

VACCINE

The present invention relates to fusion proteins comprising an antigen derived from the so-called tumour rejection antigen PRAME (also known as DAGE) linked to an immunological fusion partner which provides T helper epitopes, such as, for example protein D from *Haemophilus influenzae* B, methods for preparing the same and for formulating vaccines and use of the same for treating a range of cancers, including, but not limited to melanoma, breast, bladder, lung cancer such as NSCLC, sarcoma, ovarian cancer, head and neck cancer, renal cancer, colorectal carcinoma, multiple myeloma, leukemia including acute leukemia and oesophageal carcinoma.

In a further embodiment, the present invention relates to fusion partner proteins comprising protein D derivatives and methods for preparing same.

Among different groups of tumour-associated antigens, cancer testis antigens are of interest for immunotherapy because of their broad tumour-specific expression and the fact that generally these antigens are not expressed in healthy cells. More than 50 cancer/testis antigens have been described so far and, for many of them, epitopes recognized by T lymphocytes have been identified. PRAME is a cancer testis antigen and is in under investigation as a potential immunotherapy.

In immunotherapy the cancer antigen is introduced to the patient usually as a vaccine, for example containing an antigen as a protein or an immunogenic fragment thereof, or as DNA encoding for the protein or as a vector containing said DNA, which stimulates the patient's immune system to attack tumours expressing the same antigen.

If the appropriate response is stimulated, T lymphocytes (T cells) attack antigens directly, and provide control of the immune response. B cells and T cells develop that are specific for one antigen type. When the immune system is exposed to a different antigen, different B cells and T cells are formed. As lymphocytes develop, they normally learn to recognize the body's own tissues (self) as different from tissues and particles not normally found in the body (non-self). Once B cells and T cells are formed, a few of those cells will

multiply and provide "memory" for the immune system. This allows the immune system to respond faster and more efficiently the next time it is exposed to the same antigen.

Certain experiments seem to indicate that cancer testis antigens can stimulate the memory mechanisms in the immune system.

It is hypothesized by some that PRAME is involved in cell death or cell cycles. It has been shown by some groups to be expressed in melanoma and a wide variety of tumours including lung, kidney and head and neck. Interestingly it also seems to be expressed in 40-60% leukemia such as acute lymphoid leukemia and acute myeloid leukemia, see for example Exp Hematol. 2000 Dec;28(12):1413-22. In patients it has been observed that over expression of PRAME seems to be associated with higher survival and lower rates of relapse in comparison to those who do not over express the protein.

The antigen and its preparation are described in US patent No. 5, 830, 753. PRAME is found in the Annotated Human Gene Database H-Inv DB under the accession numbers: U65011.1, BC022008.1, AK129783.1, BC014974.2, CR608334.1, AF025440.1, CR591755.1, BC039731.1, CR623010.1, CR611321.1, CR618501.1, CR604772.1, CR456549.1, and CR620272.1.

Protein D is a surface protein of the gram-negative bacterium, *Haemophilus influenza B*. Information on immunological fusion partners derived from protein D can be obtained from WO 91/18926.

Fusion proteins of a portion of an antigen and a heterologous fusion partner are sometimes prepared to increase the immunogenicity of the antigen and/or aid production of the protein in appropriate quantities and/or purity see for example WO 99/40188 which describes a fusion protein of MAGE and, for example protein D a surface protein of the gram-negative bacterium, *Haemophilus influenza B*. The fusion protein is prepared recombinantly and the protein D secretion sequence can be incorporated into the fusion protein to potentially assist secretion and solubilisation of the final product.

Brief Description of the Figures and Constructs/Sequences

Figure 1	SDS-page analysis of Construct 3 and 4
Figure 2	SDS-page analysis of Construct 3a and 4a
Figure 3	CD4 response (AS01B adjuvant)
Figure 4	CD8 response (AS01B adjuvant - 20070499)
Figure 5	CD4 response (AS15 adjuvant)
Figure 6	CD8 response (AS15 adjuvant)
Figure 7	A marked up amino acid sequence of examples of constructs of the present invention
Figure 8	Alignment between LipoD-MAGE3-His (SEQ ID NO:43) and pD1/3-PRAME-His (SEQ ID NO:10)
Figure 9	Alignment between the shared sequence of the original protein D from <i>Haemophilus influenzae</i> (SEQ ID NO:45) and the LipoD-MAGE3-His (SEQ ID NO:43)
Figure 10	Alignment between the shared sequence of the original protein D from <i>Haemophilus influenzae</i> (SEQ ID NO:41), the pD-MAGE3-His (SEQ ID NO:45) and a construct of pD1/3-PRAME-His which does not include the amino acids 2-D and 3-P (SEQ ID NO:44)
Figure 11	SDS page analysis of pD1/3-PRAME with or without secretion signal (SS) and His-tail (His) in pET21 vector.
Figure 12	Sequence of protein D 1/3 MAGE-A3-His (SEQ ID NO:44)
Construct 1 (Example 1)	MDP- 20-127 - Protein D – PRAME – TSGHHHHHH (plasmid TCMP14) (nucleotide sequence SEQ ID NO: 1; amino acid sequence SEQ ID NO:2)
Construct 2 (Example 2)	MDP- 20-127 - Protein D – PRAME – no His tail (plasmid TCMP14) (nucleotide sequence SEQ ID NO: 3; amino acid sequence SEQ ID NO:4)
Construct 3 (Example 3)	MDP- 20-127 - Protein D – PRAME – LEHHHHHH (plasmid pET21) (nucleotide sequence SEQ ID NO: 5; amino acid sequence SEQ ID NO:6)
Construct 4 (Example 4)	MDP- 20-127 - Protein D – PRAME – no His tail (plasmid pET21) (nucleotide sequence SEQ ID NO: 7; amino acid sequence SEQ ID NO:8)
Construct 3a (Example 3a)	MDP- 20-127 - Protein D – PRAME – HHHHHH (plasmid pET26) (nucleotide sequence SEQ ID NO: 9; amino acid sequence SEQ ID NO:10)
Construct 4a (Example 4a)	MDP- 20-127 - Protein D – PRAME – no His tail (plasmid pET26) (nucleotide sequence SEQ ID NO: 11; amino acid sequence SEQ ID NO:12)
Sequence 1 (SEQ ID NO:1)	is the DNA sequence for Example 1

Sequence 2 (SEQ ID NO:2)	is the amino acid sequence for Example 1
Sequence 3 (SEQ ID NO:3)	is the DNA sequence for Example 2
Sequence 4 (SEQ ID NO:4)	is the amino acid sequence for Example 2
Sequence 5 (SEQ ID NO:5)	is the DNA sequence for Example 3
Sequence 6 (SEQ ID NO:6)	is the amino acid sequence for Example 3
Sequence 7 (SEQ ID NO:7)	is the DNA sequence for Example 4
Sequence 8 (SEQ ID NO:8)	is the amino acid sequence for Example 4
Sequence 9 (SEQ ID NO:9)	is the codon optimized DNA sequence for Example 3a
Sequence 10 (SEQ ID NO:10)	is the amino acid sequence for Example 3a
Sequence 11 (SEQ ID NO:11)	is the codon optimized DNA sequence for Example 4a
Sequence 12 (SEQ ID NO:12)	is the amino acid sequence for Example 4a
SEQ ID NO:13	VLDGLDVLL (PRA ¹⁰⁰⁻¹⁰⁸)
SEQ ID NO:14	SLYSFPEPEA (PRA ¹⁴²⁻¹⁵¹)
SEQ ID NO:15	ALYVDSLFFL (PRA ³⁰⁰⁻³⁰⁹)
SEQ ID NO:16	LYVDSLFFL (PRA ³⁰¹⁻³⁰⁹)
SEQ ID NO:17	SLLQHLIGL (PRA ⁴²⁵⁻⁴³³)
SEQ ID NO:18 to 35	Oligonucleotides used in the examples of the present invention
SEQ ID NO: 36	TCC ATG ACG TTC CTG ACG TT (CpG 1826;)
SEQ ID NO: 37	TCT CCC AGC GTG CGC CAT (CpG 1758)
SEQ ID NO: 38	ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG TCG TCG TTT TGT CGT TTT GTC GTT (CpG 2006)
SEQ ID NO: 39	TCC ATG ACG TTC CTG ATG CT (CpG 1668)
SEQ ID NO: 40	TCG ACG TTT TCG GCG CGC GCC G (CpG 5456)
SEQ ID NO:41	AA 1 to 127 of Protein D from <i>Haemophilus influenzae</i>
SEQ ID NO:42	1-Met; 2-Asp; 3-Pro; followed by AA 20 to 127 of Protein D
SEQ ID NO:43	Amino acid sequence of protein D 1/3- MAGE-A3 -His
SEQ ID NO:44	Amino acids from protein D from <i>Haemophilus influenzae</i> for use in a pD1/3-

	PRAME-His sequence
SEQ ID NO:45	Amino acids from protein D from Haemophilus influenzae for use in a pD1/3-MAGE-His sequence

Summary of the invention

The present invention provides a fusion protein comprising:

- a) PRAME or an immunogenic fragment thereof, and
 - b) a heterologous fusion partner derived from protein D,
- wherein the said fusion protein does not include the secretion sequence (signal sequence) of protein D.

The present invention further provides a fusion partner protein as described herein derived from protein D, in which the fusion partner protein does not include the secretion sequence or signal sequence of protein D.

The present invention further provides a fusion protein as described herein and an antigen or fragment thereof.

The present invention further provides a fusion partner protein derived from protein D, in which the fusion partner protein comprises or consists of amino acids 20 to 127 of protein D. In one embodiment of the present invention, one or more amino acids from the protein D fusion partner protein as described herein may be deleted or may be replaced by substitution. The amino acids may be substituted with conservative substitutions as defined herein, or other amino acids may be used. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or more amino acids may be substituted.

The protein D fusion partner protein as described herein may additionally or alternatively contain deletions or insertions within the amino acid sequence when compared to the wild-type protein D sequence. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or more amino acids may be inserted or deleted.

The term "secretion sequence" or "signal sequence" or "secretion signal" of protein D, in the context of this application, is intended to refer to approximately amino acids 1 to

16, 17, 18 or 19 of the naturally occurring protein. In one embodiment, the secretion or signal sequence or secretion signal of protein D refers to the N-terminal 19 amino acids of protein D. The terms "secretion sequence" or "signal sequence" or "secretion signal" are used interchangeably in the present specification.

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The fusion partner protein of the present invention may comprise the remaining full length protein D protein, or may comprise approximately the remaining N-terminal third of protein D. For example, the remaining N-terminal third of protein D may comprise approximately or about amino acids 20 to 127 of protein D. In one embodiment, the protein D sequence for use in the present invention comprises amino acids 20 to 127 of protein D. In a further embodiment, the present invention comprises or consists of any of the sequences starting from any of the following amino acids of the protein D sequence: 17, 18, 19, 20, 21, or 22; and terminating at any on the following amino acids of the protein D sequence: 125, 126, 127, 128, 129, 130, 131, 132, 133, 134, 135, 136, 137, 138, 139 or 140.

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By "remaining" in this context is meant the sequence of the protein D protein without the secretion or signal sequence as described herein.

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In one embodiment of the present invention in which the fusion protein comprises PRAME or an immunogenic fragment thereof, the protein D derivative of the present invention comprises approximately the first 1/3 of the protein, more specifically the amino acids 20 to 127. In an alternative embodiment of the present invention in which the fusion protein comprises PRAME or an immunogenic fragment thereof, the protein D comprises approximately the first 1/3 of the protein in which the N-terminal 109 amino acids of protein D are used. In one embodiment of the present invention the protein D portion does not include the secretion sequence of the protein. In one embodiment of the present invention the protein D derivative is not lipidated.

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In one embodiment, the present invention provides a protein D construct, as described herein, as a fusion partner protein. The protein D construct may be a fusion partner protein for a construct additionally comprising a PRAME or MAGE-A3 construct

as described herein or may be a fusion partner protein for a construct additionally comprising another cancer antigen or any other antigen.

It seems that for fusion proteins comprising PRAME or an immunogenic fragment thereof and protein D, or for fusion proteins comprising protein D, or for a fusion partner protein comprising protein D, that the presence of the secretion sequence (or signal sequence) may detrimentally affect the amount of fusion protein produced.

PRAME

In one aspect the fusion protein of the present invention comprises a fusion partner protein as described herein and a PRAME antigen or immunogenic fragment thereof. Generally the PRAME protein has 509 amino acids and in one embodiment all 509 amino acids of PRAME may be used. Several cytotoxic T lymphocyte (CTL) epitopes have been identified on PRAME, for example:

VLDGLDVLL (PRA¹⁰⁰⁻¹⁰⁸; SEQ ID NO:13);
SLYSFPEPEA (PRA¹⁴²⁻¹⁵¹; SEQ ID NO:14);
ALYVDSLFFL (PRA³⁰⁰⁻³⁰⁹; SEQ ID NO:15);
LYVDSLFFL (PRA³⁰¹⁻³⁰⁹; SEQ ID NO:16) and
SLLQHLIGL (PRA⁴²⁵⁻⁴³³; SEQ ID NO:17).

Generally it is desirable to include as many of these epitopes as possible into the antigen to generate a strong immune response and ensure the antigen is as immunogenic as possible. Although, it may be possible to compensate for a lower immunogenicity of a given construct by employing a formulation with a potent immunological adjuvant. Strong adjuvants are discussed below in more detail.

In one aspect the invention provides the PRAME portion of the fusion protein comprising, consisting of or consisting essentially of full length protein.

However, the invention also extends to PRAME constructs with conservative substitutions. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or more amino acids may be substituted. The PRAME construct as described herein may additionally or alternatively contain deletions or insertions within the amino acid sequence when compared to the wild-

type PRAME sequence. In one embodiment, 1, 2, 3, 4, 5, 6, 7, 8, 9 or more amino acids may be inserted or deleted.

Conservative substitutions are well known and are generally set up as the default scoring matrices in sequence alignment computer programs. These programs include PAM250 (Dayhoft M.O. *et al.*, (1978), "A model of evolutionary changes in proteins", In "Atlas of Protein sequence and structure" 5(3) M.O. Dayhoft (ed.), 345-352), National Biomedical Research Foundation, Washington, and Blosum 62 (Steven Henikof and Jorja G. Henikof (1992), "Amino acid substitution matrices from protein blocks"), Proc. Natl. Acad. Sci. USA 89 (Biochemistry): 10915-10919.

In general terms, substitution within the following groups are conservative substitutions, but substitutions between groups are considered non-conserved. The groups are:

- i) Aspartate/asparagine/glutamate/glutamine
- ii) Serine/threonine
- iii) Lysine/arginine
- iv) Phenylalanine/tyrosine/tryptophane
- v) Leucine/isoleucine/valine/methionine
- vi) Glycine/alanine

Generally the PRAME sequence/amino acids used in the fusion proteins of the invention will be greater than 80%, such as 85, 90, 95 and more specifically 99% identical to naturally occurring PRAME. However, those skilled in the art are aware that amino acid residues generated as a result of the cloning process may be retained in the recombinantly synthesized proteins. If these do not detrimentally affect the characteristics of the product, it is optional whether or not they are removed.

In one aspect the invention provides a fusion protein as described herein comprising, consisting of or consisting essentially of full length PRAME protein. In a further aspect the PRAME portion of the fusion protein of the present invention comprises, consists of or consists essentially of one or more of the following epitopes:

VLDGLDVLL (PRA¹⁰⁰⁻¹⁰⁸; SEQ ID NO:13);

SLYSFPEPEA (PRA¹⁴²⁻¹⁵¹; SEQ ID NO:14);
ALYVDSLFFL (PRA³⁰⁰⁻³⁰⁹; SEQ ID NO:15);
LYVDSLFFL (PRA³⁰¹⁻³⁰⁹; SEQ ID NO:16) and
SLLQHLIGL (PRA⁴²⁵⁻⁴³³; SEQ ID NO:17).

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Fusion proteins

In a further embodiment of the present invention, a tumour antigen other than PRAME or in addition to PRAME may be used in a fusion protein as described herein. In one embodiment, a fusion protein is provided comprising a fusion partner protein as described herein and one or more of the following tumour antigens or tumour antigen derivatives or an immunogenic portion thereof which is able to direct an immune response to the antigen: a MAGE antigen, for example a MAGE-A antigen such as MAGE 1, MAGE 2, MAGE 3, MAGE 4, MAGE 5, MAGE 6, MAGE 7, MAGE 8, MAGE 9, MAGE 10, MAGE 11, MAGE 12. These antigens are sometimes known as MAGE A1, MAGE A2, MAGE A3, MAGE A4, MAGE A5, MAGE A6, MAGE A7, MAGE A8, MAGE A9, MAGE A 10, MAGE A11 and/or MAGE A12 (The MAGE A family). In one embodiment, an antigen from one of two further MAGE families may be used: the MAGE B and MAGE C group. The MAGE B family includes MAGE B1 (also known as MAGE Xp1, and DAM 10), MAGE B2 (also known as MAGE Xp2 and DAM 6) MAGE B3 and MAGE B4 – the Mage C family currently includes MAGE C1 and MAGE C2.

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The MAGE antigen for use in the present invention may comprise the full length MAGE antigen. Alternatively, the MAGE antigen may comprise an immunogenic portion of MAGE in which 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids may be deleted from or substituted in the amino acid sequence. In one embodiment of the present invention, 2 amino acids may be deleted from the N-terminus of the MAGE sequence. In one embodiment of the present invention in which the antigen is MAGE-A3 or an immunogenic portion thereof, the sequence of MAGE-A3 may be from amino acid 3 to 314 of MAGE-A3.

