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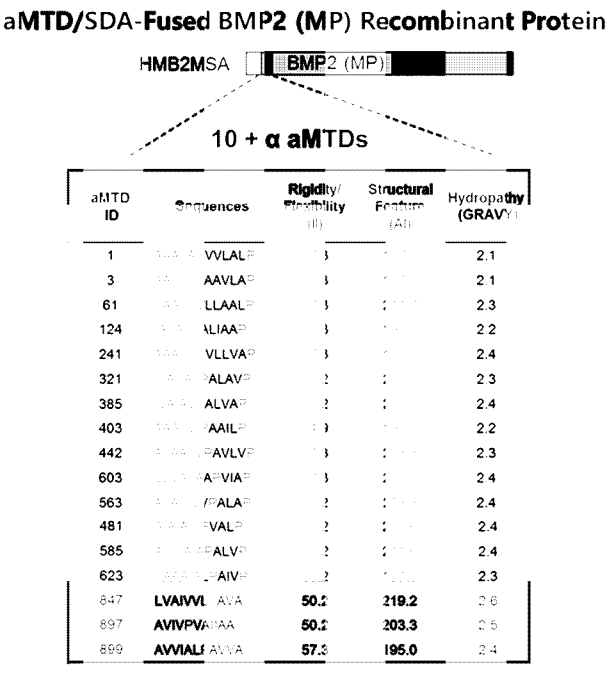
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(54) Title: CELL-PERMEABLE BONE MORPHOGENETIC PROTEIN (CP-BMP) RECOMBINANT PROTEIN AND USE THEREOF



(57) Abstract: The present invention provides the CP-BMP recombinant protein with technical advantages as an intracellular protein therapy for the treatment of bone defects caused by osteogenesis imperfecta, osteoporosis, fracture and osteoctomy in that it could resolve cell-/tissue-permeability and bio-transfer function.

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Description

Title of Invention: CELL-PERMEABLE BONE MORPHOGENETIC PROTEIN (CP-BMP) RECOMBINANT PROTEIN AND USE THEREOF

Technical Field

[1] The present invention relates to cell-permeable bone morphogenetic protein (CP-BMP) recombinant protein and use thereof. The recombinant protein of the present invention has on improved cell-permeability and biological activity as an intracellular protein therapy for regenerating of defected bone caused by osteogenesis imperfecta, osteoporosis, fracture and osteotomy.

Background Art

[2] Bone is a unique tissue that undergoes continuous remodeling throughout life and retains the potential for regeneration even in adult. Bone regeneration is required for bone defects caused by fracture and osteoporosis. Bone morphogenetic proteins (BMPs) are multifunctional growth factors that belong to the transforming growth factor (TGF) superfamily. About 30 BMP-related proteins have been identified and can be subdivided into several groups based on their structures and functions. Especially, BMP2, BMP4 and BMP7 could induce chondrocyte-derived osteoprogenitor (CDOP) cell differentiation, and are important in bone formation and regeneration.

[3] BMPs are synthesized as pre-pro peptides consisting of a signal peptide (SP), latency associated peptide (LAP) and mature peptide (MP). After the synthesis, SP and LAP are processed by enzymatic cleavage, where the C-terminal mature domain is released and secreted. BMPs bind to two-types of BMP receptors and signals through Smad-dependent (canonical) and Smad-independent (non-canonical) pathways. In the canonical pathway, BMP type I receptors phosphorylate receptor-regulated Smads (R-Smads). Phosphorylated R-Smads form a complex compound with common-partner Smads (Co-Smads), translocate into the nucleus and regulate the transcription of osteogenic-related genes.

[4] There are four phases in the process of bone fracture repair: i) inflammatory response, ii) endochondral formation (soft callus formation and osteoblast recruitment), iii) primary bone formation (hard callus formation and mineralization), and iv) secondary bone formation (remodeling). The bone healing process involves various associated factors including BMPs and TGF-β. The effect of BMPs in recombinant systems demonstrates their abilities to enhance fracture healing and skeletal defect repairs in a variety of animal models. Osteogenic potential of BMPs has allowed for their successful use as therapeutic agents for fracture healing, where enhancing bone

regeneration has become general practice in spine fusion surgeries and fracture repair. The responsible genes and associated transcription factors for osteogenesis are also activated to express within a few hours of BMP treatment.

[5] The FDA has approved the use of recombinant human BMPs (rhBMPs) including BMP2. However, rhBMPs have rapid systemic clearance and short biological half-life (7 to 16 minutes systemically and up to 8 days locally) and possible negative side-effects (ex. cancer risk) due to high dosage of BMP.

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Disclosure of Invention

Technical Problem

[45] Macromolecule, such as bone morphogenetic proteins (BMPs), cannot be translocated across the cell membrane. Therefore, there was a need to develop macromolecule intracellular transduction technology (MITT), which enables the translocation of macromolecules into the cell/tissues.

[46] In the previous studies, MITT-based hydrophobic CPPs named membrane translocating sequence (MTS) and membrane translocating motif (MTM), derived from the hydrophobic signal peptide of fibroblast growth factor 4 (FGF4) have been reported and used to deliver biologically active peptides and proteins, such as BMP, systemically in animals.

[47] However, they could not effectively deliver BMP *in vivo* and *in vitro*, and their delivery efficiency was in sufficient due to protein aggregation, low solubility/yield and poor cell-/tissue-permeability.

Solution to Problem

[48] To resolve these problems, newly designed advanced macromolecule transduction domain (aMTD)-enabled macromolecule intracellular transduction technology (MITT) has been adopted for the development of novel protein using BMP against bone formation and regeneration.

[49] For MITT, six critical factors (length, bending potential, instability index, aliphatic index, GRAVY, amino acid composition) have been determined through analysis of baseline hydrophobic CPPs. Advanced macromolecule transduction domain (aMTD), newly designed based on these six critical factors, could optimize cell-/tissue-permeability of cargo proteins that have a therapeutic effects and develop them as protein-based drugs. Further, in order to increase solubility and yield of recombinant protein, solubilization domains (SDs) additionally fused to the aMTD-cargo recombinant protein, thereby notably increased the solubility and yield of the recombinant protein.

[50] One embodiment of the present invention provides a cell-permeable bone morphogenetic protein (CP-BMP), which comprises a BMP being one of BMP2 and BMP7 and an advanced macromolecule transduction domain (aMTD) being composed of 9 to 13 amino acid sequences and having improved cell or tissue permeability,

[51] wherein the aMTD is fused to one end or both ends of the BMP and has the following features of:

[52] (a) being composed of 3 or more amino acids selected from the group consisting of Ala, Val, Ile, Leu, and Pro;

[53] (b) having proline as amino acids corresponding to any one or more of positions 5 to

8, and 12 of its amino acid sequence; and

[54] (c) having an instability index of 40 to 60; an aliphatic index of 180 to 220; and a grand average of hydropathy (GRAVY) of 2.1 to 2.6, as measured by Protparam.

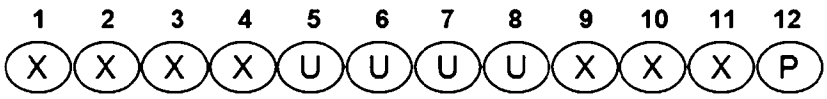
[55] According to one embodiment, one or more solubilization domain (SD)(s) are further fused to the end(s) of one or more of the BMP and the aMTD.

[56] According to another embodiment, the aMTD may have α-Helix structure.

[57] According to still another embodiment, the aMTD may be composed of 12 amino acid sequences and represented by the following general formula:

[58] [General formula]

[59]



[60] wherein X(s) refers to Alanine (A), Valine (V), Leucine (L) or Isoleucine (I); one of U refers to proline and the other U(s) refer to A, V, L or I; and P refers to proline.

[61] Another embodiment of the present invention provides a CP-BMP recombinant protein which is represented by any one of the following structural formula:

[62] A-B-C, A-C-B, B-A-C, B-C-A, C-A-B, C-B-A and A-C-B-C

[63] wherein A is an advanced macromolecule transduction domain (aMTD) having improved cell or tissue permeability, B is a BMP having one of BMP2 and BMP7, and C is a solubilization domain (SD); and

[64] the aMTD is composed of 9 to 13 amino acid sequences and has the following features of:

[65] (a) being composed of 3 or more amino acids selected from the group consisting of Ala, Val, Ile, Leu, and Pro;

[66] (b) having proline as amino acids corresponding to any one or more of positions 5 to 8, and 12 of its amino acid sequence;

[67] (c) having an instability index of 40 to 60; an aliphatic index of 180 to 220; and a grand average of hydropathy (GRAVY) of 2.1 to 2.6, as measured by Protparam; and

[68] (d) having α-Helix structure.

[69] According to one embodiment of the present invention, the BMP may have an amino acid sequence selected from the group consisting of SEQ ID NOs: 815 to 818.

[70] According to another embodiment of the present invention, the BMP may be encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 819 to 823.

[71] According to still another embodiment of the present invention, the BMP may further include a ligand selectively binding to a receptor of a cell, a tissue, or an organ.

[72] According to still another embodiment of the present invention, the aMTD may have an amino acid sequence selected from the group consisting of SEQ ID NOs: 1 to 240.

[73] According to still another embodiment of the present invention, the aMTD may be encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 241 to 481.

[74] According to still another embodiment of the present invention, the SD(s), independently, may have an amino acid sequence selected from the group consisting of SEQ ID NOs: 799 to 805.

[75] According to still another embodiment of the present invention, the SD(s), independently, may be encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 806 to 812.

[76] According to still another embodiment of the present invention, the BMP recombinant protein may have a histidine-tag affinity domain additionally fused to one end thereof.

[77] According to still another embodiment of the present invention, the histidine-tag affinity domain may have an amino acid sequence of SEQ ID NO: 813.

[78] According to still another embodiment of the present invention, the histidine-tag affinity domain may be encoded by a polynucleotide sequence of SEQ ID NO: 814.

[79] According to still another embodiment of the present invention, the fusion may be formed via a peptide bond or a chemical bond.

[80] According to still another embodiment of the present invention, the CP-BMP recombinant protein may be used for the regeneration of defected bone.

[81] Still another embodiment of the present invention provides a polynucleotide sequence encoding the CP-BMP recombinant protein.

[82] According to one embodiment of the present invention, the polynucleotide sequence may be selected from the group consisting of SEQ ID NOs: 824 and 825.

[83] According to another embodiment of the present invention, the polynucleotide sequence may be selected from the group consisting of SEQ ID NOs: 826 and 827.

[84] Still another embodiment of the present invention provides a recombinant expression vector including the polynucleotide sequence.

[85] Still another embodiment of the present invention provides a transformant transformed with the recombinant expression vector.

[86] Still another embodiment of the present invention provides a preparing method of the CP-BMP recombinant protein including preparing the recombinant expression vector; preparing the transformant using the recombinant expression vector; culturing the transformant; and recovering the recombinant protein expressed by the culturing.

[87] Still another embodiment of the present invention provides a composition including the CP-BMP recombinant protein as an active ingredient.

[88] Still another embodiment of the present invention provides a pharmaceutical composition for regenerating of defected bone including the CP-BMP recombinant protein

as an active ingredient; and a pharmaceutically acceptable carrier.

[89] Still another embodiment of the present invention provides use of the CP-BMP recombinant protein as a medicament for regenerating of defected bone.

[90] Still another embodiment of the present invention provides a medicament including the CP-BMP recombinant protein.

[91] Still another embodiment of the present invention provides use of the CP-BMP recombinant protein in the preparation of a medicament for regenerating of defected bone.

[92] Still another embodiment of the present invention provides a method of regenerating of defected bone, the method including preparing defected bone; and treating the defected bone with an therapeutically effective amount of the CP-BMP recombinant protein.

[93] According to one embodiment of the present invention, the subject may be a mammal.

[94] Unless defined otherwise, all terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the present invention belongs. Although a certain method and a material is described herein, it should not be construed as being limited thereto, any similar or equivalent method and material to those may also be used in the practice or testing of the present invention. All publications mentioned herein are incorporated herein by reference in their entirety to disclose and describe the methods and/or materials in connection with which the publications are cited. It must be noted that as used herein and in the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise.

[95] A "peptide" refers to a chain-type polymer formed by amino acid residues which are linked to each other via peptide bonds, and used interchangeably with "polypeptide." Further, a "polypeptide" includes a peptide and a protein.

[96] Further, the term "peptide" includes amino acid sequences that are conservative variations of those peptides specifically exemplified herein. The term "conservative variation," as used herein, denotes the replacement of an amino acid residue by another, biologically similar residue. Examples of conservative variations include substitution of one hydrophobic residue, such as isoleucine, valine, leucine, alanine, cysteine, glycine, phenylalanine, proline, tryptophan, tyrosine, norleucine, or methionine for another, or substitution of one polar residue for another, for example, substitution of arginine for lysine, glutamic acid for aspartic acid, or glutamine for asparagine, and the like. Neutral hydrophilic amino acids which may be substituted for one another include asparagine, glutamine, serine, and threonine.

[97] The term "conservative variation" also includes use of a substituted amino acid in

place of an unsubstituted parent amino acid, provided that antibodies raised to the substituted polypeptide also immunoreacts with the unsubstituted polypeptide. Such conservative substitutions are within the definition of the classes of the peptides according to one embodiment of the present invention.

[98] A person having ordinary skill in the art may make similar substitutions to obtain peptides having higher cell permeability and a broader host range. For example, one embodiment of the present invention provides peptides corresponding to amino acid sequences (e.g. SEQ ID NOs: 1 to 240) provided herein, as well as analogues, homologs, isomers, derivatives, amidated variations, and conservative variations thereof, as long as the cell permeability of the peptide remains.

[99] Minor modifications to primary amino acid sequence of the peptides according to one embodiment of the present invention may result in peptides which have substantially equivalent or enhanced cell permeability, as compared to the specific peptides described herein. Such modifications may be deliberate, as by site-directed mutagenesis, or may be spontaneous.

[100] All peptides may be synthesized using L-amino acids, but D forms of all of the peptides may be synthetically produced. In addition, C-terminal derivatives, such as C-terminal methyl esters and C-terminal amidates, may be produced in order to increase the cell permeability of the peptide according to one embodiment of the present invention.

[101] All of the peptides produced by these modifications are included herein, as long as in the case of amidated versions of the peptide, the cell permeability of the original peptide is altered or enhanced such that the amidated peptide is therapeutically useful. It is envisioned that such modifications are useful for altering or enhancing cell permeability of a particular peptide.

[102] Furthermore, deletion of one or more amino acids may also result in a modification to the structure of the resultant molecule without any significant change in its cell permeability. This may lead to the development of a smaller active molecule which may also have utility. For example, amino- or carboxyl-terminal amino acids which may not be required for the cell permeability of a particular peptide may be removed.

[103] The term "gene" refers to an arbitrary nucleic acid sequence or a part thereof having a functional role in protein coding or transcription, or regulation of other gene expression. The gene may be composed of all nucleic acids encoding a functional protein or a part of the nucleic acid encoding or expressing the protein. The nucleic acid sequence may include a gene mutation in exon, intron, initiation or termination region, promoter sequence, other regulatory sequence, or a unique sequence adjacent to the gene.

[104] The term "primer" refers to an oligonucleotide sequence that hybridizes to a com-

plementary RNA or DNA target polynucleotide and serves as the starting points for the stepwise synthesis of a polynucleotide from mononucleotides by the action of a nucleotidyltransferase as occurs, for example, in a polymerase chain reaction.

[105] The term "coding region" or "coding sequence" refers to a nucleic acid sequence, a complement thereof, or a part thereof which encodes a particular gene product or a fragment thereof for which expression is desired, according to the normal base pairing and codon usage relationships. Coding sequences include exons in genomic DNA or immature primary RNA transcripts, which are joined together by the cellular biochemical machinery to provide a mature mRNA. The anti-sense strand is the complement of the nucleic acid, and the coding sequence may be deduced therefrom.

[106] One embodiment of the present invention provides a CP-BMP recombinant protein, which comprises a BMP being one of BMP2 and BMP7, and an advanced macromolecule transduction domain (aMTD) being composed of 9 to 13 amino acid sequences, preferably 10 to 12 amino acid sequences, and having improved cell or tissue permeability,

[107] wherein the aMTD is fused to one end or both ends of the BMP and has the following features of:

[108] (a) being preferably composed of 3 or more amino acids selected from the group consisting of Ala, Val, Ile, Leu, and Pro;

[109] (b) having proline as amino acid sequences corresponding to any one or more of positions 5 to 8, and 12 of its amino acids, and preferably one or more of positions 5 to 8 and position 12 of its amino acid sequence; and

[110] (c) having an instability index of preferably 40 to 60 and more preferably 41 to 58; an aliphatic index of preferably 180 to 220 and more preferably 185 to 225; and a grand average of hydropathy (GRAVY) of preferably 2.1 to 2.6 and more preferably 2.2 to 2.6 as measured by Protparam (see http://web.expasy.org/protparam/).

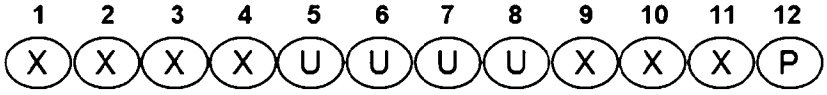
[111] According to one embodiment, one or more solubilization domain (SD)(s) are further fused to one or more of the BMP and the aMTD, preferably one end or both ends of the BMP, and more preferably the C-terminus of the BMP.

[112] According to another embodiment, the aMTD may have α-Helix structure.

[113] According to still another embodiment, the aMTD may be preferably composed of 12 amino acid sequences and represented by the following general formula:

[114] [General formula]

[115]



[116] wherein X(s) refers to Alanine (A), Valine (V), Leucine (L) or Isoleucine (I); one of U refers to proline and the other U(s) refer to A, V, L or I; and P refers to proline.

[117] Still another embodiment of the present invention provides a CP-BMP recombinant protein which is represented by any one of structural formula A-B-C, A-C-B, B-A-C, B-C-A, C-A-B, C-B-A and A-C-B-C, and preferably by A-B-C or A-C-B-C:

[118] wherein A is an advanced macromolecule transduction domain (aMTD) having improved cell or tissue permeability, B is a BMP having one of BMP2 and BMP7, and C is a solubilization domain (SD); and

[119] the aMTD is composed of 9 to 13, preferably 10 to 12 amino acid sequences and has the following features of:

[120] (a) being composed of 3 or more amino acids selected from the group consisting of Ala, Val, Ile, Leu, and Pro;

[121] (b) having proline as amino acids corresponding to any one or more of positions 5 to 8, and 12 of its amino acid sequence, and preferably, one or more of positions 5 to 8 and position 12 of its amino acid sequence;

[122] (c) having an instability index of preferably 40 to 60 and more preferably 41 to 58; an aliphatic index of preferably 180 to 220 and more preferably 185 to 225; and a grand average of hydropathy (GRAVY) of preferably 2.1 to 2.6 and more preferably 2.2 to 2.6, as measured by Protparam (see http://web.expasy.org/protparam/); and

[123] (d) preferably having α-Helix structure.

[124] In one embodiment of the present invention, the BMP may have an amino acid sequence selected from the group consisting of SEQ ID NOs: 815 to 818. The BMP may have one selected from the group consisting of BMP2 (M form), BMP2 (L form), BMP7 (M form) and BMP7 (L form). The BMP may be preferably BMP2 (M form) of SEQ ID NO: 815 or a BMP7 (M form) of SEQ ID NO: 817.

[125] In another embodiment of the present invention, the BMP may be encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 819 to 823. The BMP may be preferably BMP2 (M form) encoded by a polynucleotide sequence of SEQ ID NO: 819, BMP2 (M form) for codon-optimization encoded by a polynucleotide sequence of SEQ ID NO: 820 or BMP7 (M form) encoded by a polynucleotide sequence of SEQ ID NO: 822. The BMP may be more preferably BMP2 (M form) encoded by a polynucleotide sequence of SEQ ID NO: 819 or BMP2 (M form) for codon-optimization encoded by a polynucleotide sequence of SEQ ID NO: 820.

[126] When the CP-BMP recombinant protein is intended to be delivered to a particular cell, tissue, or organ, the BMP may form a fusion product, together with an extracellular domain of a ligand capable of selectively binding to a receptor which is specifically expressed on the particular cell, tissue, or organ, or monoclonal antibody (mAb) capable of specifically binding to the receptor or the ligand and a modified form thereof.

[127] The binding of the peptide and a biologically active substance may be formed either

by indirect linkage by a cloning technique using an expression vector at a nucleotide level or by direct linkage via chemical or physical covalent or non-covalent bond of the peptide and the biologically active substance.

[128] In still another embodiment of the present invention, the BMP may preferably further include a ligand selectively binding to a receptor of a cell, a tissue, or an organ.

[129] In one embodiment of the present invention, the aMTD may have an amino acid sequence selected from the group consisting of SEQ ID NOs: 1 to 240. The aMTD may be preferably $aMTD_1$ of SEQ ID NO: 1, $aMTD_3$ of SEQ ID NO: 3, $aMTD_{24}$ of SEQ ID NO: 12, $aMTD_{61}$ of SEQ ID NO: 17, $aMTD_{123}$ of SEQ ID NO: 33, $aMTD_{124}$ of SEQ ID NO: 34, $aMTD_{241}$ of SEQ ID NO: 56, $aMTD_{321}$ of SEQ ID NO: 74, $aMTD_{385}$ of SEQ ID NO: 91, $aMTD_{403}$ of SEQ ID NO: 94, $aMTD_{442}$ of SEQ ID NO: 101, $aMTD_{481}$ of SEQ ID NO: 110, $aMTD_{563}$ of SEQ ID NO: 131, $aMTD_{585}$ of SEQ ID NO: 136, $aMTD_{603}$ of SEQ ID NO: 139, $aMTD_{623}$ of SEQ ID NO: 143, $aMTD_{847}$ of SEQ ID NO: 200 and $aMTD_{897}$ of SEQ ID NO: 228 and $aMTD_{899}$ of SEQ ID NO: 229, and more preferably $aMTD_{24}$ of SEQ ID NO: 12 and $aMTD_{442}$ of SEQ ID NO: 101.

[130] In still another embodiment of the present invention, the aMTD may be encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 241 to 481. The aMTD may be preferably $aMTD_1$ encoded by a polynucleotide sequence of SEQ ID NO: 241, $aMTD_3$ encoded by a polynucleotide sequence of SEQ ID NO: 243, $aMTD_{24}$ encoded by a polynucleotide sequence of SEQ ID NO: 252, $aMTD_{61}$ encoded by a polynucleotide sequence of SEQ ID NO: 257, $aMTD_{123}$ encoded by a polynucleotide sequence of SEQ ID NO: 273, $aMTD_{124}$ encoded by a polynucleotide sequence of SEQ ID NO: 274, $aMTD_{241}$ encoded by a polynucleotide sequence of SEQ ID NO: 296, $aMTD_{321}$ encoded by a polynucleotide sequence of SEQ ID NO: 314, $aMTD_{385}$ encoded by a polynucleotide sequence of SEQ ID NO: 331, $aMTD_{403}$ encoded by a polynucleotide sequence of SEQ ID NO: 334, $aMTD_{442}$ encoded by a polynucleotide sequence of SEQ ID NO: 341, $aMTD_{442}$ for codon-optimization encoded by a polynucleotide sequence of SEQ ID NO: 481, $aMTD_{481}$ encoded by a polynucleotide sequence of SEQ ID NO: 350, $aMTD_{563}$ encoded by a polynucleotide sequence of SEQ ID NO: 371, $aMTD_{585}$ encoded by a polynucleotide sequence of SEQ ID NO: 376, $aMTD_{603}$ encoded by a polynucleotide sequence of SEQ ID NO: 379, $aMTD_{623}$ encoded by a polynucleotide sequence of SEQ ID NO: 383, $aMTD_{847}$ encoded by a polynucleotide sequence of SEQ ID NO: 440, $aMTD_{897}$ encoded by a polynucleotide sequence of SEQ ID NO: 468 and $aMTD_{899}$ encoded by a polynucleotide sequence of SEQ ID NO: 469, and more preferably $aMTD_{24}$ encoded by a polynucleotide sequence of SEQ ID NO: 252, $aMTD_{442}$ encoded by a polynucleotide sequence of SEQ ID NO: 341 and $aMTD_{442}$ for codon-optimization encoded by a polynucleotide sequence of SEQ ID NO: 481.

[131] In still another embodiment of the present invention, the SD(s) may have an amino acid sequence independently selected from the group consisting of SEQ ID NOs: 799 to 805. The SD(s) may has one or more selected from the group consisting of SDA, SDB, SDB' (SDB for deimmunization), SDC, SDD, SDE and SDF. The SD may be preferably SDA of SEQ ID NO: 799, SDB of SEQ ID NO: 800, SDB' of SEQ ID NO: 805 or SDC of SEQ ID NO: 801, and more preferably, SDB of SEQ ID NO: 800 and SDB' of SEQ ID NO: 805 which have superior structural stability.

[132] In still another embodiment of the present invention, the SDs may be encoded by a polynucleotide sequence independently selected from the group consisting of SEQ ID NOs: 806 to 812. The SD may be preferably SDA encoded by a polynucleotide sequence of SEQ ID NO: 806, SDB encoded by a polynucleotide sequence of SEQ ID NO: 807, SDB' encoded by a polynucleotide sequence of SEQ ID NO: 812, or SDC encoded by a polynucleotide sequence of SEQ ID NO: 808, and more preferably, SDB and SDB' having superior structural stability, which is encoded by a polynucleotide sequence of SEQ ID NO: 807 and SEQ ID NO: 812.

[133] In still another embodiment of the present invention, the CP-BMP recombinant protein may be preferably selected from the group consisting of:

[134] 1) a recombinant protein, in which BMP protein having an amino acid sequence of SEQ ID NOs: 815 and 818 is fused to the N-terminus or the C-terminus of aMTD having any one amino acid sequence selected from the group consisting of SEQ ID NOs: 1 to 240, preferably SEQ ID NOs: 1, 3, 12, 17, 33, 34, 56, 74, 91, 94, 101, 110, 131, 136, 139, 143, 200, 228 and 229 and more preferably SEQ ID NO: 12 and 101;

[135] 2) a recombinant protein, in which SD having any one amino acid sequence selected from the group consisting of SEQ ID NOs: 799 to 805, preferably SEQ ID NOs: 799, 800, 801 and 805, and more preferably SEQ ID NO: 800 and 805 is further fused to the N-terminus or the C-terminus of the BMP protein in the recombinant protein of 1); and

[136] 3) a recombinant protein, in which one or more of a histidine tag having an amino acid sequence of SEQ ID NO: 813 is further fused to the N-terminus or the C-terminus of the aMTD in the recombinant protein of 1) or 2).

[137] The BMPs may exhibit a physiological phenomenon-related activity or a therapeutic purpose-related activity by intracellular or *in* vivo delivery. The recombinant expression vector may include a tag sequence which makes it easy to purify the recombinant protein, for example, consecutive histidine codon, maltose binding protein codon, Myc codon, etc., and further include a fusion partner to enhance solubility of the recombinant protein, etc. Further, for the overall structural and functional stability of the recombinant protein or flexibility of the proteins encoded by respective genes, the recombinant expression vector may further include one or more glycine, proline, and spacer amino acid or polynucleotide sequences including AAY amino acids. Fur-

thermore, the recombinant expression vector may include a sequence specifically digested by an enzyme in order to remove an unnecessary region of the recombinant protein, an expression regulatory sequence, and a marker or reporter gene sequence to verify intracellular delivery, but is not limited thereto.

[138] In still another embodiment of the present invention, the CP-BMP recombinant protein may have a histidine-tag affinity domain additionally fused to one end thereof. Preferably, the histidine-tag may be fused to the N-terminus of BMP, aMTD or SD. More preferably, the histidine-tag may be fused to the N-terminus of aMTD or BMP.

[139] In still another embodiment of the present invention, the histidine-tag affinity domain may have an amino acid sequence of SEQ ID NO: 813.

[140] In still another embodiment of the present invention, the histidine-tag affinity domain may be encoded by a polynucleotide sequence of SEQ ID NO: 814.

[141] In still another embodiment of the present invention, the fusion may be formed via a peptide bond or a chemical bond.

[142] The chemical bond may be preferably selected from the group consisting of disulfide bonds, diamine bonds, sulfide-amine bonds, carboxyl-amine bonds, ester bonds, and covalent bonds.

[143] In still another embodiment of the present invention, the CP-BMP recombinant protein may be used for the regeneration of defected bone. The CP-BMP recombinant protein may act on tissues or bone defected by osteogenesis imperfecta, osteoporosis, fracture and osteotomy to efficiently help cell differentiation, leading to bone re-generation or formation.

[144] Still another embodiment of the present invention provides a polynucleotide sequence encoding the CP-BMP recombinant protein.

[145] The polynucleotide sequence may be present in a vector in which the polynucleotide sequence is operably linked to regulatory sequences capable of providing for the ex-pression of the polynucleotide sequence by a suitable host cell.

[146] According to one embodiment of the present invention, the polynucleotide sequence may be selected from the following groups:

[147] 1) a polynucleotide sequence, in which any one polynucleotide sequence selected from the group consisting of SEQ ID NOs: 241 to 481, preferably SEQ ID NOs: 241, 243, 252, 257, 273, 481, 274, 296, 314, 331, 334, 341, 350, 371, 375, 379, 383, 440, 468, 469 and 481, and more preferably SEQ ID NOs: 12 and 341, is operably linked with and a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 815 to 818; and

[148] 2) a polynucleotide sequence, in which any one polynucleotide sequence selected from the group consisting of SEQ ID NOs: 806 to 812, preferably SEQ ID NOs: 806, 807, 808 and 812, and more preferably SEQ ID NOs: 807 and 812 is further operably

linked to the polynucleotide sequence of 1).

[149] Within the expression vector, the term "operably linked" is intended to mean that the polynucleotide sequence of interest is linked to the regulatory sequence(s) in a manner which allows for expression of the polynucleotide sequence. The term "regulatory sequence" is intended to include promoters, enhancers, and other expression control elements. Such operable linkage with the expression vector can be achieved by conventional gene recombination techniques known in the art, while site-directed DNA cleavage and linkage are carried out by using conventional enzymes known in the art.

[150] The expression vectors may contain a signal sequence or a leader sequence for membrane targeting or secretion, as well as regulatory sequences such as a promoter, an operator, an initiation codon, a termination codon, a polyadenylation signal, an enhancer and the like. The promoter may be a constitutive or an inducible promoter. Further, the expression vector may include one or more selectable marker genes for selecting the host cell containing the expression vector, and may further include a polynucleotide sequence that enables the vector to replicate in the host cell in question.

[151] The expression vector constructed according to the present invention may be the vector where the polynucleotide encoding the CP-BMP recombinant protein (where an aMTD is fused to the N-terminus or C-terminus of a BMP protein) is inserted within the multiple cloning sites (MCS), preferably NdeI/SalI, NdeI/BamHI, NdeI/NotI and NdeI/HindIII site of a pET-22b(+) vector, a pET-26b(+) vector or a pET-28a(+) vector (Novagen, Darmstadt, Germany).

[152] In still another embodiment of the present invention, the polynucleotide encoding the SD being additionally fused to the N-terminus or C-terminus of a BMP protein may be inserted into a cleavage site of restriction enzyme (NdeI, EcoRI, SalI, XhoI, NotI, HindIII, etc.) within the multiple cloning sites (MCS) of a pET-22b(+) vector, a pET-26b(+) vector or a pET-28a(+) vector.

[153] In still another embodiment of the present invention, the polynucleotide is cloned into a pET-22b(+) vector, a pET-26b(+) vector or a pET-28a(+) vector bearing a His-tag sequence so as to fuse six histidine residues to the N-terminus of the CP-BMP recombinant protein to allow easy purification.

[154] According to one embodiment of the present invention, the polynucleotide sequence may be represented by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 824 and 825.

[155] According to another embodiment of the present invention, the polynucleotide sequence may be further fused with SD, and may be represented by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 826 and 827.

[156] According to still another embodiment of the present invention, the polynucleotide sequence may be fused with a histidine-tag affinity domain, and may be a polynu-

cleotide sequence of SEQ ID NOs: 828 and 832.

[157] Preferably, the CP-BMP recombinant protein of another embodiment of the present invention may be composed of an amino acid sequence selected from the group consisting of SEQ ID NOs: 824, 826 and 828.

[158] Still another embodiment of the present invention provides a recombinant expression vector including the polynucleotide sequence.

[159] Preferably, the vector may be inserted in a host cell and recombined with the host cell genome, or refers to any nucleic acid including a nucleotide sequence competent to replicate spontaneously as an episome. Such a vector may include a linear nucleic acid, a plasmid, a phagemid, a cosmid, an RNA vector, a viral vector, etc.

[160] Preferably, the vector may be genetically engineered to incorporate the nucleic acid sequence encoding the recombinant protein in an orientation either N-terminal and/or C-terminal to a nucleic acid sequence encoding a peptide, a polypeptide, a protein domain, or a full-length protein of interest, and in the correct reading frame so that the recombinant protein consisting of aMTD, BMP, and preferably SD may be expressed. Expression vectors may be selected from those readily available for use in prokaryotic or eukaryotic expression systems. Preferably, a pET-22b(+) vector, a pET-26b(+) vector or a pET-28a(+) vector may be used.

[161] Standard recombinant nucleic acid methods may be used to express a genetically engineered recombinant protein. The nucleic acid sequence encoding the recombinant protein according to one embodiment of the present invention may be cloned into a nucleic acid expression vector, e.g., with appropriate signal and processing sequences and regulatory sequences for transcription and translation, and the protein may be synthesized using automated organic synthetic methods. Synthetic methods of producing proteins are described in, for example, the literature [Methods in Enzymology, Volume 289: Solid-Phase Peptide Synthesis by Gregg B. Fields (Editor), Sidney P. Colowick, Melvin I. Simon (Editor), Academic Press (1997)].

[162] In order to obtain high level expression of a cloned gene or nucleic acid, for example, a cDNA encoding the recombinant protein of the present invention, the recombinant protein sequence may be typically subcloned into an expression vector that includes a strong promoter for directing transcription, a transcription/translation terminator, and in the case of a nucleic acid encoding a protein, a ribosome binding site for translational initiation. Suitable bacterial promoters are well known in the art and are described, e.g., in the literatures [Sambrook & Russell, Molecular Cloning: A Laboratory Manual, 3d Edition, Cold Spring Harbor Laboratory, N.Y. (2001); and Ausube, et al., Current Protocols in Molecular Biology, Greene Publishing Associates and Wiley Interscience, N. Y. (1989)]. Bacterial expression systems for expression of the recombinant protein of the present invention are available in, e.g., *E.* coli, *Bacillus*

sp., and *Salmonella* (Palva et al., Gene 22: 229-235 (1983); Mosbach et al., Nature 302: 543-545 (1983)). Kits for such expression systems are commercially available. Eukaryotic expression systems for mammalian cells, yeast, and insect cells are well known in the art and are also commercially available. The eukaryotic expression vector may be preferably an adenoviral vector, an adeno-associated vector, or a retroviral vector.

[163] Generally, the expression vector for expressing the cell permeable recombinant protein according to one embodiment of the present invention in which the cargo protein, i.e. BMP, is attached to the N-terminus, C-terminus, or both termini of aMTD may include regulatory sequences including, for example, a promoter, operably attached to a sequence encoding the advanced macromolecule transduction domain. Non-limiting examples of inducible promoters that may be used include steroid-hormone responsive promoters (e.g., ecdysone-responsive, estrogen-responsive, and glutacorticoid-responsive promoters), tetracycline "Tet-On" and "Tet-Off" systems, and metal-responsive promoters.

[164] The recombinant protein may be introduced into an appropriate host cell, e.g., a bacterial cell, a yeast cell, an insect cell, or a tissue culture cell. The recombinant protein may also be introduced into embryonic stem cells in order to generate a transgenic organism. Large numbers of suitable vectors and promoters are known to those skilled in the art and are commercially available for generating the recombinant protein of the present invention.

[165] Known methods may be used to construct vectors including the polynucleotide sequence according to one embodiment of the present invention and appropriate transcriptional/translational control signals. These methods include *in vitro* recombinant DNA techniques, synthetic techniques, and *in vivo* recombination/genetic recombination. For example, these techniques are described in the literatures [Sambrook & Russell, Molecular Cloning: A Laboratory Manual, 3d Edition, Cold Spring Harbor Laboratory, N. Y. (2001); and Ausubel et al., Current Protocols in Molecular Biology Greene Publishing Associates and Wiley Interscience, N.Y. (1989)].

[166] Still another embodiment of the present invention provides a transformant transformed with the recombinant expression vector.

[167] The transformation includes transfection, and refers to a process whereby a foreign (extracellular) DNA, with or without an accompanying material, enters into a host cell. The "transfected cell" refers to a cell into which the foreign DNA is introduced into the cell, and thus the cell harbors the foreign DNA. The DNA may be introduced into the cell so that a nucleic acid thereof may be integrated into the chromosome or replicable as an extrachromosomal element. The cell introduced with the foreign DNA, etc. is called a transformant.

[168] As used herein, 'introducing' of a protein, a peptide, an organic compound into a cell may be used interchangeably with the expression of 'carrying,' 'penetrating,' 'transporting,' 'delivering,' 'permeating' or 'passing.'

[169] It is understood that the host cell refers to a eukaryotic or prokaryotic cell into which one or more DNAs or vectors are introduced, and refers not only to the particular subject cell but also to the progeny or potential progeny thereof. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

[170] The host cells may be preferably bacterial cells, and as the bacterial cells, there are, in principle, no limitations. They may be eubacteria (gram-positive or gram-negative) or archaebacteria, as long as they allow genetic manipulation for insertion of a gene of interest, preferably for site-specific integration, and they may be cultured on a manufacturing scale. Preferably, the host cells may have the property to allow cultivation to high cell densities.

[171] Examples of bacterial host cells that may be used in the preparation of the recombinant protein are *E.* coli (Lee, 1996; Hannig and Makrides, 1998), *Bacillus* subtilis, *Pseudomonas* fluorescens (Squires et al., 2004; Retallack et al., 2006) as well as various *Corynebacterium* (US 2006/0003404 A1) and *Lactococcus* lactis (Mierau et al., 2005) strains. Preferably, the host cells are *Escherichia* coli cells.

[172] More preferably, the host cell may include an RNA polymerase capable of binding to a promoter regulating the gene of interest. The RNA polymerase may be endogenous or exogenous to the host cell.

[173] Preferably, host cells with a foreign strong RNA polymerase may be used. For example, *Escherichia* coli strains engineered to carry a foreign RNA polymerase (e.g. like in the case of using a T7 promoter a T7-like RNA polymerase in the so-called "T7 strains") integrated in their genome may be used. Examples of T7 strains, e.g. BL21(DE3), HMS174(DE3), and their derivatives or relatives (see Novagen, pET System manual, 11th edition), may be widely used and commercially available. Preferably, BL21-CodonPlus(DE3)-RIL or BL21-CodonPlus(DE3)-RIPL (Agilent Technologies) may be used. These strains are DE3 lysogens containing the T7 RNA polymerase gene under control of the lacUV5 promoter. Induction with IPTG allows production of T7 RNA polymerase which then directs the expression of the gene of interest under the control of the T7 promoter.

[174] The host cell strains, *E.* coli BL21(DE3) or HMS174(DE3), which have received their genome-based T7 RNA polymerase via the phage DE3, are lysogenic. It is preferred that the T7 RNA polymerase contained in the host cell has been integrated by a method which avoids, or preferably excludes, the insertion of residual phage

sequences in the host cell genome since lysogenic strains have the disadvantage to potentially exhibit lytic properties, leading to undesirable phage release and cell lysis.

[175] Still another embodiment of the present invention provides a preparing method of the CP-BMP recombinant protein including preparing the recombinant expression vector; preparing the transformant using the recombinant expression vector; culturing the transformant; and recovering the recombinant protein expressed by culturing.

[176] Culturing may be preferably in a mode that employs the addition of a feed medium, this mode being selected from the fed-batch mode, semi-continuous mode, or continuous mode, and the bacterial expression host cells may include a DNA construct, integrated in their genome, carrying the DNA sequence encoding the protein of interest under the control of a promoter that enables expression of said protein.

[177] There are no limitations in the type of the culture medium. The culture medium may be semi-defined, i.e. containing complex media compounds (e.g. yeast extract, soy peptone, casamino acids), or it may be chemically defined, without any complex compounds. Preferably, a defined medium may be used. The defined media (also called minimal or synthetic media) are exclusively composed of chemically defined substances, i.e. carbon sources such as glucose or glycerol, salts, vitamins, and, in view of a possible strain auxotrophy, specific amino acids or other substances such as thiamine. Most preferably, glucose may be used as a carbon source. Usually, the carbon source of the feed medium serves as the growth-limiting component which controls the specific growth rate.

[178] Host cells may be disrupted by any convenient method, including freeze-thaw cycling, sonication, mechanical disruption, or the use of cell lysing agents. The literature [Scopes, Protein Purification: Principles and Practice, New York: Springer-Verlag (1994)] describes a number of general methods for purifying recombinant (and non-recombinant) proteins. The methods may include, e.g., ion-exchange chromatography, size-exclusion chromatography, affinity chromatography, selective precipitation, dialysis, and hydrophobic interaction chromatography. These methods may be adapted to devise a purification strategy for the cell permeable recombinant protein. If the cell permeable recombinant protein includes a purification handle, such as an epitope tag or a metal chelating sequence, affinity chromatography may be used to easily purify the protein.

[179] The amount of the protein produced may be evaluated by detecting the advanced macromolecule transduction domain directly (e.g., using Western analysis) or indirectly (e.g., by assaying materials derived from the cells for specific DNA binding activity, such as by electrophoretic mobility shift assay). Proteins may be detected prior to purification, during any stage of purification, or after purification. In some implementations, purification or complete purification may not be necessary.

[180] The recombinant protein prepared by the method according to one embodiment of the present invention may be an improved cell/tissue-permeable recombinant BMP, and induces differentiation of osteoblasts to regenerate defected bones.

[181] The cell permeable recombinant proteins prepared by the method according to one embodiment of the present invention may be preferably used for regeneration of defected bone, which osteogenesis imperfecta, osteoporosis, bone fracture and osteotomy.

[182] The osteogenesis imperfecta (OI), also known as "brittle bone disease" or Lobstein syndrome, is a debilitating and rare congenital bone disease that affects about one in every 15,000 people. Though phenotypes vary among OI types, common symptoms include incomplete ossification of bones and teeth, reduced bone mass, brittle bones, and pathologic fractures. These common symptoms of OI are thought to be caused by gene mutations which result in deficiencies in Type-I collagen or other proteins involved in bone matrix deposition or homeostasis.

[183] The osteoporosis is a disease in which bones become fragile and more likely to fracture. Usually the bone loses density, which measures the amount of calcium and minerals in the bone. Osteoporosis is the most common type of bone disease. Bone is living tissue. Existing bone is constantly being replaced by new bone. Osteoporosis occurs when the body fails to form enough new bone, when too much existing bone is reabsorbed by the body, or both.

[184] The bone fracture is a medical condition in which there is a damage in the continuity of the bone. A bone fracture can be the result of high force impact or stress, or a minimal trauma injury as a result of certain medical conditions that weaken the bones, such as osteoporosis, bone cancer, or osteogenesis imperfecta, where the fracture is then properly termed a pathologic fracture.

[185] The osteotomy is a surgical operation in which a bone is cut to shorten, lengthen, or change its alignment. In some osteotomies, the bone is cut and an implant is provided in the bone to change the alignment of the bone.

[186] The CP-BMP recombinant proteins according to one embodiment of the present invention may be used to bone regeneration, which the bone including but not limited to a tibia, fibula, femur, pelvis, humerus, ulna, radius, metacarpal and metatarsal.

[187] The cell permeable recombinant proteins may be delivered to the interior of the cell, eliminating the need to transfect or transform the cell with a recombinant vector. The cell permeable recombinant proteins according to one embodiment of the present invention may be used *in vitro* to investigate protein function or may be used to maintain cells in a desired state.

[188] Still another embodiment of the present invention provides a composition including the CP-BMP recombinant protein as an active ingredient. The composition may

include CP-BMP2, CP-BMP7 or both CP-BMP2 and CP-BMP7 as an active ingredient. Preferably, the composition may include CP-BMP 2 or CP-BMP7, and more preferably, both CP-BMP2 and CP-BMP7 for effective bone regeneration.

[189] Still another embodiment of the present invention provides a pharmaceutical composition for regenerating of defected bone including the CP-BMP recombinant protein as an active ingredient; and a pharmaceutically acceptable carrier.

[190] Preferably, the composition may be for injectable (e.g. intraperitoneal, intravenous, subcutaneous, and intra-arterial, etc.) and may include the active ingredient in an amount of 75 to 600 ug/defected site, preferably 75 to 300 ug/defected site, more preferably 75 to 150 ug /defected site.

[191] In the treatment of adult humans, the range of 75 to 150 ug/defected site/day in single or divided dose, is especially preferred. However, it will be understood that the concentration of the CP-BMP recombinant protein actually administered will be determined by a physician, in the light of the relevant circumstances, including the condition to be treated, the chosen route of administration, the age, weight, and response of the individual patient, and the severity of the patient's symptoms, and therefore the above dosage ranges are not intended to limit the scope of the invention in any way. In some instances dosage levels below the lower limit of the aforesaid range may be more than adequate, while in other cases still larger doses may be employed without causing any harmful side effect, provided that such larger doses are first divided into several smaller doses for administration throughout the day.

[192] Still another embodiment of the present invention provides use of the CP-BMP recombinant protein as a medicament for regenerating of defected bone.

[193] Still another embodiment of the present invention provides a medicament including the CP-BMP recombinant protein.

[194] Still another embodiment of the present invention provides use of the CP-BMP recombinant protein for the preparation of a medicament for regenerating of defected bone.

[195] Still another embodiment of the present invention provides a method of regenerating of defected bone, preparing defected bone; and treating the defected bone with an therapeutically effective amount of the CP-BMP recombinant protein. According to the method, the CP-BMP recombinant protein may be administrated or treated to the site of defected bone, and can induces bone regeneration and formation.

[196] In one embodiment of the present invention, the subject may be preferably a mammal.

[197] The pharmaceutical composition according to one embodiment of the present invention may be prepared by using pharmaceutically suitable and physiologically acceptable additives, in addition to the active ingredient, and the additives may include

excipients, disintegrants, sweeteners, binders, coating agents, blowing agents, lubricants, glidants, flavoring agents, etc.

[198] For administration, the pharmaceutical composition may be preferably formulated by further including one or more pharmaceutically acceptable carriers in addition to the above-described active ingredient.

[199] Dosage forms of the pharmaceutical composition may include granules, powders, tablets, coated tablets, capsules, suppositories, liquid formulations, syrups, juice, suspensions, emulsions, drops, injectable liquid formulations, etc. For formulation of the composition into a tablet or capsule, for example, the active ingredient may be combined with any oral, non-toxic pharmaceutically acceptable inert carrier, such as ethanol, glycerol, water, etc. If desired or necessary, suitable binders, lubricants, disintegrants, and colorants may be additionally included as a mixture.

[200] Examples of the suitable binder may include, but are not limited to, starch, gelatin, natural sugars such as glucose or beta-lactose, corn sweeteners, natural and synthetic gums such as acacia, tragacanth, or sodium oleate, sodium stearate, magnesium stearate, sodium benzoate, sodium acetate, sodium chloride, etc. Examples of the disintegrant may include, but are not limited to, starch, methyl cellulose, agar, bentonite, xanthan gum, etc. For formulation of the composition into a liquid preparation, a pharmaceutically acceptable carrier which is sterile and biocompatible may be used, such as saline, sterile water, a Ringer's solution, buffered saline, an albumin infusion solution, a dextrose solution, a maltodextrin solution, glycerol, and ethanol, and these materials may be used alone or in any combination thereof. If necessary, other common additives, such as antioxidants, buffers, bacteriostatic agents, etc., may be added. Further, diluents, dispersants, surfactants, binders, and lubricants may be additionally added to prepare injectable formulations such as aqueous solutions, suspensions, and emulsions, or pills, capsules, granules, or tablets. Furthermore, the composition may be preferably formulated, depending upon diseases and ingredients, using any appropriate method known in the art, as disclosed in Remington's Pharmaceutical Science, Mack Publishing Company, Easton PA.

[201] Preferably, the treatment or treating mean improving or stabilizing the subject's condition or disease; or preventing or relieving the development or worsening of symptoms associated with the subject's condition or disease.

[202] The subject and patient are used herein interchangeably. They refer to a human or another mammal (e.g., mouse, rat, rabbit, dog, cat, cattle, swine, sheep, horse or primate) that can be afflicted with or is susceptible to a disease or disorder (e.g., PD) but may or may not have the disease or disorder. In certain embodiments, the subject is a human being.

[203] Preferably, the amount effective or effective amount is the amount of an active in-

gredient or a pharmaceutical composition disclosed herein that when administered to a subject for treating a disease, is sufficient to effect such treatment of the disease. Any improvement in the patient is considered sufficient to achieve treatment. An effective amount of an active ingredient or a pharmaceutical composition disclosed herein, used for the regeneration of defected bone can vary depending upon the manner of administration, the age, body weight, and general health of the patient. Ultimately, the prescribers or researchers will decide the appropriate amount and dosage regimen.

[204] In the treatment or prevention method according to one embodiment of the present invention, the composition including the CP-BMP recombinant protein as an active ingredient may be administered in a common manner via oral, buccal, rectal, intravenous, intra-arterial, intraperitoneal, intramuscular, intrasternal, percutaneous, topical, intraocular or subcutaneous route, more preferably via subcutaneous or intravenous injection route.

Advantageous Effects of Invention

[205] One embodiment of the present invention provides artificially constructed aMTD sequences based on the critical factors (CFs) that overcome the limitations of prior arts (MTM/MTS/MTD), such as limited diversity and unpredictable cell-permeability. Based on the CFs that assure the cell-permeability, the aMTD displays these sequences shows up to 109.9 relative fold enhanced ability compared to prior arts thereof to deliver biologically active macromolecules into live cells. Therefore, one embodiment of the present invention would allow their practically effective applications in molecule delivery, drug delivery, protein therapy, intracellular protein therapy, protein replacement therapy, peptide therapy, gene delivery and so on.

[206] With enhanced solubility and yield, aMTD/SD-fused BMP recombinant protein could be produced in large quantities. In addition, effective cell-permeability of the recombinant protein overcomes the limitations of previously developed bone regeneration. Therefore, CP-BMP recombinant protein of the present invention would allow practical applications to efficiently bone regeneration for recovery of bone defected by osteogenesis imperfect, osteoporosis, facture and osteoectomy.

Brief Description of Drawings

[207] FIG. 1 shows Structure of aMTD- or rPeptide-Fused Recombinant Proteins. A schematic diagram of the His-tagged CRA recombinant proteins is illustrated and constructed according to one embodiment of the present invention. The his-tag for affinity purification (white), aMTD or rPeptide (gray) and cargo A (CRA, black) are shown.

[208] FIGs. 2a to 2c show Construction of Expression Vectors for aMTDs- or rPeptide-Fused Recombinant Proteins. These FIGs. show the agarose gel electrophoresis analysis showing plasmid DNA fragments at 645 bp insert encoding aMTDs or

rPeptide-fused CRA cloned into the pET-28a(+) vector according to one embodiment of the present invention.

[209] FIGs. 3a to 3d show Inducible Expression of aMTD- or rPeptide-Fused Recombinant Proteins. Expressed recombinant aMTD- or random peptide-fused CRA recombinant proteins were transformed in *E.* coli BL21(DE3) strain. Expression of recombinant proteins in *E.* coli before (-) and after (+) induction with IPTG was monitored by SDS-PAGE, and stained with Coomassie blue.

[210] FIGs. 4a and 4b show Purification of aMTD- or rPeptide-Fused Recombinant Proteins. Expressed recombinant proteins were purified by Ni^{2+} affinity chromatography under the natural condition. Purification of recombinant proteins displayed through SDS-PAGE analysis.

[211] FIGs. 5a to 5u show Determination of aMTD-Mediated Cell-Permeability. Cell- permeability of a negative control (A: rP38) and reference hydrophobic CPPs (MTM12 and MTD85) are shown. The cell-permeability of each aMTD and/or rPeptide is visually compared to that of the cargo protein lacking peptide sequence (HCA). Gray shaded area represents untreated RAW 264.7 cells (vehicle); thin light gray line represents the cells treated with equal molar concentration of FITC (FITC only); dark thick line indicates the cells treated with FITC-his-tagged CRA protein (HCA); and the cells treated with the FITC-proteins (HMCA) fused to negative control (rP38), reference CPP (MTM12 or MTD85) or new hydrophobic CPP (aMTD) are shown with light thick line and indicated by arrows.

[212] FIGs. 6a to 6c show Determination of rPeptide-Mediated Cell-Permeability. The cell-permeability of each aMTD and/or rPeptide was visually compared to that of the cargo protein lacking peptide sequence (HCA). Gray shaded area represents untreated RAW 264.7 cells (vehicle); thin light gray line represents the cells treated with equal molar concentration of FITC (FITC only); dark thick line indicates the cells treated with FITC-his-tagged CRA protein (HCA); and the cells treated with the FITC-proteins fused to rPeptides are shown with light thick line and indicated by arrows.

[213] FIGs. 7a to 7k shows Visualized Cell-Permeability of aMTD-Fused Recombinant Proteins. NIH3T3 cells were treated with FITC-labeled protein (10 uM) fused to aMTD for 1 hour at 37℃. Cell-permeability of the proteins was visualized by laser scanning confocal microscopy (LSM700 version).

[214] FIGs. 8a to 8b show Visualized Cell-Permeability of rPeptide-Fused Recombinant Proteins. Cell-permeability of rPeptide-fused recombinant proteins was visualized by laser scanning confocal microscopy (LSM700 version).

[215] FIGs. 9a to 9c show Relative Cell-Permeability of aMTD-Fused Recombinant Proteins Compared to Negative Control (rP38). The FIGs. show graphs comparing the cell-permeability of the recombinant proteins fused to aMTDs and a negative control

(A: rP38).

[216] FIGs. 10a to 10c show Relative Cell-Permeability of aMTD-Fused Recombinant Proteins Compared to Reference CPP (MTM12). The FIGs. show graphs comparing the cell-permeability of the recombinant proteins fused to aMTDs and a reference CPP (MTM12).

[217] FIGs. 11a to 11c show Relative Cell-Permeability of aMTD-Fused Recombinant Proteins Compared to Reference CPP (MTD85). The FIGs. show graphs comparing the cell-permeability of the recombinant proteins fused to aMTDs and a reference CPP (MTD85).

[218] FIG. 12 shows Relative Cell-Permeability of rPeptide-Mediated Recombinant Proteins Compared to Average that of aMTDs. The FIG. shows graphs comparing the cell-permeability of the recombinant proteins fused to rPeptides and that (average value: aMTD AVE) of aMTDs.

[219] FIGs. 13a and 13b show Association of Cell-Permeability with Amino Acid Composition in aMTD Sequences. These graphs display delivery potential (Geometric Mean) of aMTDs influenced with amino acid composition (A, I, V and L).

[220] FIGs. 14a and 14b show Association of Cell-Permeability with Critical Factors in aMTDs. These graphs show the association of cell-permeability with critical factors [bending potential: proline position (PP), rigidity/flexibility: instability index (II), structural feature: aliphatic index (AI) and hydropathy: grand average of hydropathy (GRAVY)].

[221] FIGs. 15a and 15b show Relative Relevance of aMTD-Mediated Cell-Permeability with Critical Factors. Cell-permeability of 10 high and 10 low ranked aMTDs in their delivery potential were examined for their association with the critical factors [bending potential: proline position (PP), rigidity/flexibility: instability index (II), structural feature: aliphatic index (AI) and hydropathy: grand average of hydropathy (GRAVY)].

[222] FIG. 16 shows Relative Relevance of rPeptide-Mediated Cell-Permeability with Hydropathy Range (GRAVY). This graph and a chart illustrate relative relevance of rPeptide-mediated cell-permeability with its hydropathy range (GRAVY).

[223] FIG. 17 shows Structural Features of BMP2 and BMP7. A structural composition of BMP families is illustrated and structure design for BMP2/7 recombinant proteins in present invention is based on their basic structure.

[224] FIG. 18a shows Schematic Diagram of His-Tagged BMP2/7 (M form) Recombinant Proteins. Design of BMP2/7 (M form) recombinant proteins containing histidine tag for affinity purification, BMP2/7 (MP), $aMTD_{24}$, solubilization domain A (SDA), and/or solubilization domain B (SDB).

[225] FIG. 18b shows Schematic Diagram of His-Tagged BMP2/7 (L form) Recombinant Proteins. Design of BMP2/7 (L form) recombinant proteins containing histidine tag for

affinity purification, BMP2/7 (LAP+MP), $aMTD_{24}$, solubilization domain A (SDA), and/or solubilization domain B (SDB).

[226] FIG. 19a shows Construction of Expression for His-Tagged BMP2 (M form) Recombinant Proteins. This figure show the agarose gel electrophoresis analysis show plasmid DNA fragments encoding BMP2 (MP) cloned into the pET-28a(+) vector according to one embodiment of the present invention aMTD-fused BMP2 (MP) and SD.

[227] FIG. 19b shows Construction of Expression for His-Tagged BMP7 (M form) Recombinant Proteins. These agarose gel electrophoresis analysis show plasmid DNA fragments encoding BMP7 (MP) cloned into the pET-28a(+) vector according to one embodiment of the present invention aMTD fused BMP7 (MP) and SD.

[228] FIG. 19c shows Construction of Expression for His-Tagged BMP2 (L form) Recombinant Proteins. These agarose gel electrophoresis analysis show plasmid DNA fragments encoding BMP2 (LAP+MP: LP) cloned into the pET-28a(+) vector according to one embodiment of the present invention aMTD fused BMP2 (LP) and SD.

[229] FIG. 19d shows Construction of Expression for His-Tagged BMP7 (L form) Recombinant Proteins. These agarose gel electrophoresis analysis show plasmid DNA fragments encoding BMP7 (LAP+MP: LP) cloned into the pET-28a(+) vector according to one embodiment of the present invention aMTD fused BMP7 (LP) and SD.

[230] FIG. 20a shows Inducible Expression and Purification of BMP2 (M form/L form) Recombinant Proteins. Expression of BMP2 recombinant proteins in *E. coli* before (-) and after (+) induction with IPTG, and purification by Ni^{2+} affinity chromatography (P) were confirmed by SDS-PAGE which stained with Coomassie Brilliant Blue.

[231] FIG. 20b shows Inducible Expression and Purification of BMP7 (M form / L form) Recombinant Proteins. Expression of BMP7 recombinant proteins in *E. coli* before (-) and after (+) induction with IPTG, and purification by Ni^{2+} affinity chromatography (P) were confirmed by SDS-PAGE which stained with Coomassie Brilliant Blue.

[232] FIG. 21 shows Structural Changes of BMP2/7 (L form) Recombinant Proteins. Additional designs (A, B, C) of BMP2/7 (LAP+MP: LP) recombinant recombinant proteins contained histidine tag for affinity purification (white), BMP2/7 (LP), $aMTD_{123}$ (black), solubilization domain A (SDA), and/or solubilization domain B (SDB) or solubilization domain C (SDC).

[233] FIG. 22 shows Construction of Expression for Newly Designed BMP2/7 (L form) Recombinant Proteins. These agarose gel electrophoresis analysis show plasmid DNA fragments encoding newly designed BMP2 (LAP+MP: LP) cloned into the pET-28a(+) vector according to one embodiment of the present invention aMTD fused BMP2 (LP)

and SD.

[234] FIG. 23a shows Inducible Expression and Purification of Newly Designed BMP2 (L form) Recombinant Proteins. Expression of BMP2 (L form) recombinant proteins before (-) and after (+) induction with IPTG, and purification by Ni^{2+} affinity chromatography (P) were confirmed by SDS-PAGE analysis which stained with Coomassie Brilliant Blue.

[235] FIG. 23b shows Inducible Expression and Purification of Newly Designed BMP7 (L form) Recombinant Proteins. Expression of BMP7 (L form) recombinant proteins before (-) and after (+) induction with IPTG, and purification by Ni^{2+} affinity chromatography (P) were confirmed by SDS-PAGE analysis which stained with Coomassie Brilliant Blue.

[236] FIG. 24 shows aMTD-Mediated Cell-Permeability of BMP2/7 (M form) Recombinant Proteins. RAW 264.7 cells were exposure to FITC-labeled BMP2/7 recombinant proteins (10 uM) compared with control protein fused with/without aMTD and solubilization domain A or B (10 uM) for 1 hour, treated with proteinase K to remove cell associated but non-internalized proteins and analyzed by FACS. Gray shaded area represents untreated RAW 264.7 cells (vehicle) and equimolar concentration of unconjugated FITC (FITC-only, green)-treated cells were served as control.

[237] FIG. 25 shows aMTD-Mediated Intracellular Delivery and Localization of BMP2/7 (M form) Recombinant Proteins. Fluorescence confocal laser scanning microscopy shows intracellular localization of BMP2/7 (M form) recombinant proteins in NIH3T3 cells after incubated with 10 uM of FITC-conjugated BMP2/7 recombinant proteins, unconjugated FITC (FITC-only) or protein physiological buffer (vehicle) for 1 hour. Nomarski images are provided to show their cell morphology.

[238] FIG. 26 shows Tissue Distribution of CP-BMP2/7 Recombinant Proteins. Cryosection of saline-perfused organs were prepared from mice 1 hour after the intraperitoneal injection of the BMP2/7 recombinant proteins, vehicle, or FITC only. The images from fluorescence microscopy shows distribution of BMP2/7 recombinant proteins in various organs.

[239] FIG. 27 shows Morphological Differentiation in C2C12 Myoblasts with CP-BMP2/7 Recombinant Proteins. The images of cells show the morphology of the C2C12 myoblasts after treatment of BMP2/7 (M form) recombinant proteins with dose variation. (x100 magnification). The C2C12 cells were treated with the BMP2/7 recombinant proteins for 7 days. The proteins were freshly replaced every day. To compare the effect of CP-BMP2/7 recombinant proteins, the morphology is compared with BMP2/7 (M form) recombinant proteins.

[240] FIG. 28 shows Stimulatory Effect of CP-BMP2/7 Recombinant Proteins on Smad

Signaling in C2C12 Cells. The C2C12 cells were treated for 15 minutes with 10 uM BMP2/7 (M form) recombinant proteins and then extracted protein in these cells. The cell lysates were analyzed for phosphorylated Smad-1/5/8 and β-actin expression.

[241] FIG. 29 shows Stimulatory Effect of CP-BMP2/7 Recombinant Proteins on ALP Activity in MC3T3-E1 Cells. The BMP2/7 recombinant proteins (10 uM) were continuously treated for 5 days and then measured ALP activity.

[242] FIG. 30 shows Osteogenic Differentiation of C2C12 Myoblasts by Using Combinational Treatment of CP-BMP2 and CP-BMP7 Recombinant Proteins. The images of cells, which were continuously treated with vehicle (control) or 1 uM of CP-BMP2/7 recombinant proteins (x100 magnification) for 7 days.

[243] FIG. 31 shows ALP Activity of C1C12 Myoblasts by Using Combinational Treatment of CP-BMP2 and CP-BMP7 Recombinant Proteins. The CP-BMP2/7 recombinant proteins (10 uM) were continuously treated for 5 days and then measured ALP activity.

[244] FIG. 32 shows Osteoblastic Effect of CP-BMP2/7 Recombinant Protein in Calvarial Injection Mouse Models. Hematoxylin and Eosin (H&E)-stained calvarial bone sections in diluent, BMP2/7 recombinant protein treated groups (x400). Arrows indicate newly formed bone matrix.

[245] FIG. 33 shows Relative Activity of CP-BMP2/7 recombinant proteins on New Bone Formation in Calvarial Injection Mouse Models. The graph compared the newly formed ECM thickness of aMTD/SD-fused CP-BMP2/7 recombinant proteins or aMTD lacking SD-fused BMP2/7 recombinant proteins with protein physiological buffer (diluent).

[246] FIG. 34 shows Structure of CP-BMP2 Recombinant Proteins fused various aMTDs and SDA.

[247] FIGs. 35a and 35b show Inducible Expression and Purification of Newly Designed CP-BMP2 Recombinant Proteins. Expression of CP-BMP2 recombinant proteins before (-) and after (+) induction with IPTG, and purification by Ni^{2+} affinity chromatography (P) were confirmed by SDS-PAGE analysis which stained with Coomassie Brilliant Blue.

[248] FIG. 36a shows aMTD-Mediated Cell-Permeability of CP-BMP2 Recombinant Proteins fused various aMTDs.

[249] FIG. 36b shows Quantitative aMTD-Mediated Cell-Permeability of CP-BMP2 Recombinant Proteins fused various aMTDs.

[250] FIG. 37 shows Stimulatory Effect of CP-BMP2 Recombinant Proteins on ALP Activity in C3H10T1/2 Cells.

[251] FIG. 38 shows aMTD-Mediated Cell-Permeability of CP-BMP2 Recombinant Proteins in RAW 264.7 cells.

[252] FIG. 39 shows aMTD-Mediated Intracellular Delivery and Localization of CP-BMP2/7 Recombinant Proteins.

[253] FIG. 40a shows Schematic Diagram of BMP2 Recombinant Protein Effects in C2C12 Trans-differentiation into Osteoblasts.

[254] FIG. 40b shows CP-BMP2 Recombinant Protein Inhibits Myotube Formation.

[255] FIG. 41 shows CP-BMP2 Recombinant Protein Improves ALP Activity.

[256] FIG. 42 shows CP-BMP2 Recombinant Protein Induces Smad-mediated Osteogenic Differentiation.

[257] FIG. 43 shows CP-BMP2 Recombinant Protein and BMPR II Co-localize in Golgi, ER and/or Nucleus.

[258] FIG. 44a shows CP-BMP2 Recombinant Protein Induces New Bone Formation (Calvarial Injection Assay).

[259] FIG. 44b shows Quantification of New Bone Formation Induced by CP-BMP2 Recombinant Protein (Calvarial Injection Assay).

[260] FIG. 45a shows CP-BMP2 Recombinant Protein Enhances Bone Regeneration (Calvarial Critical-sized Defect Model).

[261] FIG. 45b shows Quantification of Bone Regeneration Induced by CP-BMP2 Recombinant Protein (Calvarial Critical-sized Defect Model).

[262] FIG. 46a shows CP-BMP2 Recombinant Protein Significantly Induces Bone Regeneration with Reduced Administration Frequency.

[263] FIG. 46b shows Quantification of Bone Regeneration Induced by CP-BMP2 Recombinant Protein with Reduced Administration Frequency.

[264] FIG. 47a shows CP-BMP2 Recombinant Protein Induces Bone Regeneration with Dose-dependent Manner.

[265] FIG. 47b shows Quantification of Bone Regeneration Induced by CP-BMP2 Recombinant Protein with Dose-dependent Manner.

[266] FIG. 48 shows Structures of rhBMP2, rBMP2 and CP-BMP2 Recombinant Proteins.

[267] FIG. 49a shows Horses Characteristics Used Equine Bone Defect Model.

[268] FIG. 49b shows Design of Equine Bone Defect Model.

[269] FIG. 50 shows CP-BMP2 Recombinant Protein Have Similar Osteogenic Activity To Original BMP2 And Much Better Therapeutic Applicability Even Without Scaffold.

[270] FIG. 51 shows CP-BMP2 Recombinant Protein Is Very Safe Protein *in* vivo (Single Dose Acute Toxicity).

[271] FIGs. 52a and 52b show CP-BMP2 Recombinant Protein Is Very Safe Protein *in* vivo (Repeated Dose Toxicity Assay).

[272] FIG. 53 shows CP-BMP2 Recombinant Protein Shows Longer Persistency Than Plain-BMP2 Recombinant Protein *In* Vivo Bioavailability.

[273] FIG. 54 shows CP-BMP2 recombinant protein Is More Stable Than Plain-BMP2 Re-

combinant Protein In Blood.

[274] FIG. 55 shows CP-BMP2 Recombinant Protein Shows Longer Persistence Than Non-CP-BMP2 Recombinant Protein.

Mode for the Invention

[275] **1. Analysis of Reference Hydrophobic** CPPs to Identify 'Critical Factors' for Development of Advanced MTDs

[276] Previously reported MTDs were selected from a screen of more than 1,500 signal peptide sequences. Although the MTDs that have been developed did not have a common sequence or sequence motif, they were all derived from the hydrophobic (H) regions of signal sequences (HRSSs) that also lack common sequences or motifs except their hydrophobicity and the tendency to adopt alpha-helical conformations. The wide variation in H-region sequences may reflect prior evolution for proteins with membrane translocating activity and subsequent adaptation to the SRP/Sec61 machinery, which utilizes a methionine-rich signal peptide binding pocket in SRP to accommodate a wide-variety of signal peptide sequences.

[277] Previously described hydrophobic CPPs (e.g. MTS/MTM and MTD) were derived from the hydrophobic regions present in the signal peptides of secreted and cell surface proteins. The prior art consists first, of ad hoc use of H-region sequences (MTS/MTM), and second, of H-region sequences (with and without modification) with highest CPP activity selected from a screen of 1,500 signal sequences (MTM). Second prior art, the modified H-region derived hydrophobic CPP sequences had advanced in diversity with multiple number of available sequences apart from MTS/MTM derived from fibroblast growth factor (FGF) 4. However, the number of MTDs that could be modified from naturally occurring secreted proteins are somewhat limited. Because there is no set of rules in determining their cell-permeability, no prediction for the cell-permeability of modified MTD sequences can be made before testing them.

[278] The hydrophobic CPPs, like the signal peptides from which they originated, did not conform to a consensus sequence, and they had adverse effects on protein solubility when incorporated into protein cargo. We therefore set out to identify optimal sequence and structural determinants, namely critical factors (CFs), to design new hydrophobic CPPs with enhanced ability to deliver macromolecule cargoes including proteins into the cells and tissues while maintaining protein solubility. These newly developed CPPs, advanced macromolecule transduction domains (aMTDs) allowed almost infinite number of possible designs that could be designed and developed based on the critical factors. Also, their cell-permeability could be predicted by their character analysis before conducting any *in vitro* and/or *in* vivo experiments. These critical factors below have been developed by analyzing all published reference hy-

drophobic CPPs.

[279]

[280] **1-1. Analysis of Hydrophobic** CPPs

[281] Seventeen different hydrophobic CPPs (Table 1) published from 1995 to 2014 (Table 2) were selected. After physiological and chemical properties of selected hydrophobic CPPs were analyzed, 11 different characteristics that may be associated with cell-permeability have been chosen for further analysis. These 11 characteristics are as follows: sequence, amino acid length, molecular weight, pI value, bending potential, rigidity/flexibility, structural feature, hydropathy, residue structure, amino acid composition and secondary structure of the sequences (Table 3).

[282] Table 1 shows the Summary of Published Hydrophobic Cell-Penetrating Peptides which were Chosen.

[283] [Table 1]

#	Pepides	Origin	Protein	Ref.
1	MTM	Homo sapiens	NP_001998 Kaposi fibroblast growth factor (K-FGF)	1
2	MTS	Homo sapiens	NP_001998 Kaposi fibroblast growth factor (K-FGF)	2
3	MTD10	Streptomyces coelicolor	NP_625021 Glycosyl hydrolase	8
4	MTD13	Streptomyces coelicolor	NP_639877 Putative secreted protein	3
5	MTD47	Streptomyces coelicolor	NP_627512 Secreted protein	4
6	MTD56	Homo sapiens	P23274 Peptidyl-prolyl cis-trans isomerase B precursor	5
7	MTD73	Drosophila melanogaster	AAA17887 Spatzle (spz) protein	5
8	MTD77	Homo sapiens	NP_003231 Kaposi fibroblast growth factor (K-FGF)	6
9	MTD84	Phytophthora cactorum	AAK63068 Phytotoxic protein PcF precusor	4
10	MTD85	Streptomyces coelicolor	NP_629842 Peptide transport system peptide binding protein	7
11	MTD86	Streptomyces coelicolor	NP_629842 Peptide transport system secreted peptide binding protein	7
12	MTD103	Homo sapiens	TMBV19 domain Family member B	8
13	MTD132	Streptomyces coelicolor	NP_628377 P60-family secreted protein	4
14	MTD151	Streptomyces coelicolor	NP_630126 Secreted chitinase	8
15	MTD173	Streptomyces coelicolor	NP_624384 Secreted protein	4
16	MTD174	Streptomyces coelicolor	NP_733505 Large, multifunctional secreted protein	8
17	MTD181	Neisseria meningitidis Z2491	CAB84257.1 Putative secreted protein	4

[284] Table 2 shows the Summarizes Reference Information.

[285] [Table 2]

#	References					
	Title	Journal	Year	Vol	Issue	Page
1	Inhibition of Nuclear Translocation of Transcription Factor NF-kB by a Synthetic peptide Containing a Cell Membrane-permeable Motif and Nuclear Localization Sequence	JOURNAL OF BIOLOGICAL CHEMISTRY	1995	270	24	14255
2	Epigenetic Regulation of Gene Structure and Function with a Cell-Permeable Cre Recombinase	NATURE BIOTECHNOLOGY	2001	19	10	929
3	Cell-Permeable NM23 Blocks the Maintenance and Progression of Established Pulmonary Metastasis	CANCER RESEARCH	2011	71	23	7216
4	Antitumor Activity of Cell-Permeable p18INK4c With Enhanced Membrane and Tissue Penetration	MOLECULAR THERAPY	2012	20	8	1540
5	Antitumor Activity of Cell-Permeable RUNX3 Protein in Gastric Cancer Cells	CLINICAL CANCER RESEARCH	2012	19	3	680
6	The Effect of Intracellular Protein Delivery on the Anti-Tumor Activity of Recombinant Human Endostatin	BIOMATERIALS	2013	34	26	6261
7	Partial Somatic to Stem Cell Transformations Induced By Cell-Permeable Reprogramming Factors	SCIENTIFIC REPORTS	2014	4	10	4361
8	Cell-Permeable Parkin Proteins Suppress Parkinson Disease-Associated Phenotypes in Cultured Cells and Animals	PLOS ONE	2014	9	7	17

[286] Table 3 shows the Characteristics of Published Hydrophobic Cell-Penetrating Peptides (A) which were Analyzed.

[287] [Table 3]

#	Peptides	Sequence	Length	Molecular Weight	pI	Bending Potential	Rigidity/ Flexibility (Instability Index : II)	Structural Feature (Aliphatic Index : AI)	Hydropathy (GRAVY)	Residue Structure	A/a Composition						Secondary Structure	Cargo	Ref.
											A	V	L	I	P	G			
1	MTM	AAVALLPAVLLALLAP	16	1,515.9	5.6	Bending	45.5	220.0	2.4	Aliphatic Ring	6	2	6	0	2	0	Helix	p50	1
2	MTS	AAVLLPVLLAAP	12	1,147.4	5.6	Bending	57.3	211.7	2.3	"	4	2	4	0	2	0	No-Helix	CRE	2
3	MTD10	LGGAVVAAPVAAAVAP	16	1,333.5	5.5	Bending	47.9	140.6	1.8	"	7	4	1	0	2	2	Helix	Parkin	8
4	MTD13	LAAAALAVLPL	11	1,022.3	5.5	Bending	26.6	213.6	2.4	"	5	1	4	0	1	0	No-Helix	RUNX3	3
5	MTD47	AAAVPVLVAA	10	881.0	5.6	Bending	47.5	176.0	2.4	"	5	3	1	0	1	0	No-Helix	CMYC	4
6	MTD56	VLLAAALIA	9	854.1	5.5	No -Bending	8.9	250.0	3.0	"	4	1	3	1	0	0	Helix	ES	5
7	MTD73	PVLLLLA	7	737.9	6.0	No -Bending	36.1	278.6	2.8	"	1	1	4	0	1	0	Helix	ES	5
8	MTD77	AVALLILAV	9	882.1	5.6	No -Bending	30.3	271.1	3.3	"	3	2	3	1	0	0	Helix	NM23	6
9	MTD84	AVALVAVVAVA	11	982.2	5.6	No -Bending	9.1	212.7	3.1	"	5	5	1	0	0	0	Helix	OCT4	4
10	MTD85	LLAAAAALLLA	11	1,010.2	5.5	No -Bending	9.1	231.8	2.7	"	6	0	5	0	0	0	No-Helix	RUNX3	7
11	MTD86	LLAAAAALLLA	11	1,010.2	5.5	No -Bending	9.1	231.8	2.7	"	6	0	5	0	0	0	No-Helix	SOX2	7
12	MTD103	LALPVLLLA	9	922.2	5.5	Bending	51.7	271.1	2.8	"	2	1	5	0	1	0	Helix	p18	8
13	MTD132	AVVVPAIVLAAP	12	1,119.4	5.6	Bending	50.3	195.0	2.4	"	4	4	1	1	2	0	No-Helix	LIN28	4
14	MTD151	AAAPVAAVP	9	1,031.4	5.5	Bending	73.1	120.0	1.6	"							No-Helix	Parkin	8
15	MTD173	AVIPILAVP	9	892.1	5.6	Bending	48.5	216.7	2.4	"	2	2	1	2	2	0	Helix	KLF4	4
16	MTD174	LILLLPAVALP	12	1,011.8	5.5	Bending	79.1	257.3	2.6	"							Helix	Parkin	8
17	MTD181	AVLLLPAAA	9	838.0	5.6	Bending	51.7	206.7	2.4	"	4	1	3	0	1	0	No-Helix	SOX2	4
		AVE	10.8 ±2.4	1,011 ±189.6	5.6 ±0.1	Proline Presence	40.1 ±21.9	217.9 ±43.6	2.5 ±0.4										

[288] Two peptide/protein analysis programs were used (ExPasy: SoSui: http://harrier.nagahama-i-bio.ac.jp/sosui/sosui_submit.html) to determine various indexes and structural features of the peptide sequences and to design new sequence.

Followings are important factors analyzed.

[289]

[290] **1-2. Characteristics of Analyzed Peptides: Length, Molecular Weight and pI Value**

[291] Average length, molecular weight and pI value of the peptides analyzed were 10.8±2.4, 1,011±189.6 and 5.6±0.1, respectively (Table 4)

[292] Table 4 shows the Summarizes Critical Factors (CFs) of Published Hydrophobic Cell-Penetrating Peptides (A) which were Analyzed.

[293] [Table 4]

- Length: **10.8 ± 2.4**
- Molecular Weight: **1,011 ± 189.6**
- pI: **5.6 ± 0.1**
- Bending Potential (BP): **Proline presences In the middle and/or the end of peptides, or No Proline.**
- Instability Index (II): **40.1 ± 21.9**
- Residue Structure & Aliphatic Index (AI): **217.9 ± 43.6**
- Hydropathy (GRAVY): **2.5 ± 0.4**
- Aliphatic Ring: **Non-polar hydrophobic & aliphatic amino acid (A, V, L, I).**
- Secondary Structure: **α-Helix is favored but not required.**

[294]

[295] **1-3. Characteristics of Analyzed Peptides: Bending Potential** - Proline Position (PP)

[296] Bending potential (bending or no-bending) was determined based on the fact whether proline (P) exists and/or where the amino acid(s) providing bending potential to the peptide in recombinant protein is/are located. Proline differs from the other common amino acids in that its side chain is bonded to the backbone nitrogen atom as well as the alpha-carbon atom. The resulting cyclic structure markedly influences protein architecture which is often found in the bends of folded peptide/protein chain.

[297] Eleven out of 17 were determined as 'Bending' peptide which means that proline is present in the middle of sequence for peptide bending and/or located at the end of the peptide for protein bending. As indicated above, peptide sequences could penetrate the plasma membrane in a "bent" configuration. Therefore, bending or no-bending potential is considered as one of the critical factors for the improvement of current hydrophobic CPPs.

