



(51) International Patent Classification:

C07K 14/705 (2006.01) C07K 14/725 (2006.01)
C07K 16/00 (2006.01) C07K 14/74 (2006.01)
C12N 15/62 (2006.01)

(21) International Application Number:

PCT/EP2024/065725

(22) International Filing Date:

07 June 2024 (07.06.2024)

(25) Filing Language:

English

(26) Publication Language:

English

(30) Priority Data:

23178392.9 09 June 2023 (09.06.2023) EP

(71) Applicant: **DANMARKS TEKNISKE UNIVERSITET**
[DK/DK]; Anker Engelunds Vej 101, 2800 Kongens Lyngby (DK).

(72) Inventors: **HADRUP, Sine Reker**; c/o Danmarks Tekniske
Universitet, Anker Engelunds Vej 101, 2800 Kongens
Lyngby (DK). **BASAVARAJU, Yogesh**; c/o Danmarks

Tekniske Universitet, Anker Engelunds Vej 101, 2800 Kongens Lyngby (DK).

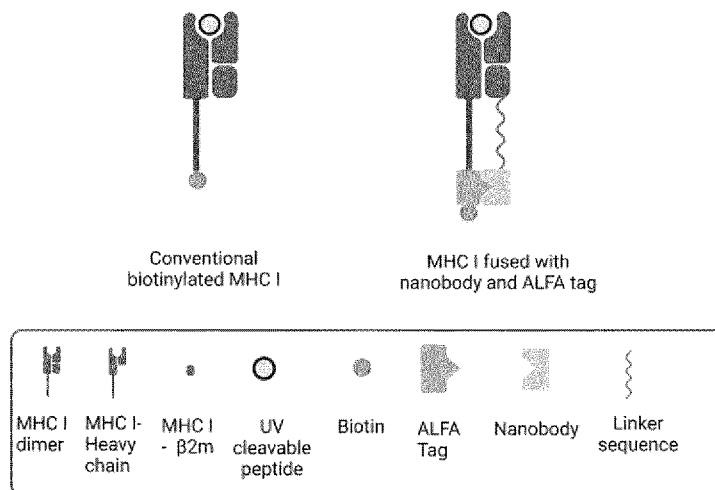
(74) Agent: **GUARDIAN IP CONSULTING I/S**; Diplomvej,
Building 381, 2800 Kgs. Lyngby (DK).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CV, CZ, DE, DJ, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IQ, IR, IS, IT, JM, JO, JP, KE, KG, KH, KN, KP, KR, KW, KZ, LA, LC, LK, LR, LS, LU, LY, MA, MD, MG, MK, MN, MU, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, WS, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, CV, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SC, SD, SL, ST, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ,

(54) Title: ENHANCING PROTEIN-DIMER STABILITY WITH A NANOBODY AS A FUSION PARTNER

Figure 1



(57) Abstract: The invention relates to a modified mammalian dimeric receptor protein having enhanced protein stability in solution. The two polypeptide chains of the modified dimeric receptor are expressed as fusion proteins, whereby one polypeptide chain is covalently fused to a peptide comprising a single chain nanobody, while the second polypeptide chain is covalently fused to a peptide comprising an antigenic peptide cognate for the nanobody. The invention further relates to methods of screening ligands for the modified dimeric receptor, and methods for introducing and presenting antigenic ligands in said receptors. The invention further relates to the use of a nanobody and an antigenic peptide cognate to said nanobody to stabilise or enhance selective pairing of the polypeptides of a dimeric fusion protein.

RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, ME, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:

— *of inventorship (Rule 4.17(iv))*

Published:

— *with international search report (Art. 21(3))*
— *with sequence listing part of description (Rule 5.2(a))*
— *in black and white; the international application as filed contained color or greyscale and is available for download from PATENTSCOPE*

TITLE: Enhancing protein-dimer stability with a nanobody as a fusion partner**FIELD OF THE INVENTION**

The invention relates to a modified mammalian dimeric receptor protein having enhanced protein stability in solution. The two polypeptide chains of the modified dimeric receptor are expressed as fusion proteins, whereby one polypeptide chain is covalently fused to a peptide comprising a nanobody, while the second polypeptide chain is covalently fused to a peptide comprising an antigenic peptide cognate for the nanobody. The invention further relates to methods of screening ligands for the modified dimeric receptor, and methods for introducing and presenting antigenic ligands in said receptors.

The invention further relates to the use of a nanobody and an antigenic peptide cognate to said nanobody to stabilise or enhance selective pairing of the polypeptides of a dimeric fusion protein.

BACKGROUND OF THE INVENTION

Protein expression and purification is a foundation for biopharmaceuticals and over the decades a large number of creative solutions have been applied to generate stable recombinant protein expression which aids in better protein quality, higher yield and enhanced activity. Among the challenges faced in this field is to express mammalian dimeric receptor proteins in a soluble form in order to facilitate their use in screening methods to identify their ligands and to study cellular response mechanisms. Common structural features of such receptor proteins are the one of more extracellular domains and a membrane spanning domain that forms part of one or both chains of the dimeric receptor and serves to anchor the receptor to a membrane. Expression of soluble forms of such receptors, from which the membrane spanning domain(s) have been deleted, has relied on the inherent affinity of such modified chains of the dimeric receptor to associate and form a receptor that exhibits its native functional properties. Both the functional properties of such soluble receptors and their long term stability is critical for their eventual use in screening assays.

The MHC class I and II heterodimers are examples of receptors that have been expressed in soluble form. Strategies to improve their T-cell binding/staining efficacy in screening assays have focused on creating multimers, where the individual heterodimers are bound to a multimerization domain, for example streptavidin or dextran. Screening methods employing MHC class I and II heterodimers rely on loading the MHC *in vitro* with a desired peptide to facilitate T cell screening, where the success of loading or peptide exchange can be compromised by insufficient dimer stability.

Exploitation of the full potential of soluble mammalian dimeric receptor proteins in a wide range of screening methods requires the development of structurally modified

forms of such receptors that are both stable and fully retain their natural biological functions. The present invention provides modified soluble mammalian dimeric receptor proteins that meet such needs.

SUMMARY OF THE INVENTION

5 According to a first embodiment is provided:

a soluble mammalian dimeric receptor protein devoid of a transmembrane anchoring domain comprising modified first and second receptor polypeptides, wherein:

(a) the first receptor polypeptide is fused to a first peptide comprising a nanobody, and

10 (b) the second receptor polypeptide is fused to a second peptide comprising an antigenic peptide cognate to said nanobody, and wherein stability of the dimeric receptor protein is enhanced by non-covalent bonds between the nanobody and the cognate antigenic peptide relative to corresponding unmodified first and second receptor polypeptides devoid of a transmembrane anchoring domain from which said modified chains were derived.

15 According to a second embodiment is provided:

a method of detecting one or more antigenic peptide-responsive T cells in a sample, the method comprising the steps of:

20 (a) providing a preparation of soluble mammalian dimeric receptor proteins according to the first embodiment, wherein each of said receptor proteins is a soluble MHCI or soluble MHCII,

(b) loading each said MHCI or MHCII with at least one antigenic peptide to form a MHCI-peptide complex or a MHCII-peptide,

(c) conjugating each said MHCI-peptide complex or MHCII-peptide complex with a carrier coupled to a fluorophore to form a tetrameric or multimeric complex;

25 (d) staining a sample of cells to be interrogated with said conjugated MHCI-peptide tetrameric or multimeric complexes or said conjugated MHCII-peptide tetrameric or multimeric complexes obtained in step (c), and

(e) detecting the binding of said tetrameric or multimeric complexes to the one or more antigenic peptide responsive cells in the sample.

30

A third embodiment of the invention provides a method of producing a soluble mammalian dimeric receptor protein comprising a modified first and second receptor polypeptide devoid of a transmembrane anchoring domain according to the invention, the method comprising the steps of:

35 (a) providing a first nucleic acid coding sequence encoding the first receptor polypeptide having a C-terminally fused first peptide comprising a nanobody, and

- (b) providing a second nucleic acid coding sequence encoding the second receptor polypeptide having a C-terminally fused second peptide comprising an antigenic peptide cognate to said nanobody,
- (c) providing a first vector comprising said first nucleic acid coding sequence and a
 5 second vector comprising said second nucleic acid coding sequence, or alternatively a third vector comprising said first nucleic acid coding sequence transcriptionally linked to said second nucleic acid coding sequence, wherein expression of said first and second nucleic acid coding sequences is operably linked to a promoter;
- (d) expressing said modified first and second receptor polypeptides in host cells
 10 comprising respectively said first and said second vector or host cells comprising said third vector;
- (e) recovering and contacting the expressed first and second receptor polypeptides to form the soluble mammalian dimeric receptor protein,
- wherein stability of the soluble mammalian dimeric receptor protein derived from said
 15 modified first and second receptor polypeptides is enhanced as compared to a corresponding unmodified soluble mammalian dimeric receptor protein from which the soluble mammalian dimeric receptor protein of the invention is derivable.

According to a fourth embodiment is provided:

- 20 a method of loading or displacing placeholder peptides in soluble dimeric MHCI or MHCII receptor proteins according to the first embodiment with a second antigenic peptide comprising the steps of:
- (a) providing a preparation of the soluble MHCI or MHCII of the first embodiment, or the tetrameric or multimeric complex thereof,
- 25 (b) combining said preparation with the second antigenic peptide,
- (c) recovering the soluble MHCI or MHCII, or tetrameric or multimeric complexes thereof loaded with said second antigenic peptide.

- 30 According to a fifth embodiment is provided the use of a nanobody and antigenic peptide to said nanobody to stabilise a soluble mammalian dimeric receptor protein or to enhance selective pairing of the receptor polypeptides of a mammalian dimeric receptor protein.

- A sixth embodiment of the invention provides a dimeric receptor protein,
 35 wherein the dimeric receptor protein is a modified mammalian T cell receptor protein (TRC) comprising first and second receptor polypeptides, wherein:
- (a) the first polypeptide is a TRC beta-chain having a C-terminally fused first peptide comprising a nanobody, and
- (b) the second polypeptide is an TRC alpha-chain having a C-terminally fused second

peptide comprising a first antigenic peptide cognate to said nanobody,
or

(c) said first polypeptide is a TRC alpha-chain having a C-terminally fused first peptide comprising a nanobody, and

- 5 (d) said second polypeptide is an TRC beta-chain having a C-terminally fused second peptide comprising a first antigenic peptide cognate to said nanobody,
wherein pairing affinity of the TCR beta and alpha chain according to (a) and (b) or (c) and (d) is enhanced by non-covalent bonds between the nanobody and the cognate peptide relative to a corresponding TCR alpha chain and beta chain of an unmodified
10 mammalian T cell receptor protein from said modified mammalian T cell receptor protein is derivable.

A seventh embodiment of the invention provides for the use of a nanobody and an antigenic peptide cognate to said nanobody to obtain selective pairing of an alpha chain and a beta chain of a modified TCR according to the sixth embodiment of the invention,
15 when expressed in a host cell (e.g. T cell).

DESCRIPTION OF THE INVENTION

Brief description of the figures:

Figure 1: Cartoon showing a conventionally biotinylated MHCI soluble heterodimer (left panel); and a modified MHCI soluble heterodimer comprising a MHCI heavy chain having
20 a C-terminally fused antigenic peptide ALFA-tag [MHCI-HC-ALFA], and a MHCI β 2m chain having a C-terminally fused antigenic peptide-cognate nanobody [B2M-NB] (right panel). A biotinylated Avi-tag is fused to the C-terminus of the ALFA-tag on the heavy chain of the modified MHCI soluble heterodimer. Components of the modified MHCI soluble heterodimer are shown in the lower panel.

25 **Figure 2:** Cartoon illustrating the relative stability of (A) conventional biotinylated soluble MHCI heterodimer (upper panel) and (B) a modified soluble MHCI heterodimer of the invention following UV irradiation-induced peptide exchange – where the modification of the heterodimer prevents dissociation of the MHCI β 2m chain seen in the unstable conventional biotinylated soluble MHCI heterodimer (A). The higher stability translates into a higher recovery of the modified soluble MHCI heterodimer of the
30 invention during the peptide-exchange process. Lower panel shows components of the modified soluble MHCI heterodimer.

Figure 3: Image of a stained gel of electrophoretically-separated samples showing: B2M-NB inclusion bodies of the expected size of 25.9KDa [KiloDaltons] in lanes 1-2; and
35 HLA A0201-ALFA inclusion bodies having the expected size of 36.7KDa in lanes 3-4,

where the samples were treated with or without β -mercaptoethanol. Left lane shows molecular weight markers.

Figure 4: Akta pure chromatogram image showing separation of re-folded MHCI heterodimers composed of B2M-NB and HLA A0201-ALFA monomers derived from *E. coli* inclusion bodies): purification peak 1 (P1) at 8.12mL comprises aggregated MHCI heavy chains [HLA A0201-ALFA]; peak 2 (P2) at 14.15 comprises the re-folded MHCI heterodimer [HLA A0201-NB:ALFA]. Peak 3 (P3) at 16.22mL comprises B2-NB monomers which elute last. mAU is a milli-absorbance unit of proteins at 280nm.

Figure 5: Image of a stained gel of electrophoretically-separated samples comprising in lanes 1-3: aggregated MHCI heavy chains [HLA A0201-ALFA] from Peak 1; in lanes 4-6: denatured HLA A0201-NB:ALFA (61.7 kDa) comprising the monomers HLA A0201-ALFA at 36.7KDa and B2M-NB at 25.9KDa; in lanes 7-9: B2-NB monomers at 25.9KDa from Peak 3. Samples treated with streptavidin in lanes 2, 5 and 8 (+); and in lane 5: biotin-labelled dimer, HLA A0201-NB:ALFA, that binds to streptavidin migrates at the higher molecular weight of >60kDa instead of 36.7Kda indicating 100% biotinylation efficiency. Left lane shows molecular weight markers.

Figure 6: Flow cytometric analysis (using FlowJo v10.8.1) of CMV antigen-specific T cells incubated with 0.1 or 0.5 or 1 μ L of soluble tetramers of [A] HLA A0201-WT or [B] HLA A0201-NB:ALFA employing combinatorial staining using allophycocyanin- (APC-) and Phycoerythrin- (PE-) streptavidin fluorophores.

Figure 7: Graph showing the mean fluorescence intensity of CMV antigen-specific T cells incubated with a 0.1 or 0.5 or 1 μ L staining volume of soluble tetramers of either HLA A0201-WT or HLA A0201-NB:ALFA employing combinatorial staining using allophycocyanin- (APC-) and Phycoerythrin- (PE-) streptavidin fluorophores (derived from the Flow cytometric of figure 6).

Figure 8: Graph showing the mean fluorescence intensity of viral (CMV) antigen-specific T cells incubated with soluble tetramers of either HLA A0201-WT or HLA A0201-NB:ALFA over time when incubated at: (A) 4°C or (B) 25°C; where the soluble tetramers are loaded with a peptide comprising a CMV antigen.

Figure 9: Histogram plot showing the fluorescence intensity resulting from the detection of CMV antigen-specific T cells using allophycocyanin – (APC) and Phycoerythrin (PE) streptavidin fluorophores, following incubation with [A] 0.1 μ L; [B] 0.5 μ L; [C] 1 μ L of soluble tetramers of HLA A0201-WT [light gray plot] or HLA A0201-NB:ALFA [dark gray plot].

Figure 10: Plasmid map of pET 3D vector: The pET 3D vector is part of the pET series of bacterial expression vectors. All MHC I genes are cloned at NcoI and BamHI sites, driven by a T7 promoter. The plasmid has an Ampicillin resistance gene for selection and a high-copy-number origin of replication for propagation.

5 **Figure 11:** Cartoon showing DNA expression constructs for a MHCI-WT β 2M chain and four HLA-WT heavy chain allele; showing their insertion orientation and site of integration when cloned into pET3D vector at the NcoI and BamHI restriction sites.

Figure 12: Cartoon showing DNA expression constructs for a MHCI B2M-NB and four alleles of the heavy chain HLA-[AO201]ALFA expression constructs; showing their
10 insertion orientation and site of integration when cloned into pET3D at the NcoI and BamHI restriction sites.

Figure 13: Plasmid map of the pDTU vector used for protein expression in mammalian host cells. The pDTU vector is a lentiviral vector having all the necessary features for lentivirus production and viral transduction such as 5'- and 3' Long Terminal Repeat (LTR), an ampicillin resistance gene for selection, a high-copy-number origin of
15 replication and a cloning site of XbaI and BamHI, driven by an EF-1 α promoter. A GFP expression gene or puromycin expression gene is added at the end of the expression construct which acts as a selection marker with GFP expression analyzed on flow cytometry or puromycin antibiotic selection. This fluorescence or antibiotic selection
20 ensures the selection of transduced cells. The expression cassette is followed with a Woodchuck Hepatitis virus posttranscriptional regulatory element (WPRE) for enhanced expression.

Figure 14: Plasmid map of pDTU plasmid with puromycin as selection marker, replacing GFP after the BamHI cloning site. The plasmid has the same other features
25 as pDTU shown in Figure 13.

Figure 15: Cartoon showing a wild-type biotinylated MHC II soluble heterodimer (MHCII-WT, left panel); a modified MHC II soluble heterodimer comprising a MHCII β chain having a C-terminally fused aLzip, and a MHCII α chain having a C-terminally fused bLzip (MHCII-LZIP, middle panel); and a modified MHC II soluble heterodimer
30 comprising a MHCII β chain having a C-terminally fused antigenic peptide ALFA-tag, and a MHCII α chain having a C-terminally fused antigenic peptide-cognate nanobody (MHCII-NB-ALFA; right panel). A biotinylated Avi-tag is linked to the C-terminus of the WT-type and modified α chain of the MHC II soluble heterodimers. Components of the modified MHC II soluble heterodimers are shown in the lower panel.

Figure 16: Cartoon showing gene constructs encoding the α and β chains of [A] MHC II-WT, [B] MHC II-NB:ALFA and [C] MHCII-LZIP comparator, and their cloning sites. The encoded truncated alleles of the MHCII-WT or modified MHC II β and α chains (DRB1*01:01-beta and DRB1-alpha) are separated by 2A self-cleaving peptides for inducing ribosomal skipping during translation (T2A and P2A). Expression of the gene constructs is driven by an EF1 α promoter, together with a downstream EGFP transgene for monitoring transduction. An WPRE element inserted 3-prime of the EGFP transgene ensures stable integration and high expression of the inserted gene constructs. In [B] the DRB1*01:01-beta chain has a C-terminally fused ALFA-nanobody, and the DRB1-alpha chain has a C-terminally fused ALFA-tag. In [C] the DRB1*01:01-beta chain has a C-terminally fused acidic leucine zipper (aLzip), and the DRB1-alpha chain has a C-terminally fused basic leucine zipper (bLzip).

Figure 17: Cartoon showing gene constructs encoding the β and α chains of [A] TCR-WT and [B] TCR-NB:ALFA and their cloning sites. The encoded truncated TCR β and α chains are separated by 2A self-cleaving peptides for inducing ribosomal skipping during translation (T2A and P2A). Expression of the gene constructs is driven by EF1 α promoter, together with a downstream EGFP transgene for monitoring transduction. An WPRE element inserted 3-prime of the EGFP transgene ensures stable integration and high expression of the inserted gene construct. In [B] the TCR β chain has a C-terminally fused ALFA-nanobody, and the TCR α chain has a C-terminally fused ALFA-tag.

Figure 18: Plasmid map of an insect cell vector (pMT/BiP/V5-His A) with inducible metallothionein promoter (MT) followed by a BiP signal sequence for extracellular expression of recombinant proteins in *Drosophila* S2 cells and a multiple cloning site (MCS) to clone a gene of interest.

Figure 19: Plasmid map of pCoHygro, an insect cell selection plasmid which has a hygromycin resistance gene driven by copia promoter. This plasmid is used in combination with pMT/BiP/V5-His A for stable transfection and selection of insect cells.