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In another embodiment, the tumour antigen or derivative for use in the present invention may be PRAME, BAGE, LAGE 1, LAGE 2 (also known as NY-ESO-1), SAGE, HAGE, XAGE, PSA, PAP, PSCA, P501S (also known as prostein), HASH1, HASH2, Cripto,

B726, NY-BR1.1, P510, MUC-1, Prostase, STEAP, tyrosinase, telomerase, survivin, CASB616, P53, and/or Her-2/neu or an immunogenic portion thereof which is able to direct an immune response to the antigen.

5 In a further embodiment of the invention, the tumour antigen may comprise or consist of one of the following antigens, or an immunogenic portion thereof which is able to direct an immune response to the antigen: SSX-2; SSX-4; SSX-5; NA17; MELAN-A; P790; P835; B305D; B854; CASB618 (as described in WO00/53748); CASB7439 (as described in WO01/62778); C1491; C1584; and C1585.

10 In one embodiment, the antigen for use in the present invention may comprise or consist of P501S. P501S, also named prostatein (Xu et al., Cancer Res. 61, 2001, 1563-1568), is known as SEQ ID NO. 113 of WO98/37814 and is a 553 amino acid protein.

15 Immunogenic fragments and portions thereof comprising at least 20, preferably 50, more preferably 100 contiguous amino acids as disclosed in the above referenced patent application may be used in fusion proteins of the present invention. Preferred fragments are disclosed in WO 98/50567 (PS108 antigen) and as prostate cancer-associated protein (SEQ ID NO: 9 of WO 99/67384). Other preferred fragments are amino acids 51-553, 34-553 or 55-553 of the full-length P501S protein.

20 In one embodiment, the antigen may comprise or consist of WT-1 expressed by the Wilm's tumor gene; or an immunogenic portion thereof which is able to direct an immune response to the antigen; or the N-terminal fragment WT-1F comprising about or approximately amino acids 1-249 of WT-1.

25 In a further embodiment, the antigen may comprise or consist of the antigen expressed by the Her-2/neu gene, or a fragment thereof or an immunogenic portion thereof which is able to direct an immune response to the antigen. In one embodiment, the Her-2/neu antigen may be one of the following fusion proteins which are described in WO00/44899.

30 The antigen for use in the present invention may comprise or consist of "HER-2/neu ECD-ICD fusion protein," also referred to as "ECD-ICD" or "ECD-ICD fusion protein," which refers to a fusion protein (or fragments thereof) comprising the extracellular domain (or

fragments thereof) and the intracellular domain (or fragments thereof) of the HER-2/neu protein. In one embodiment, this ECD-ICD fusion protein does not include a substantial portion of the HER-2/neu transmembrane domain, or does not include any of the HER-2/neu transmembrane domain.

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In a further embodiment, the antigen may comprise or consist of "HER-2/neu ECD-PD fusion protein," also referred to as "ECD-PD" or "ECD-PD fusion protein," or the "HER-2/neu ECD-APD fusion protein," also referred to as "ECD-APD" or "ECD-APD fusion protein," which refers to fusion proteins (or fragments thereof) comprising the extracellular domain (or fragments thereof) and phosphorylation domain (or fragments thereof, e.g., APD) of the HER-2/neu protein. In one embodiment, the ECD-PD and ECD-APD fusion proteins do not include a substantial portion of the HER-2/neu transmembrane domain, or does not include any of the HER-2/neu transmembrane domain.

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The fusion proteins of the PRAME antigen and protein D fusion partner protein as described herein may be chemically conjugated, but are preferably expressed as recombinant fusion proteins, which may allow increased levels of PRAME protein to be produced in an expression system as compared to PRAME alone without fusion partner, such as protein D or modified protein D proteins.

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Additionally or alternatively, the tumour antigens described herein and the fusion partner protein of the present invention may be chemically conjugated or may be expressed as recombinant fusion proteins, which may allow increased levels of PRAME protein or another tumour antigen to be produced in an expression system as compared to PRAME or another tumour antigen alone without fusion partner, such as protein D or modified protein D proteins.

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Fusion proteins of the present invention, as described herein, may additionally comprise one or more linker sequences between the fusion partner protein and the tumour antigen or immunogenic portion thereof; or between the fusion partner protein and a His tail or other affinity tag (if present); or between the tumour antigen or immunogenic portion thereof and a His tail or other affinity tag(if present). The amino acids in the linker sequences may be unrelated to the sequences of the antigen and/or fusion partner.

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Fusion proteins of the present invention, as described herein, may additionally comprise amino acids Met-Asp-Pro at the N-terminal end of the fusion protein sequence. The Met amino acid may be from the original protein D sequence or may be from an unrelated sequence.

The fusion partner may assist in expressing the protein (expression enhancer) at higher yields than the native recombinant protein. The fusion partner protein D, due to its foreign nature, may be particularly immunogenic *in vivo* and assist the fusion protein comprising PRAME or another tumour antigen by providing T helper epitopes, preferably T helper epitopes recognised by CD4 T-cells. Such CD4-T cells may be believed to contribute to generating a favourable immune response, in particular, a CD8 cytolytic T-cell response.

In one embodiment, the fusion partner may act as both an expression enhancing partner and an immunological fusion partner.

In one aspect the invention provides a fusion protein wherein the N-terminal portion of protein D (as described above or herein) is fused to the N-terminus of PRAME or an immunogenic fragment thereof. More specifically the fusion with the protein D fragment and the N-terminus of PRAME is effected such that the PRAME replaces the C-terminal-fragment of protein D that has been excised. Thus the N-terminus of protein D becomes the N-terminus of the fusion protein.

In a further aspect the invention provides a fusion protein wherein the N-terminal portion of protein D (as described above or herein) is fused to the N-terminus or another portion of a tumour antigen or an immunogenic fragment thereof. More specifically the fusion with the protein D fragment and the N-terminus or other portion of a tumour antigen may be effected such that the PRAME or other tumour antigen or derivative thereof as described herein replaces the C-terminal-fragment of protein D that has been excised. Thus the N-terminus of protein D becomes the N-terminus of the fusion protein.

Other fusion partners or fragments thereof may be included in fusion proteins of the invention or may replace the protein D element of the present invention, for example in embodiments comprising the PRAME antigen or a fragment or portion thereof as described herein. Examples of other fusion partners include:

- the non-structural protein from influenzae virus, NS1 (hemagglutinin) - typically the N terminal 81 amino acids are utilised, although different fragments may be used provided they include T-helper epitopes,
- LYTA derived from *Streptococcus pneumoniae*, which synthesize an N-acetyl-L-alanine amidase, amidase LYTA, (coded by the *lytA* gene {Gene, 43 (1986) page 265-272} such as the repeat portion of the Lyta molecule found in the C terminal end for example starting at residue 178 such as residues 188 - 305.

Purification of hybrid proteins containing the C-LYTA fragment at its amino terminus has been described {Biotechnology: 10, (1992) page 795-798.

Fusion proteins of the invention may include an affinity tag, such as for example, a histidine tail comprising between 5 to 9 such as 6 histidine residues. These residues may, for example be on the terminal portion of protein D (such as the N-terminal of protein D) and/or the may be fused to the terminal portion of the PRAME antigen or derivative thereof, or the tumour antigen or derivative thereof as described herein. Generally however the histidine tail will be located on terminal portion of the PRAME antigen or derivative thereof, or the tumour antigen or derivative thereof as described herein such as the C-terminal end of the PRAME antigen or derivative thereof, or the tumour antigen or derivative thereof as described herein. Histidine tails may be advantageous in aiding purification.

The present invention also provides a nucleic acid encoding the proteins of the present invention. Such sequences can be inserted into a suitable expression vector and used for DNA/RNA vaccination or expressed in a suitable host. Microbial vectors expressing the nucleic acid may be used as vaccines. Such vectors include for example, poxvirus, adenovirus, alphavirus, listeria and monophage.

A DNA sequence encoding the proteins of the present invention can be synthesized using standard DNA synthesis techniques, such as by enzymatic ligation as described by D.M. Roberts *et al.* in *Biochemistry* 1985, 24, 5090-5098, by chemical synthesis, by *in vitro* enzymatic polymerization, or by PCR technology utilising for example a heat stable polymerase, or by a combination of these techniques.

Enzymatic polymerisation of DNA may be carried out *in vitro* using a DNA polymerase such as DNA polymerase I (Klenow fragment) in an appropriate buffer containing the nucleoside triphosphates dATP, dCTP, dGTP and dTTP as required at a temperature of 10°-37°C, generally in a volume of 50µl or less. Enzymatic ligation of DNA fragments may be carried out using a DNA ligase such as T4 DNA ligase in an appropriate buffer, such as 0.05M Tris (pH 7.4), 0.01M MgCl₂, 0.01M dithiothreitol, 1mM spermidine, 1mM ATP and 0.1mg/ml bovine serum albumin, at a temperature of 4°C to ambient, generally in a volume of 50ml or less. The chemical synthesis of the DNA polymer or fragments may be carried out by conventional phosphotriester, phosphite or phosphoramidite chemistry, using solid phase techniques such as those described in 'Chemical and Enzymatic Synthesis of Gene Fragments - A Laboratory Manual' (ed. H.G. Gassen and A. Lang), Verlag Chemie, Weinheim (1982), or in other scientific publications, for example M.J. Gait, H.W.D. Matthes, M. Singh, B.S. Sproat, and R.C. Titmas, *Nucleic Acids Research*, 1982, 10, 6243; B.S. Sproat, and W. Bannwarth, *Tetrahedron Letters*, 1983, 24, 5771; M.D. Matteucci and M.H. Caruthers, *Tetrahedron Letters*, 1980, 21, 719; M.D. Matteucci and M.H. Caruthers, *Journal of the American Chemical Society*, 1981, 103, 3185; S.P. Adams *et al.*, *Journal of the American Chemical Society*, 1983, 105, 661; N.D. Sinha, J. Biernat, J. McMannus, and H. Koester, *Nucleic Acids Research*, 1984, 12, 4539; and H.W.D. Matthes *et al.*, *EMBO Journal*, 1984, 3, 801.

The process of the invention may be performed by conventional recombinant techniques such as described in Maniatis *et al.*, *Molecular Cloning - A Laboratory Manual*; Cold Spring Harbor, 1982-1989.

In particular, the process may comprise the steps of :

- i) preparing a replicable or integrating expression vector capable, in a host cell, of expressing a DNA polymer comprising a nucleotide sequence that encodes the protein or

- an immunogenic derivative thereof;
- ii) transforming a host cell with said vector;
- iii) culturing said transformed host cell under conditions permitting expression of said DNA polymer to produce said protein; and
- iv) recovering said protein.

The term 'transforming' is used herein to mean the introduction of foreign DNA into a host cell. This can be achieved for example by transformation, transfection or infection with an appropriate plasmid or viral vector using e.g. conventional techniques as described in Genetic Engineering; Eds. S.M. Kingsman and A.J. Kingsman; Blackwell Scientific Publications; Oxford, England, 1988. The term 'transformed' or 'transformant' will hereafter apply to the resulting host cell containing and expressing the foreign gene of interest.

The expression vectors are novel and also form part of the invention.

The replicable expression vectors may be prepared in accordance with the invention, by cleaving a vector compatible with the host cell to provide a linear DNA segment having an intact replicon, and combining said linear segment with one or more DNA molecules which, together with said linear segment encode the desired product, such as the DNA polymer encoding the protein of the invention, or derivative thereof, under ligating conditions.

Thus, the DNA polymer may be preformed or formed during the construction of the vector, as desired.

The choice of vector will be determined in part by the host cell, which may be prokaryotic or eukaryotic but are generally *E. coli* or CHO cells. Suitable vectors may include plasmids for example TMCP14 or pET21 or pET26, pcDNA3, bacteriophages, cosmids and recombinant viruses.

The preparation of the replicable expression vector may be carried out conventionally with appropriate enzymes for restriction, polymerisation and ligation of the DNA, by procedures described in, for example, Maniatis *et al.* cited above.

5 The recombinant host cell is prepared, in accordance with the invention, by transforming a host cell with a replicable expression vector of the invention under transforming conditions. Suitable transforming conditions are conventional and are described in, for example, Maniatis *et al.* cited above, or "DNA Cloning" Vol. II, D.M. Glover ed., IRL Press Ltd, 1985.

10 The choice of transforming conditions is determined by the host cell. Thus, a bacterial host such as *E. coli* may be treated with a solution of CaCl_2 (Cohen *et al.*, Proc. Nat. Acad. Sci., 1973, 69, 2110) or with a solution comprising a mixture of RbCl , MnCl_2 , potassium acetate and glycerol, and then with 3-[N-morpholino]-propane-sulphonic acid,
15 RbCl and glycerol. Mammalian cells in culture may be transformed by calcium co-precipitation of the vector DNA onto the cells. The invention also extends to a host cell transformed with a replicable expression vector of the invention.

20 The DNA may be codon optimized by standard techniques to further facilitate expression of the relevant host. In one embodiment of the present invention there is provided DNA encoding a fusion protein comprising a PRAME antigen or portion or fragment thereof as described herein, in which the nucleotide sequence of the PRAME antigen or portion or fragment thereof is codon-optimised. In one embodiment, the protein
25 D nucleotide sequence is not codon-optimised.

30 Culturing the transformed host cell under conditions permitting expression of the DNA polymer is carried out conventionally, as described in, for example, Maniatis *et al.* and "DNA Cloning" cited above. Thus, preferably the cell is supplied with nutrient and cultured at a temperature below 50°C.

The proteins of the present invention may be expressed in prokaryotes or eukaryotes such as yeast but are often expressed in *E. coli*. Particular strains of *E. coli* such as AR58 and BLR DE3 may be employed.

5 Generally a selection marker of, for example kanamycine resistance or ampicillin resistance is incorporated to facilitate identification of the successful incorporation of the recombinant gene/construct into the expression system.

10 The product is recovered by conventional methods according to the host cell and according to the localisation of the expression product (intracellular or secreted into the culture medium or into the cell periplasm). In one embodiment of the present invention the expression product is intracellular. In one embodiment of the present invention the expression product is an insoluble protein. Thus, where the host cell is bacterial, such as *E. coli* it may, for example, be lysed physically, chemically or enzymatically and the
15 protein product isolated from the resulting lysate. Where the host cell is mammalian, the product may generally be isolated from the nutrient medium or from cell free extracts. Conventional protein isolation techniques include selective precipitation, adsorption chromatography, and affinity chromatography including a monoclonal antibody affinity column.

20 In one embodiment of the invention there is provided a process for producing a fusion protein as described herein comprising the step of expressing in a cell a fusion protein comprising a fusion partner protein as described herein. The cell may be a bacterium. In one embodiment in which the cell is a bacterium, the bacterium may be *E. coli*.
25 The process of the present invention may comprise the step of expressing a fusion protein as described herein in a cell as an insoluble protein. The process may further comprise the step of lysing the cell and purifying the expressed fusion protein from the lysed cells.

30 In one embodiment of the invention there is provided a fusion protein obtained by or obtainable by a method or process described herein.

The proteins of the present invention are provided either soluble in a liquid form or in a lyophilised form.

It is generally expected that each human dose will comprise 1 to 1000 µg of protein, and preferably 30 - 300 µg.

The present invention also provides pharmaceutical composition such as vaccine comprising a fusion protein of the present invention in a pharmaceutically acceptable excipient.

The vaccine may optionally contain one or more other tumour-associated antigens or polypeptides, or preferably be combined with other cancer vaccines based on a tumour-associated antigen. For example, these tumour-associated antigens could be antigens as described herein and/or may be members belonging to the MAGE, LAGE and GAGE families or WT-1. In one embodiment the tumour-associated antigen may comprise or consist of the MAGE-A3 antigen.

Vaccine preparation is generally described in Vaccine Design ("The subunit and adjuvant approach" (eds. Powell M.F. & Newman M.J). (1995) Plenum Press New York). Encapsulation within liposomes is described by Fullerton, US Patent 4,235,877.

The proteins of the present invention may be preferably adjuvanted in the vaccine formulation of the invention. Suitable adjuvants may include an aluminium salt such as aluminium hydroxide gel (alum) or aluminium phosphate, but may also be a salt of calcium, iron or zinc, or may be an insoluble suspension of acylated tyrosine, or acylated sugars, cationically or anionically derivatised polysaccharides, or polyphosphazenes. Other known adjuvants include CpG containing oligonucleotides. The oligonucleotides are characterised in that the CpG dinucleotide is unmethylated. Such oligonucleotides are well known and are described in, for example WO 96/02555.

In the formulation of the inventions it may be desirable that the adjuvant composition induces an immune response preferentially of the TH1 type. In one embodiment there is provided an adjuvant system including, for example a combination of

monophosphoryl lipid A, preferably 3-de-O-acylated monophosphoryl lipid A (3D-MPL) together with an aluminium salt. The adjuvant may optionally also include CpG oligonucleotides to preferentially induce a TH1 response.

5 An enhanced system that may be used in the present invention comprises the combination of a monophosphoryl lipid A and a saponin derivative particularly the combination of QS21 and 3D-MPL as disclosed in WO 94/00153, or, for example a less reactogenic composition where the QS21 is quenched with cholesterol as disclosed in WO 96/33739.

10 A formulation that may be used in formulations of the present invention, comprising QS21 3D-MPL & tocopherol, for example in an oil in water emulsion, is described in WO 95/17210.