[298]

[299] **1-4. Characteristics of Analyzed Peptides: Rigidity/Flexibility - Instability Index (II)**

[300] Since one of the crucial structural features of any peptide is based on the fact whether the motif is rigid or flexible, which is an intact physicochemical characteristic of the peptide sequence, instability index (II) of the sequence was determined. The index value representing rigidity/flexibility of the peptide was extremely varied (8.9 to 79.1), but average value was 40.1±21.9 which suggested that the peptide should be somehow flexible, but not too much rigid or flexible (Table 3).

[301]

[302] **1-5. Characteristics of Analyzed Peptides: Structural Features - Structural Feature (Aliphatic Index: AI) and** Hydropathy (Grand Average of Hydropathy: GRAVY)

[303] Alanine (V), valine (V), leucine (L) and isoleucine (I) contain aliphatic side chain and are hydrophobic - that is, they have an aversion to water and like to cluster. These amino acids having hydrophobicity and aliphatic residue enable them to pack together to form compact structure with few holes. Analyzed peptide sequence showed that all composing amino acids were hydrophobic (A, V, L and I) except glycine (G) in only one out of 17 (MTD10 - Table 3) and aliphatic (A, V, L, I, and P). Their hydropathic index (Grand Average of Hydropathy: GRAVY) and aliphatic index (AI) were 2.5±0.4 and 217.9±43.6, respectively. Their amino acid composition is also indicated in the Table 3.

[304]

[305] **1-6. Characteristics of Analyzed Peptides: Secondary Structure (Helicity)**

[306] As explained above, the CPP sequences may be supposed to penetrate the plasma membrane directly after inserting into the membranes in a "bent" configuration with hydrophobic sequences having α-helical conformation. In addition, our analysis strongly indicated that bending potential was crucial for membrane penetration. Therefore, structural analysis of the peptides was conducted to determine whether the sequences were to form helix or not. Nine peptides were helix and eight were not (Table 3). It seems to suggest that helix structure may not be required.

[307]

[308] **1-7. Determination of Critical Factors** (CFs)

[309] In the 11 characteristics analyzed, the following 6 are selected namely "Critical Factors" for the development of new hydrophobic CPPs - advanced MTDs: amino acid length, bending potential (proline presence and location), rigidity/flexibility (instability index: II), structural feature (aliphatic index: AI), hydropathy (GRAVY) and amino acid composition/residue structure (hydrophobic and aliphatic A/a) (Tables 3 and

Table 4).

[310]

[311] **2. Analysis of Selected Hydrophobic** CPPs to Optimize 'Critical Factors'

[312] Since the analyzed data of the 17 different hydrophobic CPPs (analysis A, Tables 3 and 4) previously developed during the past 2 decades showed high variation and were hard to make common- or consensus- features, analysis B (Tables 5 and 6) and C (Tables 7 and 8) were also conducted to optimize the critical factors for better design of improved CPPs - aMTDs. Therefore, 17 hydrophobic CPPs have been grouped into two groups and analyzed the groups for their characteristics in relation to the cell permeable property. The critical factors have been optimized by comparing and contrasting the analytical data of the groups and determining the common homologous features that may be critical for the cell permeable property.

[313]

[314] **2-1. Selective Analysis (B) of Peptides Used to Biologically Active Cargo Protein for in vivo**

[315] In analysis B, eight CPPs were used with each biologically active cargo *in* vivo. Length was 11±3.2, but 3 out of 8 CPPs possessed little bending potential. Rigidity/ Flexibility (instability index: II) was 41±15, but removing one [MTD85: rigid, with minimal II (9.1)] of the peptides increased the overall instability index to 45.6±9.3. This suggested that higher flexibility (40 or higher II) is potentially be better. All other characteristics of the 8 CPPs were similar to the analysis A, including structural feature and hydropathy (Tables 5 and 6).

[316] Table 5 shows the Characteristics of Published Hydrophobic Cell-Penetrating Peptides (B): Selected CPPs That were Used to Each Cargo In Vivo.

[317] [Table 5]

#	Peptides	Sequence	Length	Molecular Weight	pI	Bending Potential	Rigidity/ Flexibility (Instability Index : II)	Structural Feature (Aliphatic Index : AI)	Hydropathy (GRAVY)	Residue Structure	A/a Composition						Secondary Structure	Cargo	Ref.
											A	V	L	I	P	G			
1	MTM	AAVALLPAVLLALLAP	16	1,515.9	5.6	Bending	45.5	220.0	2.4	Aliphatic Ring	6	2	6	0	2	0	Helix	p50	1
2	MTS	AAVLLPVLLAAP	12	1,147.4	5.6	Bending	57.3	211.7	2.3	"	4	2	4	0	2	0	No-Helix	CRE	2
3	MTD10	LGGAVVAAPVAAAVAP	16	1,333.5	5.5	Bending	47.9	140.6	1.8	"	7	4	1	0	2	2	Helix	Parkin	8
4	MTD73	PVLLLLA	7	737.9	6.0	No -Bending	36.1	278.6	2.8	"	1	1	4	0	1	0	Helix	ES	6
5	MTD77	AVALLILAV	9	882.1	5.6	No -Bending	30.3	271.1	3.3	"	3	2	3	1	0	0	Helix	NM23	3
6	MTD85	LLAAAAALLLA	11	1,010.2	5.5	No -Bending	9.1*	231.8	2.7	"	6	0	5	0	0	0	No-Helix	RUNX3	5
7	MTD103	LALPVLLLA	9	922.2	5.5	Bending	51.7	271.1	2.8	"	2	1	5	0	1	0	Helix	p18	4
8	MTD132	AVVVPAIVLAAP	12	1,119.4	5.6	Bending	50.3	195.0	2.4	"	4	4	1	1	2	0	No-Helix	LIN28	7
		AVE	11 ±3.2	1,083 ±252	5.6 ±0.1	Proline Presence	41 ± 15	227 ± 47	2.5 ± 0.4										

* Removing the MTD85 increases II to 45.6±9.3.

[318] Table 6 shows the Summarized Critical Factors of Published Hydrophobic Cell-Penetrating Peptides (B).

[319] [Table 6]

- Length: **11 ± 3.2**
- Molecular Weight: **1,083 ± 252**
- pI: **5.6 ± 0.1**
- Bending Potential (BP): **Proline presences in the middle and/or the end of peptides, or No Proline.**
- Instability Index (II): **41.0 ± 15** (* Removing the MTD85 increases II to 45.6 ± 9.3)
- Residue Structure & Aliphatic Index (AI): **227 ± 47**
- Hydropathy (GRAVY): **2.5 ± 0.4**
- Aliphatic Ring: **Non-polar hydrophobic & aliphatic amino acid (A, V, L, I).**
- Secondary Structure: **α-Helix is favored but not required.**

[320]

[321] **2-2. Selective Analysis (C) of Peptides That Provided Bending Potential and Higher Flexibility**

[322] To optimize the 'Common Range and/or Consensus Feature of Critical Factor' for the practical design of aMTDs and the random peptides (rPs or rPeptides), which were to prove that the 'Critical Factors' determined in the analysis A, B and C were correct to improve the current problems of hydrophobic CPPs - protein aggregation, low solubility/yield, and poor cell-/tissue-permeability of the recombinant proteins fused to the MTS/MTM or MTD, and non-common sequence and non-homologous structure of the peptides, empirically selected peptides were analyzed for their structural features and physicochemical factor indexes.

[323] Hydrophobic CPPs which did not have a bending potential, rigid or too much flexible sequences (too much low or too much high Instability Index), or too low or too high hydrophobic CPPs were unselected, but secondary structure was not considered because helix structure of sequence was not required.

[324] In analysis C, eight selected CPP sequences that could provide a bending potential and higher flexibility were finally analyzed (Table 7 and 8). Common amino acid length is 12 (11.6±3.0). Proline is presence in the middle of and/or the end of sequence. Rigidity/Flexibility (II) is 45.5 to 57.3 (Avg: 50.1±3.6). AI and GRAVY representing structural feature and hydrophobicity of the peptide are 204.7±37.5 and 2.4±0.3, respectively. All peptides are consisted with hydrophobic and aliphatic amino

acids (A, V, L, I, and P). Therefore, analysis C was chosen as a standard for the new design of new hydrophobic CPPs - aMTDs.

[325] Table 7 shows the Characteristics of Published Hydrophobic Cell-Penetrating Peptides (C): Selected CPPs that Provided Bending Potential and Higher Flexibility.

[326] [Table 7]

#	Peptides	Sequence	Length	Molecular Weight	pI	Bending Potential	Rigidity/ Flexibility (Instability Index : II)	Structural Feature (Aliphatic Index : AI)	Hydropathy (GRAVY)	Residue Structure	A/a Composition						Secondary Structure	Cargo	Ref.
											A	V	L	I	P	G			
1	MTM	AAVALLPAVLLALLAP	16	1515.9	5.6	Bending	45.5	220.0	2.4	Aliphatic Ring	6	2	6	0	2	0	Helix	p50	1
2	MTS	AAVLLPVLLAAP	12	1147.4	5.6	Bending	57.3	211.7	2.3	"	4	2	4	0	2	0	No-Helix	CRE	2
3	MTD10	LGGAVVAAPVAAAVAP	16	1333.5	5.5	Bending	47.9	140.6	1.8	"	7	4	1	0	2	2	Helix	Parkin	8
4	MTD47	AAAVPVLVAA	10	881.0	5.6	Bending	47.5	176.0	2.4	"	5	3	1	0	1	0	No-Helix	CMYC	4
5	MTD103	LALPVLLLA	9	922.2	5.5	Bending	51.7	271.1	2.8	"	2	1	5	0	1	0	Helix	p18	8
6	MTD132	AVVVPAIVLAAP	12	1119.4	5.6	Bending	50.3	195.0	2.4	"	4	4	1	1	2	0	No-Helix	LIN28	4
7	MTD173	AVIPILAVP	9	892.1	5.6	Bending	48.5	216.7	2.4	"	2	2	1	2	2	0	Helix	KLF4	4
8	MTD181	AVLLLPAAA	9	838.0	5.6	Bending	51.7	206.7	2.4	"	4	1	3	0	1	0	No-Helix	SOX2	4
		AVE	11.6 ±3.0	1081.2 ±244.6	5.6 ±0.1	Proline Presence	50.1 ±3.6	204.7 ±37.5	2.4 ±0.3										

[327] Table 8 shows the Summarized Critical Factors of Published Hydrophobic Cell-Penetrating Peptides (C).

[328] [Table 8]

- Length: **11.6 ± 3.0**
- Molecular Weight: **1,081.2 ± 224.6**
- pI: **5.6 ± 0.1**
- Bending Potential (BP): **Proline presences in the middle and/or the end of peptides.**
- Instability Index (II): **50.1 ± 3.6**
- Residue Structure & Aliphatic Index (AI): **204.7 ± 37.5**
- Hydropathy (GRAVY): **2.4 ± 0.3**
- Aliphatic Ring: **Non-polar hydrophobic & aliphatic amino acid (A, V, L, I).**
- Secondary Structure: **α-Helix is favored but not required.**

[329]

[330] **3. New Design of Improved Hydrophobic** CPPs - aMTDs Based on the Optimized Critical Factors

[331] **3-1. Determination of Common Sequence and/or Common Homologous Structure**

[332] As mentioned above, H-regions of signal sequence (HOURSS)-derived CPPs (MTS/MTM and MTD) do not have a common sequence, sequence motif, and/or common-structural homologous feature. According to one embodiment of the present invention, the aim is to develop improved hydrophobic CPPs formatted in the common sequence- and structural-motif which satisfy newly determined 'Critical Factors' to have 'Common Function,' namely, to facilitate protein translocation across the membrane with similar mechanism to the analyzed reference CPPs. Based on the analysis A, B and C, the common homologous features have been analyzed to determine the critical factors that influence the cell-permeability. The range value of each critical factor has been determined to include the analyzed index of each critical factor from analysis A, B and C to design novel aMTDs (Table 9). These features have been confirmed experimentally with newly designed aMTDs in their cell-permeability.

[333] Table 9 shows the Comparison The Range/Feature of Each Critical Factor Between The Value of Analyzed CPPs and The Value Determined for New Design of Novel aMTDs Sequences.

[334] [Table 9]

Summarized Critical Factors of aMTD		
Critical Factor	Selected CPPs	Newly Designed CPPs
	Range	Range
Bending Potential (Proline Position: PP)	Proline presences in the middle and/or at the end of peptides	Proline presences in the middle (5', 6', 7' or 8') and at the end of peptides
Rigidity / Flexibility (Instability Index: II)	45.5 - 57.3 (50.1 ± 3.6)	40 - 60
Structural Feature (Aliphatic Index: AI)	140.6 - 220.0 (204.7 ± 37.5)	180 - 220
Hydropathy (Grand Average of Hydropathy GRAVY)	1.8 - 2.8 (2.4 ± 0.3)	2.1 - 2.6
Length (Number of Amino Acid)	11.6 ± 3.0	9 - 13
Amino acid Composition	A, V, I, L, P	A, V, I, L, P

[335] In Table 9, universal common features and sequence/structural motif are provided. Length is 9 to 13 amino acids, and bending potential is provided with the presence of proline in the middle of sequence (at 5', 6', 7' or 8' amino acid) for peptide bending and at the end of peptide for recombinant protein bending and Rigidity/Flexibility of aMTDs is II > 40 are described in Table 9.

[336]

[337] **3-2. Critical Factors for Development of advanced** MTDs

[338] Recombinant cell-permeable proteins fused to the hydrophobic CPPs to deliver therapeutically active cargo molecules including proteins into live cells had previously been reported, but the fusion proteins expressed in bacteria system were hard to be purified as a soluble form due to their low solubility and yield. To address the crucial weakness for further clinical development of the cell-permeable proteins as protein-based biotherapeutics, greatly improved form of the hydrophobic CPP, named as advanced MTD (aMTD) has newly been developed through critical factors-based peptide analysis. The critical factors used for the current invention of the aMTDs are herein (Table 9).

[339] 1. Amino Acid Length: 9 to 13

[340] 2. Bending Potential (Proline Position: PP)

[341] : Proline presences in the middle (from 5' to 8' amino acid) and at the end of sequence

[342] 3. Rigidity/Flexibility (Instability Index: II): 40 to 60

[343] 4. Structural Feature (Aliphatic Index: AI): 180 to 220

[344] 5. Hydropathy (GRAVY): 2.1 to 2.6

[345] 6. Amino Acid Composition: Hydrophobic and Aliphatic amino acids to A, V, L, I and P

[346]

[347] **3-3. Design of Potentially Best** aMTDs That All Critical Factors are Considered and Satisfied

[348] After careful consideration of six critical factors derived from analysis of unique features of hydrophobic CPPs, advanced macromolecule transduction domains (aMTDs) have been designed and developed based on the common 12 amino acid platform which satisfies the critical factors including amino acid length (9 to 13) determined from the analysis.

[349] [General formula]

[350]

1	2	3	4	5	6	7	8	9	10	11	12
X	X	X	X	U	U	U	U	X	X	X	P

[351] Unlike previously published hydrophobic CPPs that require numerous experiments to determine their cell-permeability, newly developed aMTD sequences could be designed by performing just few steps as follows using above mentioned platform to follow the determined range value/feature of each critical factor.

[352] First, prepare the 12 amino acid sequence platform for aMTD. Second, place proline (P) in the end (12') of sequence and determine where to place proline in one of four U(s) in 5', 6', 7', and 8. Third, alanine (A), valine (V), leucine (L) or isoleucine (I) is

placed in either X(s) and/or U(s), where proline is not placed. Lastly, determine whether the amino acid sequences designed based on the platform, satisfy the value or feature of six critical factors to assure the cell permeable property of aMTD sequences. Through these processes, numerous novel aMTD sequences have been constructed. The expression vectors for preparing non-functional cargo recombinant proteins fused to each aMTD, expression vectors have been constructed and forcedly expressed in bacterial cells. These aMTD-fused recombinant proteins have been purified in soluble form and determined their cell-permeability quantitatively. aMTD sequences have been newly designed, numbered from 1 to 240, as shown in Tables 10 to 15. In Tables 10 to 15, sequence ID Number is a sequence listings for reference, and aMTD numbers refer to amino acid listing numbers that actually have been used at the experiments. For further experiments, aMTD numbers have been used. In addition, polynucleotide sequences shown in the sequence lists have been numbered from SEQ ID NO : 241 to SEQ ID NO : 480.

[353] Tables 10 to 15 show the 240 new hydrophobic aMTD sequences that were developed to satisfy all critical factors.

[354] [Table 10]

Sequence ID Number	aMTD	Sequences	Length	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Residue Structure
1	1	AAALAPVVLALP	12	57.3	187.5	2.1	Aliphatic
2	2	AAAVPLLAVVVP	12	41.3	195.0	2.4	Aliphatic
3	3	AALLVPAAVLAP	12	57.3	187.5	2.1	Aliphatic
4	4	ALALLPVAALAP	12	57.3	195.8	2.1	Aliphatic
5	5	AAALLPVALVAP	12	57.3	187.5	2.1	Aliphatic
6	11	VVALAPALAALP	12	57.3	187.5	2.1	Aliphatic
7	12	LLAAVPAVLLAP	12	57.3	211.7	2.3	Aliphatic
8	13	AAALVPVVALLP	12	57.3	203.3	2.3	Aliphatic
9	21	AVALLPALLAVP	12	57.3	211.7	2.3	Aliphatic
10	22	AVVLVPVLAAAP	12	57.3	195.0	2.4	Aliphatic
11	23	VVLVLPAAAAVP	12	57.3	195.0	2.4	Aliphatic
12	24	IALAAPALIVAP	12	50.2	195.8	2.2	Aliphatic
13	25	IVAVAPALVALP	12	50.2	203.3	2.4	Aliphatic
14	42	VAALPVVAVVAP	12	57.3	186.7	2.4	Aliphatic
15	43	LLAAPLVVAAVP	12	41.3	187.5	2.1	Aliphatic
16	44	ALAVPVALLVAP	12	57.3	203.3	2.3	Aliphatic
17	61	VAALPVLLAALP	12	57.3	211.7	2.3	Aliphatic
18	62	VALLAPVALAVP	12	57.3	203.3	2.3	Aliphatic
19	63	AALLVPALVAVP	12	57.3	203.3	2.3	Aliphatic

[355] [Table 11]

Sequence ID Number	aMTD	Sequences	Length	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Residue Structure
20	64	AIVALPVAVLAP	12	50.2	203.3	2.4	Aliphatic
21	65	IAIVAPVVALAP	12	50.2	203.3	2.4	Aliphatic
22	81	AALLPALAALLP	12	57.3	204.2	2.1	Aliphatic
23	82	AVVLAPVAAVLP	12	57.3	195.0	2.4	Aliphatic
24	83	LAVAAPLALALP	12	41.3	195.8	2.1	Aliphatic
25	84	AAVAAPLLLALP	12	41.3	195.8	2.1	Aliphatic
26	85	LLVLPAAALAAP	12	57.3	195.8	2.1	Aliphatic
27	101	LVALAPVAAVLP	12	57.3	203.3	2.3	Aliphatic
28	102	LALAPAALALLP	12	57.3	204.2	2.1	Aliphatic
29	103	ALIAAPILALAP	12	57.3	204.2	2.2	Aliphatic
30	104	AVVAAPLVLALP	12	41.3	203.3	2.3	Aliphatic
31	105	LLALAPAALLAP	12	57.3	204.1	2.1	Aliphatic
32	121	AIVALPALALAP	12	50.2	195.8	2.2	Aliphatic
33	123	AAIIVPAALLAP	12	50.2	195.8	2.2	Aliphatic
34	124	IAVALPALIAAP	12	50.3	195.8	2.2	Aliphatic
35	141	AVIVLPALAVAP	12	50.2	203.3	2.4	Aliphatic
36	143	AVLAVPAVLVAP	12	57.3	195.0	2.4	Aliphatic
37	144	VLAIVPAVALAP	12	50.2	203.3	2.4	Aliphatic
38	145	LLAVVPAVALAP	12	57.3	203.3	2.3	Aliphatic
39	161	AVIALPALIAAP	12	57.3	195.8	2.2	Aliphatic
40	162	AVVALPAALIVP	12	50.2	203.3	2.4	Aliphatic
41	163	LALVLPAALAAP	12	57.3	195.8	2.1	Aliphatic
42	164	LAAVLPALLAAP	12	57.3	195.8	2.1	Aliphatic
43	165	ALAVPVALAIVP	12	50.2	203.3	2.4	Aliphatic
44	182	ALIAPVVALVAP	12	57.3	203.3	2.4	Aliphatic
45	183	LLAAPVVIALAP	12	57.3	211.6	2.4	Aliphatic
46	184	LAAIVPAIIAVP	12	50.2	211.6	2.4	Aliphatic
47	185	AALVLPLIIAAP	12	41.3	220.0	2.4	Aliphatic
48	201	LALAVPALAALP	12	57.3	195.8	2.1	Aliphatic
49	204	LIAALPAVAALP	12	57.3	195.8	2.2	Aliphatic
50	205	ALALVPAIAALP	12	57.3	195.8	2.2	Aliphatic
51	221	AAILAPIVALAP	12	50.2	195.8	2.2	Aliphatic
52	222	ALLIAPAAVIAP	12	57.3	195.8	2.2	Aliphatic
53	223	AILAVPIAVVAP	12	57.3	203.3	2.4	Aliphatic
54	224	ILAAVPIALAAP	12	57.3	195.8	2.2	Aliphatic
55	225	VAALLPAAAVLP	12	57.3	187.5	2.1	Aliphatic
56	241	AAAVVPVLLVAP	12	57.3	195.0	2.4	Aliphatic
57	242	AALLVPALVAAP	12	57.3	187.5	2.1	Aliphatic
58	243	AAVLLPVALAAP	12	57.3	187.5	2.1	Aliphatic
59	245	AAALAPVLALVP	12	57.3	187.5	2.1	Aliphatic
60	261	LVLVPLLAAAAP	12	41.3	211.6	2.3	Aliphatic
61	262	ALIAVPAIIVAP	12	50.2	211.6	2.4	Aliphatic
62	263	ALAVIPAAAILP	12	54.9	195.8	2.2	Aliphatic
63	264	LAAAPVVIVIAP	12	50.2	203.3	2.4	Aliphatic
64	265	VLAIAPLLAAVP	12	41.3	211.6	2.3	Aliphatic
65	281	ALIVLPAAVAVP	12	50.2	203.3	2.4	Aliphatic
66	282	VLAVAPALIVAP	12	50.2	203.3	2.4	Aliphatic
67	283	AALLAPALIVAP	12	50.2	195.8	2.2	Aliphatic
68	284	ALIAPAVALIVP	12	50.2	211.7	2.4	Aliphatic
69	285	AIVLLPAAVVAP	12	50.2	203.3	2.4	Aliphatic

[356] [Table 12]

Sequence ID Number	aMTD	Sequences	Length	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Residue Structure
70	301	VIAAPVLAVLAP	12	57.3	203.3	2.4	Aliphatic
71	302	LALAPALALLAP	12	57.3	204.2	2.1	Aliphatic
72	304	AIILAPIAAIAP	12	57.3	204.2	2.3	Aliphatic
73	305	IALAAPILLAAP	12	57.3	204.2	2.2	Aliphatic
74	321	IVAVALPALAVP	12	50.2	203.3	2.3	Aliphatic
75	322	VVAIVLPALAAP	12	50.2	203.3	2.3	Aliphatic
76	323	IVAVALPVALAP	12	50.2	203.3	2.3	Aliphatic
77	324	IVAVALPAALVP	12	50.2	203.3	2.3	Aliphatic
78	325	IVAVALPAVALP	12	50.2	203.3	2.3	Aliphatic
79	341	IVAVALPAVLAP	12	50.2	203.3	2.3	Aliphatic
80	342	VIVALAPAVLAP	12	50.2	203.3	2.3	Aliphatic
81	343	IVAVALPALVAP	12	50.2	203.3	2.3	Aliphatic
82	345	ALLIVAPVAVAP	12	50.2	203.3	2.3	Aliphatic
83	361	AVVIVAPAVIAP	12	50.2	195.0	2.4	Aliphatic
84	363	AVLAVAPALIVP	12	50.2	203.3	2.3	Aliphatic
85	364	LVAAVAPALIVP	12	50.2	203.3	2.3	Aliphatic
86	365	AVIVVAPALLAP	12	50.2	203.3	2.3	Aliphatic
87	381	VVAIVLPAVAAP	12	50.2	195.0	2.4	Aliphatic
88	382	AAALVIPAILAP	12	54.9	195.8	2.2	Aliphatic
89	383	VIVALAPALLAP	12	50.2	211.6	2.3	Aliphatic
90	384	VIVAIAPALLAP	12	50.2	211.6	2.4	Aliphatic
91	385	IVAIAVPALVAP	12	50.2	203.3	2.4	Aliphatic
92	401	AALAVIPAAILP	12	54.9	195.8	2.2	Aliphatic
93	402	ALAAVIPAAILP	12	54.9	195.8	2.2	Aliphatic
94	403	AAALVIPAAILP	12	54.9	195.8	2.2	Aliphatic
95	404	LAAAVIPAAILP	12	54.9	195.8	2.2	Aliphatic
96	405	LAAAVIPVAILP	12	54.9	211.7	2.4	Aliphatic
97	421	AAILAAPLIAVP	12	57.3	195.8	2.2	Aliphatic
98	422	VVAILAPLLAAP	12	57.3	211.7	2.4	Aliphatic
99	424	AVVVAAPVLALP	12	57.3	195.0	2.4	Aliphatic
100	425	AVVAIAPVLALP	12	57.3	203.3	2.4	Aliphatic
101	442	ALAALVPAVLVP	12	57.3	203.3	2.3	Aliphatic
102	443	ALAALVPVALVP	12	57.3	203.3	2.3	Aliphatic
103	444	LAAALVPVALVP	12	57.3	203.3	2.3	Aliphatic
104	445	ALAALVPALVVP	12	57.3	203.3	2.3	Aliphatic
105	461	IAAVIVPAVALP	12	50.2	203.3	2.4	Aliphatic
106	462	IAAVLVPAVALP	12	57.3	203.3	2.4	Aliphatic
107	463	AVAILVPLLAAP	12	57.3	211.7	2.4	Aliphatic
108	464	AVVILVPLAAAP	12	57.3	203.3	2.4	Aliphatic
109	465	IAAVIVPVAALP	12	50.2	203.3	2.4	Aliphatic
110	481	AIAIAIVPVALP	12	50.2	211.6	2.4	Aliphatic
111	482	ILAVAAIPVAVP	12	54.9	203.3	2.4	Aliphatic
112	483	ILAAAIIPAALP	12	54.9	204.1	2.2	Aliphatic
113	484	LAVVLAAPAIVP	12	50.2	203.3	2.4	Aliphatic
114	485	AILAAIVPLAVP	12	50.2	211.6	2.4	Aliphatic
115	501	VIVALAVPALAP	12	50.2	203.3	2.4	Aliphatic
116	502	AIVALAVPVLAP	12	50.2	203.3	2.4	Aliphatic
117	503	AAIIIVLPAALP	12	50.2	220.0	2.4	Aliphatic
118	504	LIVALAVPALAP	12	50.2	211.7	2.4	Aliphatic
119	505	AIIIVIAPAAAP	12	50.2	195.8	2.3	Aliphatic

[357] [Table 13]

Sequence ID Number	aMTD	Sequences	Length	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Residue Structure
120	521	LAALIVVPAVAP	12	50.2	203.3	2.4	Aliphatic
121	522	ALLVIAVPAVAP	12	57.3	203.3	2.4	Aliphatic
122	524	AVALIVVPALAP	12	50.2	203.3	2.4	Aliphatic
123	525	ALAIVVAPVAVP	12	50.2	195.0	2.4	Aliphatic
124	541	LLALIIAPAAAP	12	57.3	204.1	2.1	Aliphatic
125	542	ALALIIVPAVAP	12	50.2	211.6	2.4	Aliphatic
126	543	LLAALIAPAALP	12	57.3	204.1	2.1	Aliphatic
127	544	IVALIVAPAAVP	12	43.1	203.3	2.4	Aliphatic
128	545	VVLVLAAPAAVP	12	57.3	195.0	2.3	Aliphatic
129	561	AAVAIVLPAVVP	12	50.2	195.0	2.4	Aliphatic
130	562	ALIAAIVPALVP	12	50.2	211.7	2.4	Aliphatic
131	563	ALAVIVVPALAP	12	50.2	203.3	2.4	Aliphatic
132	564	VAIALIVPALAP	12	50.2	211.7	2.4	Aliphatic
133	565	VAIVLVAPAVAP	12	50.2	195.0	2.4	Aliphatic
134	582	VAVALIVPALAP	12	50.2	203.3	2.4	Aliphatic
135	583	AVILALAPIVAP	12	50.2	211.6	2.4	Aliphatic
136	585	ALIVAIAPALVP	12	50.2	211.6	2.4	Aliphatic
137	601	AAILIAVPIAAP	12	57.3	195.8	2.3	Aliphatic
138	602	VIVALAAPVLAP	12	50.2	203.3	2.4	Aliphatic
139	603	VLVALAAPVIAP	12	57.3	203.3	2.4	Aliphatic
140	604	VALIAVAPAVVP	12	57.3	195.0	2.4	Aliphatic
141	605	VIAAVLAPVAVP	12	57.3	195.0	2.4	Aliphatic
142	622	ALIVLAAPVAVP	12	50.2	203.3	2.4	Aliphatic
143	623	VAAAIALPAIVP	12	50.2	187.5	2.3	Aliphatic
144	625	ILAAAAAPLIVP	12	50.2	195.8	2.2	Aliphatic
145	643	LALVLAAPAIVP	12	50.2	211.6	2.4	Aliphatic
146	645	ALAVVALPAIVP	12	50.2	203.3	2.4	Aliphatic
147	661	AAILAPIVAALP	12	50.2	195.8	2.2	Aliphatic
148	664	ILIAIAIPAAAP	12	54.9	204.1	2.3	Aliphatic
149	665	LAIVLAAPVAVP	12	50.2	203.3	2.3	Aliphatic
150	666	AAIAIIAPAIVP	12	50.2	195.8	2.3	Aliphatic
151	667	LAVAIVAPALVP	12	50.2	203.3	2.3	Aliphatic
152	683	LAIVLAAPAVLP	12	50.2	211.7	2.4	Aliphatic
153	684	AAIVLALPAVLP	12	50.2	211.7	2.4	Aliphatic
154	685	ALLVAVLPAALP	12	57.3	211.7	2.3	Aliphatic
155	686	AALVAVLPVALP	12	57.3	203.3	2.3	Aliphatic
156	687	AILAVALPLLAP	12	57.3	220.0	2.3	Aliphatic
157	703	IVAVALVPALAP	12	50.2	203.3	2.4	Aliphatic
158	705	IVAVALLPALAP	12	50.2	211.7	2.4	Aliphatic
159	706	IVAVALLPAVAP	12	50.2	203.3	2.4	Aliphatic
160	707	IVALAVLPAVAP	12	50.2	203.3	2.4	Aliphatic
161	724	VAVLAVLPALAP	12	57.3	203.3	2.3	Aliphatic
162	725	IAVLAVAPAVLP	12	57.3	203.3	2.3	Aliphatic
163	726	LAVAIIAPAVAP	12	57.3	187.5	2.2	Aliphatic
164	727	VALAIALPAVLP	12	57.3	211.6	2.3	Aliphatic
165	743	AIAIALVPVALP	12	57.3	211.6	2.4	Aliphatic
166	744	AAVVIVAPVALP	12	50.2	195.0	2.4	Aliphatic
167	746	VAIIVVAPALAP	12	50.2	203.3	2.4	Aliphatic
168	747	VALLAIAPALAP	12	57.3	195.8	2.2	Aliphatic
169	763	VAVLIAVPALAP	12	57.3	203.3	2.3	Aliphatic

[358] [Table 14]

Sequence ID Number	aMTD	Sequences	Length	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Residue Structure
170	764	AVALAVLPAVVP	12	57.3	195.0	2.3	Aliphatic
171	765	AVALAVVPAVLP	12	57.3	195.0	2.3	Aliphatic
172	766	IVVIAVAPAVAP	12	50.2	195.0	2.4	Aliphatic
173	767	IVVAAVVPALAP	12	50.2	195.0	2.4	Aliphatic
174	783	IVALVPAVAIAP	12	50.2	203.3	2.5	Aliphatic
175	784	VAALPAVALVVP	12	57.3	195.0	2.4	Aliphatic
176	786	LVAIAPLAVLAP	12	41.3	211.7	2.4	Aliphatic
177	787	AVALVPVIVAAP	12	50.2	195.0	2.4	Aliphatic
178	788	AIAVAIAPVALP	12	57.3	187.5	2.3	Aliphatic
179	803	AIALAVPVLALP	12	57.3	211.7	2.4	Aliphatic
180	805	LVLIAAAPIALP	12	41.3	220.0	2.4	Aliphatic
181	806	LVALAVPAAVLP	12	57.3	203.3	2.3	Aliphatic
182	807	AVALAVPALVLP	12	57.3	203.3	2.3	Aliphatic
183	808	LVVLAAAPLAVP	12	41.3	203.3	2.3	Aliphatic
184	809	LIVLAAPALAAP	12	50.2	195.8	2.2	Aliphatic
185	810	VIVLAAPALAAP	12	50.2	187.5	2.2	Aliphatic
186	811	AVVLAVPALAVP	12	57.3	195.0	2.3	Aliphatic
187	824	LIIVAAAPAVAP	12	50.2	187.5	2.3	Aliphatic
188	825	IVAVIVAPAVAP	12	43.2	195.0	2.5	Aliphatic
189	826	LVALAAPIIAVP	12	41.3	211.7	2.4	Aliphatic
190	827	IAAVLAAPALVP	12	57.3	187.5	2.2	Aliphatic
191	828	IALLAAPIIAVP	12	41.3	220.0	2.4	Aliphatic
192	829	AALALVAPVIVP	12	50.2	203.3	2.4	Aliphatic
193	830	IALVAAPVALVP	12	57.3	203.3	2.4	Aliphatic
194	831	IIVAVAPAAIVP	12	43.2	203.3	2.5	Aliphatic
195	832	AVAAIVPVIVAP	12	43.2	195.0	2.5	Aliphatic
196	843	AVLVLVAPAAAP	12	41.3	219.2	2.5	Aliphatic
197	844	VVALLAPLIAAP	12	41.3	211.8	2.4	Aliphatic
198	845	AAVVIAPLLAVP	12	41.3	203.3	2.4	Aliphatic
199	846	IAVAVAAPLLVP	12	41.3	203.3	2.4	Aliphatic
200	847	LVAIVVLPAVAP	12	50.2	219.2	2.6	Aliphatic
201	848	AVAIVVLPAVAP	12	50.2	195.0	2.4	Aliphatic
202	849	AVILLAPLIAAP	12	57.3	220.0	2.4	Aliphatic
203	850	LVIALAAPVALP	12	57.3	211.7	2.4	Aliphatic
204	851	VLAVVLPAVALP	12	57.3	219.2	2.5	Aliphatic
205	852	VLAVAAPAVLLP	12	57.3	203.3	2.3	Aliphatic
206	863	AAVVLLPIIAAP	12	41.3	211.7	2.4	Aliphatic
207	864	ALLVIAPAIAVP	12	57.3	211.7	2.4	Aliphatic
208	865	AVLVIAVPAIAP	12	57.3	203.3	2.5	Aliphatic
209	867	ALLVVIAPLAAP	12	41.3	211.7	2.4	Aliphatic
210	868	VLVAAILPAAIP	12	54.9	211.7	2.4	Aliphatic
211	870	VLVAAVLPIAAP	12	41.3	203.3	2.4	Aliphatic
212	872	VLAAAVLPLVVP	12	41.3	219.2	2.5	Aliphatic
213	875	AIAIVVPAVAVP	12	50.2	195.0	2.4	Aliphatic
214	877	VAIIAVPAVVAP	12	57.3	195.0	2.4	Aliphatic
215	878	IVALVAPAAVVP	12	50.2	195.0	2.4	Aliphatic
216	879	AAIVLLPAVVVP	12	50.2	219.1	2.5	Aliphatic
217	881	AALIVVPAVAVP	12	50.2	195.0	2.4	Aliphatic
218	882	AIALVVPAVAVP	12	57.3	195.0	2.4	Aliphatic
219	883	LAIVPAAIAALP	12	50.2	195.8	2.2	Aliphatic

[359] [Table 15]

Sequence ID Number	aMTD	Sequences	Length	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Residue Structure
220	885	LVAIAPAVAVLP	12	57.3	203.3	2.4	Aliphatic
221	887	VLAVAPAVAVLP	12	57.3	195.0	2.4	Aliphatic
222	888	ILAVVAIPAAAP	12	54.9	187.5	2.3	Aliphatic
223	889	ILVAAAPIAALP	12	57.3	195.8	2.2	Aliphatic
224	891	ILAVAAIPAALP	12	54.9	195.8	2.2	Aliphatic
225	893	VIAIPAILAAAP	12	54.9	195.8	2.3	Aliphatic
226	895	AIIIVVPAIAAP	12	50.2	211.7	2.5	Aliphatic
227	896	AILIVVAPIAAP	12	50.2	211.7	2.5	Aliphatic
228	897	AVIVPVAIIAAP	12	50.2	203.3	2.5	Aliphatic
229	899	AVVIALPAVVAP	12	57.3	195.0	2.4	Aliphatic
230	900	ALVAVIAPVVAP	12	57.3	195.0	2.4	Aliphatic
231	901	ALVAVLPAVAVP	12	57.3	195.0	2.4	Aliphatic
232	902	ALVAPLLAVAVP	12	41.3	203.3	2.3	Aliphatic
233	904	AVLAVVAPVVAP	12	57.3	186.7	2.4	Aliphatic
234	905	AVIAVAPLVVAP	12	41.3	195.0	2.4	Aliphatic
235	906	AVIALAPVVVAP	12	57.3	195.0	2.4	Aliphatic
236	907	VAIALAPVVVAP	12	57.3	195.0	2.4	Aliphatic
237	908	VALALAPVVVAP	12	57.3	195.0	2.3	Aliphatic
238	910	VAALLPAVVVAP	12	57.3	195.0	2.3	Aliphatic
239	911	VALALPAVVVAP	12	57.3	195.0	2.3	Aliphatic
240	912	VALLAPAVVVAP	12	57.3	195.0	2.3	Aliphatic
				52.6 ± 5.1	201.7 ± 7.8	2.3 ± 0.1	

[360] **3-4. Design of the Peptides That Did Not Satisfy at Least One Critical Factor**

[361] To demonstrate that present invention of new hydrophobic CPPs - aMTDs, which satisfy all critical factors described above, are correct and rationally designed, the peptides which do not satisfy at least one critical factor have also been designed. Total of 31 rPeptides (rPs) are designed, developed and categorized as follows: no bending peptides, either no proline in the middle as well at the end and/or no central proline; rigid peptides (II < 40); too much flexible peptides; aromatic peptides (aromatic ring presences); hydrophobic, with non-aromatic peptides but have amino acids other than A, V, L, I, P or additional proline residues; hydrophilic, but non-aliphatic peptides.

[362] **3-4-1. Peptides That Do Not Satisfy the Bending Potential**

[363] Table 16 shows the peptides that do not have any proline in the middle (at 5', 6', 7' or 8') and at the end of the sequences. In addition, Table 16 describes the peptides that do not have proline in the middle of the sequences. All these peptides are supposed to have no-bending potential.

[364] [Table 16]

Group	rPeptide ID	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)
No-Bending Peptides (No Proline at 5, 6, 7 or 8 and/or 12)	931	AVLIAPAILAAA	12	6	57.3	204.2	2.5
	936	ALLILAAAVAAP	12	12	41.3	204.2	2.4
	152	LAAAVAAVAALL	12	None	9.2	204.2	2.7
	27	LAIVAAAAALVA	12	None	2.1	204.2	2.8
	935	ALLILPAAAVAA	12	6	57.3	204.2	2.4
	670	ALLILAAAVAAL	12	None	25.2	236.6	2.8
	934	LILAPAAVVAAA	12	5	57.3	195.8	2.5
	37	TTCSQQQYCTNG	12	None	53.1	0.0	-1.1
	16	NNSCTTYTNGSQ	12	None	47.4	0.0	-1.4
	113	PVAVALLIAVPP	12	1,11,12	57.3	195.0	2.1

[365] **3-4-2. Peptides That Do Not Satisfy the Rigidity/Flexibility**

[366] To prove that rigidity/flexibility of the sequence is a crucial critical factor, rigid (Avg. II: 21.8±6.6) and too high flexible sequences (Avg. II: 82.3±21.0) were also designed. Rigid peptides that instability index is much lower than that of new aMTDs (II: 41.3 to 57.3, Avg. II: 53.3±5.7) are shown in Table 17. Bending, but too high flexible peptides that II is much higher than that of new aMTDs are also provided in Table 18.

[367] [Table 17]

Group	rPeptide ID	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)
Rigid Peptides (II < 50)	226	ALVAAIPALAIP	12	6	20.4	195.8	2.2
	6	VIAMIPAAFWVA	12	6	15.7	146.7	2.2
	750	LAIAAIAPLAIP	12	8,12	22.8	204.2	2.2
	26	AAIALAAPLAIV	12	8	18.1	204.2	2.5
	527	LVLAAVAPIAIP	12	8,12	22.8	211.7	2.4
	466	IIAAAAPLAIIP	12	7,12	22.8	204.2	2.3
	167	VAIAIPAALAIP	12	6,12	20.4	195.8	2.3
	246	VVAVPLLVAFAA	12	5	25.2	195.0	2.7
	426	AAALAIPLAIIP	12	7,12	4.37	204.2	2.2
	606	AAAIAAIPIIIP	12	8,12	4.4	204.2	2.4
	66	AGVLGGPIMGVP	12	7,12	35.5	121.7	1.3
	248	VAAIVPIAALVP	12	6,12	34.2	203.3	2.5
	227	LAAIVPIAAAVP	12	6,12	34.2	187.5	2.2
	17	GGCSAPQTTCSN	12	6	51.6	8.3	-0.5
	67	LDAEVPLADDVP	12	6,12	34.2	130.0	0.3

[368] [Table 18]

Group	rPeptide ID	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GARVY)
Bending Peptides but Too High Flexibility	692	PAPLPPVVILAV	12	1,3,5,6	105.5	186.7	1.8
	69	PVAVLPPAALVP	12	1,6,7,12	89.4	162.5	1.6
	390	VPLLVPVVPVVP	12	2,6,9,12	105.4	210.0	2.2
	350	VPILVPVVPVVP	12	2,6,9,12	121.5	210.0	2.2
	331	VPVLVPLVPVVP	12	2,6,9,12	105.4	210.0	2.2
	9	VALVPAALILPP	12	5,11,12	89.4	203.3	2.1
	68	VAPVLPAAPLVP	12	3,6,9,12	105.5	162.5	1.6
	349	VPVLVPVVPVVP	12	2,6,9,12	121.5	201.6	2.2
	937	VPVLVPLPVPVV	12	2,6,8,10	121.5	210.0	2.2
	938	VPVLLPVVVPVP	12	2,6,10,12	121.5	210.0	2.2
	329	LPVLVPVVPVVP	12	2,6,9,12	121.5	210.0	2.2
	49	VVPAAPAVPVVP	12	3,6,9,12	121.5	145.8	1.7
	772	LPVAPVIPIIVP	12	2,5,8,12	79.9	210.8	2.1
	210	ALIALPALPALP	12	6,9,12	89.4	195.8	1.8
	28	AVPLLPLVPAVP	12	3,6,9,12	89.4	186.8	1.8
	693	AAPVLPVAVPIV	12	3,6,10	82.3	186.7	2.1
	169	VALVAPALILAP	12	6,12	73.4	211.7	2.4
	29	VLPPLPVLPVLP	12	3,4,6,9,12	121.5	202.5	1.7
	190	AAILAPAVIAPP	12	6,11,12	89.4	163.3	1.8

[369] **3-4-3. Peptides That Do Not Satisfy the Structural Features**

[370] New hydrophobic CPPs - aMTDs are consisted with only hydrophobic and aliphatic amino acids (A, V, L, I and P) with average ranges of the indexes - AI: 180 to 220 and GRAVY: 2.1 to 2.6 (Table 9). Based on the structural indexes, the peptides which contain an aromatic residue (W, F or Y) are shown in Table 19 and the peptides which are hydrophobic with non-aromatic sequences but have amino acids residue other than A, V, L, I, P or additional proline residues are designed (Table 20). Finally, hydrophilic and/or bending peptides which are consisted with non-aliphatic amino acids are shown in Table 21.

[371] [Table 19]

Group	rPeptide ID	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)
Aromatic Peptides (Aromatic Ring Presences)	30	WFFAGPIMLIWP	12	6, 12	9.2	105.8	1.4
	33	AAAILAPAFLAV	12	7	57.3	171.7	2.4
	131	WIIAPWWLAWIA	12	5	51.6	179.2	1.9
	922	WYVIPVLPLVVP	12	8,12	41.3	194.2	2.2
	71	FMWMWFPFMWYP	12	7,12	71.3	0.0	0.6
	921	IWWFVVLPLVVP	12	8,12	41.3	194.2	2.2

[372] [Table 20]

Group	rPeptide ID	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GARVY)
Hydrophobic but Non Aromatic Peptides	436	VVMLVVPAVMLP	12	7,12	57.3	194.2	2.6
	138	PPAALLAILAVA	12	1,2	57.3	195.8	2.2
	77	PVALVLVALVAP	12	1,12	41.3	219.2	2.5
	577	MLMIALVPMIAV	12	8	18.9	195.0	2.7
	97	ALLAAPPALLAL	12	6,7	57.3	204.2	2.1
	214	ALIVAPALMALP	12	6,12	60.5	187.5	2.2
	59	AVLAAPVVAALA	12	6	41.3	187.5	2.5
	54	LAVAAPPVVALL	12	6,7	57.3	203.3	2.3

[373] [Table 21]

Group	rPeptide ID	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)
Hydrophilic Peptides but Non Aliphatic	949	SGNSCOOCGNSS	12	None	41.7	0.0	-1.1
	39	CYNTSPCTGCCY	12	6	52.5	0.0	0.0
	19	YVSCCTYTNGSO	12	None	47.7	0.0	-1.0
	947	CYYNOOSNNNNO	12	None	59.6	0.0	-2.4
	139	TGSTNSPTCTST	12	7	53.4	0.0	-0.7
	18	NYCCTPTTNGOS	12	6	47.9	0.0	-0.9
	20	NYCNTCPTYGOS	12	7	47.4	0.0	-0.9
	635	GSTGGSOONNOY	12	None	31.9	0.0	-1.9
	40	TYNTSCTPGTCY	12	8	49.4	0.0	-0.6
	57	ONNCNTSSOGGG	12	None	52.4	0.0	-1.6
	159	CYSGSTSONOPP	12	11,12	51.0	0.0	-1.3
	700	GTSNTCOSNONS	12	None	19.1	0.0	-1.6
	38	YYNOSTCGGOCY	12	None	53.8	0.0	-1.0

[374] **3-5. Summary of Newly Designed Peptides**

[375] Total of 457 sequences have been designed based on the critical factors. Designed potentially best aMTDs (hydrophobic, flexible, bending, aliphatic and 12-A/a length peptides) that do satisfy all range/feature of critical factors are 316. Designed rPeptides that do not satisfy at least one of the critical factors are 141 that no bending peptide sequences are 26; rigid peptide (II<40) sequences are 23; too much flexible peptides are 24; aromatic peptides (aromatic ring presences) are 27; hydrophobic, but non-aromatic peptides are 23; and hydrophilic, but non-aliphatic peptides are 18.

[376]

[377] **4. Preparation of Recombinant Report Proteins Fused to** aMTDs and rPeptides

[378] Recombinant proteins fused to aMTDs and others [rPeptides, reference hydrophobic CPP sequences (MTM and MTD)] were expressed in a bacterial system, purified with single-step affinity chromatography and prepared as soluble proteins in physiological condition. These recombinant proteins have been tested for the ability of their cell-

permeability by utilizing flow cytometry and laser scanning confocal microscopy.

[379]

[380] **4-1. Selection of Cargo Protein for Recombinant Proteins Fused to Peptide Sequences**

[381] For clinical/non-clinical application, aMTD-fused cargo materials would be biologically active molecules that could be one of the following: enzymes, transcription factors, toxic, antigenic peptides, antibodies and antibody fragments. Furthermore, biologically active molecules could be one of these following macromolecules: enzymes, hormones, carriers, immunoglobulin, membrane-bound proteins, transmembrane proteins, internal proteins, external proteins, secreted proteins, virus proteins, native proteins, glycoproteins, fragmented proteins, disulfide bonded proteins, recombinant proteins, chemically modified proteins and prions. In addition, these biologically active molecules could be one of the following: nucleic acid, coding nucleic acid sequence, mRNAs, antisense RNA molecule, carbohydrate, lipid and glycolipid.

[382] According to these pre-required conditions, a non-functional cargo to evaluate aMTD-mediated protein uptake has been selected and called as Cargo A (CRA) that should be soluble and non-functional. The domain (A/a 289 to 840; 184 A/a length) is derived from protein S (Genbank ID: CP000113.1).

[383]

[384] **4-2. Construction of Expression Vector and Preparation of Recombinant Proteins**

[385] Coding sequences for recombinant proteins fused to each aMTD are cloned NdeI (5') and SalI (3') in pET-28a(+) (Novagen, Darmstadt, Germany) from PCR-amplified DNA segments. PCR primers for the recombinant proteins fused to aMTD and rPeptides are represented by SEQ ID NOs: 482 to 798. Structure of the recombinant proteins is displayed in FIG. 1.

[386] The recombinant proteins were forcedly expressed in *E.* coli BL21(DE3) cells grown to an OD_{600} of 0.6 and induced for 2 hours with 0.7 mM isopropyl-β-D-thiogalactopyranoside (IPTG). The proteins were purified by Ni^{2+} affinity chromatography as directed by the supplier (Qiagen, Hilden, Germany) in natural condition. After the purification, purified proteins were dissolved in a physiological buffer such as DMEM medium.

[387] [Table 22]

▶ Potentially Best aMTDs (Hydrophobic, Flexible, Bending, Aliphatic & Helical)	: 240
▶ Random Peptides	: 31
- No Bending Peptides (No Proline at 5 or 6 and/or 12)	: 02
- No Bending Peptides (No Central Proline)	: 01
- Rigid Peptides (II<50)	: 09
- Too Much Flexible Peptides	: 09
- Aromatic Peptides (Aromatic Ring Presences)	: 01
- Hydrophobic, But Non-Aromatic Peptides	: 02
- Hydrophilic, But Non-Aliphatic Peptides	: 07

[388]

[389] **4-3. Expression of** aMTD- or Random Peptide (rP)- Fused Recombinant Proteins

[390] The present invention also relates to the development method of aMTD sequences having cell-permeability. Using the standardized six critical factors, 316 aMTD sequences have been designed. In addition, 141 rPeptides are also developed that lack one of these critical factors: no bending peptides: i) absence of proline both in the middle and at the end of sequence or ii) absence of proline either in the middle or at the end of sequence, rigid peptides, too much flexible peptides, aromatic peptides (aromatic ring presence), hydrophobic but non-aromatic peptides, and hydrophilic but non-aliphatic peptides (Table 22).

[391] These rPeptides are devised to be compared and contrasted with aMTDs in order to analyze structure/sequence activity relationship (SAR) of each critical factor with regard to the peptides' intracellular delivery potential. All peptide (aMTD or rPeptide)-containing recombinant proteins have been fused to the CRA to enhance the solubility of the recombinant proteins to be expressed, purified, prepared and analyzed.

[392] These designed 316 aMTDs and 141 rPeptides fused to CRA were all cloned (FIG. 2) and tested for inducible expression in *E.* coli (FIG. 3). Out of these peptides, 240 aMTDs were inducibly expressed, purified and prepared in soluble form (FIG. 4). In addition, 31 rPeptides were also prepared as soluble form (FIG. 4).

[393] To prepare the proteins fused to rPeptides, 60 proteins were expressed that were 10 out of 26 rPeptides in the category of no bending peptides (Table 16); 15 out of 23 in the category of rigid peptides [instability index (II) < 40] (Table 17); 19 out of 24 in the category of too much flexible peptides (Table 18); 6 out of 27 in the category of aromatic peptides (Table 19); 8 out of 23 in the category of hydrophobic but non-aromatic peptides (Table 20); and 12 out of 18 in the category of hydrophilic but non-aliphatic peptides (Table 21).

[394]

[395] **4-4. Quantitative Cell-Permeability of** aMTD-Fused Recombinant Proteins

[396] The aMTDs and rPeptides were fluorescently labeled and compared based on the critical factors for cell-permeability by using flow cytometry and confocal laser

scanning microscopy (FIGs. 5 to 8). The cellular uptake of the peptide-fused non-functional cargo recombinant proteins could quantitatively be evaluated in flow cytometry, while confocal laser scanning microscopy allows intracellular uptake to be assessed visually. The analysis included recombinant proteins fused to a negative control [rP38] that has opposite characteristics (hydrophilic and aromatic sequence: YYNQSTCGGQCY) to the aMTDs (hydrophobic and aliphatic sequences). Relative cell-permeability (relative fold) of aMTDs to the negative control was also analyzed (Table 23 and FIG. 9).

[397] Table 23 shows the Comparison Analysis of Cell-Permeability of aMTDs with a Negative Control (A: rP38).

[398] [Table 23]

	Negative Control rP38
aMTD The Average of 240 aMTDs	19.6 ± 1.6* (Best: 164.2)

*Relative Fold (aMTD in Geo Mean in its comparison to rP38)

[399] Relative cell-permeability (relative fold) of aMTDs to the reference CPPs [B: MTM12 (AAVLLPVLLAAP), C: MTD85 (AVALLILAV)] was also analyzed (Tables 40 and 41)

[400] Table 24 shows Comparison Analysis of Cell-Permeability of aMTDs with a Reference CPP (B: MTM12).

[401] [Table 24]

	MTM12
aMTD The Average of 240 aMTDs	13.1±1.1* (Best: 109.9)

*Relative Fold (aMTD in Geo Mean in its comparison to MTM12)

[402] Table 25 shows the Comparison Analysis of Cell-Permeability of aMTDs with a Reference CPP (C: MTD85).

[403] [Table 25]

	MTD85
aMTD The Average of 240 aMTDs	6.6±0.5* (Best: 55.5)

*Relative Fold (aMTD in Geo Mean in its comparison to MTD85)

[404] Geometric means of negative control (histidine-tagged rP38-fused CRA recombinant protein) subtracted by that of naked protein (histidine-tagged CRA protein) lacking any peptide (rP38 or aMTD) was standardized as relative fold of 1. Relative cell-permeability of 240 aMTDs to the negative control (A type) was significantly increased by up to 164 fold, with average increase of 19.6±1.6 (Tables 26 to 31).

[405] [Table 26]

Sequence ID Number	aMTD	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Relative Ratio (Fold)		
								A	B	C
1	899	AVVIALPAVVAP	12	7	57.3	195.0	2.4	164.2	109.9	55.5
2	908	VALALAPVVVAP	12	7	57.3	195.0	2.3	150.6	100.8	50.9
3	910	VAALLPAVVVAP	12	6	57.3	195.0	2.3	148.5	99.4	50.2
4	810	VIVLAAPALAAP	12	7	50.2	187.5	2.2	120.0	80.3	40.6
5	904	AVLAVVAPVVAP	12	8	57.3	186.7	2.4	105.7	70.8	35.8
6	321	IVAVALPALAVP	12	7	50.2	203.3	2.3	97.8	65.2	32.9
7	851	VLAVVLPAVALP	12	7	57.3	219.2	2.5	96.6	64.7	32.7
8	911	VALALPAVVVAP	12	6	57.3	195.0	2.3	84.8	56.8	28.7
9	852	VLAVAAPAVLLP	12	7	57.3	203.3	2.3	84.6	56.6	28.6
10	803	AIALAVPVLALP	12	7	57.3	211.7	2.4	74.7	50.0	25.3
11	888	ILAVVAIPAAAP	12	8	54.9	187.5	2.3	71.0	47.5	24.0
12	825	IVAVIVAPAVAP	12	8	43.2	195.0	2.5	69.7	46.6	23.6
13	895	AIIIVVPAIAAP	12	7	50.2	211.7	2.5	60.8	40.7	20.6
14	896	AILIVVAPIAAP	12	8	50.2	211.7	2.5	57.5	38.5	19.4
15	727	VALAIALPAVLP	12	8	57.3	211.6	2.3	54.7	36.7	18.5
16	603	VLVALAAPVIAP	12	8	57.3	203.3	2.4	54.1	36.1	18.2
17	847	LVAIVVLPAVAP	12	8	50.2	219.2	2.6	50.2	33.4	16.9
18	826	LVALAAPIIAVP	12	7	41.3	211.7	2.4	49.2	32.9	16.6
19	724	VAVLAVLPALAP	12	8	57.3	203.3	2.3	47.5	31.8	16.1
20	563	ALAVIVVPALAP	12	8	50.2	203.3	2.4	47.1	31.4	15.9
21	811	AVVLAVPALAVP	12	7	57.3	195.0	2.3	46.5	31.1	15.7
22	831	IIVAVAPAAIVP	12	7	43.2	203.3	2.5	46.3	31.0	15.7
23	829	AALALVAPVIVP	12	8	50.2	203.3	2.4	44.8	30.0	15.2
24	891	ILAVAAIPAALP	12	8	54.9	195.8	2.2	44.7	29.9	15.1
25	905	AVIAVAPLVVAP	12	7	41.3	195.0	2.4	44.0	29.5	14.9
26	564	VAIALIVPALAP	12	8	50.2	211.7	2.4	43.6	29.1	14.7
27	124	IAVALPALIAAP	12	6	50.3	195.8	2.2	43.6	29.0	14.7
28	827	IAAVLAAPALVP	12	8	57.3	187.5	2.2	43.0	28.8	14.6
29	2	AAAVPLLAVVVP	12	5	41.3	195.0	2.4	40.9	27.2	13.8
30	385	IVAIAVPALVAP	12	7	50.2	203.3	2.4	38.8	25.9	13.1
31	828	IALLAAPIIAVP	12	7	41.3	220.0	2.4	36.8	24.6	12.4
32	806	LVALAVPAAVLP	12	7	57.3	203.3	2.3	36.7	24.6	12.4
33	845	AAVVIAPLLAVP	12	7	41.3	203.3	2.4	35.8	24.0	12.1
34	882	AIALVVPAVAVP	12	7	57.3	195.0	2.4	35.0	23.4	11.8
35	545	VVLVLAAPAAVP	12	8	57.3	195.0	2.3	34.6	23.1	11.7
36	161	AVIALPALIAAP	12	6	57.3	195.8	2.2	34.5	23.0	11.6
37	481	AIAIAIVPVALP	12	8	50.2	211.6	2.4	34.3	23.0	11.6
38	900	ALVAVIAPVVAP	12	8	57.3	195.0	2.4	34.3	22.9	11.6
39	223	AILAVPIAVVAP	12	6	57.3	203.3	2.4	33.0	22.1	11.2
40	824	LIIVAAAPAVAP	12	8	50.2	187.5	2.3	32.8	21.9	11.1
41	562	ALIAAIVPALVP	12	8	50.2	211.7	2.4	32.7	21.8	11.0
42	222	ALLIAPAAVIAP	12	6	57.3	195.8	2.2	32.6	21.7	11.0
43	61	VAALPVLLAALP	12	5	57.3	211.7	2.3	31.2	20.8	10.5
44	582	VAVALIVPALAP	12	8	50.2	203.3	2.4	30.6	20.4	10.3
45	889	ILVAAAPIAALP	12	7	57.3	195.8	2.2	30.3	20.3	10.3
46	787	AVALVPVIVAAP	12	6	50.2	195.0	2.4	29.3	19.6	9.9
47	703	IVAVALVPALAP	12	8	50.2	203.3	2.4	29.2	19.5	9.9
48	705	IVAVALLPALAP	12	8	50.2	211.7	2.4	28.6	19.1	9.7
49	885	LVAIAPAVAVLP	12	6	57.3	203.3	2.4	28.3	19.0	9.6
50	3	AALLVPAAVLAP	12	6	57.3	187.5	2.1	27.0	18.0	9.1
51	601	AAILIAVPIAAP	12	8	57.3	195.8	2.3	26.8	17.9	9.0
52	843	AVLVLVAPAAAP	12	8	41.3	219.2	2.5	26.4	17.7	8.9
53	403	AAALVIPAAILP	12	7	54.9	195.8	2.2	25.2	16.8	8.5
54	544	IVALIVAPAAVP	12	8	43.1	203.3	2.4	23.4	15.6	7.9
55	522	ALLVIAVPAVAP	12	8	57.3	203.3	2.4	22.7	15.2	7.7

[406] [Table 27]

Sequence ID Number	aMTD	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Relative Ratio (Fold)		
								A	B	C
56	805	LVLIAAAPIALP	12	8	41.3	220.0	2.4	22.3	14.9	7.6
57	464	AVVILVPLAAAP	12	7	57.3	203.3	2.4	22.3	14.9	7.5
58	405	LAAAVIPVAILP	12	7	54.9	211.7	2.4	22.2	14.8	7.5
59	747	VALLAIAPALAP	12	8	57.3	195.8	2.2	22.0	14.8	7.5
60	501	VIVALAVPALAP	12	8	50.2	203.3	2.4	21.5	14.4	7.3
61	661	AAILAPIVAALP	12	6	50.2	195.8	2.2	21.4	14.3	7.2
62	786	LVAIAPLAVLAP	12	6	41.3	211.7	2.4	21.2	14.2	7.2
63	625	ILAAAAAPLIVP	12	8	50.2	195.8	2.2	20.9	13.9	7.0
64	442	ALAALVPAVLVP	12	7	57.3	203.3	2.3	20.4	13.6	6.9
65	912	VALLAPAVVVAP	12	6	57.3	195.0	2.3	19.9	13.3	6.7
66	165	ALAVPVALAIVP	12	5	50.2	203.3	2.4	19.8	13.2	6.7
67	422	VVAILAPLLAAP	12	7	57.3	211.7	2.4	19.6	13.1	6.6
68	686	AALVAVLPVALP	12	8	57.3	203.3	2.3	19.5	13.1	6.6
69	343	IVAVALPALVAP	12	7	50.2	203.3	2.3	19.4	12.9	6.5
70	323	IVAVALPVALAP	12	7	50.2	203.3	2.3	19.1	12.8	6.4
71	461	IAAVIVPAVALP	12	7	50.2	203.3	2.4	19.0	12.7	6.4
72	21	AVALLPALLAVP	12	6	57.3	211.7	2.3	18.9	12.6	6.4
73	404	LAAAVIPAAILP	12	7	54.9	195.8	2.2	18.9	12.6	6.4
74	261	LVLVPLLAAAAP	12	5	41.3	211.6	2.3	18.5	12.3	6.2
75	524	AVALIVVPALAP	12	8	50.2	203.3	2.4	18.3	12.2	6.2
76	225	VAALLPAAAVLP	12	6	57.3	187.5	2.1	18.3	12.2	6.2
77	264	LAAAPVVIVIAP	12	5	50.2	203.3	2.4	18.2	12.1	6.1
78	1	AAALAPVVLALP	12	6	57.3	187.5	2.1	17.7	11.8	6.0
79	382	AAALVIPAILAP	12	7	54.9	195.8	2.2	17.7	11.8	6.0
80	463	AVAILVPLLAAP	12	7	57.3	211.7	2.4	17.6	11.7	5.9
81	322	VVAIVLPALAAP	12	7	50.2	203.3	2.3	17.6	11.7	5.9
82	503	AAIIIVLPAALP	12	8	50.2	220.0	2.4	17.6	11.8	5.9
83	870	VLVAAVLPIAAP	12	8	41.3	203.3	2.4	16.6	11.1	5.6
84	241	AAAVVPVLLVAP	12	6	57.3	195.0	2.4	16.6	11.0	5.6
85	726	LAVAIIAPAVAP	12	8	57.3	187.5	2.2	16.5	11.0	5.6
86	341	IVAVALPAVLAP	12	7	50.2	203.3	2.3	16.4	10.9	5.5
87	542	ALALIIVPAVAP	12	8	50.2	211.6	2.4	16.2	10.8	5.5
88	361	AVVIVAPAVIAP	12	7	50.2	195.0	2.4	16.0	10.7	5.4
89	224	ILAAVPIALAAP	12	6	57.3	195.8	2.2	15.8	10.6	5.3
90	482	ILAVAAIPVAVP	12	8	54.9	203.3	2.4	15.8	10.6	5.3
91	64	AIVALPVAVLAP	12	6	50.2	203.3	2.4	15.8	10.6	5.3
92	484	LAVVLAAPAIVP	12	8	50.2	203.3	2.4	15.6	10.4	5.3
93	868	VLVAAILPAAIP	12	8	54.9	211.7	2.4	14.9	10.0	5.0
94	541	LLALIIAPAAAP	12	8	57.3	204.1	2.1	14.8	9.9	5.0
95	666	AAIAIIAPAIVP	12	8	50.2	195.8	2.3	14.7	9.9	5.0
96	665	LAIVLAAPVAVP	12	8	50.2	203.3	2.3	14.7	9.9	5.0
97	363	AVLAVAPALIVP	12	7	50.2	203.3	2.3	14.7	9.8	4.9
98	242	AALLVPALVAAP	12	6	57.3	187.5	2.1	14.6	9.7	4.9
99	384	VIVAIAPALLAP	12	7	50.2	211.6	2.4	14.0	9.4	4.7
100	877	VAIIAVPAVVAP	12	7	57.3	195.0	2.4	14.0	9.4	4.7
101	863	AAVVLLPIIAAP	12	7	41.3	211.7	2.4	13.8	9.3	4.7
102	525	ALAIVVAPVAVP	12	8	50.2	195.0	2.4	13.8	9.2	4.7
103	875	AIAIVVPAVAVP	12	7	50.2	195.0	2.4	13.8	9.2	4.7
104	285	AIVLLPAAVVAP	12	6	50.2	203.3	2.4	13.3	8.9	4.5
105	281	ALIVLPAAVAVP	12	6	50.2	203.3	2.4	13.3	8.9	4.5
106	867	ALLVVIAPLAAP	12	8	41.3	211.7	2.4	13.2	8.8	4.4
107	766	IVVIAVAPAVAP	12	8	50.2	195.0	2.4	12.9	8.6	4.4
108	342	VIVALAPAVLAP	12	7	50.2	203.3	2.3	12.7	8.5	4.3
109	881	AALIVVPAVAVP	12	7	50.2	195.0	2.4	12.7	8.5	4.3
110	505	AIIIVIAPAAAP	12	8	50.2	195.8	2.3	12.4	8.3	4.2

[407] [Table 28]

Sequence ID Number	aMTD	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Relative Ratio (Fold)		
								A	B	C
111	763	VAVLIAVPALAP	12	8	57.3	203.3	2.3	12.3	7.2	4.2
112	706	IVAVALLPAVAP	12	8	50.2	203.3	2.4	12.0	7.0	4.1
113	687	AILAVALPLLAP	12	8	57.3	220.0	2.3	12.0	7.0	4.1
114	643	LALVLAAPAIVP	12	8	50.2	211.6	2.4	11.8	7.9	4.0
115	282	VLAVAPALIVAP	12	6	50.2	203.3	2.4	11.8	7.9	4.0
116	543	LLAALIAPAALP	12	8	57.3	204.1	2.1	11.7	7.8	4.0
117	325	IVAVALPAVALP	12	7	50.2	203.3	2.3	11.7	7.8	4.0
118	846	IAVAVAAPLLVP	12	8	41.3	203.3	2.4	11.7	6.8	4.0
119	383	VIVALAPALLAP	12	7	50.2	211.6	2.3	11.6	7.7	3.9
120	381	VVAIVLPAVAAP	12	7	50.2	195.0	2.4	11.5	7.7	3.9
121	808	LVVLAAAPLAVP	12	8	41.3	203.3	2.3	11.5	7.6	3.9
122	865	AVLVIAVPAIAP	12	8	57.3	203.3	2.5	11.3	7.5	3.8
123	725	IAVLAVAPAVLP	12	8	57.3	203.3	2.3	11.2	7.5	3.8
124	844	VVALLAPLIAAP	12	7	41.3	211.8	2.4	11.2	7.5	3.8
125	897	AVIVPVAIIAAP	12	5	50.2	203.3	2.5	11.2	7.5	3.8
126	605	VIAAVLAPVAVP	12	8	57.3	195.0	2.4	11.0	7.4	3.7
127	744	AAVVIVAPVALP	12	8	50.2	195.0	2.4	11.0	7.3	3.7
128	221	AAILAPIVALAP	12	6	50.2	195.8	2.2	10.9	7.3	3.7
129	622	ALIVLAAPVAVP	12	8	50.2	203.3	2.4	10.6	7.1	3.6
130	401	AALAVIPAAILP	12	7	54.9	195.8	2.2	10.6	7.1	3.6
131	324	IVAVALPAALVP	12	7	50.2	203.3	2.3	10.3	6.9	3.5
132	878	IVALVAPAAVVP	12	7	50.2	195.0	2.4	10.3	6.9	3.5
133	302	LALAPALALLAP	12	5	57.3	204.2	2.1	10.2	6.8	3.4
134	685	ALLVAVLPAALP	12	8	57.3	211.7	2.3	10.2	5.9	3.4
135	848	AVAIVVLPAVAP	12	8	50.2	195.0	2.4	10.0	6.7	3.4
136	602	VIVALAAPVLAP	12	8	50.2	203.3	2.4	9.9	5.8	3.4
137	788	AIAVAIAPVALP	12	8	57.3	187.5	2.3	9.8	6.6	3.3
138	145	LLAVVPAVALAP	12	6	57.3	203.3	2.3	9.5	6.3	3.2
139	11	VVALAPALAALP	12	6	57.3	187.5	2.1	9.5	6.3	3.2
140	141	AVIVLPALAVAP	12	6	50.2	203.3	2.4	9.4	6.3	3.2
141	521	LAALIVVPAVAP	12	8	50.2	203.3	2.4	9.4	6.3	3.2
142	425	AVVAIAPVLALP	12	7	57.3	203.3	2.4	9.4	6.3	3.2
143	365	AVIVVAPALLAP	12	7	50.2	203.3	2.3	9.3	6.2	3.1
144	263	ALAVIPAAAILP	12	6	54.9	195.8	2.2	9.0	6.0	3.0
145	345	ALLIVAPVAVAP	12	7	50.2	203.3	2.3	8.9	5.9	3.0
146	850	LVIALAAPVALP	12	8	57.3	211.7	2.4	8.8	5.9	3.0
147	144	VLAIVPAVALAP	12	6	50.2	203.3	2.4	8.8	5.9	3.0
148	767	IVVAAVVPALAP	12	8	50.2	195.0	2.4	8.5	5.0	2.9
149	185	AALVLPLIIAAP	12	6	41.3	220.0	2.4	8.5	5.7	2.9
150	849	AVILLAPLIAAP	12	7	57.3	220.0	2.4	8.3	4.8	2.8
151	864	ALLVIAPAIAVP	12	7	57.3	211.7	2.4	8.2	4.8	2.8
152	162	AVVALPAALIVP	12	6	50.2	203.3	2.4	8.2	5.5	2.8
153	164	LAAVLPALLAAP	12	6	57.3	195.8	2.1	8.2	5.5	2.8
154	907	VAIALAPVVVAP	12	7	57.3	195.0	2.4	8.1	5.4	2.8
155	444	LAAALVPVALVP	12	7	57.3	203.3	2.3	8.1	5.4	2.7
156	443	ALAALVPVALVP	12	7	57.3	203.3	2.3	8.0	5.3	2.7
157	901	ALVAVLPAVAVP	12	7	57.3	195.0	2.4	7.7	5.1	2.6
158	887	VLAVAPAVAVLP	12	6	57.3	195.0	2.4	7.7	5.1	2.6
159	746	VAIIVVAPALAP	12	8	50.2	203.3	2.4	7.6	4.4	2.6
160	902	ALVAPLLAVAVP	12	5	41.3	203.3	2.3	7.6	5.1	2.6
161	565	VAIVLVAPAVAP	12	8	50.2	195.0	2.4	7.5	5.0	2.5
162	245	AAALAPVLALVP	12	6	57.3	187.5	2.1	7.5	5.0	2.5
163	743	AIAIALVPVALP	12	8	57.3	211.6	2.4	7.4	4.9	2.5
164	465	AVVILVPLAAAP	12	7	57.3	203.3	2.4	7.4	4.9	2.5
165	104	AVVAAPLVLALP	12	6	41.3	203.3	2.3	7.3	4.9	2.5

[408] [Table 29]

Sequence ID Number	aMTD	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Relative Ratio (Fold)		
								A	B	C
166	707	IVALAVLPAVAP	12	8	50.2	203.3	2.4	7.3	4.9	2.5
167	872	VLAAAVLPLVVP	12	8	41.3	219.2	2.5	7.3	4.9	2.5
168	583	AVILALAPIVAP	12	8	50.2	211.6	2.4	7.3	4.8	2.4
169	879	AAIVLLPAVVVP	12	7	50.2	219.1	2.5	7.2	4.8	2.4
170	784	VAALPAVALVVP	12	5	57.3	195.0	2.4	7.1	4.7	2.4
171	893	VIAIPAILAAAP	12	5	54.9	195.8	2.3	7.0	4.7	2.4
172	13	AAALVPVVALLP	12	6	57.3	203.3	2.3	7.0	4.7	2.4
173	809	LIVLAAPALAAP	12	7	50.2	195.8	2.2	7.0	4.7	2.4
174	445	ALAALVPALVVP	12	7	57.3	203.3	2.3	6.9	4.6	2.3
175	81	AALLPALAALLP	12	5	57.3	204.2	2.1	6.9	4.6	2.3
176	667	LAVAIVAPALVP	12	8	50.2	203.3	2.3	6.9	4.6	2.3
177	906	AVIALAPVVVAP	12	7	57.3	195.0	2.4	6.8	4.6	2.3
178	483	ILAAAIIPAALP	12	8	54.9	204.1	2.2	6.8	4.5	2.3
179	485	AILAAIVPLAVP	12	8	50.2	211.6	2.4	6.8	4.5	2.3
180	421	AAILAAPLIAVP	12	7	57.3	195.8	2.2	6.7	4.5	2.3
181	585	ALIVAIAPALVP	12	8	50.2	211.6	2.4	6.6	4.4	2.2
182	424	AVVVAAPVLALP	12	7	57.3	195.0	2.4	6.6	4.4	2.2
183	364	LVAAVAPALIVP	12	7	50.2	203.3	2.3	6.5	4.3	2.2
184	402	ALAAVIPAAILP	12	7	54.9	195.8	2.2	6.4	4.3	2.2
185	462	IAAVLVPAVALP	12	7	57.3	203.3	2.4	6.3	4.2	2.1
186	265	VLAIAPLLAAVP	12	6	41.3	211.6	2.3	6.0	4.0	2.0
187	301	VIAAPVLAVLAP	12	6	57.3	203.3	2.4	6.0	4.0	2.0
188	183	LLAAPVVIALAP	12	6	57.3	211.6	2.4	6.0	4.0	2.0
189	243	AAVLLPVALAAP	12	6	57.3	187.5	2.1	5.9	3.9	2.0
190	664	ILIAIAIPAAAP	12	8	54.9	204.1	2.3	5.7	3.8	1.9
191	783	IVALVPAVAIAP	12	6	50.2	203.3	2.5	5.7	3.8	1.9
192	502	AIVALAVPVLAP	12	8	50.2	203.3	2.4	5.6	3.7	1.9
193	262	ALIAVPAIIVAP	12	6	50.2	211.6	2.4	5.5	3.7	1.9
194	683	LAIVLAAPAVLP	12	8	50.2	211.7	2.4	5.5	3.2	1.9
195	830	IALVAAPVALVP	12	7	57.3	203.3	2.4	5.3	3.5	1.8
196	764	AVALAVLPAVVP	12	8	57.3	195.0	2.3	5.0	3.4	1.7
197	807	AVALAVPALVLP	12	7	57.3	203.3	2.3	5.0	3.3	1.7
198	184	LAAIVPAIIAVP	12	6	50.2	211.6	2.4	4.8	3.2	1.6
199	305	IALAAPILLAAP	12	6	57.3	204.2	2.2	4.8	3.2	1.6
200	101	LVALAPVAAVLP	12	6	57.3	203.3	2.3	4.5	3.0	1.5
201	304	AIILAPIAAIAP	12	6	57.3	204.2	2.3	4.4	3.0	1.5
202	604	VALIAVAPAVVP	12	8	57.3	195.0	2.4	4.3	2.5	1.5
203	645	ALAVVALPAIVP	12	8	50.2	203.3	2.4	4.3	2.9	1.5
204	201	LALAVPALAALP	12	6	57.3	195.8	2.1	4.2	2.8	1.4
205	163	LALVLPAALAAP	12	6	57.3	195.8	2.1	4.1	2.4	1.4
206	832	AVAAIVPVIVAP	12	7	43.2	195.0	2.5	4.1	2.7	1.4
207	182	ALIAPVVALVAP	12	6	57.3	203.3	2.4	4.0	2.7	1.4
208	23	VVLVLPAAAAVP	12	6	57.3	195.0	2.4	4.0	2.6	1.3
209	105	LLALAPAALLAP	12	6	57.3	204.1	2.1	4.0	2.6	1.3
210	561	AAVAIVLPAVVP	12	8	50.2	195.0	2.4	3.9	2.6	1.3
211	765	AVALAVVPAVLP	12	8	57.3	195.0	2.3	3.8	2.2	1.3
212	684	AAIVLALPAVLP	12	8	50.2	211.7	2.4	3.5	2.1	1.2
213	143	AVLAVPAVLVAP	12	6	57.3	195.0	2.4	3.3	2.2	1.1
214	504	LIVALAVPALAP	12	8	50.2	211.7	2.4	3.3	2.2	1.1
215	22	AVVLVPVLAAAP	12	6	57.3	195.0	2.4	3.1	2.1	1.1
216	5	AAALLPVALVAP	12	6	57.3	187.5	2.1	3.1	2.1	1.0
217	283	AALLAPALIVAP	12	6	50.2	195.8	2.2	3.1	2.0	1.0
218	65	IAIVAPVVALAP	12	6	50.2	203.3	2.4	3.0	2.0	1.0
219	883	LAIVPAAIAALP	12	6	50.2	195.8	2.2	3.0	2.0	1.0
220	123	AAIIVPAALLAP	12	6	50.2	195.8	2.2	2.9	2.0	1.0

[409] [Table 30]

Sequence ID Number	aMTD	Sequences	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Relative Ratio (Fold)		
								A	B	C
221	284	ALIAPAVALIVP	12	5	50.2	211.7	2.4	2.8	1.8	0.9
222	205	ALALVPAIAALP	12	6	57.3	195.8	2.2	2.6	1.7	0.9
223	42	VAALPVVAVVAP	12	5	57.3	186.7	2.4	2.5	1.7	0.8
224	121	AIVALPALALAP	12	6	50.2	195.8	2.2	2.5	1.7	0.8
225	25	IVAVAPALVALP	12	6	50.2	203.3	2.4	2.4	1.6	0.8
226	24	IALAAPALIVAP	12	6	50.2	195.8	2.2	2.3	1.6	0.8
227	204	LIAALPAVAALP	12	6	57.3	195.8	2.2	2.2	1.5	0.8
228	12	LLAAVPAVLLAP	12	6	57.3	211.7	2.3	2.2	1.5	0.7
229	43	LLAAPLVVAAVP	12	5	41.3	187.5	2.1	2.1	1.4	0.7
230	103	ALIAAPILALAP	12	6	57.3	204.2	2.2	2.1	1.4	0.7
231	82	AVVLAPVAAVLP	12	6	57.3	195.0	2.4	2.1	1.4	0.7
232	4	ALALLPVAALAP	12	6	57.3	195.8	2.1	2.0	1.3	0.7
233	85	LLVLPAAALAAP	12	5	57.3	195.8	2.1	1.9	1.3	0.7
234	63	AALLVPALVAVP	12	6	57.3	203.3	2.3	1.9	1.3	0.7
235	44	ALAVPVALLVAP	12	5	57.3	203.3	2.3	1.6	1.1	0.5
236	84	AAVAAPLLLALP	12	6	41.3	195.8	2.1	1.5	1.0	0.5
237	62	VALLAPVALAVP	12	6	57.3	203.3	2.3	1.4	0.9	0.5
238	83	LAVAAPLALALP	12	6	41.3	195.8	2.1	1.4	0.9	0.5
239	102	LALAPAALALLP	12	5	57.3	204.2	2.1	1.4	0.9	0.5
240	623	VAAAIALPAIVP	12	8	50.2	187.5	2.3	0.8	0.6	0.3
								19.6±1.6	13.1±1.1	6.6±0.5

[410] Moreover, compared to reference CPPs (B type: MTM12 and C type: MTD85), novel 240 aMTDs averaged of 13±1.1 (maximum 109.9) and 6.6±0.5 (maximum 55.5) fold higher cell-permeability, respectively (Tables 26 to 31).