Figure 20: Cartoon showing gene constructs encoding a truncated HLA DM α chain with a C terminal FLAG tag (Fig. A) and a truncated HLA DM β chain with a C terminal KT3 tag (Fig. B) that were cloned in frame with MT promoter at the BglII-XbaI restriction sites in pMT/BiP/V5-His A.

Figure 21: Plasmid map of the pCDNA3 (Thermofisher) mammalian expression vector having a CMV promoter, multiple cloning site (MCS), bGH poly adenylation sequence and a Neomycin resistance marker providing Geneticin resistance. This plasmid is an alternative to pDTU plasmid for expression of MHC II transgenes.

Figure 22: Cartoon showing a conventional soluble IgG antibody (left panel); and a modified soluble antibody comprising a first heavy chain having a C-terminally fused antigenic peptide ALFA-tag , and a second heavy chain having a C-terminally fused antigenic peptide-cognate nanobody (right panel). Components of the modified IgG antibody soluble heterodimer are shown in the lower panel.

Abbreviations, terms and definitions:

Amino acid sequence identity: The term "sequence identity" as used herein, indicates a quantitative measure of the degree of similarity between two amino acid sequences of essentially equal length. The two sequences to be compared must be aligned to give a best possible fit, by means of the insertion of gaps or alternatively, truncation at the ends of the protein sequences. The sequence identity can be calculated as $((N_{\text{ref}} - N_{\text{dif}}) / N_{\text{ref}}) \times 100$, wherein N_{dif} is the total number of non-identical residues in the two sequences when aligned and wherein N_{ref} is the number of residues in one of the sequences. Sequence identity calculations are preferably automated using the BLAST program e.g. the BLASTP program (Pearson W.R and D.J. Lipman (1988)) (www.ncbi.nlm.nih.gov/cgi-bin/BLAST). Sequence alignment may be performed using program MAFFT24 (Multiple Alignment using Fast Fourier Transform; Katoh et al 2019) using default parameters (SCORING MATRIX: blosum62, gap opening penalty: 1.53, gap extension penalty 0.123).

Preferably, the numbers of substitutions, insertions, additions or deletions of one or more amino acid residues in the polypeptide as compared to its comparator polypeptide is limited, i.e. no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 substitutions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 insertions, no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 additions, and no more than 1, 2, 3, 4, 5, 6, 7, 8, 9, or 10 deletions. Preferably the substitutions are conservative amino acid substitutions: limited to exchanges within members of group 1: Glycine, Alanine, Valine, Leucine, Isoleucine; group 2: Serine, Cysteine, Selenocysteine, Threonine, Methionine; group 3: Proline; group 4: Phenylalanine, Tyrosine, Tryptophan; Group 5: Aspartate, Glutamate, Asparagine, Glutamine; Group 6: Histidine. Lysine, Arginine.

ALFA is a rationally designed antigenic peptide tag called Alfa tag.

B2M is β -2 microglobulin.

B2M-NB is a MHCI- β 2m chain C-terminally fused to an antigenic peptide-cognate nanobody.

CMV is Cytomegalovirus.

Devoid is a term used with respect to a soluble dimeric receptor protein of the invention, where said soluble dimeric receptor protein is defined as being devoid of transmembrane domain(s), said transmembrane domain(s) being domain(s) in a corresponding dimeric receptor protein found in nature; and from which said soluble dimeric receptor protein is derivable, for example by providing soluble dimeric receptor protein comprising recombinantly expressed first and second receptor polypeptides lacking said transmembrane domain(s).

ELISA is Enzyme linked ImmunoSorbent Assay

First peptide or second peptide is defined herein as a peptide having a molecule mass and an amino acid sequence length that accommodates a nanobody, or an antigenic peptide cognate to said nanobody respectively, and any additional peptide spacer and peptide tag according to the present invention.

FBS is Fetal Bovine Serum

HLA A0201-ALFA is an example of MHCI-HC-ALFA comprising correspondingly modified human MHCI heavy chain.

HLA A0201-NB:ALFA is an example of MHCI-NB:AP comprising corresponding human MHCI heavy chain and MHCI- β 2m chain fusion proteins

HLA A0201-WT is soluble MHCI complex comprising a truncated human MHCI heavy chain and a MHCI- β 2m chain.

HLA-DM is a soluble human leukocyte antigen DM which is an intracellular protein involved in the mechanism of antigen presentation on antigen presenting cells (APCs) of the immune system.

IPTG is Isopropyl thiogalactoside

IB is an inclusion body

Kd is dissociation constant is the rate constant of dissociation at equilibrium, defined as the ratio koff / kon.

MFI is Mean fluorescence intensity

MHCI is Major Histocompatibility Complex I.

MHCI-HC-AP is a truncated MHCI heavy-chain having a C-terminally fused antigenic peptide (AP), wherein AP can be an ALFA-tag.

MHCI-NB:AP is a soluble MHC I heterodimer composed of the fusion monomers 'MHC I-HC-AP' and 'B2M-NB'.

MHCII is Major Histocompatibility Complex II

5 MHCII β -AP is a truncated MHCII β -chain with an antigenic-peptide fused to the C-terminus of the β -chain, wherein AP can be an ALFA-tag.

MHCII α -NB is a truncated MHCII α -chain having a C-terminally fused antigenic peptide-cognate nanobody.

NB is a nanobody.

PAGE is Polyacrylamide gel electrophoresis

10 PBS is Phosphate buffered saline

PBMC is a peripheral blood mononuclear cell

SDS is Sodium Dodecyl Sulphate

TCR is a T cell receptor

WT is wild type

15

Detailed description of the invention:

The present invention provides modified soluble mammalian dimeric receptor proteins that are characterized by enhanced stability, as well as retaining the functional properties of their native counterparts from which they were derived.

20 In a first embodiment, the invention provides a soluble mammalian dimeric receptor protein comprising modified first and second receptor polypeptides devoid of a transmembrane anchoring domain, wherein:

(a) the first receptor polypeptide is fused to a first peptide comprising a nanobody, and

25 (b) the second receptor polypeptide is fused to a second peptide comprising an antigenic peptide cognate to said nanobody, and wherein stability of the dimeric receptor protein is enhanced by non-covalent bonds between the nanobody and the cognate peptide relative to corresponding unmodified first and second receptor polypeptides devoid of a transmembrane anchoring domain from which said modified chains were derived.

30 A soluble mammalian dimeric receptor protein according to the invention comprises structural domains corresponding to the extracellular domains of its counterpart mammalian receptor protein found in nature, but lacking domains for cell membrane

anchoring, such as one or more transmembrane domains. The membrane anchoring domains that are lacking from said soluble mammalian receptor protein can be detected and delineated using DeepTMHMM (Hallgren et al., 2022), a deep learning model for transmembrane topology prediction and classification. The receptor protein is defined as
 5 being soluble in the sense that it remains in solution in an aqueous medium. The soluble mammalian dimeric receptor protein comprises a core of two receptor polypeptide chains that either share a homologous structure or are heterologous in structure, and where the two receptor polypeptide chains are capable of forming a homodimer or a heterodimer, respectively. The two chains of the homodimeric or heterodimeric receptor
 10 protein according to the invention associate and are held together by means of non-covalent bonds.

The receptor polypeptide chains of the soluble mammalian dimeric receptor protein of the invention differ from their counterpart found in nature in that each chain (i.e. the
 15 first receptor polypeptide and the second receptor polypeptide) is a fusion polypeptide. A fusion polypeptide as defined herein, is a polypeptide composed of two or more fused domains that is encoded by a single gene, where the gene comprises coding sequences for the respective fused domains that are joined together in the single gene. Accordingly, the first receptor polypeptide of the receptor protein is fused to a first
 20 peptide comprising a nanobody; and the second receptor polypeptide is fused to a second peptide comprising an antigenic peptide cognate to said nanobody.

The nanobody as defined herein is a single-domain antibody consisting of a single monomeric variable antibody domain that is able to bind selectively to a specific antigen
 25 and preferably has a molecular weight of only 12–15 kDa. In a preferred embodiment the nanobody has an affinity for its cognate antigenic peptide measurable as a dissociation constant (K_d) in the range of 10pM to 500nM; 10pM to 200nM; 10pM to 10nM; 10pM to 500pM; 20pM to 200pM; 20pM to 100pM (listed in order of increasing preference), for example 20pM to 200nM; or a K_d of <20pM; <30pM; <40pM; <50pM;
 30 <60pM; <70pM; <80pM; <90pM; <100pM; <200pM; <300pM; <400pM; <500pM; <600pM; <700pM; <800pM; <1nM; <10nM; <20nM; <50nM, most preferably <100nM, when measured in PBS pH 7.4 containing 0.002% (v/v) Tween-20 (PBS-T) at 20°C. A suitable method and instrumentation for measuring said affinity is Surface Plasmon resonance (SPR) as described in Example 17 (by ref to Götzke et al., 2019, on
 35 page 10 [Affinity determinations]).

The affinity of the nanobody for its cognate antigenic peptide is epitope driven (in contrast to a charge driven leucine zipper) and can therefore stabilize the soluble mammalian dimeric receptor protein of the invention in different media and buffers independent of salt concentration.

The antigenic peptide as defined herein is a peptide that is cognate to said nanobody, i.e. an antigenic peptide to which the nanobody is capable of binding, preferably with a dissociation constant as defined above. Such antigenic peptide is preferably 4 – 15 amino acids in length, more preferably at least 9 amino acids in length. Suitable nanobodies and their cognate antigenic peptides may be selected from among ALFA-tag, EPEA-tag/C-tag, Spot-tag, Moon-tag, 6E-tag, BC2-tag, or Pep-tag (Table 1).

TABLE 1: Nanobody:cognate antigenic peptide pairs

Nanobody	Protein sequence SEQ ID NO.:	Cognate antigenic peptide	Peptide sequence SEQ ID NO.:
ALFA nanobody	4	ALFA tag: SRLEELRRRLTE	2
C-tag nanobody	43	C-tag: EPEA	42
Moon-tag nanobody	45	Moon-tag: KNEQELLELDKWASL	44
6E-tag nanobody	47	6E-tag: QADQEAKELARQIS	46
BC2-tag nanobody	49	BC2 tag: PDRKAAVSHWQQ	48
Pep-tag nanobody	51	Pep tag: AVERYLKDQQLGIW	50
BC-2 Nanobody	49	Spot tag PDRVRAVSHWSS	66

10

Preferably the nanobody is either directly fused to the C-terminus of the first receptor polypeptide, or alternatively the fusion may include a spacer amino acid sequence between the nanobody and the C-terminus of the first receptor polypeptide. The spacer sequence may be included to confer flexibility between the two fused domains, and/or it may serve to align the nanobody with its cognate antigenic peptide in the second receptor polypeptide when the two polypeptide chains for the dimeric receptor protein associate and non-covalently bind.

15

The antigenic peptide is fused at the C-terminus of the second receptor polypeptide. The fusion may include two spacer amino acid sequences located at each end of the antigenic peptide or only one spacer sequence located between the antigenic peptide and the second receptor polypeptide. Preferably, one spacer is located at the N-terminal end of the antigenic peptide. Again, the one or two spacers are used to confer flexibility

20

between the fused domains, and/or to align the antigenic peptide with the cognate nanobody in the first receptor polypeptide when the two receptor polypeptide chains for the dimeric receptor protein are associated.

- 5 The amino acid spacers fused to the first or second receptor polypeptide chain are preferably enriched in small or polar amino acids such as Gly and Ser to provide good flexibility and solubility. The length of the linker is generally between 3 and 21 amino acid residues, preferably 6 to 12 residues; for example having the sequence: GGS, GGGS [SEQ ID NO.:69], GGSGGS [SEQ ID NO.:70], or GGSGGSGGS [SEQ ID NO.:71].

10

A peptide tag may also be fused to the C-terminal amino acid residue of the first or second receptor fusion polypeptides, for example the C-terminal amino acid residue of the nanobody or the cognate antigenic peptide. A suitable peptide tag is the avi-tag since this can bind biotin and facilitate capture with streptavidin. Suitable peptide tags are listed in Table 2.

15

Table 2: Soluble mammalian dimeric receptor protein peptide tags

Peptide tag	Protein sequence SEQ ID NO.:
Avi-tag	52
His tag	53
Myc tag	54
FLAG tag (-1, -2, -3)	55, 56, or 57
HA tag (-1, -2, -3)	58, 59, or 60

20

The interaction between the nanobody and its cognate antigenic peptide in the soluble mammalian dimeric receptor protein of the invention enhances the stability of the dimeric receptor protein as compared to a corresponding soluble mammalian dimeric receptor protein from which the receptor protein of the invention is derivable. Preferably the stability of the soluble mammalian dimeric receptor protein is increased by at least 5, 10, 15, 20, 30, 40, 50, 60, or 70 % (in order of increasing preference) when compared to a corresponding soluble mammalian dimeric receptor protein from which it is derivable, as measured indirectly by employing the stability assays described in examples 6, 13 and 16.

25

30

In one aspect, the soluble mammalian dimeric receptor protein according to the invention is a soluble Major Histocompatibility Complex I (MHCI). Soluble MHCI is derived from native MHCI by deletion of the transmembrane anchoring domain from each of the heavy-chain and the $\beta 2m$ chain of the corresponding native heterodimer.

The soluble MHC I receptor protein of the invention is characterized by a MHC I heavy-chain having an antigenic peptide fused at the C-terminus of the heavy-chain [MHC I-HC-AP], and a MHC I β 2m chain having an antigenic peptide-cognate nanobody fused at the C-terminus of the β 2m chain [B2M-NB].

- 5 Alternatively, said soluble MHC I receptor protein is characterized by a MHC I heavy-chain having a C-terminally fused antigenic peptide-cognate nanobody [MHC I-HC-NB], and a MHC I β 2m chain having a C-terminally fused antigenic peptide [B2M-AP].

10 Examples of suitable antigenic peptide:antigenic peptide-cognate nanobody pairs include ALFA-tag, C-tag, Spot-tag, Moon-tag, Pep-tag, 6E-tag and each their cognate nanobody (Table 1). MHC I-NB:ALFA is an example of a soluble MHC I receptor of the invention comprising the heavy-chain [MHC I-HC-ALFA] having the antigenic peptide ALFA-tag [A0201-ALFA: SEQ ID NO.: 10], and the β 2m chain [B2M-NB; SEQ ID NO.: 8] having an ALFA tag-cognate nanobody, as shown in figure 1. A spacer sequence, as
15 described above, may be fused to the MHC I heavy-chain or β 2m chain at one or both ends of the antigenic-peptide or nanobody amino acid sequence. A soluble mammalian dimeric receptor protein according to the invention can be engineered starting from any MHC I allele, as described in example 1.

20 The soluble MHC I according to the invention may additionally be loaded with a UV cleavable peptide in the peptide-binding groove of the MHC molecule, as illustrated in example 3 (as described by Toebe et al., 2006). Alternatively, the soluble MHC I may be loaded with a desired specific second antigenic peptide ligand, preferably comprising 8 – 10 amino acids, as illustrated in figure 2 and example 4.

25

Table 3: MHC I dimeric receptor protein UV degradable peptides

UV degradable peptides 1, 2, 3, 4, 5: amino acid sequence*	Amino Acid Sequence SEQ ID NO.:
STAPGJLEY	61
KILGFVFJV	62
RIYRJGATR	63
FLRGRARJGL	64
AARGJTLAM	65

* The peptides may comprise β -amino acid 3-amino-3-(2-nitrophenyl)-propionic acid], or α -amino acid(2-nitro)-phenylglycine in place of any of the conventional α -amino acid, herein denoted as (J).

30

The soluble MHC I according to the invention may be provided as a heterodimer, in which form the soluble MHC I may be considered to be a monomer, or alternatively it can be

- provided in multimeric form by conjugation to a carrier. Suitable carriers, such as streptavidin or dextran can be bound to an amino acid residue in a C-terminal portion of the heavy-chain [such as MHCI-HC-ALFA] or the β 2m chain [such as B2M-NB] of the soluble MHCI. For example the soluble MHCI of the invention can have an Avi-tag and
- 5 be provided in biotinylated form that can be labelled by non-covalent binding with fluorophore-labelled streptavidin (as carrier), or alternatively labelled and tetramerized or multimerized by binding to fluorophore-labelled streptavidin-conjugated dextran as illustrated in example 4 and figure 2. Suitable fluorophores include allophycocyanin (APC), Phycoerythrin (PE), Alexa Fluor 488, Alexa Fluor594, Alexa Fluor647, Brilliant
- 10 violet 421, Brilliant violet 510, Brilliant violet 570, Brilliant violet 605, Brilliant violet 650, Brilliant violet 711, Brilliant violet 785, Fluorescein isothiocyanate (FITC), Peridinin-Chlorophyll-Protein (PerCP), and Cyanine dye conjugates such as APC-Cy7, PE-Cy5, PE-Cy7, PerCP/Cy5.5.
- 15 The soluble MHCI according to the invention is characterised by an enhanced T cell staining efficiency of at least 5, 10, 15, 20, 30, 40, 50, 60, or 70 % (in order of increased preference, for example at least 40%) when compared to a corresponding soluble MHCI from which it is derivable, as illustrated in example 5 and figure 7. Furthermore, the soluble MHCI according to the invention is characterised by an enhanced heterodimeric
- 20 stability of at least 5, 10, 15, 20, 30, 40, 50, 60 or 70 % (in order of increased preference, for example at least 40%) when compared to a corresponding soluble MHCI from which it is derivable as can be measured employing the stability assays described in example 6 and illustrated in figure 8.
- 25 In one aspect, the soluble mammalian dimeric receptor protein according to the invention is a soluble Major Histocompatibility Complex II (MHCII). Soluble MHCII is derived from native MHCII by deletion of the transmembrane anchoring domain from each of the alpha-chain and the beta-chain of the native heterodimer, as depicted in figure 15. The soluble MHCII receptor protein of the invention is characterized by the
- 30 resulting truncated MHCII beta-chain having an antigenic-peptide fused to the C-terminus of the beta-chain [MHCII beta-AP], and the resulting truncated MHCII alpha-chain having a C-terminally fused antigenic-peptide-cognate nanobody [MHCII alpha-NB].
- Alternatively, the resulting truncated MHCII beta-chain has a C-terminally fused
- 35 antigenic-peptide-cognate nanobody [MHCII beta-NB] and the resulting truncated MHCII alpha-chain has a C-terminally fused antigenic-peptide [MHCII alpha-AP].

Examples of suitable antigenic-peptide:antigenic-peptide-cognate nanobody pairs include ALFA-tag, EPEA-tag or C-tag, Spot-tag, Moon-tag, Pep-tag, 6E-tag and each

their cognate nanobody [Table 1]. MHCII-NB:ALFA is an example of a soluble MHCII receptor of the invention comprising the MHCII beta-chain [MHCII beta-ALFA] having the antigenic-peptide ALFA-tag and the MHCII alpha-chain [MHCII α -NB] having an ALFA tag-cognate nanobody. A spacer sequence, as described above, may be inserted into the MHCII alpha-chain or beta-chain at the N-terminal (and optionally C-terminal) end of the antigenic-peptide or nanobody amino acid sequence. Soluble mammalian dimeric MHCII receptor protein according to the invention can be engineered starting from any MHCII allele, as described in example 8; and expressed as a fusion protein, for example having SEQ ID NO.: 24.

The modified soluble MHCII of the invention can be provided with a placeholder peptide that can be substituted with a desired second antigenic peptide (for peptide presentation). For example, the truncated MHCII beta-chain can be expressed with a CLIP peptide fused to the N-terminus of the beta-chain by means of a cleavable linker (e.g. 3C cleavage site (LEVLFQGP; SEQ ID NO.: 67) or a thrombin cleavage site (LVPRGS; SEQ ID NO.: 68; example 8). In this case, the modified soluble MHCII of the invention can subsequently be loaded with a desired specific second antigenic peptide, preferably comprising 13 - 30 amino acids, by substituting the placeholder peptide using human leukocyte antigen DM [HLA-DM] as described in example 9 and 10.