15 Another adjuvant formulation that may be used in formulations of the present invention is QS21, 3D-MPL & CpG or equivalent thereof, for example in an oil in water emulsion or as a liposomal formulation.

20 Accordingly in one embodiment of the present invention there is provided a vaccine comprising a fusion protein or fusion partner protein as described herein and an adjuvant, for example as described above.

Combination of PRAME and MAGE

25 In one embodiment of the present invention there is provided a composition comprising (a) an antigen component comprising a PRAME antigen or fusion protein as described herein and (b) an antigen component comprising a MAGE antigen or fusion protein as described herein. In one embodiment, the composition may further comprise an adjuvant as described herein.

30 The MAGE antigen for use in the combination may comprise the full length MAGE antigen. Alternatively, the MAGE antigen may comprise an immunogenic portion of MAGE in which 1, 2, 3, 4, 5, 6, 7, 8, 9, 10 or more amino acids may be deleted from or substituted in the amino acid sequence. In one embodiment of the present invention, 2

amino acids may be deleted from the N-terminus of the MAGE sequence. In one embodiment of the present invention in which the antigen is MAGE-A3 or an immunogenic portion thereof, the sequence of MAGE-A3 may be from amino acid 3 to 314 of MAGE-A3.

5

For the combination described above, either or both of the PRAME and/or MAGE antigens may be part of a fusion protein or proteins as described herein, or the antigens may be present in other fusion proteins or may be presented as antigen alone.

10 In one embodiment of the present invention there is provided a composition comprising a fusion protein comprising a PRAME antigen and fusion partner protein as described herein and a fusion protein comprising a MAGE-A3 antigen and fusion partner protein as described herein. In an alternative embodiment, the fusion protein comprising the MAGE-A3 antigen comprises or consists of the MAGE-A3 antigen and a fusion partner
15 protein comprising approximately the first 109 amino acids of protein D, in which one or two or more amino acids from protein D are optionally substituted, and in which the signal sequence of protein D is optionally present, in addition to the first 109 amino acids of protein D.

20 The fusion proteins of the present invention may additionally optionally comprise one or more amino acids as "linkers" between the sequences of the antigen and the fusion partner protein or between the antigen and a His tail, if present. The amino acids may be unrelated to the sequences of the antigen and/or fusion partner.

25 Fusion proteins of the present invention, as described herein, may additionally comprise amino acids Met-Asp-Pro at the N-terminal end of the fusion protein sequence. The Met amino acid may be from the original protein D sequence or may be from an unrelated sequence.

30 In one embodiment, the sequence of a fusion protein comprising MAGE-A3 and protein D for use in the present invention is shown in Figure 12 and SEQ ID NO:43.

The present invention also extends to methods of preparing said vaccines/compositions.

EXAMPLES

- 5 Four fusion constructs were prepared and will be referred to herein as Examples/constructs 1, 2, 3 and 4. A codon optimized construct was prepared from example 3 and is designated as example 3a herein. A codon optimized construct was prepared from example 4 and is designated as example 4a herein.
- 10 In Examples 3a and 4a the sequence in respect of the protein D portion of the molecule is the same. However, certain codons in the PRAME region were modified, to further improve expression and, in Example 3a, the linker between PRAME and the his tail has been removed.

TABLE A Fusion Protein Structures and Plasmid Details of the Examples/Constructs 1 to 4

N-terminal -----C-Terminal					
Example/ Construct No.	amino acids inserted by cloning process	fusion partner	cancer antigen	Linker/His tail amino acid seq.	plasmid used
1	MDP	20-127 protein D	PRAME	TSGHHHHHH	TCMP14
2	MDP	20-127 protein D	PRAME	-	TCMP14
3	MDP	20-127 protein D	PRAME	LEHHHHHH	pET21
4	MDP	20-127 protein D	PRAME	-	pET21
3a	MDP	20-127 protein D	PRAME	HHHHHH	pET26
4a	MDP	20-127 protein D	PRAME	-	pET26

5 The fusion proteins of the above examples comprise the amino acids 20-127 of protein D. The amino acids Met, Asp and Pro were included at the N-terminal of the protein D fragment (ie amino acids MDP-20-127 Protein D). It is thought that these three additional amino acids may aid the stability of the protein and/or increase the level of the protein expression thereof. Amino acid 127 of protein D is fused to the N-terminal of full length PRAME (ie amino acid 127 of protein D is fused to N-terminal of PRAME). A histidine tag tail, to aid purification, was included in three of the six proteins. The exact sequence of the tail is dependent the plasmid used.

15 Three different types of plasmids, TCMP14 and pET21 or pET26 were constructed: for each plasmid, DNA encoding for fusion protein was included with and without a histidine tail.

Unless stated otherwise the general strategy below was used in the preparation of each of the examples.

Cloning strategy for the generation of PD1/3-PRAME (with or without His-tag) recombinant protein using TCMP14 vector:

Amplification of the sequences presented in the plasmid TCMP14 were done using a three steps PCR strategy. The vector pHIC348 containing the DNA sequence encoding the entire protein D gene has been obtained from Dr. A. Forsgren, Department of Medical Microbiology, University of Lund, Malmö General Hospital, Malmö, Sweden. The DNA sequence of protein D has been published by Janson et al. (1991) { Janson H, LO Heden, A Grubb, M Ruan, & A Forsgren. 1991. Infect Immun 59:119-125}. The expression vector pMG81 is a derivative of pBR322, in which bacteriophage λ derived control elements for transcription and translation of foreign inserted genes were introduced (Shatzman et al., 1983) { Shatzman A, YS Ho, & M Rosenberg. 1983. Experimental Manipulation of Gene Expression. Inouya (ed) pp 1-14. Academic NY}. In addition, the Ampicillin resistance gene was exchanged with the Kanamycin resistance gene. The coding sequence for the portion of NS1 protein (amino acid 4 to 81) was substituted for a multiple cloning sites to get pMG81 MCS. The coding sequence for the 1/3 protein D (amino acid 20 to 127) was cloned into pMG81 MCS using BamHI and NcoI restriction sites to get pMG81-1/3PD. First, PCR amplification of the section corresponding to amino acid 20-127 of protein D was done using pMG81-1/3PD vector as template and oligonucleotide sense:

5' ATA TAA CAT ATG GAT CCA AGC AGC CAT TCA TCA AAT 3' (CAN008; SEQ ID NO: 18) and antisense:

5' CCA CAA ACG CCT TCG TTC CAT GGT TTC AAA GTT TTC TGT c 3' (CAN037; SEQ ID NO: 19).

PRAME cDNA obtained from the Ludwig Institute, Brussels, Belgium was inserted in the BstXI-NotI sites of the pCDNA1 vector (Invitrogen) to generate pCDNA-1-PRAME recombinant vector. PCR amplification of the section corresponding to amino acid of PRAME protein was done using pCDNA-1-PRAME vector (GSKBio) as template and oligonucleotide sense:

5' GAC AGA AAA CTT TGA AAC CAT GGA ACG AAG GCG TTT GTG G 3' (CAN036; SEQ ID NO: 20) and antisense:

5' AGA GAG ACT AGT CTA GTT AGG CAT GAA ACA GGG GCA CAG 3' (CAN029; SEQ ID NO: 21) or

5' GGA GGA ACT AGT GTT AGG CAT GAA ACA GGG GCA CAG 3' (CAN002; SEQ ID NO: 22) depending if a his-tail (CAN002) or not (CAN029) was added. The final PRAME sequence inserted in the TCMP14 plasmid was obtained following a PCR amplifications using the 1/3PD and PRAME gene templates that were generated in the preliminary steps for template and oligonucleotide sense: CAN008, and antisense: CAN029 or CAN002 depending if an his-tail was present (CAN002) or not (CAN029). NdeI at 5' end and SpeI at 3' end sites were also added for cloning of the fragment into TCMP14 vector.

Construction of the vector design to express the recombinant protein 1/3PD-PRAME with or without His-tag recombinant protein using pET21 vector :

A recombinant cDNA plasmid called pcDNA1-PRAME (as described in the previous strategy) containing the coding sequence for PRAME gene and the vector PMG81-1/3PD (as described in the previous strategy) containing the N-terminal portion of the protein D coding sequence were used. The cloning strategy included the following steps.

a) First, the 1/3PD sequence without secretion signal (secretion or signal sequence) was PCR amplified from plasmid PMG81-1/3PD using the oligonucleotide sense: 5' AGAGAGCATATGAGCAGCCATTCATCAAATATGGCG (CAN040; SEQ ID NO: 22),

and the antisense:

5' ACGTGGGCGGCCGCGGTTTCAAAGTTTTCTGTCAATTTCTAA (CAN032; SEQ ID NO: 23);

NdeI at the 5' end and NotI at the 3' end sites were also added for cloning of the fragment into pET21b(+) vector.

b) The PRAME sequence was PCR amplified from plasmid pcDNA1-PRAME using the oligonucleotide sense:

5' TTGTTGGCGGCCGCAATGGAACGAAGGCGTTTGTGGGGT (CAN033; SEQ ID NO: 25), and the antisense:

5' GGAGGACTCGAGGTTAGGCATGAAACAGGGGCACAG (CAN034; SEQ ID NO: 26); NotI at the 5' end and XhoI at the 3' end sites were also added for cloning of the fragment into

pET21b vector. This amplification resulted in the addition at the C-terminal of the protein of two amino acids, Leu and Glu, followed by 6 His in pET21b(+) plasmid. For the generation of the protein without His-tag, a stop codon (TAG) was added at the 3' end of the PRAME gene by using CAN033 and CAN035 (antisense:

5' GGAGGACTCGAGCTAGTTAGGCATGAAACAGGGGCACAG (CAN035; SEQ ID NO: XX) instead of CAN033 and CAN034.

c) Cloning into pET21b(+) plasmid (Invitrogen) of the above amplified fragments.

d) Removal of NotI site between 1/3PD and PRAME by using QuikChange II Site-Directed Mutagenesis Kit (Stratagene) and the oligonucleotide sense:

5' CAGAAAACCTTTGAAACCATGGAACGAAGGCG (CAN106; SEQ ID NO: XX), and the antisense:

5' cgccttcgttccatggtttcaaagttttctg (CAN107; SEQ ID NO: XX).

e) Addition of two amino acids Asp and Pro following the Met at position 1 at the N-terminal of the protein D 1/3 by mutagenesis and using the oligonucleotide sense:

5' GGAGATATACATATGGATCCAAGCAGCCATTCATCAAATATGG (CAN104; SEQ ID NO: XX) and the antisense:

5' CCATATTTGATGAATGGCTGCTTGGATCCATATGTATATCTCC (CAN105; SEQ ID NO: XX).

Construction of the vector design to express the recombinant protein 1/3PD-PRAME codon optimized (without or with His tag) in pET26 vector:

The PRAME gene was codon optimized and cloned in pGA4 backbone with the addition of NotI and XhoI sites in the 5' end and the 3' end of the optimized gene respectively.

This plasmid, named 0606420pGA4, was used to clone the gene in fusion with the PD1/3 in the pET26 vector using the following steps.

a) Removal of the NotI / XhoI fragment corresponding to the optimized PRAME sequence with a stop codon at the 3' end of the gene from 0606420pGA4 plasmid.

b) Cloning of the optimized PRAME fragment into a pET26b(+) plasmid which contain the 1/3PD previously cloned NdeI/NotI with CAN040 and CAN032 oligonucleotides as described above and where Asp and Pro amino acids were added in N-terminal by mutagenesis method with CAN104 and CAN105 oligonucleotides.

c) Removal of the NotI site by mutagenesis with oligonucleotides: sense

5' GACAGAAAACCTTTGAAACCATGGAACGTCGTCGTCTGTGG (CAN123; SEQ ID NO: XX) and antisense

5' CCACAGACGACGACGTTCCATGGTTTCAAAGTTTCTGTGTC (CAN124; SEQ ID NO: XX). This resulted in 1/3PD-PRAME codon optimized fusion protein without His-tail.

d) The plasmid was then used as a template for the generation of 1/3PD-PRAME codon optimized with 6 His plasmid. PCR amplification of the fusion protein was done with oligonucleotides sense

5' GGAATTCATATGGATCCAAGCAGCCATTC (CAN199; SEQ ID NO: XX) and a antisense

5' GGAGCTCTCGAGTCAGTGGTGGTGGTGGTGGTTCGGCATAAAGCACGGGC (CAN198; SEQ ID NO: XX); NdeI at the 5' end, XhoI at the 3' end sites followed by 6 His and a stop codon were also added for cloning of the fragment into pET26b(+) vector.

e) Cloning of the amplified fragment in pET26b(+) plasmid from Invitrogen.

For the production of the fusion protein, the DNA construct has been cloned into the expression vector TCMP14. This plasmid utilizes signals from lambda phage DNA to drive the transcription and translation of inserted foreign genes. The vector contains the lambda PL promoter PL, operator OL and two utilization sites (NutL and NutR) to relieve transcriptional polarity effects when N protein is provided (Gross et al., 1985. Mol. & Cell. Biol. 5:1015).

The plasmid expressing the pD-PRAME fusion protein was designed so the PRAME amino acids were added to the C-terminal of a 108 amino acids derivative of pD without its signal sequence (secretion or signal sequence) (i.e. residues 20-127). To this construction, three unrelated amino acids (Met and Asp and a Proline) were added at the N-terminal of the derivative of pD, and for certain constructions a his tail at the C-terminal of the PRAME amino acids was included (see table A above). This construct could alternatively be described as containing 109 amino acids derivative of pD, if the N-terminal Met is considered to come from the pD sequence.

Host Strain and Transformation

Hosts from *E. Coli* strain AR58 (Mott et al, Proc. Natl. Acad. Sci. USA, vol 82, pp 88-92, January 1985, Biochemistry) were transformed with plasmid DNA for Examples/ constructs 1 and 2.

The AR58 lysogenic *E. coli* strain used for the production of Examples/constructs 1 and 2 is a derivative of the standard NIH *E.coli* K12 strain N99 (F⁻ su⁻ galK2, lacZ⁻ thr⁻). It contains a defective lysogenic lambda phage (galE::TN10, l Kil- cI857 DH1). The Kil-

phenotype prevents the shut off of host macromolecular synthesis. The *ci857* mutation confers a temperature sensitive lesion to the *ci* repressor. The *DH1* deletion removes the lambda phage right operon and the hosts *bio*, *uvr3*, and *chlA* loci. The AR58 strain was generated by transduction of N99 with a P lambda phage stock previously grown on an SA500 derivative (*galE::TN10*, 1 Kil- *ci857* *DH1*). The introduction of the defective lysogen into N99 was selected with tetracycline by virtue of the presence of a *TN10* transposon coding for tetracyclin resistance in the adjacent *galE* gene. N99 and SA500 are *E. coli* K12 strains derived from Dr. Martin Rosenberg's laboratory at the National Institutes of Health.

Vectors containing the PL promoter, are introduced into an *E. coli* lysogenic host to stabilize the plasmid DNA. Lysogenic host strains contain replication-defective lambda phage DNA integrated into the genome (Shatzman et al., 1983; In Experimental Manipulation of Gene Expression. Inouya (ed) pp 1-14. Academic Press NY). The lambda phage DNA directs the synthesis of the *ci* repressor protein which binds to the OL repressor of the vector and prevents binding of RNA polymerase to the PL promoter and thereby transcription of the inserted gene. The *ci* gene of the expression strain AR58 contains a temperature sensitive mutation so that PL directed transcription can be regulated by temperature shift, i.e. an increase in culture temperature inactivates the repressor and synthesis of the foreign protein is initiated. This expression system allows controlled synthesis of foreign proteins especially of those that may be toxic to the cell (Shimatake & Rosenberg, 1981. Nature 292:128).

Hosts from *E. Coli* strain BLR (DE3) Novagen, WI, USA (catalogue number: 69053-4) BLR (DE3) Novagen, WI, USA (catalogue number: 69053-4) BLR is a *recA*⁻ derivative of BL21 that improves plasmid monomer yields and may help stabilize target plasmids containing repetitive sequences or whose products may cause the loss of the DE3 prophage (1, 2) were transformed with plasmid DNA from examples/constructs 3 and 4.

Each of transformation was carried out by standard methods with CaCl₂-treated cells (Hanahan D. « Plasmid transformation by Simanis. » In Glover, D. M. (Ed), DNA cloning. IRL Press London. (1985): p. 109-135.).

Growth and induction of bacterial host strain

• Culture

Bacteria were grown on 20 ml of Luria-Bertani (LB) broth (BD) + 1% (w/v) glucose (Laboratoire MAT, catalogue number: GR-0101) + antibiotic (Carbenicillin 100 µg/ml for pET21b, kanamycin 40 µg/ml for TCMP14). Cultures were incubated at 33°C, for AR58 cells and at 37°C, for BLR (DE3) cells until an O.D._{600nm} around 0.8.

• Induction

At O.D._{600nm} around 0.8, the cultures BLR (DE3) were induced at 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG; EMD Chemicals Inc., catalogue number: 5815) and incubated for 2 hours or 3 hours at 37°C although solubility may be increased if a lower temperature is used.

At O.D._{600nm} around 0.8, the cultures AR58 were induced by heat activation at 37°C and incubated for 7 hours.

The bacterial growth was adequate for the two expression systems.

• Extraction and Purification of the Protein

Upon expression of the polypeptide in culture, cells are typically harvested by centrifugation then disrupted by physical or chemical means (if the expressed polypeptide is not secreted into the media) and the resulting crude extract retained to isolate the polypeptide of interest. BugBuster™ Protein Extraction Reagent is used under conditions recommended by the suppliers (Novagen).