[411] [Table 31]

	Negative Control rP38	MTM12	MTD85
aMTD The Average of 240 aMTDs	19.6±1.6* (Best: 164.2)	13.1±1.1* (Best: 109.9)	6.6±0.5* (Best: 55.5)

*Relative Fold (aMTD in Geo Mean in its comparison to rP38, MTM12 or MTD85)

[412] In addition, cell-permeabilities of 31 rPeptides have been compared with that of 240 aMTDs (0.3±0.04; Tables 32 and 33).

[413] [Table 32]

Number	ID	Sequence	Length	Proline Position (PP)	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Relative Ratio to aMTD AVE
1	692	PAPLPPVVILAV	12	1,3,5,6	105.5	186.7	1.8	0.74
2	26	AAIALAAPLAIV	12	8	18.1	204.2	2.5	0.65
3	113	PVAVALLIAVPP	12	1,11,12	57.3	195.0	2.1	0.61
4	466	IIAAAAPLAIIP	12	7,12	22.8	204.2	2.3	0.52
5	167	VAIAIPAALAIP	12	6,12	20.4	195.8	2.3	0.50
6	97	ALLAAPPALLAL	12	6,7	57.3	204.2	2.1	0.41
7	390	VPLLVPVVPVVP	12	2,6,9,12	105.4	210.0	2.2	0.41
8	426	AAALAIPLAIIP	12	7,12	4.37	204.2	2.2	0.40
9	214	ALIVAPALMALP	12	6,12	60.5	187.5	2.2	0.33
10	68	VAPVLPAAPLVP	12	3,6,9,12	105.5	162.5	1.6	0.32
11	39	CYNTSPCTGCCY	12	6	52.5	0.0	0.0	0.29
12	934	LILAPAAVVAAA	12	5	57.3	195.8	2.5	0.28
13	938	VPVLLPVVVPVP	12	2,6,10,12	121.5	210.0	2.2	0.28
14	329	LPVLVPVVPVVP	12	2,6,9,12	121.5	210.0	2.2	0.23
15	606	AAAIAAIPIIIP	12	8,12	4.4	204.2	2.4	0.20
16	49	VVPAAPAVPVVP	12	3,6,9,12	121.5	145.8	1.7	0.18
17	139	TGSTNSPTCTST	12	7	53.4	0.0	-0.7	0.17
18	772	LPVAPVIPIIVP	12	2,5,8,12	79.9	210.8	2.1	0.16
19	921	IWWFVVLPLVVP	12	8,12	41.3	194.2	2.2	0.14
20	66	AGVLGGPIMGVP	12	7,12	35.5	121.7	1.3	0.13
21	693	AAPVLPVAVPIV	12	3,6,10	82.3	186.7	2.1	0.13
22	18	NYCCTPTTNGQS	12	6	47.9	0.0	-0.9	0.10
23	16	NNSCTTYTNGSQ	12	None	47.4	0.0	-1.4	0.08
24	227	LAAIVPIAAAVP	12	6,12	34.2	187.5	2.2	0.08
25	17	GGCSAPQTTCSN	12	6	51.6	8.3	-0.5	0.08
26	67	LDAEVPLADDVP	12	6,12	34.2	130.0	0.3	0.08
27	635	GSTGGSQQNNQY	12	None	31.9	0.0	-1.9	0.07
28	29	VLPPLPVLPVLP	12	3,4,6,9,12	121.5	202.5	1.7	0.07
29	57	QNNCNTSSQGGG	12	None	52.4	0.0	-1.6	0.06
30	700	GTSNTCQSNQNS	12	None	19.1	0.0	-1.6	0.05
31	38	YYNQSTCGGQCY	12	ND	53.8	0.0	-1.0	0.05
							AVE	0.3±0.04

[414] [Table 33]

	Relative Ratio to aMTD AVE*
rPeptide The Average of 31 aMTDs	0.3±0.04

*Out of 240 aMTDs, average relative fold of aMTD had been 19.6 fold compared to type A (rP38).

[415] In summary, relatively cell-permeability of aMTDs has shown maximum of 164.0, 109.9 and 55.5 fold higher to rP38, MTM12 and MTD85, respectively. In average of total 240 aMTD sequences, 19.6±1.6, 13.1±1.1 and 6.6±0.5 fold higher cell-permeability are shown to the rP38, MTM12 and MTD85, respectively (Tables 26 to 31). Relative cell-permeability of negative control (rP38) to the 240 aMTDs is only 0.3±0.04 fold.

[416]

[417] **4-5. Intracellular Delivery and Localization of** aMTD-Fused Recombinant Proteins

[418] Recombinant proteins fused to the aMTDs were tested to determine their intracellular delivery and localization by laser scanning confocal microscopy with a negative control (rP38) and previous published CPPs (MTM12 and MTD85) as the positive control references. NIH3T3 cells were exposed to 10 uM of FITC-labeled protein for 1 hour at 37℃, and nuclei were counterstained with DAPI. Then, cells were examined by confocal laser scanning microscopy (FIG. 7). Recombinant proteins fused to aMTDs clearly display intracellular delivery and cytoplasmic localization (FIG. 7) that are typically higher than the reference CPPs (MTM12 and MTD85). The rP38-fused re-combinant protein did not show internalized fluorescence signal (FIG. 7a). In addition, as seen in FIG. 8, rPeptides (his-tagged CRA recombinant proteins fused to each rPeptide) display lower- or non- cell-permeability.

[419]

[420] **4-6. Summary of Quantitative and Visual Cell-Permeability of Newly Developed** aMTDs

[421] Histidine-tagged aMTD-fused cargo recombinant proteins have been greatly enhanced in their solubility and yield. Thus, FITC-conjugated recombinant proteins

have also been tested to quantitate and visualize intracellular localization of the proteins and demonstrated higher cell-permeability compared to the reference CPPs.

[422] In the previous studies using the hydrophobic signal-sequence-derived CPPs - MTS/ MTM or MTDs, 17 published sequences have been identified and analyzed in various characteristics such as length, molecular weight, pI value, bending potential, rigidity, flexibility, structural feature, hydropathy, amino acid residue and composition, and secondary structure of the peptides. Based on these analytical data of the sequences, novel artificial and non-natural peptide sequences designated as advanced MTDs (aMTDs) have been invented and determined their functional activity in intracellular delivery potential with aMTD-fused recombinant proteins.

[423] aMTD-fused recombinant proteins have promoted the ability of protein transduction into the cells compared to the recombinant proteins containing rPeptides and/or reference hydrophobic CPPs (MTM12 and MTD85). According to the results, it has been demonstrated that critical factors of cell-penetrating peptide sequences play a major role to determine peptide-mediated intracellular delivery by penetrating plasma membrane. In addition, cell-permeability can considerably be improved by following the rational that all satisfy the critical factors.

[424]

[425] **5. Structure/Sequence Activity Relationship** (SAR) of aMTDs on Delivery Potential

[426] After determining the cell-permeability of novel aMTDs, structure/sequence activity relationship (SAR) has been analyzed for each critical factor in selected some of and all of novel aMTDs (FIGs. 13 to 16 and Table 34).

[427] [Table 34]

Rank of Delivery Potential	Rigidity/ Flexibility (II)	Sturctural Feature (AI)	Hydropathy (GRAVY)	Relative Ratio (Fold)			Amino Acid Composition			
				A	B	C	A	V	I	L
1~10	55.9	199.2	2.3	112.7	75.5	38.1	4.0	3.5	0.4	2.1
11~20	51.2	205.8	2.4	56.2	37.6	19.0	4.0	2.7	1.7	1.6
21~30	49.1	199.2	2.3	43.6	28.9	14.6	4.3	2.7	1.4	1.6
31~40	52.7	201.0	2.4	34.8	23.3	11.8	4.2	2.7	1.5	1.6
41~50	53.8	201.9	2.3	30.0	20.0	10.1	4.3	2.3	1.1	2.3
51~60	51.5	205.2	2.4	23.5	15.7	7.9	4.4	2.1	1.5	2.0
222~231	52.2	197.2	2.3	2.2	1.5	0.8	4.5	2.1	1.0	2.4
232~241	54.1	199.7	2.2	1.7	1.2	0.6	4.6	1.7	0.2	3.5

[428]

[429] **5-1.** Proline Position: In regards to the bending potential (proline position: PP), aMTDs with its proline at 7' or 8' amino acid in their sequences have much higher cell-permeability compared to the sequences in which their proline position is at 5' or 6' (FIGs. 14a and 15a).

[430]

[431] **5-2.** Hydropathy: In addition, when the aMTDs have GRAVY (Grand Average of Hydropathy) ranging in 2.1 to 2.2, these sequences display relatively lower cell-permeability, while the aMTDs with 2.3 to 2.6 GRAVY are shown significantly higher one (FIGs. 14b and 15b).

[432]

[433] **5-3.** rPeptide SAR: To the SAR of aMTDs, rPeptides have shown similar SAR correlations in the cell-permeability, pertaining to their proline position (PP) and hydropathy (GRAVY). These results confirm that rPeptides with high GRAVY (2.4 to 2.6) have better cell-permeability (FIG. 16).

[434]

[435] **5-4. Analysis of Amino Acid Composition**: In addition to proline position and hydropathy, the difference of amino acid composition is also analyzed. Since aMTDs are designed based on critical factors, each aMTD-fused recombinant protein has equally two proline sequences in the composition. Other hydrophobic and aliphatic amino acids - alanine, isoleucine, leucine and valine - are combined to form the rest of aMTD peptide sequences.

[436] Alanine: In the composition of amino acids, the result does not show a significant difference by the number of alanine in terms of the aMTD's delivery potential because all of the aMTDs have three to five alanines. However, four alanine compositions show the most effective delivery potential (geometric mean) (FIG. 13a).

[437] Leucine and Isoleucine: Also, the compositions of isoleucine and leucine in the aMTD sequences show inverse relationship between the number of amino acid (I and L) and delivery potential of aMTDs. Lower number of isoleucine and leucine in the sequences tends to have higher delivery potential (geometric mean) (FIGs. 13a and 13b).

[438] Valine: Conversely, the composition of valine of aMTD sequences shows positive correlation with their cell-permeability. When the number of valine in the sequence is low, the delivery potential of aMTD is also relatively low (FIG. 13b).

[439] Ten aMTDs having the highest cell-permeability are selected (average geometric mean: 2584±126). Their average number of valine in the sequences is 3.5; 10 aMTDs having relatively low cell-permeability (average geometric mean: 80±4) had average of 1.9 valine amino acids. The average number of valine in the sequences is lowered as their cell-permeability is also lowered as shown in FIG. 13b. Compared to higher cell-permeable aMTDs group, lower sequences had average of 1.9 in their valine composition. Therefore, to obtain high cell-permeable sequence, an average of 2-4 valines should be composed in the sequence.

[440]

[441] **5-5. Conclusion of** SAR Analysis: As seen in FIG. 15, all 240 aMTDs have been

examined for these association of the cell-permeability and the critical factors: bending potential (PP), rigidity/flexibility (II), structure feature (AI), and hydropathy (GRAVY), amino acid length and composition. Through this analysis, cell-permeability of aMTDs tends to be lower when their central proline position is at 5' or 6' and GRAVY is 2.1 or lower (FIG. 15). Moreover, after investigating 10 higher and 10 lower cell-permeable aMTDs, these trends are clearly shown to confirm the association of cell-permeability with the central proline position and hydropathy.

[442]

[443] **6. Experimental Confirmation of Index Range/Feature of Critical Factors**

[444] The range and feature of five out of six critical factors have been empirically and experimentally determined that are also included in the index range and feature of the critical factors initially proposed before conducting the experiments and SAR analysis. In terms of index range and feature of critical factors of newly developed 240 aMTDs, the bending potential (proline position: PP), rigidity/flexibility (Instability Index: II), structural feature (Aliphatic Index: AI), hydropathy (GRAVY), amino acid length and composition are all within the characteristics of the critical factors derived from analysis of reference hydrophobic CPPs.

[445] Therefore, our hypothesis to design and develop new hydrophobic CPP sequences as advanced MTDs is empirically and experimentally proved and demonstrated that critical factor-based new aMTD rational design is correct.

[446] [Table 35]

Summarized Critical Factors of aMTD		
Critical Factor	Newly Designed CPPs	Analysis of Experimental Results
	Range	Range
Bending Potential (Proline Position: PP)	Proline presences in the middle (5', 6', 7' or 8') and at the end of peptides	Proline presences in the middle (5', 6', 7' or 8') and at the end of peptides
Rigidity / Flexibility (Instability Index: II)	40 - 60	41.3 - 57.3
Structural Feature (Aliphatic Index: AI)	180 - 220	187.5 - 220.0
Hydropathy (Grand Average of Hydropathy GRAVY)	2.1 - 2.6	2.2 - 2.6
Length (Number of Amino Acid)	9 - 13	12
Amino acid Composition	A, V, I, L, P	A, V, I, L, P

[447]

[448] **7. Discovery and Development of Protein-Based New** Biotherapeutics with MITT Enabled by aMTDs for Protein Therapy

[449] 240 aMTD sequences have been designed and developed based on the critical factors. Quantitative and visual cell-permeability of 240 aMTDs (hydrophobic, flexible, bending, aliphatic and 12 a/a-length peptides) are all practically determined.

[450] To measure the cell-permeability of aMTDs, rPeptides have also been designed and tested. As seen in FIGs. 13 to 15, there are vivid association of cell-permeability and the critical factors of the peptides. Out of these critical factors, we are able to configure that the most effective cell-permeable aMTDs have the amino acid length of 12; composition of A, V, L, I and P; multiple proline located at either 7' or 8' and at the end (12'); instability index ranged of 41.3 to 57.3; aliphatic index ranged of 187.5 to 220.0; and hydropathy (GRAVY) ranged of 2.2 to 2.6.

[451] These examined critical factors are within the range that we have set for our critical factors; therefore, we are able to confirm that the aMTDs that satisfy these critical factors have relatively high cell-permeability and much higher intracellular delivery potential compared to reference hydrophobic CPPs reported during the past two decades.

[452] It has been widely evident that many human diseases are caused by proteins with deficiency or over-expression that causes mutations such as gain-of-function or loss-of-function. If biologically active proteins could be delivered for replacing abnormal proteins within a short time frame, possibly within an hour or two, in a quantitative manner, the dosage may be regulated depending on when and how proteins may be needed. By significantly improving the solubility and yield of novel aMTD in present invention (Table 31), one could expect its practical potential as an agent to effectively deliver therapeutic macromolecules such as proteins, peptides, nucleic acids, and other chemical compounds into live cells as well as live mammals including human. Therefore, newly developed MITT utilizing the pool (240) of novel aMTDs can be used as a platform technology for discovery and development of protein-based biotherapeutics to apprehend intracellular protein therapy after determining the optimal cargo-aMTD relationship.

[453]

[454] **8. Novel Hydrophobic** CPPs - aMTDs for Development of CP-BMP2/7 Recombinant Proteins

[455] **8-1. Selection of** aMTD for Cell-Permeability

[456] From 240 aMTDs and, 2 aMTDs were selected and used for the construction of CP-BMP2/7 recombinant proteins. 2 aMTDs used are shown in the following Table 36.

[457] Various hydrophobic CPPs - aMTDs have been used to enhance the delivery of cargo proteins (BMP2 or BMP7) to mammalian cells and tissues.

[458] [Table 36]

SEQ ID NO	aMTD ID	Amino Acid Sequences
12	24	IALAAPALIVAP
33	123	AAIIVPAALLAP

[459]

[460] **8-2. Selection of** Solubilization Domain (SD) for Structural Stability

[461] Recombinant cargo protein (BMP2 or BMP7) fused to hydrophobic CPP could be expressed in bacteria system, purified with single-step affinity chromatography, but protein dissolved in physiological buffers (e.q. PBS, DMEM or RPMI1640 etc.) was highly insoluble and had extremely low yield as a soluble form. Therefore, an additional non-functional protein domain (solubilization domain: SD) has been applied to fuse with the recombinant protein for improving the solubility, yield and eventually cell and tissue permeability.

[462] According to the specific aim, the selected domains are SDA to SDF (Table 37). The aMTD/SD-fused BMP2/7 recombinant proteins have been determined for their stability.

[463] The solubilization domains (SDs) and aMTDs have greatly influenced in increasing solubility/yield and cell-/tissue-permeability of the protein. Therefore, we have developed highly soluble and highly stable BMP2/7 recombinant protein fused with SD(s) (SDA, SDB or/and SDC) and aMTDs.

[464] Table 37 shows the Characteristics of Solubilization Domains.

[465] [Table 37]

SD	Genbank ID	Origin	Protein (kDa)	pI	Instability Index (II)	GRAVY
A	CP000113.1	Bacteria	23	4.6	48.1	-0.1
B	BC086945.1	Rat	11	4.9	43.2	-0.9
C	CP012127.1	Human	12	5.8	30.7	-0.1
D	CP012127.1	Bacteria	23	5.9	26.3	-0.1
E	CP011550.1	Human	11	5.3	44.4	-0.9
F	NG_034970	Human	34	7.1	56.1	-0.2

[466]

[467] **8-3. Construction of Expression Vector**

[468] BMP2 and BMP7 are synthesized as pre-pro peptides consisting of a signal peptide (SP), latency associated peptide (LAP) and mature peptide (MP). After the synthesis, SP and LAP are later processed by enzymatic cleavage, where the C-terminal mature domain is released and secreted (FIG. 17). In one embodiment of the present invention,

BMP2 and BMP7 may be prepared an L form consisting of LAP and MP, and an M form consisting of only MP. 16 different types of recombinant protein with or without the aMTD and solubilization domains (SDs) for BMP2/7 were designed.

[469] BMP2 recombinant protein structures for M form were labeled as follows: (2M-1) a BMP2 fused with His-tag, (2M-2) a BMP2 fused with His-tag and aMTD, (2M-3) a BMP2 fused with His-tag, aMTD and SDA, (2M-3C) a BMP2 fused with His-tag and SDA, (2M-4) a BMP2 fused with His-tag, aMTD and SDB, and (2M-4C) a BMP2 fused with His-tag and SDB, and BMP7 recombinant protein structures for M form were labeled as follows: (7M-1) a BMP7 fused with His-tag, (7M-2) a BMP7 fused with His-tag and aMTD, (7M-3) a BMP7 fused with His-tag, aMTD and SDA, (7M-3C) a BMP7 fused with His-tag and SDA, (7M-4) a BMP7 fused with His-tag, aMTD and SDB, and (7M-4C) a BMP7 fused with His-tag and SDB (FIG. 18a). Among them, (2/7M-3) and (2/7M-4) structures were used as candidate proteins having the biological efficacy of CP-BMP recombinant protein, and (2/7M-1), (2/7M-2), (2/7M-3C) and (2/7M-4C) were used as control groups (Non-CP-BMP) with respect to (2/7M-3) and (2/7M-4).

[470] BMP2 recombinant protein structures for L form were labeled as follows: (2L-1) a BMP2 fused with His-tag, (2L-2) a BMP2 fused with His-tag and aMTD, (2L-3) a BMP2 fused with His-tag, aMTD and SDA, (2L-4) a BMP2 fused with His-tag, aMTD and SDB, (2L-5) a BMP2 fused with His-tag, aMTD and two SDB, (2L-5C) a BMP2 fused with His-tag and two SDB, (2L-6) a BMP2 fused with His-tag, aMTD, SDA and SDB, (2L-6C) a BMP2 fused with His-tag, SDA and SDB, (2L-7) a BMP2 fused with His-tag, aMTD and SDC, and (2L-7C) a BMP2 fused with His-tag and SDC, and BMP7 recombinant protein structure for L form were labeled as follows: (7L-1) a BMP7 fused with His-tag, (7L-2) a BMP7 fused with His-tag and aMTD, (7L-3) a BMP7 fused with His-tag, aMTD and SDA, (7L-4) a BMP7 fused with His-tag, aMTD and SDB, (7L-5) a BMP7 fused with His-tag, aMTD and two SDB, (7L-5C) a BMP7 fused with His-tag and two SDB, (7L-6) a BMP7 fused with His-tag, aMTD, SDA and SDB, (7L-6C) a BMP7 fused with His-tag, SDA and SDB, (7L-7) a BMP7 fused with His-tag, aMTD and SDC, and (7L-7C) a BMP7 fused with His-tag and SDC (FIGs. 18b and 23b). Among them, (2/7L-3), (2/7L-4), (2/7L-5), (2/7L-6) and (2/7L-7) structures were used as candidate proteins having the biological efficacy of CP-BMP2/7 recombinant protein, and (2/7L-1), (2/7L-2), (2/7L-5C), (2/7L-6C) and (2/7L-7C) were used as control groups (Non-CP-BMP) with respect to (2/7L-3), (2/7L-4), (2/7L-5), (2/7L-6) and (2/7L-7).

[471]

[472] **8-4. Preparation of** BMP2/7 Recombinant Proteins

[473] The BMP2/7 recombinant proteins were successfully induced by adding IPTG and

purified. The solubility and yield of the BMP2/7 recombinant proteins were determined.

[474] The solubility and yields of BMP2/7 (M form) recombinant proteins fused with SD (2/7M-3 and 2/7M-4) were significant increased, which compared to a BMP2/7 (M form) recombinant proteins without SDs (2/7M-1 and 2/7M-2) (FIGs. 20a and 20b). The solubility and yields of BMP2/7 (L form) recombinant proteins fused with SDs (2/7L-5 and 2/7L-6) were significant increased, which BMP (L form) recombinant proteins without SD (L-1 and L-2) or with SDs (2/7L-3, 2/7L-4 and 2/7L-7) (FIGs. 20a, 20b, 23a and 23b). The results suggested that the BMP2/7 recombinant proteins fused with SDA or/and SDB displayed a significant improvement of solubility and yields.

[475] Taken together, since L form consisting of LAP and MP has a larger size than M form consisting of MP, the BMP recombinant proteins fused with same aMTD and SD may be different cell-permeability depending on L form or M form. BMP requires MP to act on cells, and therefore, in this experiment, BMP recombinant protein consisting of MP (BMP (M form) recombinant protein) was used.

[476]

[477] **9. Determination of Cell-Permeability of** BMP2/7 (M form) Recombinant Proteins

[478] The cell-permeability of the BMP2/7 (M form) recombinant proteins was investigated. BMP2/7 (M form) recombinant proteins were labeled fluorescence dye, FITC (fluorescein isothiocyanate), then cell permeability of the BMP2/7 (M form) recombinant proteins was evaluated in RAW 264.7 cells and NIH3T3 cells. The RAW 264.7 cells and the NIH3T3 cells were cultured in DMEM containing 10% fetal bovine serum (FBS) and 500 mg/mL of 5% penicillin/streptomycin (P/S). The RAW 264.7 cells analyzed by FACS (fluorescence-activated cell sorting) showed a gain in fluorescence, indicative of the presence of FITC-labeled BMP2/7 (M form) recombinant proteins as compared with control that only FITC or vehicle (diluent). For FACS analysis, cells ($1x10^4$) were analyzed using a CellQues Pro cytometric analysis software (FACS Calibur, Beckton-Dickinson, San Diego CA, USA). The cell-permeability of aMTD/SD-fused BMP2/7 (M form) recombinant proteins was examined, respectively (FIG. 24).

[479] In the NIH3T3 cells, the cell-permeability and intracellular localization of the fluorescent signal was determined by confocal laser scanning microscopy (LSM700, Zeiss, Germany; FIG. 25). The intracellular localization of the fluorescent signal was determined by confocal laser scanning microscopy and by Nomarski interference microscope image of the same cells (LSM700, Zeiss, Germany). These results suggest that the BMP2/7 (M form) recombinant protein attaching aMTD is enhanced its cell-permeability and therefore, aMTD is critical for systemic delivery of the protein *in*

vitro.

[480] Accordingly, BMP2/7 recombinant proteins (2M-3, 7M-3, 2M-4 and 7M-4) having superior cell permeability were selected as candidates with biological efficacy (cell-permeability BMP2/7 recombinant protein, CP-BMP2/7).

[481]

[482] **10. Determination of Tissue-Permeability of CP-**BMP2/7 Recombinant Proteins

[483] Tissue-permeability of CP-BMP2/7 recombinant proteins was investigated by intraperitoneal injection of a FITC-labeled aMTD/SD-fused BMP2/7 recombinant protein into ICR mouse. Tissues obtained from each organ (brain, heart, lung, liver, spleen and kidney) after the injection of the protein show that the aMTD-fused CP-BMP2/7 recombinant protein is delivered into each organ (FIG. 26). Thus, these results suggest that the CP-BMP2/7 recombinant protein attaching aMTD is enhanced its tissue-permeability and therefore, aMTD is critical for systemic delivery of the protein *in* vivo.

[484]

[485] **11. Determination of Biological Activity of CP-**BMP2/7 Recombinant Proteins in vitro

[486] To investigate biological activity of the CP-BMP2/7 recombinant protein, MC3T3-E1 cell (preosteoblast), C3H10T-1/2 cell (multiple mesenchymal stem cell) and C2C12 cell (myoblast) were examined for osteogenic differentiation *in vitro*.

[487]

[488] **11-1.** Inbihition of Myotube Formation

[489] C2C12 myoblasts are known to differentiate into myotubes under the starvation condition (<2 % of FBS or horse serum in media), and the treatment of BMPs suppress myogenesis and lead to osteogenesis (FIG. 40a). To examine the effect of CP-BMP2/7 recombinant proteins on the osteogenic differentiation, the C2C12 cells incubated with various dose of the CP-BMP2/7 recombinant proteins in serum free condition for 2 hours, and continuously exposed in 2% FBS media for 7 days (FIG. 27). These results suggest that the CP-BMP2/7 recombinant proteins inhibit myotube formation of the C2C12 cells.

[490]

[491] **11-2. Activation of** Smad Signaling Pathway

[492] To confirm biological activity of CP-BMP2/7 recombinant proteins, the activation of Smad-signaling was investigated. For starvation of cells, confluent C2C12 cells were incubated with serum free DMEM media, and then 10 uM of CP-BMP2/7 recombinant proteins were separately treated for 15 minutes. The cells were lysed, and Smad phosphorylation was examined (FIG. 28). Further, C3H10T1/2 mesenchymal stem cells and MC3T3-E1 preosteoblasts were treated with the BMP2/7 recombinant proteins, and ac-

tivation of Smad-signaling was examined in the cells. These results indicate that the CP-BMP2/7 recombinant proteins activate Smad.

[493]

[494] **11-3. Alkaline** phosphatase (ALP) Activity

[495] Alkaline phosphatase (ALP) is a widely accepted bone marker and activated by BMP stimulation. To confirm biological activity of CP-BMP2/7 recombinant proteins, ALP activity of the MC3T3-E1 cells was investigated (FIG. 29). Further, C3H10T1/2 mesenchymal stem cells and C2C12 cells were treated with the BMP2/7 recombinant proteins, and ALP activity was examined in the cells. These results indicate that the CP-BMP2/7 recombinant proteins increase ALP activity.

[496]

[497] **11-4. Combinational Treatment of CP-**BMP2 and CP-BMP7 recombinant proteins

[498] Synergistic effect of CP-BMP2 and CP-BMP7 recombinant proteins on osteogenic differentiation of C2C12 myoblasts was evaluated with inhibition effect of myotube formation and ALP activity (FIGs. 30 and 31). Further, C3H10T1/2 mesenchymal stem cells and MC3T3-E1 preosteoblasts were treated with the BMP2 or CP-BMP7 recombinant protein, and ALP activity was examined in the cells. These results indicate that combination treatment of the CP-BMP2 and CP-BMP7 recombinant proteins remarkably increase alkaline phosphatase (ALP) expression and significantly inhibit myotube formation, compared to single treatment of CP-BMP2/7 recombinant proteins.

[499]

[500] **12. Determination of Biological Activity of CP-**BMP2/7 Recombinant Proteins in vivo

[501] To investigate the effect of CP-BMP2/7 recombinant proteins on new bone formation of calvaria in vivo, CP-BMP2/7 recombinant proteins were locally injected to defected calvaria of mouse by subcutaneous injection. After 4 weeks, new bone formation was determined by using H&E staining (FIGs. 32 and 33). These results indicate that the CP-BMP2/7 recombinant proteins activate differentiation of osteoblast to form new bone.

[502]

[503] **13. Determination of Optimal** aMTD for CP-BMP2 Recombinant Protein

[504] **13-1. Selection of** aMTD for Cell-Permeability

[505] To improve cell-permeability and activity of the CP-BMP2 recombinant protein, CP-BMP2 recombinant proteins fused with different aMTDs were prepared (FIG. 34). From 240 aMTDs, 17 aMTDs were selected and used for the construction of CP-BMP2 recombinant proteins. 17 aMTDs used are shown in the following Table 38. However, the $aMTD_{481}$-fused CP-BMP2 recombinant protein was not prepared.

[506] [Table 38]

SEQ ID NO	aMTD ID	Amino Acid Sequences
1	1	AAALAPVVLALP
3	3	AALLVPAAVLAP
17	61	VAALPVLLALP
34	124	IAVALPALIAAP
74	321	IVAVALPALAVP
91	385	IVAIAVPALVAP
94	403	AAALVIPAAILP
101	442	ALAALVPAVLVP
110	481	AIAIAIVPVALP
131	563	ALAVIVVPALAP
136	585	ALIVAIAPALVP
139	603	VLVALAAPVIAP
143	623	VAAAIALPAIVP
200	847	LVAIVVLPAVAP
228	897	AVIVPVAIIAAP
229	899	AVVIALPAVVAP

[507]

[508] **13-2. Preparation of CP-**BMP2 Recombinant Proteins

[509] The 12 different types of CP-BMP2 recombinant proteins with the aMTD and SD were successfully induced by adding IPTG and purified (FIGs. 35a and 35b). These results indicate that different aMTDs-fused CP-BMP2 recombinant proteins expressed, and have high solubility and yield. However, the $aMTD_1$, $aMTD_{847}$ or $aMTD_{899}$-fused CP-BMP2 recombinant protein was not expressed.

[510]

[511] **13-3. Determination of Cell-Permeability of CP-**BMP2 Recombinant Proteins

[512] The cell-permeability of the 13 different types of CP-BMP2 recombinant proteins was investigated (FIGs. 36a and 36b). These results indicate that 13 different types of CP-BMP2 recombinant protein have high cell-permeability.

[513]

[514] **13-4. Determination of Biological Activity of CP-**BMP2 Recombinant Proteins

[515] The biological activity of the 4 different types of CP-BMP2 recombinant proteins

was investigated (FIG. 37). ALP activity of CP-BMP2 recombinant proteins was determined in C3H10T1/2 mesenchymal stem cells (FIG. 37). The 4 different types of CP-BMP2 recombinant proteins are each CP-BMP2 recombinant proteins fused with $aMTD_{24}$, $aMTD_{442}$, $aMTD_{563}$ and $aMTD_{623}$. Further, C2C12 myoblasts and MC3T3-E1 preosteoblasts were treated with the 4 different types of CP-BMP2 recombinant protein, and ALP activity was examined in the cells. These results indicate that the $aMTD_{442}$-fused CP-BMP2 recombinant protein has the most excellent ALP activity.

[516] In conclusion, CP-BMP2 recombinant protein attaching $aMTD_{442}$ having the excellent cell permeability was determined as an optimal CP-BMP2 recombinant protein.

[517]

[518] **14. Determination of Cell-Permeability of CP-**BMP2 Recombinant Proteins

[519] The cell-permeability of CP-BMP2 recombinant protein attaching $aMTD_{442}$ was investigated (FIGs. 38 and 39). These results suggest that the CP-BMP2 recombinant protein attaching $aMTD_{442}$ is enhanced its cell-permeability and therefore, $aMTD_{442}$ is critical for systemic delivery of the BMP.

[520]

[521] **15. Determination of Biological Activity of CP-**BMP2 Recombinant Protein in vitro

[522] To reinvestigate the biological activity of the CP-BMP2 recombinant protein which showed excellent effects on bone formation or regeneration, osteogenic differentiation was examined *in vitro*.

[523] C2C12 myoblasts were treated with the CP-BMP2 recombinant protein, and inhibition of myotube formation in the cells was observed (FIG. 40b). C3H10T1/2 mesenchymal stem cells were treated with the CP-BMP2 recombinant protein, and ALP activity of the cells was examined (FIG. 41). Further, C2C12 myoblasts and MC3T3-E1 preosteoblasts were treated with the CP-BMP2 recombinant protein, and inhibition of myotube formation and ALP activity were examined in the cells.

[524] These results suggest that the CP-BMP2 recombinant protein has excellent ability of osteogenic differentiation.

[525]

[526] **16. Determination of Mechanism of CP-**BMP2 Recombinant Protein

[527] To investigate the mechanism of the CP-BMP2 recombinant protein, C2C12 myoblasts were treated with the CP-BMP2 recombinant protein, and then signal intensity of Smad was examined (FIG. 42). Further, the C3H10T1/2 mesenchymal stem cells and MC3T3-E1 preosteoblasts were treated with the CP-BMP2 recombinant protein, and then signal intensity of Smad was examined in the cells. As a result, strong Smad activity was observed in the cells treated with the CP-BMP2 recombinant protein

(CP-BMP2), compared to the cells treated with the BMP2 recombinant protein lacking aMTD (Non-CP-BMP2).

[528] To investigate why the Smad signal induced by the CP-BMP2 recombinant protein is stronger than that of the control protein (Non-CP-BMP2), binding of CP-BMP2 recombinant protein and BMP2 receptor was examined in MC3T3-E1 preosteoblasts (FIG. 43). Further, the C2C12 myoblasts and MC3T3-E1 preosteoblasts were treated with the CP-BMP2 recombinant protein, and then signal intensity of Smad was examined in the cells. As a result, since the CP-BMP2 recombinant proteins permeating cells strongly bind to BMP receptors in intracellular ER (Endoplasmic reticulum) and golgi, strong Smad activity by the CP-BMP2 recombinant protein was observed.

[529]

[530] **17. Determination of Effect of CP-**BMP2 Recombinant Protein in vivo

[531] To investigate the effect of CP-BMP2 recombinant proteins on new bone formation, the CP-BMP2 recombinant proteins were locally injected to calvaria of mouse by subcutaneous injection. After 4 weeks, new bone formation was determined by using H&E staining (FIGs. 44a and 44b).

[532] To investigate the effect of CP-BMP2 recombinant proteins on bone regeneration, calvarial critical-sized defect model was designed in mouse. The CP-BMP2 recombinant proteins were locally injected to defected calvaria of mouse by subcutaneous injection. After 8 weeks, new bone formation was determined by using H&E staining (FIGs. 45a and 45b). Effective administration conditions of CP-BMP2 recombinant protein for bone regeneration were determined by changing administration frequency and dose of the CP-BMP2 recombinant protein (FIGs. 46a, 46b, 47a and 47b).

[533] To investigate the effect of CP-BMP2 recombinant proteins on bone regeneration, equine bone defect model was designed in horse (FIG. 49b). The CP-BMP2 recombinant proteins showed in FIG. 48 were locally injected to defected hind limb of horses by subcutaneous injection. After 9 weeks, new bone formation was determined by using CT (FIGs. 48a, 49b and 50).

[534] As a result, it was confirmed that the CP-BMP2 recombinant protein activated differentiation of osteoblast, leading to effective regeneration of defected bone, suggesting that the CP-BMP2 recombinant protein exhibits excellent effects on recovery of defected bone.

[535]

[536] **18. Determination of Toxicity of CP-**BMP2 Recombinant Protein

[537] To investigate toxicity of the CP-BMP2 recombinant protein *in* vivo, a toxicity assay was performed. In a single dose acute toxicity assay, a high concentration of the CP-

BMP2 recombinant protein was intravenously administered to a mouse once, and the toxicity assay was performed for 2 weeks (FIG. 51). In a repeated dose toxicity assay, different concentrations of the CP-BMP2 recombinant protein were intravenously administered to a mouse once daily, and the toxicity assay was performed for 2 weeks (FIGs. 52a and 52b). As a result, even though high concentrations of CP-BMP2 recombinant protein were administered, no toxicity was observed.

[538]

[539] **19. Determination of** Pharmacokinetics of CP-BMP2 Recombinant Protein

[540] To determinate of pharmacokinetics of CP-BMP2 recombinant proteins, CP-BMP2 recombinant protein was labeled with FITC.

[541] 30 mg/kg of FITC-labeled CP-BMP2 recombinant protein were intravenously administered to mouse. At each time point, PBMCs were separated from the blood and splenocytes were separated from the spleen of the mouse. The CP-BMP2 recombinant proteins were measured in the PBMCs and splenocytes (FIG. 53). Further, the blood that separated from mouse and the CP-BMP2 recombinant protein were mixed, and the concentration of CP-BMP2 recombinant protein was measured at each time point (FIG. 54). As a result, it was confirmed that the CP-BMP2 recombinant protein was stably maintained in the blood for a long period of time.

[542] 15 mg/kg of FITC-labeled CP-BMP2 recombinant proteins were subcutaneously injected into the calvarial bone of mouse, and FITC signals expressed in the calvarial bone of the mouse were measured at each time point (FIG. 55). As a result, it was confirmed that the CP-BMP2 recombinant protein was maintained in the blood and organ for a long period of time. These results suggest that the CP-BMP2 recombinant protein may exist *in* vivo for a long period of time, and the CP-BMP2 recombinant protein may also maintain its effect persistently.

[543]

[544] The following examples are presented to aid practitioners of the invention, to provide experimental support for the invention, and to provide model protocols. In no way are these examples to be understood to limit the invention.

[545]

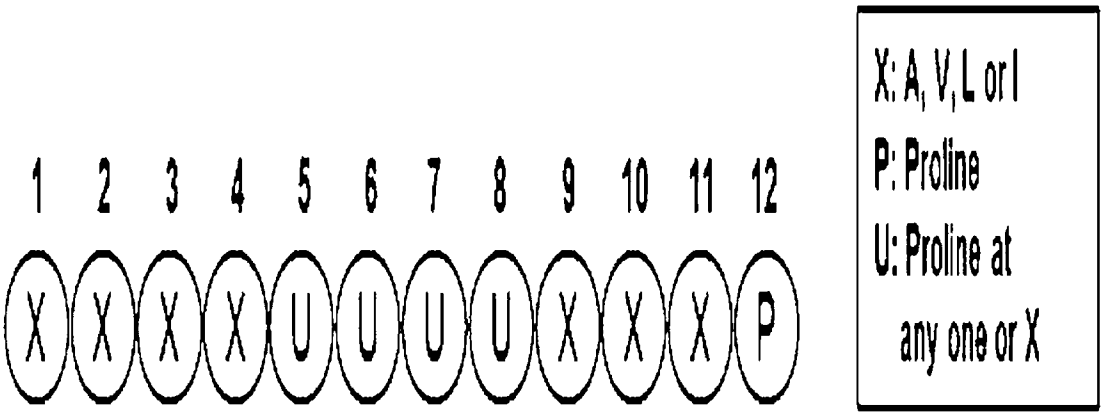
[546] **Example 1. Development of Novel Advanced Macromolecule Transduction Domain** (aMTD)

[547] H-regions of signal sequences (HOURSP)-derived CPPs (MTS/MTM and MTD) do not have a common sequence, a sequence motif, and/or a common structural homologous feature. According to one embodiment of the present invention, the aim is to develop improved hydrophobic CPPs formatted in the common sequence and structural motif that satisfy newly determined 'critical factors' to have a 'common function,' to facilitate protein translocation across the plasma membrane with similar mechanism to

the analyzed CPPs.

[548] The structural motif as follows:

[549]



[550] In Table 9, universal common sequence/structural motif is provided as follows. The amino acid length of the peptides according to one embodiment of the present invention ranges from 9 to 13 amino acids, mostly 12 amino acids, and their bending potentials are dependent with the presence and location of proline in the middle of sequence (at 5', 6', 7' or 8' amino acid) and at the end of peptide (at 12') for re-combinant protein bending. Instability index (II) for rigidity/flexibility of aMTDs is II<40, grand average of hydropathy (GRAVY) for hydropathy is around 2.2, and aliphatic index (AI) for structural features is around 200 (Table 9). Based on these standardized critical factors, new hydrophobic peptide sequences, namely advanced macromolecule transduction domain peptides (aMTDs), according to one embodiment of the present invention have been developed and summarized in Tables 10 to 15.

[551]

[552] **Example 2. Construction of Expression Vectors for Recombinant Proteins Fused to** aMTDs

[553] Our newly developed technology has enabled us to expand the method for making cell-permeable recombinant proteins. The expression vectors were designed for histidine-tagged CRA proteins fused with aMTDs or rPeptides. To construct ex-pression vectors for recombinant proteins, polymerase chain reaction (PCR) had been devised to amplify each designed aMTD or rPeptide fused to CRA.

[554] The PCR reactions (100 ng genomic DNA, 10 pmol each primer, each 0.2 mM dNTP mixture, 1X reaction buffer and 2.5 U Pfu(+) DNA polymerase (Doctor protein, Korea) was digested on the restriction enzyme site between Nde I (5') and Sal I (3') involving 35 cycles of denaturation (95°C), annealing (62°C), and extension (72°C) for 30 seconds each. For the last extension cycle, the PCR reactions remained for 5 minutes at 72°C. Then, they were cloned into the site of pET-28a(+) vectors (Novagen, Darmstadt, Germany). DNA ligation was performed using T4 DNA ligase at 4°C

overnight. These plasmids were mixed with competent cells of *E.* coli DH5-alpha strain on the ice for 10 minutes. This mixture was placed on the ice for 2 minutes after it was heat shocked in the water bath at 42℃ for 90 seconds. Then, the mixture added with LB broth media was recovered in 37℃ shaking incubator for 1 hour. Transformant was plated on LB broth agar plate with kanamycin (50 ug/mL) (Biopure, Johnson City, TN, USA) before incubating at 37℃ overnight. From a single colony, plasmid DNA was extracted, and after the digestion of Nde I and Sal I restriction enzymes, digested DNA was confirmed at 645 bp by using 1.2% agarose gels electrophoresis (FIG. 2). PCR primers for the CRA recombinant proteins fused to aMTD and random peptides (rPeptide) are summarized in Tables 23 to 30. Amino acid sequences of aMTD and rPeptide primers are shown in Tables 31 to 38.

[555]

[556] **Example 3. Inducible Expression, Purification and Preparation of Recombinant Proteins Fused to** aMTDs and rPeptides

[557] To express recombinant proteins, pET-28a(+) vectors for the expression of CRA proteins fused to a negative control [rPeptide 38 (rP38)], reference hydrophobic CPPs (MTM_{12} and MTD_{85}) and aMTDs were transformed in *E. coli* BL21(DE3) strains. Cells were grown at 37℃ in LB medium containing kanamycin (50 ug/mL) with a vigorous shaking and induced at OD_{600}=0.6 by adding 0.7 mM IPTG (Biopure) for 2 hours at 37℃. Induced recombinant proteins were loaded on 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue (InstantBlue, Expedeon, Novexin, UK) (FIG. 3).

[558] The *E.* coli cultures were harvested by centrifugation at 8,000 rpm for 10 minutes, and the supernatant was discarded. The pellet was re-suspended in the lysis buffer (50 mM NaH_2PO_4, 10 mM Imidazol, 300 mM NaCl, pH 8.0). The cell lysates were sonicated on ice using a sonicator (Sonics and Materials, Inc., Newtown, CT, USA) equipped with a probe. After centrifuging the cell lysates at 8,000 rpm for 10 minutes to pellet the cellular debris, the supernatant was incubated with lysis buffer-equilibrated Ni-NTA resin (Qiagen, Hilden, Germany) gently by open-column system (Bio-rad, Hercules, CA, USA). After washing protein-bound resin with 200 mL wash buffer (50 mM NaH_2PO_4, 20 mM Imidazol, 300 mM NaCl, pH 8.0), the bounded proteins were eluted with elution buffer (50 mM NaH_2PO_4, 250 mM Imidazol, 300 mM NaCl, pH 8.0).

[559] Recombinant proteins purified under natural condition were analyzed on 15% SDS-PAGE gel and stained with Coomassie Brilliant Blue (FIG. 4). All of the recombinant proteins were dialyzed for 8 hours and overnight against physiological buffer, a 1:1 mixture of cell culture medium (Dulbecco's Modified Eagle's Medium: DMEM, Hyclone, Logan, UT, USA) and Dulbecco's phosphate buffered saline (DPBS, Gibco,

Grand Island, NY, USA). From 316 aMTDs and 141 rPeptides cloned, 240 aMTD- and 31 rPeptide-fused recombinant proteins were induced, purified, prepared and analyzed for their cell-permeability.

[560]

[561] **Example 4. Determination of Quantitative Cell-Permeability of Recombinant Proteins**

[562] For quantitative cell-permeability, the aMTD- or rPeptide-fused recombinant proteins were conjugated to fluorescein isothiocyanate (FITC) according to the manufacturer's instructions (Sigma-Aldrich, St. Louis, MO, USA). RAW 264.7 cells were treated with 10 uM FITC-labeled recombinant proteins for 1 hour at 37℃, washed three times with cold PBS, treated with 0.25% tripsin/EDTA (Sigma-Aldrich, St. Louis, MO) for 20 minutes at 37℃ to remove cell-surface bound proteins. Cell-permeability of these recombinant proteins were analyzed by flow cytometry (Guava, Millipore, Darmstadt, Germany) using the FlowJo cytometric analysis software (FIGs. 5 to 6). The relative cell-permeability of aMTDs were measured and compared with the negative control (rP38) and reference hydrophobic CPPs (MTM12 and MTD85) (Table 31).

[563]

[564] **Example 5. Determination of Cell-Permeability and Intracellular Localization of Recombinant Proteins**

[565] For a visual reference of cell-permeability, NIH3T3 cells were cultured for 24 hours on coverslip in 24-wells chamber slides, treated with 10 uM FITC-conjugated recombinant proteins for 1 hour at 37℃, and washed three times with cold PBS. Treated cells were fixed in 4% paraformaldehyde (PFA, Junsei, Tokyo, JP) for 10 minutes at room temperature, washed three times with PBS, and mounted with VECTASHIELD Mounting Medium (Vector laboratories, Burlingame, CA, USA), and counter stained with DAPI (4',6-diamidino-2-phenylindole). The intracellular localization of the fluorescent signal was determined by confocal laser scanning microscopy (LSM700, Zeiss, Germany; FIGs. 7 and 8).

[566]

[567] **Example 6. Expression of** BMP2/7 Recombinant Proteins

[568] **<6-1> Construction of Expression Vectors for** BMP2/7 Recombinant Proteins

[569] Full-length cDNA for human BMP2 (RC214586) and BMP7 (RC203813) were purchased from Origene. Our newly developed technology, aMTD-based MITT, has enabled us to improve the method for developing cell-permeable recombinant proteins. The expression vectors were designed for BMP2/7 (M Form) recombinant proteins fused with aMTD/SD (2/7M-3 and 2/7M-4) and control proteins without aMTD- or/and SD (2/7M-1, 2/7M-2, 2/7M-3C and 2/7M-4C), and BMP2/7 (L Form) recombinant proteins fused with aMTD/SD (2/7L-3 and 2/7L-4) and control proteins without aMTD

or/and SD (2/7L-1, 2/7L-2, 2/7M-3C and 2/7M-4C).

[570] To acquire expression vectors for BMP2/7 recombinant proteins, polymerase chain reaction (PCR) had been devised to amplify these recombinant proteins. The PCR reactions (100 ng genomic DNA, 10 pmol each primer, each 0.2 mM dNTP mixture, 1X reaction buffer and 2.5 U Pfu(+) DNA polymerase (Doctor Protein, Korea) was digested on the different restriction enzyme site involving 40 cycles of denaturation (95℃), annealing (58℃), and extension (72℃) for 30 seconds each. For the last extension cycle, the PCR reactions remained for 10 minutes at 72℃. Then, they were cloned into the site of pET-28a(+) vectors (Novagen, Darmstadt, Germany). DNA ligation was performed using T4 DNA ligase (NEB, USA) at 4℃ overnight. These plasmids were mixed with competent cells of *E.* coli (BL21(DE3) codon plus RIL) strain (ATCC, USA) on the ice for 10 minutes. This mixture was placed on the ice for 2 minutes after it was heat-shocked in the water bath at 42℃ for 90 seconds. Then, the mixture added with LB broth media (ELPIS, Korea) was recovered in 37℃ shaking incubator for 1 hour. Transformant was plated on LB broth agar plate with kanamycin (50 ug/mL). From a single colony, plasmid DNA was extracted, and after the digestion of BamHI and HindIII restriction enzymes (NEB, USA), digested DNA was confirmed by using 1.2% agarose gels electrophoresis (FIGs. 19a to 19d). PCR primers for the His-tagged (or not His-tagged) BMP recombinant proteins fused to aMTD and SD are summarized in Table 39 to 42.

[571] As shown in FIGs. 19a to 19d, respective BMP2/7 (M form) recombinant expression vectors were expressed respective BMP2/7 recombinant proteins (2/7M-1, 2/7M-2, 2/7M-3, 2/7M-3C, 2/7M-4 and 2/7M-4C), and respective BMP2/7 (L form) recombinant expression vectors were expressed respective BMP2/7 recombinant protein (2/7L-1, 2/7L-2, 2/7L-3 and 2/7L-4).

[572] [Table 39]

Clone Name	Abbreviation	Priner Sequence (5'→3')	
2M-1	HB2M	Forward	ATTTATCATATGCAAGCCAAACACAAACAGCGG
		Reverse	GGTATTGGATCCCTAGCGACACCCACA
2M-2	$HM_{24}B2M$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGTGATTGTGGC GCCGCAAGCCAAACACAAACAGCGG
		Reverse	GGTATTGGATCCCTAGCGACACCCACA
2M-3	$HM_{24}B2MSA$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGTGATTGTGGC GCCGCAAGCCAAACACAAACAGCGG
		Reverse	TATGTTGGATCCGTAGCGACACCCACA
		Forward	CCCGGATCCATGCAAATATTACCGTTTTCTATAACGAA
		Reverse	CGCGTCGACTTACCTCGGCTGCACCGGCACGGAGATGAC
2M-3C	HB2MSA	Forward	ATTTATCATATGCAAGCCAAACACAAACAGCGG
		Reverse	CGCGTCGACTTACCTCGGCTGCACCGGCACGGAGATGAC
2M-4	$HM_{24}B2MSB$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGTGATTGTGGC GCCGCAAGCCAAACACAAACAGCGG
		Reverse	TATGTTGGATCCGTAGCGACACCCACA
		Forward	CCCGGATCCATGGCAGAACAAAGCGACAAGGATGTGAAG
		Reverse	CGCGTCGACTTAAAGGGTTTCCGAAGGCTTGGCTATCTT
2M-4C	HB2MSB	Forward	ATTTATCATATGCAAGCCAAACACAAACAGCGG
		Reverse	CGCGTCGACTTAAAGGGTTTCCGAAGGCTTGGCTATCTT

[573] [Table 40]

Clone Name	Abbrevlation	Priner Sequence (5'→3')	
7M-1	HB7M	Forward	ATTTATCATATGACGCCCAAGAACCAGGAAGCC
		Reverse	ATAAATGGATCCCTAGTGGCAGCCACA
7M-2	$HM_{24}B7M$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGTGG CGCCGACGCCCAAGAACCAGGAAGCC
		Reverse	ATAAATGGATCCCTAGTGGCAGCCACA
7M-3	$HM_{24}B7MSA$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGTGG CGCCGACGCCCAAGAACCAGGAAGCC
		Reverse	ATAAATGGATCCCTAGTGGCAGCCACA
		Forward	CCCGGATCCATGCAAATATTACCGTTTTCTATAACGAA
		Reverse	CGCGTCGACTTACCTCGGCTGCACCGGCACGGAGATGAC
7M-3C	HB7MSA	Forward	ATTTATCATATGACGCCCAAGAACCAGGAAGCC
		Reverse	CGCGTCGACTTACCTCGGCTGCACCGGCACGGAGATGAC
7M-4	$HM_{24}B7MSB$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGTGG CGCCGACGCCCAAGAACCAGGAAGCC
		Reverse	ATAAATGGATCCCTAGTGGCAGCCACA
		Forward	CCCGGATCCATGGCAGAACAAAGCGACAAGGATGTGAAG
		Reverse	CGCGTCGACTTAAAGGGTTTCCGAAGGCTTGGCTATCTT
7M-4C	HB7MSB	Forward	ATTTATCATATGACGCCCAAGAACCAGGAAGCC
		Reverse	CGCGTCGACTTAAAGGGTTTCCGAAGGCTTGGCTATCTT

[574] [Table 41]

Clone Name	Abbrevlation	Priner Sequence (5'→3')	
2L-1	HB2L	Forward	ATTTATCATATGCTCGTTCCGGAGCTGGGCCGC
		Reverse	GGTATTGGATCCCTAGCGACACCCACA
2L-2	$HM_{24}B2L$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGT GGCGCCGCTCGTTCCGGAGCTGGGCCGC
		Reverse	GGTATTGGATCCCTAGCGACACCCACA
2L-3	$HM_{24}B2LSA$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGT GGCGCCGCTCGTTCCGGAGCTGGGCCGC
		Reverse	TATGTTGGATCCGTAGCGACACCCACA
		Forward	CCCGGATCCATGCAAATATTACCGTTTTCTATAACGAA
		Reverse	CGCGTCGACTTACCTCGGCTGCACCGGCACGGAGATGAC
2L-4	$HM_{24}B2LSB$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGT GGCGCCGCTCGTTCCGGAGCTGGGCCGC
		Reverse	TATGTTGGATCCGTAGCGACACCCACA
		Forward	CCCGGATCCATGGCAGAACAAAGCGACAAGGATGTGAAG
		Reverse	CGCGTCGACTTAAAGGGTTTCCGAAGGCTTGGCTATCTT

[575] [Table 42]

Clone Name	Abbreviation	Priner Sequence (5'→3')	
7L-1	HB7L	Forward	ATTTATCATATGTCCGCCCTGGCCGACTTCAGC
		Reverse	ATAAATGGATCCCTAGTGGCAGCCACA
7L-2	$HM_{24}B7L$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGT GGCGCCGTCCGCCCTGGCCGACTTCAGC
		Reverse	GGTATTGGATCCCCTAGCGACACCCACA
7L-3	$HM_{24}B7LSA$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGT GGCGCCGTCCGCCCTGGCCGACTTCAGC
		Reverse	ATAAATGGATCCCTAGTGGCAGCCACA
		Forward	CCCGGATCCATGCAAATATTACCGTTTTCTATAACGAA
		Reverse	CGCGTCGACTTACCTCGGCTGCACCGGCACGGAGATGAC
7M-4	$HM_{24}B7LSB$	Forward	ATTTATCATATGATTGCGCTGGCGGCGCCGGCGCTGATTGT GGCGCCGTCCGCCCTGGCCGACTTCAGC
		Reverse	ATAAATGGATCCCTAGTGGCAGCCACA
		Forward	CCCGGATCCATGGCAGAACAAAGCGACAAGGATGTGAAG
		Reverse	CGCGTCGACTTAAAGGGTTTCCGAAGGCTTGGCTATCTT

[576]

[577] **<6-2> Expression and Purification of** Histidine-tagged BMP2/7 Recombinant Proteins

[578] *E.* coli containing the recombinant expression vectors was incubated within 1 mL of LB medium at 37℃ overnight, and then inoculated in 700 mL of LB medium, followed by incubation at 37℃, until OD_{600} reached 0.5 to 0.7 mM of isopropyl-β-D-thiogalactoside (IPTG) as a protein expression inducer was added to this culture medium, and then further incubated at 37℃ for 3 hours. This culture medium was centrifuged at 4℃ and 8,000 rpm for 15 minutes, and a supernatant was discarded to recover a cell pellet. The cell pellet thus recovered was suspended in a lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0), and then cells were disrupted by sonication. The cells were centrifuged at 15,000 rpm for 10 minutes to obtain an insoluble fraction containing recombinant proteins. Denatured recombinant proteins were lysed using denature lysis buffer (8 M Urea, 10 mM Tris, 100 mM NaH_2PO_4) and purified by adding Ni-NTA resin. Resin bound to proteins were washed 3 times with 30 mL of denature washing buffer (8 M Urea, 10 mM Tris, 20 m imidazole, 100 mM NaH_2PO_4).

BMP2/7 recombinant proteins (2/7M-1, 2/7M-2, 2/7M-3, 2/7M-3C, 2/7M-4, 2/7M-4C, 2/7L-1, 2/7L-2, 2/7L-3 and 2/7L-4) were eluted 3 times with 30 mL of denature elution buffer (8 M Urea, 10 mM Tris, 250 mM imidazole). After purification, they were dialyzed twice against a refolding buffer (550 mM Guanidine-HCl, 440 mM L-Arginine, 50 mM Tris, 100 mM NDSB, 150 mM NaCl, 2 mM reduced glutathione and 0.2 mM oxidized glutathione). Finally, they were dialyzed against a physiological buffer such as DMEM at 4℃ until the dialysis was over 300 x 10^5 times. Concentration of purified proteins was quantified using Bradford assay according to the manufacturer's instructions. After purification, they were dialyzed against DMEM as indicated above. Finally, SDS-PAGE analysis of cell lysates before (-) and after (+) IPTG induction; aliquots of Ni^{2+} affinity purified BMP2/7 recombinant proteins (P); and molecular weight standards (M) were conducted to confirm the presence of target protein.

[579]

[580] **<6-3> Determination of Solubility/Yield of** BMP2/7 Recombinant Proteins

[581] The aMTD-fused BMP2/7 recombinant proteins containing SDA or SDB are cloned, expressed, purified, and prepared in a soluble form under the denatural condition. Each BMP2/7 recombinant protein fused to aMTD and/or SD (2/7M-1, 2/7M-2, 2/7M-3, 2/7M-4, 2/7L-1, 2/7L-2, 2/7L-3 and 2/7L-4) was determined for their size (number of amino acids), yield (mg/L) and solubility on 10% SDS-PAGE gel and stained with Coomassie Brilliant Blue. Solubility was scored on a 5-point scale ranging from highly soluble proteins with little tendency to precipitate (+++++) to largely insoluble proteins (+) by measuring their turbidity (A450). Yield (mg/L) in physiological buffer condition of each recombinant protein was also determined. The cell-permeable BMP2/7 recombinant proteins were observed as a single band, where the amount of the final purified protein was up to 10 mg/mL in this protein purification procedure.

[582] As shown in FIGs. 20a and 20b, each type of BMP2/7 recombinant proteins were successfully expressed and purified. The solubility and yield of 2/7M-3 and 2/7M-4 were significantly increased compared to control protein (2/7M-1 and 2/7M-2). In contrast, 2/7L-3 and 2/7L-4 showed lower solubility and yield than 2/7M-3 and 2/7M-4, and little solubility, like the control proteins (2/7L-1 and 2/7L-2).

[583]

[584] **Example 7. Expression of New** BMP2/7 (L form) Recombinant Proteins

[585] To solve the problem with low solubility and yields of BMP2/7 (L form) recombinant protein, the type of aMTD and location of SD were changed in the aMTD/SD-fused BMP2/7 recombinant proteins. The expression vectors were designed for BMP2/7 (L Form) recombinant proteins fused with aMTD/SD (2/7L-5, 2/7L-6 and 2/7L-7) and control proteins without aMTD (2/7L-5C, 2/7L-6C and 2/7L-7C). In the

same manner as in Example 6, the recombinant expression vectors prepared by using primers described in Tables 43 and 44 were identified by gel electrophoresis, and each of the BMP recombinant proteins were expressed and purified from each of the recombinant expression vectors, and solubility and yield were measured.

[586] PCR primers for the His-tagged (or not His-tagged) BMP2/7 (L form) recombinant proteins fused to aMTD and SD are summarized in Tables 43 and 44.

[587] As shown in FIG. 22, the respective BMP2/7 (L form) recombinant expression vectors were expressed respective BMP2/7 (L form) recombinant proteins (2/7L-5, 2/7L-5C, 2/7L-6, 2/7L-6C, 2/7L-7 and 2/7L-7C).

[588] [Table 43]

Clone Name	Abbreviation	Priner Sequence (5'→3')	
2L-5	HSBB2LSBM$_{123}$	Forward	TCTTGTCATATGGCAGAACAAAGCGACAAG
		Reverse	TAAGTTGCGGCCGCTTACGCCAGCAGCGCCGCCGGCACAA TAATCGCCGCCGGAAGGGTTTTCCGAAGG
2L-5C	HSBB2LSB	Forward	TCTTGTCATATGGCAGAACAAAGCGACAAG
		Reverse	AATAACGCGGCCGCTTAAAAGGGTTTCCGAAGG
2L-6	HSAB2LSBM$_{123}$	Forward	GGGTTTCATATGATGGCAAATATTACCGTTTTC
		Reverse	TAAGTTGCGGCCGCTTACGCCAGCAGCGCCGCCGGCACAA TAATCGCCGCCGGAAGGGTTTTCCGAAGG
2L-6C	HSAB2LSB	Forward	GGGTTTCATATGATGGCAAATATTACCGTTTTC
		Reverse	AATAACGCGGCCGCTTAAAAGGGTTTCCGAAGG
2L-7	SCHB2LM$_{123}$	Forward	AATATAGGATCCCTCGTTCCGGAGCTGGGC
		Reverse	TAAGTTGCGGCCGCTTACGCCAGCAGCGCCGCCGGCACAA TAATCGCCGCCGGAAGGGTTTTCCGAAGG
2L-7C	SCHB2L	Forward	AATATAGGATCCCTCGTTCCGGAGCTGGGC
		Reverse	GTATTGGTCGACTTAGCGACACCCACAACC

[589] [Table 44]

Clone Name	Abbrevlation	Priner Sequence (5'→3')	
7L-5	HSBB7LSBM$_{123}$	Forward	TCTTGTCATATGGCAGAACAAAGCGACAAG
		Reverse	TAAGTTGCGGCCGCTTACGCCAGCAGCGCCGCCGGCACAA TAATCGCCGCCGGAAGGGTTTTCCGAAGG
7L-5C	HSBB7LSB	Forward	TCTTGTCATATGGCAGAACAAAGCGACAAG
		Reverse	AATAACGCGGCCGCTTAAAGGGTTTTCCGAAGG
7L-6	HSAB7LSBM$_{123}$	Forward	GGGTTTCATATGATGGCAAATATTACCGTTTTC
		Reverse	TAAGTTGCGGCCGCTTACGCCAGCAGCGCCGCCGGCACAA TAATCGCCGCCGGAAGGGTTTTCCGAAGG
7L-6C	HSAB7LSB	Forward	GGGTTTCATATGATGGCAAATATTACCGTTTTC
		Reverse	AATAACGCGGCCGCTTAAAGGGTTTTCCGAAGG
7L-7	SCHB7LM$_{123}$	Forward	AATGATGGATCCTCCGCCCTGGCCGACTTC
		Reverse	TAAGTTGCGGCCGCTTACGCCAGCAGCGCCGCCGGCACAA TAATCGCCGCCGGAAGGGTTTTCCGAAGG
7L-7C	SCHB7L	Forward	AATGATGGATCCTCCGCCCTGGCCGACTTC
		Reverse	TAATATGTCGACTTAGTGGCAGCCACAGGC

[590]

[591] As shown in FIGs. 23a and 23b, each type of BMP2/7 (L form) recombinant proteins were successfully expressed and purified. 2L-5, 7L-5, 2L-6 and 7L-6 were successfully expressed and purified with significantly improved solubility and yield. But, 2L-7 and 7L-7 showed very limited solubility and yield. These results demonstrate that the combinational fusion of SDA and/or SDB to BMP2/7 (L form) recombinant proteins significantly improve their solubility, while SDC fused BMP2/7 (L form) recombinant proteins showed indifference.

[592]

[593] **Example 8. Determination of Cell-Permeability of** BMP2/7 Recombinant Protein

[594] Because we first secured full set of purified BMP2/7 (M form) recombinant proteins, BMP2/7 (M form) recombinant proteins were used for further investigations including cell-/tissue-permeability and biological activity.

[595] For quantitative cell-permeability, 50 ul of 0.1 M sodium carbonate (Biosesang) was added to the each 10 mL of 10 uM aMTD/SD-fused BMP2/7 recombinant proteins (2/7M-1, 2/7M-2, 2/7M-3 and 2/7M-4), vehicle or FITC only. 50 ul/mL of 10 uM flu-

orescein isothiocynate (FITC, Sigma, F7250) was added, and left in a 4℃ shaker overnight. The FITC-aMTD/SD-fused BMP2/7 recombinant proteins were put in a dialysis membrane (Thermo), and 1 L of buffer was added thereto. For 2/7M-1, DMEM was used as a buffer, and for 2/7M-2, 2/7M-3 and 2/7M-4, saline (0.9% sodium chloride) was used as a buffer. The membranes were incubated on a 4℃ stir plate for 3 hours. The buffer was changed, followed by further incubation for 3 hours. The buffer was changed again, followed by overnight incubation. The buffer was changed again, followed by incubation for 2 hours. The proteins were filtered using a 0.2 um syringe filter, and then aliquoted and stored at -70℃ before use.

[596] RAW 264.7 cells (ATCC, USA) were treated with 10 uM of the FITC-labeled BMP2/7 recombinant proteins (2/7M-1, 2/7M-2, 2/7M-3 and 2/7M-4) for 1 hour at 37℃, washed three times with cold PBS, treated with proteinase K (10 ug/mL) for 20 minutes at 37℃ to remove cell-surface bound proteins and subjected to fluorescence-activated cell sorting (FACS) analysis (FACSCalibur; BD, Franklin Lakes, NJ).

[597] As shown in FIG. 24, the aMTD/SD-fused CP-BMP2/7 recombinant proteins (2/7M-3 and 2/7M-4) exhibited superior cell permeability, compared to the BMP2/7 recombinant proteins lacking aMTD or SD (2/7M-1 and 2/7M-2). In particular, 2M-4 and 7M-4 were found to have the highest cell permeability.

[598] For a visual reference of cell-permeability, NIH3T3 cells (ATCC, USA) were cultured for 24 hours on a coverslip in 24-wells chamber slides, treated with 10 uM of vehicle (culture medium, DMEM), FITC only or FITC-conjugated BMP2/7 recombinant proteins (2/7M-1, 2/7M-2, 2/7M-3 and 2/7M-4) for 1 hour at 37℃, and washed three times with cold PBS. Treated cells were fixed in 4% paraformaldehyde (PFA, Junsei, Tokyo, Japan) for 10 minutes at room temperature, washed three times with PBS, and mounted with VECTASHIELD Mounting Medium (Vector laboratories, Burlingame, CA, USA) with DAPI (4',6-diamidino-2-phenylindole) for nuclear staining. The intracellular localization of the fluorescent signal was determined by confocal laser scanning microscopy (top) and by Nomarski interference microscope image of the same cells (LSM700, Zeiss, Germany).

[599] As shown in FIG. 25, the aMTD/SD-fused BMP2/7 recombinant proteins (2/7M-3 and 2/7M-4) exhibited superior cell permeability, compared to the BMP2/7 recombinant proteins lacking aMTD or SD (2/7M-1 and 2/7M-2). In particular, 2M-4 and 7M-4 were found to have the highest cell permeability.

[600] Consequently, 2M-3, 7M-3, 2M-4 and 7M-4 showing excellent cell permeability as well as excellent solubility and yield were determined as CP-BMP2/7 recombinant proteins.

[601]

[602] **Example 9. Determination of Tissue-Permeability of CP-**BMP2/7 Recombinant

Proteins

[603] For tissue-permeability, ICR mouse (6-week-old, male) were injected intraperitoneally (600 ug/head) with vehicle, FITC only or FITC-conjugated BMP2/7 recombinant proteins (2M-4, 2M-4C, 7M-4 and 7M-4C). After 2 hours, the organs (brain, heart, lung, liver, spleen and kidney) were isolated, washed with O.C.T. compound (Sakura), and frozen in deep freezer. Cryosections (15 um thickness) were analyzed by fluorescence microscopy.

[604] As shown in FIG. 26, the aMTD/SD-fused CP-BMP2/7 recombinant proteins (2M-4 and 7M-4) expressed FITC signals in many organs (brain, hear, lung, liver, spleen and kidney), compared to the BMP recombinant proteins lacking aMTD (2M-4C and 7M-4C). As a result, it was confirmed that the BMP2/7 recombinant protein has cell permeability by aMTD.

[605]

[606] **Example 10. Determination of Biological Activity of CP-**BMP2/7 Recombinant Proteins in vitro

[607] **<10-1> Inhibition of** Myotube Formation

[608] C2C12 myoblasts (ATCC, USA) were cultured with high glucose DMEM (Hyclone) and 10% fetal bovine serum (FBS, Hyclone) at 37°C for growth and expansion. The C2C12 cells were plated on 24-well culture plate ($1x10^5$ cells/well) in the growth media for 24 hours. To induce the differentiation, the cells were exposed to a starvation condition with 2% of FBS in a culture media with or without BMP2/7 recombinant proteins. The BMP2/7 recombinant proteins (2M-1, 7M-1, 2M-4 and 7M-4) were treated with different concentration (0.1, 0.5, 0, 5 uM). After 3 days and 7 days of culture, the cell morphologies were photographed to determine the differentiation into either myotube formation.

[609] As shown in FIG. 27, the inhibitory effects on myotube formation of C2C12 cells were not shown at the low concentrations (0.1 and 0.5 uM) of 2M-1, 7M-1, 2M-4 and 7M-4. However, treatment of 2M-1 or 7M-1 at 1 uM significantly inhibited the myotube formation, which manifests the transition of lineage differentiation from myogenic to osteogenic. Highest concentration 5 uM of 2M-1 has shown weak cytotoxicity, while same dose of 7M-1 has shown strong inhibition of myotubes formation without any cytotoxic effect. Unlike what has been previously expected, 2M-4 and 7M-4 did not affect and differentiation of the C2C12 cells even at the high doses (1 and 5 uM). Therefore, we have selected 1 uM of BMP2/7 recombinant protein as the effective concentration for further experiments.

[610]

[611] **<10-2> Activation of** Smad Signaling Pathway

[612] To investigate the activation of BMP-Smad signaling, C2C12 cells were cultured

with high glucose DMEM (Hyclone) and 10% fetal bovine serum (FBS, Hyclone) at 37°C for growth and expansion. The cells were plated on 24-well culture plate ($1x10^5$ cells/well) in the growth media for 24 hours. The cells were incubated with serum-free medium alone (α-MEM or DMEM) containing 10 uM of BMP2/7 recombinant proteins (2/7M-3, 2/7M-3C, 2/7M-4 and 2/7M-4C) of indicated concentration for 15 minutes. The cells treated BMP2/7 recombinant proteins were lysed in a lysis buffer (RIPA buffer) containing a protease cocktail and phosphatase inhibitor cocktail (Sigam). Equal amounts of cell lysate protein were subjected to SDS-PAGE and transferred to nitrocellulose membranes. The protein transferred membranes were incubated to block non-specific binding sites in immersing the membrane in 5% skim milk. The membranes were incubated with anti-phosphorylated Smad1/5/8 (Cellsignaling) overnight at 4°C and anti-β-actin (Santacruz) at room temperature (RT) and then incubated with the appropriate horseradish peroxidase-conjugated secondary antibodies for 1 hour at RT. The blots were developed using a chemiluminescence detection system and exposed to an x-ray film.

[613] As shown in FIG. 28, p-Smad 1/5/8 activities of 2M-4 and 7M-4 were similar to that of vehicle, and 2M-3 and 7M-3 showed the excellent p-Smad 1/5/8 activity.

[614]

[615] **<10-3> ALP Activity**

[616] To investigate whether the CP-BMP2/7 recombinant proteins directly affect osteogenic activity, an ALP activity assay was performed.

[617] Mouse pre-osteoblast, MC3T3-E1 cells were cultured in the minimum essential medium (MEM) alpha modification (Hyclone) with 10 % FBS and 1 % penicillin/streptomycin. ALP activity was measured with cell lysate, according to the manufacturer's protocol. Briefly, supernatant of cell lysate was used after 13000 rpm of centrifugation for 10 min, and 10 ul of supernatant was reacted with 200 ul of ALP substrate solution for 30 minutes at 37°C. After 30 minutes, the optical density (O.D) was measured by using microplate reader at 405 nm of wave length. Different concentrations of p-Nitrophenyl Phosphate were used as standards for ALP activity, and calculated ALP activities were normalized by total protein concentration, which was obtained from bradford (Bio-rad) protein assay.

[618] As shown in FIG. 29, the treatment of CP-BMP2/7 recombinant proteins (2M-3, 7M-3, 2M-4 and 7M-4) showed ALP activity, compared to treatment of the control proteins (2/7M-3C and 2/7M-4C). In particular, 2M-3 as the BMP2 recombinant protein showed 3-folds higher ALP activity than 2M-4, and both 7M-3 and 7M-4 as the BMP7 recombinant protein showed excellent ALP activity.

[619]

[620] **<10-4> Combinational Treatment of CP-**BMP2 and CP-BMP7 Recombinant

Proteins

[621] To evaluated synergistic effect of CP-BMP2 and CP-BMP7 recombinant proteins on osteogenic differentiation of C2C12 myoblasts, inhibitory of myotube formation and ALP activity were investigated.

[622] In the same manner as in Example <10-1>, single treatment or co-treatment with each 1 uM of CP-BMP2 and/or CP-BMP7 recombinant proteins (2M-4 and 7M-4) was performed in the C2C12 cells. After 3 days and 7 days of culture, cell morphologies were photographed to determine the differentiation into either myotube formation or osteogenesis.

[623] As shown in FIG. 30, it was confirmed that co-treatment of the CP-BMP2 and CP-BMP7 recombinant proteins (2M-4 and 7M-4) inhibited myotube formation, compared to single treatment of the CP-BMP2 or CP-BMP7 recombinant protein (2M-4 or 7M-4).

[624] In the same manner as in Example <10-3>, single treatment or co-treatment with each 1 uM of CP-BMP2 and CP-BMP7 recombinant proteins (2M-4 and 7M-4) was performed ALP activity assay in the MC3T3-E1 cells.

[625] As shown in FIG. 31, it was confirmed that co-treatment of the CP-BMP2 and CP-BMP7 recombinant proteins (2M-4 and 7M-4) significantly increased in ALP activity, compared to single treatment of CP-BMP2 or CP-BMP7 recombinant protein (2M-4 or 7M-4).

[626] These results showed that sufficient exposure time of CP-BMP2/7 recombinant proteins is required for effective osteogenic differentiation. Further, combinational treatment of CP-BMP2 and CP-BMP7 synergistically induced the osteogenic differentiation of the cells.

[627]

[628] **Example 11. Determination of Biological Activity of CP-**BMP2/7 Recombinant Proteins in vivo

[629] 7.5 mg/kg of BMP2/7 recombinant proteins (2M-3, 7M-3, 2M-3C and 7M-3C) were subcutaneously injected into the calvarial bone of the B6 mouse (6 weeks, male) for 4 weeks three times a week. After 4 weeks, the calvarial bone was separated. Also, samples were decalcified using Rapidcal for 2 weeks (BBC Biochemical, Mount Vernon, WA, USA) by replacing the solution every 2 days. Samples were dehydrated with graded EtOH (70 to 100%), toluene, and paraffin. Dehydrated samples were embedded in paraffin wax and hardened into a paraffin block for sectioning. Specimens were cut to 6 um using a microtome (Shandon, Runcorn, Cheshire, GB). Sections underwent deparaffinization and hydration and stained nuclei and cytosol with Harris hematoxylin and eosin solution (H&E staining). Goldner's trichrome staining method was used to determined detailed bone tissue morphology such as min-

eralized collagen. Following dehydration, samples were mounted with mounting medium (Richard-Allan Scientific, Kalamazoo, MI, USA) and observed under an optical microscope (Nikon 2000, Japan).

[630] As shown in FIG. 32, only few lining cells were observed on the surface of calvarial bone tissue in diluent treated group. In 2M-3C or 7M-3C treated group, the BMP2/7 recombinant protein without aMTD, showed increase of extra cellular matrix (ECM) formation on the surface of calvaria tissue, which indicated that the immature bone matrix formation. On the other hands, the significant increase of ECM formation was observed in 2M-3 or 7M-3 treated group, the BMP2/7 recombinant protein fused with aMTD.

[631] As shown in FIG. 33, the new bone formation was quantified by measuring their newly formed ECM thickness. Although the 2M-3C or 7M-3C treated group showed more than 5 folds greater relative activity, 2M-3 or 7M-3 treated group showed more than 20 folds greater relative activity which compare to diluent treated group.

[632] These results showed that the fusion of aMTD to BMP2 or BMP7 recombinant proteins, CP-BMP2/7 recombinant proteins, resulted in great increase of their bioactivity such as new bone formation.

[633]

[634] **Example 12. Determination of Biological Activity of CP-**BMP2 Recombinant Proteins in vitro

[635] **<12-1> Expression and Purification of CP-**BMP2 Recombinant Proteins

[636] To improve cell-permeability and activity of the CP-BMP2 recombinant protein, different aMTD-fused CP-BMP2 recombinant proteins were prepared (FIG. 34).

[637] First, the expression vectors were designed for CP-BMP2 recombinant proteins fused with $aMTD_1$, $aMTD_3$, $aMTD_{61}$, $aMTD_{124}$, $aMTD_{241}$, $aMTD_{321}$, $aMTD_{385}$, $aMTD_{403}$, $aMTD_{442}$, $aMTD_{481}$, $aMTD_{563}$, $aMTD_{585}$, $aMTD_{603}$, $aMTD_{623}$, $aMTD_{847}$, $aMTD_{897}$, $aMTD_{899}$. The expression vectors were expressed in the same manner as in Example <6-1>, PCR primers for CP-BMP2 recombinant proteins fused with aMTD and SD are summarized in Table 45.

[638] As a result, it was confirmed that the expression vectors which CP-BMP2 recombinant protein fused with $aMTD_1$, $aMTD_3$, $aMTD_{61}$, $aMTD_{124}$, $aMTD_{241}$, $aMTD_{321}$, $aMTD_{385}$, $aMTD_{403}$, $aMTD_{442}$, aMTD563, $aMTD_{585}$, $aMTD_{603}$, $aMTD_{623}$, $aMTD_{847}$, $aMTD_{897}$ and $aMTD_{899}$, except for $aMTD_{481}$, were prepared.

