	2	2	2	2	2	1	1	1	1
L	4	4	4	5	5	5	5	4	4	4
N	1	1	1	1	1	1	1	1		
K	2	2	2	2	2	2	2	2	2	1
M	2	2	2	2	2	2	2	2	2	1
F	1	1	1	1	1	1	1	1	1	1
Q	1	1	2	2	2	2	2	2	2	2
V		1	1	1	1	2	2	2	2	2
W					1	1	1	1	1	1
D							1	1	1	1

AA Percentage by Position

	1	2	3	4	5	6	7	8	9	10
AMINO ACID										
C	13	9	9	9	9	9	9	9	9	11
T	9	9	4	4	4	4	4	4	9	11
Y	9	9	9	4	4	4	4	4	4	5
S	4	4	4	4				4	4	5
P	9	9	9	9	9	4	4	4	4	11
A	9	9	9	9	9	9	4	4	4	5
L	17	17	17	22	22	22	22	17	17	18
N	4	4	4	4	4	4	4	4		
K	9	9	9	9	9	9	9	9	9	5
M	9	9	9	9	9	9	9	9	9	5
F	4	4	4	4	4	4	4	4	4	5
Q	4	4	9	9	9	9	9	9	9	11
V		4	4	4	4	9	9	9	9	11
W					4	4	4	4	4	5
D							4	4	4	5

Figure 14

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1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20 21 22 23 24 25
W H S N L N D A T Y Q R T R A L V R T G M D P R M

AA Distribution by Position

	1	2	3	4	5	6	7	8	9
AMINO ACID									
W	1								
H	1	1							
S	1	1	1						
N	2	2	2	2	1	1			
L	2	2	2	2	2	1	1	1	1
D	1	1	1	1	1	1	2	1	1
A	2	2	2	2	2	2	2	2	1
T	2	2	2	3	3	3	3	3	3
Y	1	1	1	1	1	1	1	1	1
Q	1	1	1	1	1	1	1	1	1
R	2	2	3	3	3	3	3	3	4
V		1	1	1	1	1	1	1	1
G					1	1	1	1	1
M						1	1	1	1
P								1	1

AA Percentage by Position

	1	2	3	4	5	6	7	8	9
AMINO ACID									
W	6								
H	6	6							
S	6	6	6						
N	13	13	13	13	6	6			
L	13	13	13	13	13	6	6	6	7
D	6	6	6	6	6	6	13	6	7
A	13	13	13	13	13	13	13	13	7
T	13	13	13	19	19	19	19	19	13
Y	6	6	6	6	6	6	6	6	7
Q	6	6	6	6	6	6	6	6	7
R	13	13	19	19	19	19	19	19	25
V		6	6	6	6	6	6	6	7
G					6	6	6	6	7
M						6	6	6	7
P								6	7

Figure 15

1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
S	D	Y	E	G	R	L	I	T	Y	Q	R	T	R	A	L	V	A	S	N	E	N	M	E	T	M

AA Distribution by Position

	1	2	3	4	5	6	7	8	9
AMINO ACID									
S	1	1	1	1	1	1	1	1	1
D	2	2	1	1	1	1	1	1	1
Y	2	2	2	1	1	1	1	1	1
E	1	1	1	2	1	1	2	2	2
G	1	1	1	1	1				
R	3	3	3	3	3	3	2	2	2
L	2	2	2	2	2	2	2	1	1
I	1	1	1	1	1	1	1	1	
T	2	2	2	2	2	2	2	3	3
Q	1	1	1	1	1	1	1	1	1
A	2	2	2	2	2	2	2	2	2
V	1	1	1	1	1	1	1	1	1
N			1	1	2	2	2	2	2
M						1	1	1	2

AA Percentage by Position

	1	2	3	4	5	6	7	8	9
AMINO ACID									
S	5	5	5	5	5	5	5	5	5
D	11	11	5	5	5	5	5	5	5
Y	11	11	11	5	5	5	5	5	5
E	5	5	5	11	5	5	11	11	11
G	5	5	5	5	5				
R	16	16	16	16	16	16	11	11	11
L	11	11	11	11	11	11	11	5	5
I	5	5	5	5	5	5	5	5	
T	11	11	11	11	11	11	11	16	16
Q	5	5	5	5	5	5	5	5	5
A	11	11	11	11	11	11	11	11	11
V	5	5	5	5	5	5	5	5	5
N			5	5	11	11	11	11	11
M						5	5	5	11

Figure 16