PD1/3-Prame-his protein purification

E. coli cell paste was resuspended in 20 mM Tris buffer pH 8.5 then passed through homogenizer system (Panda from Niro Soavi S.p.A. - 2 passes – 750 bars). After addition of 2 mM MgCl₂ and Benzonase (50 U/ml), homogenate was incubated 1 hour at room temperature (RT) under gentle agitation then centrifuged 30 minutes at 15900 g and RT. Resulting pellet was resuspended in 20 mM Tris buffer pH 8.5 containing 1% Sodium Dodecyl Sulphate (SDS) and 60 mM Glutathione and incubated 30 minutes at RT under gentle agitation. After centrifugation 30 minutes at 15900 g and RT, pellet was discarded.

Centrifugation supernatant was 10-fold diluted in 20 mM Tris buffer containing 6.66 M Urea, 0.333 M sodium chloride (NaCl) and 11.11 mM Imidazole and then subjected to chromatographic separation on a Nickel ion metal affinity column (IMAC Sepharose 6 FF

- GE Healthcare) equilibrated in a 20 mM Tris buffer pH 8.5 containing 0.1% SDS, 6.0 M Urea, 0.3 M NaCl and 10 mM Imidazole. After washing of the column with 20 mM Tris buffer pH 8.5 containing 0.5% Sarcosyl, 6.0 M Urea, 0.3 M NaCl and 10 mM Imidazole, antigen was eluted from the column by increasing the concentration of Imidazole up to 40 mM in the same washing buffer. After addition of phosphate up to 50 mM, antigen positive eluate was passed through a Macro-Prep Ceramic Hydroxyapatite type II column (Bio-Rad) equilibrated in a 20 mM Tris buffer pH 8.5 containing 50 mM phosphate, 0.5% Sarcosyl, 6.0 M Urea and 0.3 M NaCl. Hydroxyapatite flow-through containing the antigen was then diafiltered against 5 mM Borate buffer pH 9.8 containing 3.15% Sucrose on an Omega 30 kDa membrane (Pall). Ultrafiltration retentate was sterilized by filtration through a 0.45/0.22 µm Cellulose acetate membrane (Sartorius). Purified material was stored at -70°C.

An alternative purification process has also been used, which differs from the above process in the following steps:

- No benzonase treatment
- No shift from SDS to sarcosyl on IMAC column (SDS from extraction up to HA step)
- The buffer used for the diafiltration was 5 mM Tris buffer pH 8.5 - 0.5 M Arginine.

This alternative purification process resulted in incomplete SDS removal with residual value around 0.05 and 0.085%.

• **Purification**

The expressed recombinant proteins were purified from supernatant fractions obtained after centrifugation of induced *E. coli* using a His-Bind metal chelation resin (QIAGEN, Chatsworth, CA) according to the instructions from the resin manufacturer.

Characterisation of the Protein

SDS-Page:

Gel: NuPAGE 4-12 % Bis-Tris Gel 1.0mm 15 or 26 wells (Invitrogen catalog number: NP0323BOX)

See Figure 1 and 2 below, which show SDS page analysis of Example 3 and 4 and 3a and 4a respectively, wherein the different recombinant 1/3pD-PRAME proteins with or

Replacement page 30

without his-tag migrate on gel at an apparent molecular weight of ~70kDa. The recombinant proteins are found as inclusion bodies in the *E. coli* cell lysate, after induction.

Preparation of samples, buffers and migration conditions were done under conditions recommended by the suppliers (Invitrogen). 10µl of all preparations were loaded (before induction (BI) and after induction (AI)) in wells corresponding to 100 µl of culture equivalent.

Fig 1 legend: SDS page analysis after Coomassie-blue staining of recombinant 1/3PD-PRAME after IPTG induction of *E. coli* BLR DE3 strain transformed with recombinant pET21. An equivalent of 100µL of culture after 2 hours of induction in BLR DE3 strain with 1mM IPTG at 25, 30 or 37°C has been loaded on gel. Clone #3 (1/3PD-PRAME / pET21) and Clone #4 (1/3PD-PRAME-His / pET21) are presented on gel before (BI) and after (AI) inductions in soluble (supernatant) and non-soluble (pellet) fractions. Lane 1 and 10: Standard Broad Range prestain (BioRad Cat#161-0318), lane 2 (clone #3, BI, supernatant), lane 3 (clone #3, BI, pellet), lane 4 (clone #3, AI, 25°C, supernatant), lane 5 (clone #3, AI, 25°C, pellet), lane 6 (clone #3, AI, 30°C, supernatant), lane 7 (clone #3, AI, 30°C, pellet), lane 8 (clone #3, AI, 37°C, supernatant), lane 9 (clone #3, AI, 37°C, pellet), lane 11 (clone #4, BI, supernatant), lane 12 (clone #4, BI, pellet), lane 13 (clone #4, AI, 25°C, supernatant), lane 14 (clone #4, AI, 25°C, pellet), lane 15 (clone #4, AI, 30°C, supernatant), lane 16 (clone #4, AI, 30°C, pellet), lane 17 (clone #4, AI, 37°C, supernatant), lane 18 (clone #4, AI, 37°C, pellet).

Fig 2 legend: SDS page analysis after Coomassie-blue staining of recombinant 1/3PD-PRAME after IPTG induction of *E. coli* BLR DE3 strain transformed with recombinant pET26. An equivalent of 100µL of culture after 2 hours of induction in BLR DE3 strain with 1mM IPTG at 25, 30 or 37°C has been loaded on gel. Clone #3a (1/3PD-PRAME codon optimized / pET26) and Clone #4a (1/3PD-PRAME-His codon optimized / pET26) are presented on gel before (BI) and after (AI) inductions in soluble (supernatant) and non-soluble (pellet) fractions. Lane 2 and 10: Standard Broad Range prestain (BioRad Cat#161-0318), lane 1 (clone #3a, BI, supernatant), lane 3 (clone #3a, BI, pellet), lane 4 (clone #3a, AI, 25°C, supernatant), lane 5 (clone #3a, AI, 25°C, pellet), lane 6 (clone #3a, AI, 30°C, supernatant), lane 7 (clone #3a, AI, 30°C, pellet), lane 8 (clone #3a, AI, 37°C,

Replacement page 30a

supernatant), lane 9 (clone #3a, AI, 37°C, pellet), lane 11 (clone #4a, BI, supernatant), lane 12 (clone #4a, BI, pellet), lane 13 (clone #4a, AI, 25°C, supernatant), lane 14 (clone #4a, AI, 25°C, pellet), lane 15 (clone #4a, AI, 30°C, supernatant), lane 16 (clone #4a, AI, 30°C, pellet), lane 17 (clone #4a, AI, 37°C, supernatant), lane 18 (clone #4a, AI, 37°C, pellet).

Western Blot

Membranes were blocked for 30 minutes at 37°C, 60RPM using 3% milk/PBS 1X fresh solution. After the blocking incubation, primary antibodies were added (rabbit anti-PRAME; GSK Biologicals SA) at dilution 1:5000 or α -6X His tag (AbCam) at dilution 1:3000 in 3% milk/PBS 1X fresh solution for 1 hour at 37°C, 60RPM. After that, membranes were washed three times 5 minutes at room temperature using 0.02% Tween20/PBS 1X. Secondary antibodies were added (perox donkey anti-IgG (H+L) rabbit (Jackson laboratory) at dilution 1:20 000 using 3% milk/PBS 1X fresh solution. Membranes were incubated for 1 hour at 37°C, 60RPM. After that, membranes were washed three times 5 minutes at room temperature using 0.02% Tween20/PBS 1X before the membrane expositions to peroxydase substrate (KH_2PO_4 , 10mM; $(\text{NH}_4)_2\text{SO}_4$, 10mM; O-dianisidine, 0.01% & hydrogen peroxide 0.045%) or alkaline phosphatase substrate (Sigma Fast) following the supplier's recommendations.

Molecular analysis:

Example/construct 1

Analysis	Entire Protein
Length	629 aa
Molecular Weight	71629.96 m.w.
1 microgram =	13.961 pMoles
Molar Extinction coefficient	67680
1 A[280] corr. to	1.06 mg/ml
A[280] of 1 mg/ml	0.94 AU
Isoelectric Point	6.41
Charge at pH 7	-5.84

Isoelectric Point	6.41
Charge at pH 7	-5.84

Example/construct 2

Analysis	Entire Protein
Length	620 aa
Molecular Weight	70561.90 m.w.
1 microgram =	14.172 pMoles
Molar Extinction coefficient	67680
1 A[280] corr. to	1.04 mg/ml
A[280] of 1 mg/ml	0.96 AU
Isoelectric Point	6.28
Charge at pH 7	-6.36

Example/construct 3

Analysis	Entire Protein
Length	628 aa
Molecular Weight	71627.01 m.w.
1 microgram =	13.961 pMoles
Molar Extinction coefficient	67680
1 A[280] corr. to	1.06 mg/ml
A[280] of 1 mg/ml	0.94 AU
Isoelectric Point	6.34
Charge at pH 7	-6.84

5

Example/construct 4

Analysis	Entire Protein
Length	620 aa
Molecular Weight	70561.90 m.w.

1 microgram =	14.172 pMoles
Molar Extinction coefficient	67680
1 A[280] corr. to	1.04 mg/ml
A[280] of 1 mg/ml	0.96 AU
Isoelectric Point	6.28
Charge at pH 7	-6.36

Example 5

Evaluation of the production of proteins with or without the secretion signal (secretion or signal sequence) of the protein D 1/3 in the fusion protein.

Table B

Protein	Expression level
PD1/3-PRAME with secretion signal	+
PD1/3-PRAME without secretion signal	+++

Fig 11: SDS page analysis after Coomassie-blue staining of recombinant 1/3PD-PRAME with or without secretion signal after IPTG induction of *E. coli* BL21 DE3 strain transformed with recombinant pET21. An equivalent of 100µL of culture after 3 hours of induction in BL21 DE3 strain with 1mM IPTG at 37°C has been loaded on gel. Those constructs are presented on gel before (BI) and after (AI) inductions in soluble (supernatant) and non-soluble (pellet) fractions. Lane 1: Standard Broad Range prestain (BioRad Cat#161-0318), lane 2 (pD1/3-PRAME + SS, BI, supernatant), lane 3 (pD1/3-PRAME + SS, BI, pellet), lane 4 (pD1/3-PRAME + SS, AI, supernatant), lane 5 (pD1/3-PRAME + SS, AI, pellet), lane 6 (pD1/3-PRAME + SS + His, BI, supernatant), lane 7 (pD1/3-PRAME + SS + His, BI, pellet), lane 8 (pD1/3-PRAME + SS + His, AI, supernatant), lane 9 (pD1/3-PRAME + SS + His, AI, pellet), lane 10 (pD1/3-PRAME w/o SS, BI, supernatant), lane 11 (pD1/3-PRAME w/o SS, BI, pellet), lane 12 (pD1/3-PRAME w/o SS, AI, supernatant), lane 13 (pD1/3-PRAME w/o SS, AI, pellet), lane 14 (pD1/3-PRAME w/o SS + His, BI, supernatant), lane 15 (pD1/3-PRAME w/o SS + His, BI, pellet), lane 16 (pD1/3-PRAME w/o SS + His, AI, supernatant), lane 17 (pD1/3-PRAME w/o SS + His, AI, pellet).

Example 6: Immunogenicity of PD-PRAME-His formulated in AS01B or AS15: dose range of antigen in a constant dose of adjuvant.

Aim: dose-range of antigen to select the best dose to use in preclinical experiments

Protocol:

6 groups of 12 CB6F1 mice received intra-muscular (IM) injections at day 0 and 14 of:

1. PBS
2. PRAME (50* μ g) in AS01B or AS15
3. PRAME (10 μ g) in AS01B or AS15
4. PRAME (2 μ g) in AS01B or AS15
5. PRAME (0.4 μ g) in AS01B or AS15
6. PRAME (0.08 μ g) in AS01B or AS15

* 44.7 μ g actually administered instead of the 50 μ g intended dose

AS01B is a liposomal adjuvant formulation comprising QS21 and 3D-MPL; AS15 is a liposomal adjuvant formulation comprising QS21, 3D-MPL and CpG.

The construct used in this example was Example/Construct 3a (pET26 with a His tail), provided in 5 mM Tris buffer pH 8.5 - 0.5 M Arginine. Protein provided in a borate buffer with sucrose may also be used.

Read-outs:

- Intracellular Cytokine Staining (ICS) 14 days post 2 injections after in vitro restimulation of spleen cells (4 pools of 3 mice per group) with the pool of peptides PRAME at 1 μ g/ml/peptide (15-mer)

CD4 response (AS01B adjuvant)

Results of ICS for CD4 cytokines for the AS01B adjuvant are shown in Figure 3. In this experiment it may be concluded that the best dose of PRAME antigen to induce a CD4 response in AS01B under these conditions seems to be 2µg.

5 *CD8 response (AS01B adjuvant)*

Results of ICS for CD8 cytokines for the AS01B adjuvant are shown in Figure 4. The data appear to show a very low CD8 response and heterogeneity of the response intra-group.

CD4 response (AS15 adjuvant)

10 Results of ICS for CD4 cytokines for the AS15 adjuvant are shown in Figure 5. These data appear to show that a similar CD4 response was induced with 44µg, 10µg, 2µg and 0.4µg of PRAME formulated in AS15; with a decreased response induced with 0.08µg PRAME

15 *CD8 response (AS15 adjuvant)*

Results of ICS for CD8 cytokines for the AS15 adjuvant are shown in Figure 6. These data appear to show no CD8 response (background in the PBS group)

Example 7

In summary, for the inventions described herein, the following summary may be used to described specific constructs of PD1/3-PRAME that have so far been generated:

Constructs used for PD1/3-PRAME

- No signal sequence of Protein D are included (amino acids 2 to 19 of protein D)
- The Methionine of Protein D is included (AA 1 of the protein D)
- Two unrelated AA (Asp and Pro) are substituted for amino acids 2-Lys and 3-Leu of Protein D
- The first 109 AA of protein D after the signal sequence of protein D are included (109 amino acids including the first Met in N-term $\pm$ AA20 to 127 of the protein D)
- AA 1 - 509 of PRAME are included (full length original sequence of PRAME)
- With or without a His tail composed of one of the following:
 - Three unrelated amino acid (Thr, Ser and Gly) + 6 His residues for cloning in TCMP14 plasmid; or
 - Two unrelated amino acid (Leu and Glu) + 6 His residues for cloning in pET21 plasmid; or
 - 6 His residues for cloning in pET26 plasmid.

pD1/3 - PRAME +/- His tail protein :

	pD 1/3 20 - 127	PRAME 1 - 509	
N term MDP	pD 1/3	PRAME	C term
N term MDP	pD 1/3	PRAME	TSG 6xHis (in TCMP14) C term
N term MDP	pD 1/3	PRAME	LE 6xHis (in pET21) C term
N term MDP	pD 1/3	PRAME	6xHis (in pET26) C term

A marked up amino acid sequence of examples of constructs of the present invention is shown in Figure 7

Alignments of the following constructs are shown in Figures 8, 9 and 10:

- Alignment between LipoD-MAGE3-His and D1/3-PRAME-His (Figure 8)
- Alignment between the shared sequence of the original protein D from *Haemophilus influenzae* and the LipoD-MAGE3-His (Figure 9)
- Alignment between the shared sequence of the original protein D from *Haemophilus influenzae*, the LipoD-MAGE3-His and the pD1/3-PRAME-His (Figure 10)

Formulation of Vaccine preparation using fusion proteins:

The fusion proteins of the invention can be formulated into vaccines which are either adjuvanted or not. In one embodiment, as an adjuvant, the formulation may comprise a mixture of 3 de -O-acylated monophosphoryl lipid A (3D-MPL) and QS21 in an oil/water emulsion. The adjuvant system SBAS2 has been previously described WO 95/17210. The adjuvant for use in the present invention may alternatively comprise 3 de -O-acylated monophosphoryl lipid A (3D-MPL), QS21 and CpG in an oil-in water formulation or in a liposomal formulation.

3D-MPL: is an immunostimulant derived from the lipopolysaccharide (LPS) of the Gram-negative bacterium *Salmonella minnesota*. MPL has been deacylated and is lacking a phosphate group on the lipid A moiety. This chemical treatment dramatically reduces toxicity while preserving the immunostimulant properties (Ribi, 1986).

It is believed that 3D-MPL combined with various vehicles may strongly enhance both the humoral and a TH1 type of cellular immunity.

QS21: is a natural saponin molecule extracted from the bark of the South American tree *Quillaja saponaria* Molina. A purification technique developed to separate the individual saponines from the crude extracts of the bark, permitted the isolation of the particular saponin, QS21, which is a triterpene glycoside demonstrating stronger adjuvant activity and lower toxicity as compared with the parent component. QS21 has been shown to activate MHC class I restricted CTLs to several subunit Ags, as well as to stimulate Ag specific lymphocytic proliferation (Kensil, 1992).

It is thought that there may be a synergistic effect of combinations of MPL and QS21 in the induction of both humoral and TH1 type cellular immune responses.

The oil/water emulsion comprises an organic phase made of 2 oils (a tocopherol and squalene), and an aqueous phase of PBS containing Tween 80 as emulsifier. The emulsion comprised 5% squalene 5% tocopherol 0.4% Tween 80 and had an average particle size of 180 nm and is known as SB62 (see WO 95/17210). The resulting oil droplets should have a size of approximately 180 nm.