[639] [Table 45]

No.	Abbreviation	Primer Sequence (5'→3')	
		Forword	Reverse
1	HM_1B2MSA	ATTTATCATATGGCGGCGGCGCTGGCGCCGGTGGTGCTGGCGCTGCCGCA AGCCAAACACAAACAGCGG	CGCGTCGACTTACCTCGGCTGCACCGGCACGGAGATGAC
2	HM_3B2MSA	ATTTATCATATGGCGGCGGCGCTGGCGCCGGTGGTGCTGGCGCTGCCGC AAGCCAAACACAAACAGCGG	
3	HM_{61}B2MSA	ATTTATCATATGGTGGCGGCGCTGCCGGTGCTGCTGGCGGCGCTGCCGCA AGCCAAACACAAACAGCGG	
4	HM_{124}B2MSA	ATTTATCATATGATTGCGGTGGCGCTGCCGGCGCTGATTGCGGCGCCGCA AGCCAAACACAAACAGCGG	
5	HM_{241}B2MSA	ATTTATCATATGGCGGCGGCGGTGGTGCCGGTGCTGCTGGTGGCGCCGC AAGCCAAACACAAACAGCGG	
6	HM_{321}B2MSA	ATTTATCATATGATTGTGGCGGTGGCGCTGCCGGCGCTGGCGGTGCCGCA AGCCAAACACAAACAGCGG	
7	HM_{385}B2MSA	ATTTATCATATGATTGTGGCGATTGCGGTGCCGGCGCTGGTGGCGCCGCA AGCCAAACACAAACAGCGG	
8	HM_{403}B2MSA	ATTTATCATATGGCGGCGGCGCTGGTGATTCCGGCGGCGATTCTGCCGCA AGCCAAACACAAACAGCGG	
9	HM_{442}B2MSA	ATTTATCATATGGCGCTGGCGGCGCTGGTGCCGGCGGTGCTGGTGCCGCA AGCCAAACACAAACAGCGG	
10	HM_{603}B2MSA	ATTTATCATATGGTGCTGGTGGCGCTGGCGGCGCCGGTGATTGCGCCGCA AGCCAAACACAAACAGCGG	
11	HM_{563}B2MSA	ATTTATCATATGGCGCTGGCGGTGATTGTGGTGCCGGCGCTGGCGCCGCA AGCCAAACACAAACAGCGG	
12	HM_{481}B2MSA	ATTTATCATATGGCGATTGCGATTGCGATTGTGCCGGTGGCGCTGCCGCA AGCCAAACACAAACAGCGG	
13	HM_{585}B2MSA	ATTTATCATATGGCGCTGATTGTGGCGATTGCGCCGGCGCTGGTGCCGCA AGCCAAACACAAACAGCGG	
14	HM_{623}B2MSA	ATTTATCATATGGTGGCGGCGGCGATTGCGCTGCCGGCGATTGTGCCGCA AGCCAAACACAAACAGCGG	
15	HM_{847}B2MSA	ATTTATCATATGCTGGTGGCGATTGTGGTGCTGCCGGCGGTGGCGCCGCA AGCCAAACACAAACAGCGG	
16	HM_{897}B2MSA	ATTTATCATATGGCGGTGATTGTGCCGGTGGCGATTATTGCGGCGCCGCA AGCCAAACACAAACAGCGG	
17	HM_{899}B2MSA	ATTTATCATATGGCGGTGGTGATTGCGCTGCCGGCGGTGGTGGCGCCGCA AGCCAAACACAAACAGCGG	

[640]

[641] The expression vectors expressed respective CP-BMP2 recombinant proteins, in the same manner as in Example <6-2>. The 12 different types of CP-BMP2 recombinant protein, except for recombinant proteins fused with $aMTD_1$, $aMTD_{847}$, $aMTD_{899}$, were expressed. Solubility and yield of the 12 different CP-BMP2 recombinant proteins were measured in the same manner as in Example <6-3>.

[642] As shown in FIGs. 35a and 35b, the 12 different types of CP-BMP2 recombinant proteins were successfully expressed and purified, and the solubility and yield of the CP-BMP2 recombinant proteins were increased. In particular, the CP-BMP2 recombinant proteins fused with $aMTD_{24}$, $aMTD_{442}$, $aMTD_{563}$ and $aMTD_{623}$ were found to have high solubility.

[643]

[644] **<12-2> Determination of Cell-Permeability of CP-**BMP2 Recombinant Proteins

[645] The cell-permeability of the 13 different types of CP-BMP2 recombinant proteins was investigated in the same manner as in Example 8. RAW 264.7 cells were treated with the 13 different types of CP-BMP2 recombinant proteins, vehicle or only FITC.

[646] As shown in FIGs. 36a and 36b, aMTD-fused CP-BMP2 recombinant proteins have excellent cell permeability. In particular, CP-BMP2 recombinant proteins fused with $aMTD_{24}$, $aMTD_{442}$, $aMTD_{563}$ and $aMTD_{623}$ were found to have high cell permeability.

[647]

[648] **<12-3> Determination of Biological Activity of CP-**BMP2 Recombinant Proteins

[649] To determine biological activity of 4 different types of CP-BMP2 recombinant protein having excellent cell permeability, an ALP assay was performed in the same manner as in Example <10-3>. C3H10T1/2 mesenchymal stem cells were treated with CP-BMP2 recombinant proteins fused with $aMTD_{24}$, $aMTD_{442}$, $aMTD_{563}$ and $aMTD_{623}$, or control (vehicle).

[650] As shown in FIG. 37, treatment of the CP-BMP2 recombinant protein showed ALP activity, compared to treatment of the control (vehicle). In particular, $aMTD_{442}$-fused CP-BMP2 recombinant protein was showed higher ALP activity. Further, in C2C12 myoblasts and MC3T3-E1 preosteoblasts, treatment of CP-BMP2 recombinant proteins fused with $aMTD_{442}$ showed higher ALP activity.

[651] As in the following Table 46, solubility, cell-permeability and biological activity of each of the CP-BMP2 recombinant proteins fused with different aMTDs ($aMTD_1$, $aMTD_3$, $aMTD_{61}$, $aMTD_{124}$, $aMTD_{241}$, $aMTD_{321}$, $aMTD_{385}$, $aMTD_{403}$, $aMTD_{442}$, $aMTD_{481}$, $aMTD_{563}$, $aMTD_{585}$, $aMTD_{603}$, $aMTD_{623}$, $aMTD_{847}$, $aMTD_{897}$, $aMTD_{899}$) were compared.

[652] [Table 46]

Solubility

Rank	aMTD	Yield (mg/L)
1	623	51
2	563	48
3	442	47
4	24	42

Cell-Permeability

Rank	aMTD	Rel. fold
1	623	12.2
2	442	11.5
3	24	8.5
4	563	7.9

Biological Activity

Rank	aMTD	ALP activity
1	442	6.92
2	563	5.24
3	623	5.23
4	24	2.97

[653]

[654] The $aMTD_{442}$-fused CP-BMP2 recombinant protein showing the most excellent biological activity as well as excellent solubility and cell-permeability was determined as an optimal CP-BMP2 recombinant protein, and this $aMTD_{442}$-fused CP-BMP2 recombinant protein was subjected to subsequent experiments.

[655]

[656] **Example 13. Determination of Cell-Permeability of CP-**BMP2 Recombinant Protein

[657] To investigate cell-permeability of the $aMTD_{442}$ fused CP-BMP2 recombinant protein, RAW 264.7 cells and NIH3T3 cells were used in the same manner as in Example 8. The RAW 264.7 cells and NIH3T3 cells were treated with the FITC-labeled CP-BMP2 recombinant proteins, vehicle, FITC only or control protein (BMP2).

[658] As shown in FIGs. 38 and 39, it was confirmed that aMTD/SD-fused CP-BMP2 recombinant protein (CP-BMP2) exhibited superior cell-permeability, compared to the BMP2 which lacking aMTD/SD. These results suggest that the CP-BMP2 recombinant protein fused with $aMTD_{442}$ is enhanced its cell-permeability and therefore, $aMTD_{442}$ is critical for systemic delivery of the BMP.

[659]

[660] **Example 14. Determination of Biological Activity of CP-**BMP2 Recombinant Protein in vitro

[661] To reinvestigate the biological activity of the CP-BMP2 recombinant protein which showed excellent effects on bone formation *in* vivo, osteogenic differentiations were examined in the C2C12 myoblasts, C3H10T1/2 mesenchymal stem cells and MC3T3-E1 preosteoblasts.

[662]

[663] **<14-1> Inhibition of** Myotube Formation

[664] In the same manner as in Example <10-1>, the C2C12 myoblasts were incubated with serum-free medium containing 1 uM of CP-BMP2 recombinant proteins or vehicle for 2 hours, and washed out with PBS. Then, the cells incubated for 7 days under 2% FBS media without any additional treatment of CP-BMP2 recombinant proteins. The cell morphologies were photographed to determine the differentiation into either myotube formation.

[665] As shown in FIG. 40b, treatment of the CP-BMP2 recombinant proteins showed inhibition of myotube formation, compared to treatment of the vehicle.

[666]

[667] **<14-2> ALP Activity**

[668] C3H10T1/2 mesenchymal stem cells (ATCC, USA) were maintained in the Roswell Park Memorial Institute medium (RPMI) 1640 (Hyclone) with 10 % FBS and 1 % penicillin/streptomycin. To induce the osteogenic differentiation, the cells were exposed to a starvation condition with a serum-free culture media. The cells were incubated with serum-free medium containing 1 uM of CP-BMP2 recombinant proteins (CP-BMP2), control protein (Non-CP-BMP2; BMP2 recombinant protein fused with his-tag and SD) or vehicle for 2 hours and washed out with PBS. The

culture media changed with 10% FBS. After 7 days of culture, ALP activity was measured in the same manner as in Example <10-3>.

[669] As shown in FIG. 41, treatment of CP-BMP2 recombinant proteins (CP-BMP2) showed 11-folds higher ALP activity than treatment of the vehicle, and 3-folds higher ALP activity than treatment of the control protein (Non-CP-BMP2).

[670] C2C12 myoblasts were incubated with serum-free medium containing 1 uM of CP-BMP2 recombinant proteins or vehicle for 2 hours, and washed out with PBS. Then, the cells incubated for 7 days under 2% FBS media without any additional treatment of 1 uM of CP-BMP2 recombinant proteins (CP-BMP2), control protein (Non-CP-BMP2; BMP2 recombinant protein fused with his-tag and SD) or vehicle for 2 hours and washed out with PBS. The culture media changed with 10% FBS. After 7 days of culture, ALP activity was measured in the same manner as in Example <10-3>.

[671] MC3T3-E1 preosteoblasts (ATCC, USA) were maintained in Alpha Modification of Eagle's Minimum Essential Media (α-MEM) (Hyclone) with 10 % FBS and 1 % penicillin/streptomycin. To induce the osteogenic differentiation, the cells were exposed to a starvation condition with a serum-free culture media included 50 mg/mL ascorbic acid, 10 mM β-glycerophosphate (SFOM). The cells were incubated with SFOM containing 1 uM of CP-BMP2 recombinant proteins (CP-BMP2), control protein (Non-CP-BMP2) or vehicle for 2 hours and washed out with PBS. After 7 days of culture, ALP activity was measured in the same manner as in Example <10-3>.

[672] As a result, in C2C12 myoblasts and MC3T3-E1 preosteoblasts, treatment of CP-BMP2 recombinant proteins (CP-BMP2) showed higher ALP activity than treatment of the vehicle and control protein (Non-CP-BMP2).

[673]

[674] **Example 15. Determination of Mechanism of CP-**BMP2 Recombinant Proteins

[675] To investigate the mechanism of the CP-BMP2 recombinant protein, intracellular activity and binding of the CP-BMP2 recombinant protein were examined.

[676]

[677] **<15-1> Activation of** Smad Signaling Pathway

[678] To investigate the activation of Smad signaling, C2C12 cells were incubated with 1 M of CP-BMP2 recombinant proteins (CP-BMP2), control protein (Non-CP-BMP2) or vehicle in the same manner as in Example <10-2>.

[679] As shown in FIG. 42, treatment of the CP-BMP2 recombinant proteins showed p-Smad 1/5/8 phosphorylation. And, treatment of the CP-BMP2 recombinant proteins showed significantly increased p-Smad 1/5/8 phosphorylation, compared to treatment of the control protein (non-CP-BMP2).

[680] To investigate the activation of Smad signaling, C3H10T1/2 mesenchymal stem cells and MC3T3-E1 preosteoblasts were incubated with 1 M of CP-BMP2 recombinant

proteins (CP-BMP2), control protein (Non-CP-BMP2) and vehicle in the same manner as in Example <10-2>.

[681] As a result, in C2C12 myoblasts and MC3T3-E1 preosteoblasts, treatment of the CP-BMP2 recombinant proteins showed p-Smad 1/5/8 phosphorylation. And, treatment of the CP-BMP2 recombinant proteins showed significantly increased p-Smad 1/5/8 phosphorylation, compared to treatment of the control protein (non-CP-BMP2).

[682]

[683] **<15-2> Binding of CP-**BMP2 Recombinant Protein and BMP Receptor

[684] To investigate why the Smad signal of the CP-BMP2 recombinant proteins is stronger than that of the control protein, co-localization of BMP2 and BMP receptor was examined.

[685] MC3T3-E1 preosteoblasts were cultured in 10% FBS-supplemented α-MEM (Modification). The cells were seeded in an 8-well slide chamber at a density of $5x10^3$ cells/well, and incubated for 24 hours. The cells were incubated in serum-free media (α-MEM) for 2 hours, and treated with 1 uM of FITC-labeled Plain-BMP2 recombinant proteins (Plain-BMP2, BMP2 fused with his-tag only), FITC-labeled CP-BMP2 recombinant proteins (CP-BMP2) or vehicle, followed by incubation for 2 hours. The cells were washed with PBS three times, and fixed in 4% paraformaldehyde for 20 minutes. The cells were washed with PBS three times, followed by permeabilization with 0.5% Triton X-100 for 15 minutes and incubation in a blocking buffer (3% BSA-0.05% Triton X-100) for 30 minutes. Anti-BMP receptor II Ab (santa cruz, dilution 1:100) was diluted with an Ab reaction buffer (1% BSA and 0.05% Triton X-100), and 100 ul thereof was treated to cells, followed by incubation for 1 hour. The cells were washed with PBS, and then incubated for 30 minutes in a PE-conjugated anti-goat IgG Ab (Bioss, dilution 1:100)-diluted Ab reaction buffer. Then, the cells were washed with PBS and treated with Cy-5.5-conjugated anti-PDI Ab (Bioss, ER marker) or Cy-5-conjugated anti-giantin Ab (Bioss, gogi marker), followed by incubation for 1 hour. The cells mounted with VECTASHIELD Mounting (with DAPI) (Vector laboratories, Burlingame, CA, USA), and the intracellular localization of the fluorescent signal was determined by confocal laser scanning microscopy (LSM800, Zeiss, Germany)

[686] As shown in FIG. 43, the CP-BMP2 recombinant protein including aMTD permeated cells, and thus co-localized with ER or golgi. However, it was confirmed that the control protein (Plain-BMP2) and vehicle did not permeate cells.

[687] As a result, since the aMTD-fused BMP (CP-BMP2 recombinant protein) is co-localized with BMP receptors in intracellular ER and golgi, strong Smad activity by CP-BMP2 recombinant protein is observed.

[688]

[689] **Example 16. Determination of Effect of CP-**BMP2 Recombinant Proteins in vivo

[690] The effect of CP-BMP2 recombinant proteins on bone formation and regeneration was investigated.

[691]

[692] **<16-1> New Bone Formation**

[693] 7.5 mg/kg of CP-BMP2 recombinant proteins (CP-BMP2), vehicle (diluent) or control protein (Non-CP-BMP2) were subcutaneously injected into the calvarial bone of the B6 mouse (6 weeks, male) for 4 weeks three times a week. After 4 weeks, the calvarial bone was separated, followed by decalcification for 3 weeks. Paraffin blocks of the calvarial bone were prepared, followed by sectioning. After deparaffination of the sections, new bone formation was determined by using Hematoxylin and Eosin (H&E) staining.

[694] As shown in FIG. 44a, only few lining cells were observed on the surface of calvarial bone tissue in diluent treated group. In non-CP-BMP2 recombinant proteins treated group, the BMP2 protein without aMTD, showed increase of extra cellular matrix (ECM) formation on the surface of calvaria tissue, which indicated that the immature bone matrix formation. On the other hands, the significant increase of ECM formation was observed in CP-BMP2 recombinant proteins treated group, the BMP2 protein fused with aMTD.

[695] As shown in FIG. 44b, the new bone formation was quantified by measuring newly formed ECM thickness. Although the non-CP-BMP2 recombinant proteins treated group showed more than 6 folds greater relative activity ($P<0.001$), CP-BMP2 recombinant proteins treated group showed more than 22 folds greater relative activity which compare to diluent treated group ($P<0.001$).

[696]

[697] **<16-2>** Calvarial Critical-Sized Defect Model

[698] The effect of CP-BMP2 recombinant proteins for bone regeneration *in vivo* was investigated by calvarial critical sized defect model using ICR mouse (6 weeks) (Dooyeol biothec, Seoul, Korea). The mice were anesthetized with Zoletil (60 mg/kg) and Xylazine (20 mg/kg) and exposed incision area by shaving scalp hair. For defect creation, head skin incision was performed; two defects on both sides of the calvaria were made by using 4 mm-diameter surgical trephine bur. Surgery sites were sutured and treated with Povidone iodine. After 24 hours of surgery, the CP-BMP2, non-CP-BMP2 or diluent was locally injected to surgery site, and the injection was repeated for 8 weeks three times a week during experimental periods. All mice were sacrificed after 9 weeks, and the calvarial bone was separated and bone regeneration was examined by x-ray and Micro-CT. The fixed calvarial tissues were exposed to soft X-rays (CMP-2, Softex Co., Tokyo, Japan) under optimized exposure condition (23 kV, 2

mA, 90 s). The exposed results were obtained by the developing film. Three-dimensional images from micro-CT scanning were analyzed with Adobe Photoshop CS6 (Adobe Systems, CA, USA) to measure regenerated bone areas.

[699] As shown in FIGs. 45a and 45b, the group treated with CP-BMP2 recombinant protein (CP-BMP2) showed 8 times higher bone regeneration than the group treated with vehicle (diluent).

[700] To determine administration frequency of the CP-BMP2 recombinant protein, to 4 groups of ICR mouse (6 weeks), each group having 6 mice, 7.5 mg/kg of CP-BMP2 recombinant proteins (CP-BMP2), vehicle (diluent) or control protein (Non-CP-BMP2) were subcutaneously injected once or three times a week for 8 weeks. The calvarial bone was separated and bone regeneration was examined by X-ray and Micro-CT.

[701] As shown in FIGs. 46a and 46b, bond regeneration was observed in all groups treated with the CP-BMP2 recombinant protein once a week or three times a week. The administration frequency was determined as once a week for 8 weeks.

[702] To determine the administration concentration of the CP-BMP2 recombinant protein, 0, 0.75, 3.75, 7.5, 15, 75, or 150 mg/kg of CP-BMP2 recombinant proteins (CP-BMP2) were subcutaneously injected to 7 groups of ICR mouse (6 weeks), each group having 10 mice, once a week for 8 weeks. The calvarial bone was separated and bone regeneration was examined by X-ray and Micro-CT.

[703] As shown in FIGs. 47a and 47b, bond regeneration was observed in all groups treated with the CP-BMP2 recombinant protein. Excellent bone regeneration effect was also observed in the groups treated with low concentration (7.5 mg/kg). Thus, the administration concentration was determined as 7.5 mg/kg.

[704] As a result, when 7.5 mg/kg of the CP-BMP2 recombinant protein was administered once a week for 8 weeks, excellent bone regeneration effect may be expected in mouse.

[705]

[706] **<16-3> Equine Bone Defect Model**

[707] To investigate the efficacy of the CP-BMP2 recombinant protein in a large animal, 3rd metatarsal bones of both hind limbs of a horse was drilled (diameter 4.5 mm X depth 10 mm) to prepare an equine hind limb hole defect model (FIGs. 48 and 49b). rBMP2 (BMP2; Cellivery, Korea) or CP-BMP2 recombinant protein (CP-BMP2) was subcutaneously injected to the defected site of the left limb, once a week for 8 weeks. Further, to compare the BMP2 administration method using a scaffold, rhBMP2 (Original; Cowell®, Korea) or CP-BMP2 recombinant protein (CP-BMP2), together with a collagen scaffold, were injected to the defected site of the right limb during operation. After operation, bone regeneration was examined by portable X-ray every week, and at 9 weeks, and the horse was sacrificed, followed by CT examination.

[708] Structures of rBMP2 (BMP2), rhBMP2 (Original), and CP-BMP2 recombinant protein (CP-BMP2) were showed in FIG. 48, and information about the horse was showed in FIG. 49a, and images of X-ray and CT were showed in FIG. 49b.

[709] As shown in FIG. 50, in the group with scaffold, the CP-BMP2 recombinant proteins (CP-BMP2) confirmed the bone regeneration effect similar to rhBMP2 (Original) (original: 1±0.35, CP-BMP2: 0.81±0.31, p=0.345). In the group without scaffold, the CP-BMP2 recombinant proteins (CP-BMP2) confirmed about 8.6-folds higher bone regeneration effect than rBMP2 (BMP2) (BMP2: 1±0.36, CP-BMP2: 8.68±1.31, $p<0.05$).

[710] As shown in FIG. 49b, completely bone regeneration by treatment of the CP-BMP2 recombinant proteins was observed from CT at 9 weeks.

[711] As a result, the CP-BMP2 recombinant protein confirmed excellent bone regeneration effect on mouse and horse, compared to the BMP2 recombinant protein lacking aMTD, suggesting efficient intracellular delivery of BMP2 by aMTD and effective regeneration of defected bone by the CP-BMP2 recombinant protein.

[712]

[713] **Example 17. Determination of Toxicity of CP-**BMP2 Recombinant Proteins

[714] To investigate toxicity of the CP-BMP2 recombinant protein *in* vivo, a toxicity assay was performed.

[715]

[716] **<17-1> Single Dose Acute Toxicity Assay**

[717] A toxicity assay was performed after single administration of ICR mouse (5 weeks) with high concentration of CP-BMP2 recombinant protein. A group was comprised of 5 male mice and 5 female mice. Each 75, 100, 150, or 200 mg/kg of the CP-BMP2 recombinant protein was intravenously administered once, or 1000 mg/kg thereof was subcutaneously administered. The survival of mouse was examined for 2 weeks. A control group was administered with a vehicle at a volume equal to the CP-BMP2 recombinant protein.

[718] In FIG. 51, the survival rate for only 5 days was for acute toxicity of CP-BMP2 recombinant protein, but the examination was actually performed for 2 weeks.

[719] As shown in FIG. 51, death of only one female mouse was observed in the group intravenously administered with 200 mg/kg of the CP-BMP2 recombinant protein, and all mice survived in other groups. Reduction in voluntary exercise was observed in the group subcutaneously administered with 1000 mg/kg of CP-BMP2 recombinant protein, but this symptom was not observed at 1 day after administration.

[720]

[721] **<17-2> Repeated Dose Toxicity Assay**

[722] A toxicity assay was performed after repeated subcutaneous administration of ICR

mouse (6 weeks) with the CP-BMP2 recombinant protein for 2 weeks. A group was comprised of 5 male mice and 5 female mice.

[723] The group treated with CP-BMP2 recombinant protein was administered with each 1.875, 3.75, or 7.5 mg/kg/day of the CP-BMP2 recombinant protein, and a control group was administered with a vehicle at a volume equal to the CP-BMP2 recombinant protein. For 2 weeks after administration, the mice were weighed. After 2 weeks, all mice were sacrificed, and measured the weights of the organs (brain, liver, heart, spleen, and kidney).

[724] As shown in FIGs. 52a and 52b, all mice in the group treated with CP-BMP2 recombinant protein and the control group survived, and there were no significant weight changes in the body weight and organs.

[725] As a result, it was confirmed that the CP-BMP2 recombinant protein has no *in* vivo toxicity.

[726]

[727] **Example 18. Determination of** Pharmacokinetics of CP-BMP2 Recombinant Proteins

[728] To determinate of pharmacokinetics of CP-BMP2 recombinant proteins, bioavailability of the CP-BMP2 recombinant proteins was investigated *in vitro* and *in* vivo.

[729]

[730] **<18-1>** Bioavailability in vivo

[731] ICR mouse (male, 6 weeks) were intravenously administered with 30 mg/kg of FITC-labeled CP-BMP2 recombinant protein (CP-BMP2) or control protein; rBMP2 (BMP2), and then the blood was collected every 10 minutes, and the spleen was separated every 2 hours.

[732] The blood was immediately put in an EDTA tube and mixed well, followed by centrifugation at 4,000 rpm and 4℃ for 5 minutes. Plasma was removed from the centrifuged blood, and only buffy coat was collected in a new microtube. 0.5 mL of RBC lysis buffer was put in the microtube, followed by vortexing. The microtube was left at room temperature for 5 minutes, followed by centrifugation at 4,000 rpm and 4℃ for 5 minutes. (When RBCs were not completely removed, 0.5 mL of RBC lysis buffer was put again, followed by vortexing). After removing a supernatant, a pellet was peripheral blood mononuclear cells (PBMCs), and added 0.3 mL of PBS, followed by pipetting.

[733] The spleen was separated into single cells using a slide glass or cell strainer in the presence of PBS. The cells were collected in a microtube, followed by centrifugation at 4,000 rpm and 4℃ for 5 minutes. After removing a supernatant, 0.5 mL of RBC lysis buffer was added thereto, followed by vortexing. The microtube was left at room tem-

perature for 5 minutes, followed by centrifugation at 4,000 rpm and 4℃ for 5 minutes. (When RBCs were not completely removed, 0.5 mL of RBC lysis buffer was put again, followed by vortexing). After removing a supernatant, a pellet was splenocytes, and added 0.5 mL of PBS, followed by pipetting. The PBMC and splenocyte were subjected to fluorescence-activated cell sorting (FACS) analysis (FACSCalibur; BD, Franklin Lakes, NJ).

[734] As shown in FIG. 53, in the PBMCs, the highest peak of the CP-BMP2 recombinant protein (CP-BMP2) was detected at 10 minutes, but no control protein (BMP2) was detected. In the splenocytes, the highest peak of the CP-BMP2 recombinant protein (CP-BMP2) was detected at 2 hours, whereas the highest peak of the control protein (BMP2) was detected at 10 minutes, but the peak was lower than that of the CP-BMP2 recombinant protein (CP-BMP2). High concentration of the CP-BMP2 recombinant protein (CP-BMP2) was detected and maintained for 8 hours, compared to the control protein (BMP2).

[735]

[736] **<18-2>** Bioavailability *ex vivo*

[737] The whole blood was obtained from ICR mouse (6 weeks), and then mixed with respective CP-BMP2 recombinant proteins (CP-BMP2) or control protein (BMP2; Plain-BMP2). A ratio of blood and protein was 7.5 ug of protein per 1 mL of blood, based on *in* vivo study. The blood and each protein were allowed to react at each time point. After reaction, plasma was separated from the blood, and histidine included in the plasma was detected by His-ELISA (Genscript Co.).

[738] As shown in FIG. 54, in *ex* vivo, high concentration was also detected for a long period of time upon treatment of CP-BMP2, compared to treatment of BMP2 (Plain-BMP2).

[739]

[740] **<18-3>** Bioavailability for duration time in vivo

[741] 15 mg/kg of Cy5-labeled CP-BMP2 recombinant protein (CP-BMP2) or control protein (Non-CP-BMP2) were subcutaneously injected into the calvarial bone of ICR mouse (6 weeks). Distribution of Cy5-labeled proteins was measured at each time point by using a bio-imaging analyzer (KODAK Image Station 4000MM).

[742] As shown in FIG. 55, high concentration of the CP-BMP2 recombinant proteins (CP-BMP2) was detected for a long period of time, compared to the control protein (Non-CP-BMP2).

[743] As a result, the CP-BMP2 recombinant protein was stably maintained *in vivo* for a long period of time, indicating that when the CP-BMP2 recombinant protein is applied to drugs, it is maintained for a long period of time to efficiently activity and functions of BMP2 *in* vivo.

[744]

[745] Those skilled in the art to which embodiments of the present invention pertain will appreciate that the embodiments of the present invention may be implemented in different forms without departing from the essential characteristics thereof. Therefore, it should be understood that the disclosed embodiments are not limitative, but illustrative in all embodiments. The scope of the present invention is made to the appended claims rather than to the foregoing description, and all variations which come within the range of equivalency of the claims are therefore intended to be embraced therein.

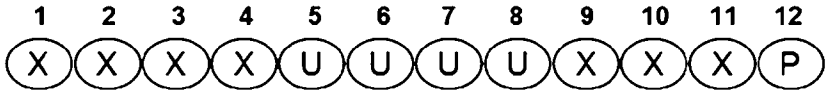
Claims

[Claim 1] A cell-permeable bone morphogenetic protein (CP-BMP) recombinant protein, which comprises a BMP being one of BMP2 and BMP7, and an advanced macromolecule transduction domain (aMTD) being composed of 9 to 13 amino acid sequences and having improved cell or tissue permeability,
wherein the aMTD is fused to one end or both ends of the BMP and has the following features of:
(a) being composed of 3 or more amino acids selected from the group consisting of Ala, Val, Ile, Leu, and Pro;
(b) having proline as amino acids corresponding to any one or more of positions 5 to 8, and 12 of its amino acid sequence; and
(c) having an instability index of 40 to 60; an aliphatic index of 180 to 220; and a grand average of hydropathy (GRAVY) of 2.1 to 2.6, as measured by Protparam.

[Claim 2] The CP-BMP recombinant protein according to claim 1, wherein one or more solubilization domain (SD)(s) are further fused to the end(s) of one or more of the BMP and the aMTD.

[Claim 3] The CP-BMP recombinant protein according to claim 1, wherein the aMTD has α-Helix structure.

[Claim 4] The CP-BMP recombinant protein according to claim 1, wherein the aMTD is composed of 12 amino acid sequences and represented by the following general formula:
[General formula]



wherein X(s) refers to Alanine (A), Valine (V), Leucine (L) or Isoleucine (I); one of U refers to proline and the other U(s) refer to A, V, L or I; and P refers to proline.

[Claim 5] A CP-BMP recombinant protein which is represented by any one of the following structural formula:
A-B-C, A-C-B, B-A-C, B-C-A, C-A-B, C-B-A and A-C-B-C
wherein A is an advanced macromolecule transduction domain (aMTD) having improved cell or tissue permeability, B is a BMP having one of BMP2 and BMP7, and C is a solubilization domain (SD); and the aMTD is composed of 9 to 13 amino acid sequences and has the following features of:

(a) being composed of 3 or more amino acids selected from the group consisting of Ala, Val, Ile, Leu, and Pro;
(b) having proline as amino acids corresponding to any one or more of positions 5 to 8, and 12 of its amino acid sequence;
(c) having an instability index of 40 to 60; an aliphatic index of 180 to 220; and a grand average of hydropathy (GRAVY) of 2.1 to 2.6, as measured by Protparam; and
(d) having α-Helix structure.

[Claim 6] The CP-BMP recombinant protein according to claim 1 or claim 5, wherein the BMP has an amino acid sequence selected from the group consisting of SEQ ID NOs: 815 to 818.

[Claim 7] The CP-BMP recombinant protein according to claim 6, wherein the BMP is encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 819 to 823.

[Claim 8] The CP-BMP recombinant protein according to claim 1 or claim 5, wherein the BMP further comprises a ligand selectively binding to a receptor of a cell, a tissue, or an organ.

[Claim 9] The CP-BMP recombinant protein according to claim 1 or claim 5, wherein the aMTD has an amino acid sequence selected from the group consisting of SEQ ID NOs: 1 to 240.

[Claim 10] The CP-BMP recombinant protein according to claim 9, wherein the aMTD is encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 241 to 481.

[Claim 11] The CP-BMP recombinant protein according to claim 2 or claim 5, wherein the SD(s), independently, have an amino acid sequence selected from the group consisting of SEQ ID NOs: 799 to 805.

[Claim 12] The CP-BMP recombinant protein of claim 11, wherein the SD(s), independently, are encoded by a polynucleotide sequence selected from the group consisting of SEQ ID NOs: 806 to 812.

[Claim 13] The CP-BMP recombinant protein according to claim 1 or claim 5, wherein the CP-BMP recombinant protein has a histidine-tag affinity domain additionally fused to one end thereof.

[Claim 14] The CP-BMP recombinant protein according to claim 13, wherein the histidine-tag affinity domain has an amino acid sequence of SEQ ID NO: 813.

[Claim 15] The CP-BMP recombinant protein of claim 14, wherein the histidine-tag affinity domain is encoded by a polynucleotide sequence of SEQ ID NO: 814.

[Claim 16] The CP-BMP recombinant protein according to claim 1 or claim 5, wherein the fusion is formed via a peptide bond or a chemical bond.

[Claim 17] The CP-BMP recombinant protein according to claim 1 or claim 5, wherein the CP-BMP recombinant protein is used for regeneration of defected bone.

[Claim 18] A polynucleotide sequence encoding the CP-BMP recombinant protein of claim 1.

[Claim 19] The polynucleotide sequence according to claim 18, wherein the polynucleotide sequence is selected from the group consisting of SEQ ID NOs: 824 and 825.

[Claim 20] A polynucleotide sequence encoding the CP-BMP recombinant protein of claim 5.

[Claim 21] The polynucleotide sequence according to claim 20, wherein the polynucleotide sequence is selected from the group consisting of SEQ ID NOs: 826 and 827.

[Claim 22] A recombinant expression vector comprising the polynucleotide sequence of claim 18 or claim 20.

[Claim 23] A transformant transformed with the recombinant expression vector of claim 22.

[Claim 24] A preparing method of the CP-BMP recombinant protein of claim 1 or claim 5 comprising:
preparing the recombinant expression vector of claim 22;
preparing a transformant using the recombinant expression vector;
culturing the transformant; and
recovering the recombinant protein expressed by the culturing.

[Claim 25] A composition comprising the CP-BMP recombinant protein of claim 1 or claim 5 as an active ingredient.

[Claim 26] A pharmaceutical composition for regenerating of defected bone comprising the CP-BMP recombinant protein of claim 1 or claim 5 as an active ingredient; and a pharmaceutically acceptable carrier.

[Claim 27] Use of the CP-BMP recombinant protein of claim 1 or claim 5 as a medicament for regenerating of defected bone.

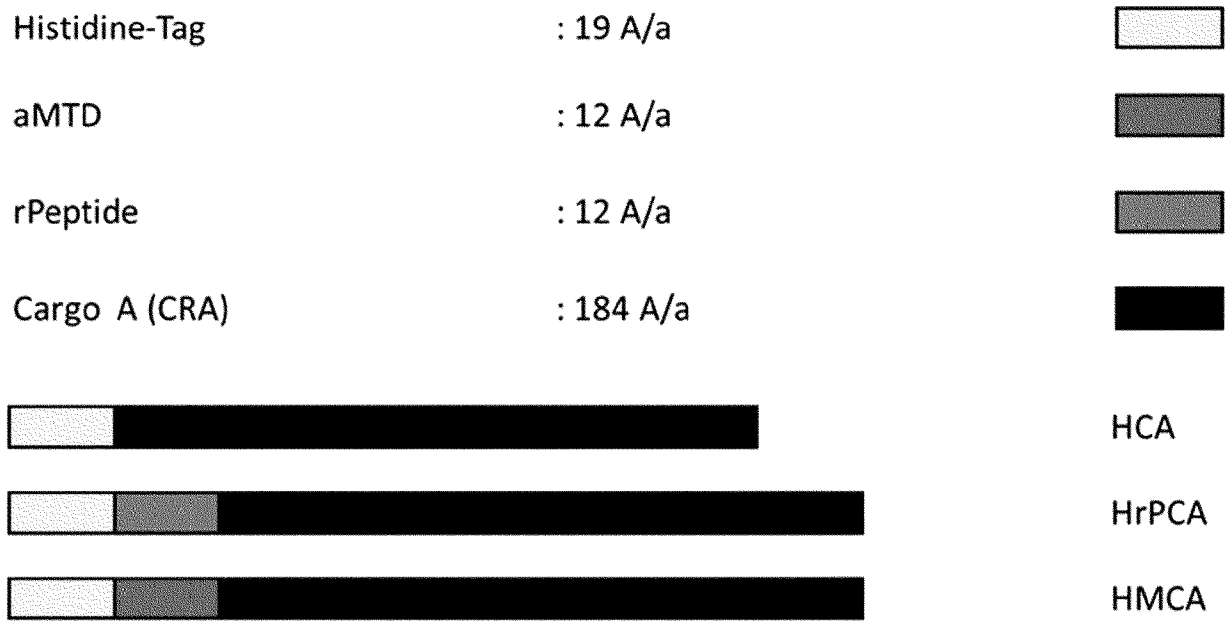
[Claim 28] A medicament comprising the CP-BMP recombinant protein of claim 1 or claim 5.

[Claim 29] Use of the CP-BMP recombinant protein of claim 1 or claim 5 for the preparation of a medicament for regenerating of defected bone.

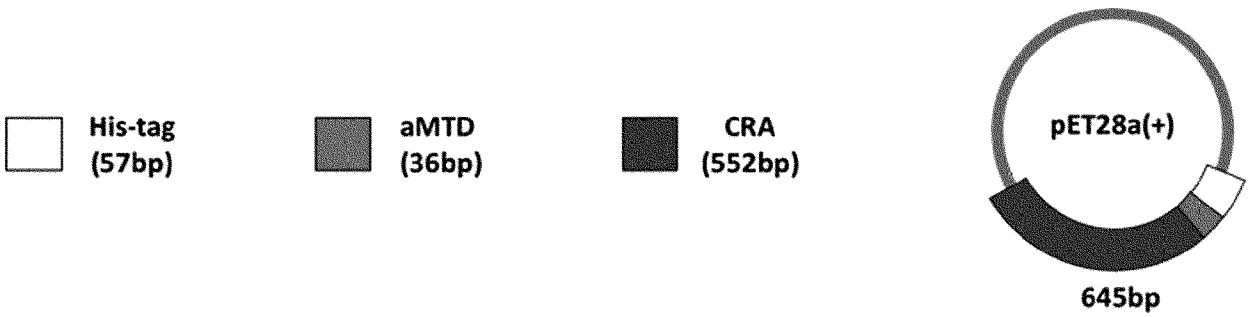
[Claim 30] A method of regenerating of defected bone:
preparing defected bone; and

treating the defected bone with an therapeutically effective amount of the CP-BMP recombinant protein according to claim 1 or claim 5.

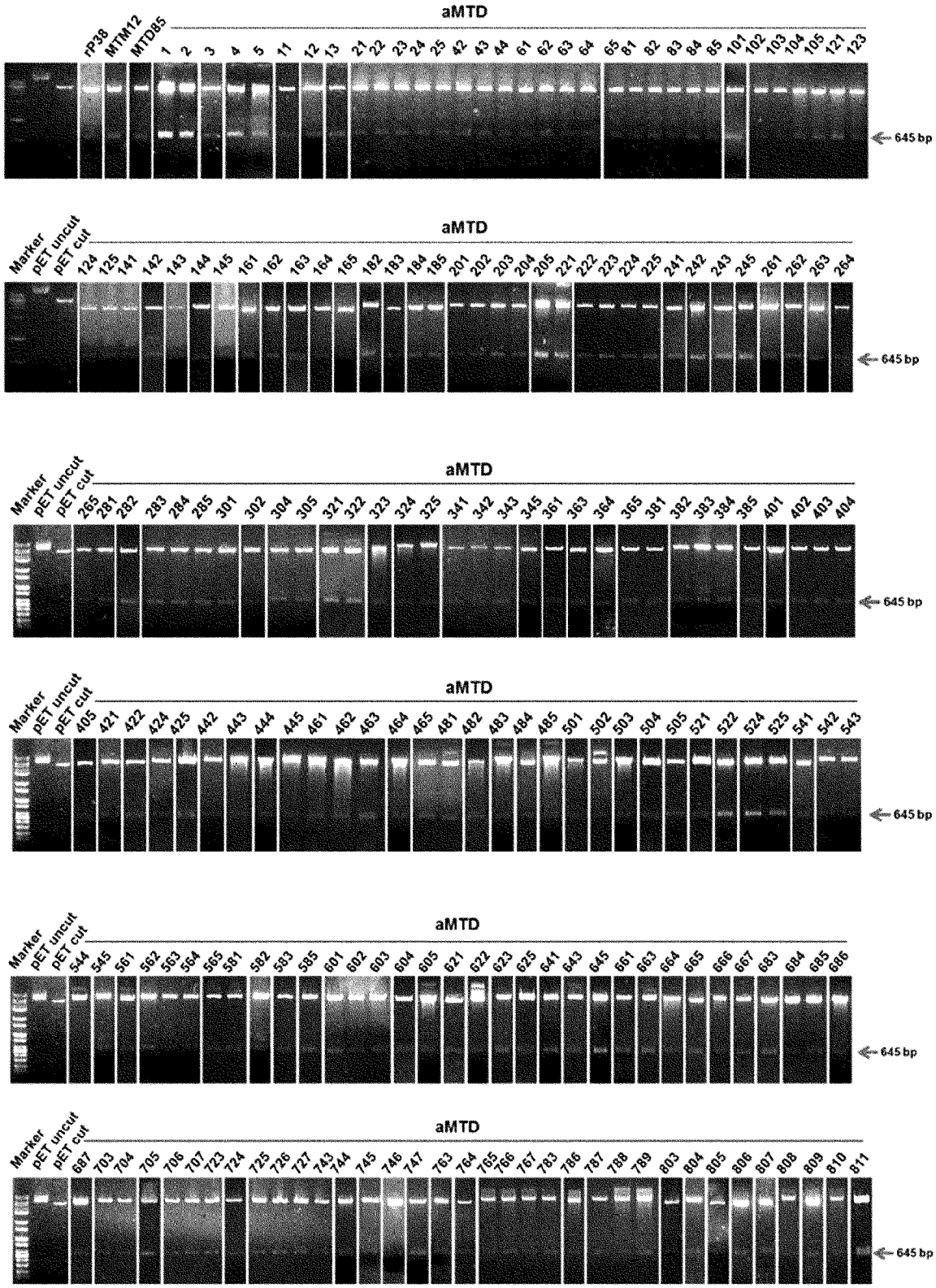
[Fig. 1]


Histidine-Tag : 19 A/a
aMTD : 12 A/a
rPeptide : 12 A/a
Cargo A (CRA) : 184 A/a
HCA
HrPCA
HMCA

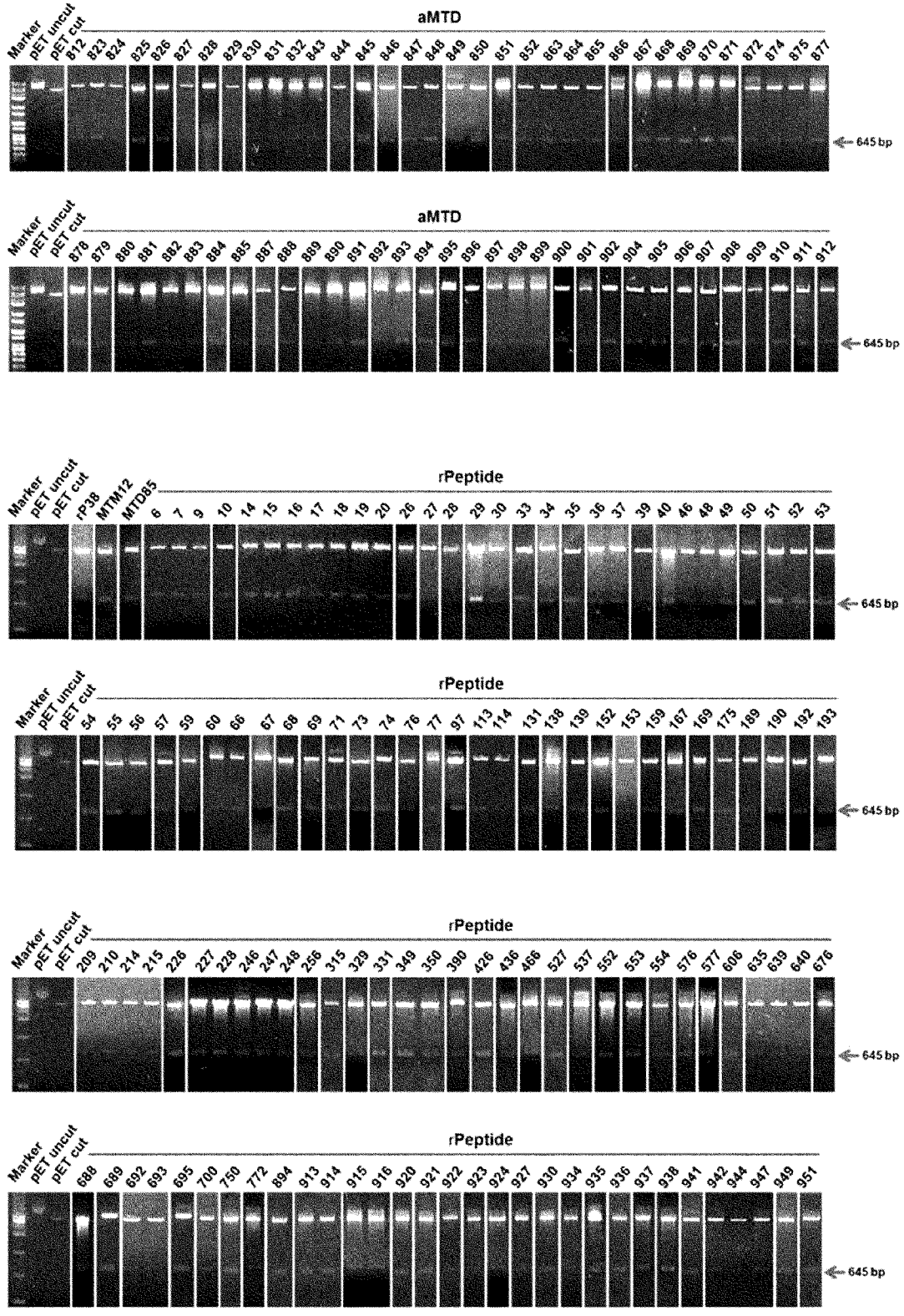
[Fig. 2a]


His-tag (57bp)
aMTD (36bp)
CRA (552bp)
pET28a(+)
645bp

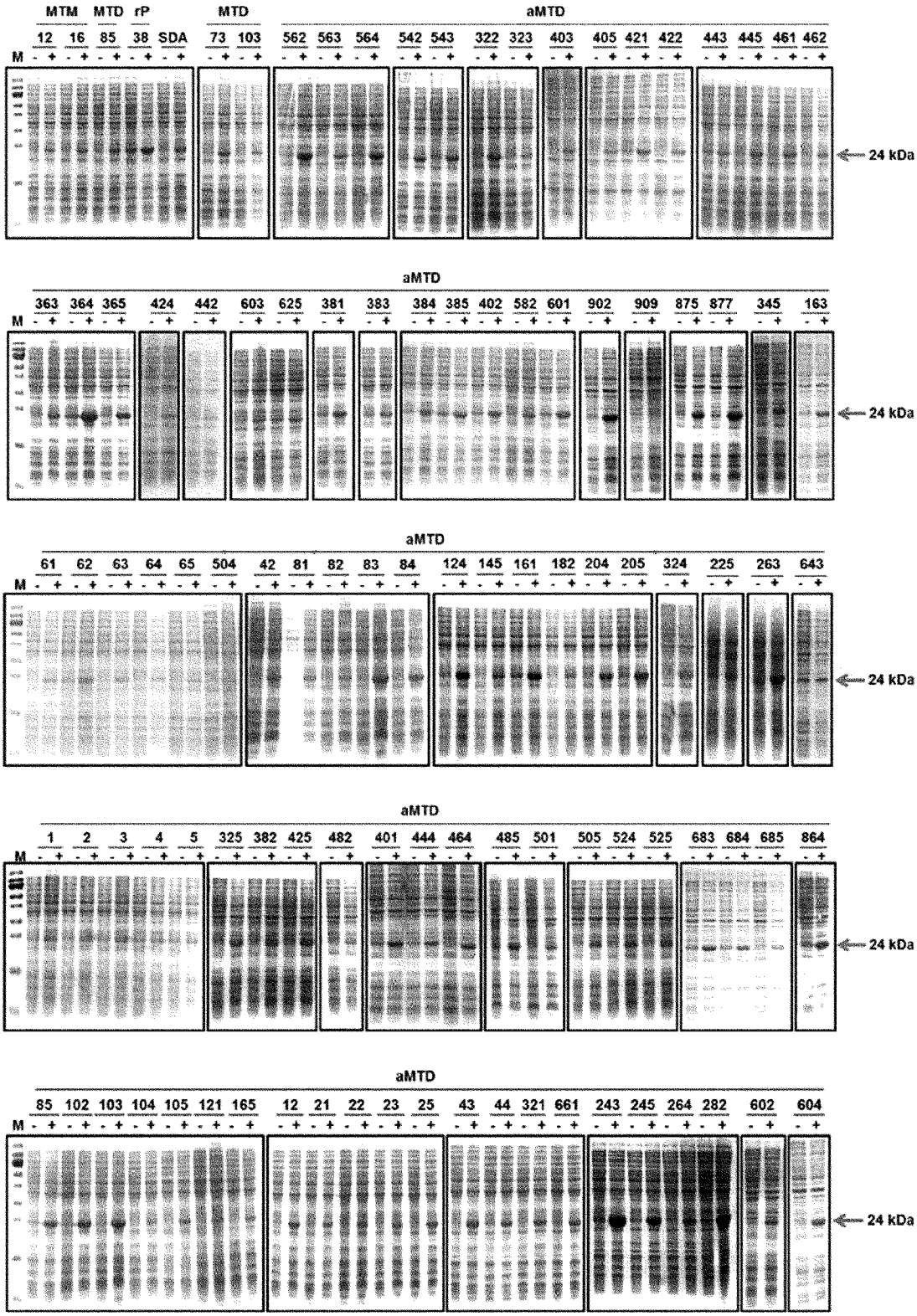
[Fig. 2b]


aMTD
rP38 MTM12 MTD85 1 2 3 4 5 11 12 13 21 22 23 24 25 42 43 44 61 62 63 64 65 81 82 83 84 85 101 102 103 104 105 121 123
645 bp
Marker pET uncut pET cut
124 125 141 142 143 144 145 161 162 163 164 165 182 183 184 185 201 202 203 204 205 221 222 223 224 225 241 242 243 245 261 262 263 264
265 281 282 283 284 285 301 302 304 305 321 322 323 324 325 341 342 343 345 361 363 364 365 381 382 383 384 385 401 402 403 404
405 421 422 424 425 442 443 444 445 461 462 463 464 465 481 482 483 484 485 501 502 503 504 505 521 522 524 525 541 542 543
544 545 561 562 563 564 565 581 582 583 585 601 602 603 604 605 621 622 623 625 641 643 645 661 663 664 665 666 667 683 684 685 686
687 703 704 705 706 707 723 724 725 726 727 743 744 745 746 747 763 764 765 766 767 783 786 787 788 789 803 804 805 806 807 808 809 810 811

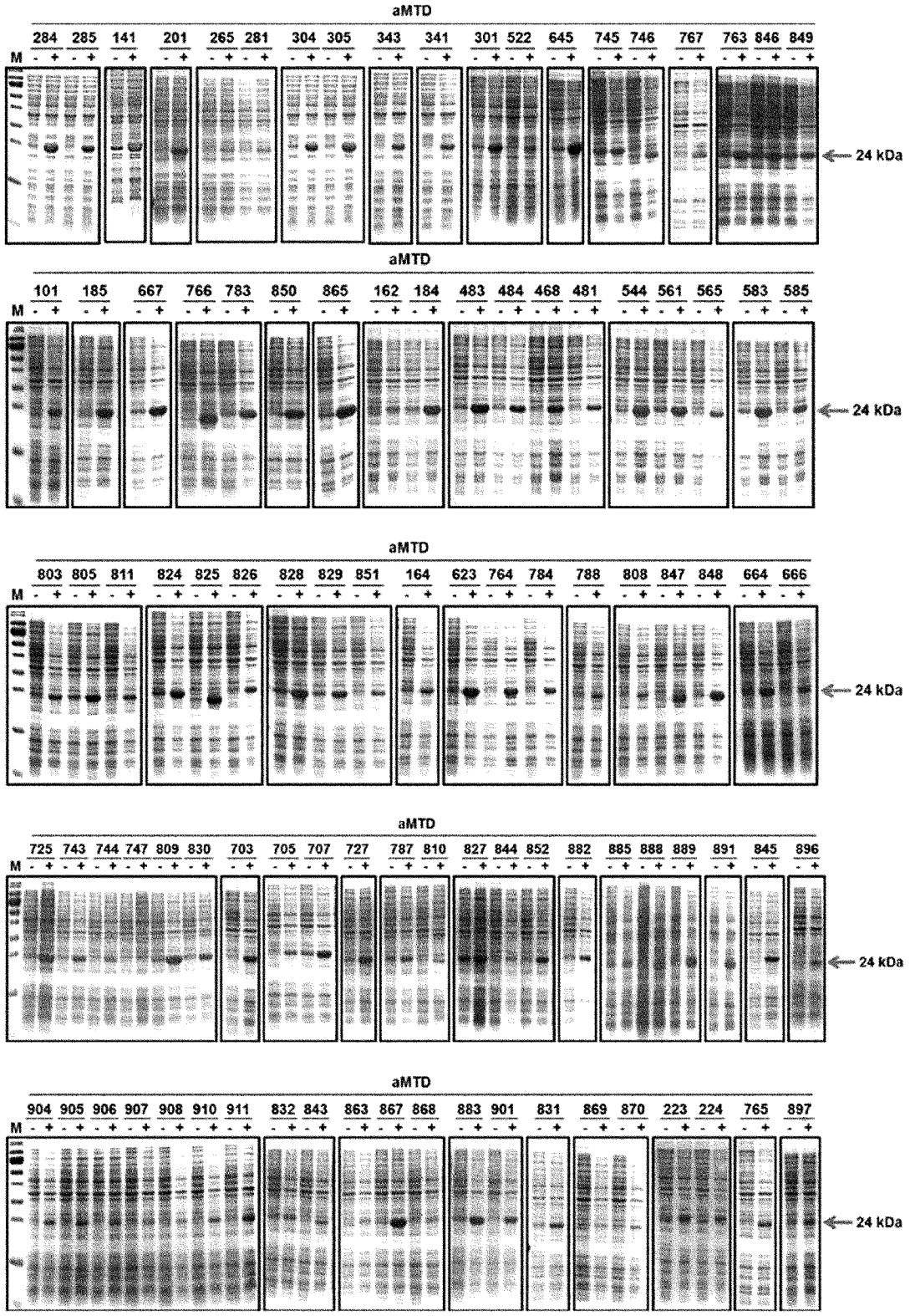
[Fig. 2c]


aMTD
Marker
pET uncut
pET cut
812 823 824 825 826 827 828 829 830 831 832 843 844 845 846 847 848 849 850 851 852 863 864 865 866 867 868 869 870 871 872 874 875 877
645 bp
aMTD
Marker
pET uncut
pET cut
878 879 880 881 882 883 884 885 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 904 905 906 907 908 909 910 911 912
645 bp
rPeptide
Marker
pET uncut
pET cut
rP38 MTM12 MTD85 6 7 9 10 14 15 16 17 18 19 20 26 27 28 29 30 33 34 35 36 37 39 40 46 48 49 50 51 52 53
645 bp
rPeptide
Marker
pET uncut
pET cut
54 55 56 57 59 60 66 67 68 69 71 73 74 76 77 97 113 114 131 138 139 152 153 159 167 169 175 189 190 192 193
645 bp
rPeptide
Marker
pET uncut
pET cut
209 210 214 215 226 227 228 246 247 248 256 315 329 331 349 350 390 426 436 466 527 537 552 553 554 576 577 606 635 639 640 676
645 bp
rPeptide
Marker
pET uncut
pET cut
688 689 692 693 695 700 750 772 894 913 914 915 916 920 921 922 923 924 927 930 934 935 936 937 938 941 942 944 947 949 951
645 bp

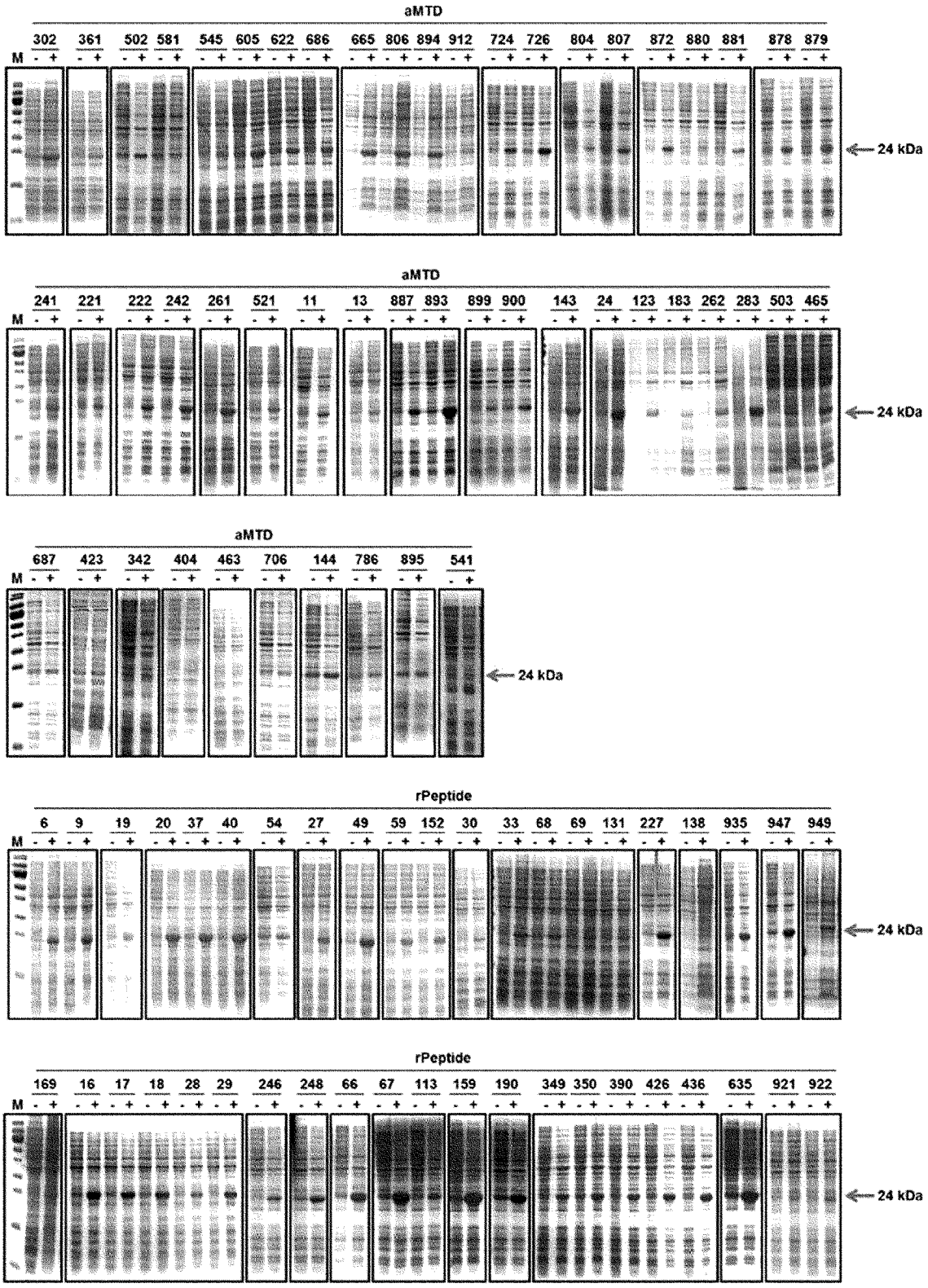
[Fig. 3a]


MTM MTD rP MTD aMTD
12 16 85 38 SDA 73 103 562 563 564 542 543 322 323 403 405 421 422 443 445 461 462
M
24 kDa
aMTD
363 364 365 424 442 603 625 381 383 384 385 402 582 601 902 909 875 877 345 163
M
24 kDa
aMTD
61 62 63 64 65 504 42 81 82 83 84 124 145 161 182 204 205 324 225 263 643
M
24 kDa
aMTD
1 2 3 4 5 325 382 425 482 401 444 464 485 501 505 524 525 683 684 685 864
M
24 kDa
aMTD
85 102 103 104 105 121 165 12 21 22 23 25 43 44 321 661 243 245 264 282 602 604
M
24 kDa

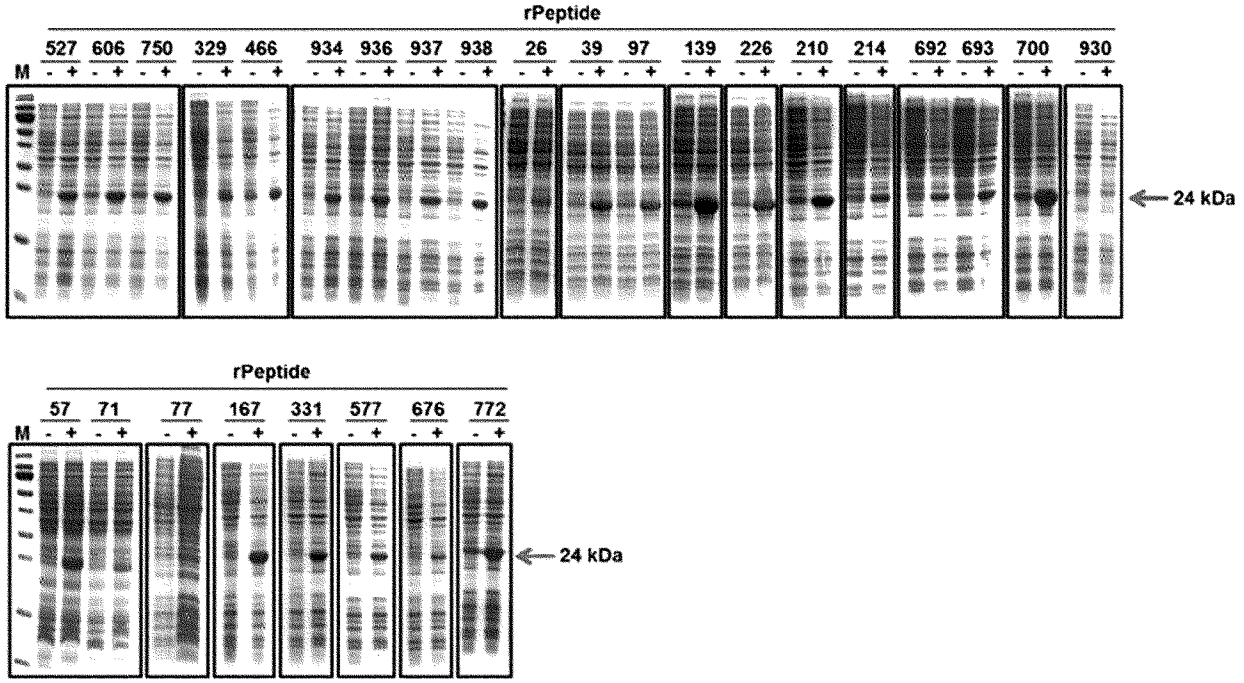
[Fig. 3b]


aMTD
284 285 141 201 265 281 304 305 343 341 301 522 645 745 746 767 763 846 849
M
24 kDa
aMTD
101 185 667 766 783 850 865 162 184 483 484 468 481 544 561 565 583 585
M
24 kDa
aMTD
803 805 811 824 825 826 828 829 851 164 623 764 784 788 808 847 848 664 666
M
24 kDa
aMTD
725 743 744 747 809 830 703 705 707 727 787 810 827 844 852 882 885 888 889 891 845 896
M
24 kDa
aMTD
904 905 906 907 908 910 911 832 843 863 867 868 883 901 831 869 870 223 224 765 897
M
24 kDa

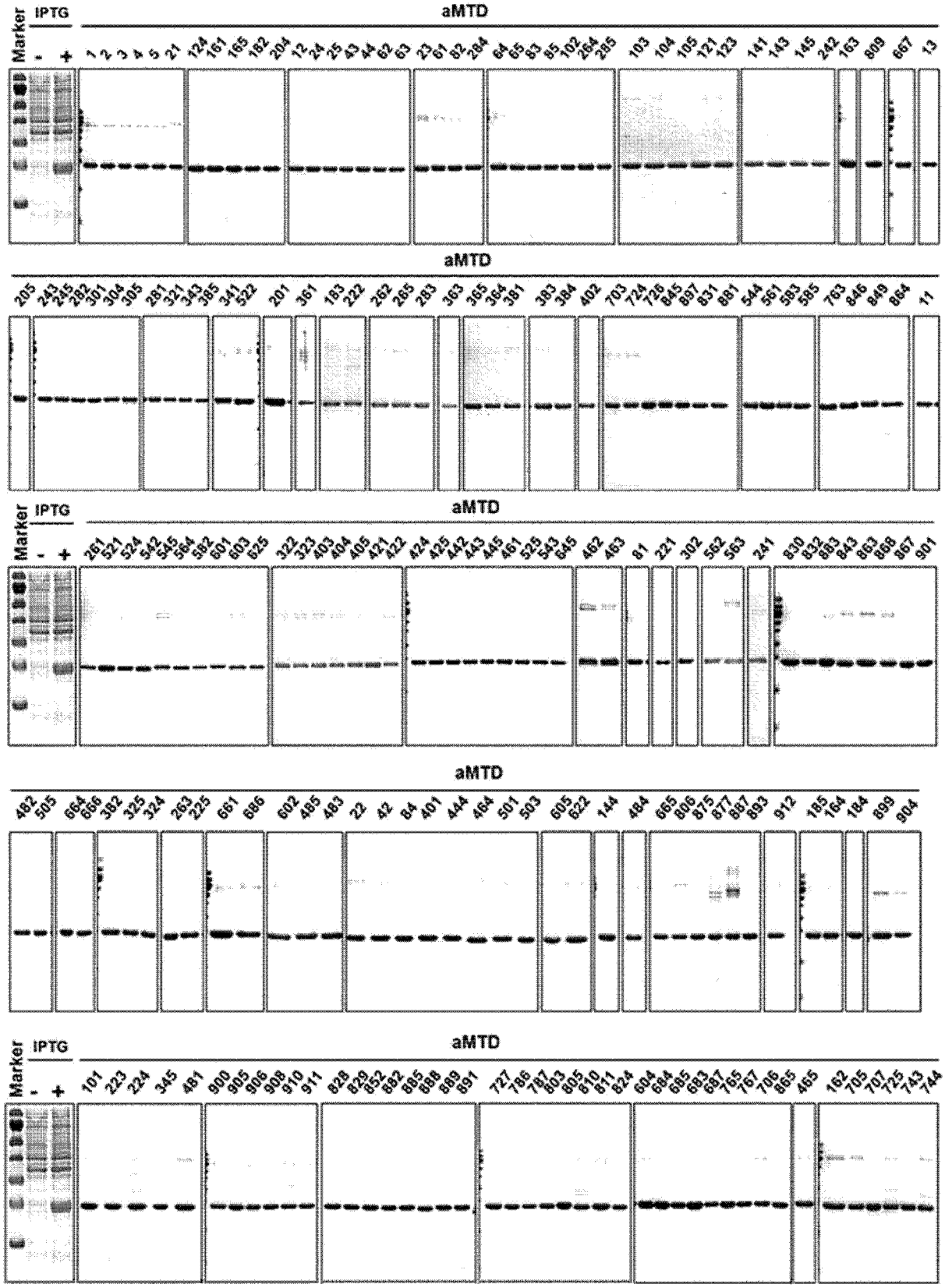
[Fig. 3c]


aMTD
302 361 502 581 545 605 622 686 665 806 894 912 724 726 804 807 872 880 881 878 879
M - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - +
24 kDa
aMTD
241 221 222 242 261 521 11 13 887 893 899 900 143 24 123 183 262 283 503 465
M - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - +
24 kDa
aMTD
687 423 342 404 463 706 144 786 895 541
M - + - + - + - + - + - + - + - + - + - +
24 kDa
rPeptide
6 9 19 20 37 40 54 27 49 59 152 30 33 68 69 131 227 138 935 947 949
M - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - +
24 kDa
rPeptide
169 16 17 18 28 29 246 248 66 67 113 159 190 349 350 390 426 436 635 921 922
M - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - +
24 kDa

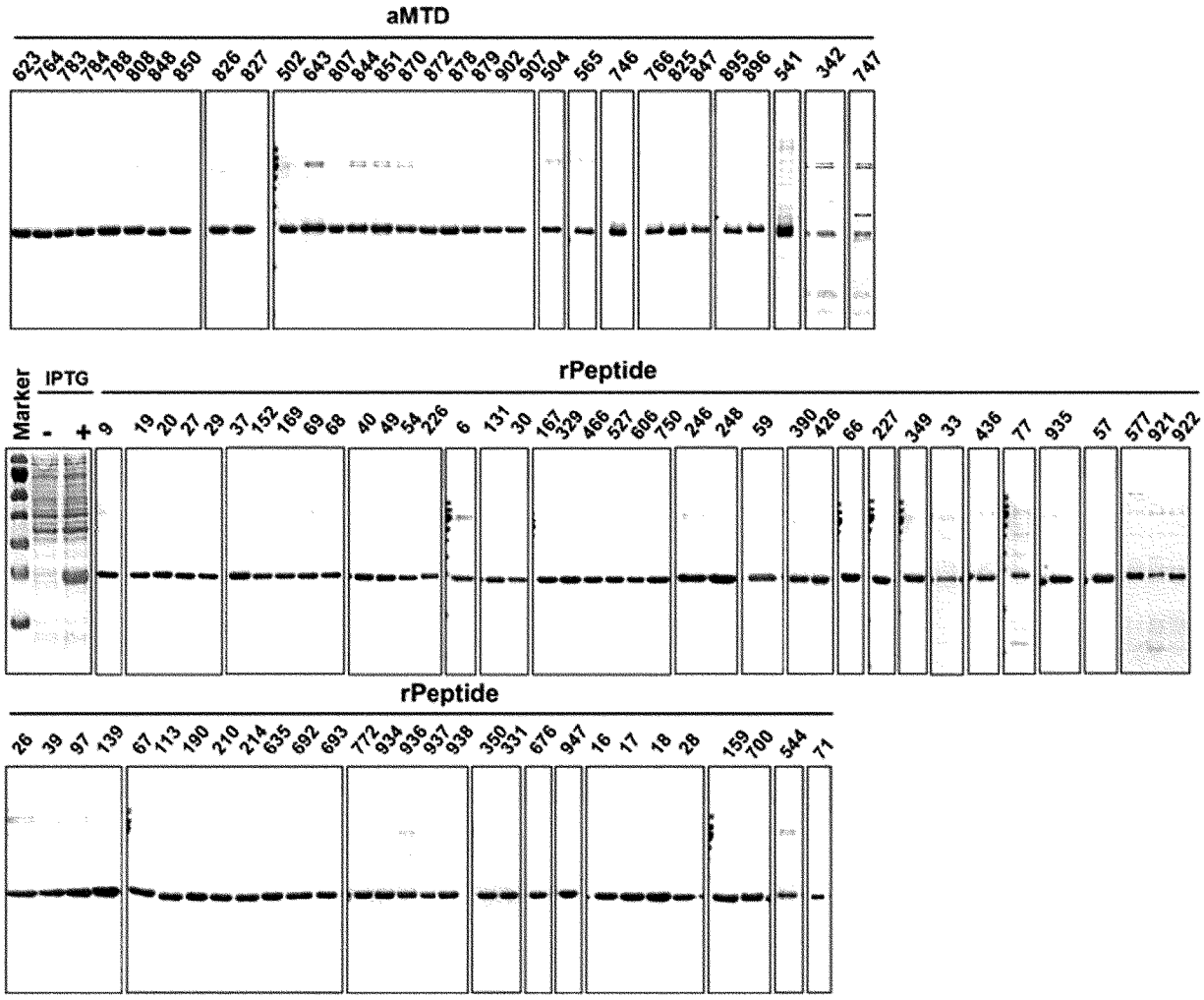
[Fig. 3d]


rPeptide
527 606 750 329 466 934 936 937 938 26 39 97 139 226 210 214 692 693 700 930
M
- + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - + - +
24 kDa
rPeptide
57 71 77 167 331 577 676 772
M
- + - + - + - + - + - + - + - +
24 kDa

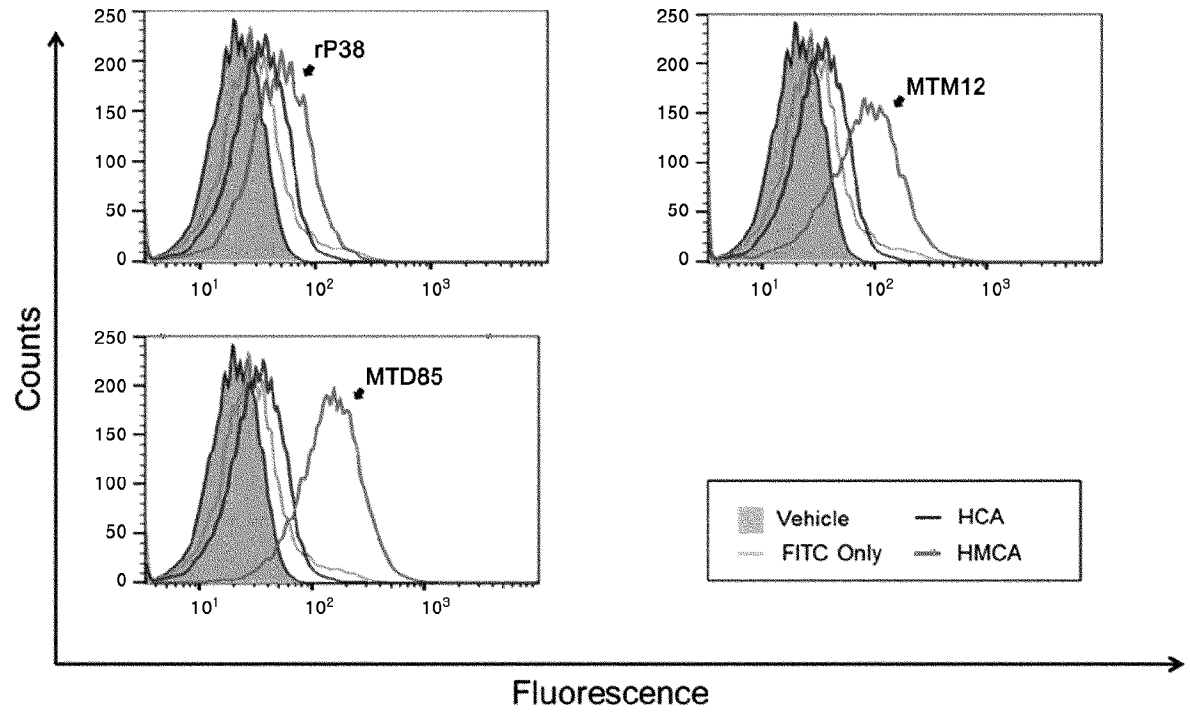
[Fig. 4a]

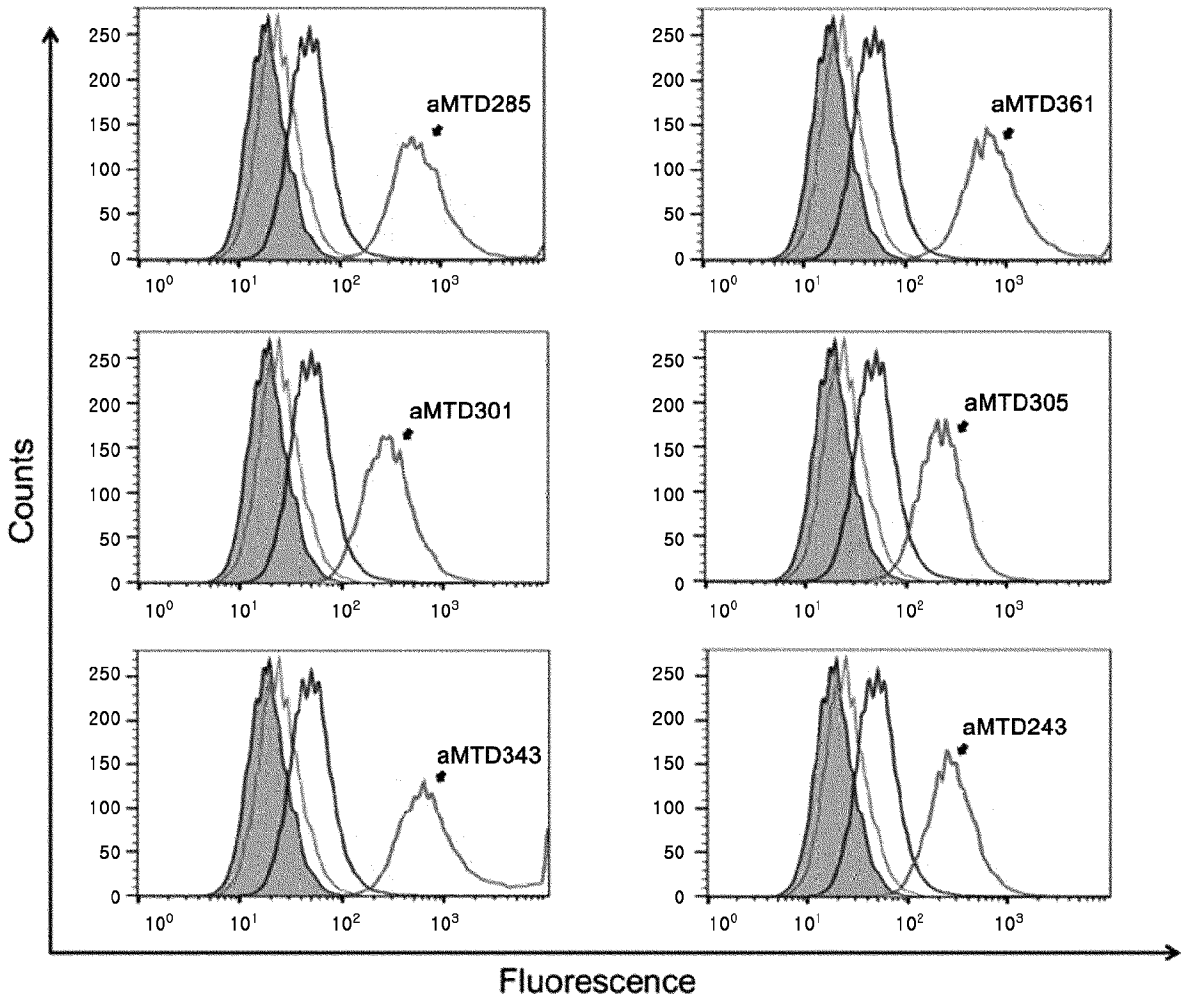

Marker
IPTG
aMTD
- +
1 2 3 4 5 21 124 161 165 182 204 12 24 25 43 44 62 63 23 61 82 284 64 65 83 85 102 264 285 103 104 105 121 123 141 143 145 242 163 809 667 13
205 243 245 282 301 304 305 281 321 343 385 341 522 201 361 183 222 262 265 283 363 365 364 381 383 384 402 703 724 726 845 897 831 881 544 561 583 585 763 846 849 864 11
261 521 524 542 545 564 582 601 603 625 322 323 403 404 405 421 422 424 425 442 443 445 461 525 543 645 462 463 81 221 302 562 563 241 830 832 883 843 863 868 867 901
482 505 664 666 382 325 324 263 225 661 686 602 485 483 22 42 84 401 444 464 501 503 605 622 144 484 665 806 875 877 887 893 912 185 164 184 899 904
101 223 224 345 481 900 905 906 908 910 911 828 829 852 882 885 888 889 891 727 786 787 803 805 810 811 824 604 684 685 683 687 765 767 705 865 465 162 705 707 725 743 744