The soluble MHCII according to the invention may be provided as a heterodimer, in which form the soluble MHCII may be considered to be a monomer, or alternatively it can be provided in multimeric form by conjugation to a carrier. For this purpose, a peptide tag is fused to a C-terminal amino acid residue of the first or second MHCII fusion polypeptides, for example the C-terminal amino acid residue of the nanobody or the cognate antigenic peptide, where the tag facilitates capture with a cognate carrier, such streptavidin or dextran. For example the soluble MHCII of the invention upon biotinylation can be bound to fluorophore labelled-streptavidin (as carrier) and optionally can be tetramerized or multimerized by non-covalent binding to fluorophore-labelled streptavidin conjugated dextran molecule, as illustrated in example 11. Suitable fluorophores include allophycocyanin (APC), Phycoerythrin (PE), Alexa Fluor 488, Alexa Fluor594, Alexa Fluor647, Brilliant violet 421, Brilliant violet 510, Brilliant violet 570, Brilliant violet 605, Brilliant violet 650, Brilliant violet 711, Brilliant violet 785, Fluorescein isothiocyanate (FITC), Peridinin-Chlorophyll-Protein (PerCP), and Cyanine dye conjugates such as APC-Cy7, PE-Cy5, PE-Cy7, PerCP/Cy5.5.

The soluble MHCII according to the invention is characterized by an enhanced T cell staining efficiency of at least 5, 10, 15, 20, 30, 40, 50, 60 or 70% (in order of increasing preference) when compared to a corresponding soluble MHCII from which it is derivable,

as illustrated in example 11. Furthermore, the soluble MHCII according to the invention is characterized by an enhanced stability of at least 5, 10, 15, 20, 30, 40, 50, 60 or 70% (in order of increasing preference) when compared to a corresponding soluble MHCII from which it is derivable, as measured employing the stability assays described in example 12.

One aspect of a soluble mammalian dimeric receptor protein according to the invention is a B cell receptor (SEQ ID NO.: 38 and SEQ ID NO.: 40). The antigenic peptide and its antigenic peptide-cognate nanobody are fused respectively to the C-terminus of the first and second polypeptide chains of the B cell receptor (e.g. IgG), respectively (figure 22).

A second embodiment of the invention provides a method of detecting one or more antigenic peptide-responsive T cells in a sample, the method comprising the steps of:

(a) providing soluble dimeric receptor proteins according to the first embodiment of the invention, wherein said proteins are soluble MHCI or soluble MHCII receptor proteins,

(b) loading each of said MHCI or MHCII receptor proteins with at least one antigenic peptide to form a MHCI-peptide complex or a MHCII-peptide complex,

(c) conjugating each of said MHCI-peptide complex or MHCII-peptide complex with a carrier coupled to a fluorophore to form a tetrameric or multimeric complex;

(d) staining a sample of Tcells to be interrogated with said conjugated MHCI-peptide tetrameric or multimeric complexes or said conjugated MHCII-peptide tetrameric or multimeric complexes of (c), and

(e) detecting the binding of said tetrameric or multimeric complexes to the one or more antigenic peptide responsive cells in the sample, for example by means of flow cytometry.

The cells to be interrogated may be PBMC cells comprising antigenic-peptide responsive cells.

The carrier may be streptavidin or dextran, each coupled to a fluorophore, where a suitable fluorophore includes allophycocyanin (APC), Phycoerythrin (PE), Alexa Fluor 488, Alexa Fluor594, Alexa Fluor647, Brilliant violet 421, Brilliant violet 510, Brilliant violet 570, Brilliant violet 605, Brilliant violet 650, Brilliant violet 711, Brilliant violet 785, Fluorescein isothiocyanate (FITC), Peridinin-Chlorophyll-Protein (PerCP), and Cyanine dye conjugates such as APC-Cy7, PE-Cy5, PE-Cy7, PerCP/Cy5.5.

A third embodiment of the invention provides a method of producing a soluble mammalian dimeric receptor protein comprising modified first and second receptor

polypeptides devoid of a transmembrane anchoring domain according to the invention, the method comprising the steps of:

- (a) providing a first nucleic acid coding sequence encoding the first receptor polypeptide having a C-terminally fused first peptide comprising a nanobody, and
- 5 (b) providing a second nucleic acid coding sequence encoding the second receptor polypeptide having a C-terminally fused second peptide comprising an antigenic peptide cognate to said nanobody,
- (c) providing a first vector comprising said first nucleic acid coding sequence and a second vector comprising said second nucleic acid coding sequence, or alternatively a
- 10 third vector comprising said first nucleic acid coding sequence linked to said second nucleic acid coding sequence, wherein expression of said first and second nucleic acid coding sequences is operably linked to a promoter;
- (d) expressing said modified first and second receptor polypeptides in host cells comprising respectively said first and said second vector or host cells comprising said
- 15 third vector;
- (e) recovering the expressed first and second receptor polypeptides, wherein stability of the dimeric receptor protein comprising said first and second receptor polypeptides is enhanced as compared to a corresponding soluble mammalian dimeric receptor protein from which the mammalian dimeric receptor protein of the
- 20 invention is derivable.

According to the third embodiment of the invention, the soluble mammalian dimeric receptor protein may be an MHCI receptor or an MHCII receptor. When the receptor is an MHCII receptor and a third vector encodes the modified first and second receptor

25 polypeptides, then the first and second receptor polypeptides can be translationally linked by a self-cleaving peptide (e.g. 2A ribosomal skipping sequence; example 8).

A fourth embodiment of the invention provides a method of loading, or displacing a placeholder peptide, in the soluble dimeric MHCI or MHCII receptor protein with a

30 second antigenic peptide comprising the steps of:

- (a) providing a solution of the soluble MHCI or MHCII of the invention or the multimeric complex thereof,
- (b) combining said solution with the second antigenic peptide,
- (c) recovering the soluble MHCI or MHCII or multimeric complex thereof loaded with
- 35 said second antigenic peptide.

The placeholder peptide may be a UV-cleavable peptide, as described in the first embodiment for an MHCI receptor of the invention, or a CLIP peptide in an MHCII receptor of the invention.

A fifth embodiment of the invention provides for the use of an antigenic peptide and respective antigenic peptide cognate nanobody to stabilise a soluble mammalian dimeric receptor protein, when the nanobody and its cognate antigenic peptide are each
5 expressed as a C-terminal fusion with one of the chains of the dimeric receptor. In a preferred embodiment the mammalian dimeric receptor protein is any of MHCI, MHCII, or B cell receptor.

A sixth embodiment of the invention provides a mammalian T cell receptor protein
10 comprising a modified alpha chain and a modified beta chain, wherein:
(a) a nanobody is fused to the C terminus of the beta chain, and
(b) an antigenic peptide cognate to said nanobody is fused to C-terminus of the alpha chain, and
wherein the dissociation constant of the modified alpha chain and modified beta chain
15 is enhanced by non-covalent bonds between the nanobody and the cognate peptide relative to a corresponding unmodified alpha chain and unmodified beta chain from which said modified chains were derived.

Suitable antigenic peptide:antigenic peptide-cognate nanobody pairs have properties that correspond to those described for the first embodiment and listed in Table 1. The
20 antigenic peptide fused to the alpha chain may include a spacer amino acid sequence located at each end of the peptide. Preferably, one spacer is located at the N-terminal end of the antigenic peptide. The spacer is preferably enriched in small or polar amino acids such as Gly and Ser, while the length of the linker is generally between 3 and 21 amino acid residues, preferably 6 to 12 residues; for example having the sequence:
25 GGS, GGGS [SEQ ID NO.:69], GGSGGS [SEQ ID NO.:70], or GGSGGSGGS [SEQ ID NO.:71].

The nanobody fused to the C terminus of the beta chain may include a spacer amino acid sequence located at the N-terminus of the nanobody or at each end of the nanobody; employing a spacer as described above.

30 In a further aspect, the invention provides T-cells expressing a mammalian T cell receptor protein comprising a modified alpha chain and a modified beta chain according to the invention.

A seventh embodiment of the invention provides for the use of a nanobody and an
35 antigenic peptide cognate to said nanobody to obtain preferential pairing of a modified alpha chain and a modified beta chain of a TCR when expressed in a host cell (e.g. T cell), when the nanobody and its cognate antigenic peptide are each expressed as a C-terminal fusion with one of the chains of the dimeric receptor.

Examples

Example 1: Structure and expression of MHCI heavy-chain and β 2m chains of the invention

The nucleotide sequences encoding the heavy-chain and β 2m chains of MHCI heterodimer alleles were obtained from Uniprot: Human β 2m chain – P61769, HLA A heavy chain – P04439, HLA B heavy chain – P01889.

DNA constructs were designed to express the truncated wild type MHCI β 2m (SEQ ID No.: 6); and the B2M-NB monomer comprising a MHCI- β 2m chain (SEQ ID No.: 6) having an antigenic peptide-cognate nanobody (SEQ ID No.: 4) fused at the C-terminus of the β 2m chain. The B2M-NB monomer has amino acid sequence SEQ ID No.: 8, encoded by nucleotide sequence SEQ ID No.: 7.

The HLA A0201-ALFA monomers each comprise a truncated MHCI heavy-chain allele comprising the antigenic peptide, ALFA-tag (SEQ ID No.: 2) fused to C-terminus of the respective heavy-chain allele by means of a spacer. DNA constructs were designed to express each of four heavy-chain alleles identified as A0201-WT (SEQ ID 26), A0301-WT (SEQ ID 28), B0801-WT (SEQ ID 30), and B4405-WT (SEQ ID 32) as illustrated in figure 11; and their respective C-terminal fusions, as illustrated in figure 12. The linker fused to the N-terminus of the ALFA-tag and nanobody comprises combinations of glycine and serine amino acids such as GGS, GGGG [SEQ ID NO.:69], GGSGGS [SEQ ID NO.:70], or GGSGGSGGS [SEQ ID NO.:71]. The amino acid sequence of the corresponding HLA HC-ALFA monomers are as follows:

HLA A0201-ALFA (SEQ ID NO.: 10) encoded by nucleotide sequence SEQ ID NO.: 9; HLA A0301-ALFA (SEQ ID NO.: 12), encoded by nucleotide sequence SEQ ID NO.: 11; HLA B0801-ALFA (SEQ ID NO.: 14), encoded by nucleotide sequence by SEQ ID NO.:13; HLA B4405-ALFA (SEQ ID NO.: 16) encoded by nucleotide sequence SEQ ID NO.: 15. The coding sequences for each monomer B2M-NB and A2-ALFA were codon-optimized for *Escherichia coli* expression, cloned behind the IPTG-inducible T7 promoter in a pET-3D bacterial vector, and expressed in *E. coli*.

Conclusions: Genetic constructs are provided for the expression of the monomeric fusion protein chains: MHCI-HC-ALFA and B2M-NB (and their WT controls) in figures 11 and 12; for subsequent assembly of stable MHCI-NB:ALFA heterodimers of the invention and their corresponding wild-type soluble MHCI heterodimer controls.

Example 2: Plasmid transformation and inclusion body preparation

The pET-3D plasmid (figure 10) further comprising gene constructs encoding the B2M-NB or HLA A0201-ALFA chains were transformed into BL21-DE3 PLYs chemically competent cells (Novagen 69451) as per manufacturers' instructions by means of heat shock. The recovered transformed cells were plated on LB agar plate containing
5 antibiotics (50µg/mL Carbenecillin-C1389 and 34µg/mL Chloramphenicol-R4408 from Sigma-Aldrich) at 37°C, overnight. A single transformed colony was cultured in LB medium with antibiotics at 37°C, and then used to inoculate a 900mL of LB media with antibiotics in a 5L conical flask. The transformed bacterial cell culture was grown to an OD of 0.5-0.6, and then B2M-NB and A2-ALFA chain expression was induced by IPTG
10 added at a final concentration of 0.5mM. The induced culture was incubated at 37°C at 200rpm for 4 hours, and the cells were then harvested by centrifugation, weighed and then re-suspended in Lysis buffer (50mM Tris-HCl pH 8.0, 25% Sucrose, 1mM EDTA, 0.2% Lysosyme) and frozen at -20/-80°C.

Inclusion bodies were isolated from each frozen lysate as follows: The lysate was
15 thawed, sonicated and then treated with 1unit/mL DNase (Thermofisher 90083) in the presence of 10mM Magnesium chloride (MgCl₂) and 1mM Manganese chloride (MnCl₂) for 30min, and then centrifuged at 40000 x g for 20min. The pellet was then washed once with detergent buffer (20mM Tris-HCl pH 7.5, 200mM NaCl, 2mM EDTA, 1% NP40, 1% Deoxycholic acid), and three times in wash buffer (1mM EDTA, 0.5% Triton X-100,
20 1mM DTT). The washed pellet was solubilized in buffer (50mM HEPES pH 6.5, 8M Urea, 0.1mM Beta-mercaptoethanol) for 48-72 hours in a rotating shaker at 4°C. The solubilized inclusion body was centrifuged at 40000 x g for 20min, and supernatant was separated and absorbance was measured at 280nm. The inclusion bodies are then checked by running on SDS-PAGE 4-20% gel (Biorad) as per Figure 3.

25 **Conclusions:** B2M-NB and A2-ALFA chains of the invention can each be expressed in *E. coli* cells, and deposited in the cell's inclusion bodies, which can subsequently be isolated and the respective chains identified following molecular size separation.

Example 3: Refolding and purification of the MHCI heterodimer of the invention

Purified inclusion bodies (comprising 2µM B2M-NB chains and 1µM HLA A0201-ALFA
30 chains) were refolded over 72 hours in refolding buffer (100mM Tris-HCl pH 8.0, 400mM L-Arginine HCl, 2mM EDTA, 5mM reduced Glutathione, 0.5mM oxidized Glutathione) in the presence of protease inhibitor tablet (Sigma-Aldrich S8830 – 1 tablet per 200mL) and 60µM UV-labile peptide (KILGFVVFJV; SEQ ID NO.: 72) specific to the MHC I allele (Rodenko et al., 2006). The refolded MHCI-NB:ALFA heterodimers were recovered by
35 centrifugation; passed through a 40µm filter to remove aggregates; concentrated using 30KDa Vivaflow 50R cassettes (Sartorius) in PBS buffer pH 7.4. The buffer-exchanged

concentrated MHCI-NB:ALFA was recovered and passed through a 4µM filter to remove aggregates; and re-concentrated in an Amicon Ultra-15 centrifugal Filter unit with 30KDa membrane (Merk-Millipore UFC903024). The re-concentrated MHCI-NB:ALFA heterodimers were biotinylated using BirA biotin-protein ligase bulk reaction kit (Avidity) as per the protocol by the manufacturer. The biotinylated MHCI-NB:ALFA was purified on a Superdex 200 gel-filtration column with PBS pH 7.4 using an AKTAPURE 25. The chromatogram is illustrated in figure 4. The MHCI-NB:ALFA heterodimers, that eluted as a peak after 14mL PBS passed through the column, were collected and concentrated. 2µg of the purified MHCI-NB:ALFA heterodimers peaks were analyzed by SDS-PAGE in the presence of streptavidin and beta-mercaptoethanol (figure 5). Purified MHCI-NB:ALFA heterodimers were then frozen at -80°C after adding glycerol to a final concentration of 10%.

Conclusions: the re-folded MHCI-NB:ALFA heterodimers of the invention exhibited a high degree of purity and close to 100% biotinylation, as determined by size exclusion chromatography.

Example 4: UV induced peptide exchange of MHCI-NB:ALFA heterodimers and formation of tetramers

MHCI-NB:ALFA heterodimers (re-folded with a UV-labile peptide and biotinylated as described in example 3) were diluted in PBS buffer to a concentration of 200µg/mL, to which an equal volume of 200µM antigenic peptide (YVLDHLIVV; SEQ ID NO.: 72) to be loaded was added. The mixture was incubated under UV light for 60 min at room temperature to induce peptide exchange (Rodenko et al., 2006); and then centrifuged to remove aggregates. The supernatant comprising the biotinylated MHCI-NB:ALFA heterodimers were then tetramerized by streptavidin-fluorophore conjugates, by adding 18.1µL of 0.1mg/ml streptavidin-fluorophore conjugate to 100µL of peptide-exchanged heterodimer. The mixture was incubated on ice for 20 - 30 min, to which 25µM free biotin was added and incubated for another 20min. MHCI-NB:ALFA tetramers were stored at -20°C after adding 10x tetramer freezing buffer (50% glycerol+ 1% BSA) to final concentration of 1x.

Conclusions: the MHCI-NB:ALFA heterodimers re-folded with a UV-labile peptide and biotinylated are competent for peptide exchange with a desired antigenic peptide and can be ligand-tetramized using streptavidin-fluorophore conjugates.

Example 5: T-cell specific staining by MHCI-NB:ALFA of the invention

Viral antigen-specific T cells, capable of recognising CMV antigen (YVLDHLIVV; SEQ ID NO.: 72) presented on the MHCI HLA A0201 chain, were washed twice with RPMI-1640

cell culture medium (Thermofisher 11875093) +10% FBS (Thermofisher 10500064). One million T cells were aliquoted into a well of a 96-well plate; centrifuged; and then washed twice with FACS buffer (PBS pH 7.4 + 2% FBS). The washed T cells were incubated with 1µL of the MHCI-NB:ALFA tetramers or MHCI-streptavidin tetramer at 37°C for 15 min, to which the following antibodies conjugated with fluorophore were then added: anti-human CD4 FITC (BD 345768), anti-human CD14 FITC (BD 345784), anti-human CD19 FITC (BD 345776), anti-human CD16 FITC (BD 335035), anti-human CD40 FITC (Serotech MCA1590F), and anti-human CD8 BV480 (BD 566121), with NIR live-dead marker (Thermofisher L10119), and then further incubated on ice for 30min (Andersen et al, 2012). The T cells were then washed with FACS buffer twice and run through a flow cytometer and analyzed on FlowJo v10.8.1 (Figure 6). The mean fluorescence intensity values of the tetramer positive population was then plotted (Figure 7). The fluorescence count of the tetramer positive population was compared by plotting as a histogram (Figure 9).

Conclusions: the MHCI-NB:ALFA tetramers of the invention loaded with antigenic peptide from CMV, with amino acid sequence YVLDHLIVV exhibited at least a 40% increase in T cell staining efficiency than corresponding soluble wild type MHC-1 tetramers.

Example 6: Stability of peptide-exchanged MHCI-NB:ALFA heterodimers of the invention

6.1 Stability of MHCI-NB:ALFA heterodimers

The MHCI-NB:ALFA and wild-type MHC-WT heterodimers in PBS buffer were each peptide-exchanged with an antigenic peptide and each then dispensed in an 1.5mL eppendorf tube and incubated at 4 or 25°C. At time=0, 12, 24, 48, 72, 96, 168 hours a 15µL aliquot was removed, and tetramerized with streptavidin-fluorophore conjugates; thawed, and centrifuged to remove aggregates. 1µL supernatant derived from each aliquot was added to a population of PBMCs; then subjected to flow cytometry and the MFI of the antigenic-peptide positive population was then plotted (Figure 8).

6.2 Stability of MHCI-NB:ALFA tetramers

The MHCI-NB:ALFA heterodimers are peptide exchanged with an antigenic peptide and tetramerized as described in example 4. The tetramers are then incubated at 4 or 25°C. When the incubation time equals 0, 12, 24, 48, 72, 96, 168 hours, then 1µL of the tetramer is taken out and added to a population of PBMCs; then subjected to flow cytometry and the MFI of the antigenic-peptide population is calculated and plotted.

Conclusions: the MHCI-NB:ALFA heterodimers of the invention loaded with an antigenic peptide exhibited improved stability and improved retention of the presented antigenic peptide compared to wild-type MHC-WT heterodimers during long-term storage (7 days) based on the staining properties of their derivative tetramers.