The adjuvant for use in the present invention may be formulated as a combination of MPL and QS21, in an oil/water emulsion or in a liposomal formulation. This preparation should be delivered in vials of 0.7 ml to be admixed with lyophilised antigen or fusion protein (vials containing from 30 to 300 µg antigen).

Immunostimulatory oligonucleotides may also be used. Examples oligonucleotides for use in adjuvants or vaccines of the present invention include CpG containing oligonucleotides, generally containing two or more dinucleotide CpG motifs separated by at least three, more often at least six or more nucleotides. A CpG motif is a cytosine nucleotide followed by a guanine nucleotide. The CpG oligonucleotides are typically deoxynucleotides. In one embodiment the internucleotide in the oligonucleotide is phosphorodithioate, or more preferably a phosphorothioate bond, although phosphodiester and other internucleotide bonds are within the scope of the invention. Also included within the scope of the invention are oligonucleotides with mixed internucleotide linkages. Methods for producing phosphorothioate oligonucleotides or phosphorodithioate are described in US5,666,153, US 5,278,302 and WO 95/26204.

Examples of oligonucleotides are as follows:

TCC ATG ACG TTC CTG ACG TT (CpG 1826; SEQ ID NO: 36)

TCT CCC AGC GTG CGC CAT (CpG 1758; SEQ ID NO: 37)

ACC GAT GAC GTC GCC GGT GAC GGC ACC ACG TCG TCG TTT TGT CGT TTT GTC GTT (CpG 2006; SEQ ID NO: 38)

TCC ATG ACG TTC CTG ATG CT (CpG 1668; SEQ ID NO: 39)

TCG ACG TTT TCG GCG CGC GCC G (CpG 5456; SEQ ID NO: 40),

the sequences may contain phosphorothioate modified internucleotide linkages.

Alternative CpG oligonucleotides may comprise one or more sequences above in that they have inconsequential deletions or additions thereto.

The CpG oligonucleotides may be synthesized by any method known in the art (for example see EP 468520). Conveniently, such oligonucleotides may be synthesized utilising an automated synthesizer.

In one embodiment of the present invention an adjuvant combination for use in the invention includes one or more of the following components: 3D-MPL and QS21 (EP 0 671 948 B1); oil in water emulsions comprising 3D-MPL and QS21 (WO 95/17210, WO 98/56414); or 3D-MPL formulated with other carriers (EP 0 689 454 B1). Other adjuvant systems that may be used in the present invention comprise a combination of 3D-MPL, QS21 and a CpG oligonucleotide as described in US6558670 and US6544518.

The final vaccine may be obtained after reconstitution of the lyophilized formulation.

References:

1. A. Roca (U. of Wisconsin), personal communication.
2. Studier, F.W. (1991) *J. Mol. Biol.* **219**, 37-44.
3. Jan H. Kessler^a *et al The Journal of Experimental Medicine*, Volume 193, Number 1, January 1, 2001 73-88,
4. Ikeda H *et al Immunity*, Feb; 6(2): 1997, 199-208

SEQ ID NO: 1

DNA sequence for Example 1

atggatccaagcagccattcatcaaatatggcgaatacccaaatgaaatcagacaaaatcattattgctc
5 accgtggtgctagecggttatttaccagagcatacgttagaatctaaagcacttgcgtttgacaaacaggc
tgattatttagagcaagatttagcaatgactaaggatggcggttagtggttattcacgatacacttttta
gatggcttgactgatgttgcgaaaaattcccacatcgatcgtaaaagatggccgttactatgtcatcg
actttaccttaaaagaaattcaaagtttagaaatgacagaaaactttgaaacctggaacgaaggcggtt
gtgggggttcattcagagccgatacatcagcatgagtggtggtgacaagcccacggagacttggtggagctg
10 gcagggcagagcctgctgaaggatgaggccctggccattgcccgcctggagttgctgcccagggagctct
tcccgcactcttcatggcagcctttgacgggagacacagccagaccctgaaggcaatggtgcaggcctg
gcccttcacctgcctccctctgggagtgctgatgaagggacaacatcttcacctggagaccttcaaagct
gtgcttgatggacttgatgtgctccttgcccaggaggttcgccccaggaggtggaaacttcaagtgtggtg
atctacggaagaactctcatcaggacttctggactgtatggtctggaaacagggccagtctgtactcatt
15 tccagagccagaagcagctcagcccatgacaaagaagcgaaaagtagatggtttgagcacagaggcagag
cagcccttcattccagtagaggtgctcgtagacctgttccctcaagggaaggtgctgtgatgaattgttct
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tgcaatgcccatgcaggatatcaagatgatcctgaaaatggtgcagctggactctattgaagatttggaa
gtgacttgtagctggaagctacccaccttggcgaaattttctccttacctggggccagatgattaatctgc
20 gtagactcctcctctcccacatccatgcactctcctacatttccccggagaaggaagagcagtatatcgc
ccagttcacctctcagttcctcagctctgcagtgctgcaggtctctctatgtggactctttatttttctt
agaggccgcctggatcagttgctcaggcacgtgatgaacccttggaaccctctcaataactaactgcc
ggctttcggaaggggatgtgatgcactctgtcccagagtcccagcgtcagtcagctaagtgtcctgagctc
aagtggggtcatgctgacggatgtaagtcccagacccctccaagctctgctggagagagcctctgccacc
25 ctccaggacctggtctttgatgagtggtggatcacggatgatcagctccttgccctcctgccttccctga
gccactgctcccagcttacaaccttaagcttctacgggaattccatctccatatctgccttgagagctc
cctgcagcaacctcatcggtgagcaatctgacccacgtgctgtatcctgtccccctggagagttatgag
gacatccatggtacctccacctggagaggcttgccctatctgcatgccaggctcaggaggtgctgtgtg
agttggggcgggccagcatggtctggcttagtgccaaccctgtcctcactgtggggacagaaccttcta
30 tgaccgcggagcccatcctgtgcccctgtttcatgcctaactagtggccaccatcaccatcaccat

SEQ ID NO: 2

Amino Acid Sequence for Example 1

35 MDPSSHSSNMANTQMKSDKIIAHRGASGYLPEHTLESKALAFQAQADYLEQDLAMTKDGRLLVVIHDHFL
DGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMERRRLWGSISQRYISMSVWTSRRLVEL
AQQSLLKDEALAIAALELLPRELFPPLFMAAFDGRHSQTLKAMVQAWPFTCLPLGVLMMKGQHLHLETFA
VLDGLDVLLAQEVRPRRWKLQVLDLRKNSHQDFWTVWSGNRASLYSFPEPEAAQPMTKKRKVDGLSTEAE
QPFIPVEVLVDLFLKEGACDELFSYLIEKVKRKKNVRLCCKKLKIFAMPMQDIKMI LKMVQLDSIEDLE
40 VTCTWKLPTLAKFSPYLGQMINLRRLLLLSHIHASSYISPEKEEQYIAQFTSQFLSLQCLQALYVDSLFFL
RGRLDQLLRHVMNPLETSLITNCRLSEGDVMHLSQSPSVSQLSVLSLSGVMLTDVSPEPLQALLERASAT

LQDLVFDECGITDDQLLALLPSLSHCSQLTTLSFYGNSISISALQSLQLHLIGLSNLTHVLYPVPLESYE
DIHGTLHLERLAYLHARLRELLCELGRPSMVWLSANPCPHCGDRTFYDPEPILCPCFMPNTSGHHHHHH

SEQ ID NO: 3

5 DNA Sequence for example 2

atggatccaagcagccattcatcaaatatggcgaatacccaaataaaatcagacaaaatcattattgctc
accgtgggtgctagcgggtatttaccagagcatatcgtagaatctaaagcacttgcggtttgcacaacaggc
tgattatttagagcaagatttagcaatgactaaggatggcggttagtggttattcacgatcacttttta
10 gatggcttgactgatgttgcaaaaaattcccacatcgatcgtaaaagatggccgttactatgtcatcg
actttaccttaaaagaaattcaaagtttagaaatgacagaaaaactttgaaaccatggaacgaaggcggtt
gtgggggttcattcagagccgatacatcagcatgagtggtggtggacaagcccacggagacttggtggagctg
gcagggcagagcctgctgaaggatgagggcctggccattgcccgcctggagttgctgcccagggagctct
tcccgcactcttcatggcagcctttgacgggagacacagccagaccctgaaggcaatgggtgcaggcctg
15 gcccttcacctgectcctctgggagtgctgatgaagggaacacatcttcacctggagaccttcaaagct
gtgcttgatggacttgatgtgctccttgcccaggaggttcgccccaggaggtggaaacttcaagtgtctg
atttacggaagaactctcatcaggacttctggactgtatggtctggaaacagggccagctctgtactcatt
tccagagccagaagcagctcagcccatagcaaaagaagcgaaaagtagatggtttgagcacagaggcagag
cagcccttcattccagtagaggtgctcgtagacctgttccctcaaggaaggtgcctgtgatgaattgttct
20 cctacctcattgagaaagtgaagcgaaagaaaaatgtactacgcctgtgctgtaagaagctgaagatttt
tgcaatgcccatgcaggatatcaagatgatacctgaaaatgggtgcagctggactctattgaagatttgaa
gtgacttgtagctggaagctaccacaccttggcgaaattttctccttacctgggcccagatgattaatctgc
gtagactcctcctctcccacatccatgcattctcctacatttccccgggagaaggaagagcagtatatcgc
ccagttcacctctcagttcctcagctctgcagtgctgcaggtctctctatgtggactctttatttttctt
25 agagggcgctggatcagttgctcaggcacgtgatgaaccccttggaacccctctcaataactaactgcc
ggctttcggaaggggatgtgatgcattctgtcccagagtcocagcgtcagtcagctaagtgtcctgagctc
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ctccaggacctgggtctttgatgagtggtgggatcacggatgatcagctccttgccctcctgccttcctga
gccactgctcccagcttacaaccttaagcttctacgggaattccatctccatatctgccttgagagctct
30 cctgcagcacctcatcggtgagcaatctgacccacgtgctgtatcctgtccccctggagagttatgag
gacatccatggtacctccacctggagaggttgccctatctgcatgccaggtcagggagttgctgtgtg
agttggggggggccagcatggtctggcttagtgccaaacctgtcctcactgtggggacagaaccttcta
tgacccggagcccatcctgtgccccctgtttcatgcctaac

35 SEQ ID NO: 4

AMINO ACIDS SEQUENCE EXAMPLE 2

MDPSSSHSSNMANTQMKSDKIIAHRGASGYLPEHTLESKALAFQAQADYLEQDLAMTKDGRLLVVIHDHFL
DGLTDVAKKFPHRHRKDGRIYVIDFTLKEIQSLEMTENFETMERRRLWGSIQSRYISMSVWTSPPRLVEL

5 AQQSLLKDEALAIAALELLPRELFPPLFMAAFDGRHSQTLKAMVQAWPFTCLPLGLVLMRGQHLHLETFKA
 VLDGLDVLLAQEVRPRRWKLQVLDLRKNHQDFWTVWSGNRASLYSFPEPEAAQPMTKRKVDGLSTEAE
 QPFI PVEVLVDLFLKEGACDELF SYLIEKVKRKNVLRCLCKKLIKIFAMPMDIKMILKMVQLDSIEDLE
 VTCTWKLP TLAKFSPYLGQMINLRRLLLSHIHASSYISPEKEEQYIAQFTSQFLSLQCLQALYVDSLFFL
 RGRLDQLLRHVMNPLETLSITNCRLSEGDVMHLSQSPSVSQLSVLSLSGVMLTDVSPPEPLQALLERASAT
 LQDLVFDECGITDDQLLALLPSLSHCSQLTTLSFYGNSISISALQSLQLHLIGLSNLTHVLYPVPLESYE
 DIHGTLHLERLAYLHARLRELLCELGRPSMVWLSANPCPHCGDRTFYDPEPILCPCFMPN

SEQ ID NO:5

10 DNA sequence for Example 3

15 atggatccaagcagccattcatcaaatatggcgaatacccaaatgaaatcagacaaaatcattattgctc
 accgtggtgctagcgggtatttaccagagcatacgtagaatctaaagcacttgcggttgccacaacaggc
 tgattatttagagcaagatttagcaatgactaaggatggcggttagtggttattcacgatcacttttta
 gatggcttgactgatgttgcaaaaaattcccatcgcgcgtatgtaagatggccgttactatgtcatcg
 actttaccttaaaagaaattcaaagtttagaaatgacagaaaaactttgaaaccatggaacgaaggcggtt
 gtgggggttcattcagagccgatacatcagcatgagtggtggaacaagcccacggagacttggtggagctg
 gcagggcagagcctgctgaaggatgaggccctggccattgcccgcctggagttgctgcccaggagctct
 tcccgccactcttcatggcagcctttgacgggagacacagccagaccctgaaggcaatggtgcaggcctg
 20 gcccttcacctgcctccctctgggagtgctgatgaagggacaacatcttcacctggagaccttcaaagct
 gtgcttgatggacttgatgtgctccttgcccaggagggtcgccccaggagggtggaacttcaagtgtggtg
 atttacggaagaactctcatcaggacttctggactgtatggtctggaacagggccagtctgtactcatt
 tccagagccagaagcagctcagcccatgacaaagaagcgaaaagtagatggtttgagcacagaggcagag
 cagcccttcattccagtagaggtgctcgtagacctgttctcaaggaaggtgcctgtgatgaattgttct
 25 cctacctcattgagaaagtgaagcgaaagaaaaatgtactacgcctgtgctgtaagaagctgaagatttt
 tgcaatgcccattgcaggatatcaagatgatcctgaaaatggtgcagctggactctattgaagatttgga
 gtgacttgtagctggaagctacccaccttggcgaaattttctccttacctgggcccagatgattaatctgc
 gtagactcctcctctcccatccatgcatcttctacatttccccggagaaggaagagcagtatatcgc
 ccagttcacctctcagttcctcagctgctgcagtgctgcaggctctctatgtggactctttatttttctt
 30 agaggccgcctggatcagttgctcaggcacgtgatgaacccttggaaccctctcaataactaactgcc
 ggctttcggaaggggatgtgatgcatctgtcccagagtcacagcgtcagtcagctaagtgtcctgagctc
 aagtggggctcatgctgaccgatgtaagtcccgagccctccaagctctgctggagagagcctctgccacc
 ctccaggacctggtctttgatgagtggtgggatcacggatgatcagctccttgccctcctgccttccctga
 gccactgctcccagcttacaaccttaagcttctacgggaattccatctccatatctgccttgagagctc
 35 cctgcagcacctcatcgggtgagcaatctgaccacgtgctgtatcctgtccccctggagagttatgag
 gacatccatggtacctccacctggagaggcttgctatctgcatgccaggctcagggagttgctgtgtg
 agttggggcgcccagcatggtctggcttagtgccaaccctgtcctcactgtggggacagaaccttcta
 tgaccggagcccatcctgtgcccctgtttcatgctaacctcgagcaccaccaccaccaccac

40 SEQ ID NO: 6

Amino acids sequence: for Example 3

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFQAQADYLEQDLAMTKDGRLLVVIHDHFL
DGLTDVAKKFPHRHRKDGRIYVIDFTLKEIQSLEMTENFETMERRRLWGSIQSRYISMSVWTSPPRLVEL
AGQSLLKDEALATAALELLPRELFPPLFMAAFDGRHSQTLKAMVQAWPFTCLPLGVLMKGQHLHLETFA
VLDGLDVLLAQEVRPRRWKLQVLDLRKNSHQDFWTWVSGNRASLYSFPEPEAAQPMTKKRKVDGLSTEAE
5 QPFI PVEVLVDLFLKEGACDELFSYLI EKVRKKNVLR LCKK LKIFAMPMDIKMILKMVQLDSIEDLE
VTCTWKLPTLAKFSPYLQMINLRRLLLSHIHASSYISPEKEEQYIAQFTSQFLSLQCLQALYVDSLFFL
RGRLDQLLRHVMNPLETSLITNCRLSEGDVMHLSQSPSVSQLSVLSLSGVMLTDVSPEPLQALLERASAT
LQDLVFDECGITDDQLLALLPSLSHCSQLTTLSTFYGNSISISALQSLQHLIGLSNLTHVLYPVPLESYE
DIHGTLHLERLAYLHARLRELLCELGRPSMVWLSANPCPHCGDRTFYDPEPILCPCFMPNLEHHHHHH
10

SEQ ID NO: 7.