[Fig. 4b]

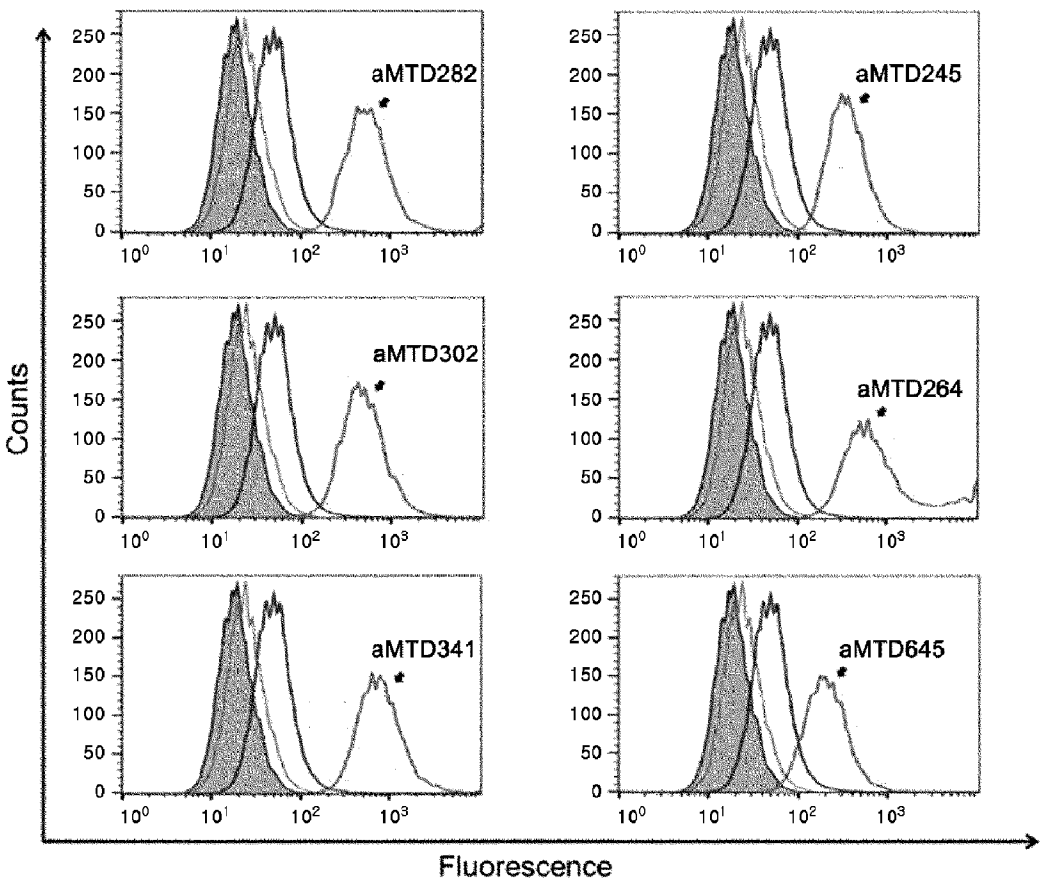

aMTD
623 764 783 784 788 808 848 850 826 827 502 643 807 844 851 870 872 878 879 902 907 504 565 746 766 825 847 895 896 541 342 747
Marker
IPTG
- +
rPeptide
9 19 20 27 29 37 152 169 69 68 40 49 54 226 6 131 30 167 329 466 527 606 750 246 248 59 390 426 66 227 349 33 436 77 935 57 577 921 922
rPeptide
26 39 97 139 67 113 190 210 214 635 692 693 772 934 936 937 938 350 331 676 947 16 17 18 28 159 700 544 71

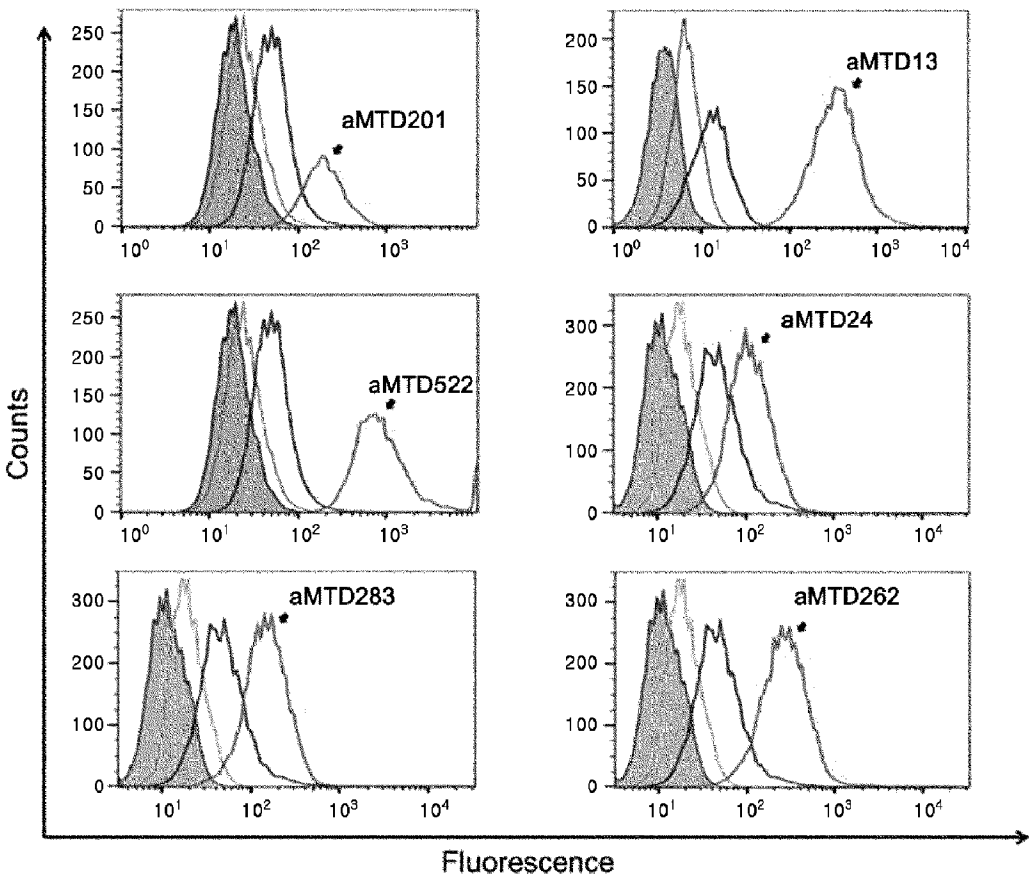
[Fig. 5a]


rP38
MTM12
MTD85
Counts
Fluorescence
Vehicle
FITC Only
HCA
HMCA

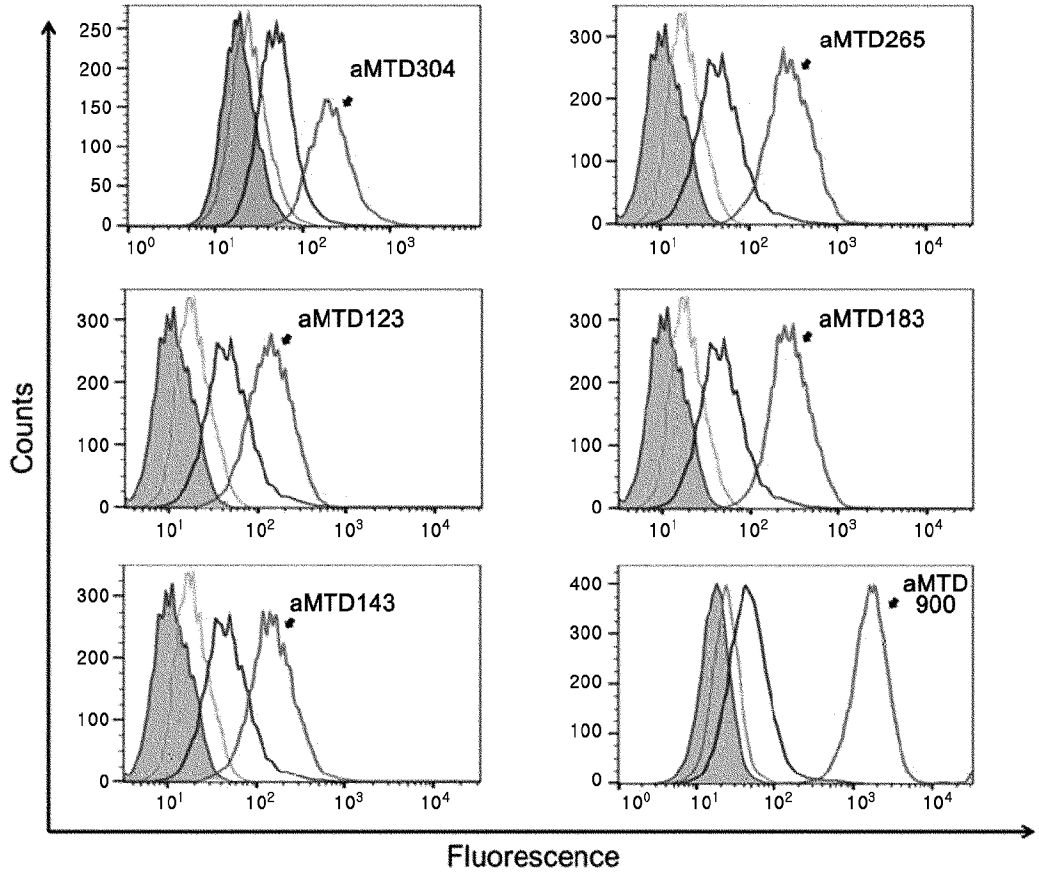

aMTD285
aMTD361
aMTD301
aMTD305
aMTD343
aMTD243
Counts
Fluorescence

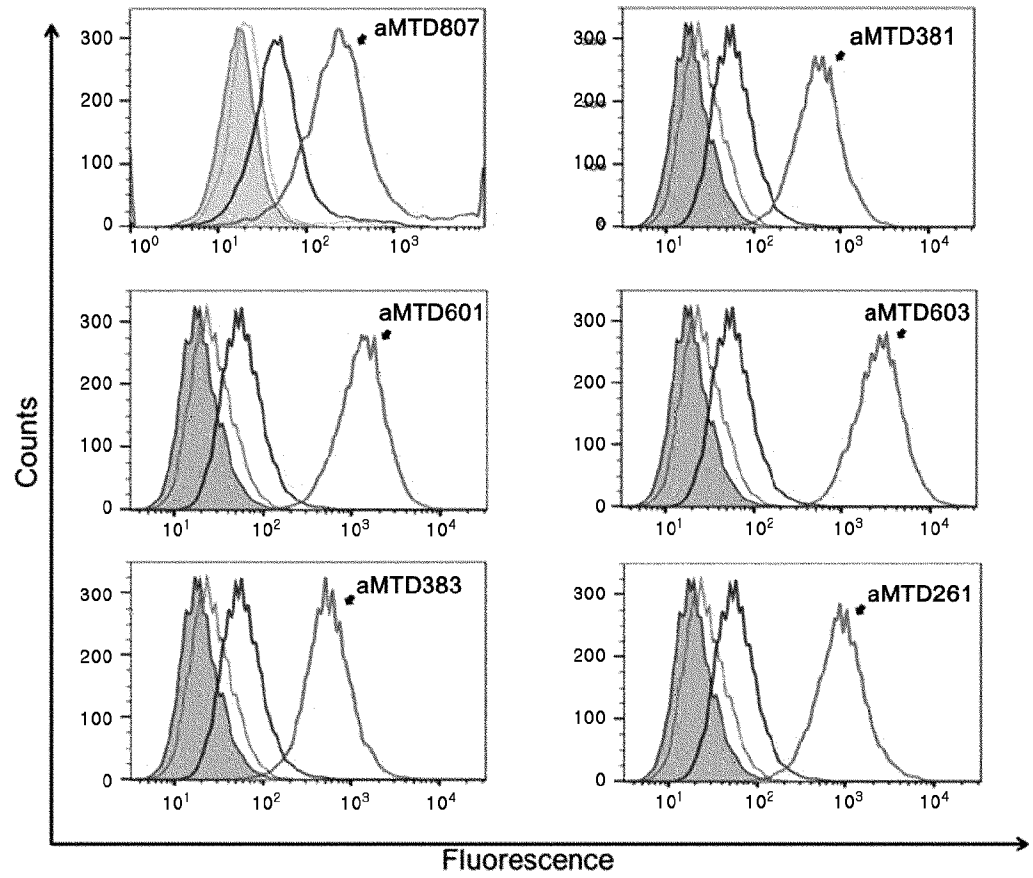
[Fig. 5b]


aMTD282
aMTD245
aMTD302
aMTD264
aMTD341
aMTD645
Counts
Fluorescence

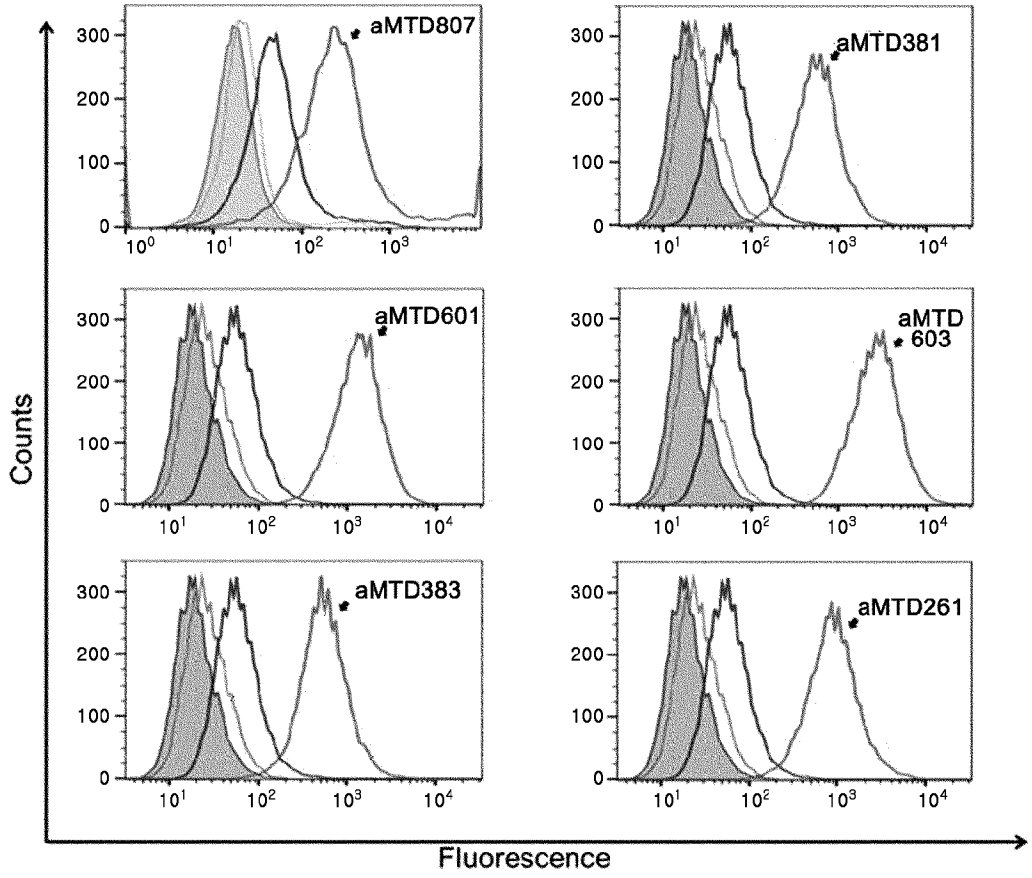

aMTD201
aMTD13
aMTD522
aMTD24
aMTD283
aMTD262
Counts
Fluorescence

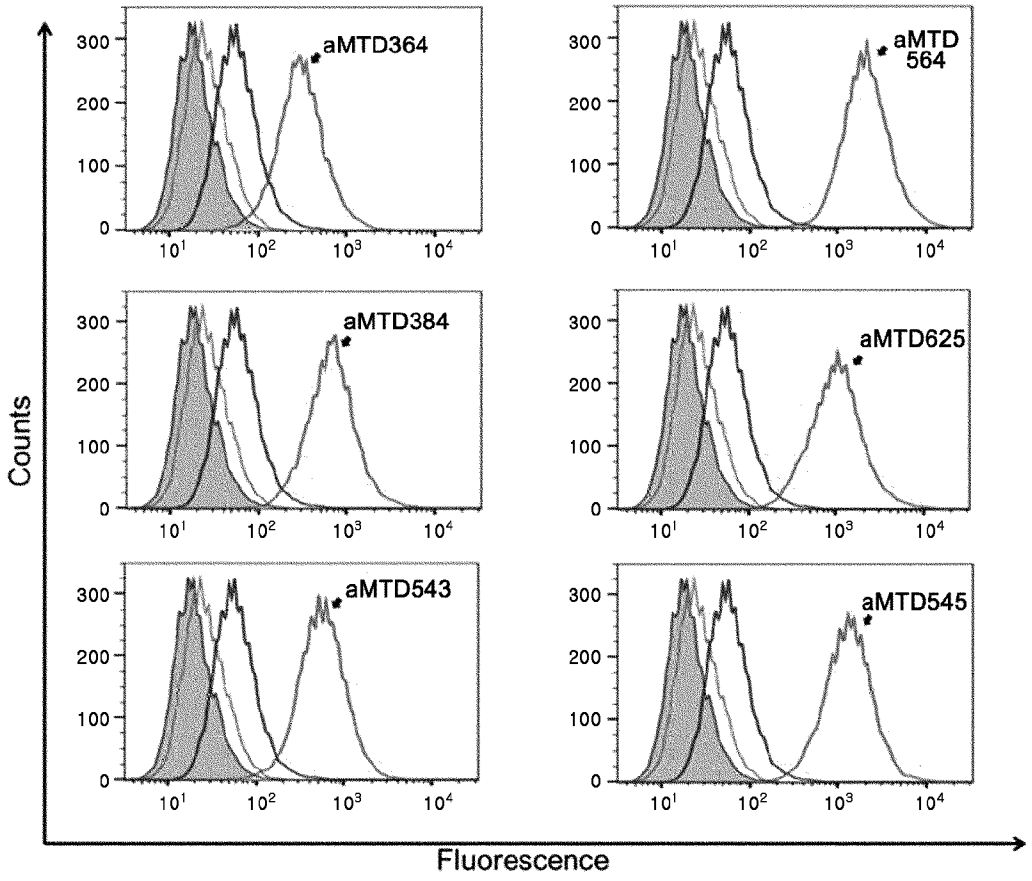
[Fig. 5c]


aMTD304
aMTD265
aMTD123
aMTD183
aMTD143
aMTD 900
Counts
Fluorescence

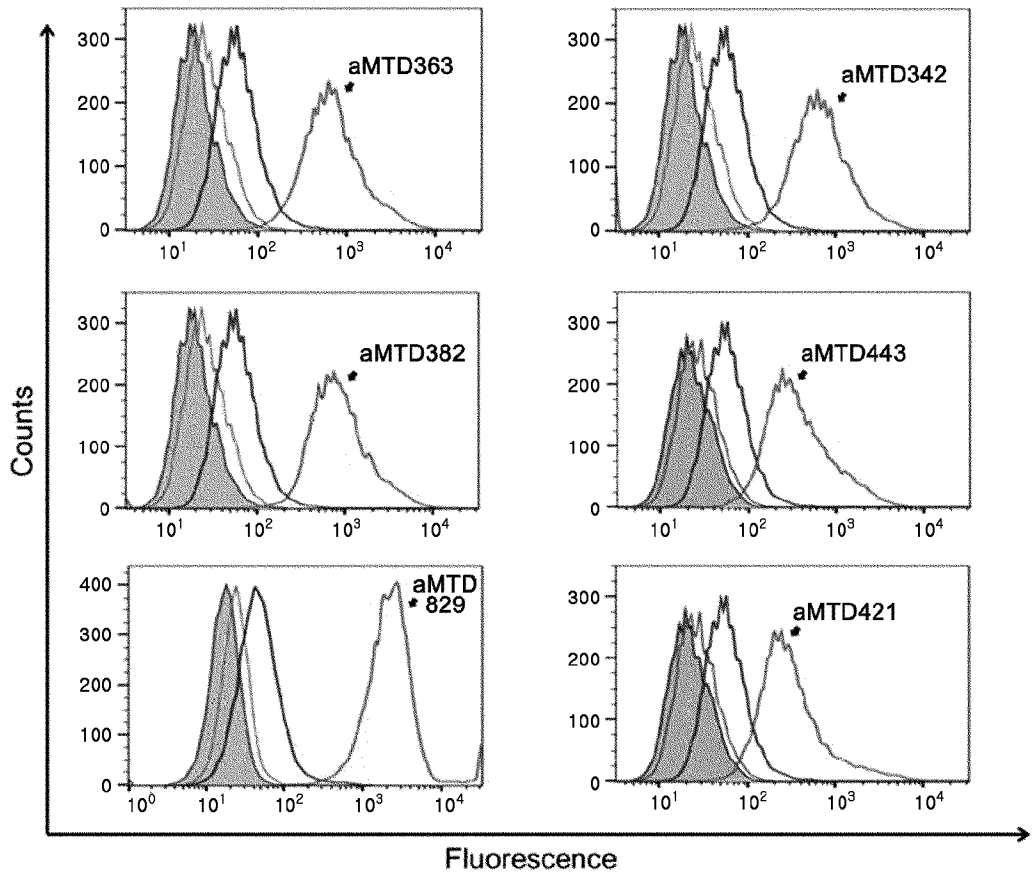

aMTD807
aMTD381
aMTD601
aMTD603
aMTD383
aMTD261
Counts
Fluorescence

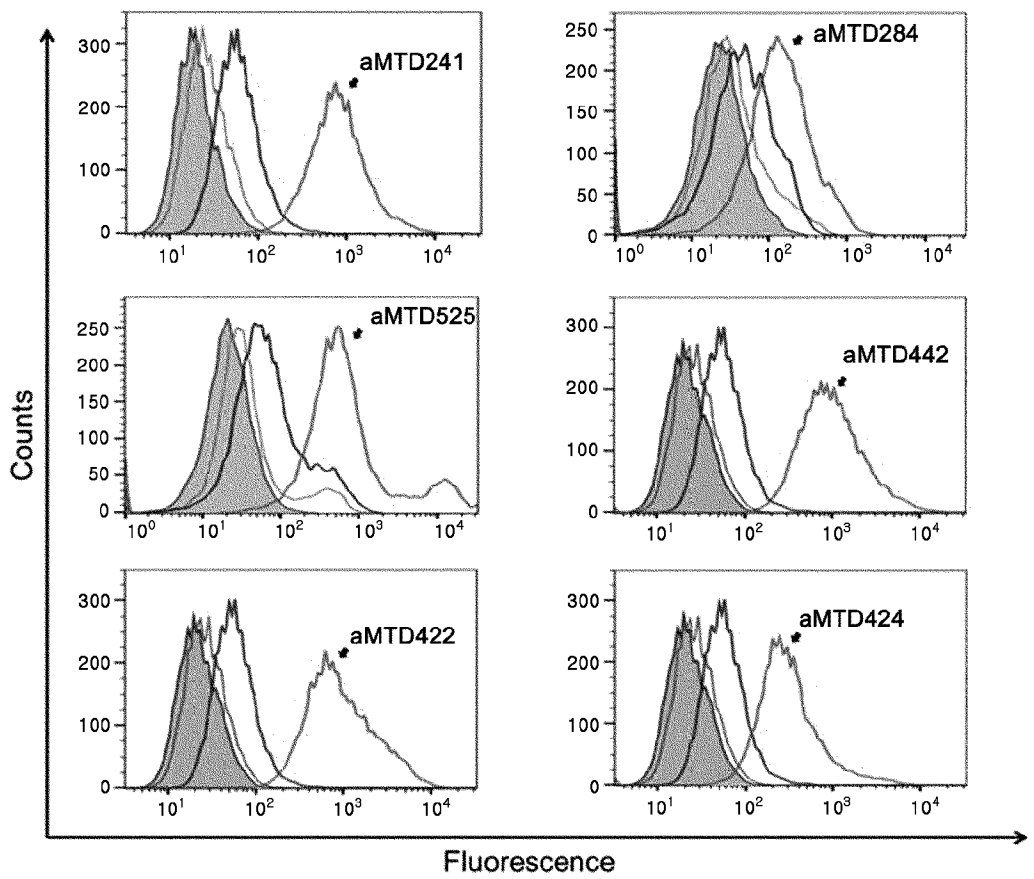
[Fig. 5d]


aMTD807
aMTD381
aMTD601
aMTD 603
aMTD383
aMTD261
Counts
Fluorescence

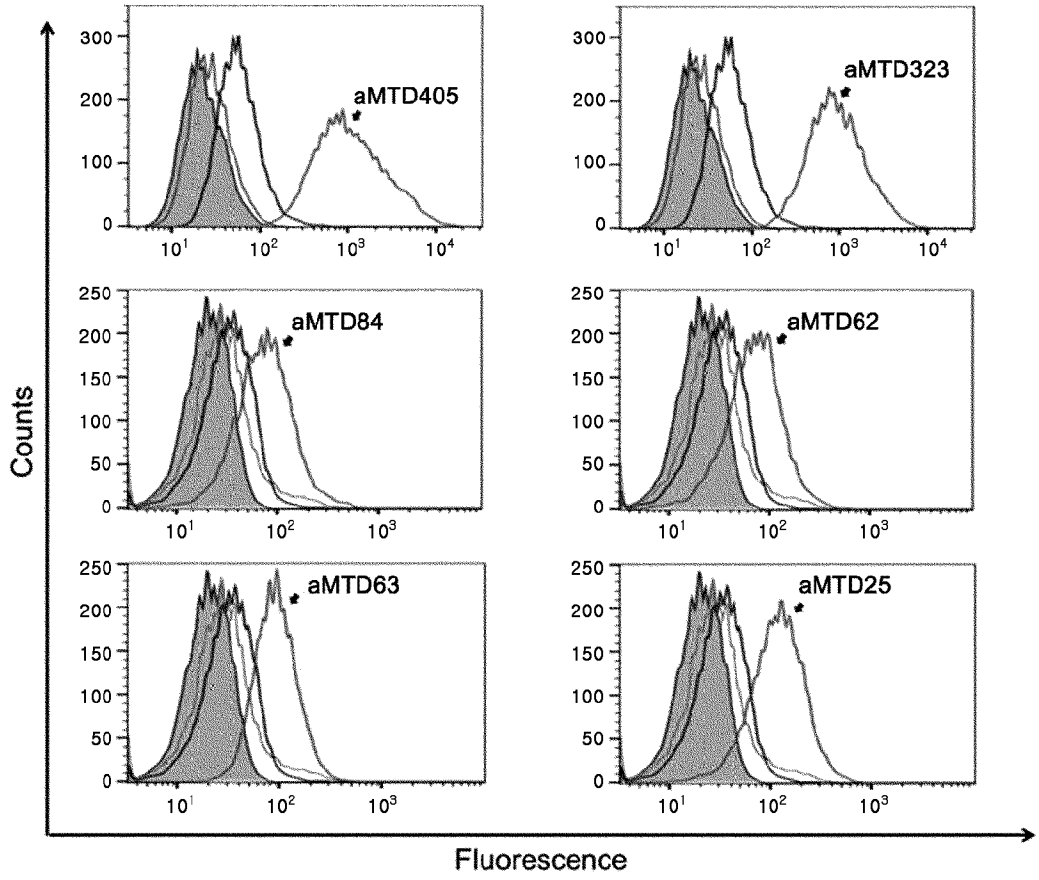

aMTD364
aMTD 564
aMTD384
aMTD625
aMTD543
aMTD545
Counts
Fluorescence

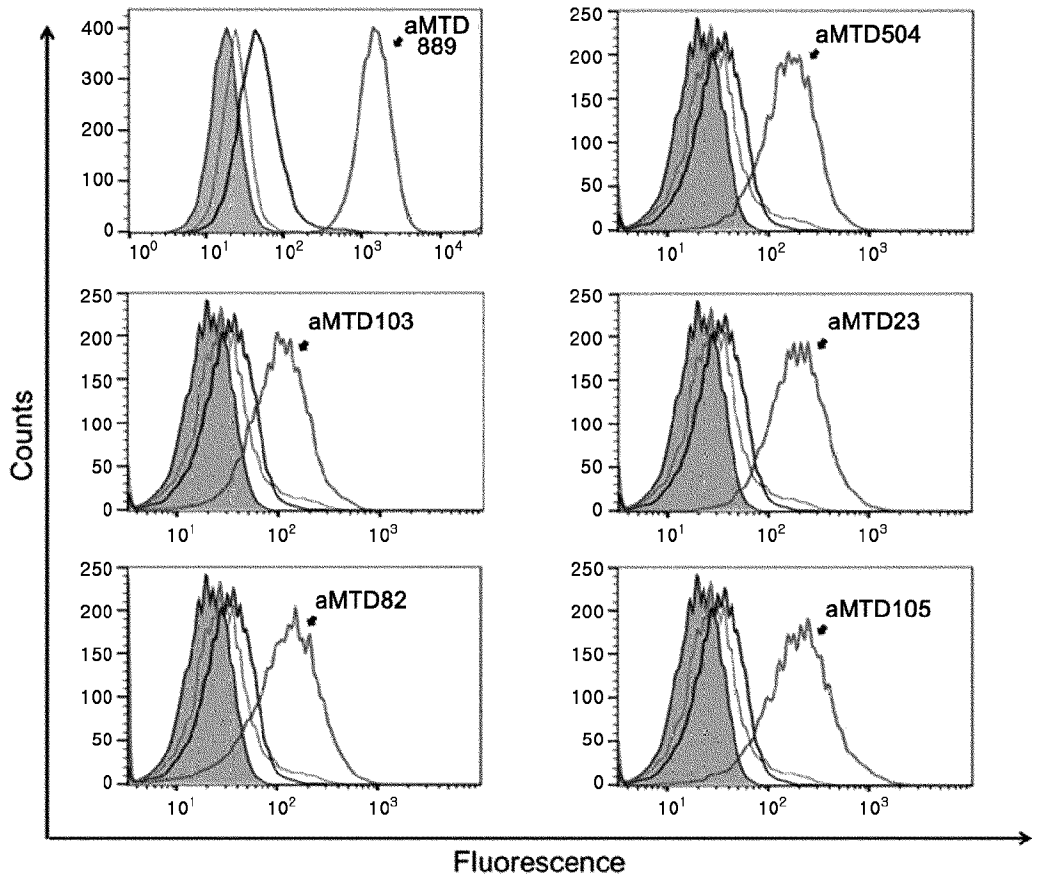
[Fig. 5e]


aMTD363
aMTD342
aMTD382
aMTD443
aMTD 829
aMTD421
Counts
Fluorescence

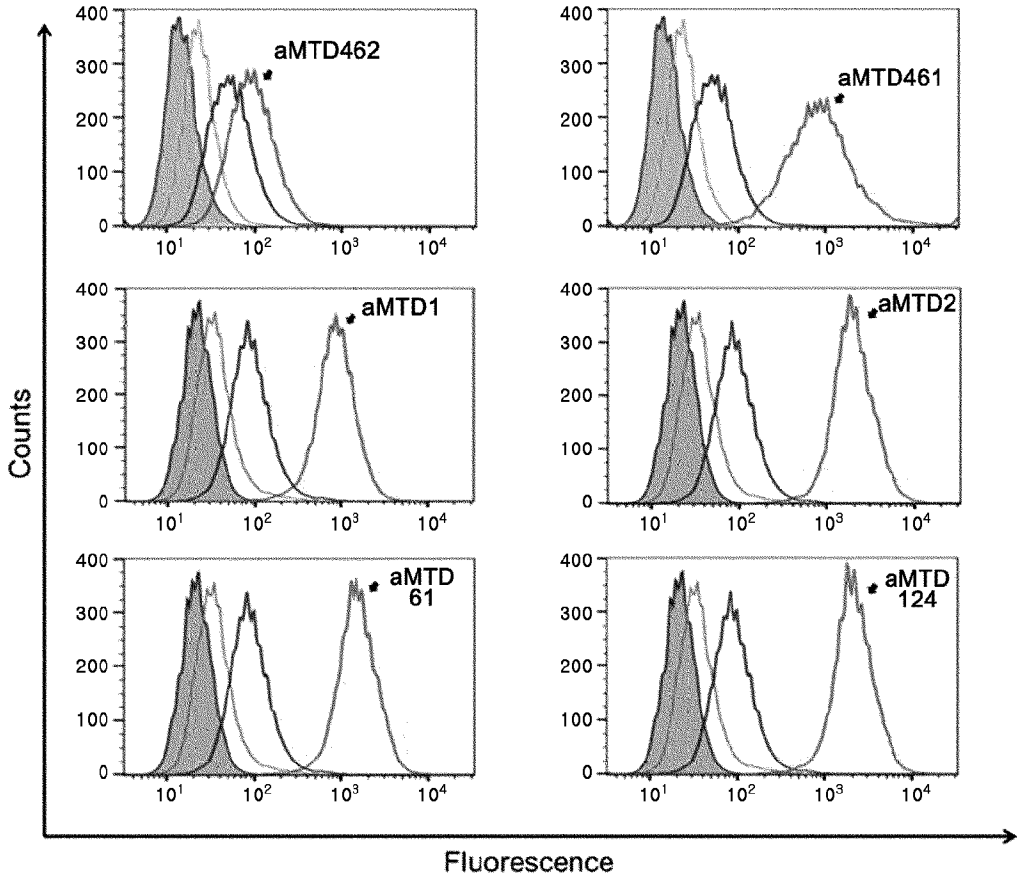

aMTD241
aMTD284
aMTD525
aMTD442
aMTD422
aMTD424
Counts
Fluorescence

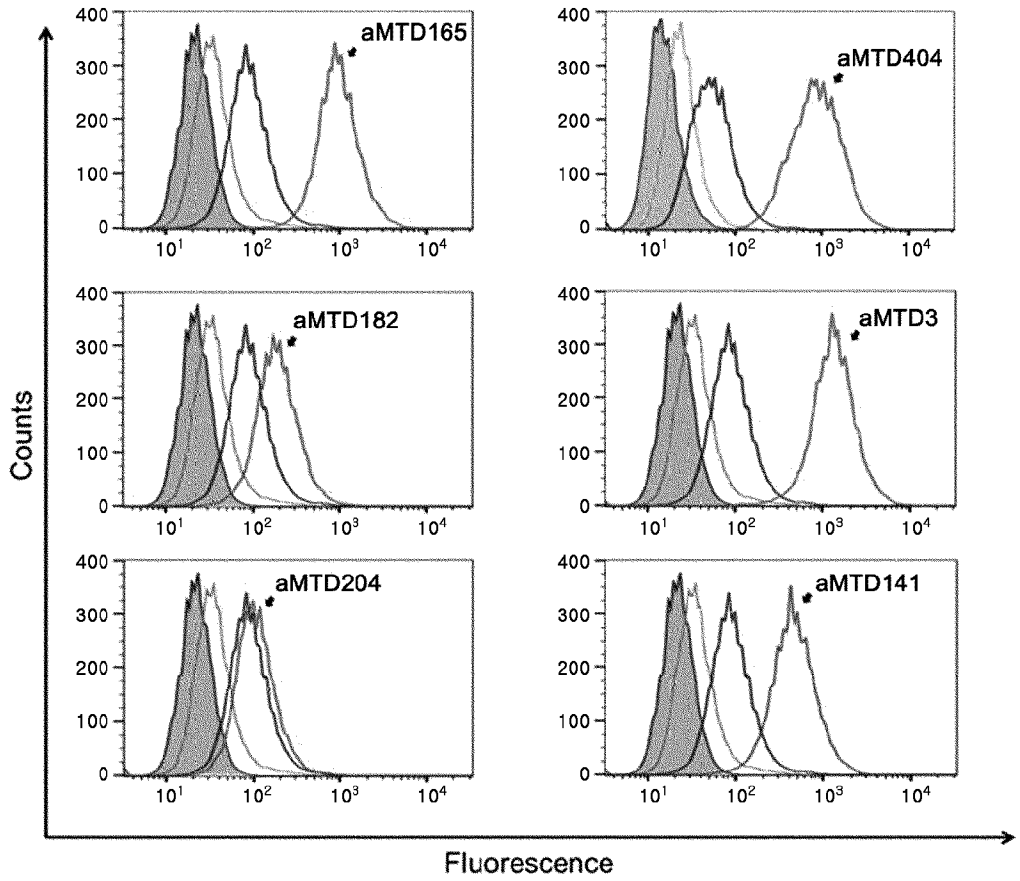
[Fig. 5f]


aMTD405
aMTD323
aMTD84
aMTD62
aMTD63
aMTD25
Counts
Fluorescence

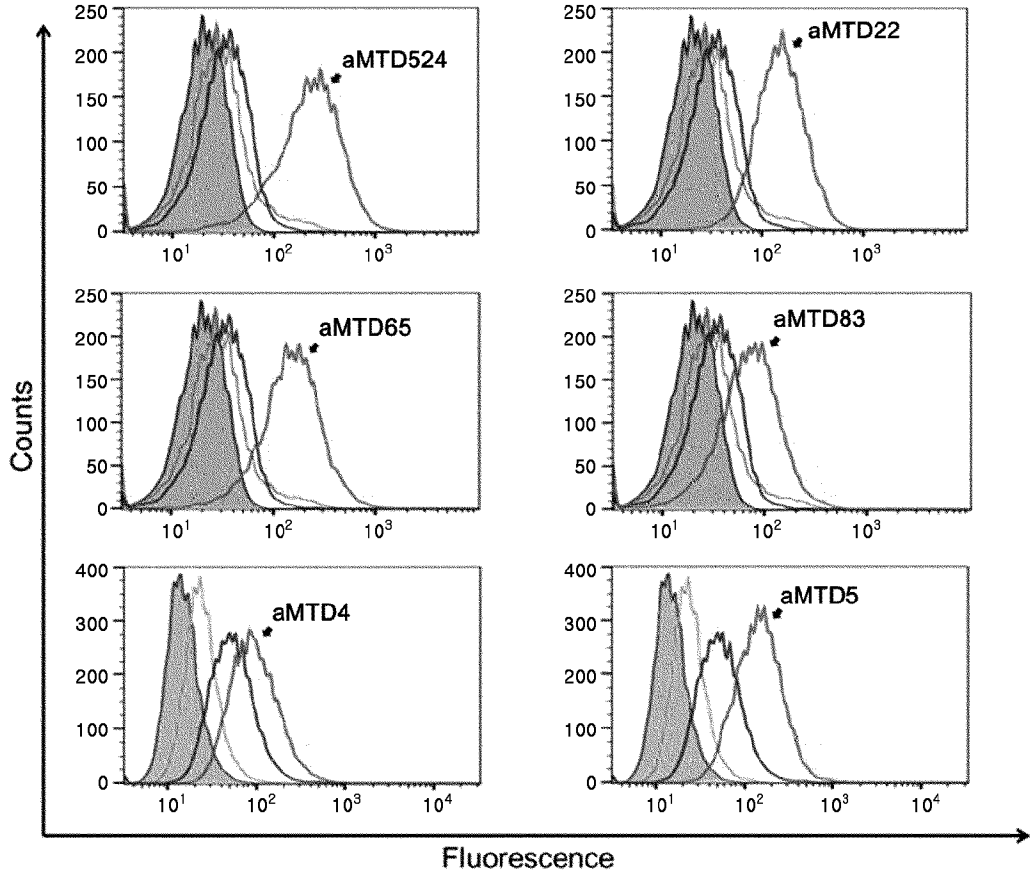

aMTD 889
aMTD504
aMTD103
aMTD23
aMTD82
aMTD105
Counts
Fluorescence

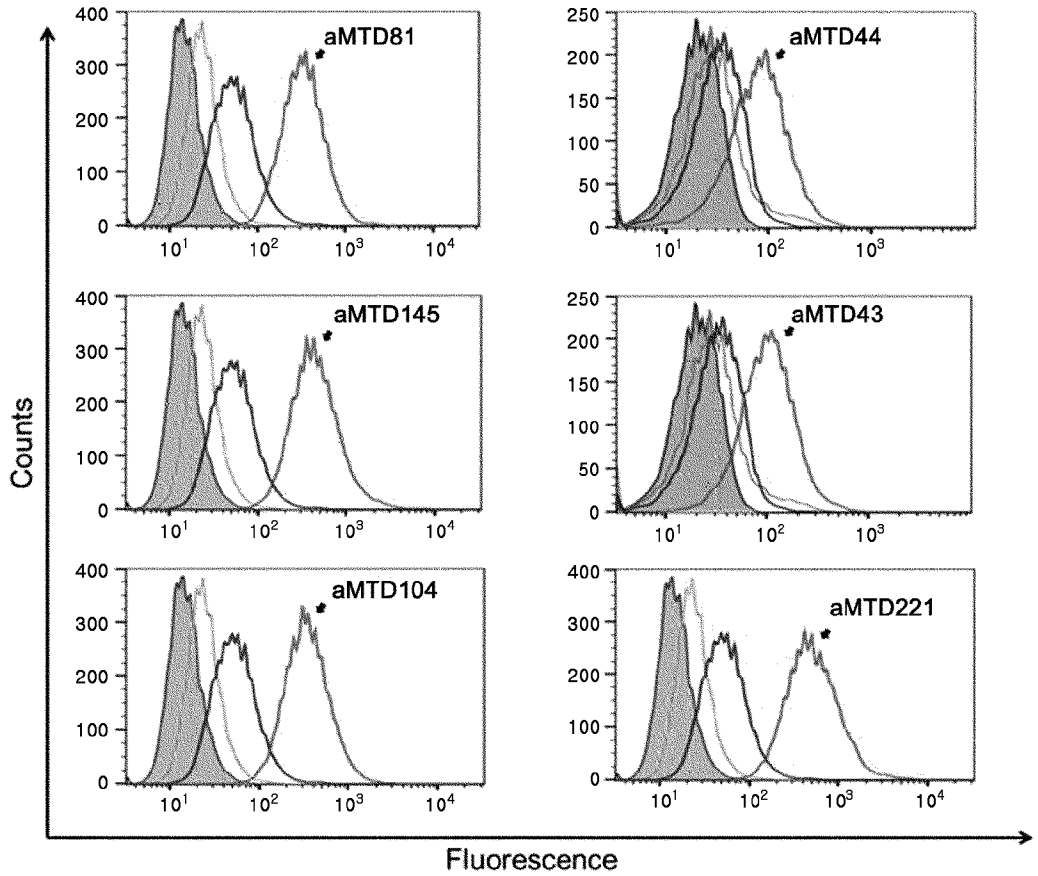
[Fig. 5g]


aMTD462
aMTD461
aMTD1
aMTD2
aMTD 61
aMTD 124
Counts
Fluorescence

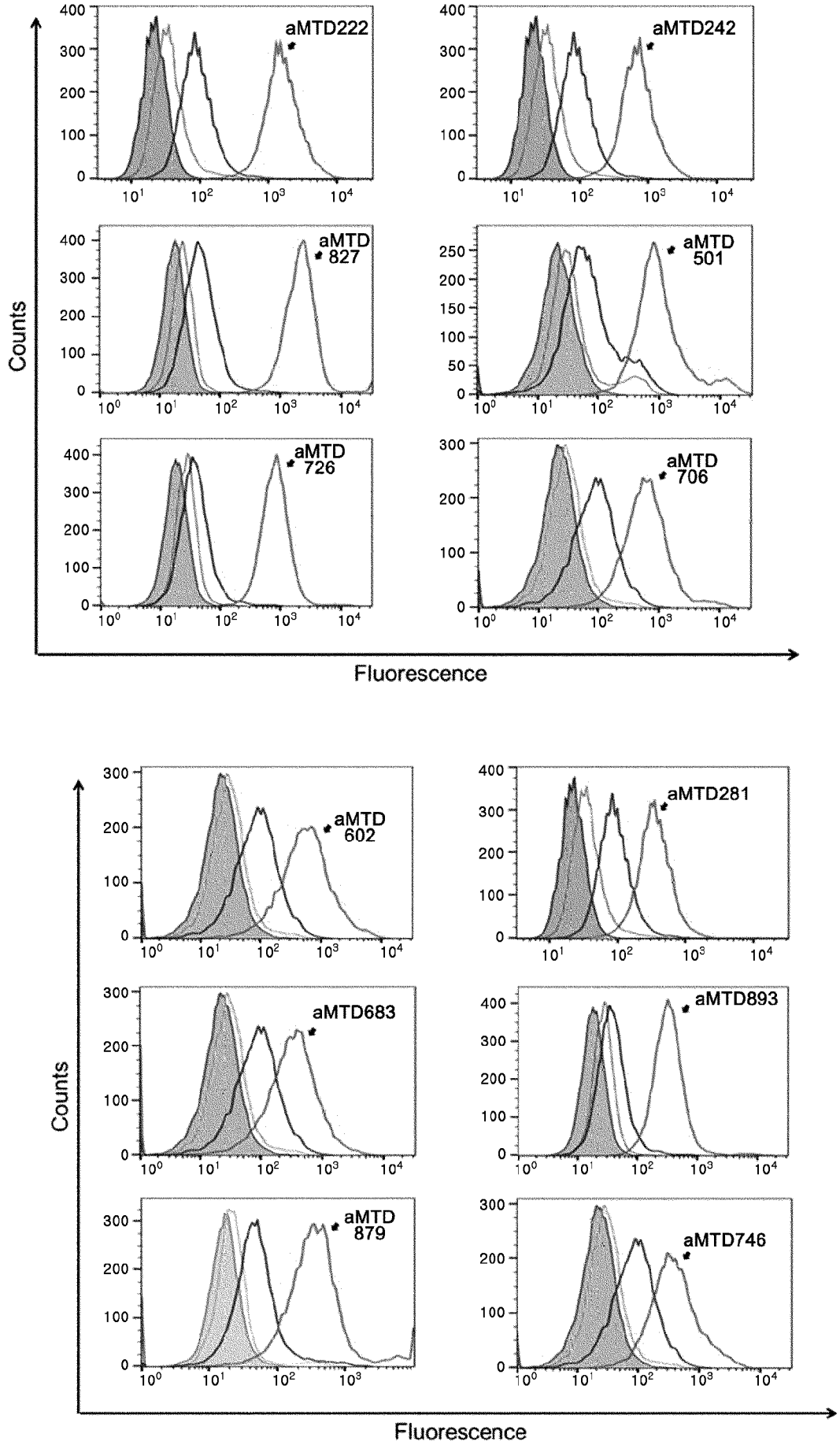

aMTD165
aMTD404
aMTD182
aMTD3
aMTD204
aMTD141
Counts
Fluorescence

[Fig. 5h]

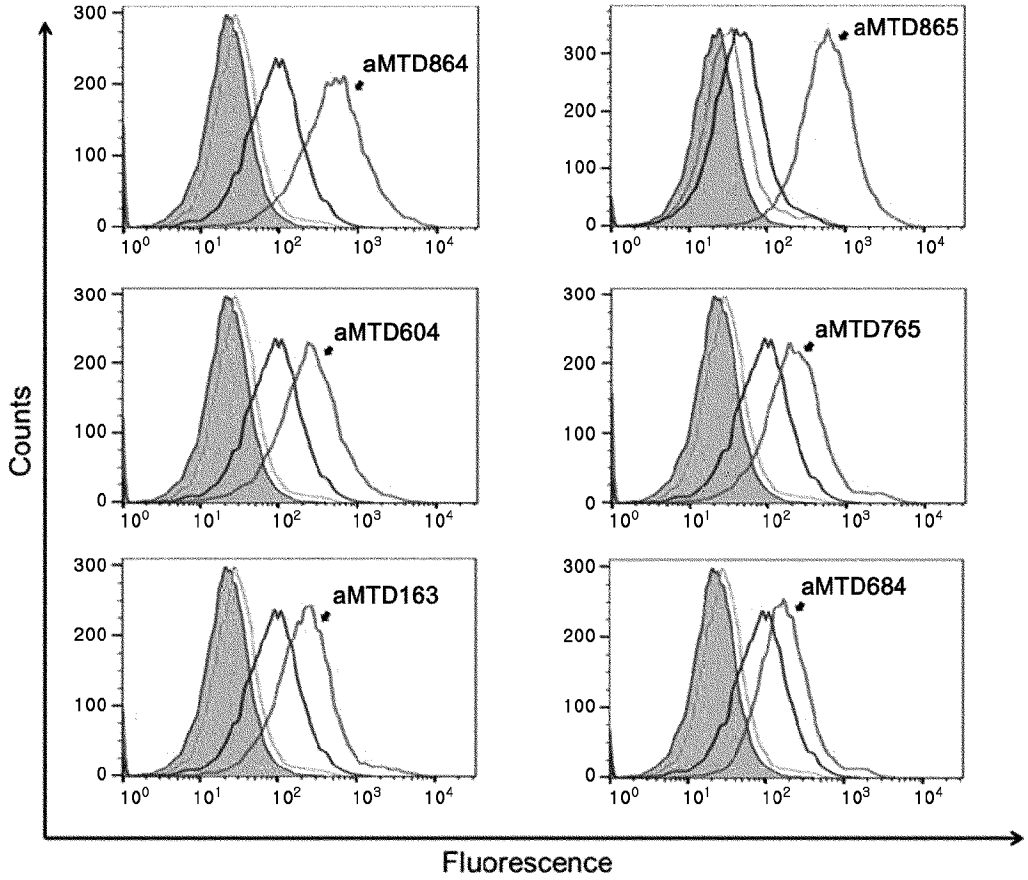

aMTD524
aMTD22
aMTD65
aMTD83
aMTD4
aMTD5
Counts
Fluorescence

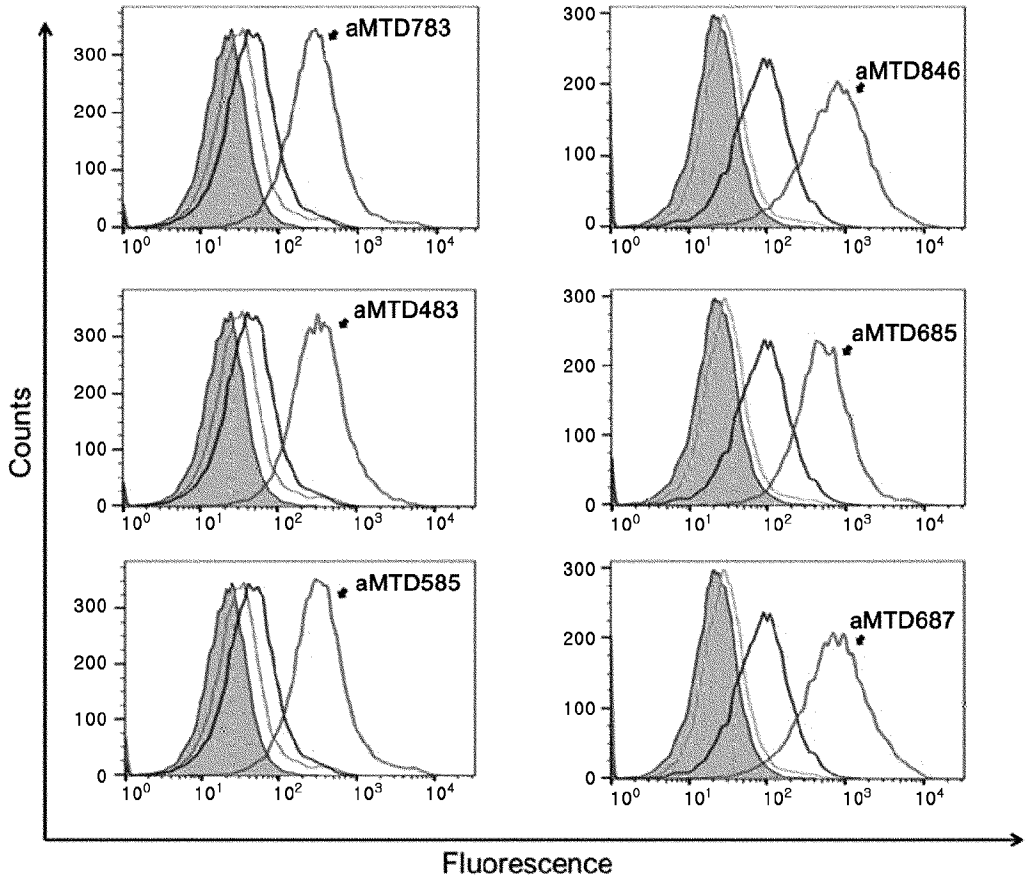

aMTD81
aMTD44
aMTD145
aMTD43
aMTD104
aMTD221
Counts
Fluorescence

[Fig. 5i]

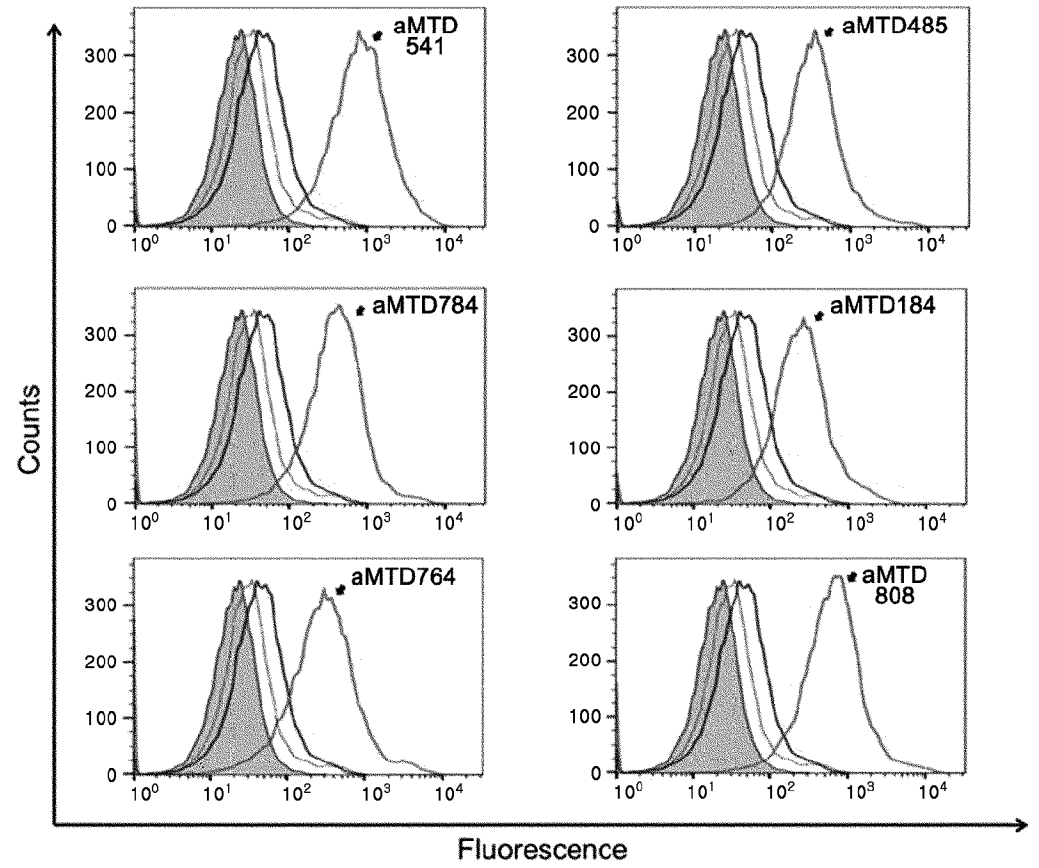

aMTD222
aMTD242
aMTD 827
aMTD 501
aMTD 726
aMTD 706
aMTD 602
aMTD281
aMTD683
aMTD893
aMTD 879
aMTD746
Counts
Fluorescence

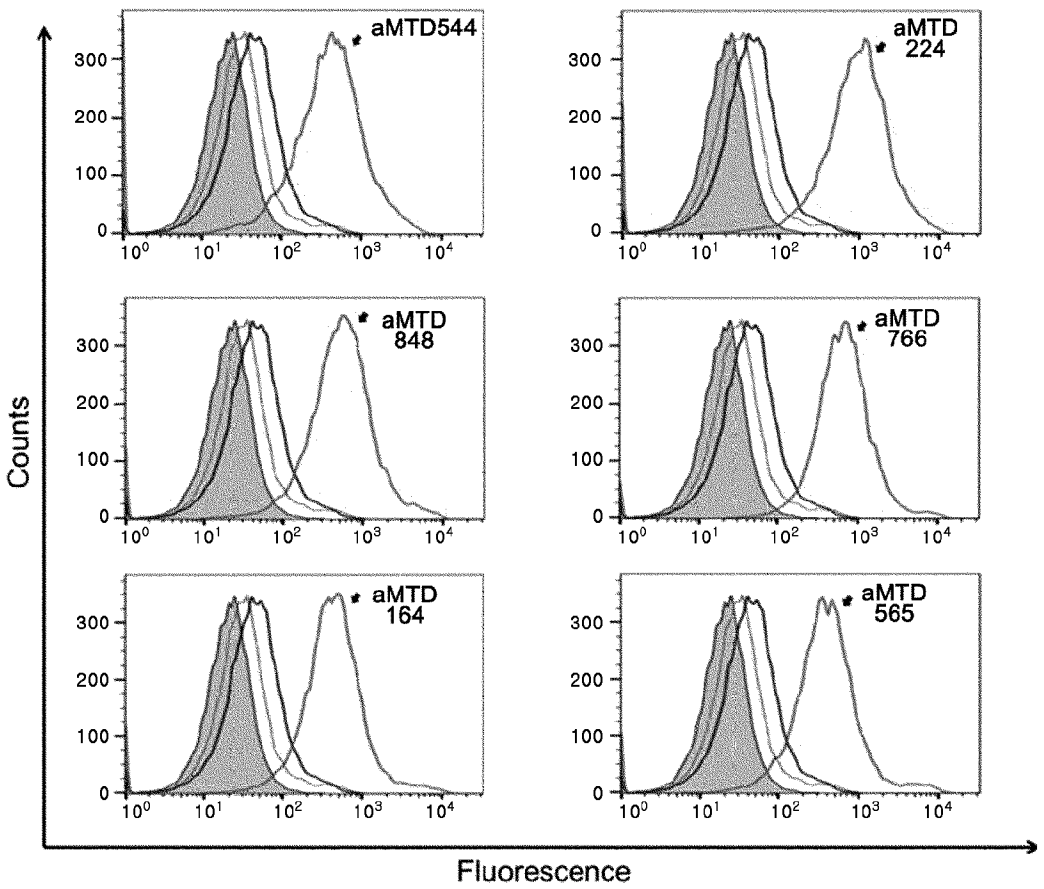
[Fig. 5j]


aMTD864
aMTD865
aMTD604
aMTD765
aMTD163
aMTD684
Counts
Fluorescence

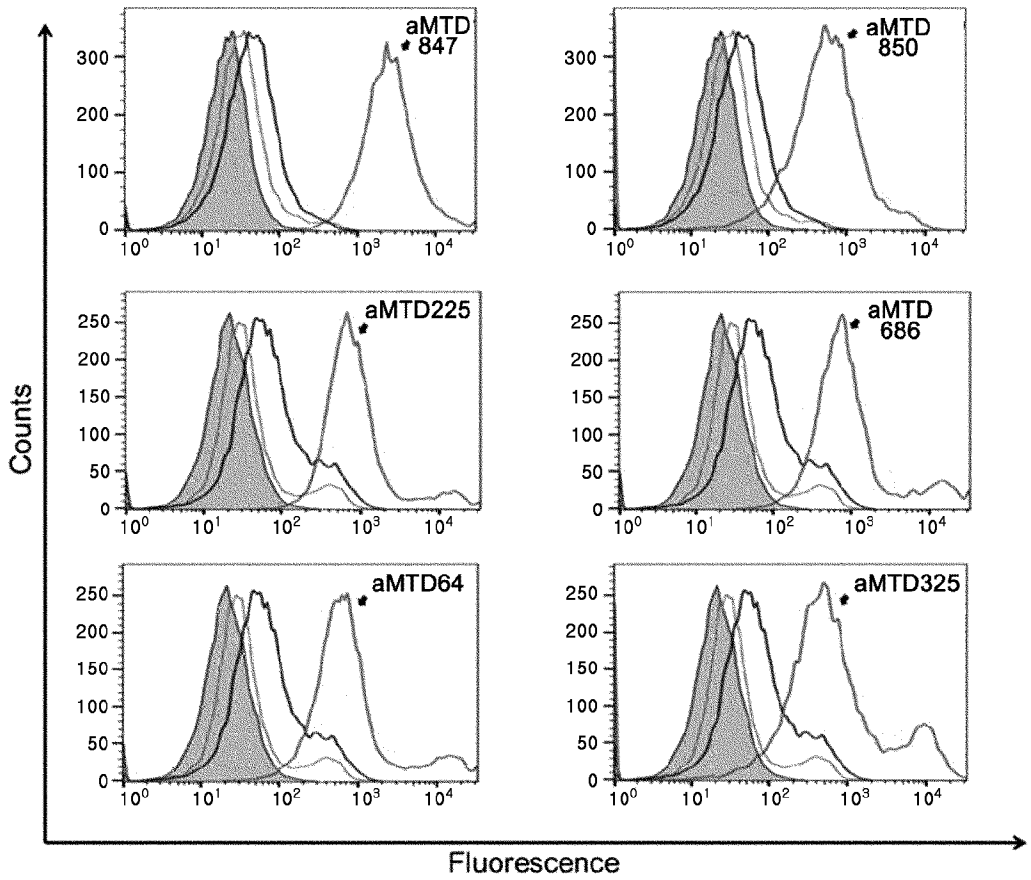

aMTD783
aMTD846
aMTD483
aMTD685
aMTD585
aMTD687
Counts
Fluorescence

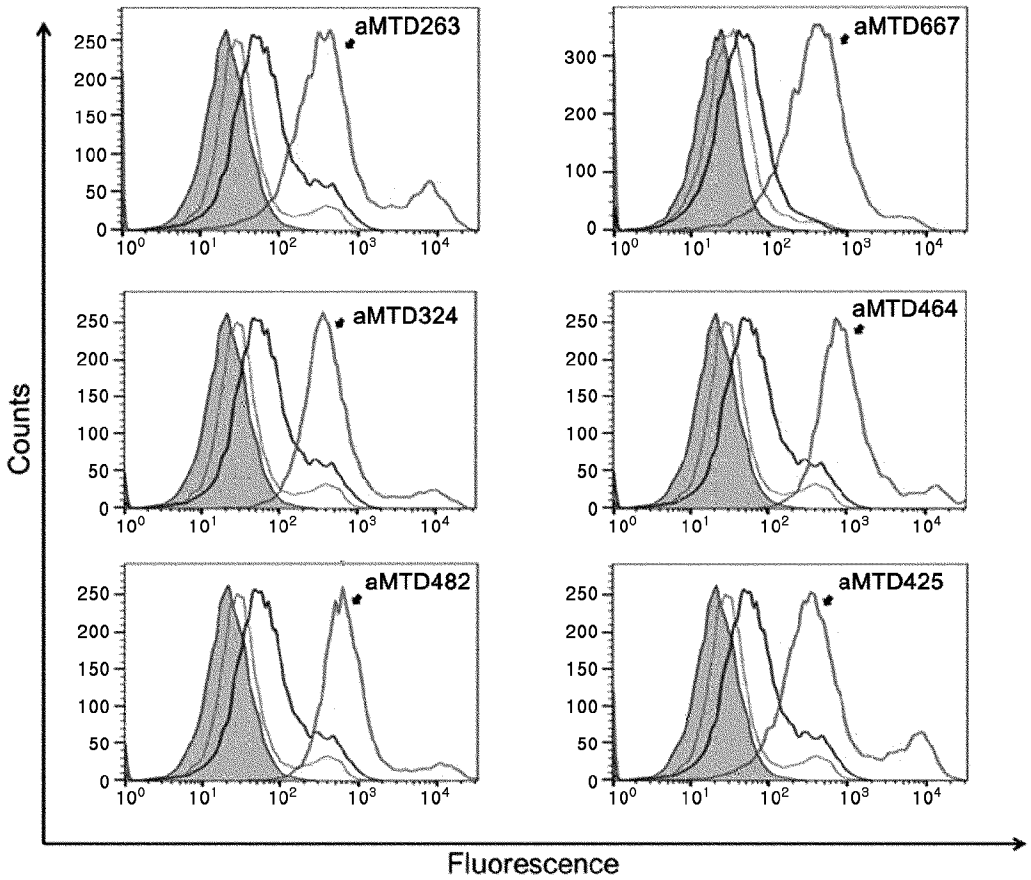
[Fig. 5k]


aMTD 541
aMTD485
aMTD784
aMTD184
aMTD764
aMTD 808
Counts
Fluorescence

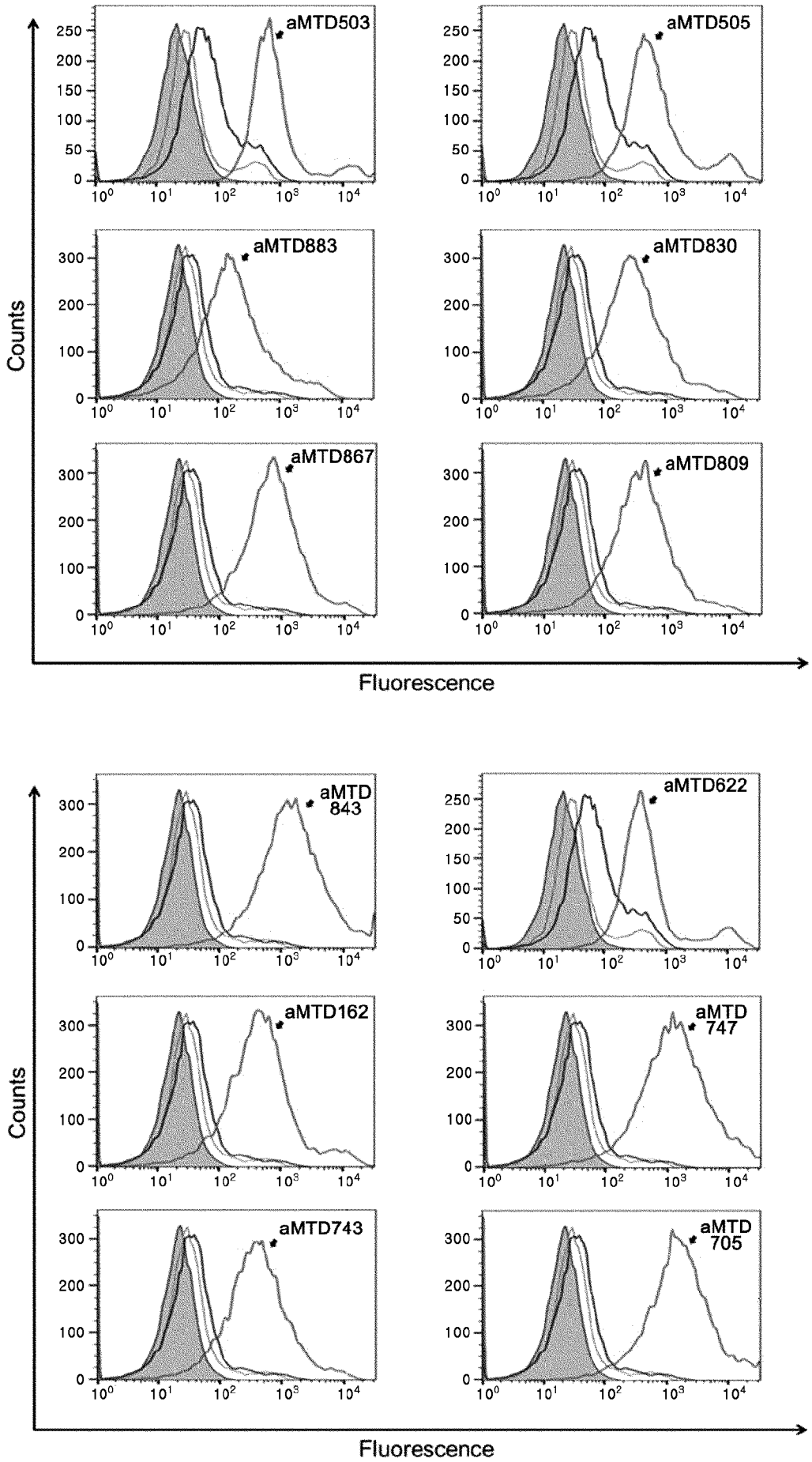

aMTD544
aMTD 224
aMTD 848
aMTD 766
aMTD 164
aMTD 565
Counts
Fluorescence

[Fig. 5l]

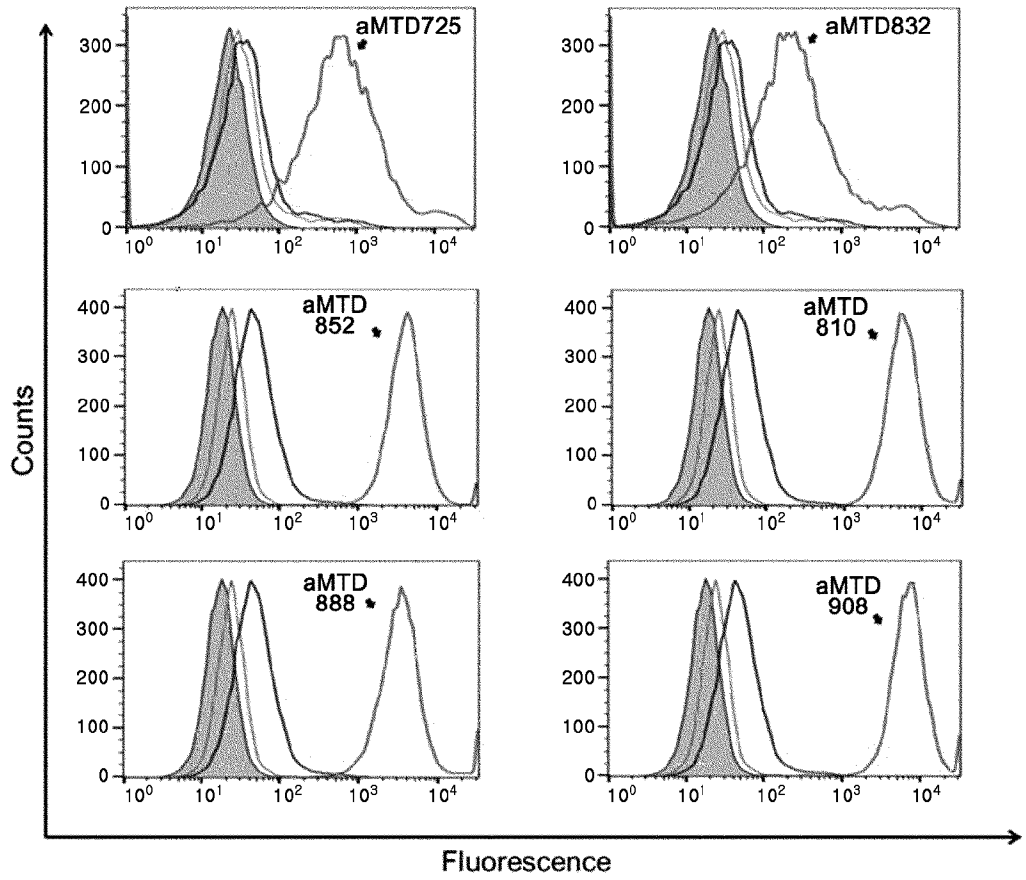

aMTD 847
aMTD 850
aMTD225
aMTD 686
aMTD64
aMTD325
Counts
Fluorescence

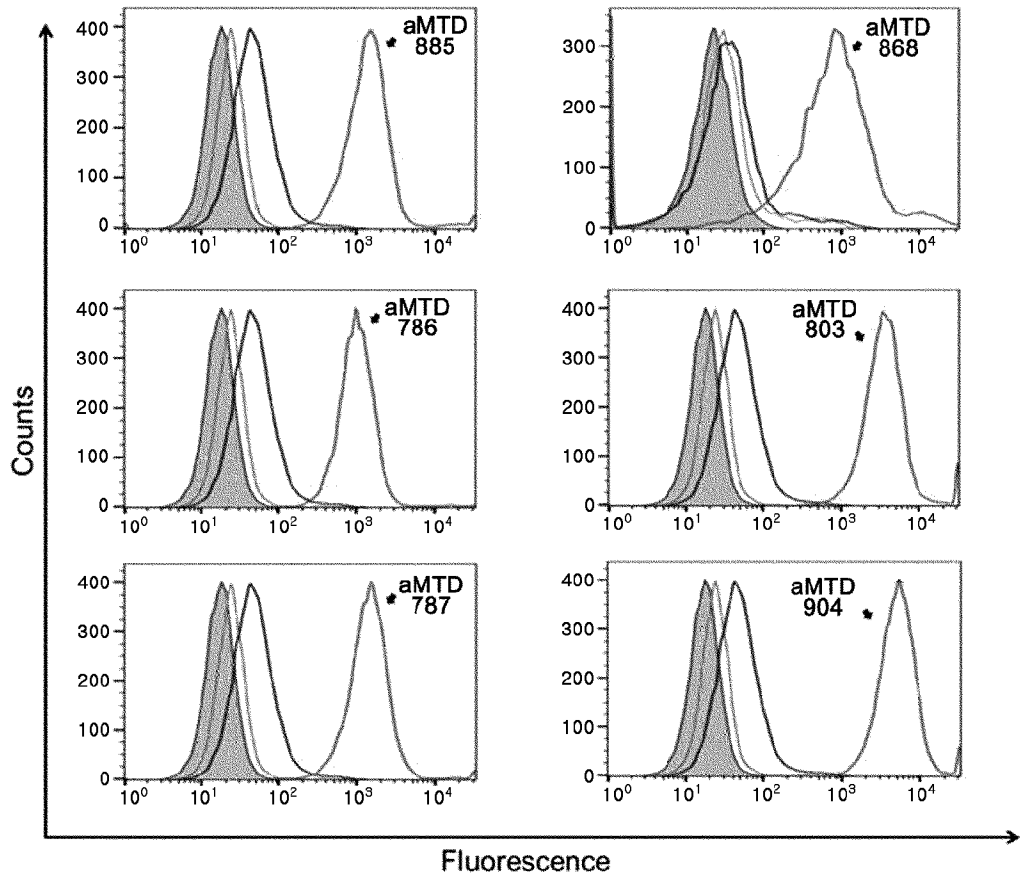

aMTD263
aMTD667
aMTD324
aMTD464
aMTD482
aMTD425
Counts
Fluorescence

[Fig. 5m]

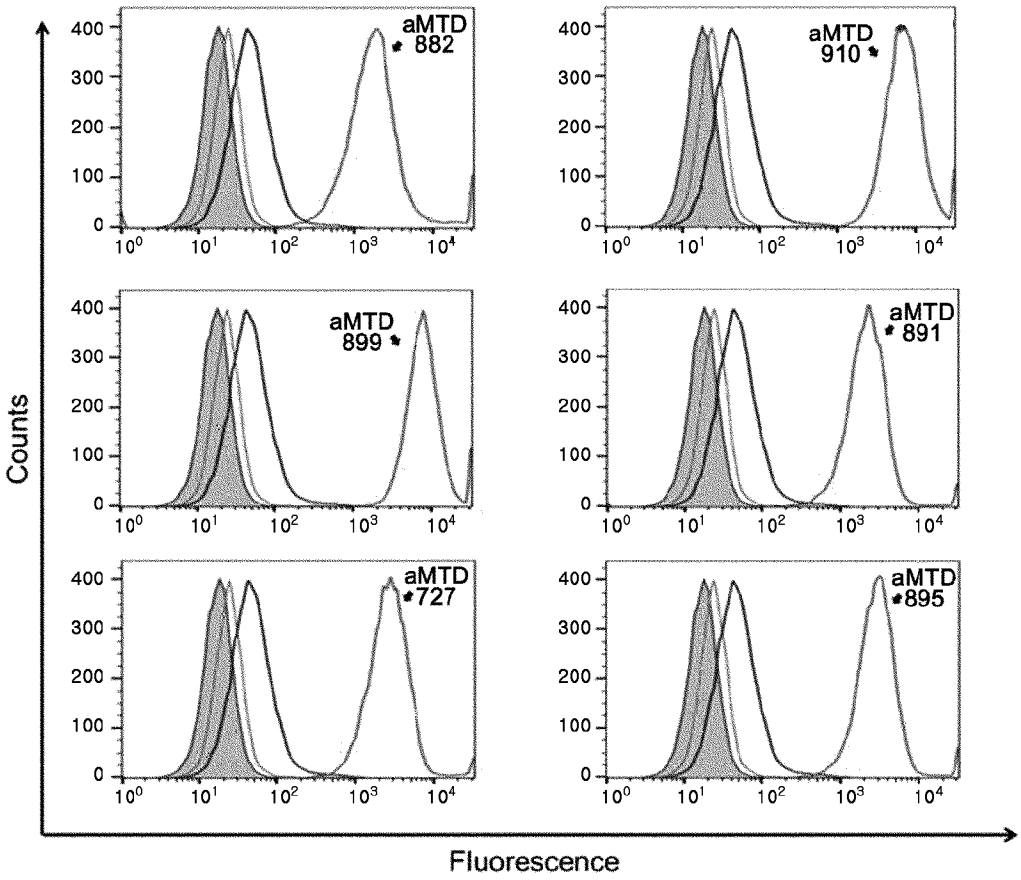

aMTD503
aMTD505
aMTD883
aMTD830
aMTD867
aMTD809
aMTD 843
aMTD622
aMTD162
aMTD 747
aMTD743
aMTD 705
Counts
Fluorescence