5 **Example 7: Plasmid isolation**

Plasmids used herein, were replicated by transformation into one shot Stbl3 chemical competent cells (C7373-03 Thermofisher) as per the manufacturers' instructions at 42°C for 45 seconds and plated on LB agar plate containing carbenicillin (100µg/mL) and incubated at 37°C incubator. The next day, a single bacterial cell colony was inoculated
10 into a conical flask containing 200mL of LB media with carbenicillin (100µg/mL) for plasmid isolation. Plasmids were isolated the next day using Qiagen Plasmid plus Midi kit (Qiagen 12945) as per manufacturers' instructions. The plasmid isolated was then measured using Nanodrop ND-1000.

Example 8: MHCII construct design, expression and purification:

15 The amino acid sequences encoding the alpha-chain and beta-chain of MHCII heterodimer alleles were obtained from Uniprot- HLA DRB1*01:01 beta chain – P01911 and HLA DRA1 alpha chain – P01903. A transgene encoding both chains of the soluble MHCII heterodimer (MHCII-alpha and MHCII-beta proteins of the invention) was expressed transiently in kidney embryonic cell line (Expi293F cells) transfected by
20 means of the ExpiFectamine 293 kit (Thermo Fisher), as follows:

8.1 Soluble MHC class II construct design:

MHCII constructs are illustrated in Figure 16. The MHCII-WT (Figure 16A) construct encodes SEQ ID NO.: 22, comprising a truncated MHCII beta chain (DRB1*01:01), having an N-terminally fused CLIP-LINKER peptide. The CLIP peptide has sequence
25 PVSKMRMATPLLQA (SEQ ID NO.: 73) and the linker contains a 3C protease cleavage site (LEVLFQGP; SEQ ID NO.:67) or a thrombin cleavage site (LVPRG; SEQ ID NO.: 68) so that the CLIP peptide can be exchanged with a selected antigenic peptide. The encoded sequence[SEQ ID NO.: 22] further comprises a truncated MHCII alpha chain (DRB1-alpha) having a C-terminally fused AVI-tag [SEQ ID NO.: 52] for biotinylation
30 and a 6X histidine tag [SEQ ID No.: 53] for affinity purification. The MHCII-NB (Figure 16B) construct encodes [SEQ ID NO.:24] comprising the same truncated MHCII beta chain (DRB1*01:01) with an N-terminally fused CLIP peptide as the MHCII-WT, but the C-terminus is fused to an ALFA-tag [SEQ ID NO.: 2], followed by the same C-terminal linker [SEQ ID NO.: 67 or 68. The encoded sequence [SEQ ID NO.:24] also comprises
35 the same truncated MHC II alpha chain as the MHCII-WT but has a C-terminally fused

ALFA cognate nanobody (ALFA nanobody; SEQ ID NO.: 4) followed by the same AVI-tag [SEQ ID NO.: 52] for biotinylation and a 6X histidine tag [SEQ ID No.: 53] for affinity purification as the MHCII-WT. The MHC II-LZIP (MHC II-LZIP) construct (Figure 16C) encodes SEQ ID NO.: 76, comprising the same MHCII beta and alpha chains as MHCII-NB-ALFA, but wherein the ALFA-tag and ALFA nanobody are replaced by the low affinity (Kd 110 +/- 12nM; Pernelle et al. 1992) acidic and basic leucine zippers [SEQ ID NO.: 76 and 78 respectively] at the C-terminus of the respective MHCII chains (Willis et al, 2021). In all three MHCII constructs, the coding sequences for the respective MHCII chain are separated by a P2A ribosomal skipping sequence, and expression of the construct is placed under the control of a strong EF1a promoter (SEQ ID No.: 41). Their protein structure is depicted by a cartoon in Figure 15.

The MHCII wild type (MHCII-WT) construct has nucleotide SEQ ID NO.: 21; and the MHCII-NB:ALFA construct has nucleotide SEQ ID NO.: 23; and the MHCII leucine zipper (MHCII-LZIP) construct comprises aL-ZIP and bL-ZIP nucleotide sequence (SEQ ID NO.: 74 and 76). The constructs were synthetically assembled and cloned at XbaI-BamHI sites into pDTU vector (figure 13) or into XbaI-EcoRV sites into pCDNA3 vector (figure 21) using Genscript. The MHCII-WT-pDTU, MHCII-LZIP-pDTU, and MHCII-NB:ALFA-pDTU or pMHCII-WT-pCDNA3, MHCII-LZIP-pCDNA3 and MHCII-NB:ALFA-pCDNA3 plasmids were isolated by plasmid isolation as per example 7. The isolated plasmids were used for MHC class II production.

8.2 Soluble MHC class II production:

The constructs encoding MHCII-NB:ALFA; truncated MHCII-WT; and MHCII-LZIP were expressed as transgenes using Expi293 expression system (Thermofisher A14635) as per manufacturer's instructions. Expi293F cells were cultured in Expi293 expression medium (Thermofisher A1435102) by maintaining an initial cell density of $0.3-0.5 \times 10^6$ cells/mL in 125mL sterile shake flask (Corning 431143) and incubated in 37°C, 8% CO₂ 85% humidity and 125 rpm. The cells were passaged at least thrice before using them for transient expression. On the day of transfection, Day-0, the plasmid DNA of MHCII-WT/pDTU and MHCII-NB:ALFA/pDTU, and MHCII-LZIP/pDTU were transfected to Expi293 culture using the Expifectamine 293 transfection reagent as per instructions from manufacturer. Transfection kit enhancers 1 and 2 were added on Day-1. Cultures were harvested on Day-6 of transfection by centrifuging at 16000 x g for 45 min and sodium azide was added to a final concentration of 0.02%. The supernatant was then filtered through 0.22µm regenerated cellulose membrane. The filtered supernatant was then supplemented with 40mM Tris-HCl pH 8.0, 300mM NaCl, 23% glycerol and 1mM PMSF.

The expressed MHCII-NB:ALFA, MHCII-LZIP and corresponding truncated MHCII:WT proteins in the filtered supernatants were purified by applying to a HisTrap FF column on AKTA pure 25. The column was then washed with 20 column volumes (CV) of wash buffer (25mM Tris-HCl pH 8.0, 300mM NaCl, 10% glycerol, 15mM imidazole). The expressed MHCII proteins were then eluted in 5 CV elution buffer (25mM Tris-HCl pH 8.0, 300mM NaCl, 10% glycerol, 250mM imidazole). MHCII protein-containing fractions were subsequently pooled, concentrated, and desalted into 1X PBS using a HiPrep 26/10 Desalting column (Cytiva). Each desalted MHCII protein was pooled and simultaneously digested with bovine thrombin (Sigma-Aldrich 605157; 0.3units of thrombin per 10 mg of MHCII protein to cleave the thrombin site making the MHCII protein available for peptide exchange) and the alpha chain was biotinylated with BirA biotin-protein ligase bulk reaction kit (5.5 µg BirA per 50 nmol of MHCII protein) overnight at room temperature. The biotinylated alpha MHCII chains were finally purified via size exclusion chromatography using a Superdex 200 10/300 GL increase column (Cytiva) equilibrated with 1X PBS. Fractions corresponding to the MHCII population (typically centered at 13 mL retention volume) were identified via SDS-PAGE, pooled, and concentrated using Amicon Ultracel 30K MWCO regenerated cellulose devices to ~2 mg/mL with a final concentration of 10% (v/v) glycerol. Aliquots were stored at ~80°C.

Example 9: HLA-DM expression and purification for catalyzing MHCII peptide exchange

The amino acid sequences encoding the α -chain and β chains of HLA-DM heterodimer were obtained from Uniprot: HLA DM beta chain – P28068 (SEQ ID NO.: 37) and HLA DM alpha chain – P28067 (SEQ ID NO.: 36). A HLA DM expressing cell line was generated using Drosophila S2 cells (Thermofisher R69007). The cell line was then expanded and induced with copper sulphate to recombinantly express HLA DM, which was purified using affinity (Anti-FLAG M2 affinity gel, Sigma A2220) chromatography followed by size-exclusion chromatography (Superdex 200). The purified HLA DM is then used in the peptide exchange reactions of MHCII.

9.1 Soluble HLA DM DNA construct design:

The HLA DM constructs encoding HLA-DM α [SEQ ID NO.: 34] and HLA-DM β [SEQ ID NO.: 36] are illustrated in Figure 20. The HLA DM α chain and β chain were truncated to remove the transmembrane domain and were replaced with a FLAG tag (DYKDDDDK; SEQ ID NO.: 55) at the C terminus end of the α chain and a SV40 large antigen KT3 tag (KPPTPPPEPET; SEQ ID NO.: 78) on the C terminus of the β chain (Sloan et al., 1995). The constructs were cloned into pMT/Bip/V5 vector (Thermofisher; Figure 18) in the multiple cloning site (MCS) at BglII and XbaI driven by inducible metallothionein (MT) promoter.

9.2 Soluble HLA DM production:

Drosophila S2 cells were cultured in complete medium – Schneider’s Drosophila medium (Thermofisher 21720-024) with 10% FBS (Thermofisher 10082-147) and 1% Penicillin-Streptomycin (Thermofisher 15070-063) for at least 3 passages before transfection. HLA DM α /pMT-BiP and HLA DM β /pMT-BiP in equimolar proportions (Figure 19) were transfected to Drosophila S2 cells as per instructions from Drosophila Expression System (DES) Inducible kit with pCoHygro (Figure 19; Thermofisher K413001) using calcium phosphate transfection kit (Thermofisher K278001). The transfected cells were then selected with 200 μ g/mL hygromycin-B (Thermofisher R220-05) for a period of 3 weeks with one passage every 3-4 days. After selection, the cells are then shifted to Sf900 II SFM (Thermofisher 21012026) in the presence of 10% FBS and 200 μ g/mL hygromycin-B at 28°C until the cell count was $>5 \times 10^6$ cells/ml. Culture conditions were then changed to shake flask with gentle shaking at 120 rpm and the FBS was weaned out from 10% to 0% slowly with 2 passages at each concentration of FBS. When the cells were in serum-free condition they were then seeded at $6-8 \times 10^6$ cells/mL and induced with 1mM CuSO₄ solution for 7 days, after which the culture was harvested by centrifuging at 3500 x g for 45 min. The supernatant was recovered and sodium azide was added to a final concentration of 0.02%. The supernatant was then filtered through 0.22 μ m regenerated cellulose membrane. Soluble HLA-DM (HLA-sDM) was then purified by affinity chromatography using AntiFLAG M2 affinity gel (Sigma-Aldrich A2220) followed by size-exclusion chromatography with Superdex200 (Cytiva). The purified HLA-DM was concentrated using Amicon Ultracel 10K MWCO regenerated cellulose devices to ~ 1 mg/mL. The aliquots were stored at -80°C.

Example 10: HLA-DM mediated peptide exchange of MHCII heterodimers of the invention

MHCII protein (5 μ M) was incubated with 100 μ M of flu peptide (PKYVKQNTLKLAT; SEQ ID NO.: 79) in an exchange buffer (100 mM sodium acetate pH 5.2, 50 mM NaCl, and 5 μ M HLA-sDM) for 18 h at 37°C. After incubation, the reaction mixture was centrifuged 3300xg for 10 min to remove aggregates, and the supernatant transferred and neutralized with 2X PBS final concentration to obtain a peptide-exchanged MHCII (pMHCII).

Example 11: Multimerization of MHCII heterodimers of the invention

11.1 Multimerization of pMHCII using dextran

1 μ M peptide-exchanged pMHCII heterodimers, obtained in example 10, were added to 0.05 μ M of fluorophore-conjugated dextran (Fina Biosolutions) and incubated on ice for

30 min. 25µM free biotin was added and incubated for another 20min (Massilamany et al., 2015). Finally, 10x multimer freezing buffer (50% glycerol+ 1% BSA) was added to get a final concentration of 1x and the pMHCII multimers were stored at 2 - 8°C until further use.

5 11.2 Tetramerization of pMHCII using streptavidin

pMHCII tetramers were produced by adding 18.1µL of 0.1mg/ml streptavidin-fluorophore conjugate to 100µL of pMHCII heterodimer (Massilamany et al., 2015). The mixture was incubated on ice for 20 - 30 min, to which 25µM free biotin was added and incubated for another 20min. pMHCII tetramers were stored at 4°C after adding 10x
10 tetramer freezing buffer (50% glycerol+ 1% BSA) to final concentration of 1x,.

Example 12: T-cell specific staining by MHCII-NB/ALFA of the invention

Multimers of the MHCII heterodimers of the invention loaded with a specific antigenic peptide provide precisely engineered reagents for the detection of T cells specific for antigens derived from pathogens, tumors, and self-proteins as illustrated in the following
15 example:

An aliquot of PBMC was washed twice with RPMI-1640 (Thermofisher 11875093) +10% FBS (Thermofisher 10500064). After counting the cells, 1-2 million cells were pipetted into a well of a 96-well plate. The plate was centrifuged at 400 x g for 5 min and the PBMCs washed twice with FACS buffer (PBS pH 7.4 + 1% FBS). The PBMCs were then
20 stained with 5µL of the MHCII-tetramers or -multimers obtained in example 11, at 37°C for 60 min and then stained with antibodies conjugated with fluorophore – anti-human CD3 BV786 (BD 565491), anti-human CD8 BV480 (BD biosciences 566121), anti-CD4 BUV395 (BD biosciences 563550) with NIR live-dead marker (Thermofisher L34976A). The plate was incubated on ice for 30min. The cells were then washed with FACS buffer
25 twice before being analyzed by flow cytometry and FlowJo v10.8.1 and the MFI was determined to compare staining using soluble MHCII-WT; MHCII-LZIP and MHCII-NB of the invention.

Example 13: Stability of peptide-loaded MHCII-NB:ALFA heterodimers of the invention

30 13.1 Stability of MHCII-NB:ALFA heterodimers:

The stability of MHCII-NB:ALFA heterodimers of the invention as compared to soluble MHCII-WT and MHCII-LZIP, when incubated in PBS buffer at 4°C or 20 °C or 37°C, was determined by withdrawing samples of the respective heterodimer for tetramerization/multimerization at – 24hr, 48hr, 72hr, 96hr, 120hr, 168hr.

Multimerization was carried out as per example 11. The resulting tetramers/multimers were then stained to PBMCs and analyzed on flow cytometry. The data is then analyzed on FlowJo v10.8.1 and the MFI compared to assess the stability of MHCII-NB relative to MHCII-WT and MHCII-LZIP.

5 13.2 Stability of MHCII-NB:ALFA multimers:

The MHCII-NB:ALFA heterodimers were peptide exchanged with an antigenic peptide and tetramerized using streptavidin or multimerized using dextran as described in example 11. The tetramers prepared were then incubated at 4°C or 20 °C or 37°C. When the incubation time equals 0, 12, 24, 48, 72, 96, 168 hours. 1µL of the tetramer was taken
10 out and added to a population of PBMCs; subjected to flow cytometry and the MFI of the antigenic-peptide population detected is calculated and plotted.

Example 14: Wild type-TCR (TCR-WT) and Modified-TCR (TCR-NB:ALFA) production employing lentiviral expression system

Transgenes encoding both chains of the WT- and the modified- TCR heterodimer (TCR-
15 alpha chain and TCR-beta chain) respectively (figure 17) were expressed in a Jurkat cell line derived from an acute T-cell leukemia patient, Clone E6-1 (ATCC, TIB-152) transduced by means of the lentiviral system, as follows:

14.1 TCR transgene design:

A wild-type transgene (SEQ ID NO.: 17) for expression of the wild-type T cell receptor
20 (TCR:WT) encodes SEQ ID NO.: 18, comprising full-length alpha and beta chains separated with a self-cleavable 2A peptide sequence (T2A – ATNFSLLKQAGDVEENPGP; SEQ ID NO.: 80) which induces ribosomal skipping during translation of the protein. The modified TCR transgene (SEQ ID NO.: 19) for expressing a modified T cell receptor (TCR-NB:ALFA) encodes SEQ ID NO.: 20, comprising full-length alpha- and beta- chains
25 separated with a self-cleavable 2A peptide sequence (T2A – ATNFSLLKQAGDVEENPGP; SEQ ID NO.: 80), wherein an ALFA-cognate nanobody [SEQ ID NO.: 4] was fused to the C terminus of the beta chain; and an ALFA tag [SEQ ID NO.: 2] was fused to the C terminus of the alpha chain by means of a GS-rich linker (SEQ ID NO.: 69 or 70).

14.2 Lentivirus production using the TCR transgene cloned vectors:

30 The TCR transgenes were cloned into a lentiviral vector, pDTU, at the Xba I and Bam HI sites in frame with the EF1α promoter (SEQ ID No.: 41) to generate two plasmids – TCR-WT/pDTU and TCR-NB:ALFA/pDTU. They were transfected into a lentivirus producer cell line (HEK293T) together with envelope and packaging plasmids. This system was used to generate sufficient lentiviral particles for the subsequent transduction and TCR

transgene construct expression in a human T lymphocyte cell line: HEK293T cells (ATCC CRL-3216) supplied by ATCC which were cultured at least twice before transfection in RPMI-1640 + 10% FBS using Trypsin-EDTA to release the adhered cells in a T-75 culture flask. The cells were seeded to ensure approximately 50% confluency in a T-75 flask 24 hours before transfection. The cells were then transfected with 15µg of the TCR-WT/pDTU and TCR-NB:ALFA/pDTU vector containing the transgene along with the packaging plasmid (Addgene – pRSV.REV, pMDLg/p.RRE and pMD2.G) using Lipofectamine 3000 (Thermofisher L3000008) as per manufacturer's instructions. The virus particles were recovered 24 and 48 hours after transfection and concentrated using Lenti-X concentrator (Clontech 631232) as per manufacturer's instructions. The concentrated virus particles comprising the transgenes encoding control TCR-WT/pDTU or TCR-NB:ALFA/pDTU were then re-suspended in 200µL PBS and stored at -80°C.

14.3 Lentiviral mediated transduction of Jurkat cells:

Jurkat cell line derived from an acute T-cell leukemia patient, Clone E6-1 (TIB-152) supplied by ATCC was cultured in ATCC-formulated RPMI-1640 +10% FBS and passaged at least twice before transduction. The cell density was maintained between 1×10^5 to 1×10^6 cells. The cells were seeded into a single well of a 24-well plate at a density of $1 - 2.5 \times 10^5$ cells/well. The cells were then transduced with the virus prepared – TCR-WT/pDTU and TCR-NB:ALFA/pDTU separately and incubated at 37°C, 5% CO₂ for 72 hours. The cells were then scaled up to 3mL in a 6 well plate. Two separate cells lines were generated by lentiviral transduction – wild type- TCR (TCR-WT) and Nanobody-TCR (TCR-NB:ALFA).

Example 15: TCR Surface expression & preferential pairing

The Jurkat cell line clone E6.1 expresses endogenous genes encoding the native TCR alpha and beta chains which compete in pairing with the transgene encoded TCR-WT beta and alpha chain respectively. Mixed pairing between said native TCR chains and transgene encoded TCR-WT chains reduces the number of functional TCR-WT on the cell surface. Whereas, in the case of TCR-NB:ALFA due to preferential pairing there will be more TCR-NB:ALFA receptor on the cell surface since the nanobody and its cognate ALFA tag will preferentially pair during the expression of the TCR-NB:ALFA. This will increase the number of functional TCRs on the cell surface compared to cells expressing the TCR-WT transgene.

The Lentivirus transduced TCR cell lines generated from Example 14 were stained with pMHCII-NB:ALFA tetramer/multimer complexes which were prepared as in example 3 and 4 and loaded with the antigenic peptide-EWRSRGFSF specific to the TCR. The pMHCII-NB:ALFA tetramers were then stained to both of the cell lines expressing TCR-

WT and TCR-NB:ALFA. The MFI of the stainings are calculated, the TCR-NB will show a greater MFI compared to TCR-WT due to preferential pairing.