DNA sequence for Example 4:

atggatccaagcagccattcatcaaatatggcgaatacccaaatagaatcagacaaaatcattattgctc
15 accgtggtgctagcgggtatttaccagagcatacgttagaatctaaagcacttgcggttgccacaacaggc
tgattatttagagcaagatttagcaatgactaaggatggcgttttagtggttattcacgatcacttttta
gatggcttgactgatgttgcaaaaaattcccatatcgatcgtaagatggcgggtactatgtcatcg
actttaccttaaaagaaattcaaagttagaaatgacagaaaactttgaaaccatggaacgaaggcggtt
gtgggggtccattcagagccgatacatcagcatgagtggtggtgacaagcccacggagacttggtggagctg
20 gcagggcagagcctgctgaaggatgaggccctggccattgcccgcctggagttgctgccaggagctct
tcccgcactcttcatggcagcctttgacgggagacacagccagaccctgaaggcaatgggtgcaggcctg
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atttacggaagaactctcatcaggacttctggactgtatggtctggaaacagggccagctctgtactcatt
25 tccagagccagaagcagctcagcccatgacaaagaagcgaaaagtagatggtttgagcacagaggcagag
cagcccttcattccagtagaggtgctcgtagacctgttcctcaagggaaggtgcctgtgatgaattgttct
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tgcaatgcccattgcaggatataagatgatcctgaaaatgggtgcagctggactctattgaagatttgga
gtgacttgtagctggaagctacccaccttggcgaaattttctccttacctgggcccagatgattaatctgc
30 gtagactcctcctctcccatccatgcattcttctacatttccccggagaaggaagagcagtatatcgc
ccagttcacctctcagttcctcagctgtcagtgccctgcaggtctctatgtggactctttatttttctt
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ggctttcggaaggggatgtgatgcattctgtcccagagtcaccagctcagtcagctaagtgtcctgagctt
aagtggggtcatgctgaccgatgtaagtcccagagccctccaagctctgctggagagagcctctgccacc
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40 tgaccgcggagcccatcctgtgcccctgtttcatgctaac

SEQ ID NO: 8

Amino Acid Sequence for Example 4

MDPSSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFQAQADYLEQDLAMTKDGRLLVVIHDHFL
DGLTDVAKKFPHRHRKDGRIYVIDFTLKEIQSLEMTENFETMERRRLWGSIQSRYISMSVWTSPPRLVEL
5 AGQSLLKDEALIAALELLPRELFPPLFMAAFDGRHSQTLKAMVQAWPFTCLPLGVLMKGQHLHLETFA
VLDGLDVLLAQEVPRRWKLQVLDLRKNHQDFWTVWSGNRASLYSFPEPEAAQPMTKKRKVDGLSTEAE
QPFIPVEVLVDLFLKEGACDELFSYLIEKVKRKKNVLRCLCKKLKIFAMPMDIKMILKMVQLDSIEDLE
VTCTWKLP TLAKFSPYLGQMINLRRLLLSHIHASSYISPEKEEQYIAQFTSQFLSLQCLQALYVDSLFFL
RGRLDQLLRHVMNPLETLSITNCRLESGDVMHLSQSPSVLSVLSLGVMLTDVSPEPLQALLERASAT
10 LQDLVFDECGITDDQLLALLPSLSHCSQLTTLSFYGNSISISALQSLLOHLIGLSNLTHVLYPVPLESYE
DIHGTLHLERLAYLHARLRELLCELGRPSMVWLSANPCPHCGDRTFYDPEPILCPCFMPN

SEQ ID NO: 9

Codon optimised DNA sequence for example 3a

15 atggatccaagcagccattcatcaaatatggcgaatacccaaatgaaatcagacaaaatcattattgctc
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SEQ ID NO: 10

Amino Acid Sequence for Example 3a

MDPSSHSSNMANTQMKSDKIIIAHRGASGYLPEHTLESKALAFQAQADYLEQDLAMTKDGRLLVVIHDHFL
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5 AGQSLLKDEALAIAALELLPRELFPPLFMAAFDGRHSQTLKAMVQAWPFTCLPLGVLMKGQHLHLETFKA
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QPFIPVEVLVDLFLKEGACDELFSLYIEKVKRKNVLRRLCCKKLKIFAMPMDIKMILKMVQLDSIEDLE
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10 LQDLVFDECGITDDQLLALLPSLSHCSQLTTLSTFYGNSISISALQSLQHLIGLSNLTHVLYPVPLESYE
DIHGTLHLERLAYLHARLRELLCELGRPSMVWLSANPCPHCGDRTFYDPEPILCPCFMPNHHHHHH

SEQ ID NO: 11

Codon optimised DNA sequence for Example 4a

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SEQ ID NO: 12

Amino Acid Sequence for Example 4a

MDPSSHSSNMANTQMKSDKII IAHRGASGYLPEHTLESKALAFQQADYLEQDLAMTKDGRLVVIHDHFL
DGLTDVAKKFPHRHRKDGRYYVIDFTLKEIQSLEMTENFETMERRRLWGSIQSRYISMSVWTSPPRLVEL
5 AGQSLLKDEALAIAALELLPRELFPPLFMAAFDGRHSQTLKAMVQAWPFTCLPLGVLMKGQHLHLETFKA
VLDGLDVLLAQEVPRRWKLQVLDLRKNSHQDFWTVWSGNRASLYSFPEPEAAQPMTKKRKVDGLSTEAE
QPFIPVEVLVDLFLKEGACDELF SYLIEKVKRKKNVLRRLCCKKLKIFAMPMQDIKMILKMVQLDSIEDLE
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10 LQDLVFDECGITDDQLLALLPSLSHCSQLTTLSFYGNSISISALQSLLQHLIGLSNLTHVLYPVPPLSEYE
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SEQUENCE LISTING

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 <212> DNA
 <213> Artificial Sequence
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 <223> MDP- 20-127 - Protein D - PRAME - TSGHHHHHH
 (plasmid TCMP14)

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20 <210> 2
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    <212> PRT
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25 <220>
    <223> MDP- 20-127 - Protein D - PRAME - TSGHHHHHH
        (plasmid TCMP14)

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    Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
35             35             40             45
    Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
        50             55             60
    Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
        65             70             75             80
40 Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
        85             90             95
    Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
        100            105            110
    Glu Arg Arg Arg Leu Trp Gly Ser Ile Gln Ser Arg Tyr Ile Ser Met

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	Leu Leu Lys Asp Glu Ala Leu Ala Ile Ala Ala Leu Glu Leu Leu Pro		
5	145	150	155
	Arg Glu Leu Phe Pro Pro Leu Phe Met Ala Ala Phe Asp Gly Arg His		
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	Ser Gln Thr Leu Lys Ala Met Val Gln Ala Trp Pro Phe Thr Cys Leu		
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10	Pro Leu Gly Val Leu Met Lys Gly Gln His Leu His Leu Glu Thr Phe		
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	Lys Ala Val Leu Asp Gly Leu Asp Val Leu Leu Ala Gln Glu Val Arg		
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	Pro Arg Arg Trp Lys Leu Gln Val Leu Asp Leu Arg Lys Asn Ser His		
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	Ile Leu Lys Met Val Gln Leu Asp Ser Ile Glu Asp Leu Glu Val Thr		
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	Leu Phe Phe Leu Arg Gly Arg Leu Asp Gln Leu Leu Arg His Val Met		
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	530	535	540	
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 25 <211> 1860
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 (plasmid TCMP14)

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<210> 4

<211> 620

25 <212> PRT

<213> Artificial Sequence

<220>

<223> MDP- 20-127 - Protein D - PRAME - no His tail

30 (plasmid TCMP14)

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 35 40 45
 Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
 40 50 55 60
 Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe
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 Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr
 85 90 95

Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met
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 Glu Arg Arg Arg Leu Trp Gly Ser Ile Gln Ser Arg Tyr Ile Ser Met
 115 120 125
 5 Ser Val Trp Thr Ser Pro Arg Arg Leu Val Glu Leu Ala Gly Gln Ser
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 Arg Glu Leu Phe Pro Pro Leu Phe Met Ala Ala Phe Asp Gly Arg His
 10 165 170 175
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 180 185 190
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 195 200 205
 15 Lys Ala Val Leu Asp Gly Leu Asp Val Leu Leu Ala Gln Glu Val Arg
 210 215 220
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 225 230 235 240
 Gln Asp Phe Trp Thr Val Trp Ser Gly Asn Arg Ala Ser Leu Tyr Ser
 20 245 250 255
 Phe Pro Glu Pro Glu Ala Ala Gln Pro Met Thr Lys Lys Arg Lys Val
 260 265 270
 Asp Gly Leu Ser Thr Glu Ala Glu Gln Pro Phe Ile Pro Val Glu Val
 275 280 285
 25 Leu Val Asp Leu Phe Leu Lys Glu Gly Ala Cys Asp Glu Leu Phe Ser
 290 295 300
 Tyr Leu Ile Glu Lys Val Lys Arg Lys Lys Asn Val Leu Arg Leu Cys
 305 310 315 320
 Cys Lys Lys Leu Lys Ile Phe Ala Met Pro Met Gln Asp Ile Lys Met
 30 325 330 335
 Ile Leu Lys Met Val Gln Leu Asp Ser Ile Glu Asp Leu Glu Val Thr
 340 345 350
 Cys Thr Trp Lys Leu Pro Thr Leu Ala Lys Phe Ser Pro Tyr Leu Gly
 355 360 365
 35 Gln Met Ile Asn Leu Arg Arg Leu Leu Leu Ser His Ile His Ala Ser
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 Ser Tyr Ile Ser Pro Glu Lys Glu Glu Gln Tyr Ile Ala Gln Phe Thr
 385 390 395 400
 Ser Gln Phe Leu Ser Leu Gln Cys Leu Gln Ala Leu Tyr Val Asp Ser
 40 405 410 415
 Leu Phe Phe Leu Arg Gly Arg Leu Asp Gln Leu Leu Arg His Val Met
 420 425 430
 Asn Pro Leu Glu Thr Leu Ser Ile Thr Asn Cys Arg Leu Ser Glu Gly
 435 440 445

Asp Val Met His Leu Ser Gln Ser Pro Ser Val Ser Gln Leu Ser Val
 450 455 460
 Leu Ser Leu Ser Gly Val Met Leu Thr Asp Val Ser Pro Glu Pro Leu
 465 470 475 480
 5 Gln Ala Leu Leu Glu Arg Ala Ser Ala Thr Leu Gln Asp Leu Val Phe
 485 490 495
 Asp Glu Cys Gly Ile Thr Asp Asp Gln Leu Leu Ala Leu Leu Pro Ser
 500 505 510
 Leu Ser His Cys Ser Gln Leu Thr Thr Leu Ser Phe Tyr Gly Asn Ser
 10 515 520 525
 Ile Ser Ile Ser Ala Leu Gln Ser Leu Leu Gln His Leu Ile Gly Leu
 530 535 540
 Ser Asn Leu Thr His Val Leu Tyr Pro Val Pro Leu Glu Ser Tyr Glu
 545 550 555 560
 15 Asp Ile His Gly Thr Leu His Leu Glu Arg Leu Ala Tyr Leu His Ala
 565 570 575
 Arg Leu Arg Glu Leu Leu Cys Glu Leu Gly Arg Pro Ser Met Val Trp
 580 585 590
 Leu Ser Ala Asn Pro Cys Pro His Cys Gly Asp Arg Thr Phe Tyr Asp
 20 595 600 605
 Pro Glu Pro Ile Leu Cys Pro Cys Phe Met Pro Asn
 610 615 620

25 <210> 5
 <211> 1884
 <212> DNA
 <213> Artificial Sequence

30 <220>
 <223> MDP- 20-127 - Protein D - PRAME - LEHHHHHH
 (plasmid pET21)

<400> 5
 35 atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc 60
 attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca 120
 cttgcggttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt 180
 cgttttagtgg ttattcacga tcacttttta gatggcctga ctgatgttgc gaaaaaatc 240
 ccacatcgtc atcgtaaaga tggcgcgttac tatgtcatcg actttacctt aaaagaaatt 300
 40 caaagtttag aaatgacaga aaactttgaa accatggaac gaaggcgttt gtgggggttc 360
 attcagagcc gatacatcag catgagtgtg tggacaagcc cacggagact tgtggagctg 420
 gcagggcaga gcctgctgaa ggatgaggcc ctggccattg ccgccctgga gttgctgccc 480
 agggagctct tcccgcact cttcatggca gcctttgacg ggagacacag ccagaccctg 540
 aaggcaatgg tgcaggcctg gcccttcacc tgcctcctc tgggagtgtc gatgaaggga 600

caacatcttc acctggagac cttcaaagct gtgcttgatg gacttgatgt gctccttgcc 660
 caggagggttc gccccaggag gtggaaactt caagtgctgg atttacggaa gaactctcat 720
 caggacttct ggactgtatg gtctggaaac agggccagtc tgtactcatt tccagagcca 780
 gaagcagctc agcccatgac aaagaagcga aaagtagatg gtttgagcac agaggcagag 840
 5 cagcccttca ttccagtaga ggtgctcgta gacctgttcc tcaaggaagg tgctgtgat 900
 gaattgttct cctacctcat tgagaaagtg aagcgaaaaga aaaatgtact acgcctgtgc 960
 tgtaagaagc tgaagatddd tgcaatgccc atgcaggata tcaagatgat cctgaaaatg 1020
 gtgcagctgg actctattga agatttggaa gtgacttgta cctggaagct acccaccttg 1080
 gcgaaatddd ctccttacct gggccagatg attaactctgc gtagactcct cctctccac 1140
 10 atccatgcat cttcctacat ttccccggag aaggaagagc agtatatcgc ccagttcacc 1200
 tctcagttcc tcagtctgca gtgcctgcag gctctctatg tggactcttt attttccctt 1260
 agaggccgcc tggatcagtt gctcaggcac gtgatgaacc ccttggaac cctctcaata 1320
 actaactgcc ggctttcgga aggggatgtg atgcatctgt ccagagtc ccagctcagt 1380
 cagctaagtg tcctgagtct aagtgggggc atgctgaccg atgtaagtcc cgagcccctc 1440
 15 caagctctgc tggagagagc ctctgccacc ctccaggacc tggctcttga tgagtgtggg 1500
 atcacggatg atcagctcct tgccctcctg ccttcctga gccactgctc ccagcttaca 1560
 accttaagct tctacgggaa ttccatctcc atatctgctt tgcagagtct cctgcagcac 1620
 ctcatcgggc tgagcaatct gaccacagt ctgtatcctg tccccctgga gagttatgag 1680
 gacatccatg gtacctcca cctggagagg cttgcctatc tgcagccag gctcagggag 1740
 20 ttgctgtgtg agttggggcg gccagcatg gtctggctta gtgccaaccc ctgtcctcac 1800
 tgtggggaca gaaccttcta tgaccggag cccatcctgt gcccctgttt catgcctaac 1860
 ctgcagcacc accaccacca ccac 1884

<210> 6

25 <211> 628

<212> PRT

<213> Artificial Sequence

<220>

30 <223> MDP- 20-127 - Protein D - PRAME - LEHHHHHH
 (plasmid pET21)

<400> 6

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

	85	90	95
	Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met		
	100	105	110
	Glu Arg Arg Arg Leu Trp Gly Ser Ile Gln Ser Arg Tyr Ile Ser Met		
5	115	120	125
	Ser Val Trp Thr Ser Pro Arg Arg Leu Val Glu Leu Ala Gly Gln Ser		
	130	135	140
	Leu Leu Lys Asp Glu Ala Leu Ala Ile Ala Ala Leu Glu Leu Leu Pro		
	145	150	155
10	Arg Glu Leu Phe Pro Pro Leu Phe Met Ala Ala Phe Asp Gly Arg His		
	165	170	175
	Ser Gln Thr Leu Lys Ala Met Val Gln Ala Trp Pro Phe Thr Cys Leu		
	180	185	190
	Pro Leu Gly Val Leu Met Lys Gly Gln His Leu His Leu Glu Thr Phe		
15	195	200	205
	Lys Ala Val Leu Asp Gly Leu Asp Val Leu Leu Ala Gln Glu Val Arg		
	210	215	220
	Pro Arg Arg Trp Lys Leu Gln Val Leu Asp Leu Arg Lys Asn Ser His		
	225	230	235
20	Gln Asp Phe Trp Thr Val Trp Ser Gly Asn Arg Ala Ser Leu Tyr Ser		
	245	250	255
	Phe Pro Glu Pro Glu Ala Ala Gln Pro Met Thr Lys Lys Arg Lys Val		
	260	265	270
	Asp Gly Leu Ser Thr Glu Ala Glu Gln Pro Phe Ile Pro Val Glu Val		
25	275	280	285
	Leu Val Asp Leu Phe Leu Lys Glu Gly Ala Cys Asp Glu Leu Phe Ser		
	290	295	300
	Tyr Leu Ile Glu Lys Val Lys Arg Lys Lys Asn Val Leu Arg Leu Cys		
	305	310	315
30	Cys Lys Lys Leu Lys Ile Phe Ala Met Pro Met Gln Asp Ile Lys Met		
	325	330	335
	Ile Leu Lys Met Val Gln Leu Asp Ser Ile Glu Asp Leu Glu Val Thr		
	340	345	350
	Cys Thr Trp Lys Leu Pro Thr Leu Ala Lys Phe Ser Pro Tyr Leu Gly		
35	355	360	365
	Gln Met Ile Asn Leu Arg Arg Leu Leu Leu Ser His Ile His Ala Ser		
	370	375	380
	Ser Tyr Ile Ser Pro Glu Lys Glu Glu Gln Tyr Ile Ala Gln Phe Thr		
	385	390	395
40	Ser Gln Phe Leu Ser Leu Gln Cys Leu Gln Ala Leu Tyr Val Asp Ser		
	405	410	415
	Leu Phe Phe Leu Arg Gly Arg Leu Asp Gln Leu Leu Arg His Val Met		
	420	425	430
	Asn Pro Leu Glu Thr Leu Ser Ile Thr Asn Cys Arg Leu Ser Glu Gly		