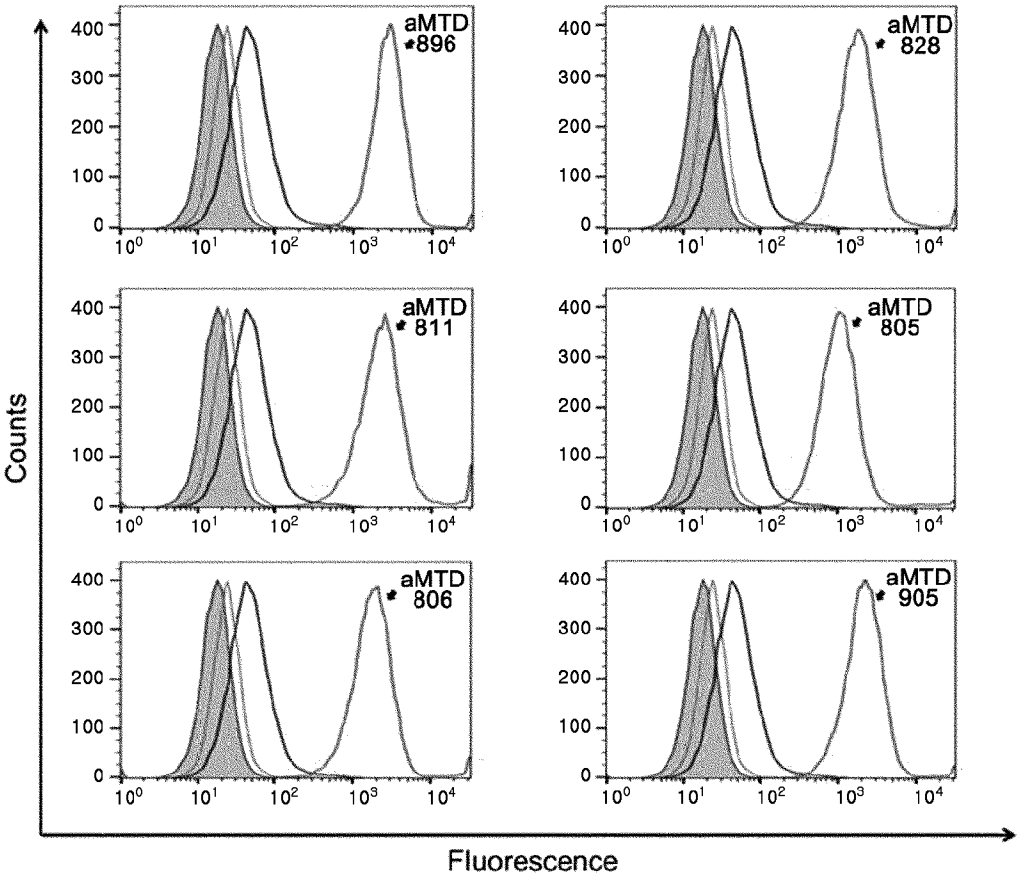
[Fig. 5n]


aMTD725
aMTD832
aMTD 852
aMTD 810
aMTD 888
aMTD 908
Counts
Fluorescence

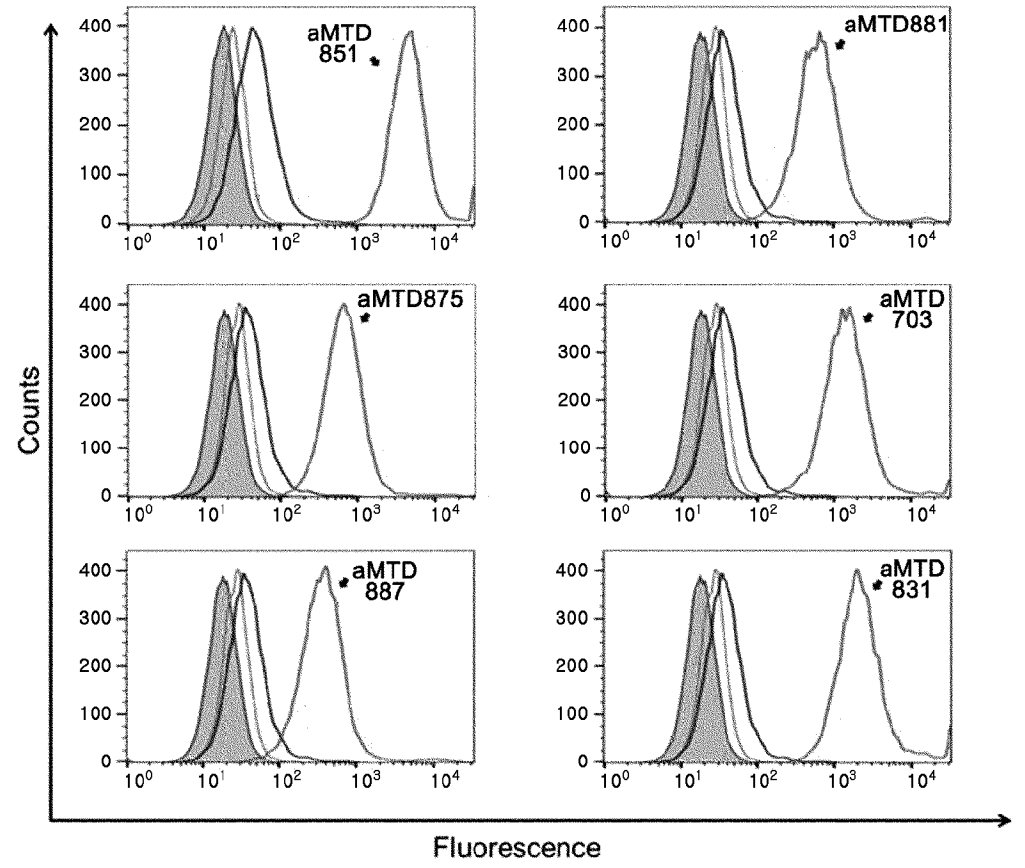

aMTD 885
aMTD 868
aMTD 786
aMTD 803
aMTD 787
aMTD 904
Counts
Fluorescence

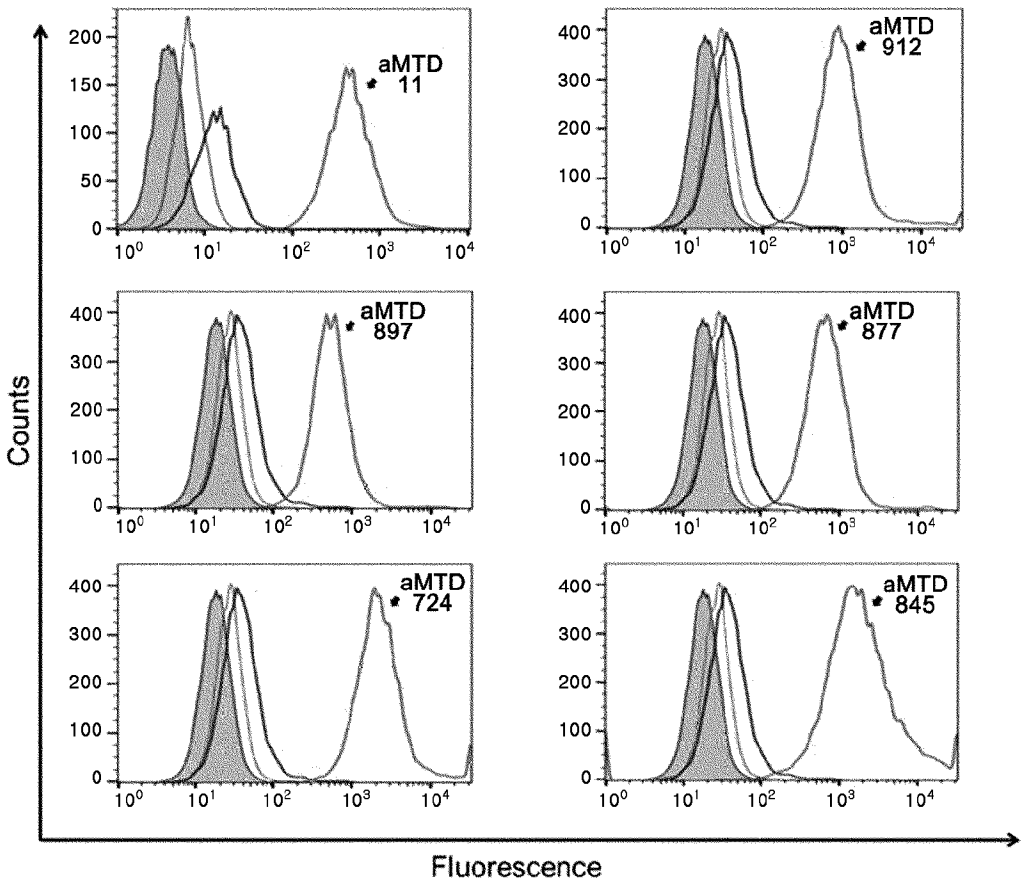
[Fig. 5o]


aMTD 882
aMTD 910
aMTD 899
aMTD 891
aMTD 727
aMTD 895
Counts
Fluorescence

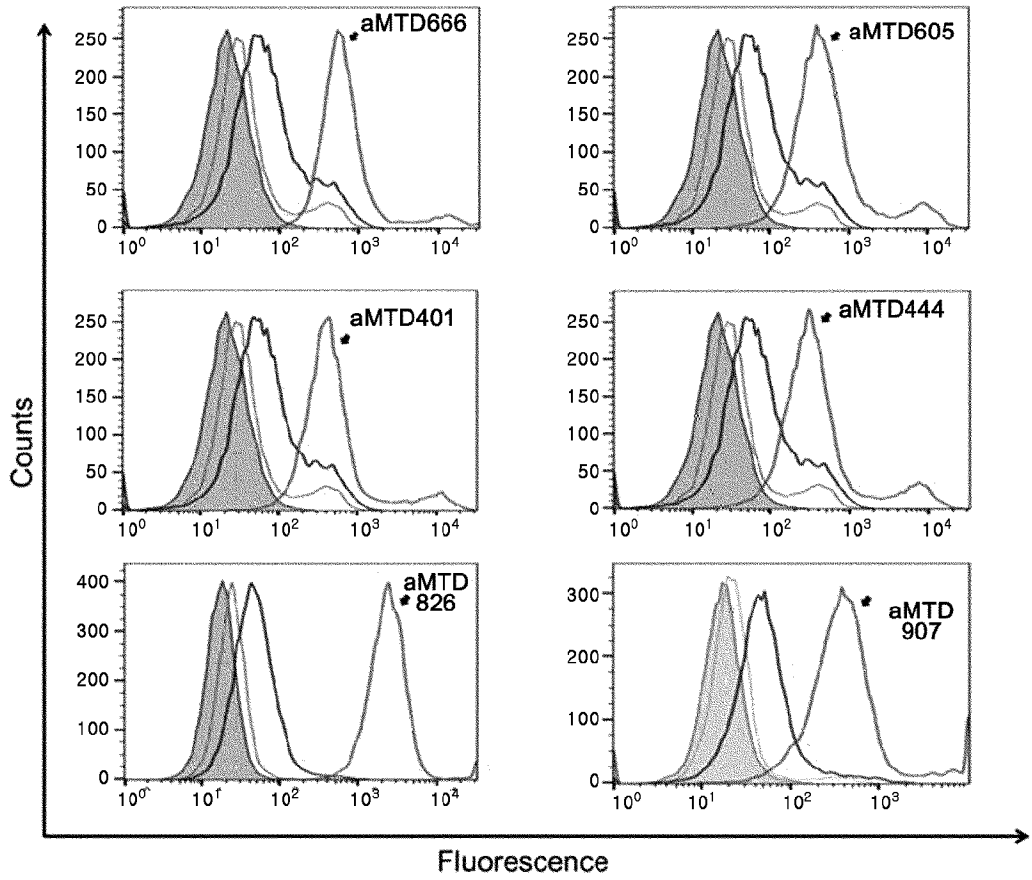

aMTD 896
aMTD 828
aMTD 811
aMTD 805
aMTD 806
aMTD 905
Counts
Fluorescence

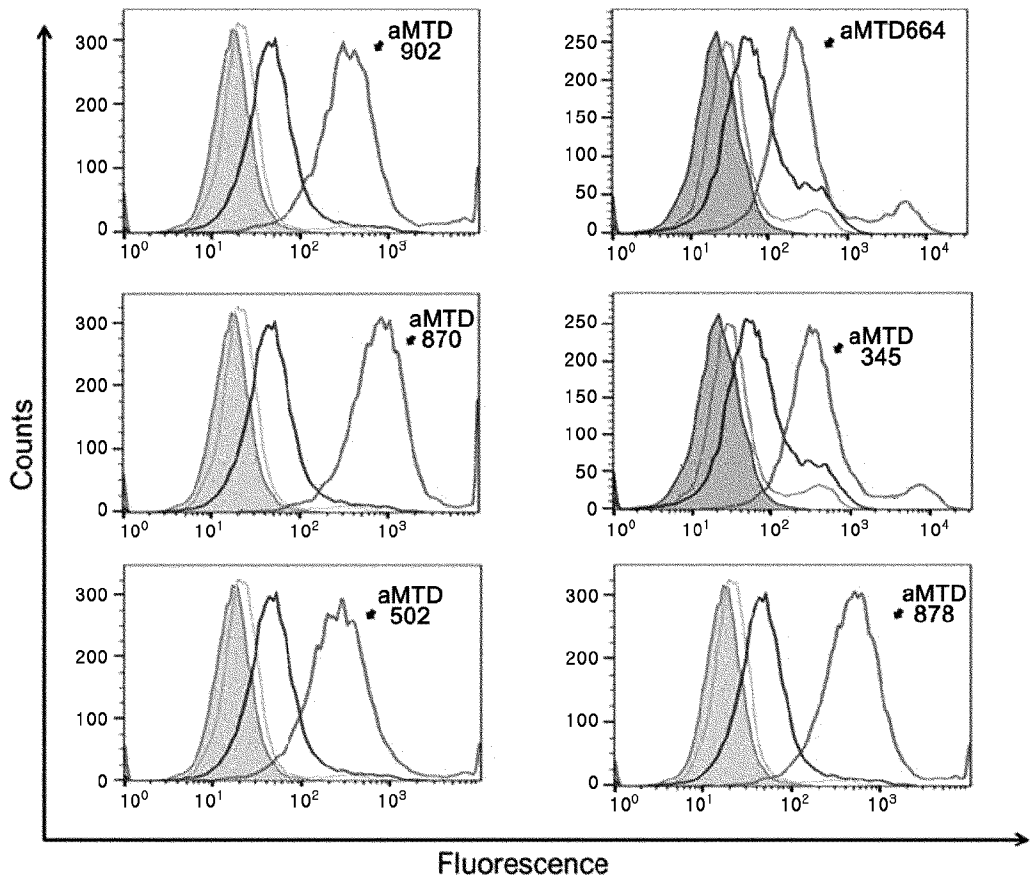
[Fig. 5p]


aMTD 851
aMTD881
aMTD875
aMTD 703
aMTD 887
aMTD 831
Counts
Fluorescence

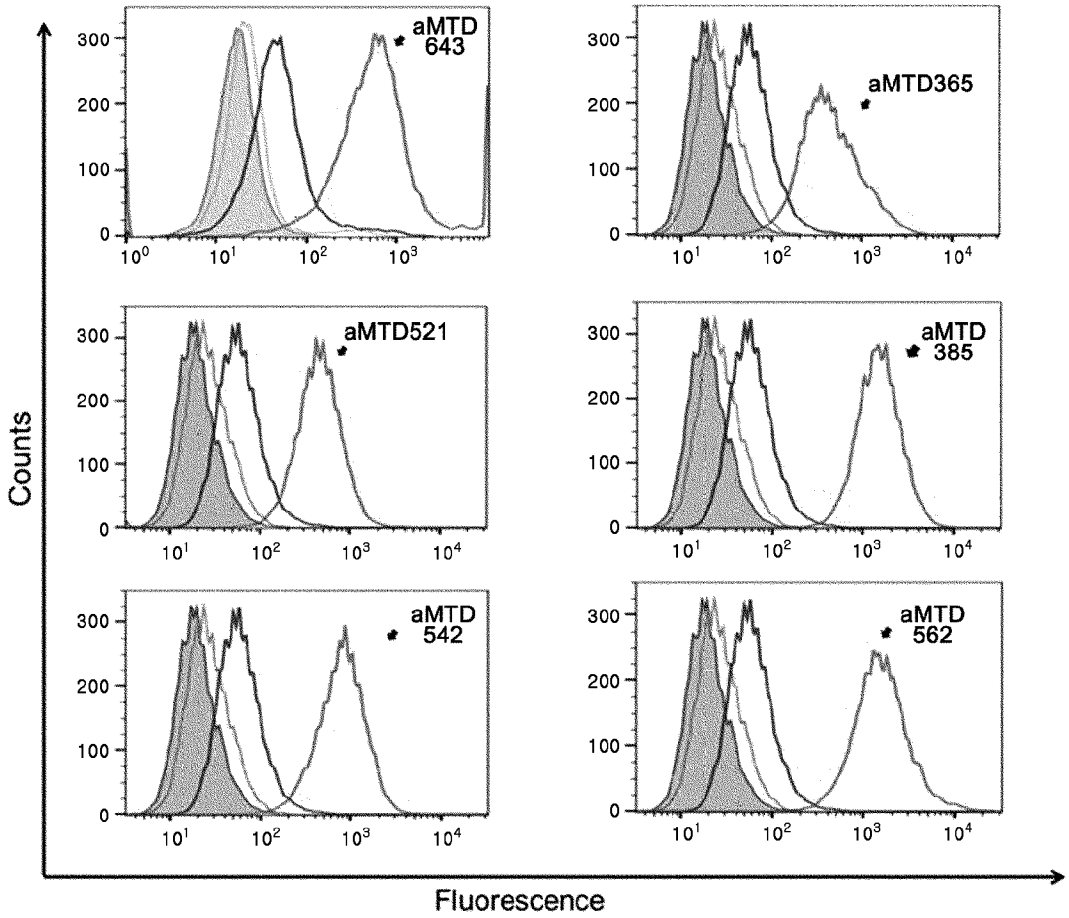

aMTD 11
aMTD 912
aMTD 897
aMTD 877
aMTD 724
aMTD 845
Counts
Fluorescence

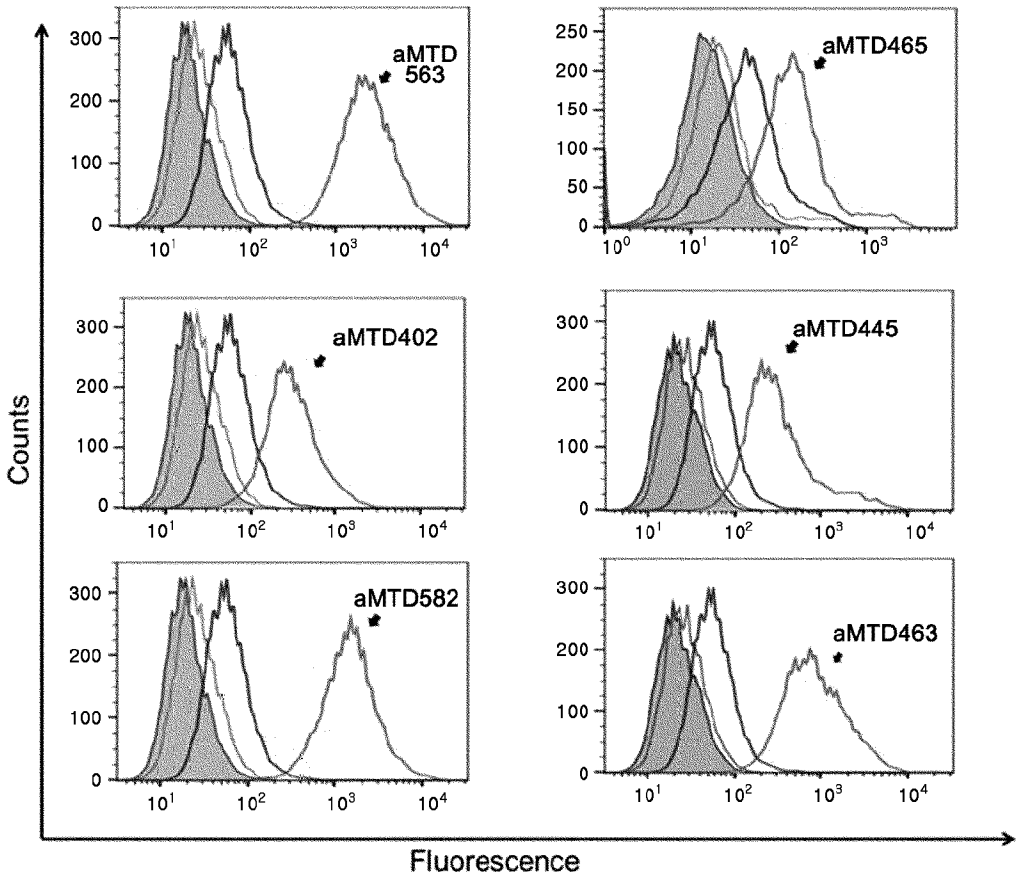
[Fig. 5q]


aMTD666
aMTD605
aMTD401
aMTD444
aMTD 826
aMTD 907
Counts
Fluorescence

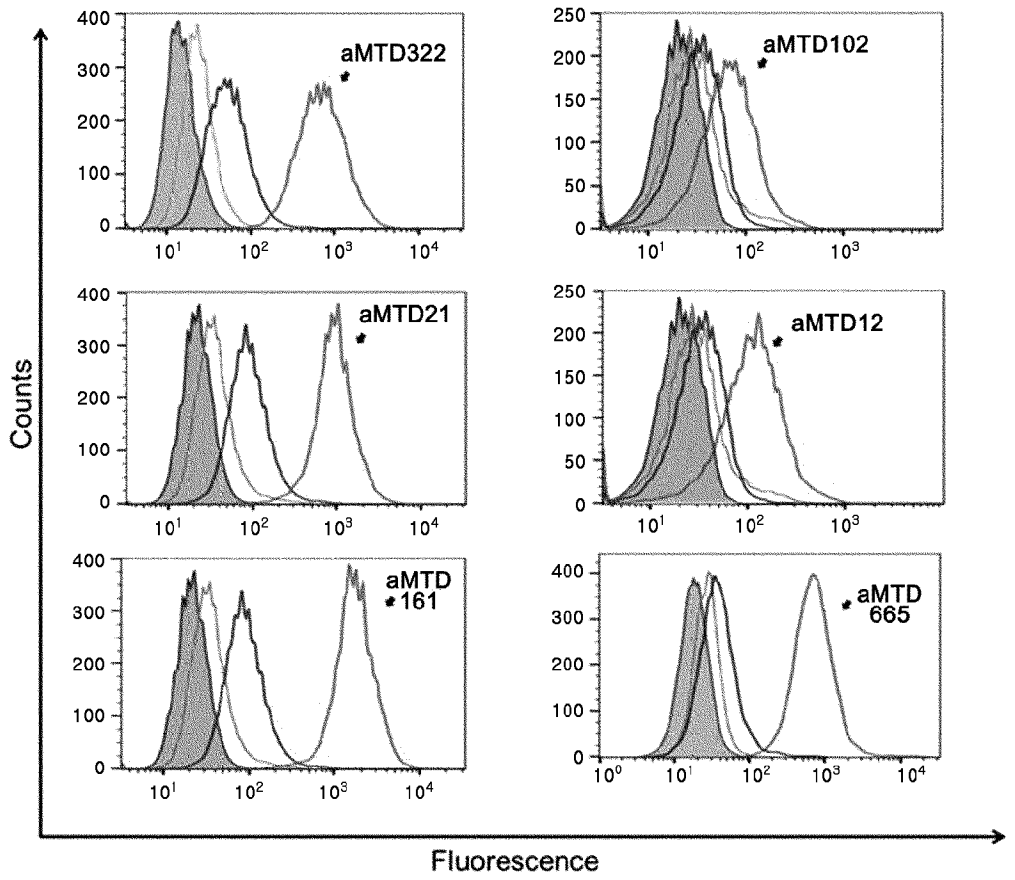

aMTD 902
aMTD664
aMTD 870
aMTD 345
aMTD 502
aMTD 878
Counts
Fluorescence

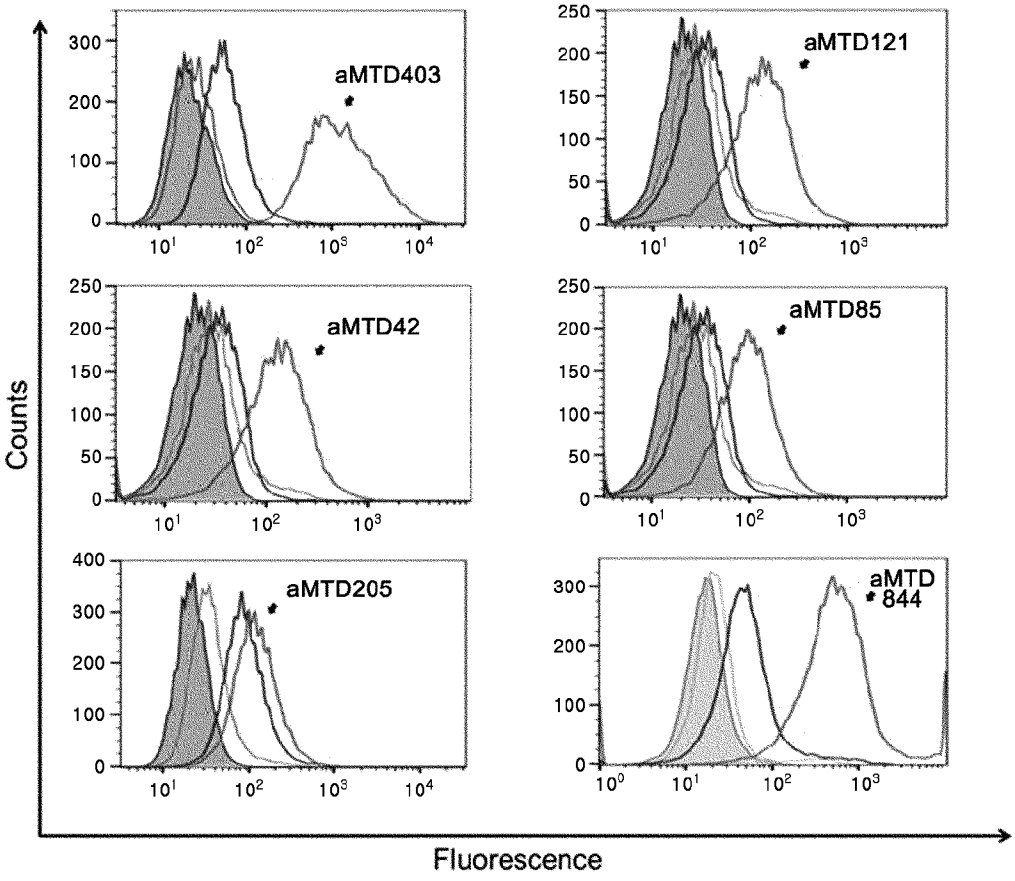
[Fig. 5r]


aMTD 643
aMTD365
aMTD521
aMTD 385
aMTD 542
aMTD 562
Counts
Fluorescence

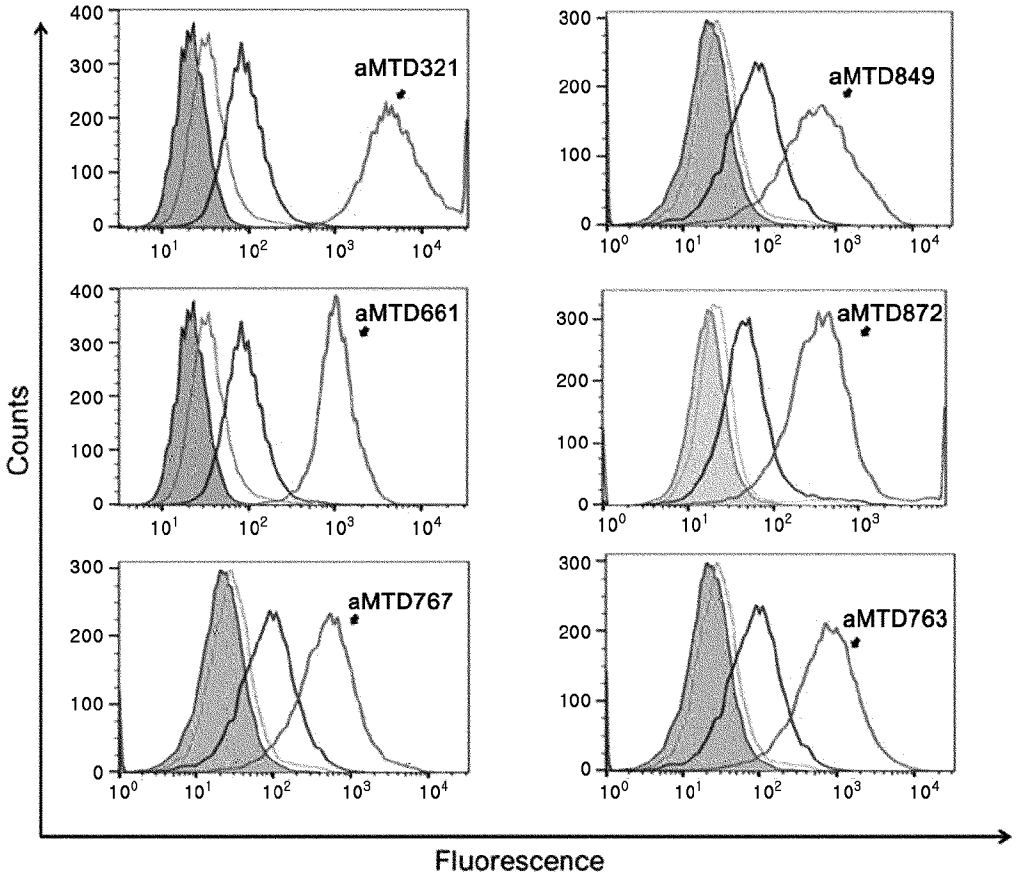

aMTD 563
aMTD465
aMTD402
aMTD445
aMTD582
aMTD463
Counts
Fluorescence

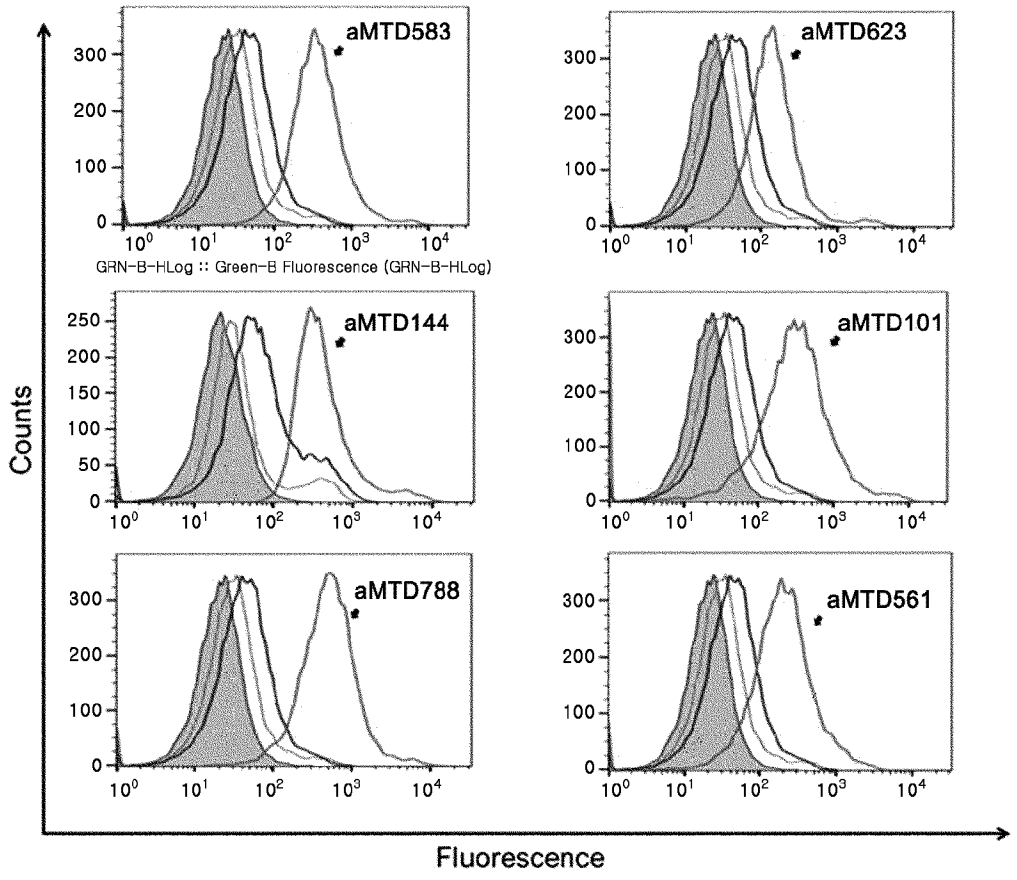
[Fig. 5s]


aMTD322
aMTD102
aMTD21
aMTD12
aMTD 161
aMTD 665
Counts
Fluorescence

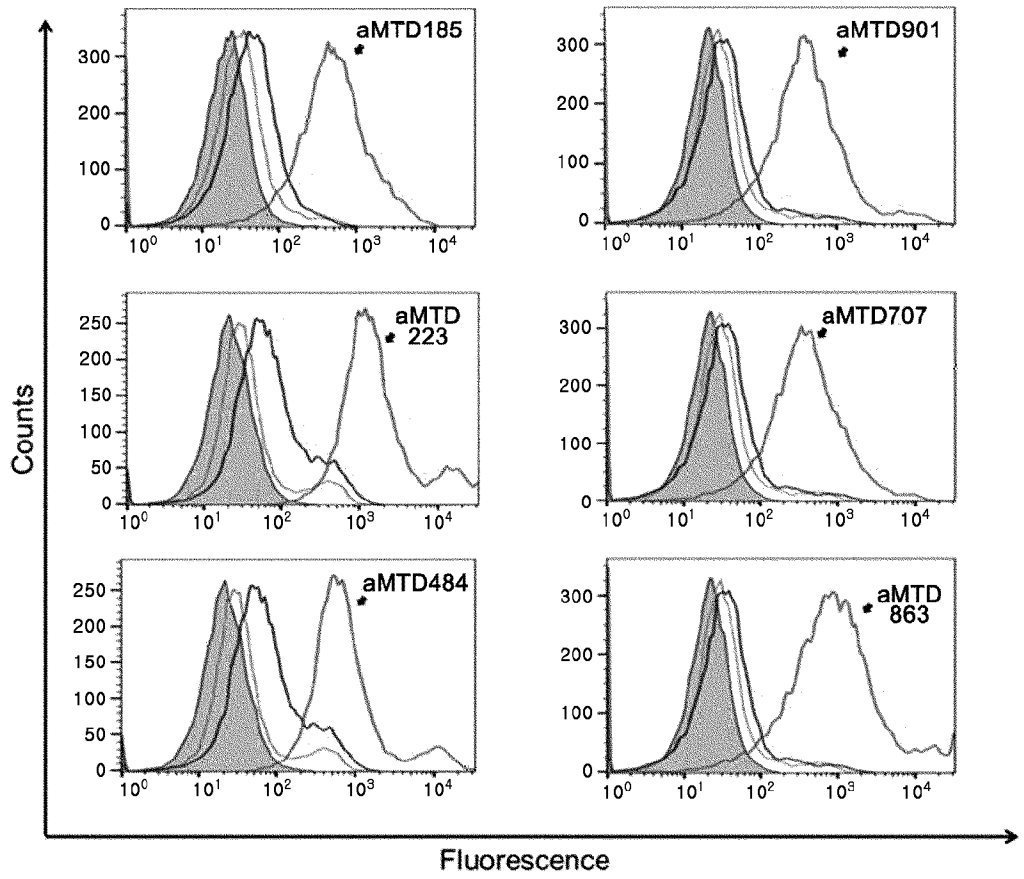

aMTD403
aMTD121
aMTD42
aMTD85
aMTD205
aMTD 844
Counts
Fluorescence

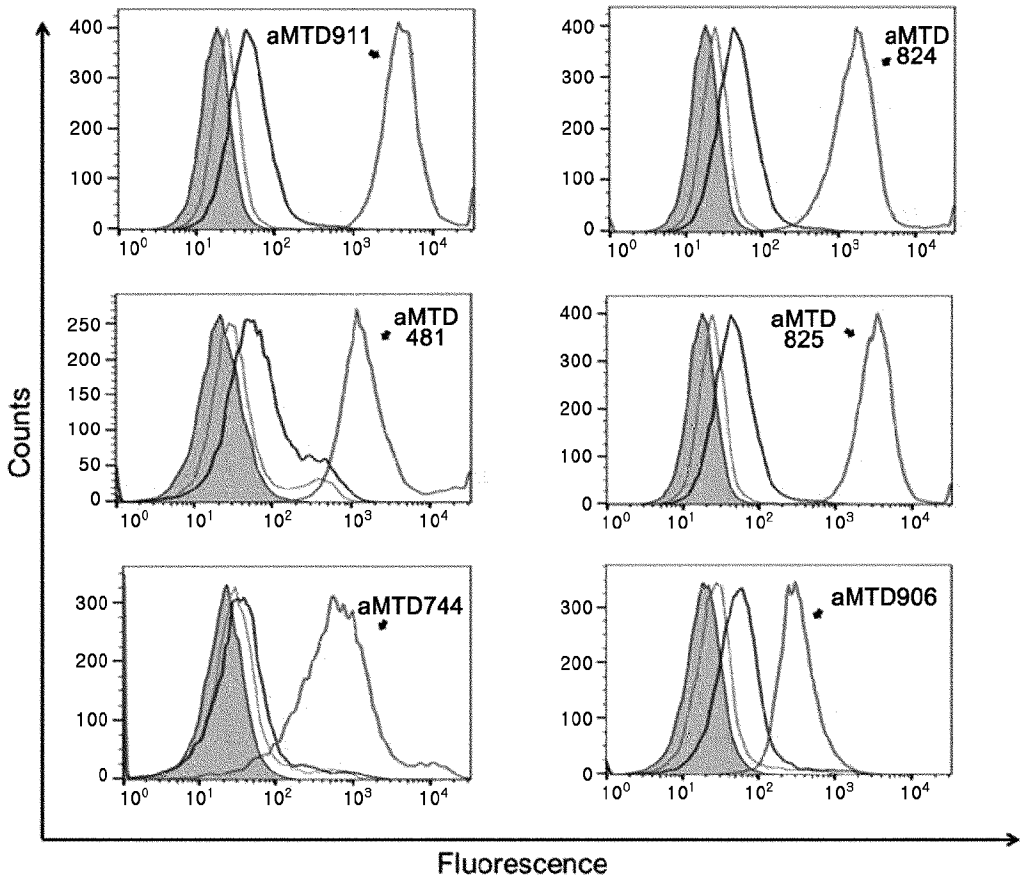
[Fig. 5t]


aMTD321
aMTD849
aMTD661
aMTD872
aMTD767
aMTD763
Counts
Fluorescence

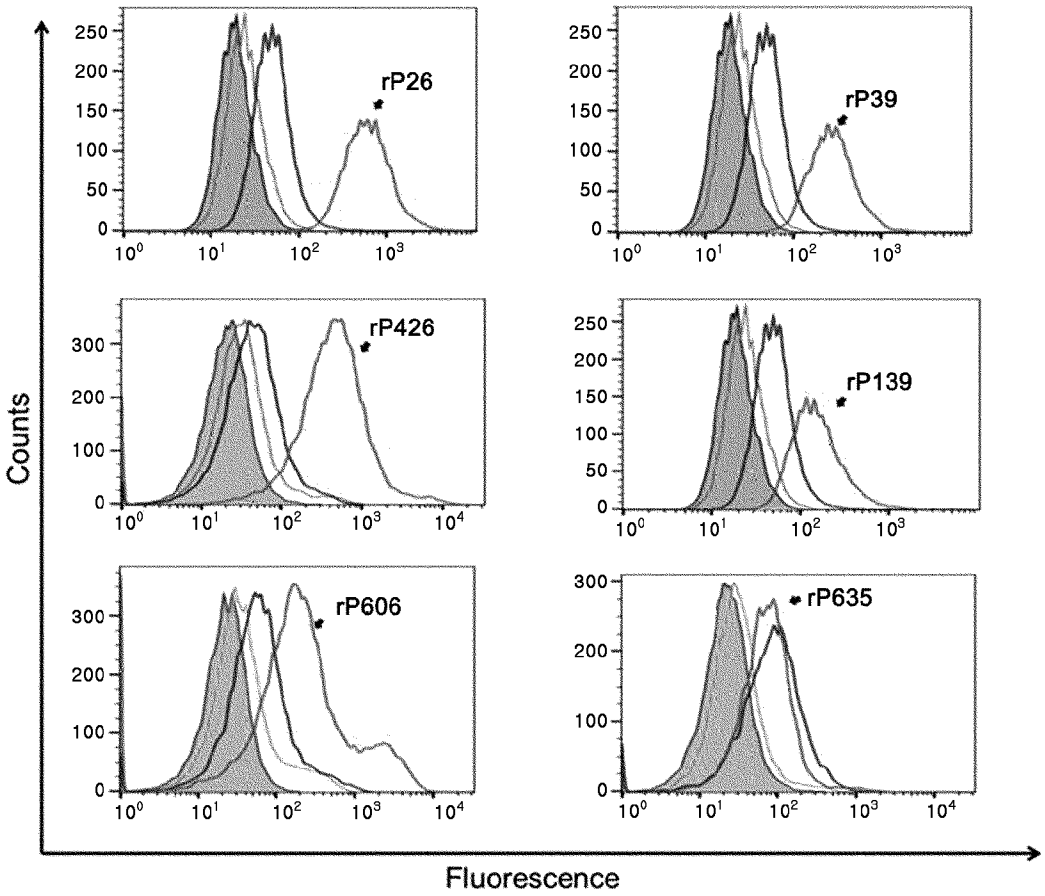

aMTD583
aMTD623
GRN-B-HLog :: Green-B Fluorescence (GRN-B-HLog)
aMTD144
aMTD101
aMTD788
aMTD561
Counts
Fluorescence

[Fig. 5u]

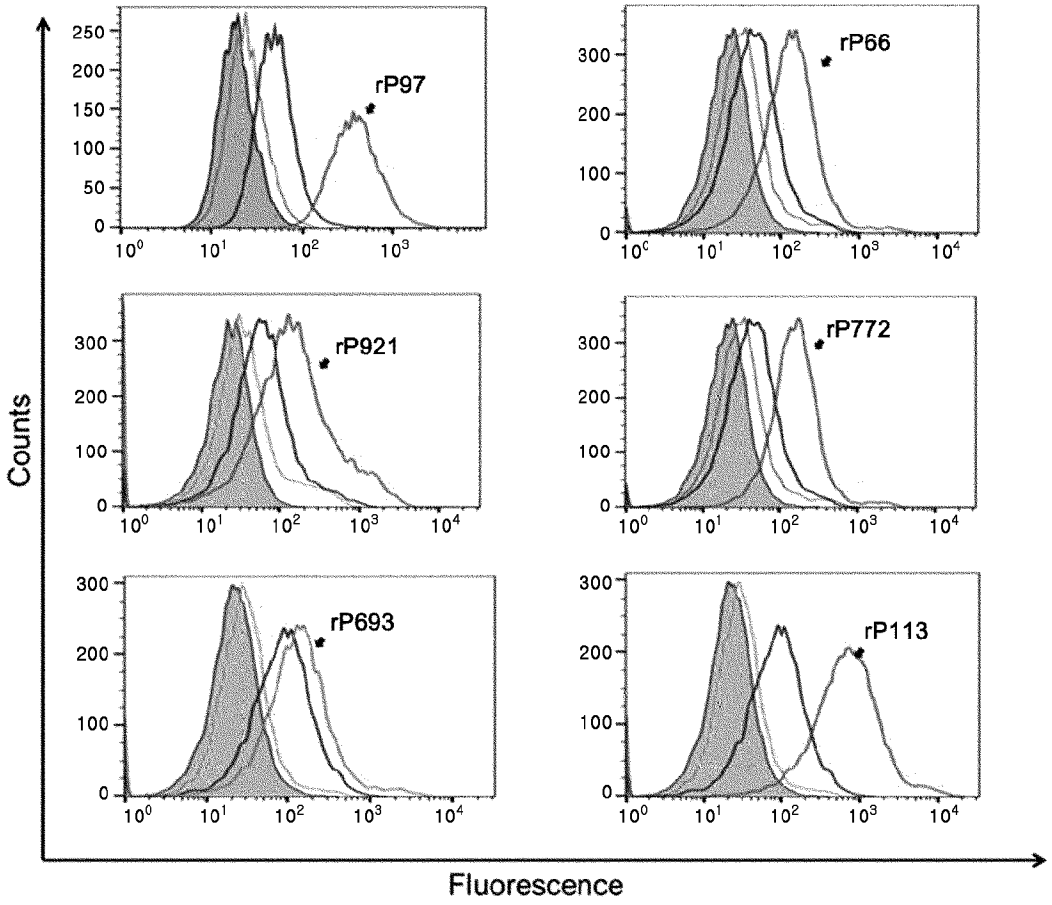

aMTD185
aMTD901
aMTD 223
aMTD707
aMTD484
aMTD 863
Counts
Fluorescence

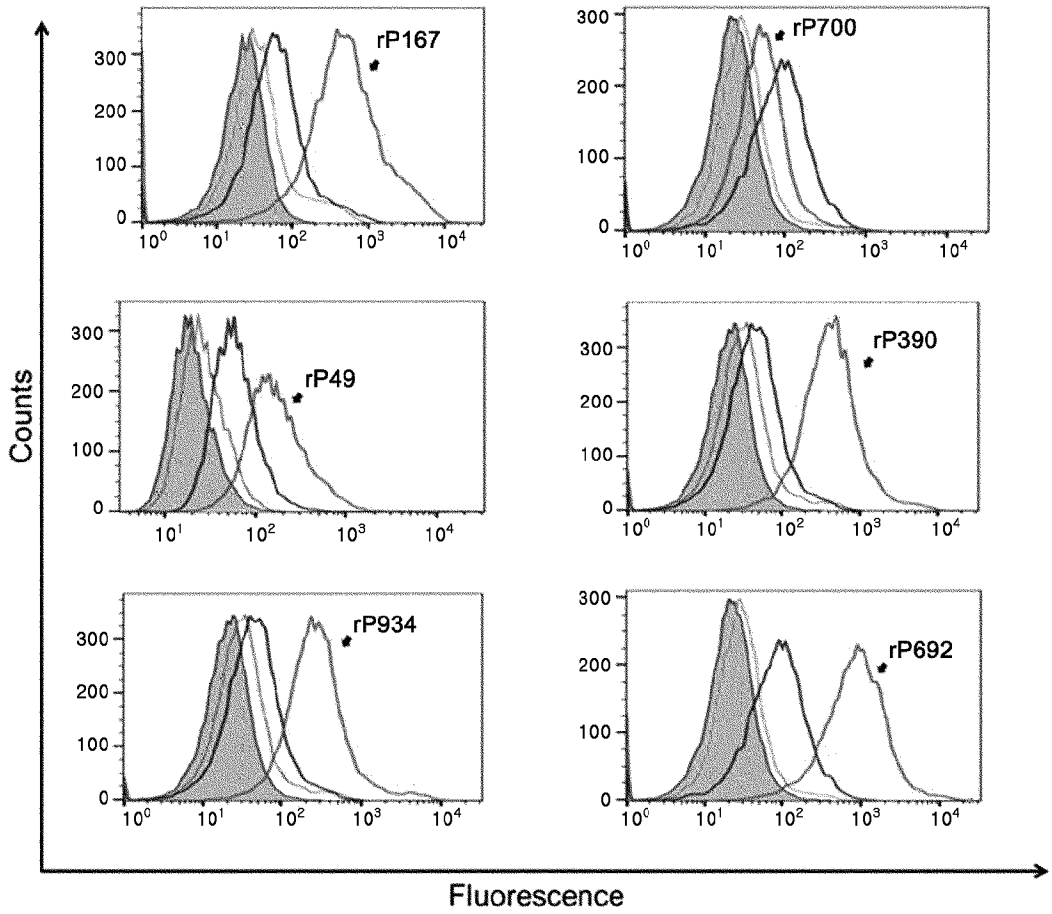

aMTD911
aMTD 824
aMTD 481
aMTD 825
aMTD744
aMTD906
Counts
Fluorescence

[Fig. 6a]

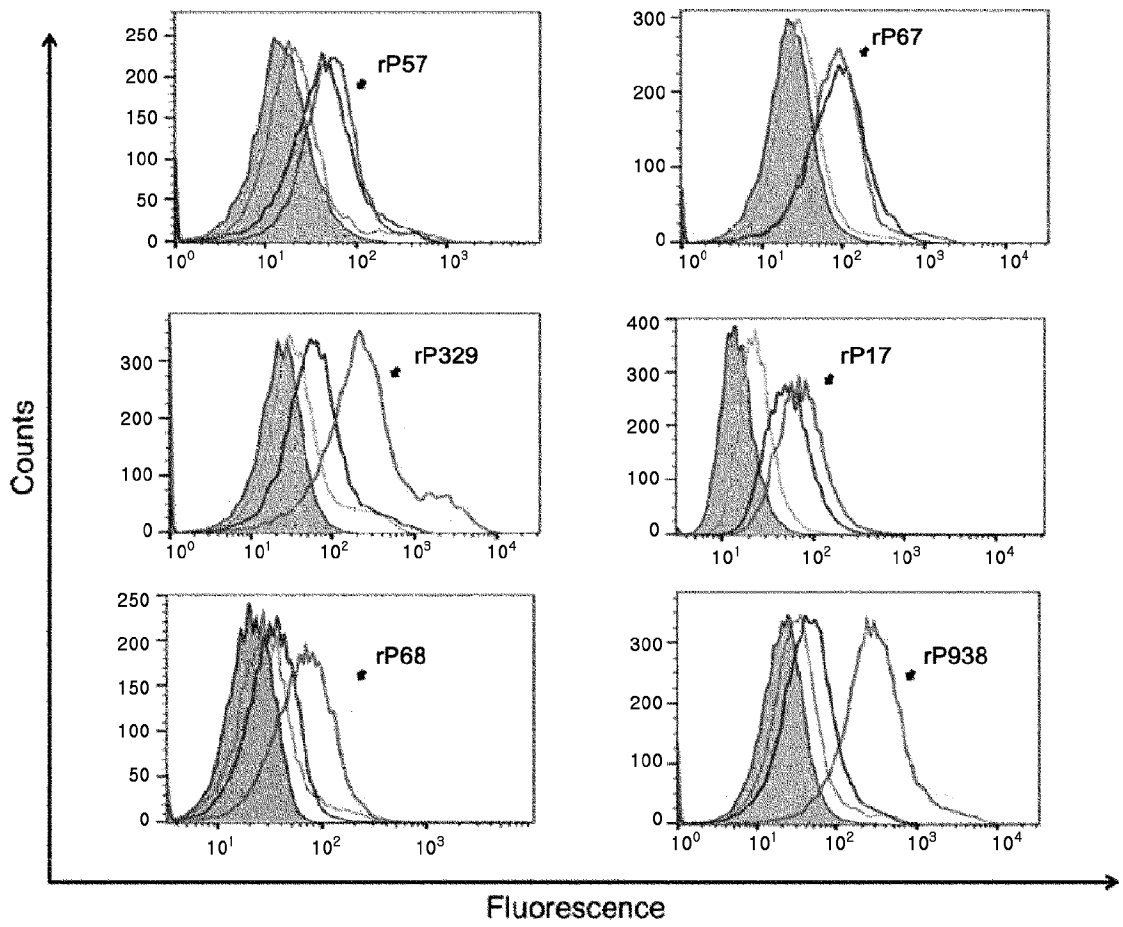


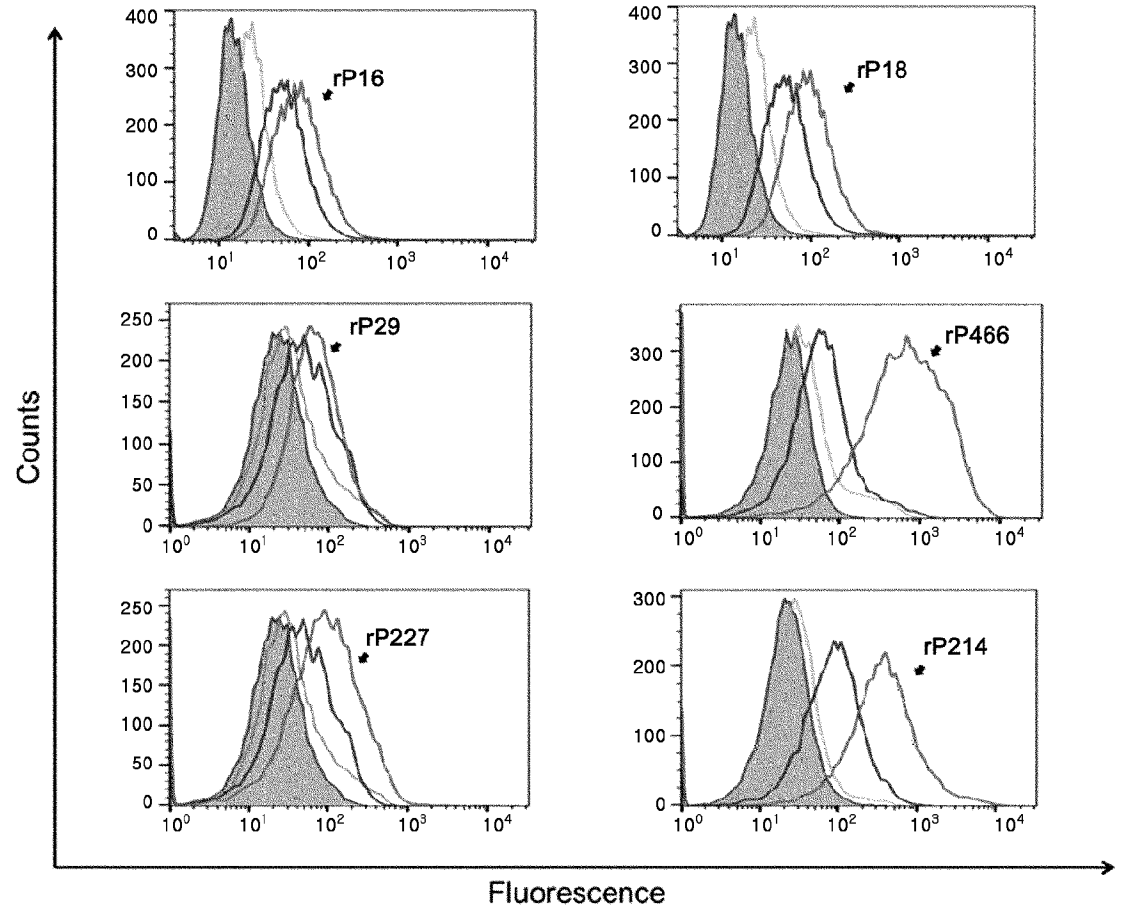
[Fig. 6b]


rP97
rP66
rP921
rP772
rP693
rP113
Counts
Fluorescence

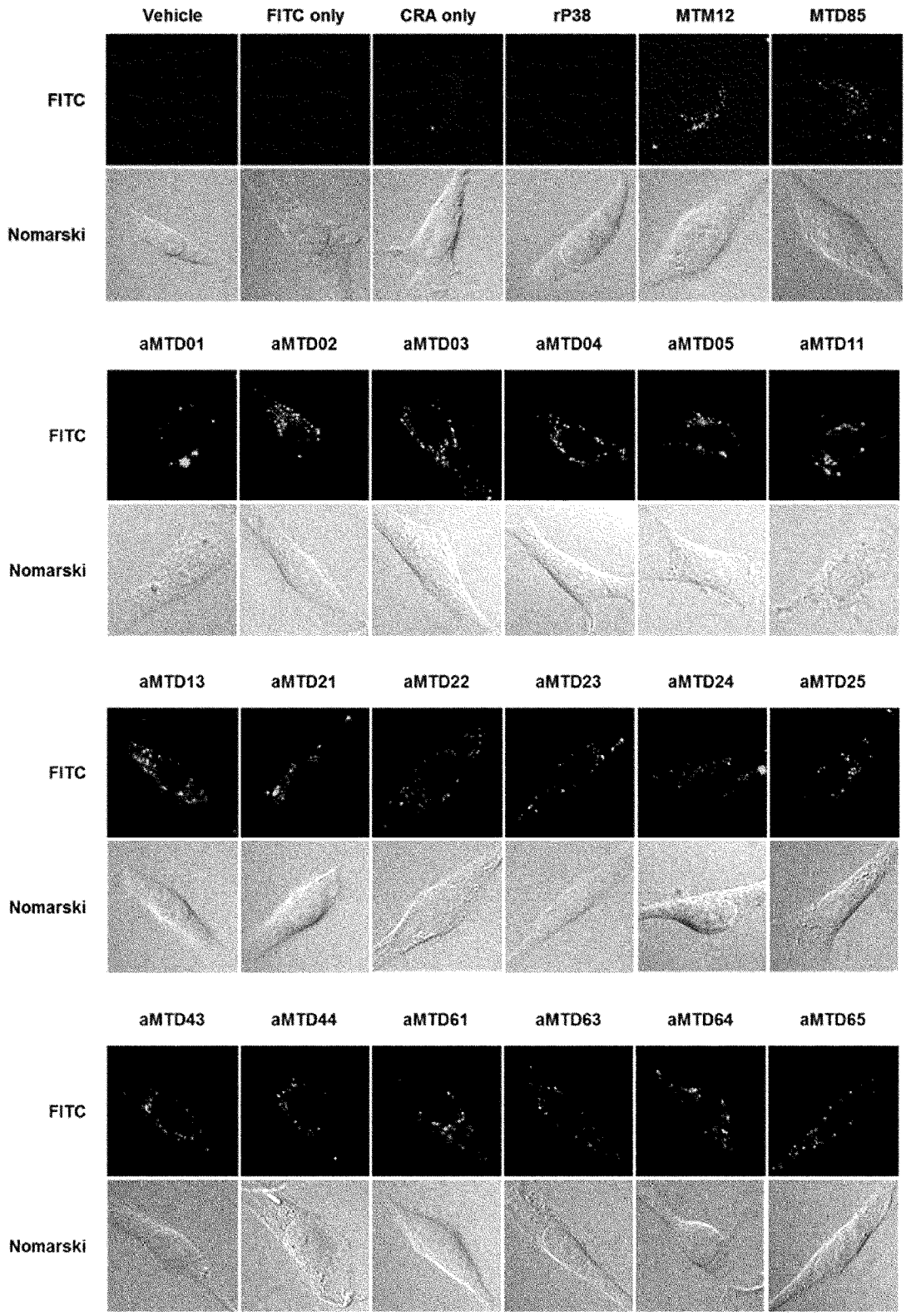

rP167
rP700
rP49
rP390
rP934
rP692
Counts
Fluorescence

[Fig. 6c]

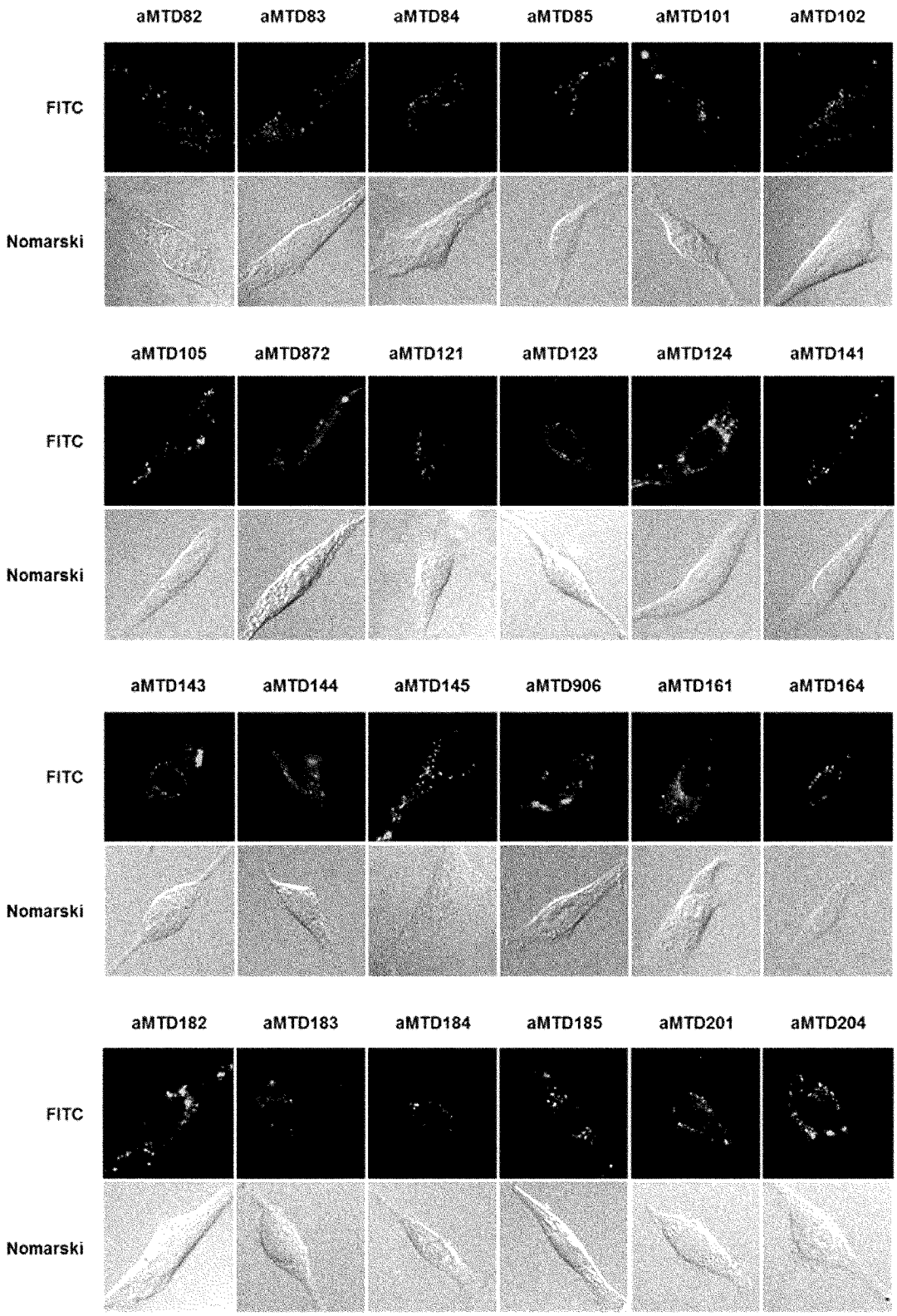

rP57
rP67
rP329
rP17
rP68
rP938
Counts
Fluorescence


rP16
rP18
rP29
rP466
rP227
rP214
Counts
Fluorescence

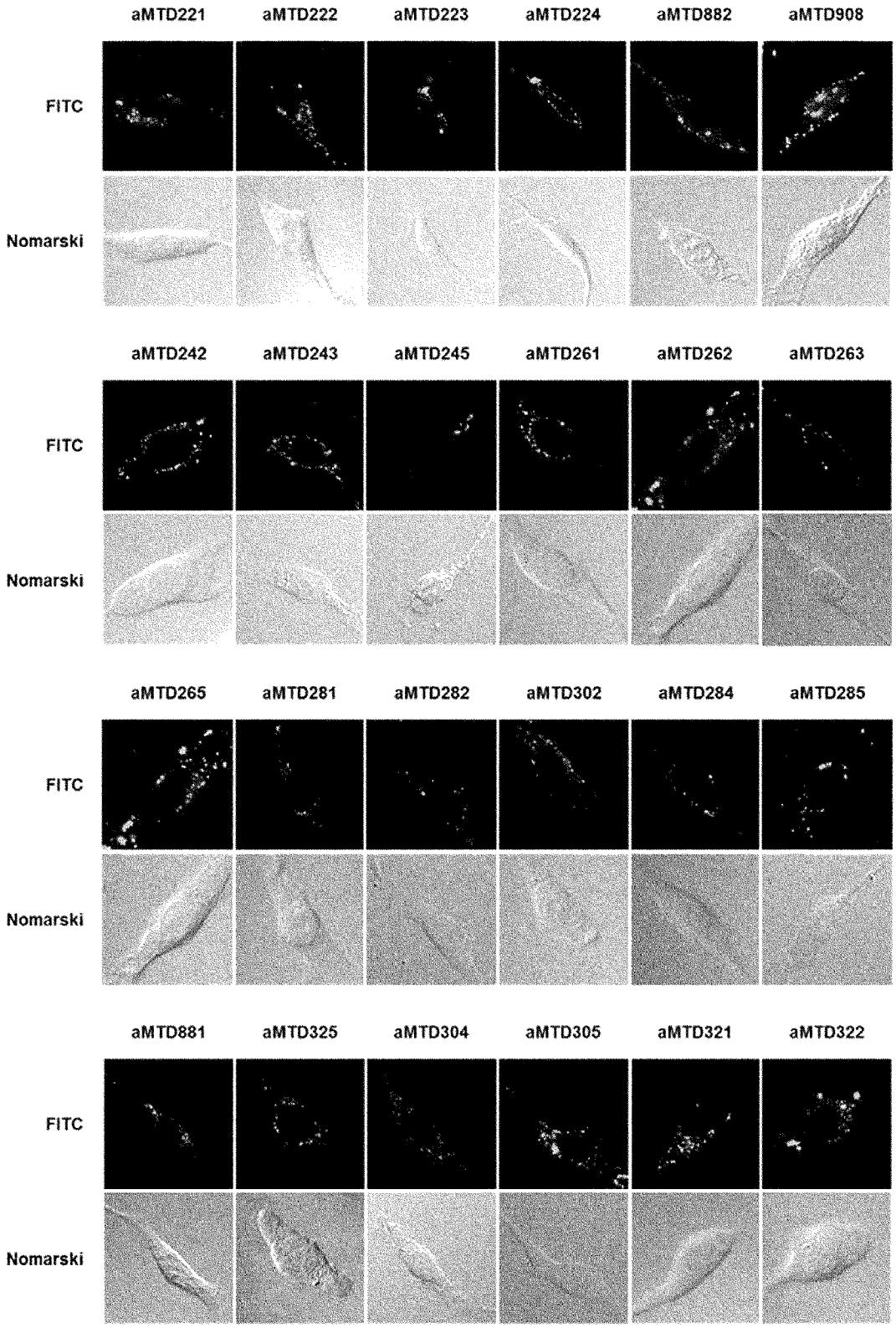
[Fig. 7a]


Vehicle
FITC only
CRA only
rP38
MTM12
MTD85
FITC
Nomarski
aMTD01
aMTD02
aMTD03
aMTD04
aMTD05
aMTD11
FITC
Nomarski
aMTD13
aMTD21
aMTD22
aMTD23
aMTD24
aMTD25
FITC
Nomarski
aMTD43
aMTD44
aMTD61
aMTD63
aMTD64
aMTD65
FITC
Nomarski

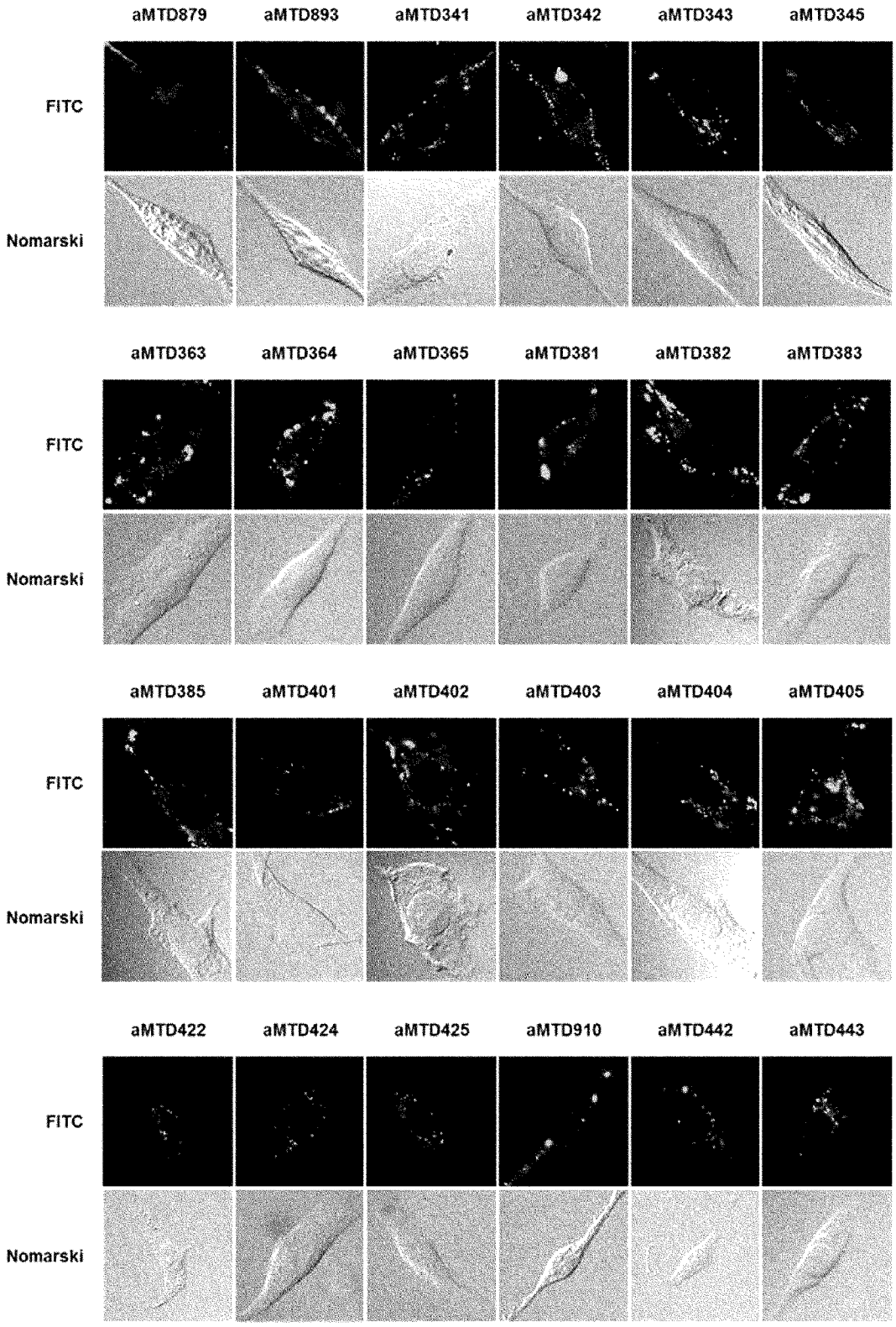
[Fig. 7b]


aMTD82
aMTD83
aMTD84
aMTD85
aMTD101
aMTD102
FITC
Nomarski
aMTD105
aMTD872
aMTD121
aMTD123
aMTD124
aMTD141
FITC
Nomarski
aMTD143
aMTD144
aMTD145
aMTD906
aMTD161
aMTD164
FITC
Nomarski
aMTD182
aMTD183
aMTD184
aMTD185
aMTD201
aMTD204
FITC
Nomarski

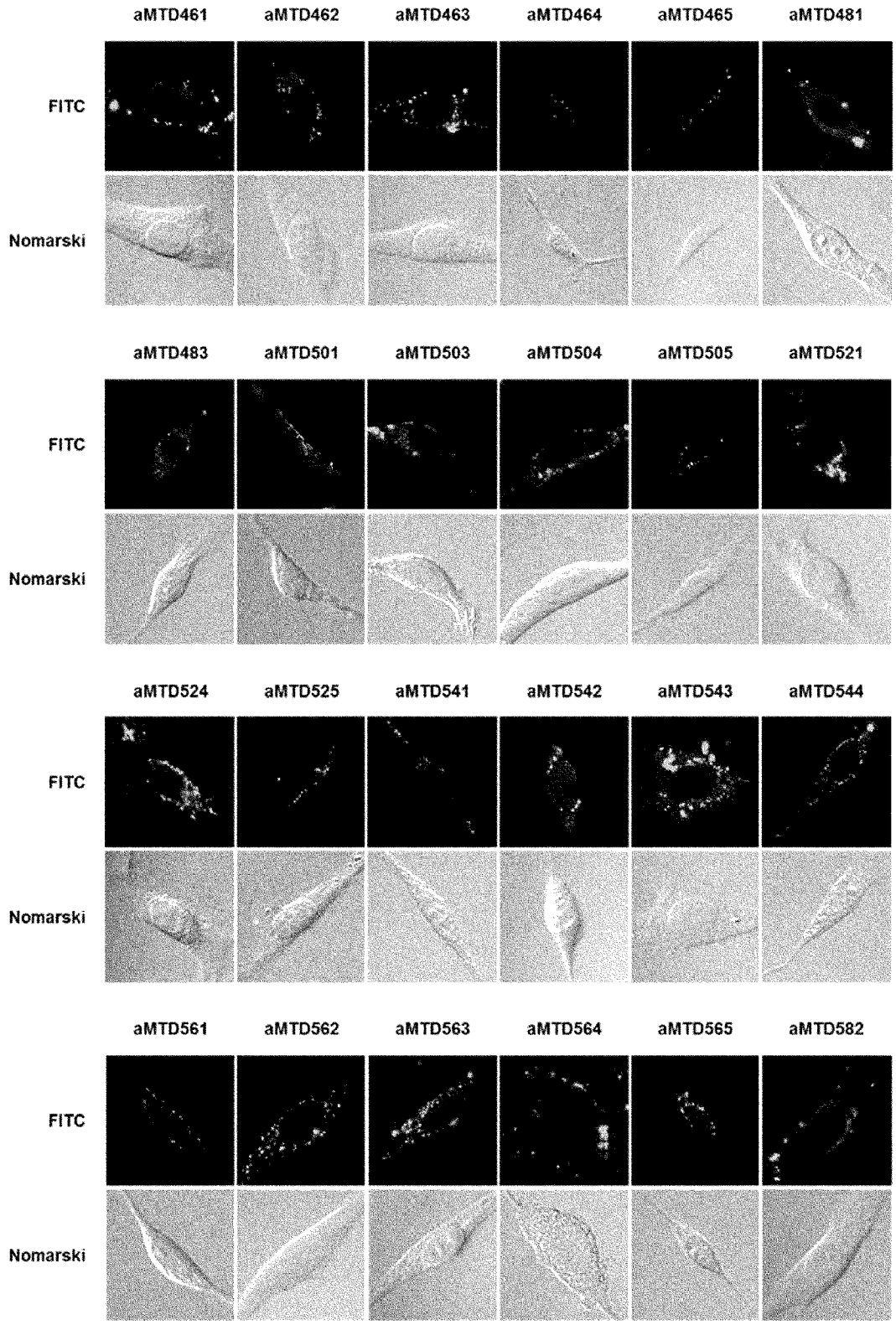
[Fig. 7c]


aMTD221
aMTD222
aMTD223
aMTD224
aMTD882
aMTD908
FITC
Nomarski
aMTD242
aMTD243
aMTD245
aMTD261
aMTD262
aMTD263
FITC
Nomarski
aMTD265
aMTD281
aMTD282
aMTD302
aMTD284
aMTD285
FITC
Nomarski
aMTD881
aMTD325
aMTD304
aMTD305
aMTD321
aMTD322
FITC
Nomarski

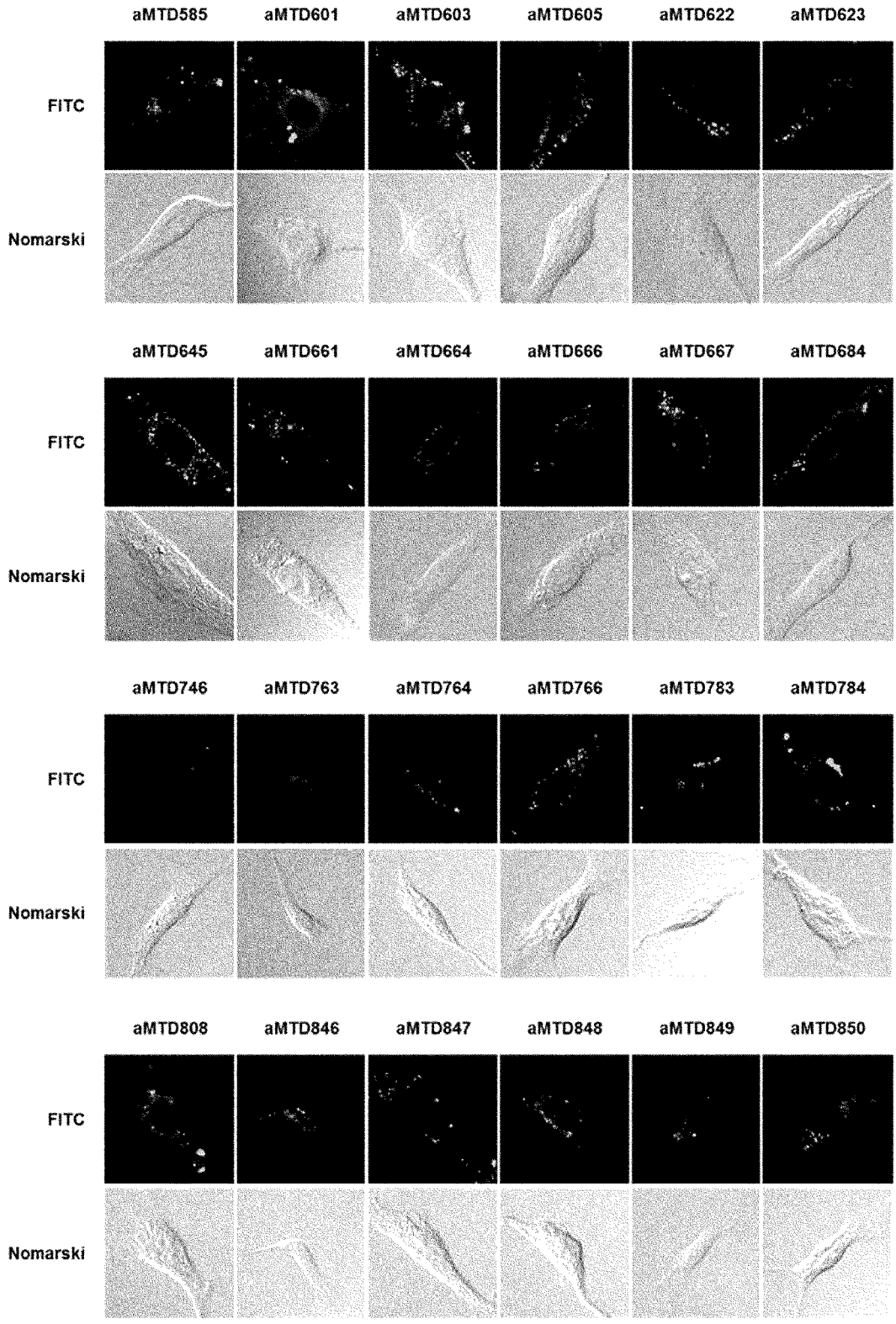
[Fig. 7d]


aMTD879
aMTD893
aMTD341
aMTD342
aMTD343
aMTD345
FITC
Nomarski
aMTD363
aMTD364
aMTD365
aMTD381
aMTD382
aMTD383
FITC
Nomarski
aMTD385
aMTD401
aMTD402
aMTD403
aMTD404
aMTD405
FITC
Nomarski
aMTD422
aMTD424
aMTD425
aMTD910
aMTD442
aMTD443
FITC
Nomarski

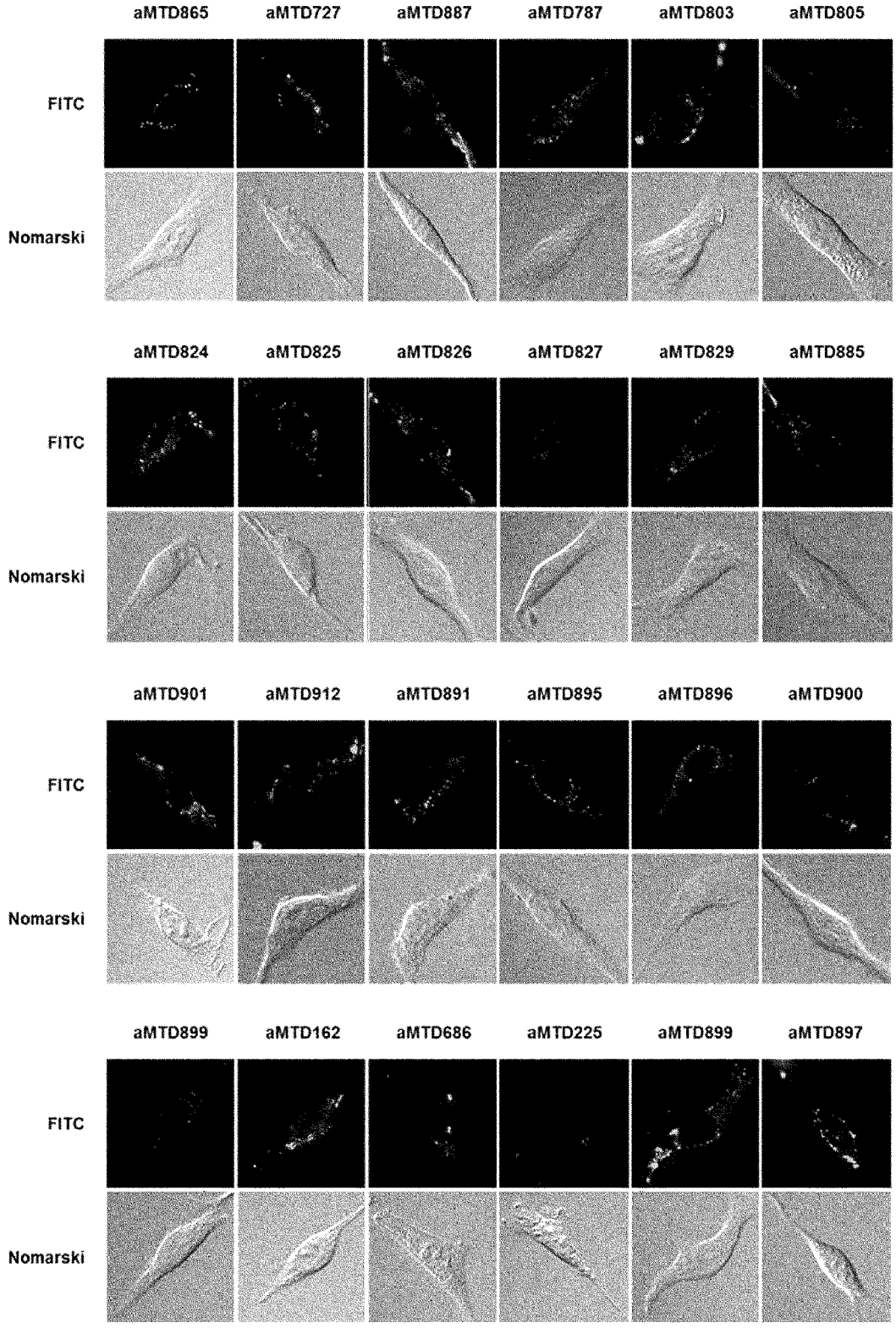
[Fig. 7e]


aMTD461
aMTD462
aMTD463
aMTD464
aMTD465
aMTD481
FITC
Nomarski
aMTD483
aMTD501
aMTD503
aMTD504
aMTD505
aMTD521
FITC
Nomarski
aMTD524
aMTD525
aMTD541
aMTD542
aMTD543
aMTD544
FITC
Nomarski
aMTD561
aMTD562
aMTD563
aMTD564
aMTD565
aMTD582
FITC
Nomarski