Example 16: Detecting MHC I dimer stability using ELISA

The ELISA method is used to detect the MHC I heavy chain with HRP-W6/32 antibody (Abcam ab33256) which recognizes the heavy chain when it is associated with $\beta 2m$, and HRP-anti- $\beta 2m$ antibody (Thermofisher MA1-33436) is used to detect only $\beta 2m$. ELISA was used to quantitate the amount of stable MHCI-WT and MHCI-NB:ALFA along with the $\beta 2m$. A 96 well Microwell MaxiSorp ELISA (Thermofischer 436110) plate was coated with 100 μ L of streptavidin in PBS (2 μ g/ml) overnight at room temperature (RT). The next day the plates were washed 4x with 300 μ L washing buffer (PBS with 0.05% tween20), and blocked for 30min with 200 μ L blocking buffer (PBS+2% BSA) at room temperature. After taking out the blocking buffer, the samples and the standards (folded MHC I-WT and folded $\beta 2m$ with a working concentration range of 100 μ g/mL to 3.1 μ g/mL serially diluted 2 fold) were added to the ELISA plate and incubated at 37°C for 1 hour. The ELISA plate was washed 4x with 300 μ L washing buffer (PBS with 0.05% tween20) and 100 μ L of antibody (HRP-W6/32 or HRP-anti- $\beta 2m$) at a final concentration of 1 μ g/mL was added to each sample and incubated for 1 hour of incubation at 37°C. The plates were washed 4x with 300 μ L washing buffer. 100 μ L ABTS substrate solution (Roche 11684302001) was added to the samples, which interacts with the peroxidase enzyme located on the detection antibody. Hereupon, the samples were incubated at RT on a shaker for 15 min, and the reaction was stopped by adding 100 μ L of 5x diluted ABTS stop solution in MiliQ water. Last, the samples were measured using the ELISA plate reader at 405 nm (Software Gen5 v3.03). MHCI-WT being less stable than MHCI-NB:ALFA will show a lower detection with W6/32 and anti- $\beta 2m$ antibody. Whereas, a higher amount of free $\beta 2m$ is detected in the MHCI-WT wash buffers compared to MHCI-NB:ALFA, in the case of MHCI NB:ALFA, dimers comprising the $\beta 2m$ and MHCI-HC are stabilized by the interaction between the nanobody and its cognate antigenic peptide (ALFA).

Example 17: Nanobody:cognate antigenic peptide affinity measurement

Affinity was determined by surface plasmon resonance (SPR) binding experiments performed on a Reichert SR 7500DC biosensor instrument at 20 °C and a flow rate of 40 μ L min⁻¹ on HC1000m SPR sensor chips (Xantec Biotechnologies) in PBS buffer containing 0.002% (v/v) Tween-20, pH 7.4. Data were analyzed using TraceDrawer v1.7.1. The HC1000m sensorchip was activated with 1-ethyl3-(3-dimethylaminiopropyl)-carbodiimide and N-hydroxysuccinimide (EDCNHS) according the manufacturers instructions. The cognate antigenic peptide (e.g. ALFA) was pre-diluted to 5 μ M in concentration buffer (50 mM sodium acetate pH 5.0) and it was

injected over the activated chip surface at a flow rate of 15 $\mu\text{L min}^{-1}$ to a final response of 150 μRIU (RIU: refractive index units; response recorded). The chip surface was subsequently inactivated with 1 M ethanolamine pH 8.5. The chips with immobilized antigenic peptide were equilibrated with PBS pH 7.4 containing 0.002% (v/v) Tween-20 (PBS-T). The nanobody (e.g. NbALFA)) were serially diluted in PBS-T and injected over the chip surface. Association was followed for 4.5 min and dissociation was followed for 15 min for each nanobody (NbALFA). For each measurement, a buffer injection was performed as a reference (buffer reference). The recorded binding was double-referenced against an empty reference channel recorded in parallel and the buffer reference. The surface was regenerated after each nanobody injection by injecting 100 mM Glycine pH 2.2, 150 mM NaCl for 2.5 min. To accurately determine the dissociation constant of each nanobody (e.g. NbALFA), the decrease in response as followed for > 7 h.

References

- 15 Hallgren J, Tsirigos KD, Damgaard Pedersen M, Juan J, Armenteros A, Marcatili P, et al. DeepTMHMM predicts alpha and beta transmembrane proteins using deep neural networks. [cited 2023 Mar 21]; Available from: <https://doi.org/10.1101/2022.04.08.487609>
- 20 Götzke H, Kilisch M, Martínez-Carranza M, Sograte-Idrissi S, Rajavel A, Schlichthaerle T, et al. The ALFA-tag is a highly versatile tool for nanobody-based bioscience applications. *Nat Commun* [Internet]. 2019;10(1):1–12. Available from: <http://dx.doi.org/10.1038/s41467-019-12301-7>
- Toebes M, Coccoris M, Bins A, Rodenko B, Gomez R, Nieuwkoop NJ, et al. Design and use of conditional MHC class I ligands. *Nat Med*. 2006;12(2):246–51.
- 25 Rodenko B, Toebes M, Hadrup SR, van Esch WJE, Molenaar AM, Schumacher TNM, et al. Generation of peptide–MHC class I complexes through UV-mediated ligand exchange. *Nat Protoc* 2006 13 [Internet]. 2006 Aug 31 [cited 2023 Mar 29];1(3):1120–32. Available from: <https://www.nature.com/articles/nprot.2006.121>
- 30 Andersen RS, Kvistborg P, Mørch Frøsig T, Pedersen NW, Lyngaa R, Bakker AH, et al. Parallel detection of antigen-specific T cell responses by combinatorial encoding of MHC multimers. *Nat Protoc* [Internet]. 2012 [cited 2023 Mar 30];7(5):891–902. Available from: <https://pubmed.ncbi.nlm.nih.gov/22498709/>
- 35 Willis RA, Ramachandiran V, Shires JC, Bai G, Jeter K, Bell DL, et al. Production of Class II MHC Proteins in Lentiviral Vector-Transduced HEK-293T Cells for Tetramer Staining Reagents. *Curr Protoc*. 2021;1(2):1–55.
- Sloan VS, Cameron P, Porter G, Gammon M, Amaya M, Zaller DM. Mediation by HLA-DM of dissociation of peptides from HLA-DR. *Nat* 1995 3756534 [Internet]. 1995 [cited 2023 Mar 29];375(6534):802–6. Available from: <https://www.nature.com/articles/375802a0>
- 40 Massilamany C, Krishnan B, Reddy J. Major Histocompatibility Complex Class II Dextramers: New Tools for the Detection of antigen-Specific, CD4 T Cells in Basic and Clinical Research. *Scand J Immunol*. 2015;82(5):399–408.

C Pernelle 1, F F Clerc, C Dureuil, L Bracco, B Tocque. An efficient screening assay for the rapid and precise determination of affinities between leucine zipper domains
Biochemistry 1993 32(43):11682-7. doi: 10.1021/bi00094a026.

Claims:

1. A soluble mammalian dimeric receptor protein devoid of a transmembrane anchoring domain comprising modified first and second receptor polypeptides, wherein:
 - 5 (a) the first receptor polypeptide is fused to a first peptide comprising a nanobody, and
 - (b) the second receptor polypeptide is fused to a second peptide comprising a first antigenic peptide cognate to said nanobody, wherein stability of the dimeric receptor protein is enhanced by non-covalent bonds between the nanobody and the cognate antigenic peptide.
- 10 2. The soluble mammalian dimeric receptor protein of claim 1, wherein said first peptide is fused to the C-terminus of said first receptor polypeptide, and wherein said second peptide is fused to the C-terminus of said second receptor polypeptide.
- 15 3. The soluble mammalian dimeric receptor protein of claim 1 or 2, wherein the soluble mammalian dimeric receptor protein is selected from among: a soluble Major Histocompatibility Complex I (MHCI), a soluble Major Histocompatibility Complex II (MHCII), and a soluble B cell receptor.
- 20 4. The soluble mammalian dimeric receptor protein of claim 3, wherein the soluble dimeric receptor protein is MHCI, and wherein:
 - 25 (a) said first receptor polypeptide is a MHCI beta-2 microglobulin (β 2m) chain having said first peptide fused at the C-terminus of said MHCI β 2m chain, and
 - (b) said second receptor polypeptide is a MHCI heavy-chain having said second peptide fused to the C-terminus of said heavy-chain, or
 - (c) said first receptor polypeptide is a MHCI heavy-chain having said first peptide fused at the C-terminus of said heavy-chain, and
 - (d) said second receptor polypeptide is an MHCI β 2m chain having said second peptide fused to the C-terminus of said MHCI β 2m chain, and
- 30 wherein said heavy-chain is devoid of a transmembrane anchoring domain.
- 35

5. The soluble mammalian dimeric receptor protein of claim 3,
wherein the soluble mammalian dimeric receptor protein is MHCII, and
wherein:
 - (a) said first receptor polypeptide is a MHCII alpha-chain having said first
peptide fused at the C-terminus of said alpha-chain, and
 - (b) said second receptor polypeptide is a MHCII beta-chain having said
second peptide fused to the C-terminus of said beta-chain,
or
 - (c) said first receptor polypeptide is a MHCII beta-chain having said first
peptide fused at the C-terminus of said beta-chain, and
 - (a) said second receptor polypeptide is an MHCII alpha chain having said
second peptide fused to the C-terminus of said MHCII alpha chain,
and
wherein said alpha-chain and said beta chain are devoid of a
transmembrane anchoring domain.
6. The soluble mammalian dimeric receptor protein of any one of claims 1 - 5,
wherein said first peptide comprises an amino acid spacer linking said first
receptor polypeptide to said nanobody, and/or
wherein said second peptide comprises an amino acid spacer linking said first
receptor polypeptide to said first antigenic peptide.
7. The soluble mammalian dimeric receptor protein of any one of claims 1 - 6,
wherein said cognate first antigenic peptide is selected from ALFA-tag, C-tag,
Spot-tag, Moon-tag, 6E-tag, BC2-tag, and Pep-tag.
8. The soluble mammalian dimeric receptor protein of any one of claims 1 - 7,
wherein the dissociation constant of the nanobody for the cognate first
antigenic peptide has a kD of from 20pM to 190nM.
9. The soluble mammalian dimeric receptor protein of any one of claims 3 - 7,
wherein a peptide binding groove of said soluble MHCI or MHCII is loaded with
a second antigenic peptide.
10. The soluble mammalian dimeric receptor protein of any one of claims 1 - 9,
wherein the receptor protein further comprises one or more detectable label.
11. A multimeric complex comprising two or more of said soluble mammalian
dimeric receptor proteins of any one of claims 1 - 10, wherein said proteins

are non-covalently or covalently linked to a multimerization domain.

12. A method of detecting one or more antigenic peptide-responsive T cells in a sample, the method comprising the steps of:
 - 5 (a) providing a preparation of soluble mammalian dimeric receptor proteins, wherein each of said proteins is the MHCI or MHCII according to any one of claims 3 - 10,
 - (b) loading each said MHCI or MHCII with at least one second antigenic peptide to form a MHCI-peptide complex or a MHCII-peptide complex,
 - 10 (c) conjugating each said MHCI-peptide complex or MHCII-peptide complex with a carrier coupled to a fluorophore to form a multimeric complex;
 - (d) staining a sample of cells to be interrogated with conjugated MHCI-peptide multimeric complexes or conjugated MHCII-peptide multimeric complexes obtained in step (c), and
 - 15 (e) detecting the binding of said multimeric complexes to the one or more second antigenic peptide responsive T cells in the sample.
13. A method of loading or displacing a placeholder peptide in the soluble dimeric MHCI or MHCII receptor protein of any one of claims 3 - 10 with a second antigenic peptide comprising the steps of:
 - 20 (a) providing a preparation of soluble dimeric receptor proteins wherein each of said proteins is the soluble MHC I or II according to any one of claims 3 - 10 or multimeric complexes thereof,
 - 25 (b) combining said preparation with the second antigenic peptide,
 - (c) recovering the soluble MHCI or II or multimeric complexes thereof loaded with said second antigenic peptide.
14. Use of a nanobody and an antigenic peptide cognate to said nanobody to
 - 30 stabilise a soluble mammalian dimeric receptor protein devoid of a transmembrane anchoring domain or to enhance selective pairing of receptor polypeptides of a mammalian dimeric receptor protein.
15. A dimeric mammalian receptor protein,
 - 35 wherein the dimeric receptor protein is a mammalian T cell receptor protein (TRC) comprising modified first and second receptor polypeptides, wherein:
 - (a) the first receptor polypeptide is a TRC beta-chain having a C-terminally fused first peptide comprising a nanobody, and

- (b) the second receptor polypeptide is a TRC alpha-chain having a C-terminally fused second peptide comprising a first antigenic peptide cognate to said nanobody,
or
5 (c) the first receptor polypeptide is a TRC alpha-chain having a C-terminally fused first peptide comprising a nanobody, and
(d) the second receptor polypeptide is an TRC beta-chain having a C-terminally fused second peptide comprising a first antigenic peptide cognate to said nanobody.
- 10 16. Use of a nanobody and an antigenic peptide cognate to said nanobody to obtain selective pairing of an alpha chain and a beta chain of a mammalian T cell receptor protein (TCR).
- 15 17. A method of producing a soluble mammalian dimeric receptor protein comprising a modified first and second receptor polypeptide devoid of a transmembrane anchoring domain according to claim 1, the method comprising the steps of:
- 20 (a) providing a first nucleic acid coding sequence encoding the first receptor polypeptide having a C-terminally fused first peptide comprising a nanobody, and
(b) providing a second nucleic acid coding sequence encoding the second receptor polypeptide having a C-terminally fused second peptide comprising an antigenic peptide cognate to said nanobody,
- 25 (c) providing a first vector comprising said first nucleic acid coding sequence and a second vector comprising said second nucleic acid coding sequence, or alternatively providing a third vector comprising said first nucleic acid coding sequence is linked to said second nucleic acid coding sequence, wherein expression of said first and second nucleic acid coding sequences is operably
- 30 linked to a promoter;
(d) expressing said modified first and second receptor polypeptides in host cells comprising respectively said first and said second vector or host cells comprising said third vector;
(e) recovering and contacting the expressed first and second receptor
- 35 polypeptides to form the soluble mammalian dimeric receptor protein.