	435		440		445
	Asp Val Met His Leu Ser Gln Ser Pro Ser Val Ser Gln Leu Ser Val				
	450		455		460
	Leu Ser Leu Ser Gly Val Met Leu Thr Asp Val Ser Pro Glu Pro Leu				
5	465		470		475
	Gln Ala Leu Leu Glu Arg Ala Ser Ala Thr Leu Gln Asp Leu Val Phe				
		485		490	495
	Asp Glu Cys Gly Ile Thr Asp Asp Gln Leu Leu Ala Leu Leu Pro Ser				
		500		505	510
10	Leu Ser His Cys Ser Gln Leu Thr Thr Leu Ser Phe Tyr Gly Asn Ser				
		515		520	525
	Ile Ser Ile Ser Ala Leu Gln Ser Leu Leu Gln His Leu Ile Gly Leu				
		530		535	540
	Ser Asn Leu Thr His Val Leu Tyr Pro Val Pro Leu Glu Ser Tyr Glu				
15	545		550		555
	Asp Ile His Gly Thr Leu His Leu Glu Arg Leu Ala Tyr Leu His Ala				
		565		570	575
	Arg Leu Arg Glu Leu Leu Cys Glu Leu Gly Arg Pro Ser Met Val Trp				
		580		585	590
20	Leu Ser Ala Asn Pro Cys Pro His Cys Gly Asp Arg Thr Phe Tyr Asp				
		595		600	605
	Pro Glu Pro Ile Leu Cys Pro Cys Phe Met Pro Asn Leu Glu His His				
		610		615	620
	His His His His				
25	625				

<210> 7

<211> 1860

30 <212> DNA

<213> Artificial Sequence

<220>

<223> MDP- 20-127 - Protein D - PRAME - no His tail

35 (plasmid pET21)

<400> 7

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attattgctc accgtggtgc tagcgggttat ttaccagagc atacgttaga atctaaagca 120
40 cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt 180
cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc 240
ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt 300
caaagtttag aaatgacaga aaactttgaa accatggaac gaaggcgttt gtgggggttc 360
attcagagcc gatacatcag catgagtgtg tggacaagcc cacggagact tgtggagctg 420

gcagggcaga gcctgctgaa ggatgaggcc ctggccattg ccgccctgga gttgctgccc 480
 agggagctct tcccgccact cttcatggca gcctttgacg ggagacacag ccagaccctg 540
 aaggcaatgg tgcaggcctg gcccttcacc tgcctccctc tgggagtgt gatgaaggga 600
 caacatcttc acctggagac cttcaaagct gtgcttgatg gacttgatgt gctccttgcc 660
 5 caggagggttc gccccaggag gtggaaactt caagtgtctg atttacggaa gaactctcat 720
 caggacttct ggactgtatg gtctggaaac agggccagtc tgtactcatt tccagagcca 780
 gaagcagctc agcccatgac aaagaagcga aaagtagatg gtttgagcac agaggcagag 840
 cagcccttca ttccagtaga ggtgctcgta gacctgttcc tcaaggaagg tgcctgtgat 900
 gaattgttct cctacctcat tgagaaagtg aagcgaaaaga aaaatgtact acgcctgtgc 960
 10 tgtaagaagc tgaagatttt tgcaatgccc atgcaggata tcaagatgat cctgaaaatg 1020
 gtgcagctgg actctattga agatttgga gtgacttgta cctggaagct acccaccttg 1080
 gcgaaatttt ctcttacct gggccagatg attaactctgc gtagactcct cctctccac 1140
 atccatgcat ctctctacat ttccccggag aaggaagagc agtatatcgc ccagttcacc 1200
 tctcagttcc tcagtctgca gtgcctgcag gctctctatg tggactcttt attttccctt 1260
 15 agaggccgcc tggatcagtt gctcaggcac gtgatgaacc ccttggaac cctctcaata 1320
 actaactgcc ggctttcgga aggggatgtg atgcatctgt cccagagtcc cagcgtcagt 1380
 cagctaagtg tctgagtct aagtggggtc atgctgaccg atgtaagtcc cgagcccctc 1440
 caagctctgc tggagagagc ctctgccacc ctccaggacc tggctcttga tgagtgtggg 1500
 atcacggatg atcagctcct tgccctcctg ccttcctga gccactgctc ccagcttaca 1560
 20 accttaagct tctacgggaa ttccatctcc atatctgct tgcagagtct cctgcagcac 1620
 ctcatcgggc tgagcaatct gaccacgtg ctgtatcctg tccccctgga gagttatgag 1680
 gacatccatg gtaccctcca cctggagagg cttgcctatc tgcagccag gctcagggag 1740
 ttgctgtgtg agttggggcg gccagcatg gtctggctta gtgccaaccc ctgtcctcac 1800
 tgtggggaca gaaccttcta tgaccggag cccatcctgt gccctgttt catgcctaac 1860
 25

<210> 8

<211> 620

<212> PRT

30 <213> Artificial Sequence

<220>

<223> MDP- 20-127 - Protein D - PRAME - no His tail
 (plasmid pET21)

35

<400> 8

Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys

1 5 10 15

Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro

40

20 25 30

Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp

35 40 45

Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val

50 55 60

	Ile	His	Asp	His	Phe	Leu	Asp	Gly	Leu	Thr	Asp	Val	Ala	Lys	Lys	Phe	65	70	75	80
	Pro	His	Arg	His	Arg	Lys	Asp	Gly	Arg	Tyr	Tyr	Val	Ile	Asp	Phe	Thr		85	90	95
5	Leu	Lys	Glu	Ile	Gln	Ser	Leu	Glu	Met	Thr	Glu	Asn	Phe	Glu	Thr	Met	100	105	110	
	Glu	Arg	Arg	Arg	Leu	Trp	Gly	Ser	Ile	Gln	Ser	Arg	Tyr	Ile	Ser	Met	115	120	125	
	Ser	Val	Trp	Thr	Ser	Pro	Arg	Arg	Leu	Val	Glu	Leu	Ala	Gly	Gln	Ser	130	135	140	
10	Leu	Leu	Lys	Asp	Glu	Ala	Leu	Ala	Ile	Ala	Ala	Leu	Glu	Leu	Leu	Pro	145	150	155	
	Arg	Glu	Leu	Phe	Pro	Pro	Leu	Phe	Met	Ala	Ala	Phe	Asp	Gly	Arg	His	165	170	175	
15	Ser	Gln	Thr	Leu	Lys	Ala	Met	Val	Gln	Ala	Trp	Pro	Phe	Thr	Cys	Leu	180	185	190	
	Pro	Leu	Gly	Val	Leu	Met	Lys	Gly	Gln	His	Leu	His	Leu	Glu	Thr	Phe	195	200	205	
	Lys	Ala	Val	Leu	Asp	Gly	Leu	Asp	Val	Leu	Leu	Ala	Gln	Glu	Val	Arg	210	215	220	
20	Pro	Arg	Arg	Trp	Lys	Leu	Gln	Val	Leu	Asp	Leu	Arg	Lys	Asn	Ser	His	225	230	235	
	Gln	Asp	Phe	Trp	Thr	Val	Trp	Ser	Gly	Asn	Arg	Ala	Ser	Leu	Tyr	Ser	245	250	255	
25	Phe	Pro	Glu	Pro	Glu	Ala	Ala	Gln	Pro	Met	Thr	Lys	Lys	Arg	Lys	Val	260	265	270	
	Asp	Gly	Leu	Ser	Thr	Glu	Ala	Glu	Gln	Pro	Phe	Ile	Pro	Val	Glu	Val	275	280	285	
	Leu	Val	Asp	Leu	Phe	Leu	Lys	Glu	Gly	Ala	Cys	Asp	Glu	Leu	Phe	Ser	290	295	300	
30	Tyr	Leu	Ile	Glu	Lys	Val	Lys	Arg	Lys	Lys	Asn	Val	Leu	Arg	Leu	Cys	305	310	315	
	Cys	Lys	Lys	Leu	Lys	Ile	Phe	Ala	Met	Pro	Met	Gln	Asp	Ile	Lys	Met	325	330	335	
35	Ile	Leu	Lys	Met	Val	Gln	Leu	Asp	Ser	Ile	Glu	Asp	Leu	Glu	Val	Thr	340	345	350	
	Cys	Thr	Trp	Lys	Leu	Pro	Thr	Leu	Ala	Lys	Phe	Ser	Pro	Tyr	Leu	Gly	355	360	365	
	Gln	Met	Ile	Asn	Leu	Arg	Arg	Leu	Leu	Leu	Ser	His	Ile	His	Ala	Ser	370	375	380	
40	Ser	Tyr	Ile	Ser	Pro	Glu	Lys	Glu	Glu	Gln	Tyr	Ile	Ala	Gln	Phe	Thr	385	390	395	
	Ser	Gln	Phe	Leu	Ser	Leu	Gln	Cys	Leu	Gln	Ala	Leu	Tyr	Val	Asp	Ser	405	410	415	

Leu Phe Phe Leu Arg Gly Arg Leu Asp Gln Leu Leu Arg His Val Met
 420 425 430
 Asn Pro Leu Glu Thr Leu Ser Ile Thr Asn Cys Arg Leu Ser Glu Gly
 435 440 445
 5 Asp Val Met His Leu Ser Gln Ser Pro Ser Val Ser Gln Leu Ser Val
 450 455 460
 Leu Ser Leu Ser Gly Val Met Leu Thr Asp Val Ser Pro Glu Pro Leu
 465 470 475 480
 Gln Ala Leu Leu Glu Arg Ala Ser Ala Thr Leu Gln Asp Leu Val Phe
 10 485 490 495
 Asp Glu Cys Gly Ile Thr Asp Asp Gln Leu Leu Ala Leu Leu Pro Ser
 500 505 510
 Leu Ser His Cys Ser Gln Leu Thr Thr Leu Ser Phe Tyr Gly Asn Ser
 515 520 525
 15 Ile Ser Ile Ser Ala Leu Gln Ser Leu Leu Gln His Leu Ile Gly Leu
 530 535 540
 Ser Asn Leu Thr His Val Leu Tyr Pro Val Pro Leu Glu Ser Tyr Glu
 545 550 555 560
 Asp Ile His Gly Thr Leu His Leu Glu Arg Leu Ala Tyr Leu His Ala
 20 565 570 575
 Arg Leu Arg Glu Leu Leu Cys Glu Leu Gly Arg Pro Ser Met Val Trp
 580 585 590
 Leu Ser Ala Asn Pro Cys Pro His Cys Gly Asp Arg Thr Phe Tyr Asp
 595 600 605
 25 Pro Glu Pro Ile Leu Cys Pro Cys Phe Met Pro Asn
 610 615 620

<210> 9
 30 <211> 1878
 <212> DNA
 <213> Artificial Sequence

 <220>
 35 <223> MDP- 20-127 - Protein D - PRAME - HHHHHH (plasmid
 pET26)

<400> 9
 atggatccaa gcagccattc atcaaatatg gcgaataccc aaatgaaatc agacaaaatc 60
 40 attattgctc accgtggtgc tagcggttat ttaccagagc atacgttaga atctaaagca 120
 cttgcgtttg cacaacaggc tgattattta gagcaagatt tagcaatgac taaggatggt 180
 cgtttagtgg ttattcacga tcacttttta gatggcttga ctgatgttgc gaaaaaattc 240
 ccacatcgtc atcgtaaaga tggccgttac tatgtcatcg actttacctt aaaagaaatt 300
 caaagtttag aaatgacaga aaactttgaa accatggaac gtcgtcgtct gtggggcagc 360

attcagagcc gttatattag catgagcgtg tggaccagcc cgcgtcgtct ggttgagctg 420
 gccggccaga gcctgctgaa agatgaagcg ctggccattg cggcgctgga gctgctgccg 480
 cgtgagctgt ttccgccgct gtttatggcg gcgtttgatg gccgtcatag ccagaccctg 540
 aaagcgatgg tgcaggcgtg gccgtttacc tgtctgccgc tgggcgtgct gatgaaaggg 600
 5 cagcatctgc atctggaaac ctttaaagcg gtgctggatg gcctggatgt gctgctggcc 660
 caggaagtgc gtccgcgtcg ttggaaactg caagtgctgg atctgcgtaa aaacagccat 720
 caggatTTTT ggaccgtgtg gagcggcaat cgtgcgagcc tgtatagctt tccggaaccg 780
 gaagcggcgc agccgatgac caaaaaacgt aaagtggatg gcctgagcac cgaagcggaa 840
 cagccgttta ttccggtgga agtgcgtggt gacctgtttc tgaaagaagg cgctgcgac 900
 10 gagctgttta gctatctgat cgaaaaagtg aaacgcaaaa aaaacgtgct gcgtctgtgc 960
 tgcaaaaaac tgaaaatctt cgcgatgccg atgcaggata ttaaaatgat cctgaaaatg 1020
 gtgcagctgg atagcattga ggacctggaa gtgacctgca cctggaaact gccgacctg 1080
 gccaaattta gcccgatatc gggccagatg attaacctgc gtgctctgct gctgtctcat 1140
 attcatgcga gcagctatat tagcccgaa aaagaagaac agtatatcgc gcagtttacc 1200
 15 agccagtttc tgagcctgca atgcctgcaa gcgctgtatg tggatagcct gttttttctg 1260
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 accaactgcc gtctgagcga aggcgatgtg atgcactcga gccagagccc gagcgttagc 1380
 cagctgtctg ttctgagcct gagcggcgtg atgctgaccg atgtgagccc ggaaccgctg 1440
 caagccctgc tggaacgtgc gagcgcgacc ctgcaagacc tgggtgtttga tgaatgcggc 1500
 20 attaccgatg atcagctgct ggccctgctg ccgagcctga gccattgcag ccagctgacc 1560
 accctgagct tttatggcaa cagcattagc attagcgcgc tgcaaagcct gctgcaacat 1620
 ctgattggcc tgagcaacct gacccatgtg ctgtatccgg tgccgctgga aagctatgaa 1680
 gatattcatg gcacctgca totggaacgt ctggcctatc tgcacgcgcg tctgcgtgag 1740
 ctgctgtgcg agctgggccc tccgagcatg gtttggctgt ctgcgaatcc gtgcccgcac 1800
 25 tgccgagatc gtacctttta tgatccggaa ccgattctgt gccctgctt tatgccgaac 1860
 caccaccacc accaccac 1878

<210> 10
 <211> 626
 30 <212> PRT
 <213> Artificial Sequence

<220>
 <223> MDP- 20-127 - Protein D - PRAME - HHHHHH (plasmid
 35 pET26)

<400> 10
 Met Asp Pro Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys
 1 5 10 15
 40 Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro
 20 25 30
 Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
 35 40 45
 Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val

	50	55	60
	Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe		
	65	70	75 80
	Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr		
5	85	90	95
	Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met		
	100	105	110
	Glu Arg Arg Arg Leu Trp Gly Ser Ile Gln Ser Arg Tyr Ile Ser Met		
	115	120	125
10	Ser Val Trp Thr Ser Pro Arg Arg Leu Val Glu Leu Ala Gly Gln Ser		
	130	135	140
	Leu Leu Lys Asp Glu Ala Leu Ala Ile Ala Ala Leu Glu Leu Leu Pro		
	145	150	155 160
	Arg Glu Leu Phe Pro Pro Leu Phe Met Ala Ala Phe Asp Gly Arg His		
15	165	170	175
	Ser Gln Thr Leu Lys Ala Met Val Gln Ala Trp Pro Phe Thr Cys Leu		
	180	185	190
	Pro Leu Gly Val Leu Met Lys Gly Gln His Leu His Leu Glu Thr Phe		
	195	200	205
20	Lys Ala Val Leu Asp Gly Leu Asp Val Leu Leu Ala Gln Glu Val Arg		
	210	215	220
	Pro Arg Arg Trp Lys Leu Gln Val Leu Asp Leu Arg Lys Asn Ser His		
	225	230	235 240
	Gln Asp Phe Trp Thr Val Trp Ser Gly Asn Arg Ala Ser Leu Tyr Ser		
25	245	250	255
	Phe Pro Glu Pro Glu Ala Ala Gln Pro Met Thr Lys Lys Arg Lys Val		
	260	265	270
	Asp Gly Leu Ser Thr Glu Ala Glu Gln Pro Phe Ile Pro Val Glu Val		
	275	280	285
30	Leu Val Asp Leu Phe Leu Lys Glu Gly Ala Cys Asp Glu Leu Phe Ser		
	290	295	300
	Tyr Leu Ile Glu Lys Val Lys Arg Lys Lys Asn Val Leu Arg Leu Cys		
	305	310	315 320
	Cys Lys Lys Leu Lys Ile Phe Ala Met Pro Met Gln Asp Ile Lys Met		
35	325	330	335
	Ile Leu Lys Met Val Gln Leu Asp Ser Ile Glu Asp Leu Glu Val Thr		
	340	345	350
	Cys Thr Trp Lys Leu Pro Thr Leu Ala Lys Phe Ser Pro Tyr Leu Gly		
	355	360	365
40	Gln Met Ile Asn Leu Arg Arg Leu Leu Leu Ser His Ile His Ala Ser		
	370	375	380
	Ser Tyr Ile Ser Pro Glu Lys Glu Glu Gln Tyr Ile Ala Gln Phe Thr		
	385	390	395 400
	Ser Gln Phe Leu Ser Leu Gln Cys Leu Gln Ala Leu Tyr Val Asp Ser		