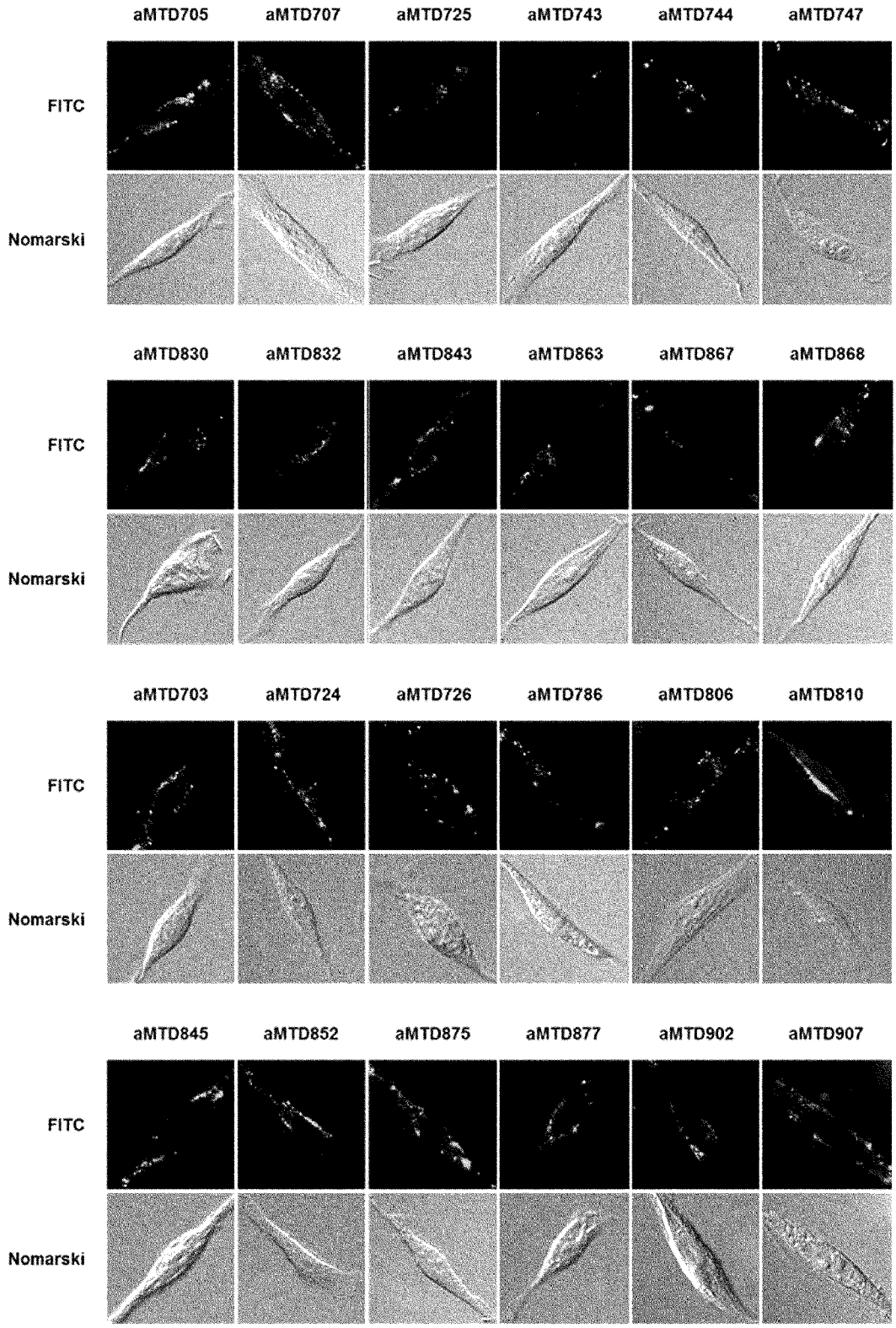
[Fig. 7f]


aMTD585
aMTD601
aMTD603
aMTD605
aMTD622
aMTD623
FITC
Nomarski
aMTD645
aMTD661
aMTD664
aMTD666
aMTD667
aMTD684
FITC
Nomarski
aMTD746
aMTD763
aMTD764
aMTD766
aMTD783
aMTD784
FITC
Nomarski
aMTD808
aMTD846
aMTD847
aMTD848
aMTD849
aMTD850
FITC
Nomarski

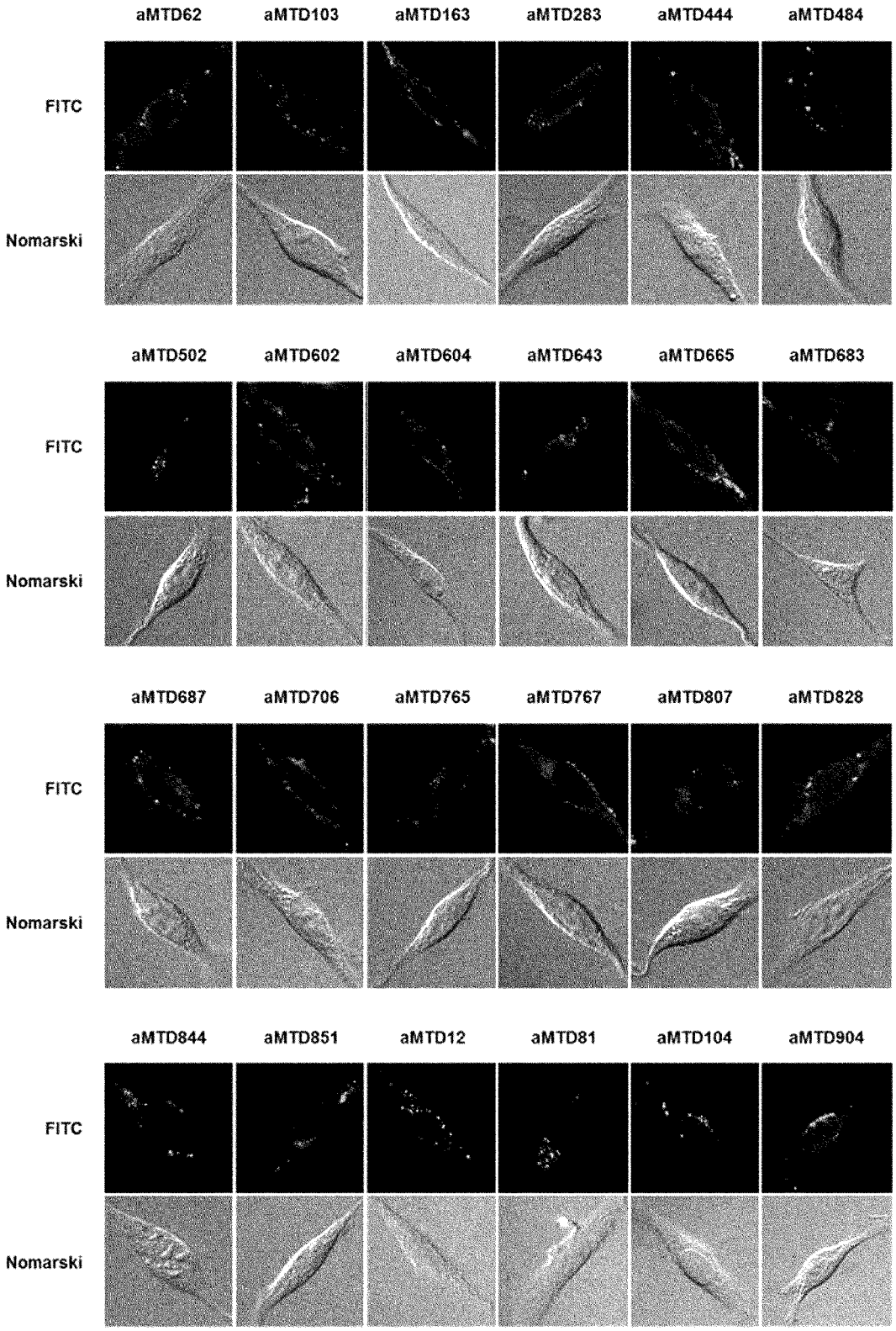
[Fig. 7g]


aMTD865
aMTD727
aMTD887
aMTD787
aMTD803
aMTD805
FITC
Nomarski
aMTD824
aMTD825
aMTD826
aMTD827
aMTD829
aMTD885
FITC
Nomarski
aMTD901
aMTD912
aMTD891
aMTD895
aMTD896
aMTD900
FITC
Nomarski
aMTD899
aMTD162
aMTD686
aMTD225
aMTD899
aMTD897
FITC
Nomarski

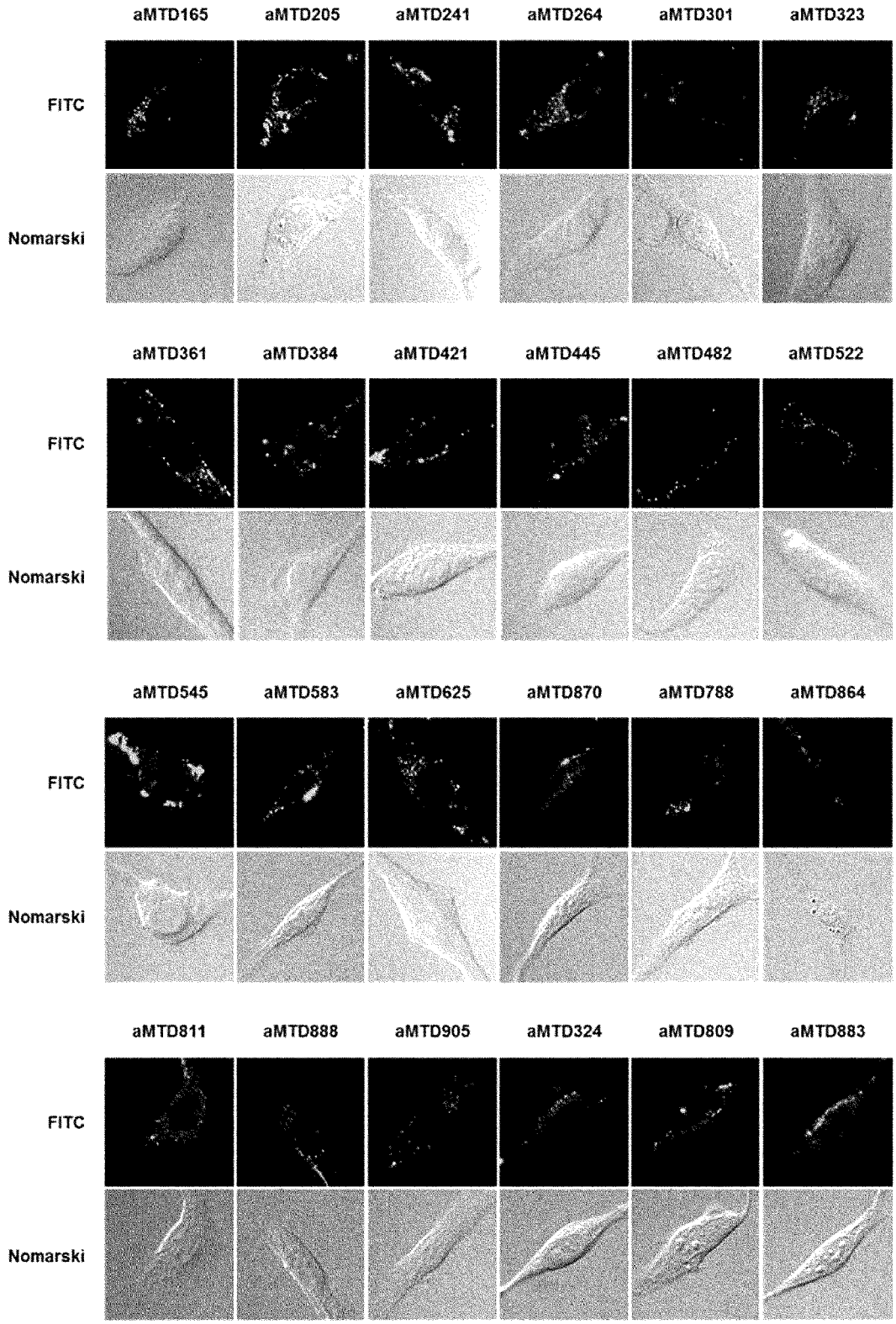
[Fig. 7h]


aMTD705
aMTD707
aMTD725
aMTD743
aMTD744
aMTD747
FITC
Nomarski
aMTD830
aMTD832
aMTD843
aMTD863
aMTD867
aMTD868
FITC
Nomarski
aMTD703
aMTD724
aMTD726
aMTD786
aMTD806
aMTD810
FITC
Nomarski
aMTD845
aMTD852
aMTD875
aMTD877
aMTD902
aMTD907
FITC
Nomarski

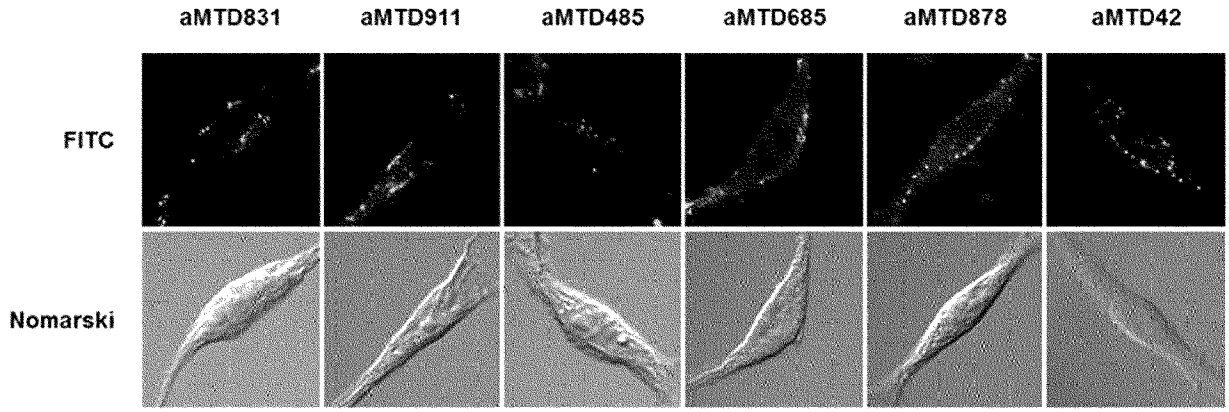
[Fig. 7i]


aMTD62
aMTD103
aMTD163
aMTD283
aMTD444
aMTD484
FITC
Nomarski
aMTD502
aMTD602
aMTD604
aMTD643
aMTD665
aMTD683
FITC
Nomarski
aMTD687
aMTD706
aMTD765
aMTD767
aMTD807
aMTD828
FITC
Nomarski
aMTD844
aMTD851
aMTD12
aMTD81
aMTD104
aMTD904
FITC
Nomarski

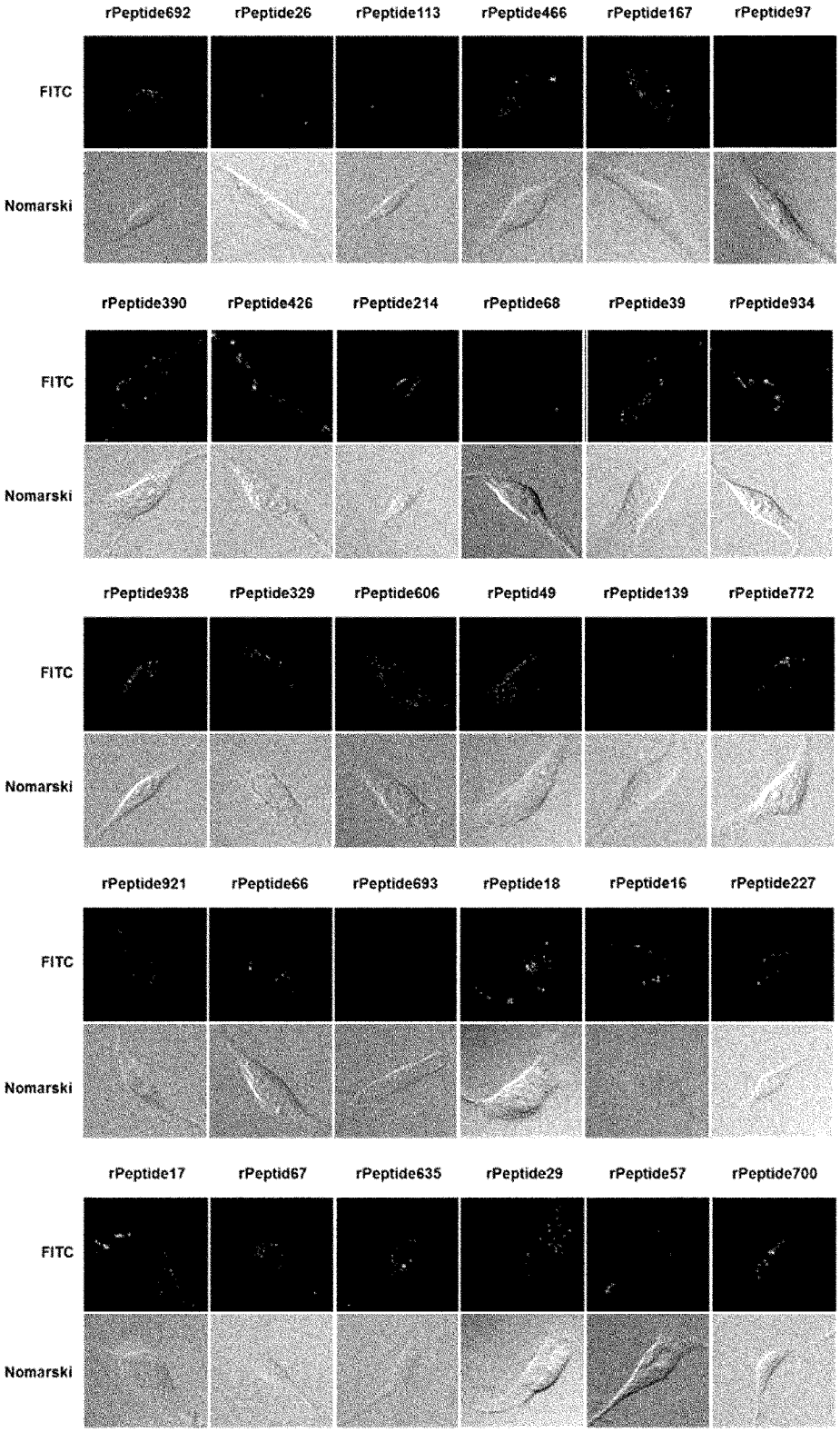
[Fig. 7j]


aMTD165
aMTD205
aMTD241
aMTD264
aMTD301
aMTD323
FITC
Nomarski
aMTD361
aMTD384
aMTD421
aMTD445
aMTD482
aMTD522
FITC
Nomarski
aMTD545
aMTD583
aMTD625
aMTD870
aMTD788
aMTD864
FITC
Nomarski
aMTD811
aMTD888
aMTD905
aMTD324
aMTD809
aMTD883
FITC
Nomarski

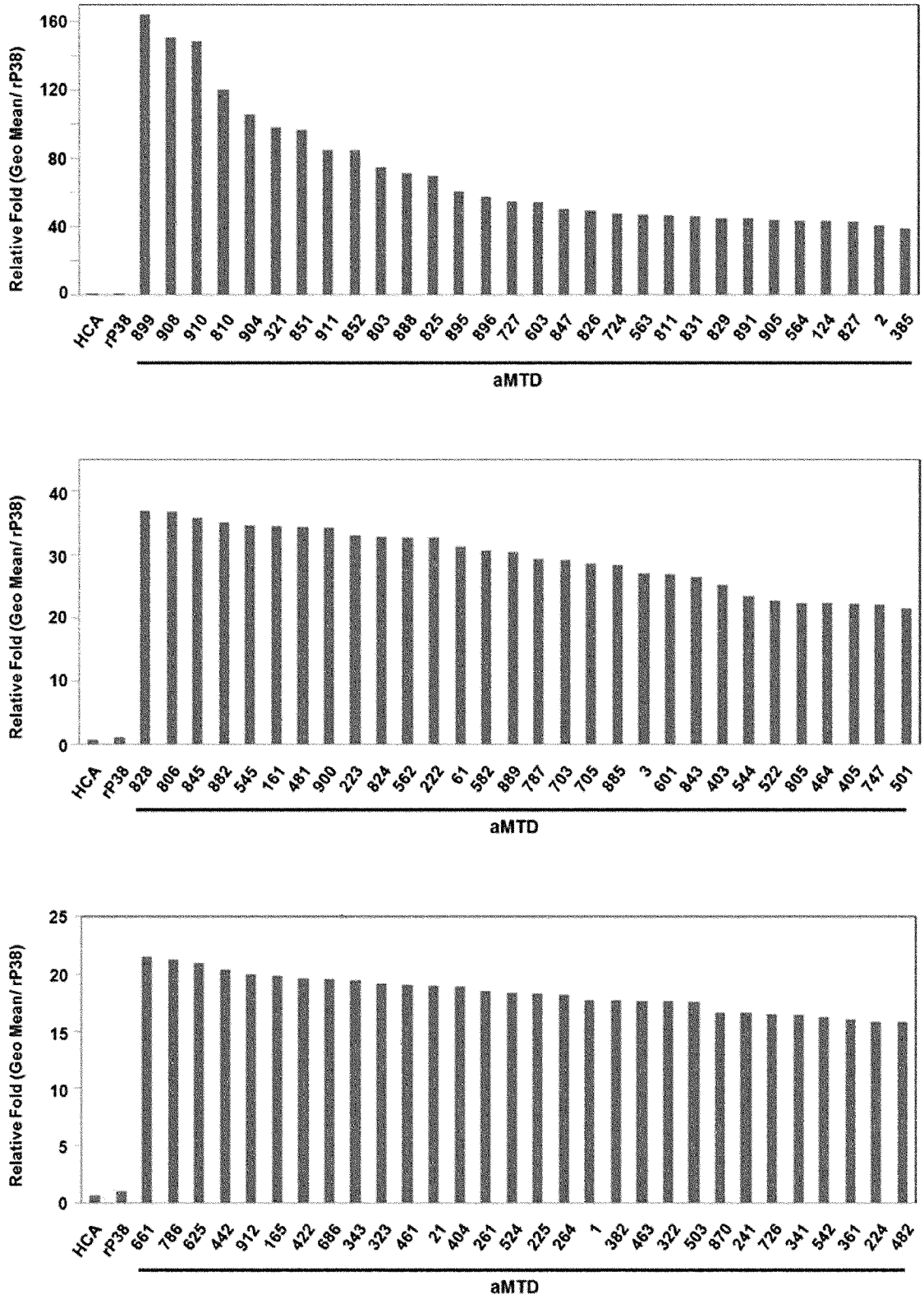
[Fig. 7k]


aMTD831
aMTD911
aMTD485
aMTD685
aMTD878
aMTD42
FITC
Nomarski

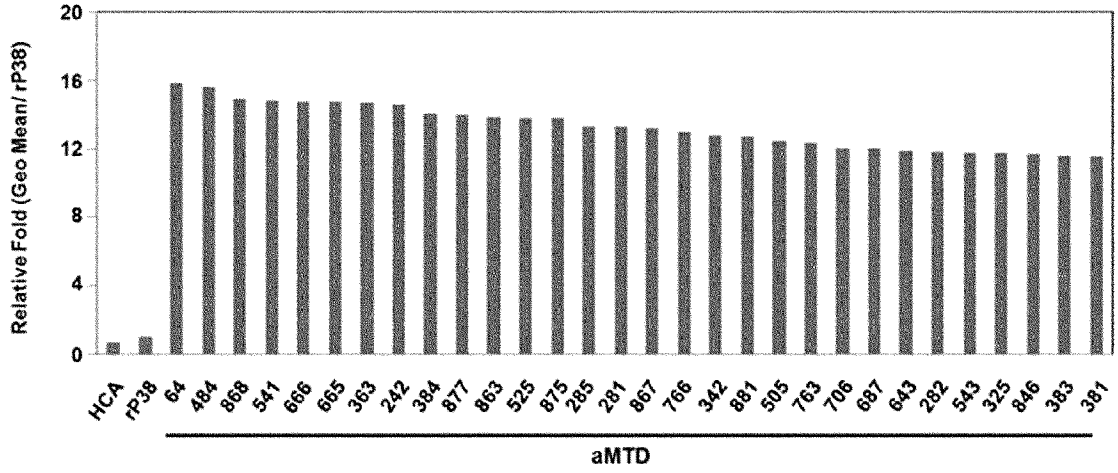
[Fig. 8]

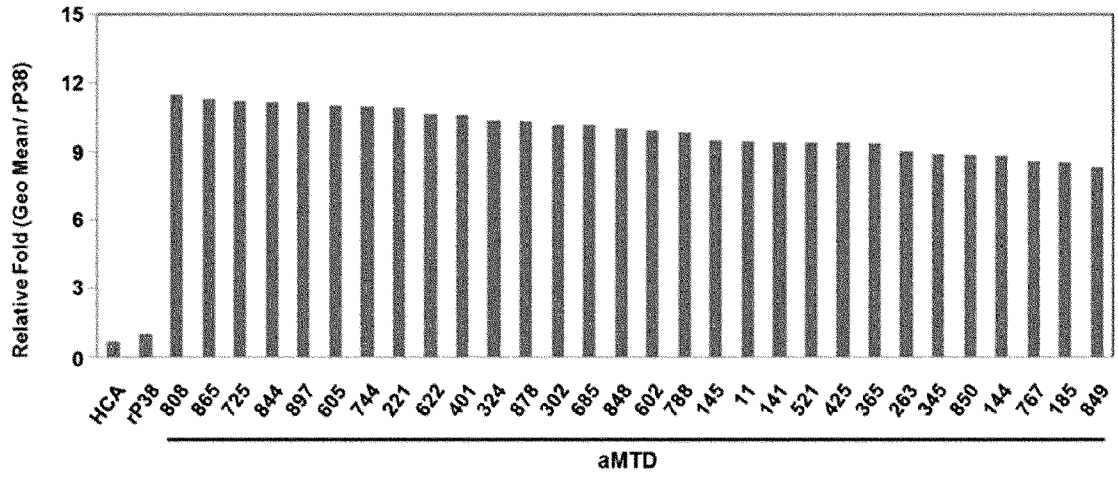

rPeptide692
rPeptide26
rPeptide113
rPeptide466
rPeptide167
rPeptide97
FITC
Nomarski
rPeptide390
rPeptide426
rPeptide214
rPeptide68
rPeptide39
rPeptide934
FITC
Nomarski
rPeptide938
rPeptide329
rPeptide606
rPeptid49
rPeptide139
rPeptide772
FITC
Nomarski
rPeptide921
rPeptide66
rPeptide693
rPeptide18
rPeptide16
rPeptide227
FITC
Nomarski
rPeptide17
rPeptid67
rPeptide635
rPeptide29
rPeptide57
rPeptide700
FITC
Nomarski

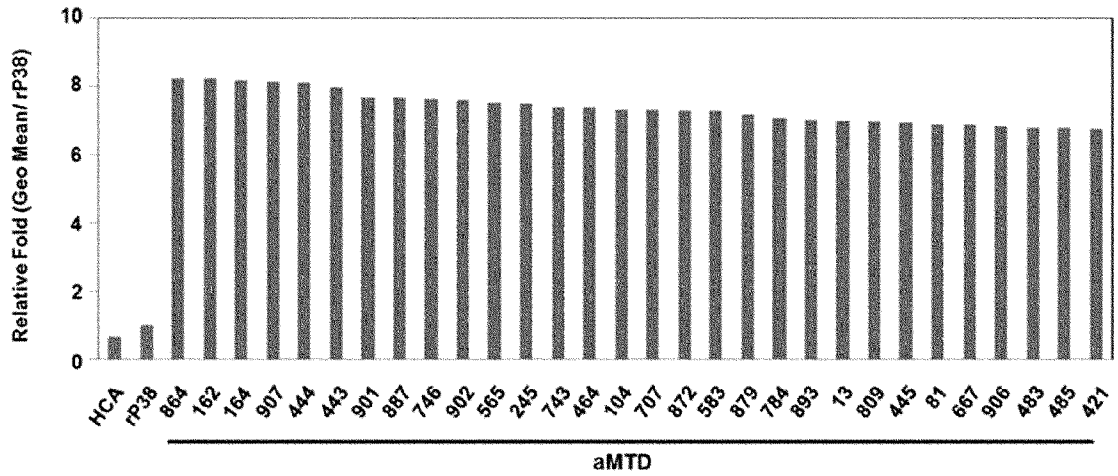
[Fig. 9a]


Relative Fold (Geo Mean/ rP38)
160
120
80
40
0
HCA
rP38
899
908
910
810
904
321
851
911
852
803
888
825
895
896
727
603
847
826
724
563
811
831
829
891
905
564
124
827
2
385
aMTD
Relative Fold (Geo Mean/ rP38)
40
30
20
10
0
HCA
rP38
828
806
845
882
545
161
481
900
223
824
562
222
61
582
889
787
703
705
885
3
601
843
403
544
522
805
464
405
747
501
aMTD
Relative Fold (Geo Mean/ rP38)
25
20
15
10
5
0
HCA
rP38
661
786
625
442
912
165
422
686
343
323
461
21
404
261
524
225
264
1
382
463
322
503
870
241
726
341
542
361
224
482
aMTD

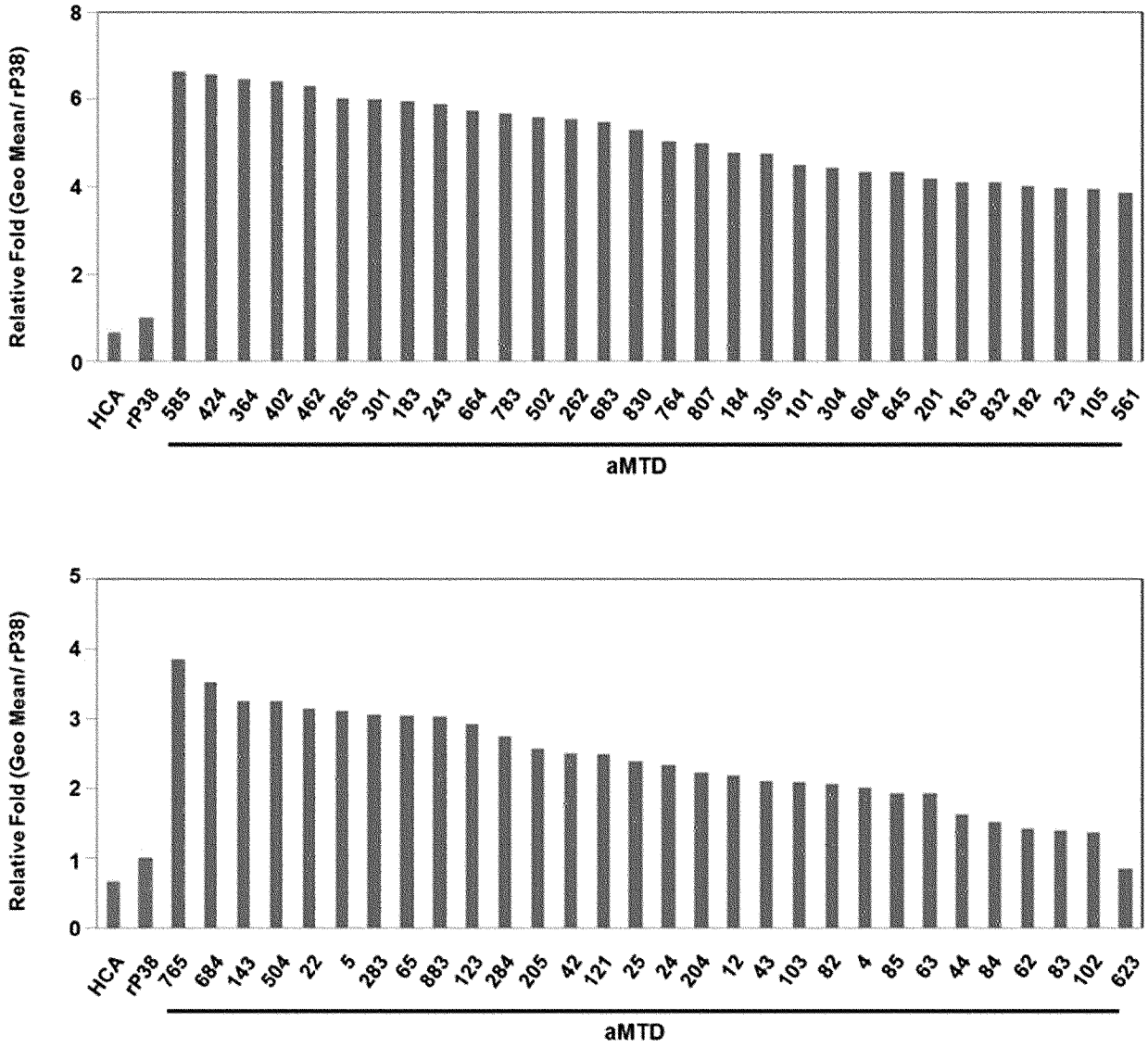
[Fig. 9b]


Relative Fold (Geo Mean/ rP38)
20
16
12
8
4
0
HCA
rP38
64
484
868
541
666
665
363
242
384
877
863
525
875
285
281
867
766
342
881
505
763
706
687
643
282
543
325
846
383
381
aMTD

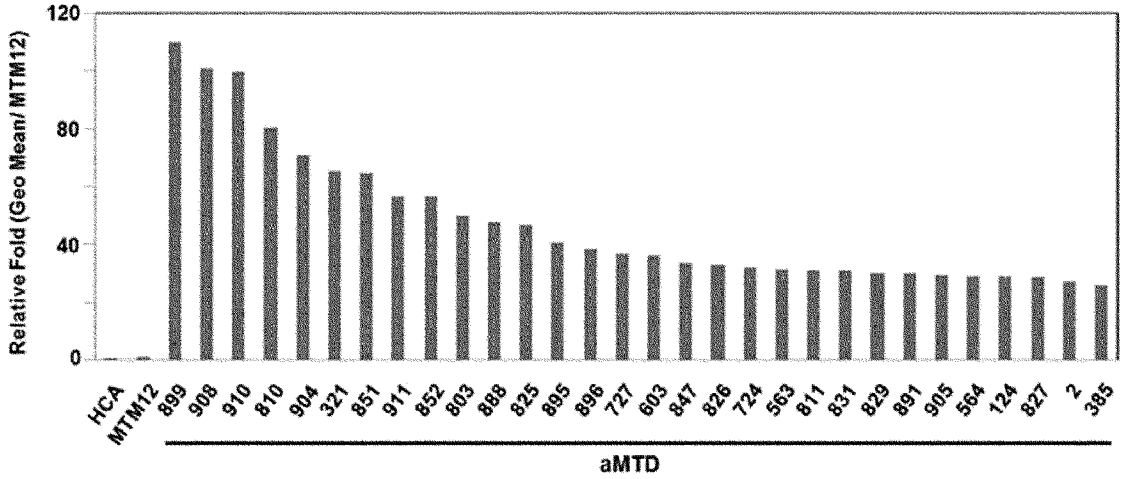

Relative Fold (Geo Mean/ rP38)
15
12
9
6
3
0
HCA
rP38
808
865
725
844
897
605
744
221
622
401
324
878
302
685
848
602
788
145
11
141
521
425
365
263
345
850
144
767
185
849
aMTD

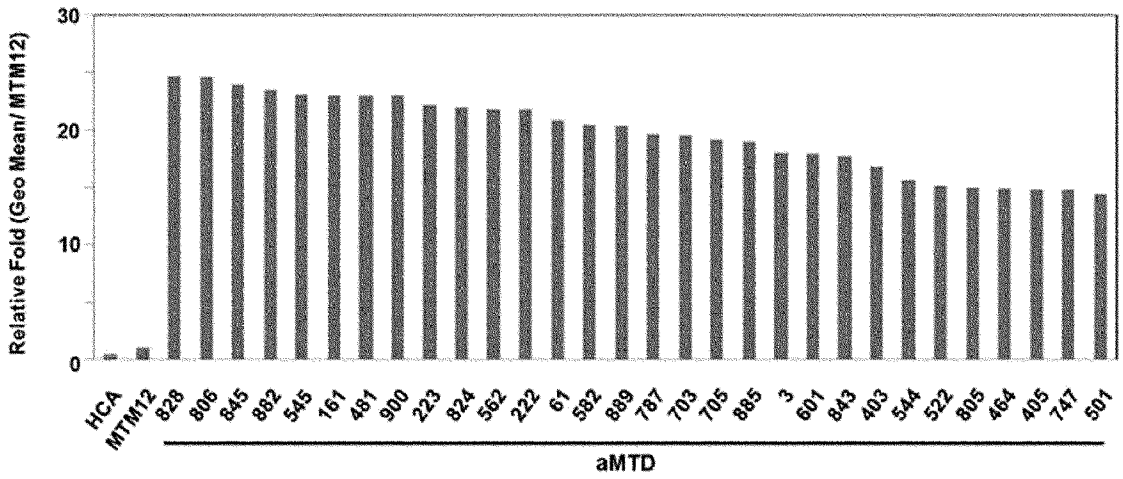

Relative Fold (Geo Mean/ rP38)
10
8
6
4
2
0
HCA
rP38
864
162
164
907
444
443
901
887
746
902
565
245
743
464
104
707
872
583
879
784
893
13
809
445
81
667
906
483
485
421
aMTD

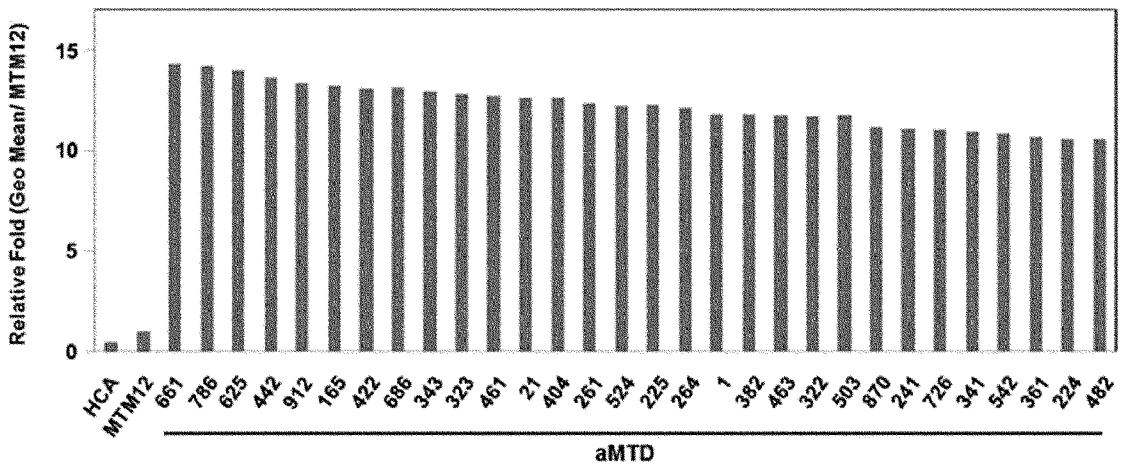
[Fig. 9c]


Relative Fold (Geo Mean/ rP38)
8
6
4
2
0
HCA
rP38
585
424
364
402
462
265
301
183
243
664
783
502
262
683
830
764
807
184
305
101
304
604
645
201
163
832
182
23
105
561
aMTD
Relative Fold (Geo Mean/ rP38)
5
4
3
2
1
0
HCA
rP38
765
684
143
504
22
5
283
65
883
123
284
205
42
121
25
24
204
12
43
103
82
4
85
63
44
84
62
83
102
623
aMTD

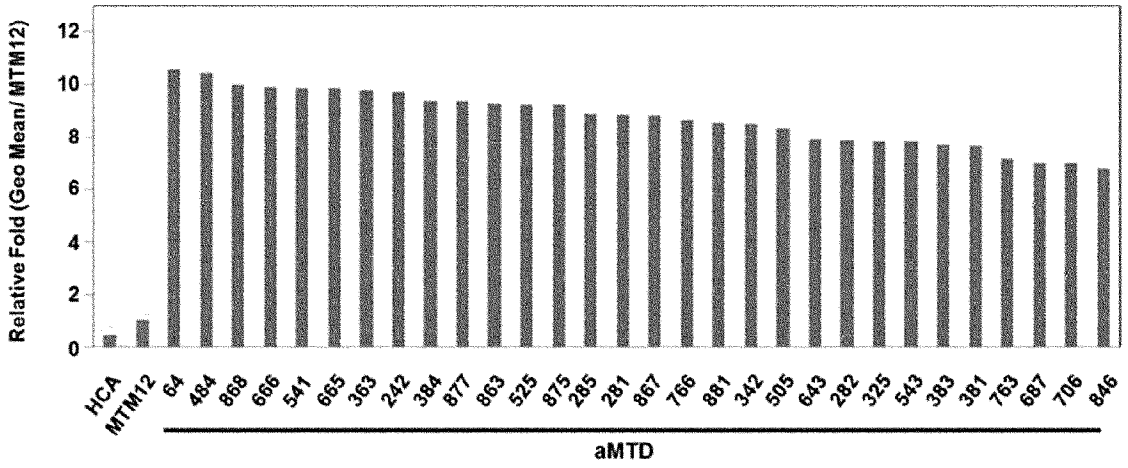
[Fig. 10a]

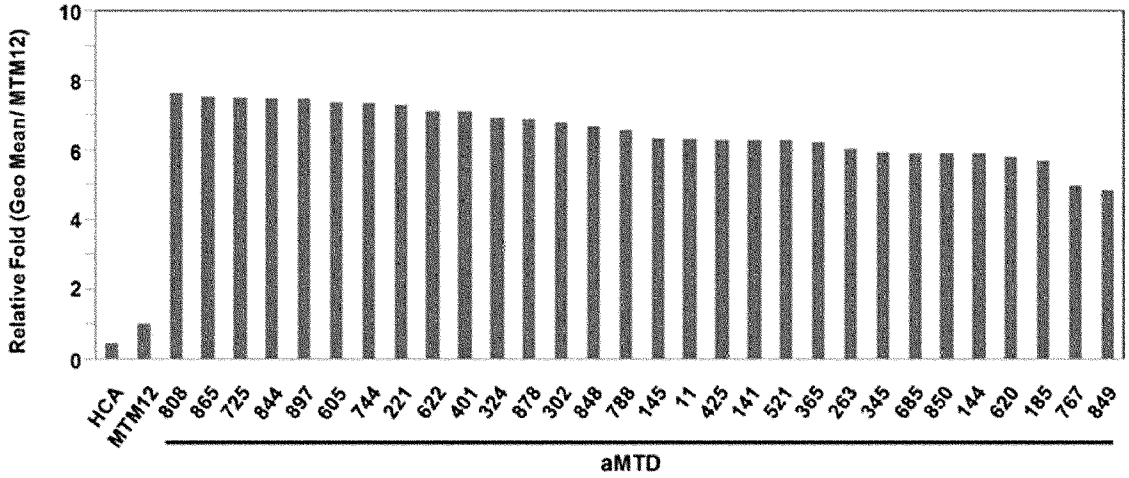

Relative Fold (Geo Mean/ MTM12)
120
80
40
0
HCA
MTM12
899
908
910
810
904
321
851
911
852
803
888
825
895
896
727
603
847
826
724
563
811
831
829
891
905
564
124
827
2
385
aMTD

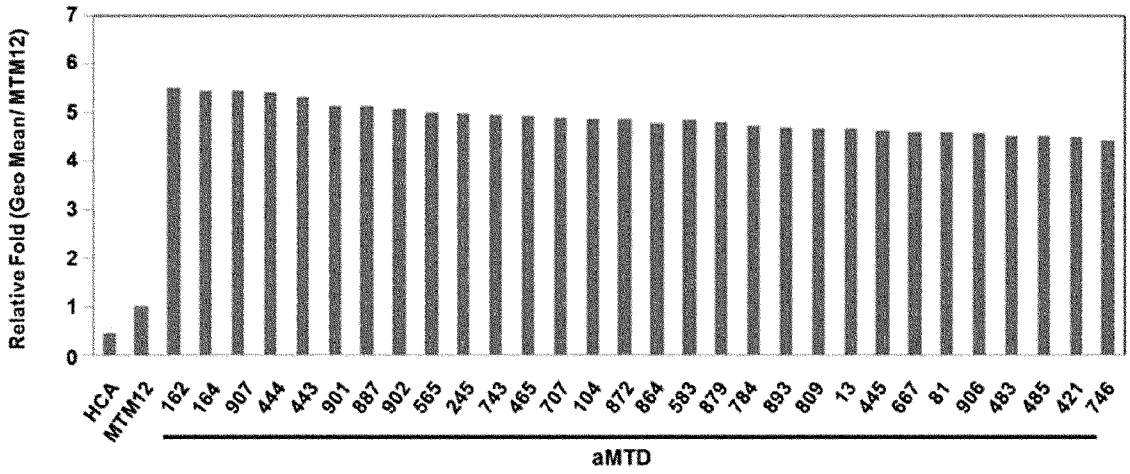

Relative Fold (Geo Mean/ MTM12)
30
20
10
0
HCA
MTM12
828
806
845
882
545
161
481
900
223
824
562
222
61
582
889
787
703
705
885
3
601
843
403
544
522
805
464
405
747
501
aMTD


Relative Fold (Geo Mean/ MTM12)
15
10
5
0
HCA
MTM12
661
786
625
442
912
165
422
686
343
323
461
21
404
251
524
225
264
1
382
463
322
503
870
241
726
341
542
361
224
482
aMTD

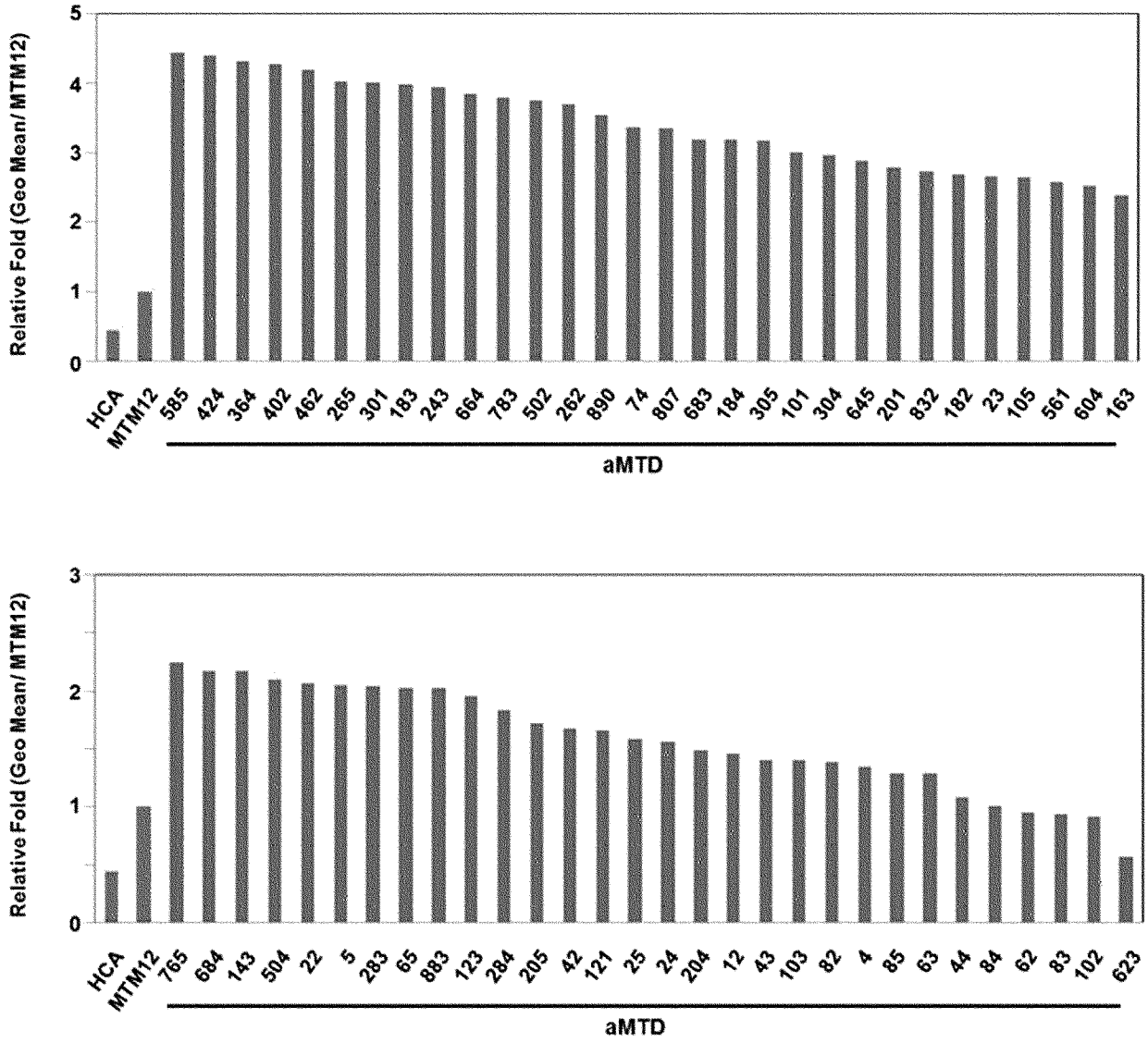
[Fig. 10b]


Relative Fold (Geo Mean/ MTM12)
12
10
8
6
4
2
0
HCA
MTM12
64
484
868
666
541
665
363
242
384
877
863
525
875
285
281
867
766
881
342
505
643
282
325
543
383
381
763
687
706
846
aMTD

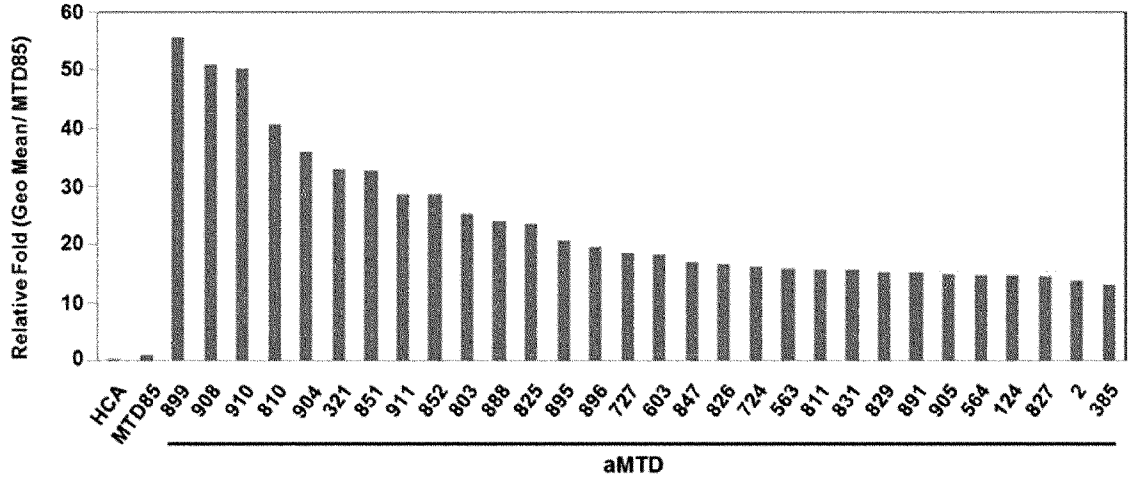

Relative Fold (Geo Mean/ MTM12)
10
8
6
4
2
0
HCA
MTM12
808
865
725
844
897
605
744
221
622
401
324
878
302
848
788
145
11
425
141
521
365
263
345
685
850
144
620
185
767
849
aMTD

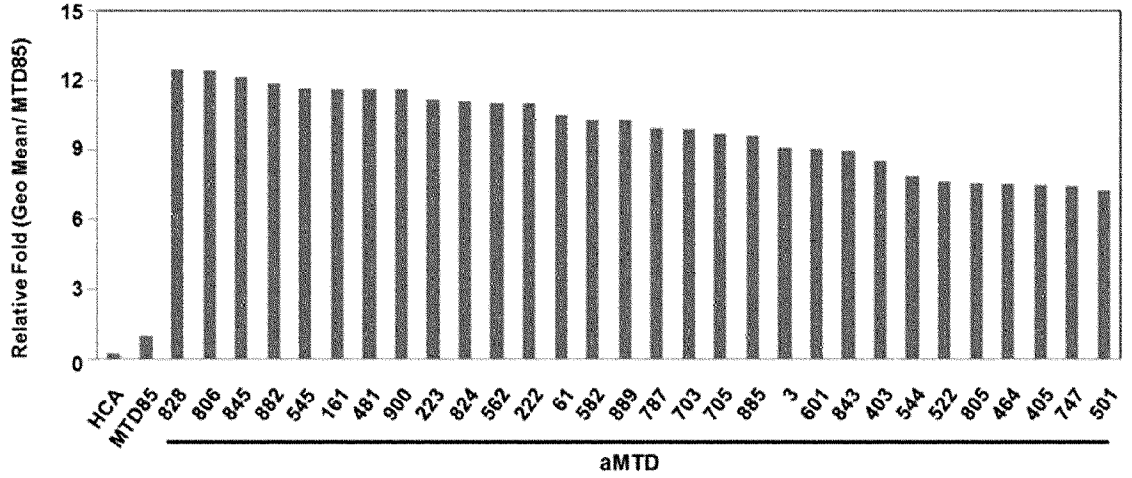

Relative Fold (Geo Mean/ MTM12)
7
6
5
4
3
2
1
0
HCA
MTM12
162
164
907
444
443
901
887
902
565
245
743
465
707
104
872
864
583
879
784
893
809
13
445
667
81
906
483
485
421
746
aMTD

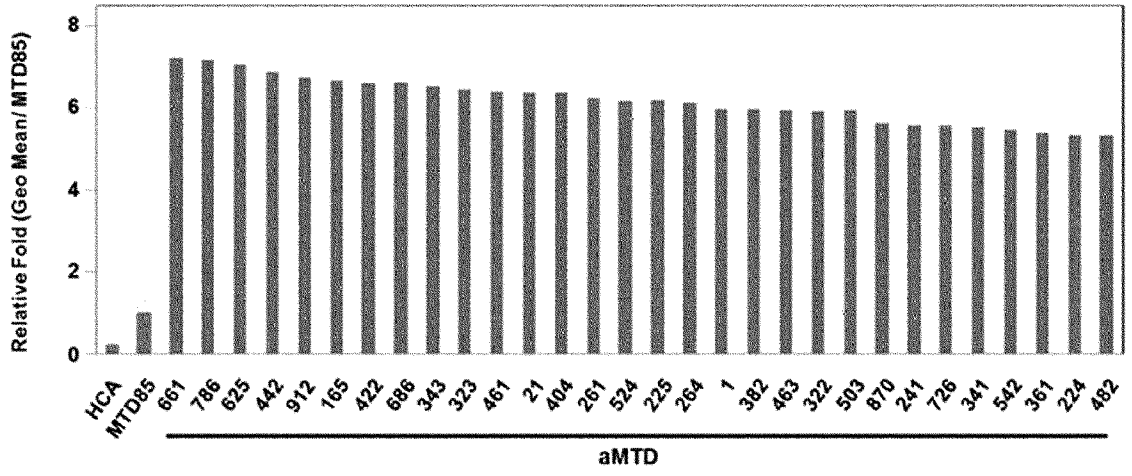
[Fig. 10c]


Relative Fold (Geo Mean/ MTM12)
5
4
3
2
1
0
HCA
MTM12
585
424
364
402
462
265
301
183
243
664
783
502
252
890
74
807
683
184
305
101
304
645
201
832
182
23
105
561
604
163
aMTD
Relative Fold (Geo Mean/ MTM12)
3
2
1
0
HCA
MTM12
765
684
143
504
22
5
283
65
883
123
284
205
42
121
25
24
204
12
43
103
82
4
85
63
44
84
62
83
102
623
aMTD

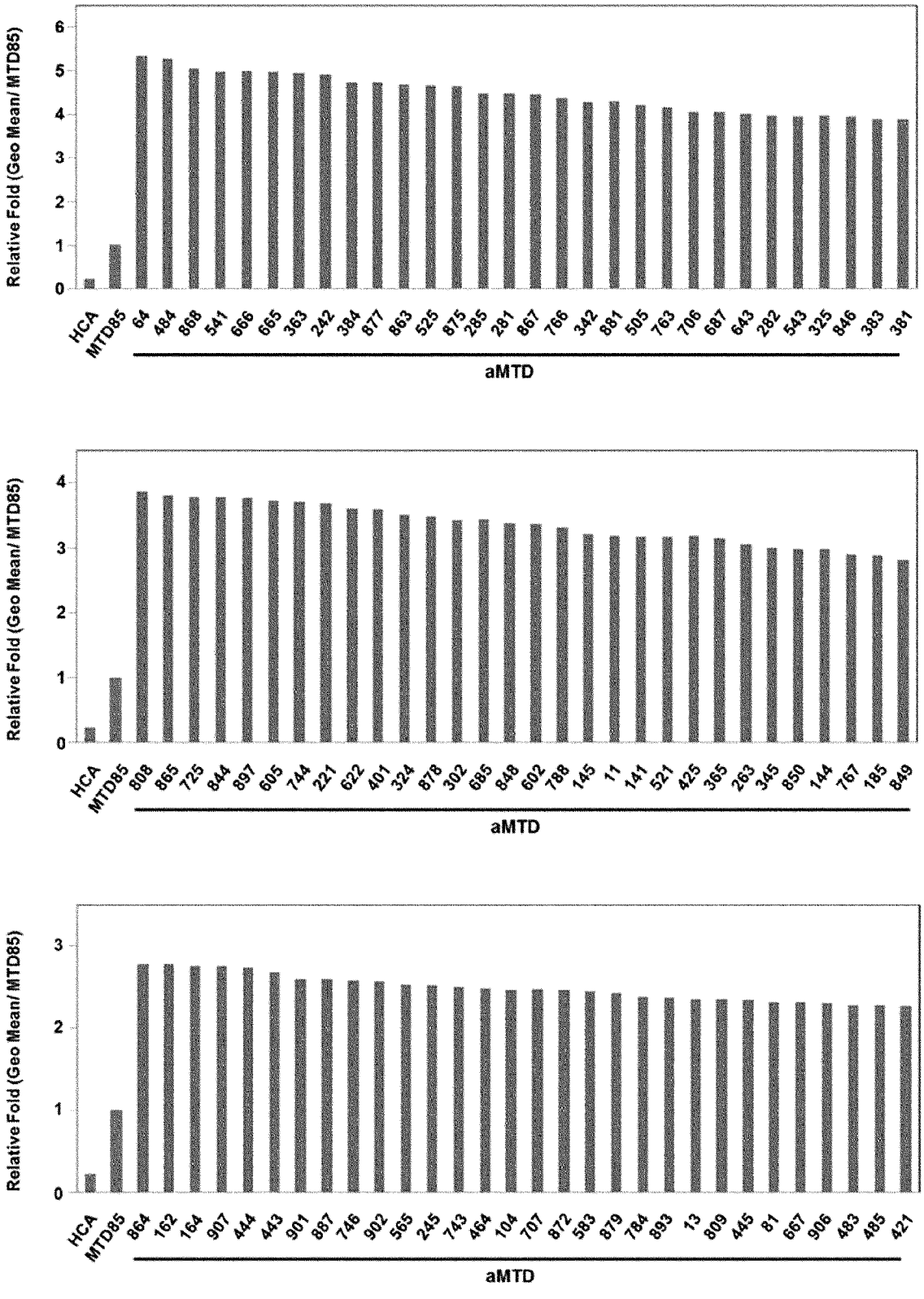
[Fig. 11a]


Relative Fold (Geo Mean/ MTD85)
60
50
40
30
20
10
0
HCA
MTD85
899
908
910
810
904
321
851
911
852
803
888
825
895
896
727
603
847
826
724
563
811
831
829
891
905
564
124
827
2
385
aMTD

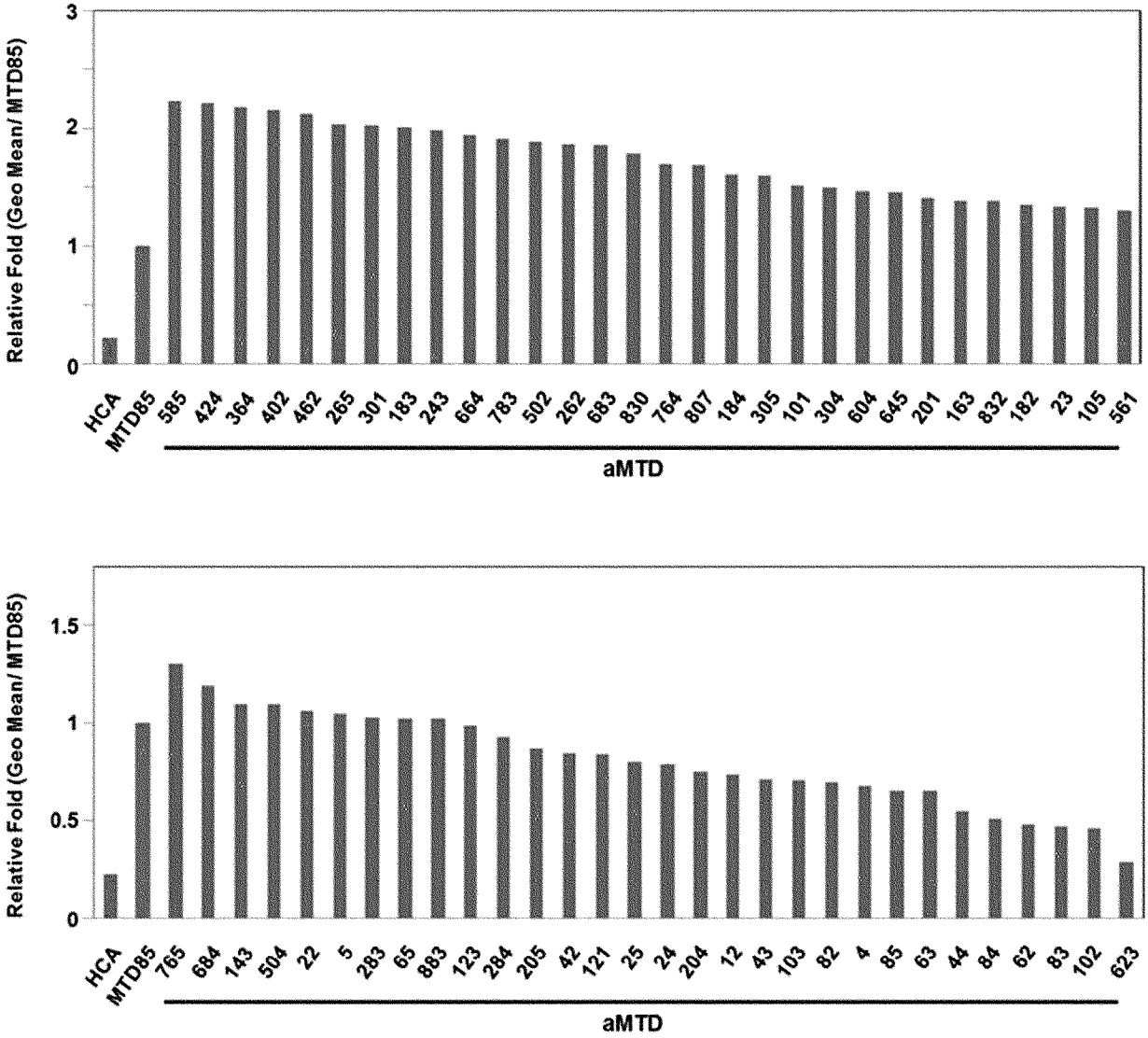

Relative Fold (Geo Mean/ MTD85)
15
12
9
6
3
0
HCA
MTD85
828
806
845
882
545
161
481
900
223
824
562
222
61
582
889
787
703
705
885
3
601
843
403
544
522
805
464
405
747
501
aMTD


Relative Fold (Geo Mean/ MTD85)
8
6
4
2
0
HCA
MTD85
661
786
625
442
912
165
422
686
343
323
461
21
404
261
524
225
264
1
382
463
322
503
870
241
726
341
542
361
224
482
aMTD

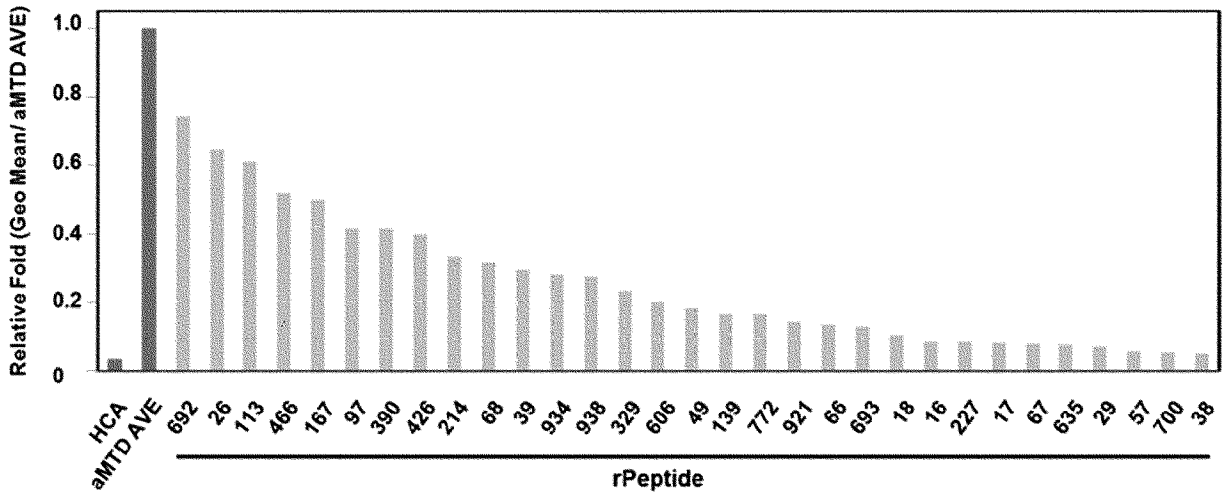
[Fig. 11b]


Relative Fold (Geo Mean/ MTD85)
0
1
2
3
4
5
6
HCA
MTD85
64
484
868
541
666
655
363
242
384
877
863
525
875
285
281
867
766
342
881
505
763
706
687
643
282
543
325
846
383
381
aMTD
Relative Fold (Geo Mean/ MTD85)
0
1
2
3
4
HCA
MTD85
808
865
725
844
897
605
744
221
622
401
324
878
302
685
848
602
788
145
11
141
521
425
365
263
345
850
144
767
185
849
aMTD
Relative Fold (Geo Mean/ MTD85)
0
1
2
3
HCA
MTD85
864
162
164
907
444
443
901
887
746
902
565
245
743
464
104
707
872
583
879
784
893
13
809
445
81
667
906
483
485
421
aMTD

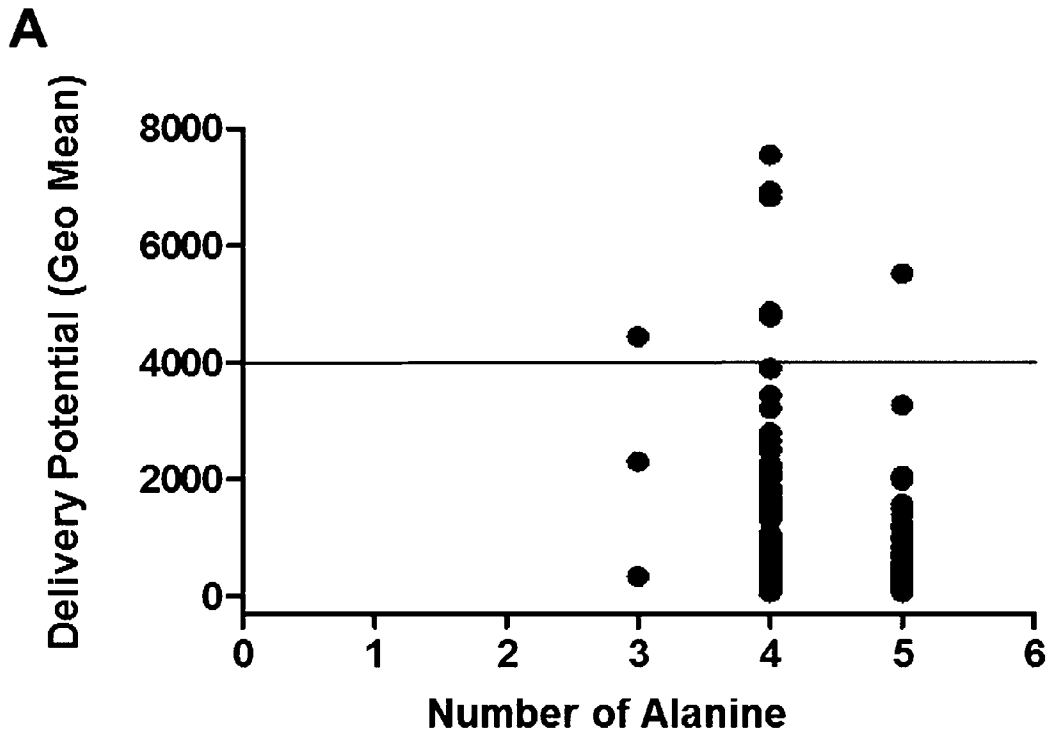
[Fig. 11c]

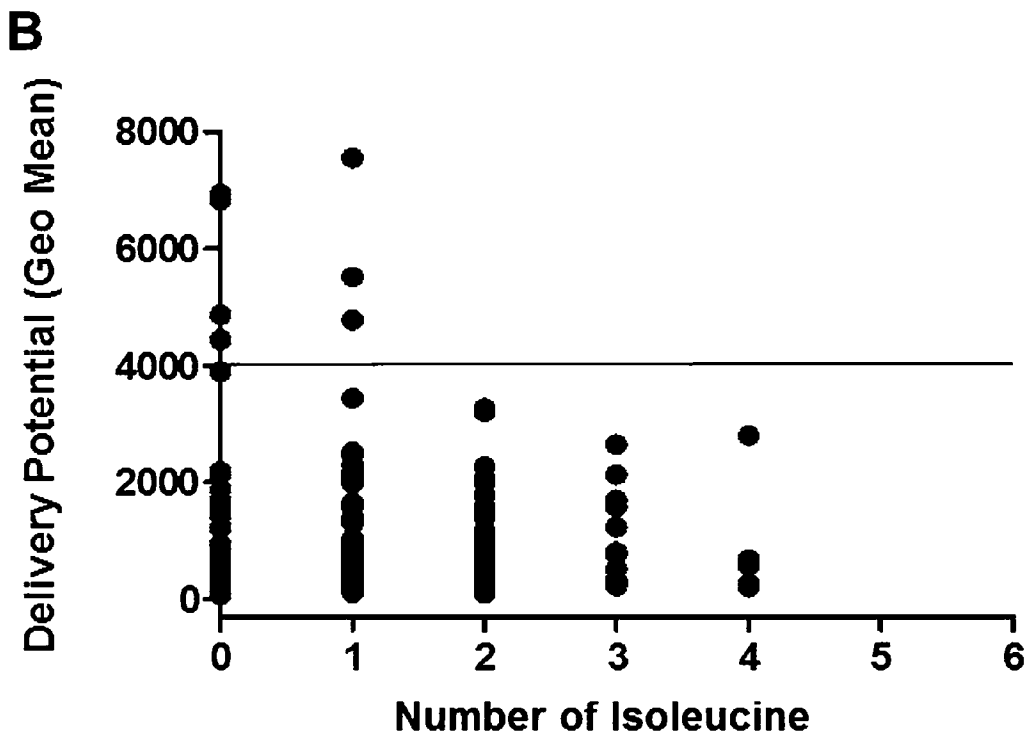


[Fig. 12]

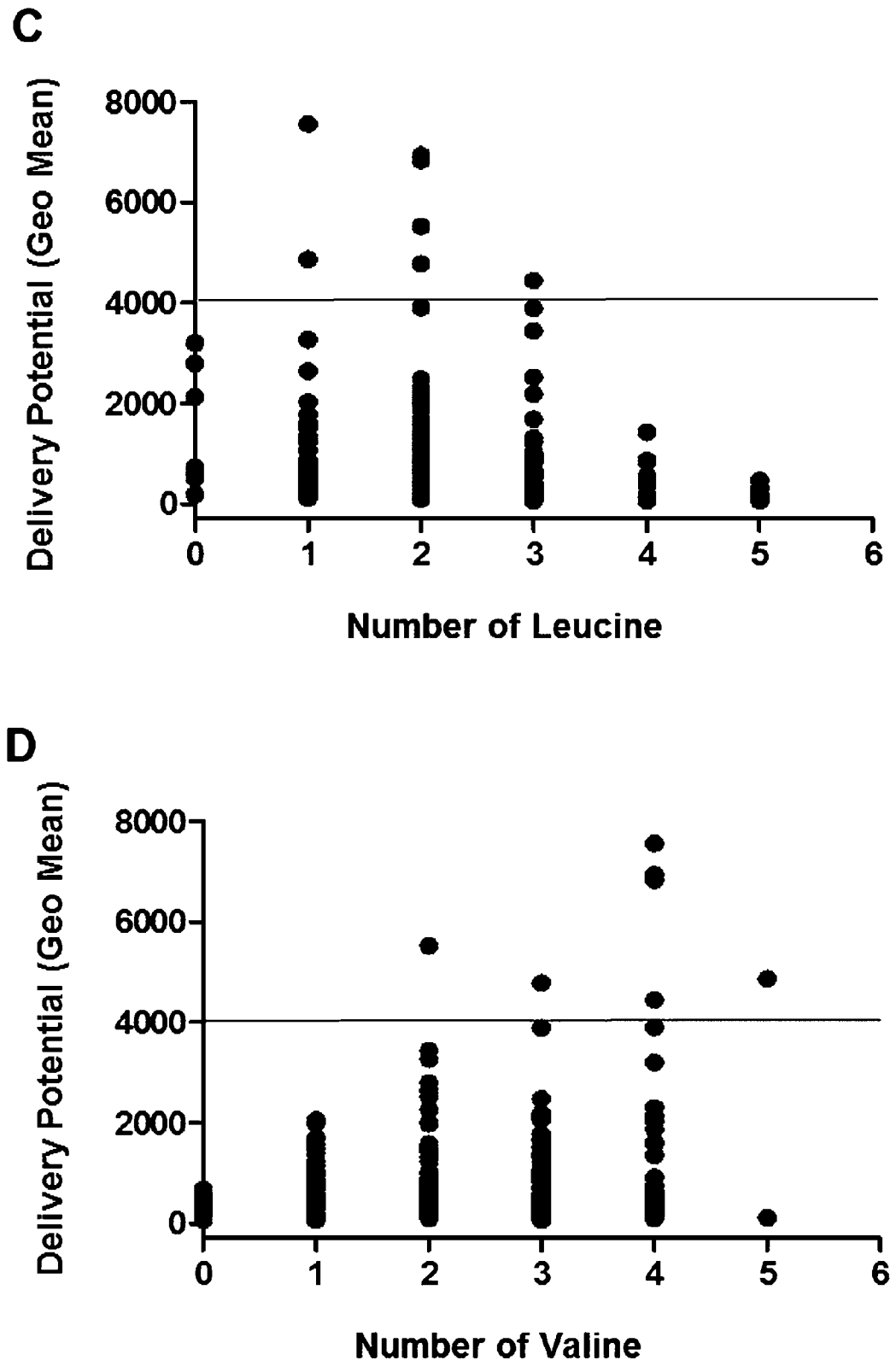


[Fig. 13a]

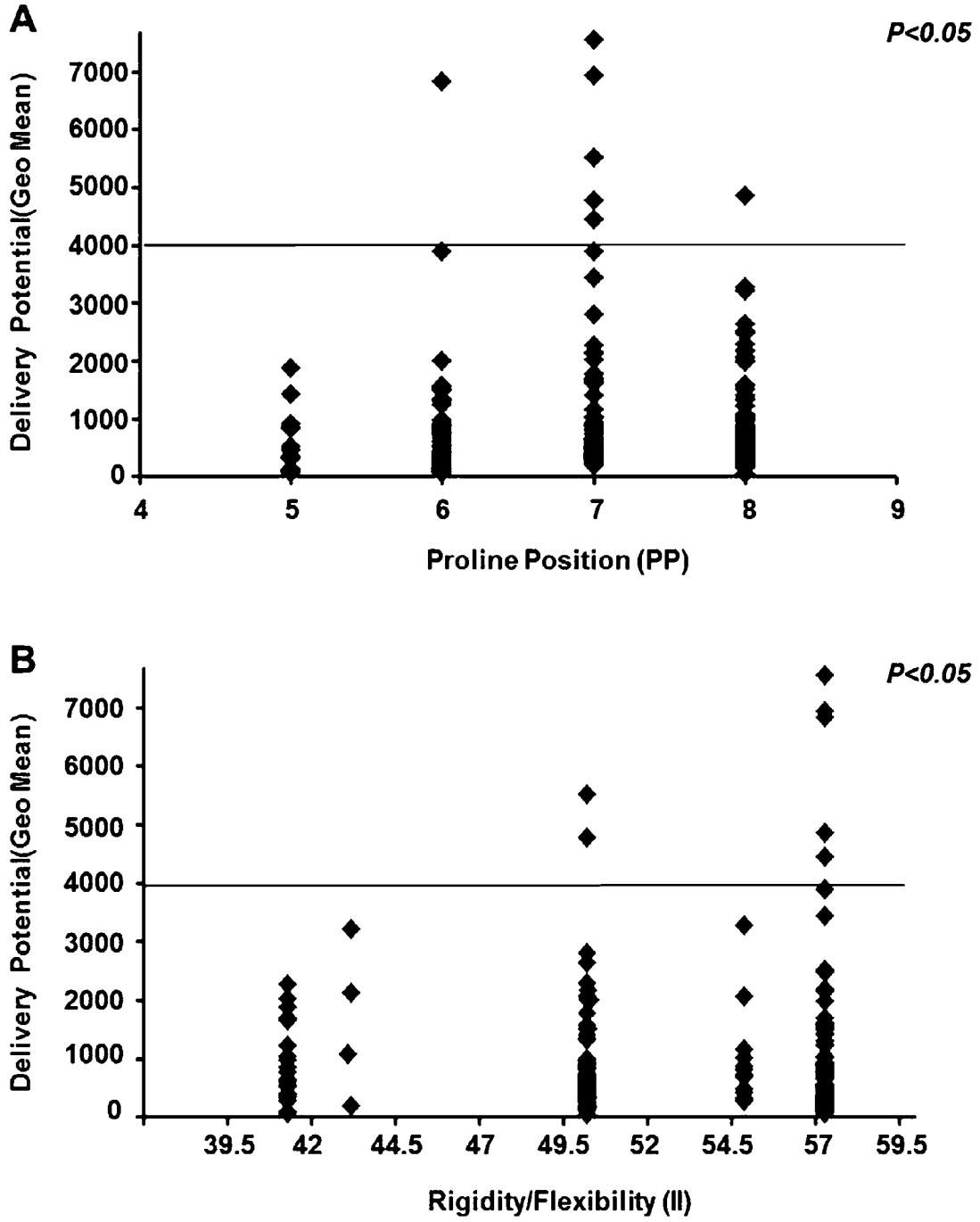

A
Delivery Potential (Geo Mean)
8000
6000
4000
2000
0
0
1
2
3
4
5
6
Number of Alanine