Figure 1

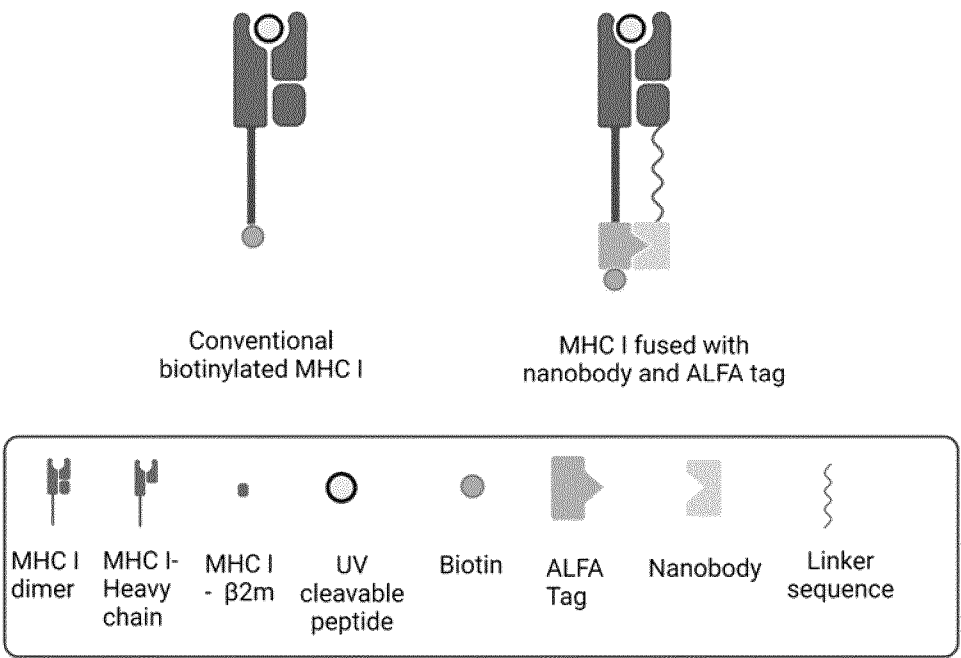


Figure 2

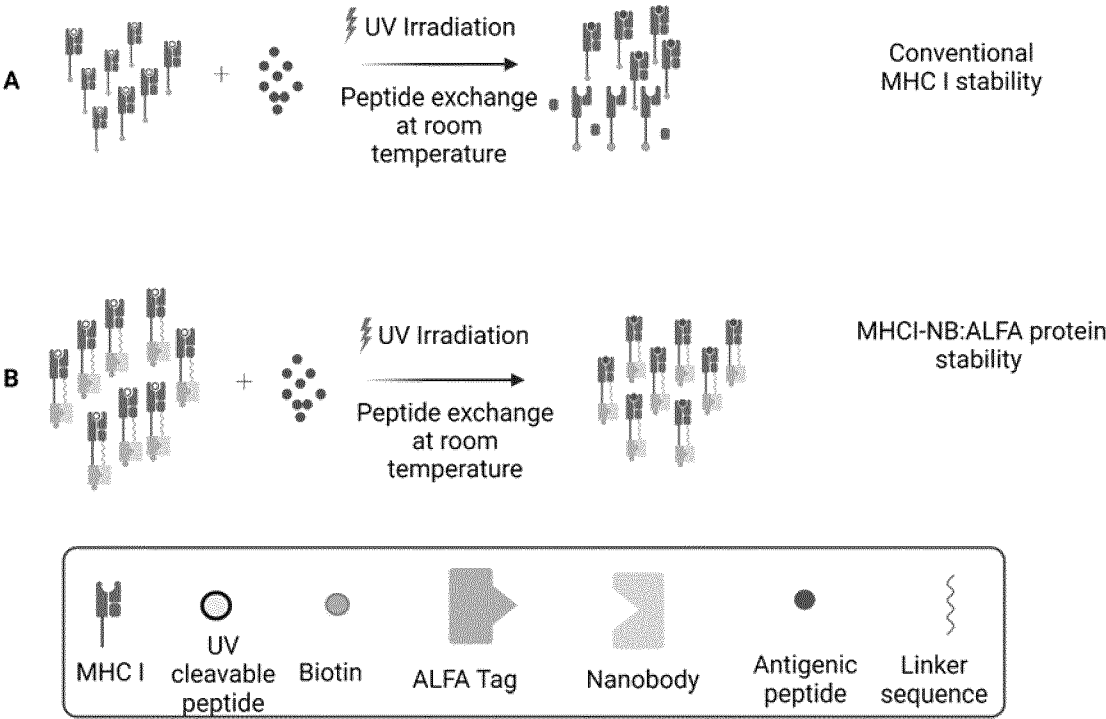


Figure 3

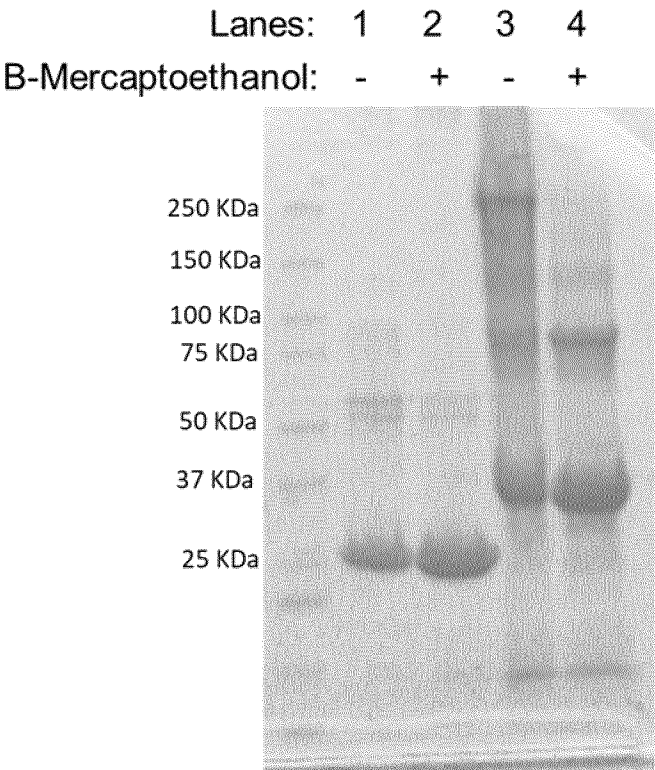


Figure 4

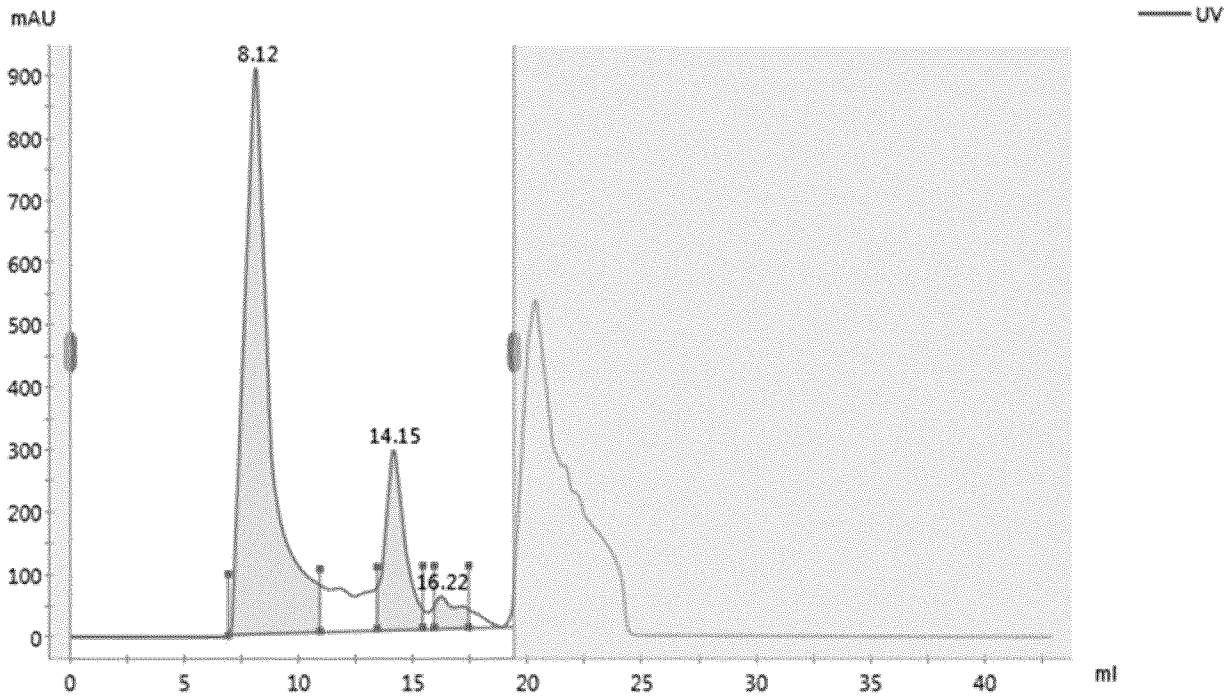


Figure 5

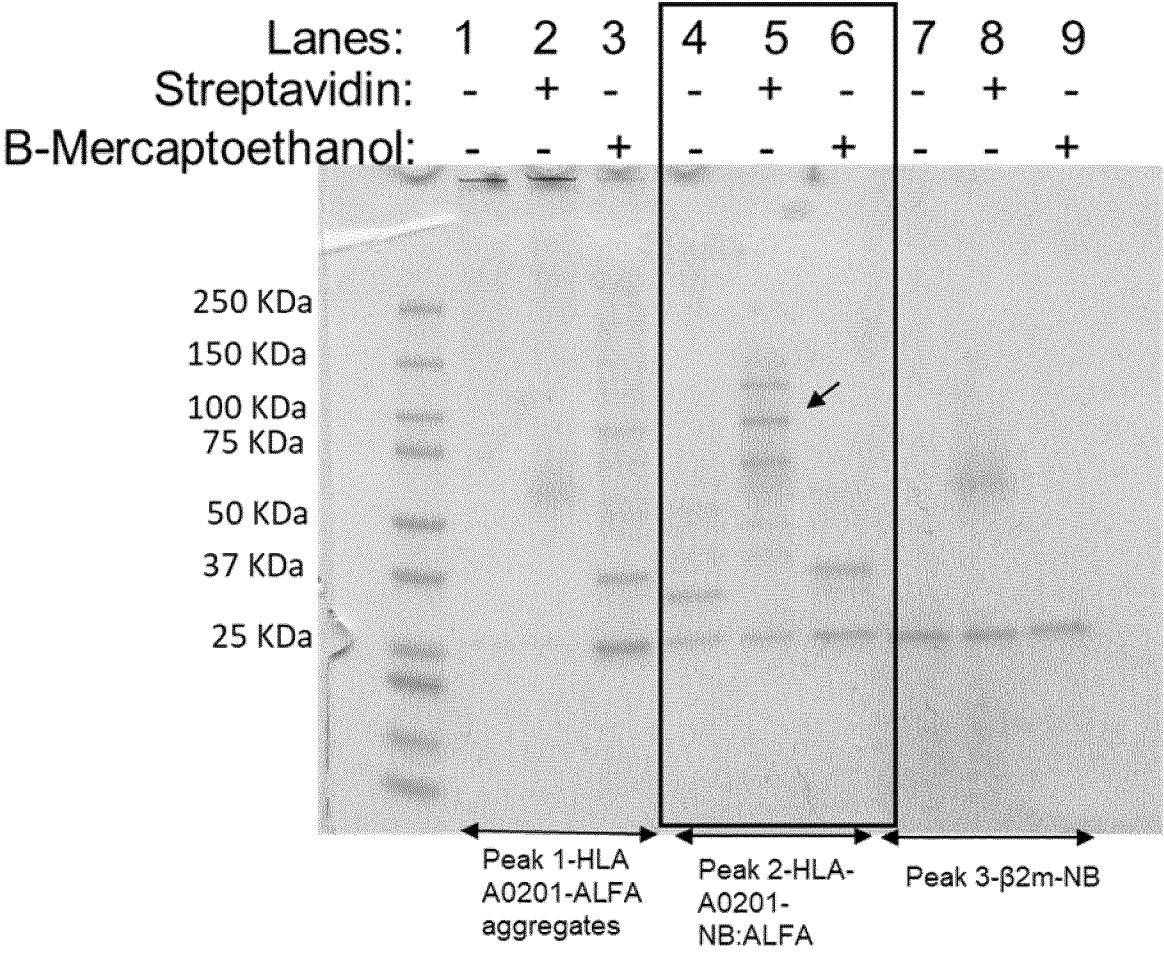


Figure 6

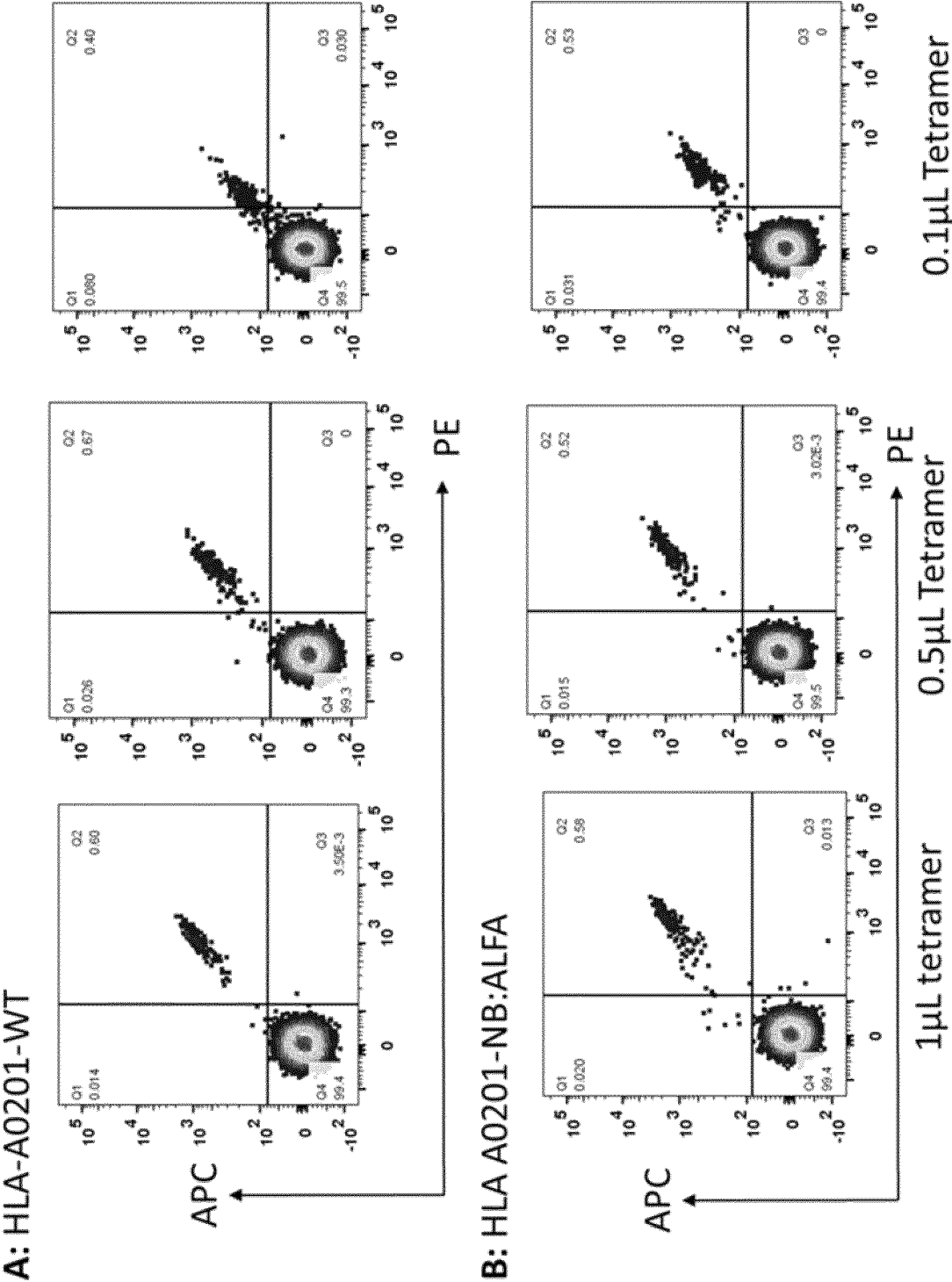


Figure 7

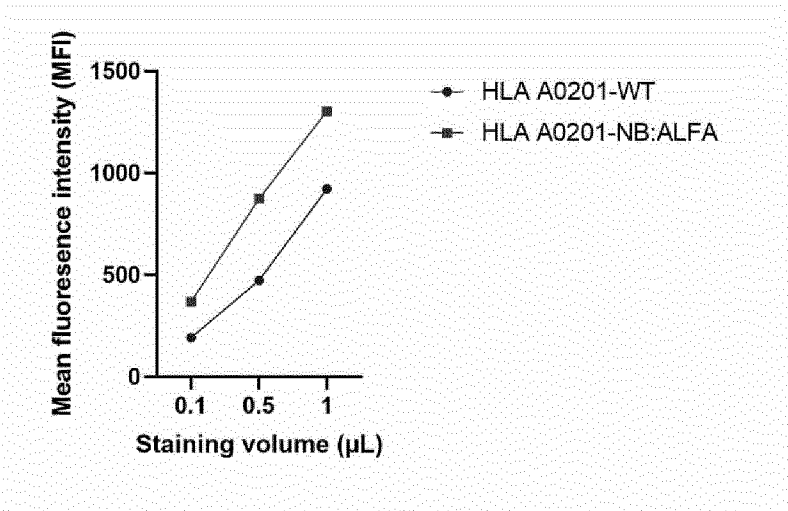


Figure 8

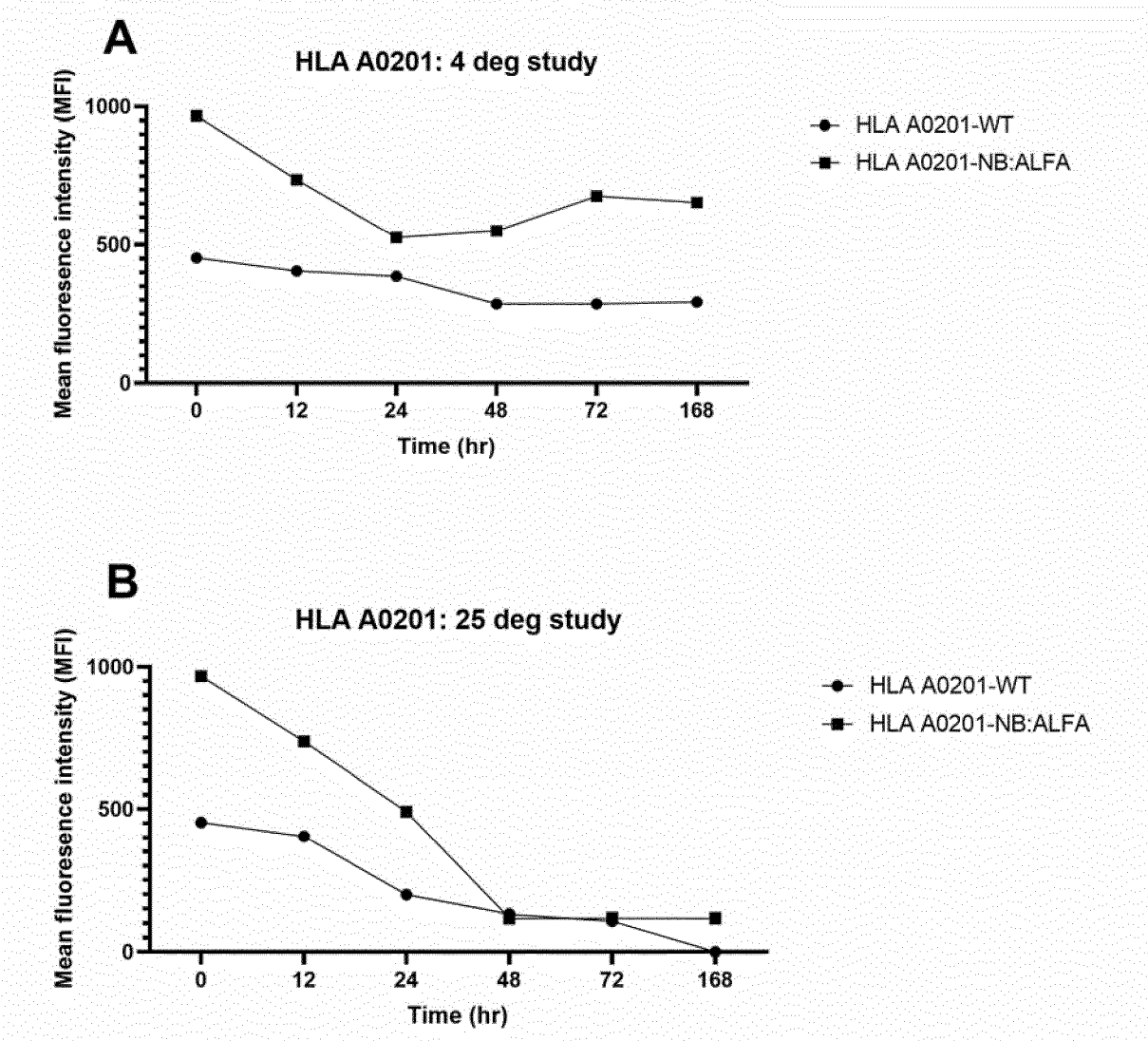


Figure 9

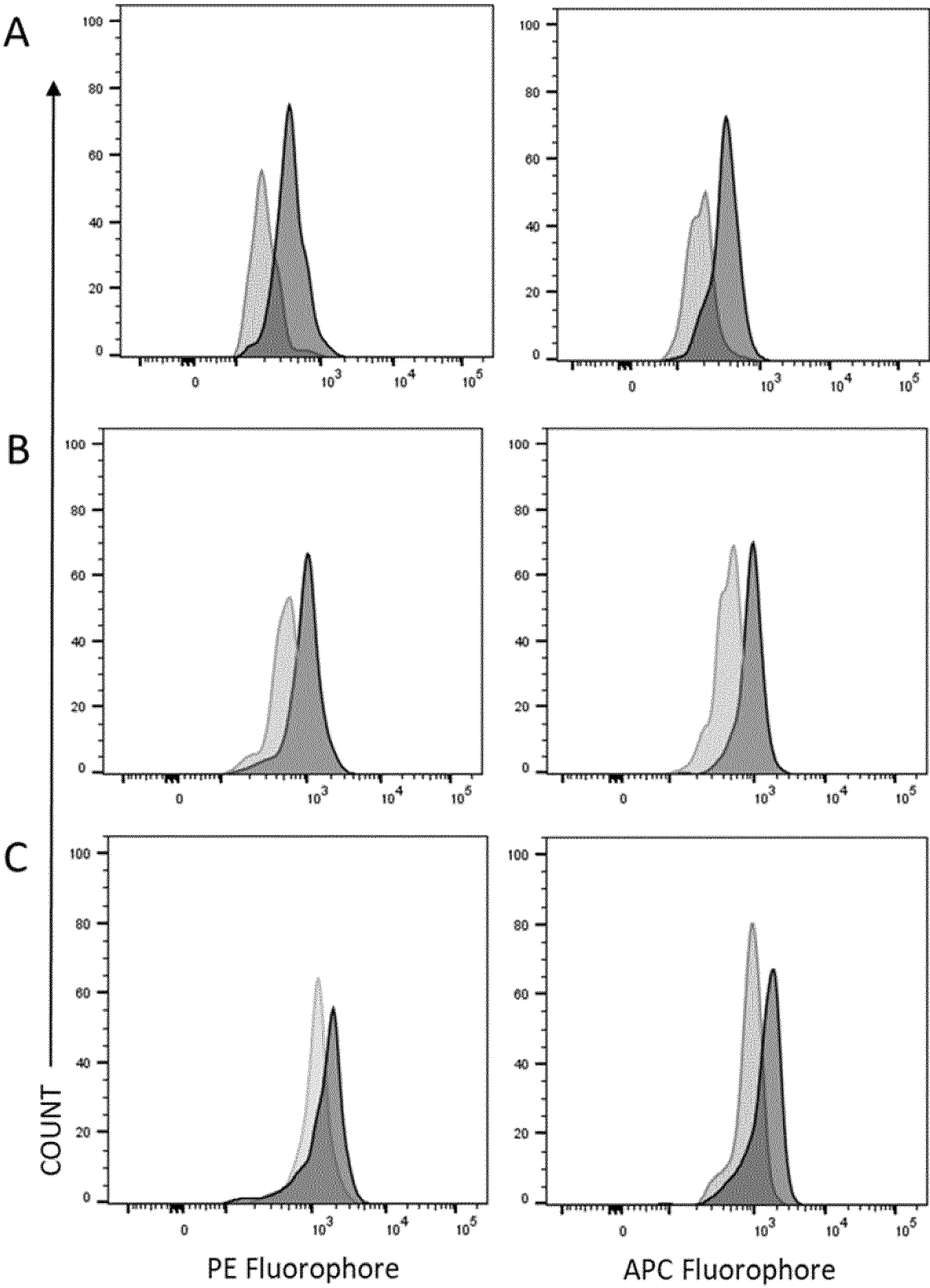


Figure 10:

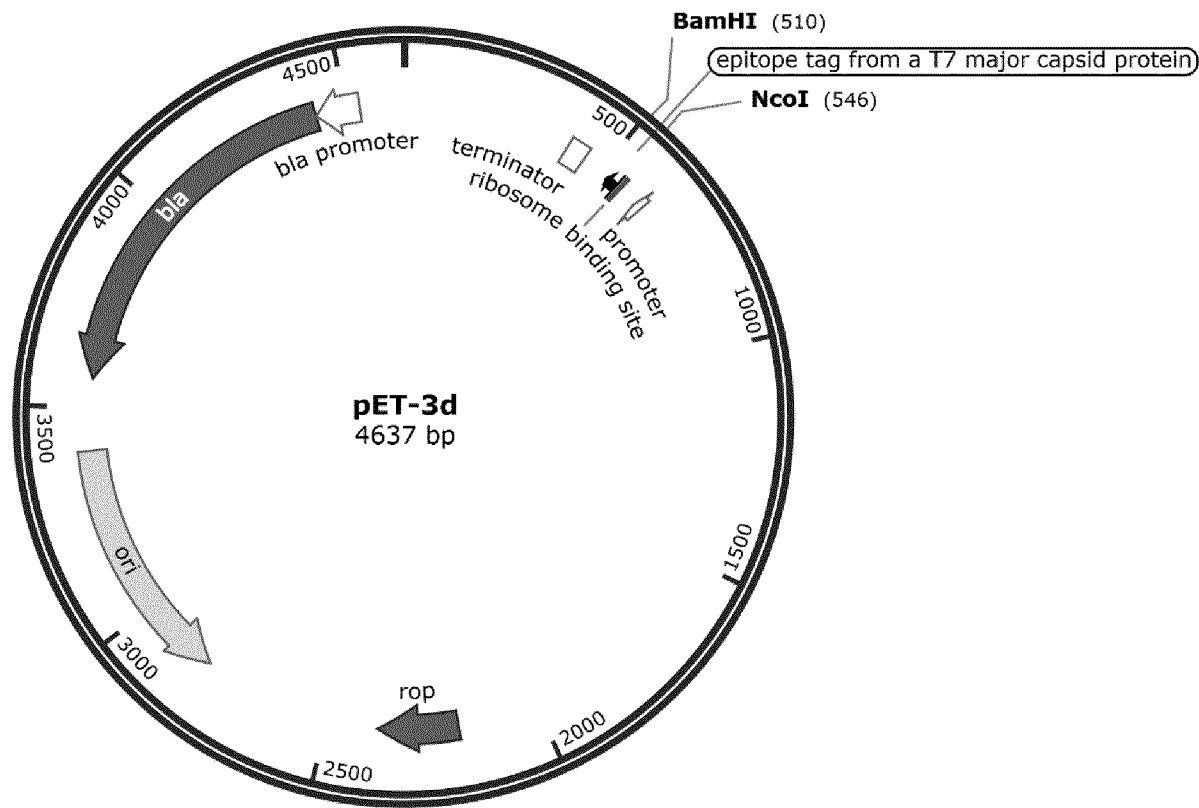


Figure 11:

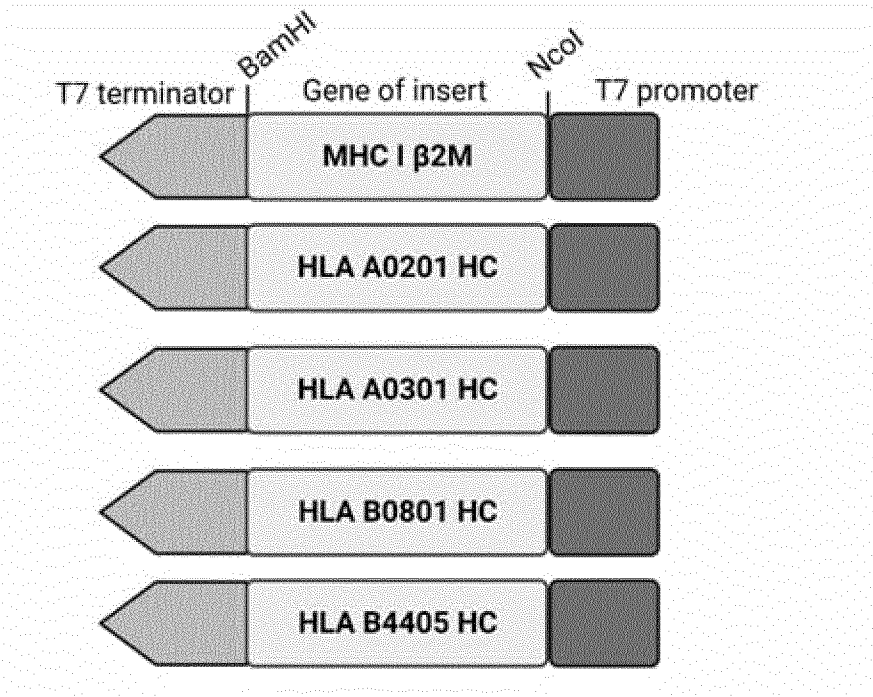


Figure 12:

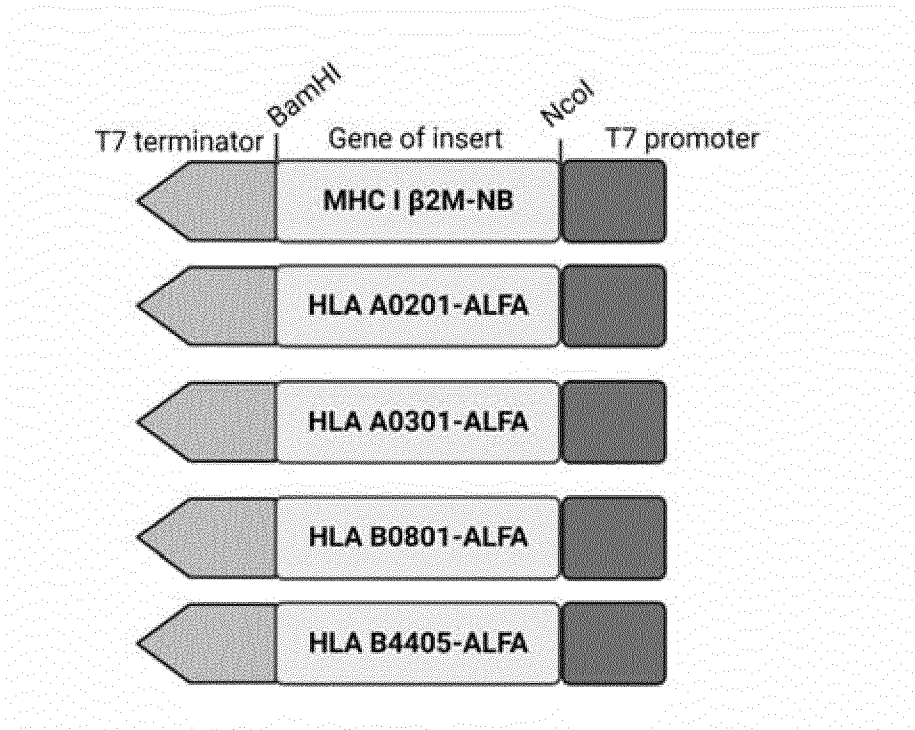


Figure 13:

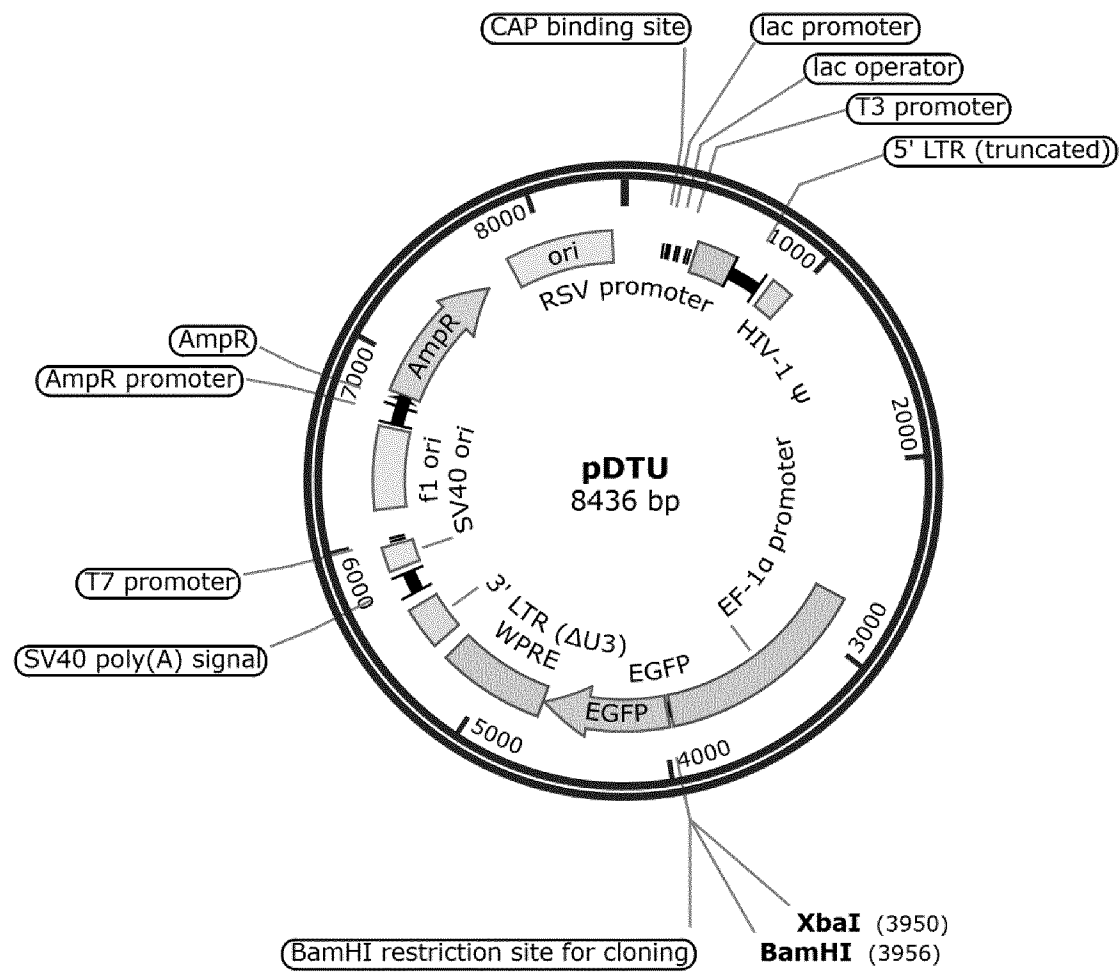


Figure 14:

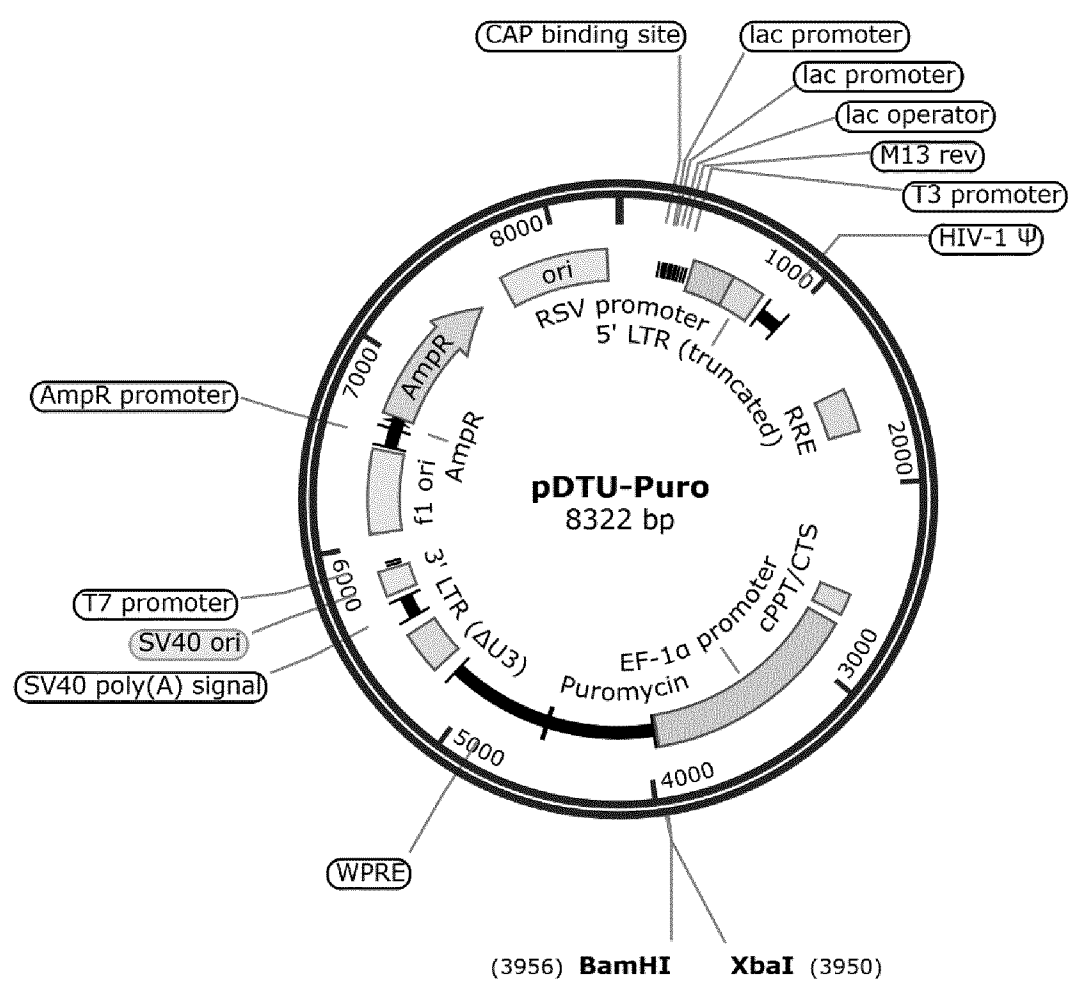


Figure 15:

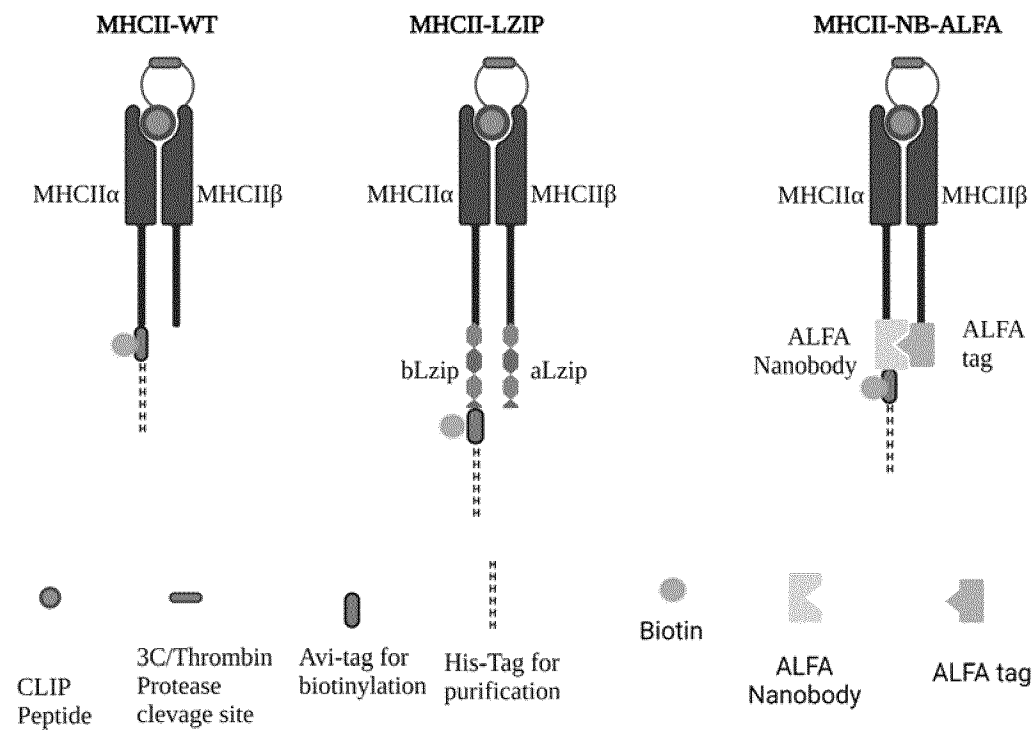


Figure 16:

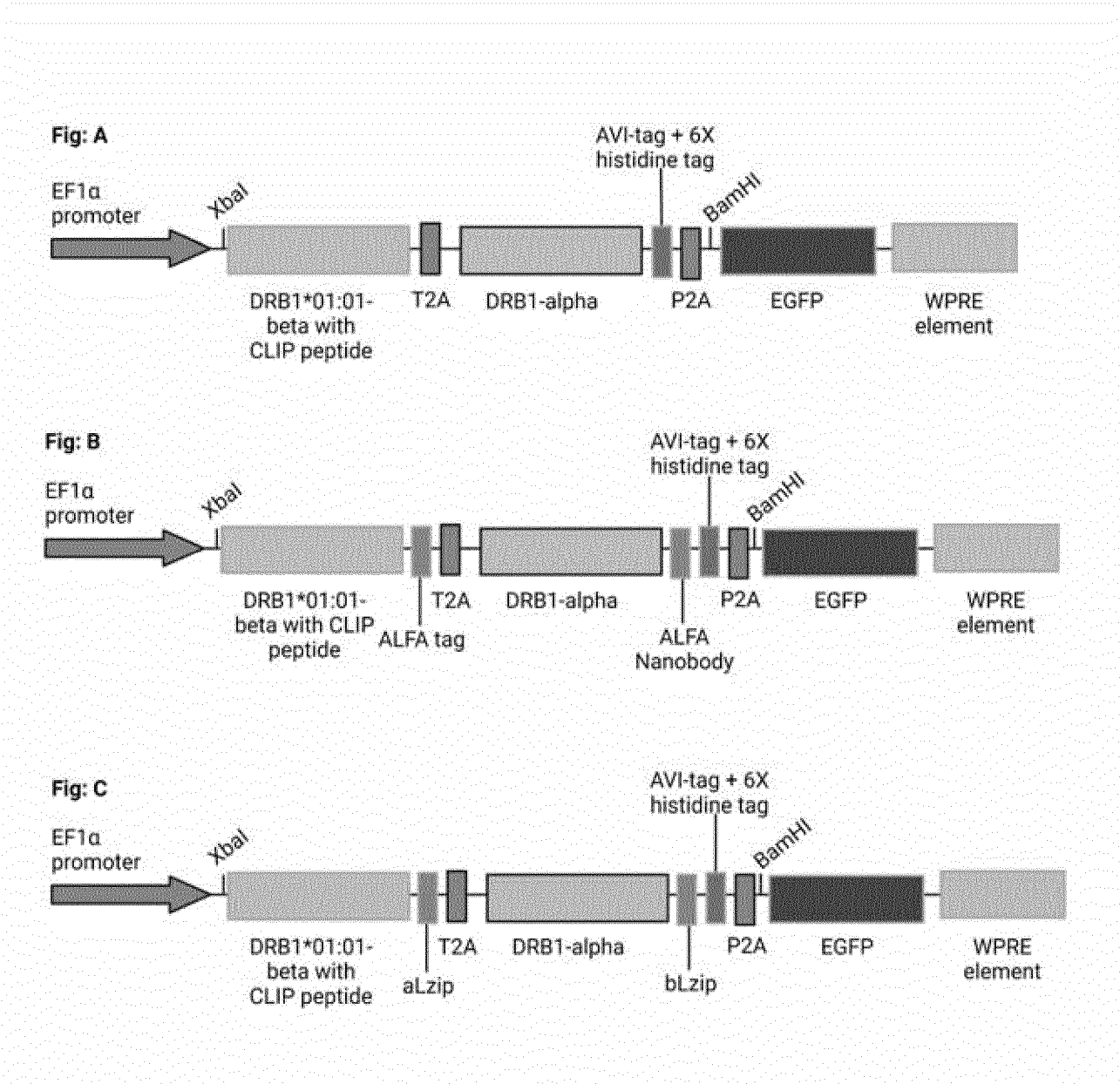


Figure 17:

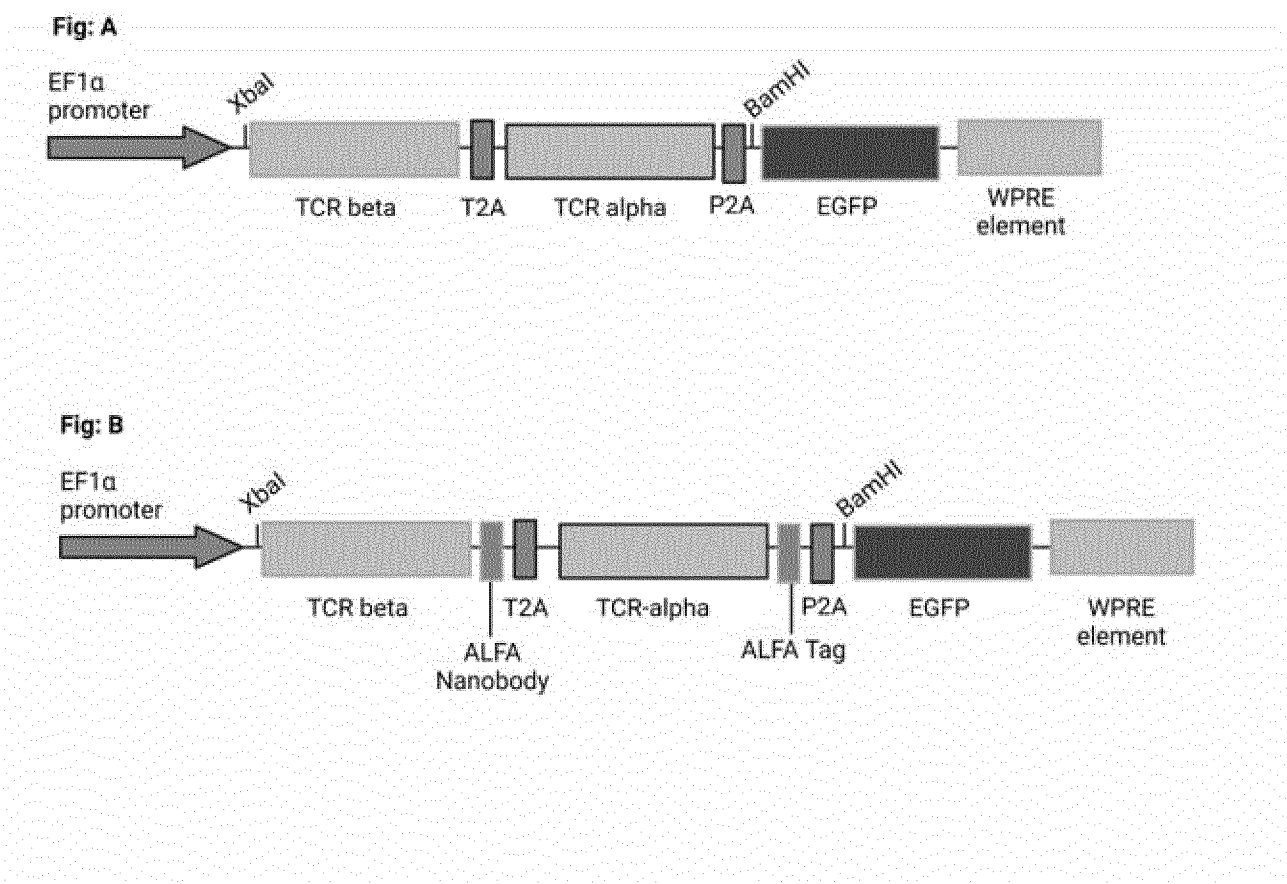


Figure 18:

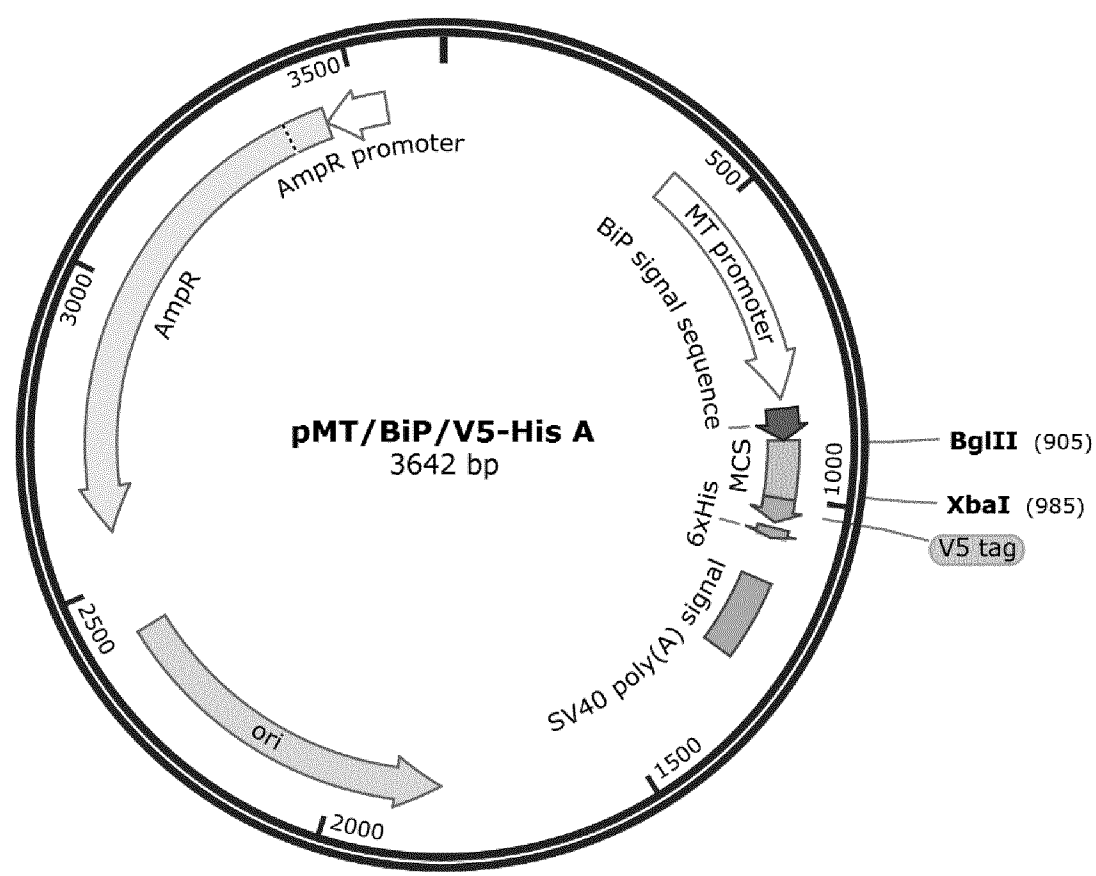


Figure 19:

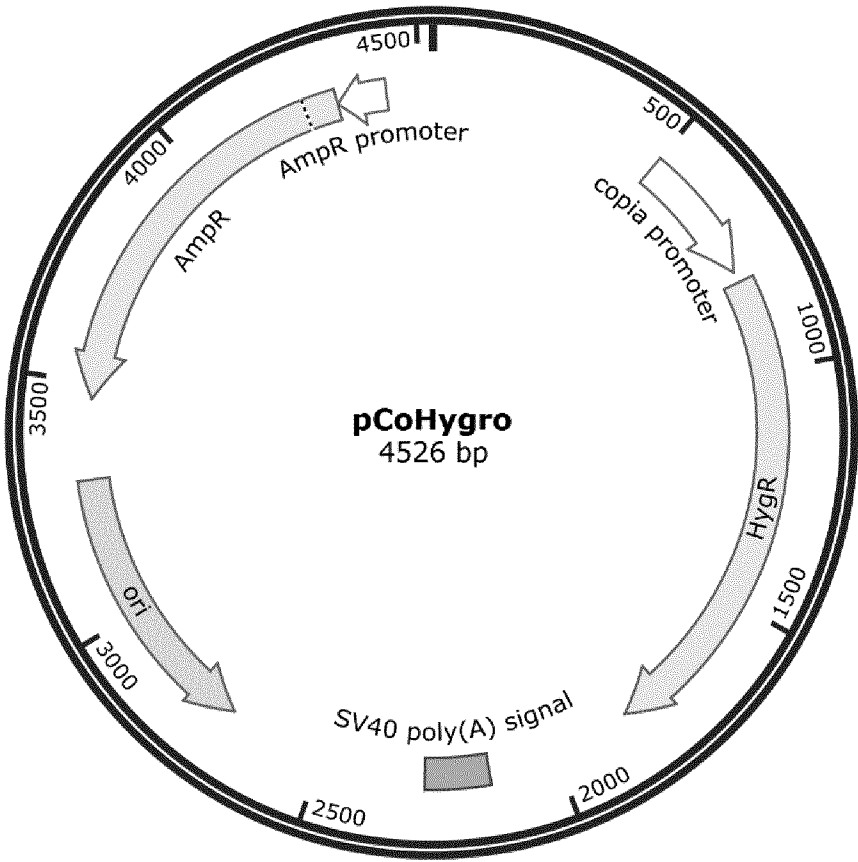


Figure 20:

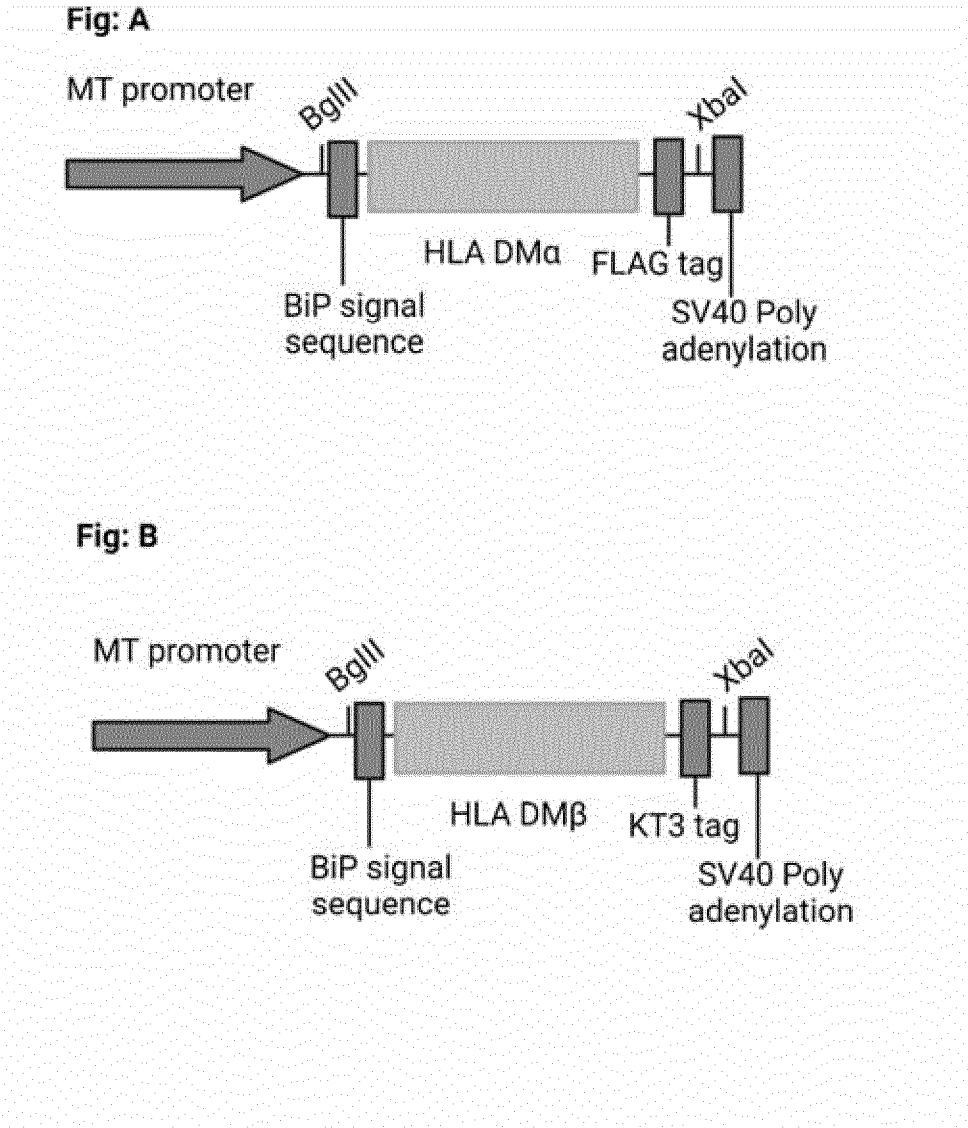


Figure 21:

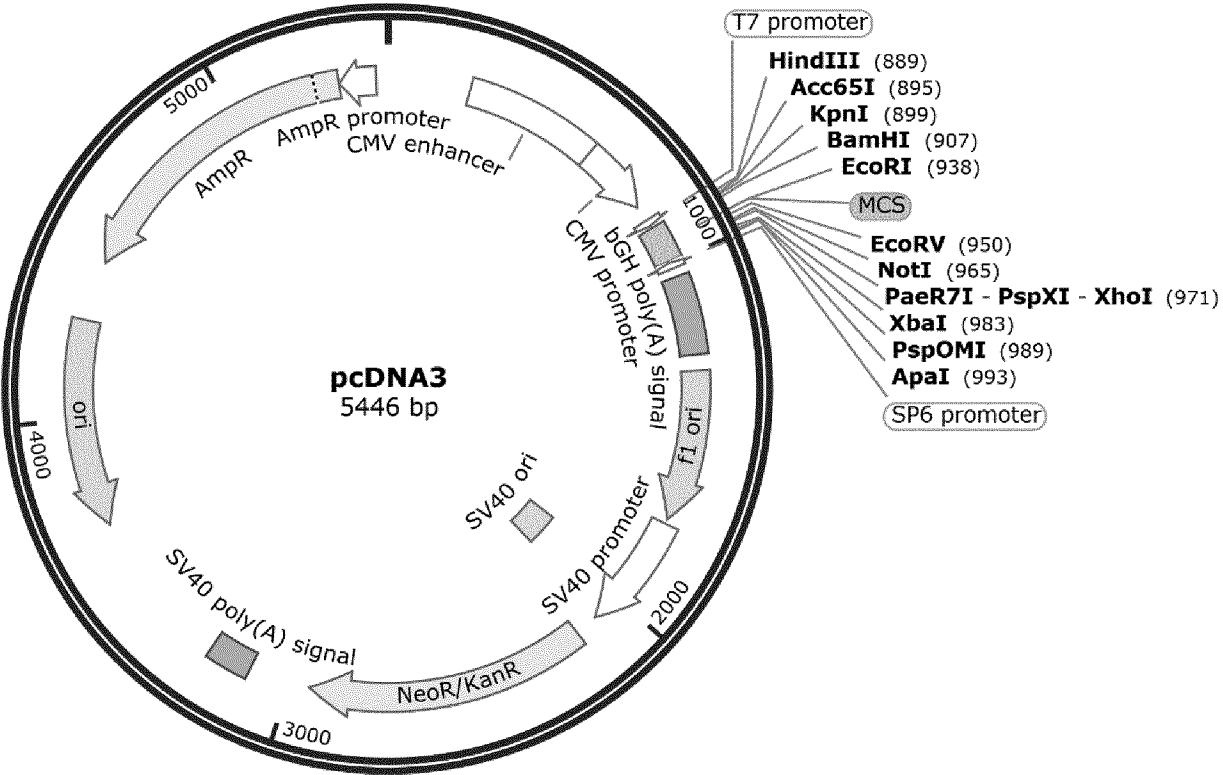
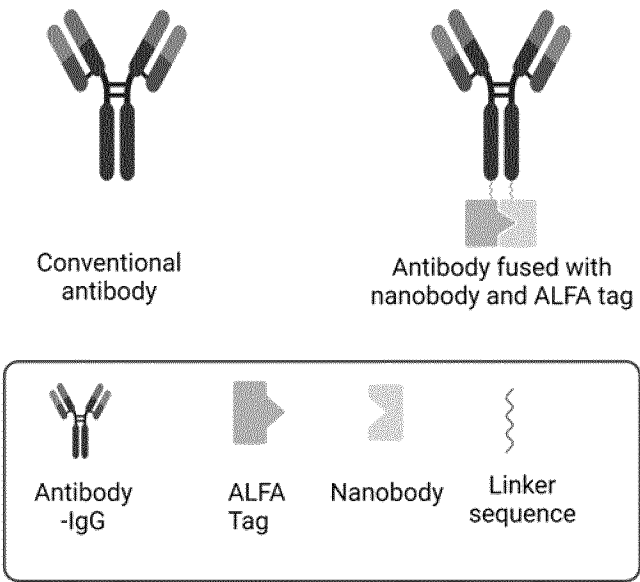


Figure 22:



INTERNATIONAL SEARCH REPORT

International application No
PCT/EP2024/065725

A. CLASSIFICATION OF SUBJECT MATTER INV. C07K14/705 C07K16/00 C12N15/62 C07K14/725 C07K14/74 ADD.		
According to International Patent Classification (IPC) or to both national classification and IPC		
B. FIELDS SEARCHED Minimum documentation searched (classification system followed by classification symbols) C07K C12N Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched Electronic data base consulted during the international search (name of data base and, where practicable, search terms used) EPO-Internal, COMPENDEX, WPI Data		
C. DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	EP 2 072 527 A1 (ALTONABIOTEC AG [DE]) 24 June 2009 (2009-06-24) abstract pages 2-3, paragraphs 15,17 page 4, paragraph 35-37 page 5, paragraphs 48,50,51,52 -----	1 - 17
A	WO 2009/004315 A1 (KING S COLLEGE LONDON [GB]; PEAKMAN MARK [GB] ET AL.) 8 January 2009 (2009-01-08) abstract page 10, line 21 - line 26 page 11, line 1 - line 26 -----	1 - 17
A	WO 2015/119576 A1 (UNIV SINGAPORE [SG]) 13 August 2015 (2015-08-13) abstract pages 18-19, paragraph 62 ----- - / - -	1 - 17
☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.		
* Special categories of cited documents : "A" document defining the general state of the art which is not considered to be of particular relevance "E" earlier application or patent but published on or after the international filing date "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified) "O" document referring to an oral disclosure, use, exhibition or other means "P" document published prior to the international filing date but later than the priority date claimed "T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention "X" document of particular relevance;; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone "Y" document of particular relevance;; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art "&" document member of the same patent family		
Date of the actual completion of the international search 27 June 2024		Date of mailing of the international search report 26/07/2024
Name and mailing address of the ISA/ European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Fax: (+31-70) 340-3016		Authorized officer Grötzing, Thilo

INTERNATIONAL SEARCH REPORT

International application No

PCT/EP2024/065725

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT		
Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2018/224459 A1 (MCNAUGHTON BRIAN R [US] ET AL) 9 August 2018 (2018-08-09) abstract page 1, paragraph 7 figure 2(A) -----	1 - 17

INTERNATIONAL SEARCH REPORT

International application No.

PCT/EP2024/065725

Box No. I Nucleotide and/or amino acid sequence(s) (Continuation of item 1.c of the first sheet)

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of a sequence listing:
 - a. ☒ forming part of the international application as filed.
 - b. ☐ furnished subsequent to the international filing date for the purposes of international search (Rule 13*ter*.1(a)).

☐ accompanied by a statement to the effect that the sequence listing does not go beyond the disclosure in the international application as filed.
2. ☐ With regard to any nucleotide and/or amino acid sequence disclosed in the international application, this report has been established to the extent that a meaningful search could be carried out without a WIPO Standard ST.26 compliant sequence listing.
3. Additional comments:

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No

PCT/EP2024/065725

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
EP 2072527 A1	24-06-2009	EP 2072527 A1	24-06-2009
		EP 2245051 A1	03-11-2010
		WO 2009080816 A1	02-07-2009
WO 2009004315 A1	08-01-2009	EP 2170944 A1	07-04-2010
		US 2010297011 A1	25-11-2010
		WO 2009004315 A1	08-01-2009
WO 2015119576 A1	13-08-2015	SG 11201606501P A	29-09-2016
		US 2017174732 A1	22-06-2017
		WO 2015119576 A1	13-08-2015
US 2018224459 A1	09-08-2018	NONE	