405 410 415
 Leu Phe Phe Leu Arg Gly Arg Leu Asp Gln Leu Leu Arg His Val Met
 420 425 430
 Asn Pro Leu Glu Thr Leu Ser Ile Thr Asn Cys Arg Leu Ser Glu Gly
 5 435 440 445
 Asp Val Met His Leu Ser Gln Ser Pro Ser Val Ser Gln Leu Ser Val
 450 455 460
 Leu Ser Leu Ser Gly Val Met Leu Thr Asp Val Ser Pro Glu Pro Leu
 465 470 475 480
 10 Gln Ala Leu Leu Glu Arg Ala Ser Ala Thr Leu Gln Asp Leu Val Phe
 485 490 495
 Asp Glu Cys Gly Ile Thr Asp Asp Gln Leu Leu Ala Leu Leu Pro Ser
 500 505 510
 Leu Ser His Cys Ser Gln Leu Thr Thr Leu Ser Phe Tyr Gly Asn Ser
 15 515 520 525
 Ile Ser Ile Ser Ala Leu Gln Ser Leu Leu Gln His Leu Ile Gly Leu
 530 535 540
 Ser Asn Leu Thr His Val Leu Tyr Pro Val Pro Leu Glu Ser Tyr Glu
 545 550 555 560
 20 Asp Ile His Gly Thr Leu His Leu Glu Arg Leu Ala Tyr Leu His Ala
 565 570 575
 Arg Leu Arg Glu Leu Leu Cys Glu Leu Gly Arg Pro Ser Met Val Trp
 580 585 590
 Leu Ser Ala Asn Pro Cys Pro His Cys Gly Asp Arg Thr Phe Tyr Asp
 25 595 600 605
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 His His
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 (plasmid pET26)
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cyllagcgg ccattcacga ccactcttca gatggcttga cggatgltgc gaaaaaatcc 240
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 caaagtttag aaatgacaga aaactttgaa accatggaac gtcgtcgtct gtggggcagc 360
 attcagagcc gttatattag catgagcgtg tggaccagcc cgcgtcgtct ggttgagctg 420
 5 gccggccaga gctgctgaa agatgaagcg ctggccattg cggcgtgga gctgctgccg 480
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 aaagcgatgg tgcaggcgtg gccgtttacc tgtctgccgc tgggcgtgct gatgaaaggc 600
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 cagccgttta ttccggtgga agtgcgtggt gacctgtttc tgaaagaagg cgctgcgcac 900
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 15 gtgcagctgg atagcattga ggacctggaa gtgacctgca cctggaaact gccgaccctg 1080
 gccaaattta gcccgatatc gggccagatg attaacctgc gtcgtctgct gctgtctcat 1140
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<210> 12
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 <212> PRT
 <213> Artificial Sequence

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<220>
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 (plasmid pET26)

40

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 20 25 30

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	35	40 45
	Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val	
	50	55 60
5	Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe	
	65	70 75 80
	Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr	
	85	90 95
	Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met	
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	Glu Arg Arg Arg Leu Trp Gly Ser Ile Gln Ser Arg Tyr Ile Ser Met	
	115	120 125
	Ser Val Trp Thr Ser Pro Arg Arg Leu Val Glu Leu Ala Gly Gln Ser	
	130	135 140
15	Leu Leu Lys Asp Glu Ala Leu Ala Ile Ala Ala Leu Glu Leu Leu Pro	
	145	150 155 160
	Arg Glu Leu Phe Pro Pro Leu Phe Met Ala Ala Phe Asp Gly Arg His	
	165	170 175
	Ser Gln Thr Leu Lys Ala Met Val Gln Ala Trp Pro Phe Thr Cys Leu	
20	180	185 190
	Pro Leu Gly Val Leu Met Lys Gly Gln His Leu His Leu Glu Thr Phe	
	195	200 205
	Lys Ala Val Leu Asp Gly Leu Asp Val Leu Leu Ala Gln Glu Val Arg	
	210	215 220
25	Pro Arg Arg Trp Lys Leu Gln Val Leu Asp Leu Arg Lys Asn Ser His	
	225	230 235 240
	Gln Asp Phe Trp Thr Val Trp Ser Gly Asn Arg Ala Ser Leu Tyr Ser	
	245	250 255
	Phe Pro Glu Pro Glu Ala Ala Gln Pro Met Thr Lys Lys Arg Lys Val	
30	260	265 270
	Asp Gly Leu Ser Thr Glu Ala Glu Gln Pro Phe Ile Pro Val Glu Val	
	275	280 285
	Leu Val Asp Leu Phe Leu Lys Glu Gly Ala Cys Asp Glu Leu Phe Ser	
	290	295 300
35	Tyr Leu Ile Glu Lys Val Lys Arg Lys Lys Asn Val Leu Arg Leu Cys	
	305	310 315 320
	Cys Lys Lys Leu Lys Ile Phe Ala Met Pro Met Gln Asp Ile Lys Met	
	325	330 335
	Ile Leu Lys Met Val Gln Leu Asp Ser Ile Glu Asp Leu Glu Val Thr	
40	340	345 350
	Cys Thr Trp Lys Leu Pro Thr Leu Ala Lys Phe Ser Pro Tyr Leu Gly	
	355	360 365
	Gln Met Ile Asn Leu Arg Arg Leu Leu Leu Ser His Ile His Ala Ser	
	370	375 380

	Ser Tyr Ile Ser Pro Glu Lys Glu Glu Gln Tyr Ile Ala Gln Phe Thr	
	385	390 395 400
	Ser Gln Phe Leu Ser Leu Gln Cys Leu Gln Ala Leu Tyr Val Asp Ser	
		405 410 415
5	Leu Phe Phe Leu Arg Gly Arg Leu Asp Gln Leu Leu Arg His Val Met	
		420 425 430
	Asn Pro Leu Glu Thr Leu Ser Ile Thr Asn Cys Arg Leu Ser Glu Gly	
		435 440 445
	Asp Val Met His Leu Ser Gln Ser Pro Ser Val Ser Gln Leu Ser Val	
10		450 455 460
	Leu Ser Leu Ser Gly Val Met Leu Thr Asp Val Ser Pro Glu Pro Leu	
	465	470 475 480
	Gln Ala Leu Leu Glu Arg Ala Ser Ala Thr Leu Gln Asp Leu Val Phe	
		485 490 495
15	Asp Glu Cys Gly Ile Thr Asp Asp Gln Leu Leu Ala Leu Leu Pro Ser	
		500 505 510
	Leu Ser His Cys Ser Gln Leu Thr Thr Leu Ser Phe Tyr Gly Asn Ser	
		515 520 525
	Ile Ser Ile Ser Ala Leu Gln Ser Leu Leu Gln His Leu Ile Gly Leu	
20		530 535 540
	Ser Asn Leu Thr His Val Leu Tyr Pro Val Pro Leu Glu Ser Tyr Glu	
	545	550 555 560
	Asp Ile His Gly Thr Leu His Leu Glu Arg Leu Ala Tyr Leu His Ala	
		565 570 575
25	Arg Leu Arg Glu Leu Leu Cys Glu Leu Gly Arg Pro Ser Met Val Trp	
		580 585 590
	Leu Ser Ala Asn Pro Cys Pro His Cys Gly Asp Arg Thr Phe Tyr Asp	
		595 600 605
	Pro Glu Pro Ile Leu Cys Pro Cys Phe Met Pro Asn	
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 <210> 15
 10 <211> 10
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 15 Ala Leu Tyr Val Asp Ser Leu Phe Phe Leu
 1 5 10

 <210> 16
 20 <211> 9
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 <400> 16
 25 Leu Tyr Val Asp Ser Leu Phe Phe Leu
 1 5

 <210> 17
 30 <211> 9
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 1 5

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<223> sense oligonucleotide CAN008

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15 ccacaaacgc cttcgttcca tggtttcaaa gttttctgtc 40

<210> 20
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<210> 21
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30 <212> DNA
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<220>
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35 <400> 21
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<210> 22
40 <211> 36
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 <210> 23
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 25 <400> 24
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 30 <212> DNA
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 cgccttcggtt ccatgggttc aaagttttct g 31
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 40 <211> 43
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<223> sense oligonucleotide CAN104

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30 <212> DNA
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<210> 34
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<210> 35
<211> 53
<212> DNA
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<220>
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<211> 18
30 <212> DNA
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<220>
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35 <400> 37
tctcccagcg tgcgccat 18

<210> 38
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<212> DNA
<213> Artificial Sequence

<220>

<223> immunostimulatory oligonucleotide CpG2006

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<210> 40
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20 25 30
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35 40 45
40 Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp
50 55 60
Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val
65 70 75 80
Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe

		85		90		95	
	Pro	His	Arg	His	Arg	Lys	Asp
				Gly	Arg	Tyr	Tyr
					Val	Ile	Asp
						Phe	Thr
		100		105		110	
	Leu	Lys	Glu	Ile	Gln	Ser	Leu
				Glu	Met	Thr	Glu
					Asn	Phe	Glu
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 15 Protein D

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	Ala	Asn
	Thr	Gln
	Met	Lys
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	10	15
20	Ser	Asp
	Lys	Ile
	Ile	Ile
	Ala	His
	Arg	Gly
	Ala	Ser
	Gly	Tyr
	Leu	Pro
	20	25
	30	
	Glu	His
	Thr	Leu
	Glu	Ser
	Lys	Ala
	Leu	Ala
	Phe	Ala
	Gln	Gln
	Ala	Asp
	35	40
	45	
	Tyr	Leu
	Glu	Gln
	Asp	Leu
	Ala	Met
	Thr	Lys
	Asp	Gly
	Arg	Leu
	Val	Val
25	50	55
	60	
	Ile	His
	Asp	His
	Phe	Leu
	Asp	Gly
	Leu	Thr
	Asp	Val
	Ala	Lys
	Lys	Phe
	65	70
	75	80
	Pro	His
	Arg	His
	Arg	Lys
	Asp	Gly
	Arg	Tyr
	Tyr	Val
	Ile	Asp
	Phe	Thr
	85	90
	95	
30	Leu	Lys
	Glu	Ile
	Gln	Ser
	Leu	Glu
	Met	Thr
	Glu	Asn
	Phe	Glu
	Thr	
	100	105
	110	

<210> 43
 35 <211> 450
 <212> PRT
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 40 <223> Protein D-MAGE-A3-His

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	Thr	Leu
	Ala	Leu
	Ser	Leu
	Leu	Ala
	Ala	Gly
	Val	Leu
	1	5
	10	15

	Ala Gly Cys Ser Ser His Ser Ser Asn Met Ala Asn Thr Gln Met Lys	
	20	25 30
	Ser Asp Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro	
	35	40 45
5	Glu His Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp	
	50	55 60
	Tyr Leu Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val	
	65	70 75 80
	Ile His Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe	
10	85	90 95
	Pro His Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr	
	100	105 110
	Leu Lys Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr Met	
	115	120 125
15	Asp Leu Glu Gln Arg Ser Gln His Cys Lys Pro Glu Glu Gly Leu Glu	
	130	135 140
	Ala Arg Gly Glu Ala Leu Gly Leu Val Gly Ala Gln Ala Pro Ala Thr	
	145	150 155 160
	Glu Glu Gln Glu Ala Ala Ser Ser Ser Ser Thr Leu Val Glu Val Thr	
20	165	170 175
	Leu Gly Glu Val Pro Ala Ala Glu Ser Pro Asp Pro Pro Gln Ser Pro	
	180	185 190
	Gln Gly Ala Ser Ser Leu Pro Thr Thr Met Asn Tyr Pro Leu Trp Ser	
	195	200 205
25	Gln Ser Tyr Glu Asp Ser Ser Asn Gln Glu Glu Glu Gly Pro Ser Thr	
	210	215 220
	Phe Pro Asp Leu Glu Ser Glu Phe Gln Ala Ala Leu Ser Arg Lys Val	
	225	230 235 240
	Ala Glu Leu Val His Phe Leu Leu Leu Lys Tyr Arg Ala Arg Glu Pro	
30	245	250 255
	Val Thr Lys Ala Glu Met Leu Gly Ser Val Val Gly Asn Trp Gln Tyr	
	260	265 270
	Phe Phe Pro Val Ile Phe Ser Lys Ala Ser Ser Ser Leu Gln Leu Val	
	275	280 285
35	Phe Gly Ile Glu Leu Met Glu Val Asp Pro Ile Gly His Leu Tyr Ile	
	290	295 300
	Phe Ala Thr Cys Leu Gly Leu Ser Tyr Asp Gly Leu Leu Gly Asp Asn	
	305	310 315 320
	Gln Ile Met Pro Lys Ala Gly Leu Leu Ile Ile Val Leu Ala Ile Ile	
40	325	330 335
	Ala Arg Glu Gly Asp Cys Ala Pro Glu Glu Lys Ile Trp Glu Glu Leu	
	340	345 350
	Ser Val Leu Glu Val Phe Glu Gly Arg Glu Asp Ser Ile Leu Gly Asp	
	355	360 365

Pro Lys Lys Leu Leu Thr Gln His Phe Val Gln Glu Asn Tyr Leu Glu
 370 375 380
 Tyr Arg Gln Val Pro Gly Ser Asp Pro Ala Cys Tyr Glu Phe Leu Trp
 385 390 395 400
 5 Gly Pro Arg Ala Leu Val Glu Thr Ser Tyr Val Lys Val Leu His His
 405 410 415
 Met Val Lys Ile Ser Gly Gly Pro His Ile Ser Tyr Pro Pro Leu His
 420 425 430
 Glu Trp Val Leu Arg Glu Gly Glu Glu Gly Gly His His His His His
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 His His
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15 <210> 44
 <211> 109
 <212> PRT
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 <223> the aa from protein D from Haemophilus influenzae
 for use in a pD1/3-PRAME-His sequence

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 Lys Ile Ile Ile Ala His Arg Gly Ala Ser Gly Tyr Leu Pro Glu His
 20 25 30
 Thr Leu Glu Ser Lys Ala Leu Ala Phe Ala Gln Gln Ala Asp Tyr Leu
 30 35 40 45
 Glu Gln Asp Leu Ala Met Thr Lys Asp Gly Arg Leu Val Val Ile His
 50 55 60
 Asp His Phe Leu Asp Gly Leu Thr Asp Val Ala Lys Lys Phe Pro His
 65 70 75 80
 35 Arg His Arg Lys Asp Gly Arg Tyr Tyr Val Ile Asp Phe Thr Leu Lys
 85 90 95
 Glu Ile Gln Ser Leu Glu Met Thr Glu Asn Phe Glu Thr
 100 105

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 <210> 45
 <211> 127
 <212> PRT
 <213> Artificial Sequence

<220>

<223> the aa from protein D from Haemophilus influenzae
for use in a pD1/3-MAGE-His sequence

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<400> 45

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	Ala	Gly	Cys	Ser	Ser	His	Ser	Ser	Asn	Met	Ala	Asn	Thr	Gln	Met	Lys
10				20					25					30		
	Ser	Asp	Lys	Ile	Ile	Ile	Ala	His	Arg	Gly	Ala	Ser	Gly	Tyr	Leu	Pro
			35				40					45				
	Glu	His	Thr	Leu	Glu	Ser	Lys	Ala	Leu	Ala	Phe	Ala	Gln	Gln	Ala	Asp
		50					55				60					
15	Tyr	Leu	Glu	Gln	Asp	Leu	Ala	Met	Thr	Lys	Asp	Gly	Arg	Leu	Val	Val
	65				70				75					80		
	Ile	His	Asp	His	Phe	Leu	Asp	Gly	Leu	Thr	Asp	Val	Ala	Lys	Lys	Phe
				85					90				95			
	Pro	His	Arg	His	Arg	Lys	Asp	Gly	Arg	Tyr	Tyr	Val	Ile	Asp	Phe	Thr
20				100					105				110			
	Leu	Lys	Glu	Ile	Gln	Ser	Leu	Glu	Met	Thr	Glu	Asn	Phe	Glu	Thr	
			115				120				125					

CLAIMS

1. A fusion protein comprising:
 - (a) cancer testis antigen PRAME;
 - (b) a heterologous fusion partner protein derived from protein D, wherein the fusion partner protein comprises amino acids 20 to 127 of protein D; and
 - (c) additional amino acids Met-Asp-Pro at the N-terminal end of the fusion protein sequencewherein the heterologous fusion partner protein derived from protein D, (b), is fused to the N-terminus of PRAME.
2. A fusion protein according to claim 1 further comprising an affinity tag.
3. A fusion protein according to claim 2, wherein the affinity tag is a histidine tail.
4. A fusion protein according to any of preceding claims additionally comprising one or more linker sequences between the fusion partner protein and PRAME; or between the fusion partner protein and affinity tag; or between PRAME and affinity tag.
5. A nucleic acid sequence encoding a fusion protein as claimed in any of claims 1 to 4.
6. A vector comprising the nucleic acid sequence of claim 5.
7. An isolated host cell transformed with the vector of claim 6.
8. A vaccine containing a fusion protein as claimed in any of claims 1 to 4 or a nucleic acid as claimed in claim 5 or a vector as claimed in claim 6.
9. A vaccine as claimed in claim 8 additionally comprising an adjuvant, and/or immunostimulatory cytokine or chemokine.
10. A vaccine as claimed in claim 9 wherein the adjuvant comprises 3D-MPL,

QS21 and/or a CpG oligonucleotide.

11. A vaccine as claimed in any of claims 8 to 10 for use in medicine.

12. Use of a protein or nucleic acid or vector according to any one of claims 1 to 6 for the manufacture of a vaccine for immunotherapeutically treating a patient suffering cancer.

13. A process for producing a fusion protein comprising the step of expressing in an isolated cell a fusion protein according to any of claims 1 to 4.

14. A process according to claim 13 in which the cell is a bacterium.

15. A process according to claim 14 in which the bacterium is *E. coli*.

16. A process according to claim 16 further comprising the step of lysing the cell and purifying the expressed fusion protein from the lysed cells.

17. A fusion protein obtained by or obtainable by the process of any of claims 13 to 16.

18. The use according to claim 12 in which the cancer is selected from melanoma, breast, bladder, lung cancer such as NSCLC, sarcoma, ovarian cancer, head and neck cancer, renal cancer, colorectal carcinoma, multiple myeloma, leukemia including acute leukemia and oesophageal carcinoma.