B
Delivery Potential (Geo Mean)
8000
6000
4000
2000
0
0
1
2
3
4
5
6
Number of Isoleucine

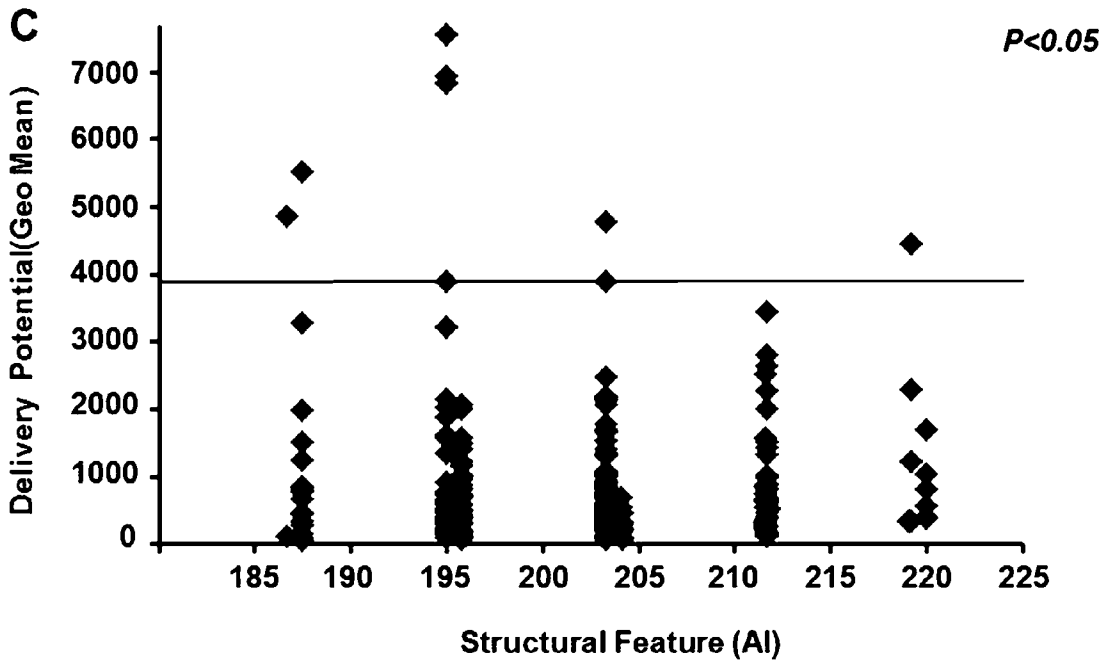
[Fig. 13b]

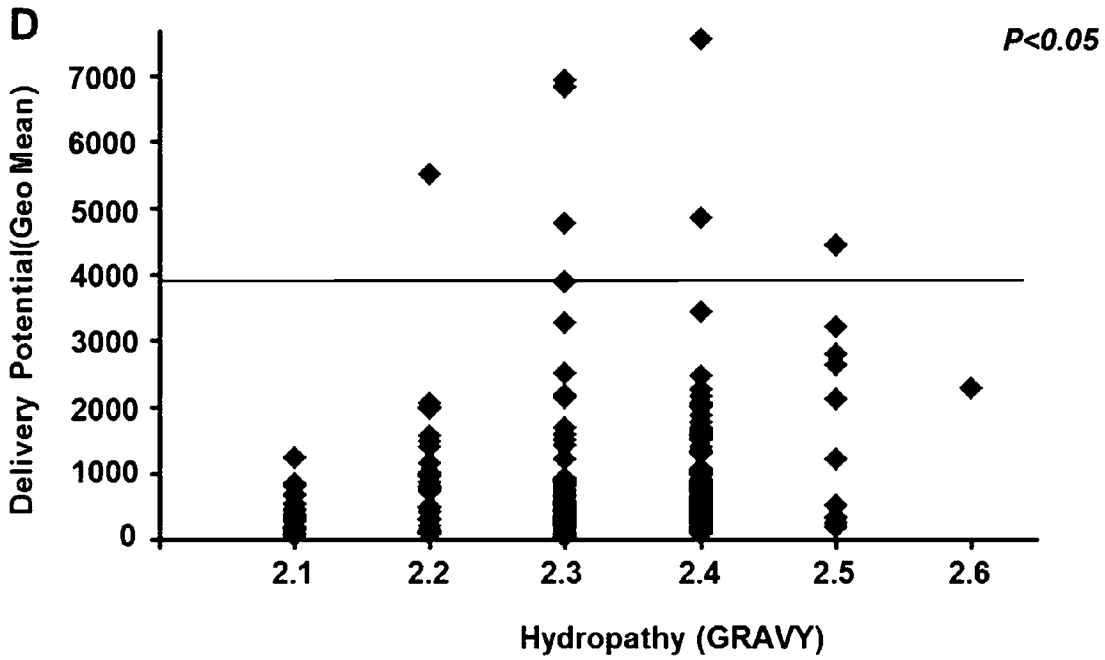

C
Delivery Potential (Geo Mean)
8000
6000
4000
2000
0
0
1
2
3
4
5
6
Number of Leucine
D
Delivery Potential (Geo Mean)
8000
6000
4000
2000
0
0
1
2
3
4
5
6
Number of Valine

[Fig. 14a]


A
P<0.05
Delivery Potential(Geo Mean)
7000
6000
5000
4000
3000
2000
1000
0
4
5
6
7
8
9
Proline Position (PP)
B
P<0.05
Delivery Potential(Geo Mean)
7000
6000
5000
4000
3000
2000
1000
0
39.5
42
44.5
47
49.5
52
54.5
57
59.5
Rigidity/Flexibility (II)

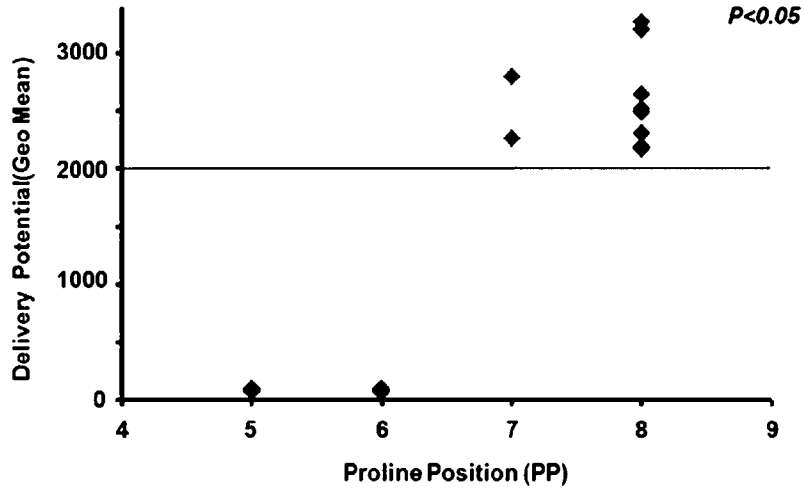
[Fig. 14b]


C
P<0.05
Delivery Potential(Geo Mean)
7000
6000
5000
4000
3000
2000
1000
0
185
190
195
200
205
210
215
220
225
Structural Feature (AI)


D
P<0.05
Delivery Potential(Geo Mean)
7000
6000
5000
4000
3000
2000
1000
0
2.1
2.2
2.3
2.4
2.5
2.6
Hydropathy (GRAVY)

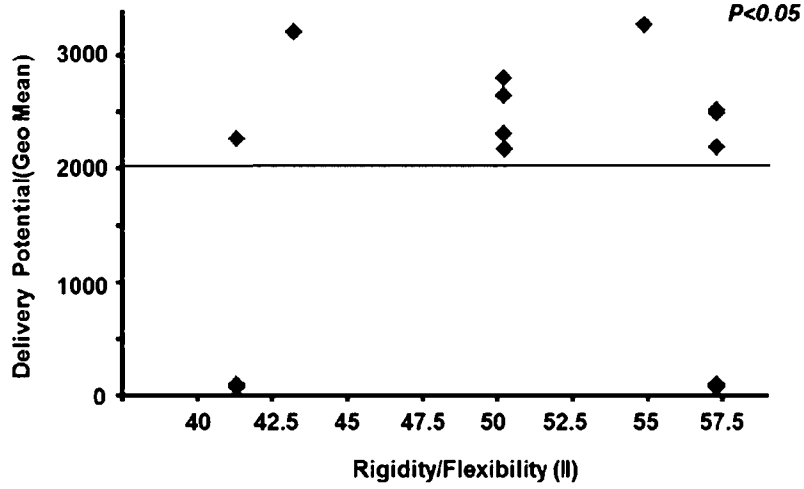
[Fig. 15a]

A



ID	Proline Position (PP)	Delivery Potential (DP)
888	8	3266
825	8	3204
895	7	2795
896	8	2645
727	8	2518
603	8	2491
847	8	2307
826	7	2262
724	8	2186
563	8	2167
43	5	97
103	6	97
4	6	93
85	5	89
63	6	89
44	5	75
84	6	70
62	6	65
83	6	64
102	5	63

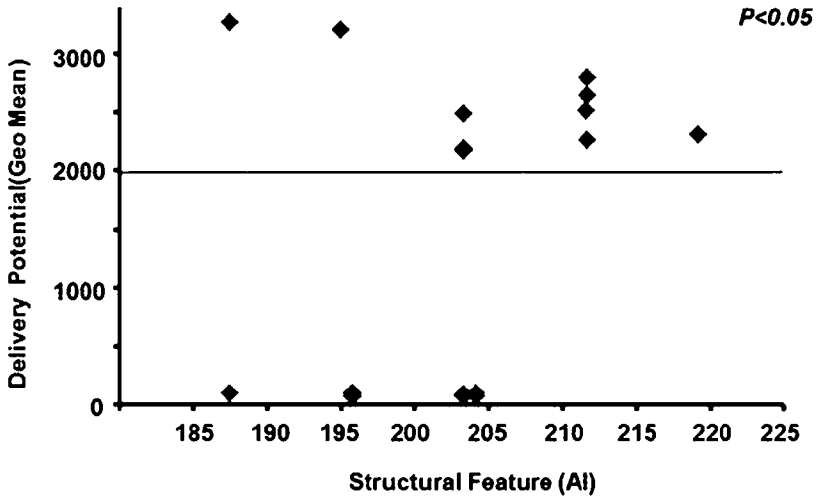
B



ID	Rigidity/ Flexibility (II)	Delivery Potential (DP)
888	54.9	3266
825	43.2	3204
895	50.2	2795
896	50.2	2645
727	57.3	2518
603	57.3	2491
847	50.2	2307
826	41.3	2262
724	57.3	2186
563	50.2	2167
43	41.3	97
103	57.3	97
4	57.3	93
85	57.3	89
63	57.3	89
44	57.3	75
84	41.3	70
62	57.3	65
83	41.3	64
102	57.3	63

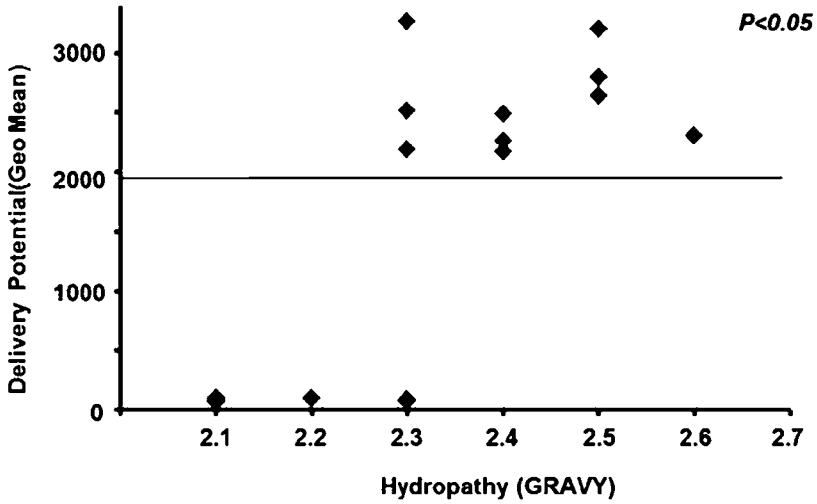
[Fig. 15b]

C



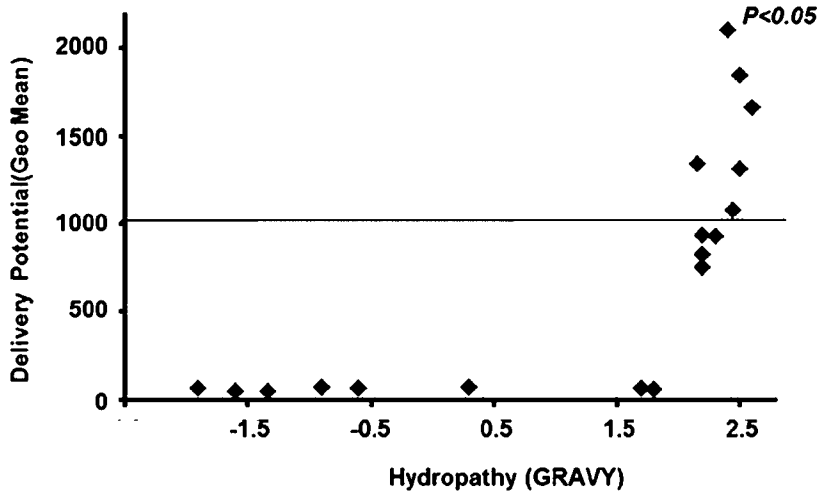
ID	Structural Feature (AI)	Delivery Potential (DP)
888	187.5	3266
825	195.0	3204
895	211.7	2795
896	211.7	2645
727	211.6	2518
603	203.3	2491
847	219.2	2307
826	211.7	2262
724	203.3	2186
563	203.3	2167
43	187.5	97
103	204.2	97
4	195.8	93
85	195.8	89
63	203.3	89
44	203.3	75
84	195.8	70
62	203.3	65
83	195.8	64
102	204.2	63

D



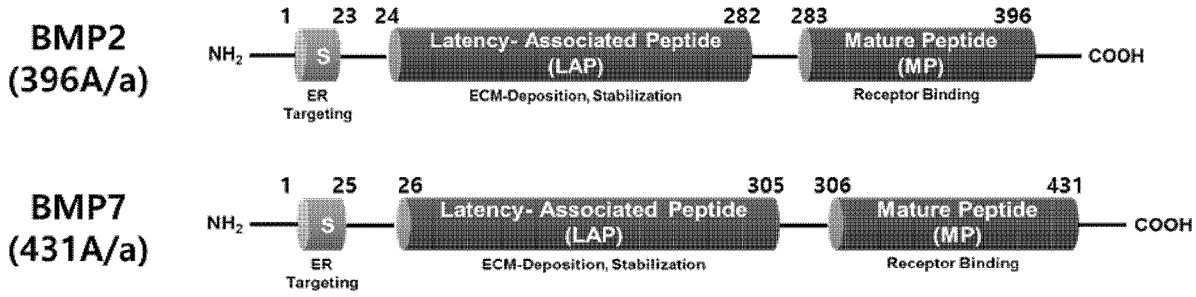
ID	Hydropathy (GRAVY)	Delivery Potential (DP)
888	2.3	3266
825	2.5	3204
895	2.5	2795
896	2.5	2645
727	2.3	2518
603	2.4	2491
847	2.6	2307
826	2.4	2262
724	2.3	2186
563	2.4	2167
43	2.1	97
103	2.2	97
4	2.1	93
85	2.1	89
63	2.3	89
44	2.3	75
84	2.1	70
62	2.3	65
83	2.1	64
102	2.1	63

[Fig. 16]

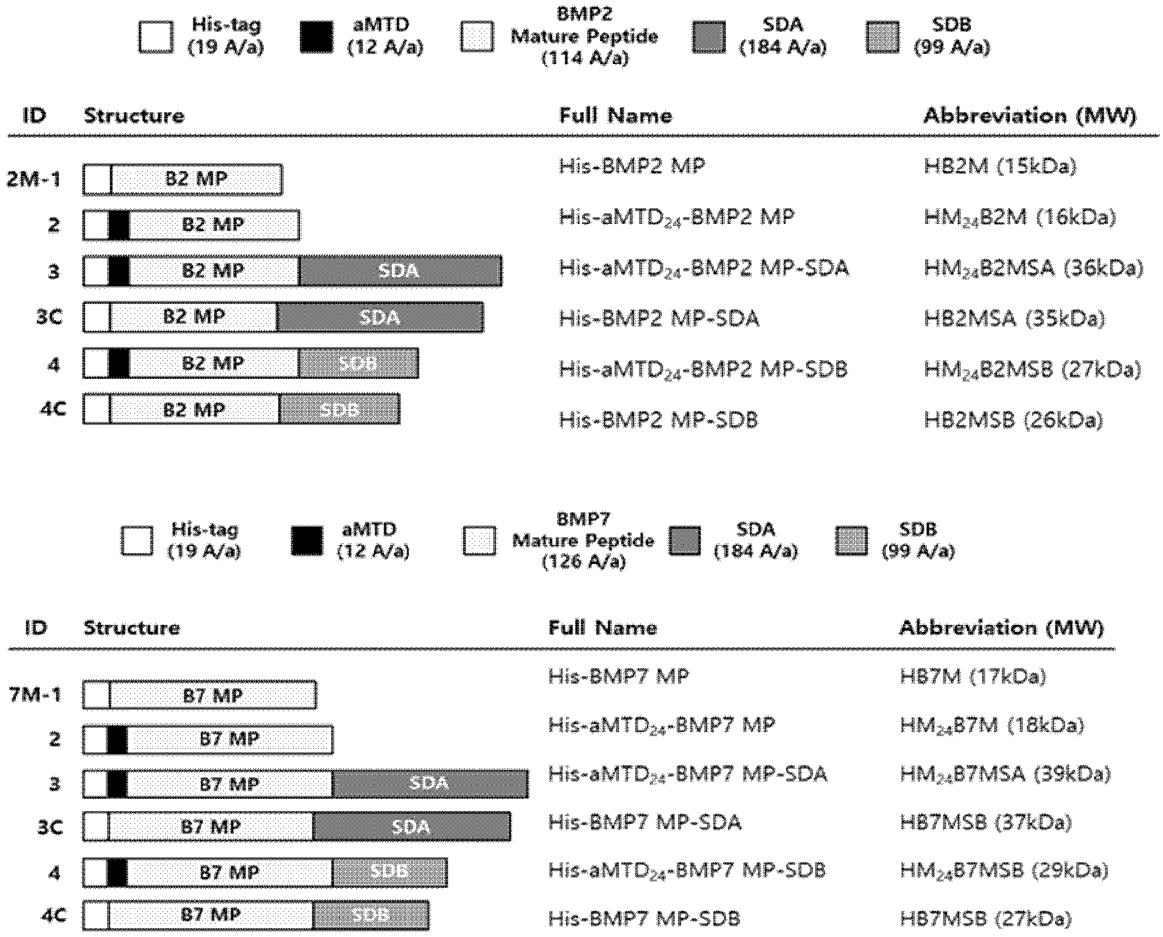


ID	Hydropathy (GRAVY)	Delivery Potential (DP)
33	2.4	2100
931	2.5	1843
436	2.6	1661
138	2.2	1339
77	2.5	1311
936	2.4	1077
226	2.2	935
30	2.3	931
6	2.2	824
750	2.2	750
67	0.3	71
20	-0.9	70
635	-1.9	67
29	1.7	63
40	-0.6	63
190	1.8	59
57	-1.6	51
159	-1.3	51
700	-1.6	47

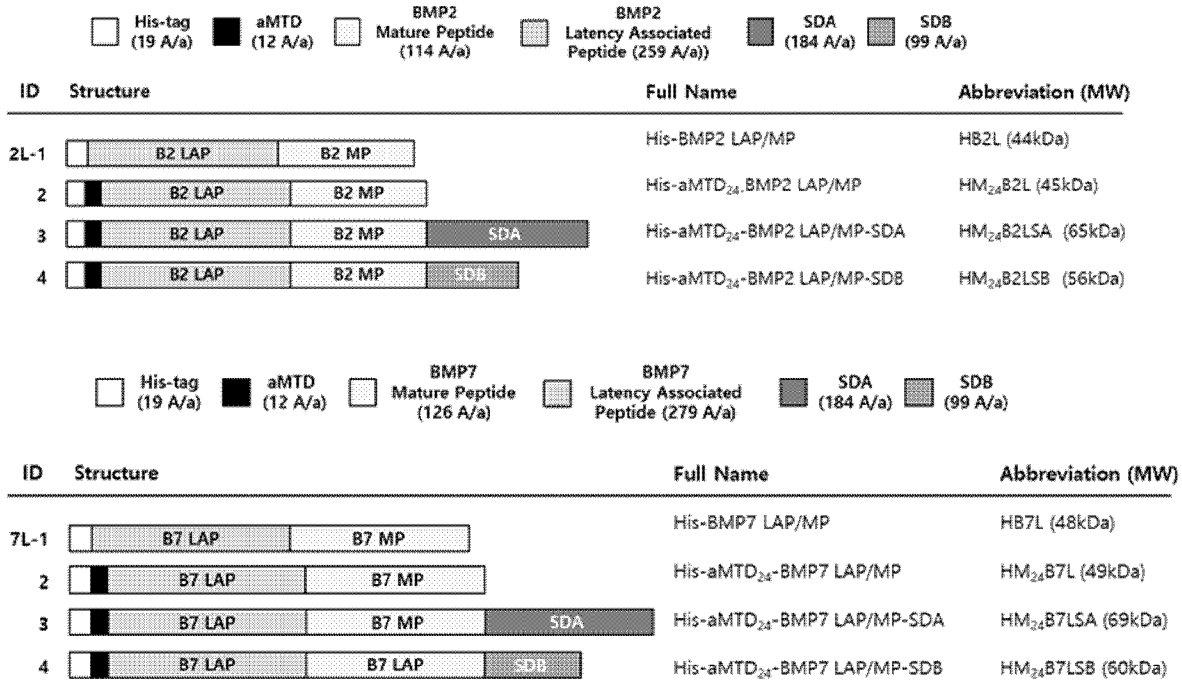
[Fig. 17]



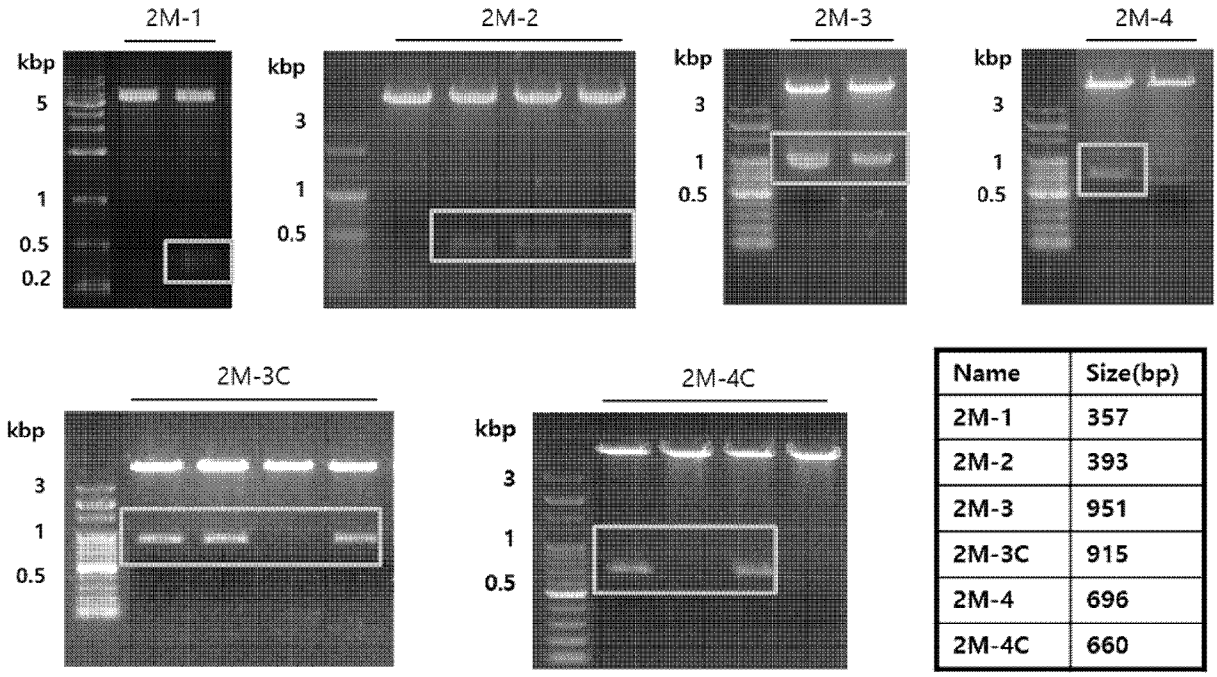
[Fig. 18a]



[Fig. 18b]

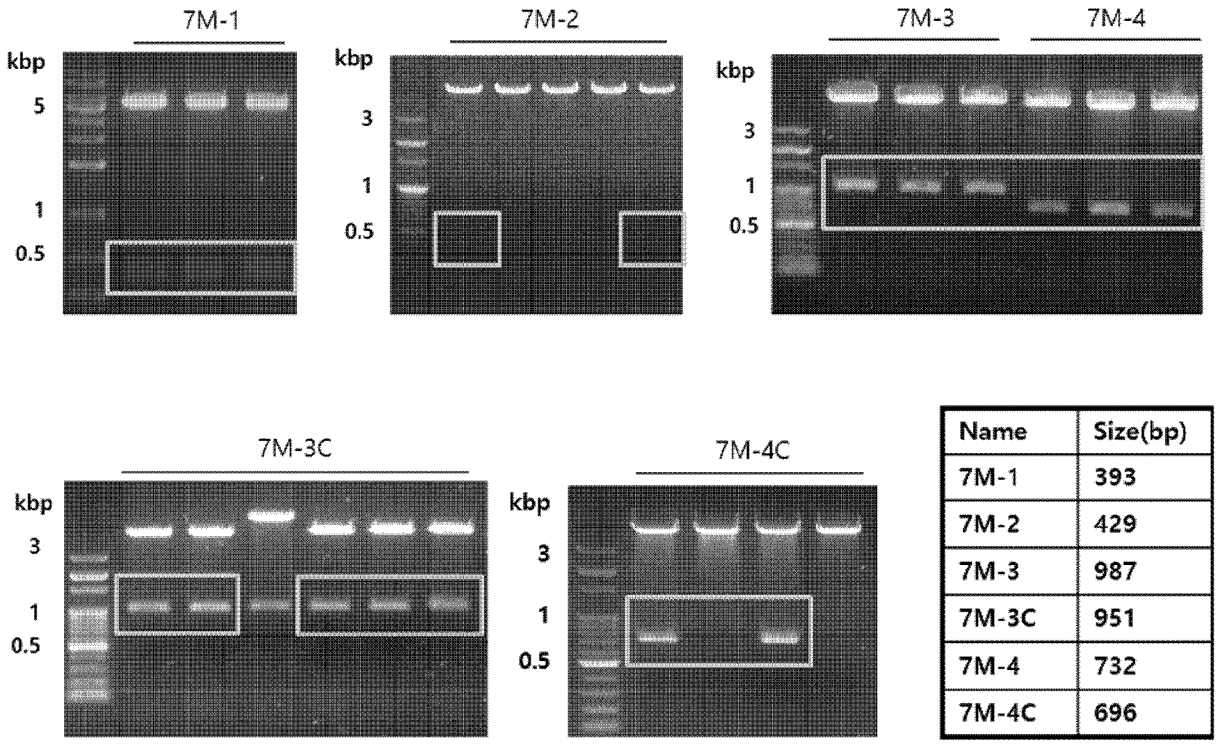


[Fig. 19a]



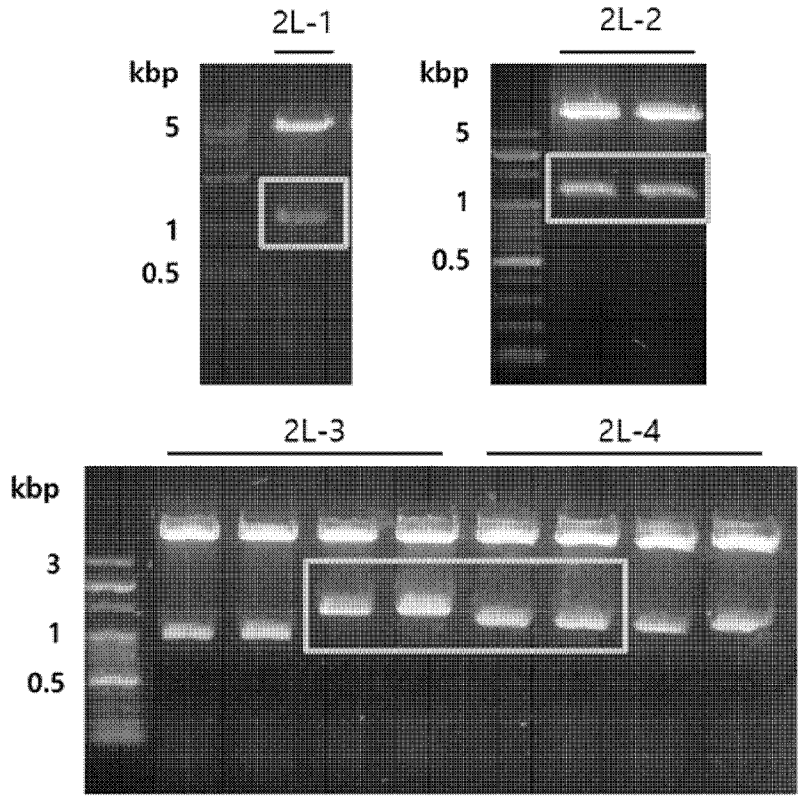
Name	Size(bp)
2M-1	357
2M-2	393
2M-3	951
2M-3C	915
2M-4	696
2M-4C	660

[Fig. 19b]



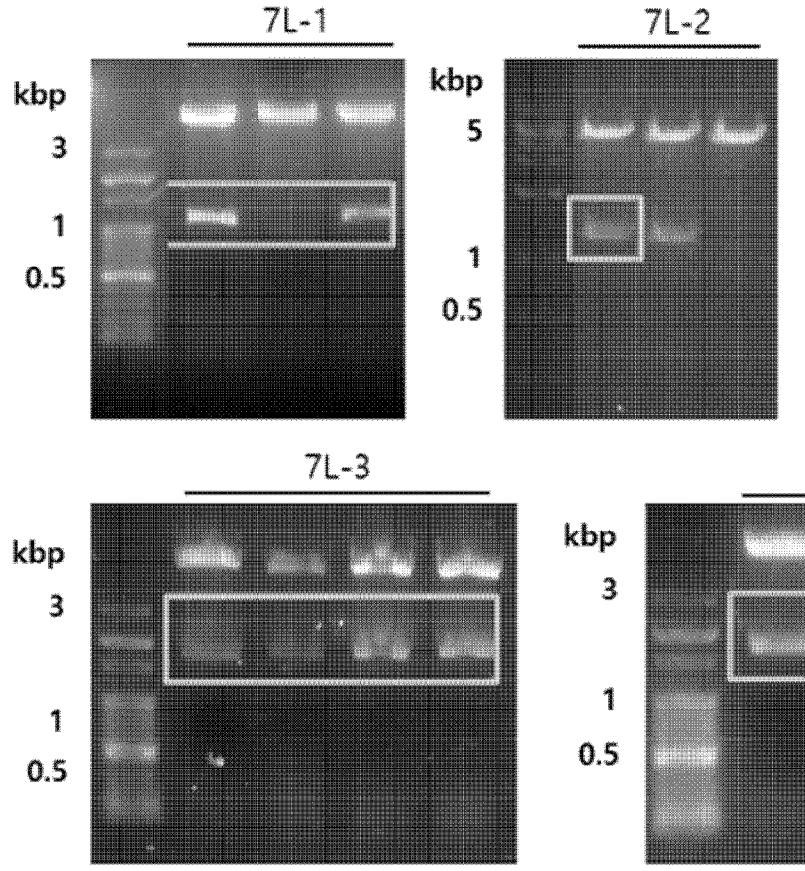
Name	Size(bp)
7M-1	393
7M-2	429
7M-3	987
7M-3C	951
7M-4	732
7M-4C	696

[Fig. 19c]



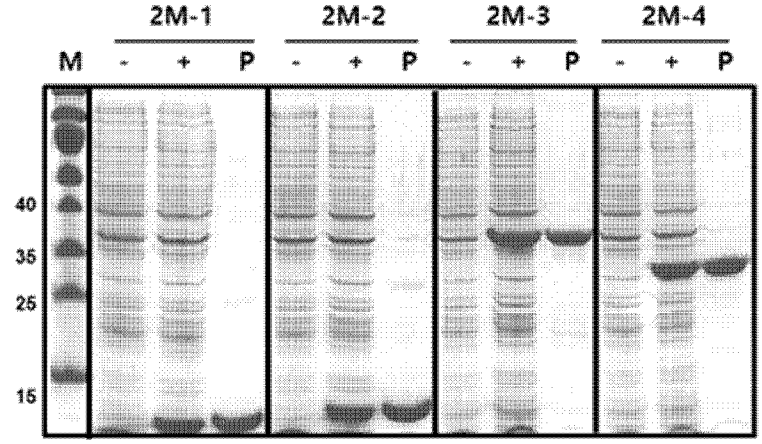
Name	Size(bp)
2L-1	1134
2L-2	1170
2L-3	1728
2L-4	1473

[Fig. 19d]

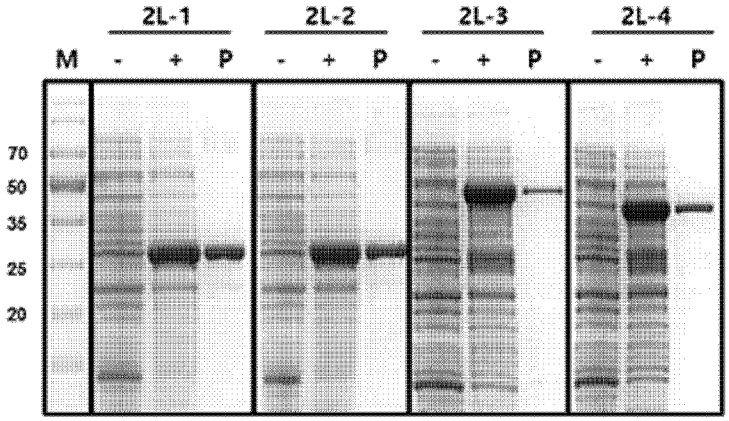


Name	Size(bp)
7L-1	1308
7L-2	1344
7L-3	1902
7L-4	1647

[Fig. 20a]

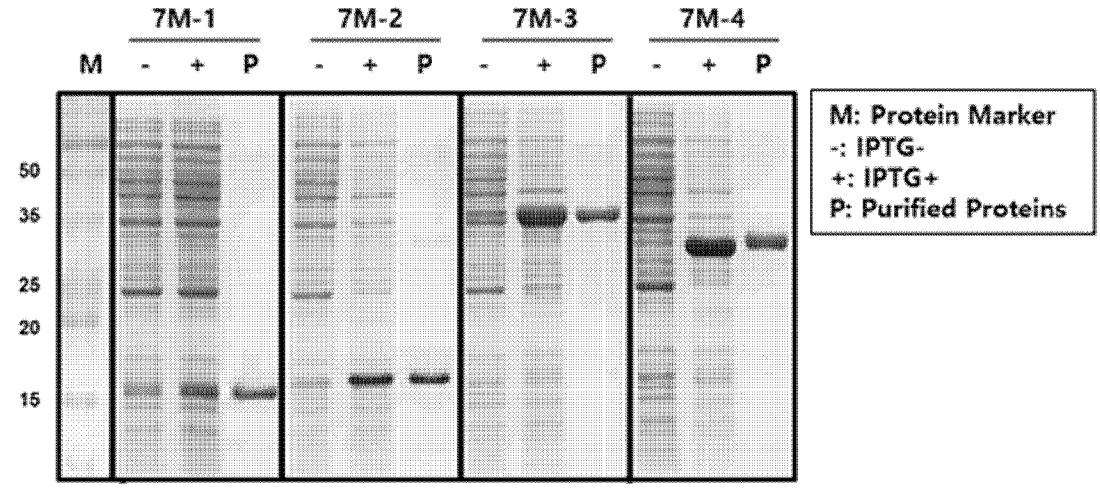


Solubility	+	+	+++++	+++++
Yield (mg/L)	8	9	42	78

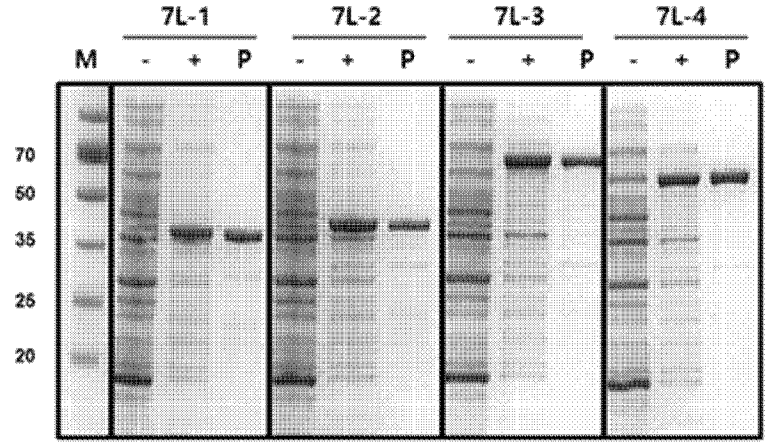


Solubility	+	+	+	+
Yield (mg/L)	0	1	1	5

[Fig. 20b]

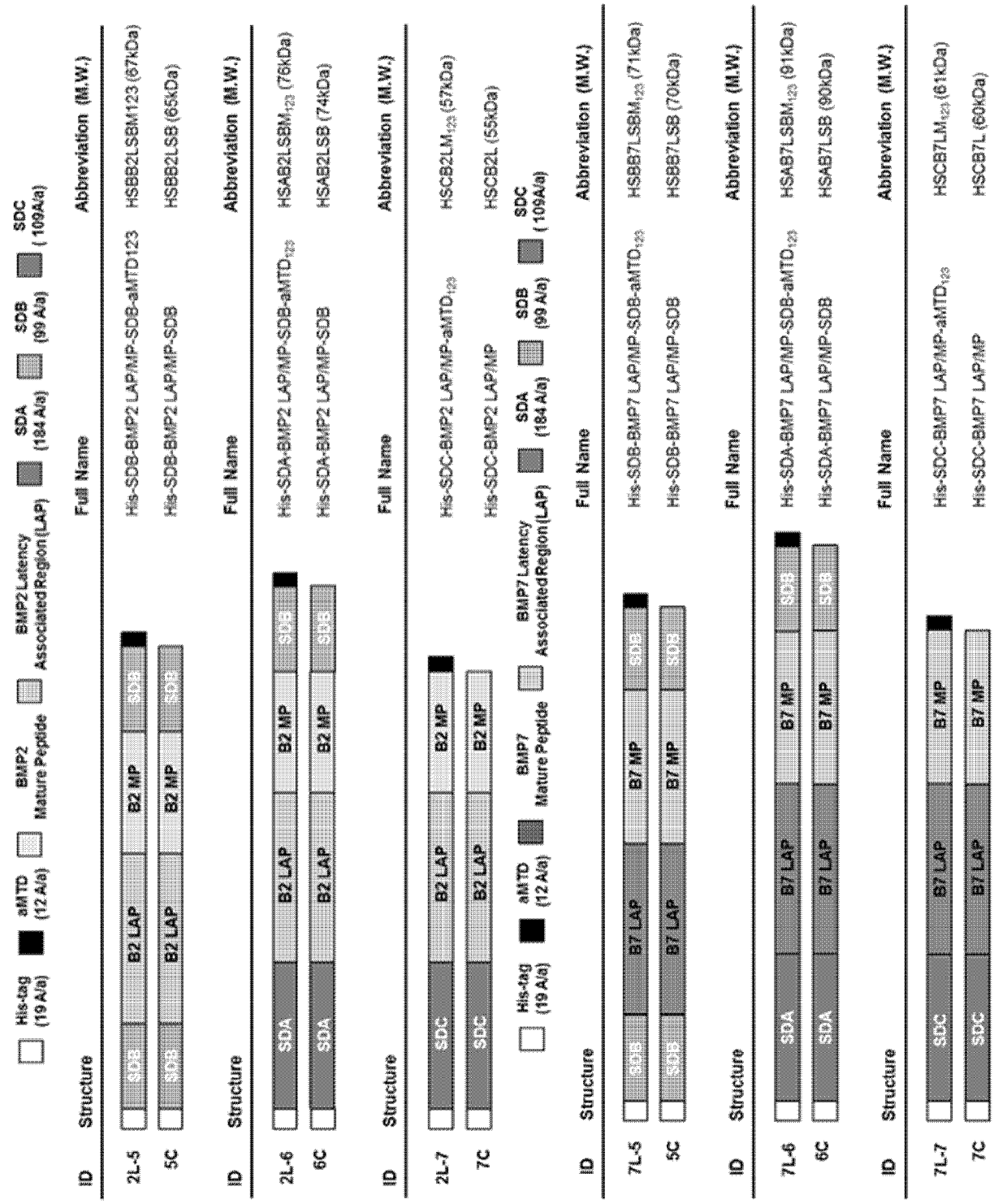


Solubility	+	+	+++++	+++++
Yield (mg/L)	0.7	0.5	60	75

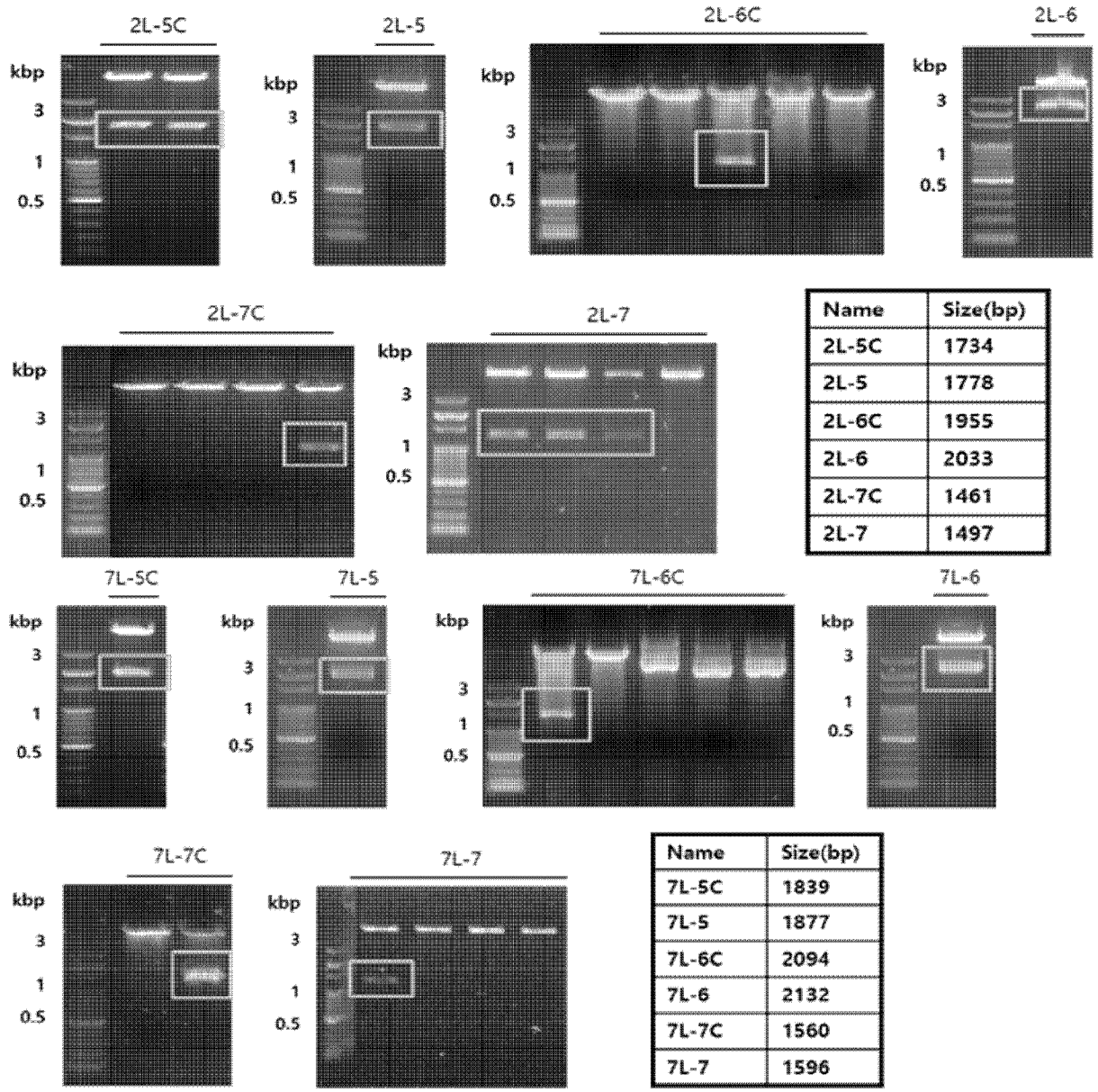


Solubility	+	+	+	++
Yield (mg/L)	0	0	0	35

[Fig. 21]


His-tag (19 A/a)
aMTD (12 A/a)
BMP2 Mature Peptide
BMP2 Latency Associated Region (LAP)
SDA (184 A/a)
SDB (99 A/a)
SDC (109A/a)
ID
Structure
Full Name
Abbreviation (M.W.)
2L-5
His-SDB-BMP2 LAP/MP-SDB-aMTD123
HSBB2LSBM123 (67kDa)
5C
His-SDB-BMP2 LAP/MP-SDB
HSBB2LSB (65kDa)
2L-6
His-SDA-BMP2 LAP/MP-SDB-aMTD123
HSAB2LSBM123 (76kDa)
6C
His-SDA-BMP2 LAP/MP-SDB
HSAB2LSB (74kDa)
2L-7
His-SDC-BMP2 LAP/MP-aMTD123
HSCB2LM123 (57kDa)
7C
His-SDC-BMP2 LAP/MP
HSCB2L (55kDa)
BMP7 Mature Peptide
BMP7 Latency Associated Region (LAP)
7L-5
His-SDB-BMP7 LAP/MP-SDB-aMTD123
HSBB7LSBM123 (71kDa)
His-SDB-BMP7 LAP/MP-SDB
HSBB7LSB (70kDa)
7L-6
His-SDA-BMP7 LAP/MP-SDB-aMTD123
HSAB7LSBM123 (91kDa)
6C
His-SDA-BMP7 LAP/MP-SDB
HSAB7LSB (90kDa)
7L-7
His-SDC-BMP7 LAP/MP-aMTD123
HSCB7LM123 (61kDa)
7C
His-SDC-BMP7 LAP/MP
HSCB7L (60kDa)

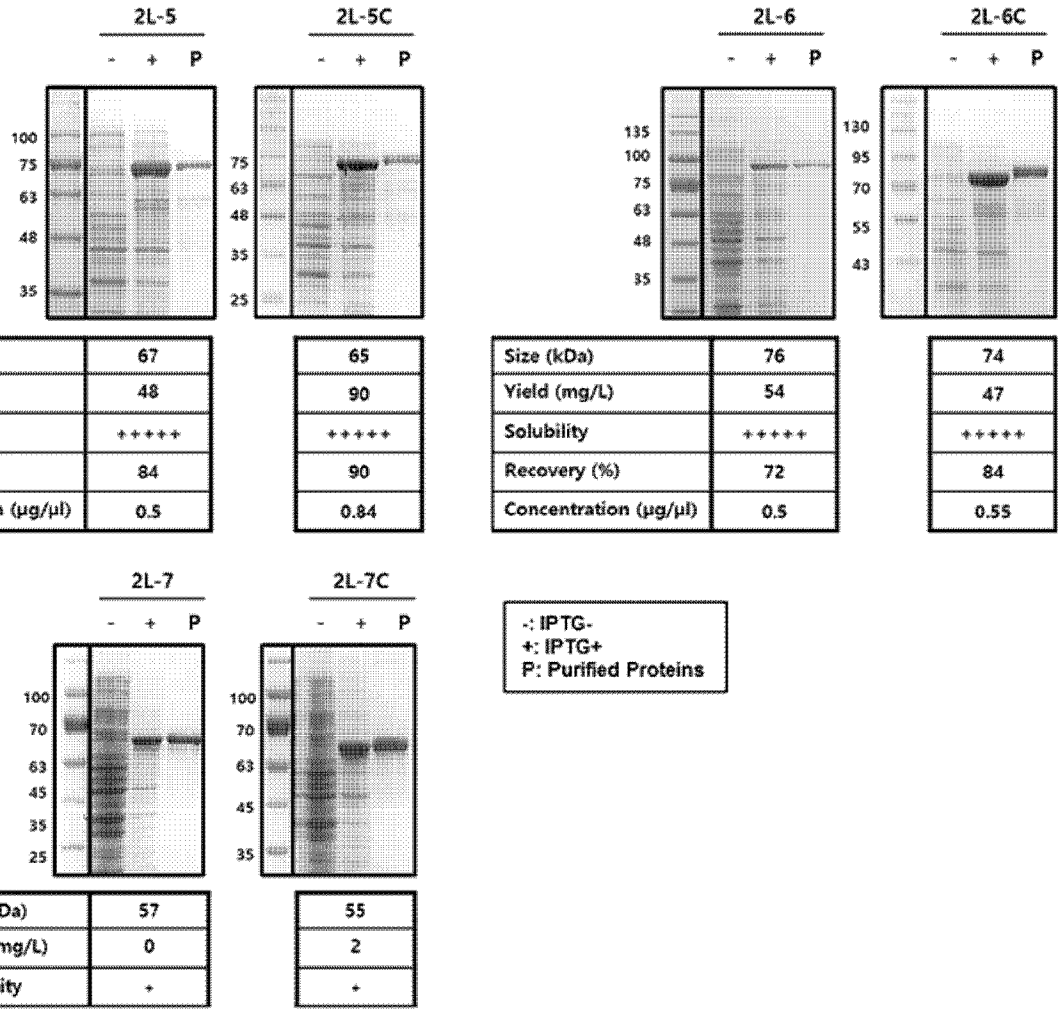
[Fig. 22]



Name	Size(bp)
2L-5C	1734
2L-5	1778
2L-6C	1955
2L-6	2033
2L-7C	1461
2L-7	1497

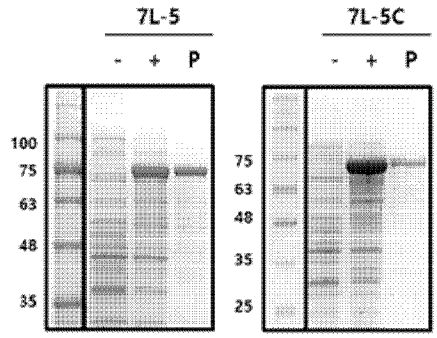
Name	Size(bp)
7L-5C	1839
7L-5	1877
7L-6C	2094
7L-6	2132
7L-7C	1560
7L-7	1596

[Fig. 23a]



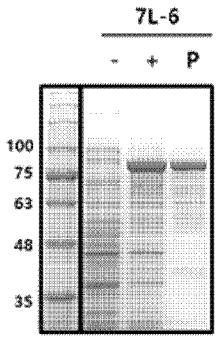
	2L-5	2L-5C	2L-6	2L-6C	2L-7	2L-7C
Size (kDa)	67	65	76	74	57	55
Yield (mg/L)	48	90	54	47	0	2
Solubility	+++++	+++++	+++++	+++++	+	+
Recovery (%)	84	90	72	84		
Concentration (μg/μl)	0.5	0.84	0.5	0.55		

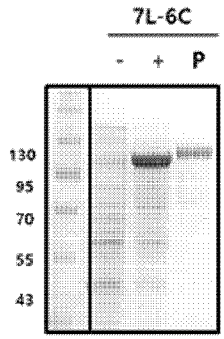
[Fig. 23b]



Size (kDa)	70
Yield (mg/L)	78
Solubility	+++++
Recovery (%)	94
Concentration (μg/μl)	0.8

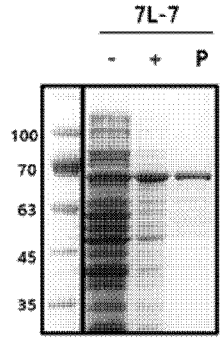
70
68
+++++
80
0.8

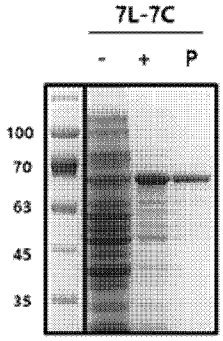




Size (kDa)	91
Yield (mg/L)	57
Solubility	+++++
Recovery (%)	85
Concentration (μg/μl)	0.6

90
34
+++++
71
0.4



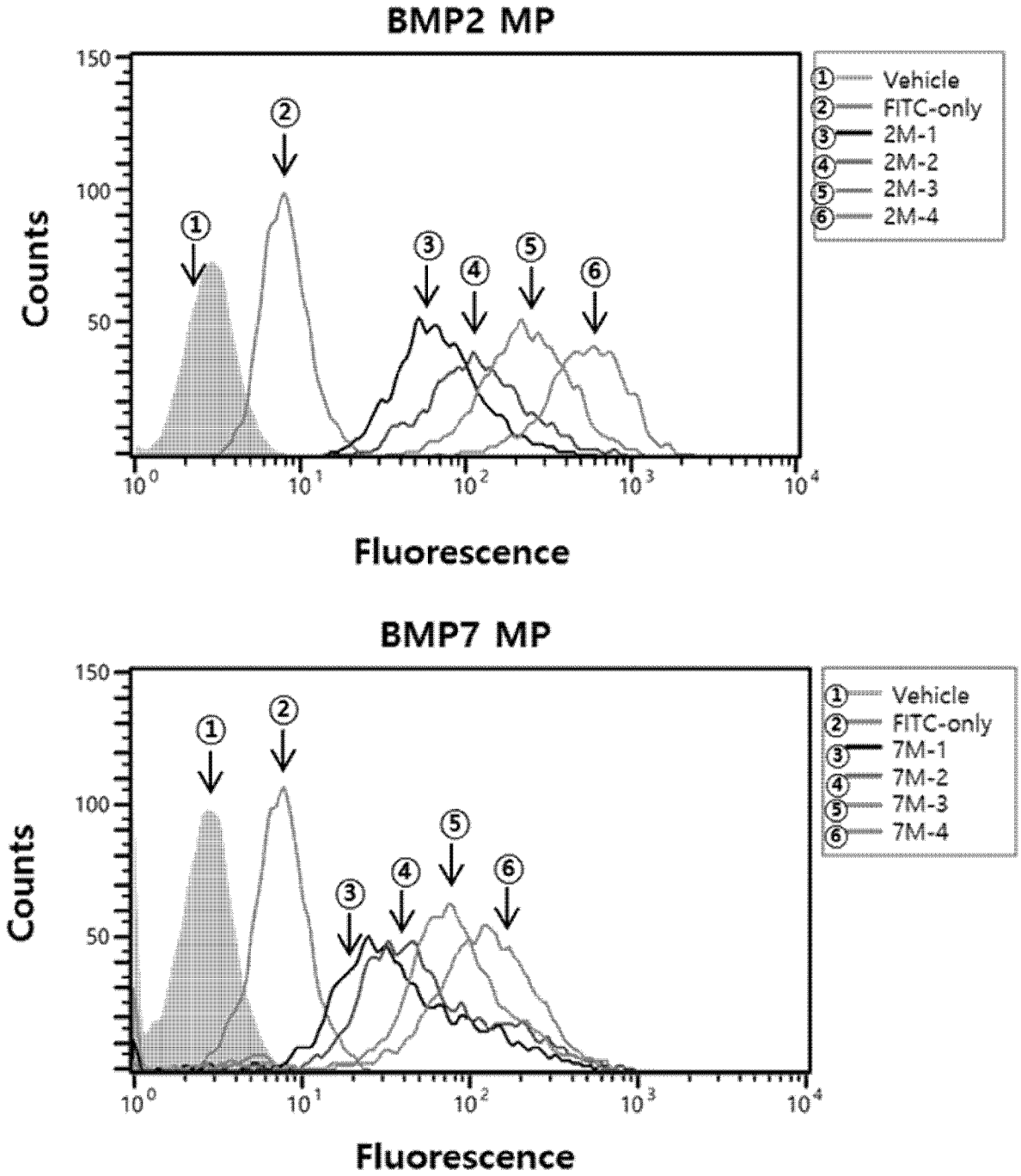


Size (kDa)	61
Yield (mg/L)	8
Solubility	+

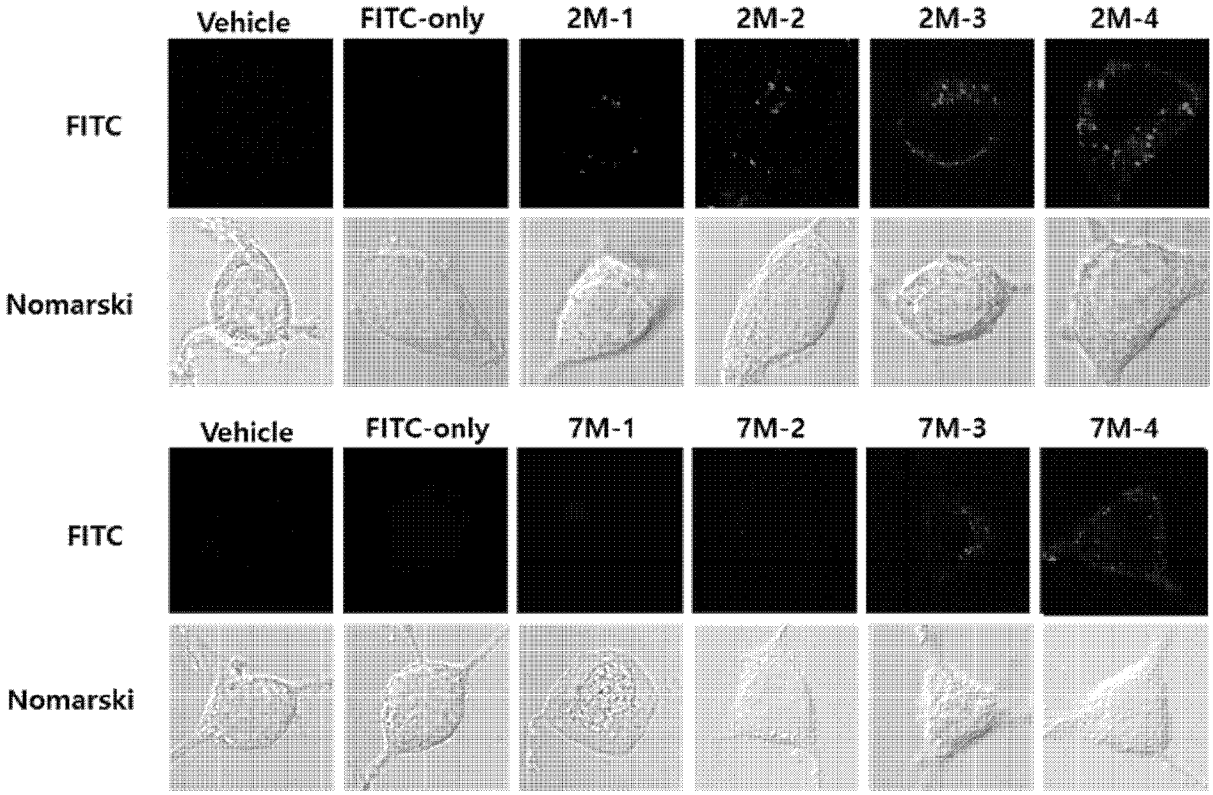
60
8
+

-: IPTG-
+: IPTG+
P: Purified Proteins

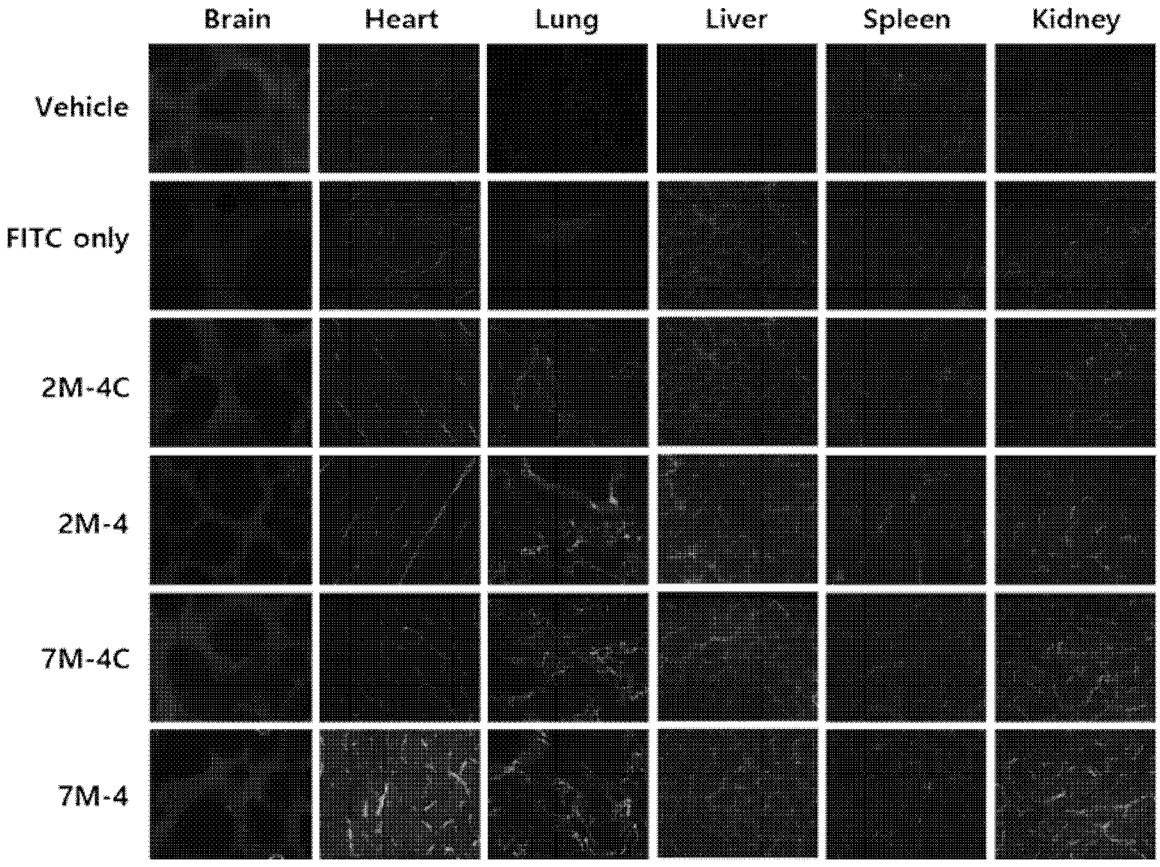
[Fig. 24]


BMP2 MP
Counts
150
100
50
10^0
10^1
10^2
10^3
10^4
Fluorescence
① Vehicle
② FITC-only
③ 2M-1
④ 2M-2
⑤ 2M-3
⑥ 2M-4
BMP7 MP
Counts
150
100
50
10^0
10^1
10^2
10^3
10^4
Fluorescence
① Vehicle
② FITC-only
③ 7M-1
④ 7M-2
⑤ 7M-3
⑥ 7M-4

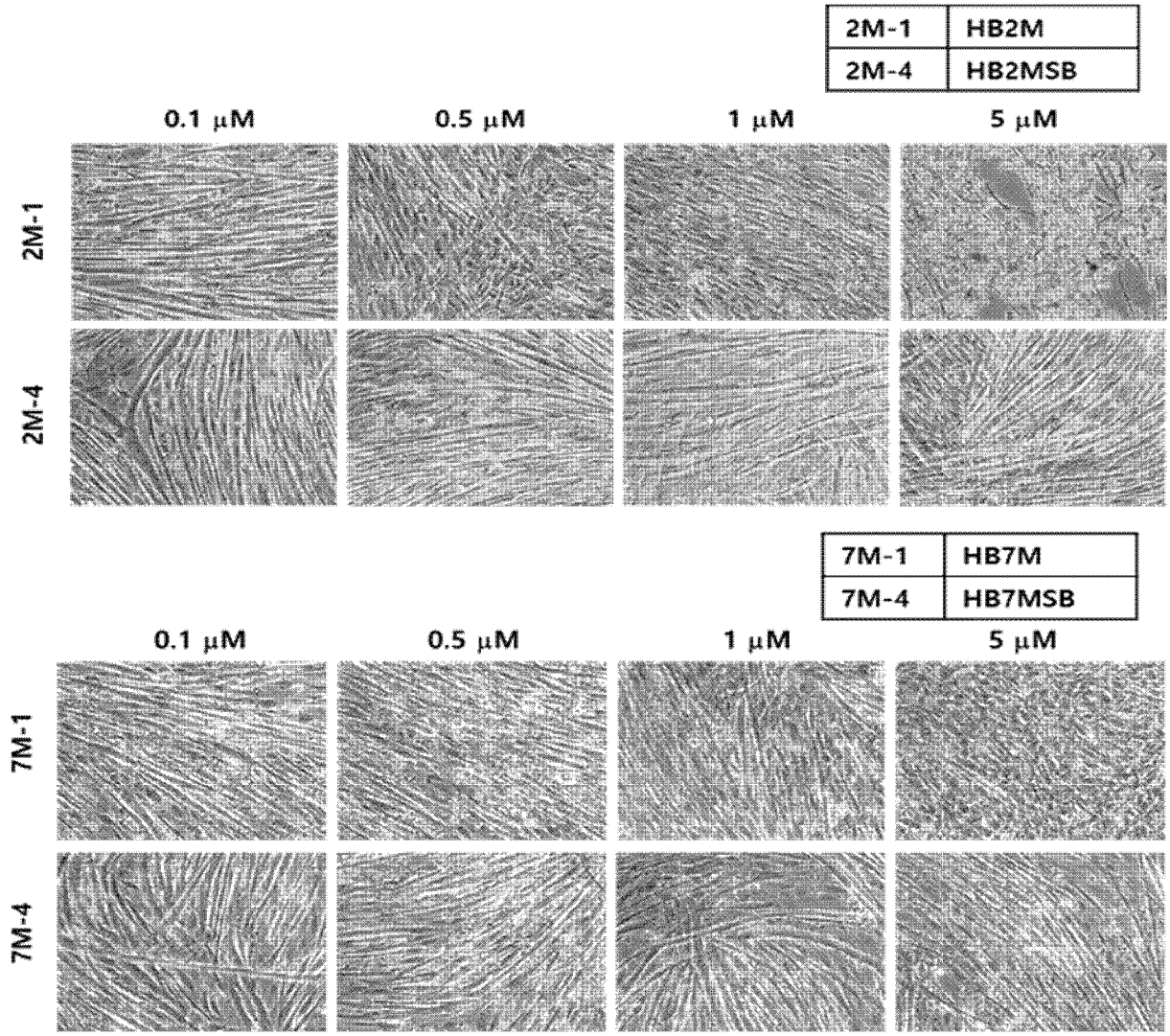
[Fig. 25]


Vehicle
FITC-only
2M-1
2M-2
2M-3
2M-4
FITC
Nomarski
Vehicle
FITC-only
7M-1
7M-2
7M-3
7M-4
FITC
Nomarski

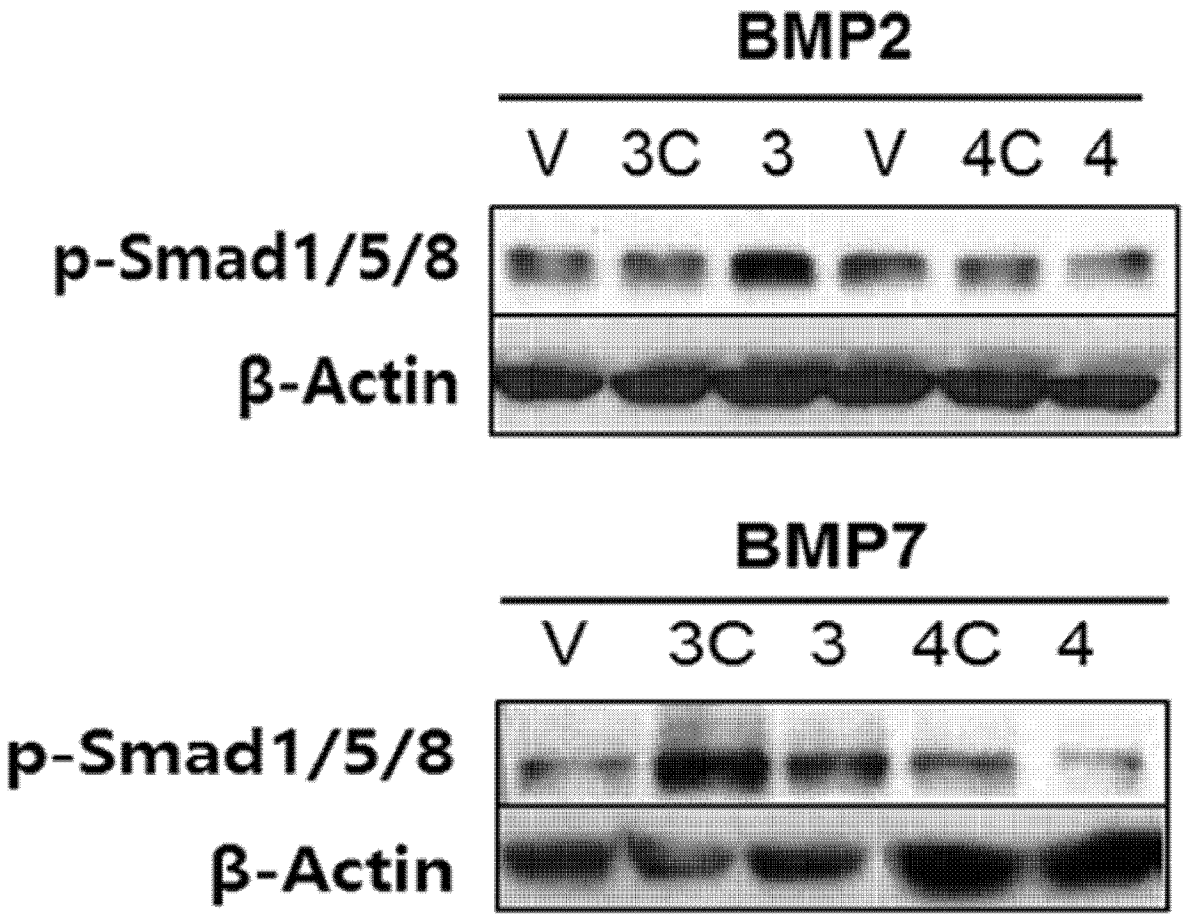
[Fig. 26]


Brain
Heart
Lung
Liver
Spleen
Kidney
Vehicle
FITC only
2M-4C
2M-4
7M-4C
7M-4

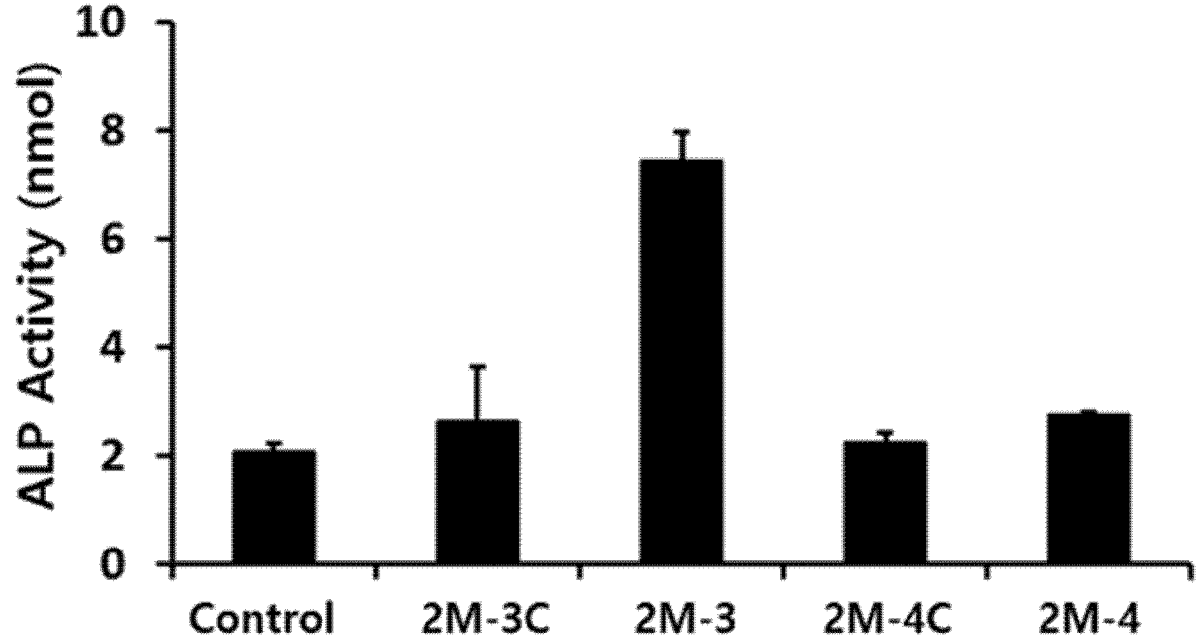
[Fig. 27]

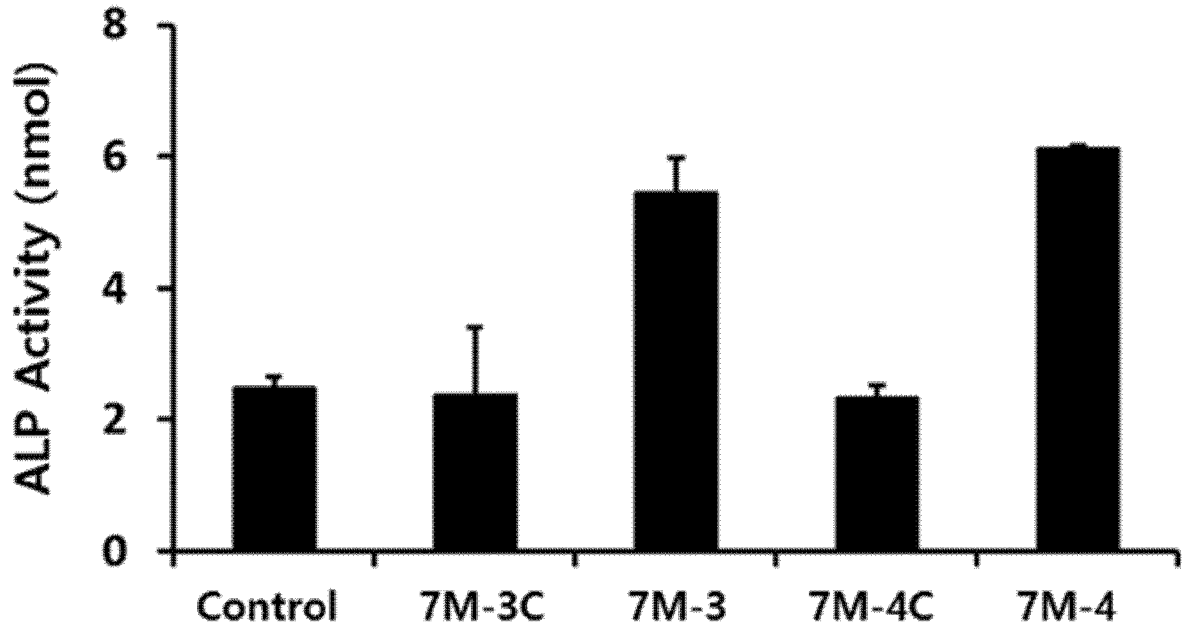

2M-1 HB2M
2M-4 HB2MSB
0.1 μM
0.5 μM
1 μM
5 μM
2M-1
2M-4
7M-1 HB7M
7M-4 HB7MSB
0.1 μM
0.5 μM
1 μM
5 μM
7M-1
7M-4

[Fig. 28]

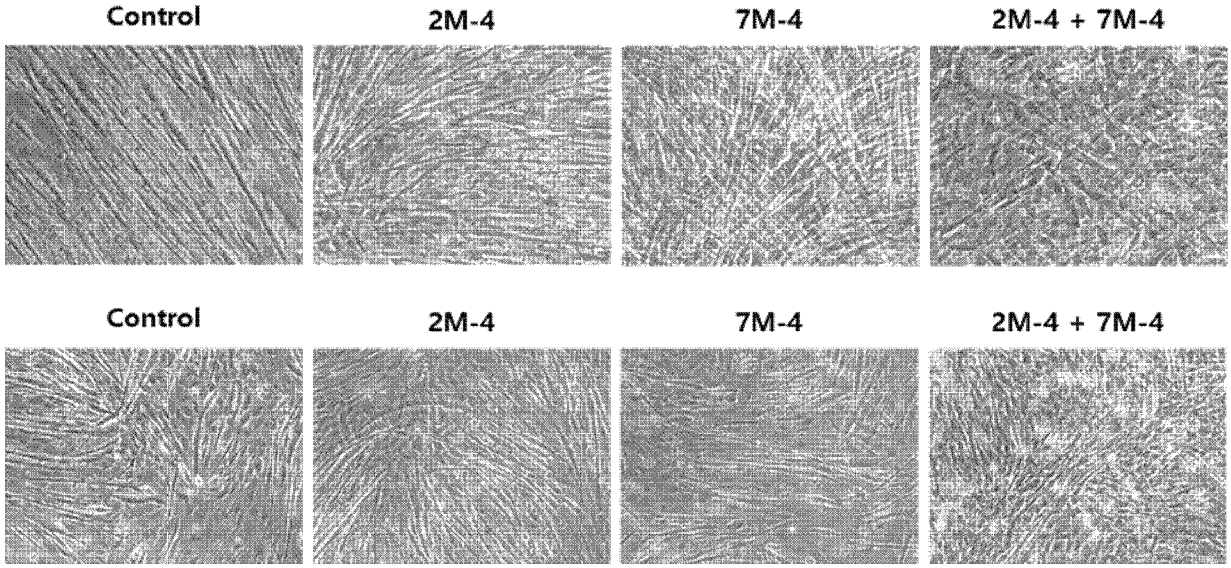

BMP2
V 3C 3 V 4C 4
p-Smad1/5/8
β-Actin
BMP7
V 3C 3 4C 4
p-Smad1/5/8
β-Actin

[Fig. 29]

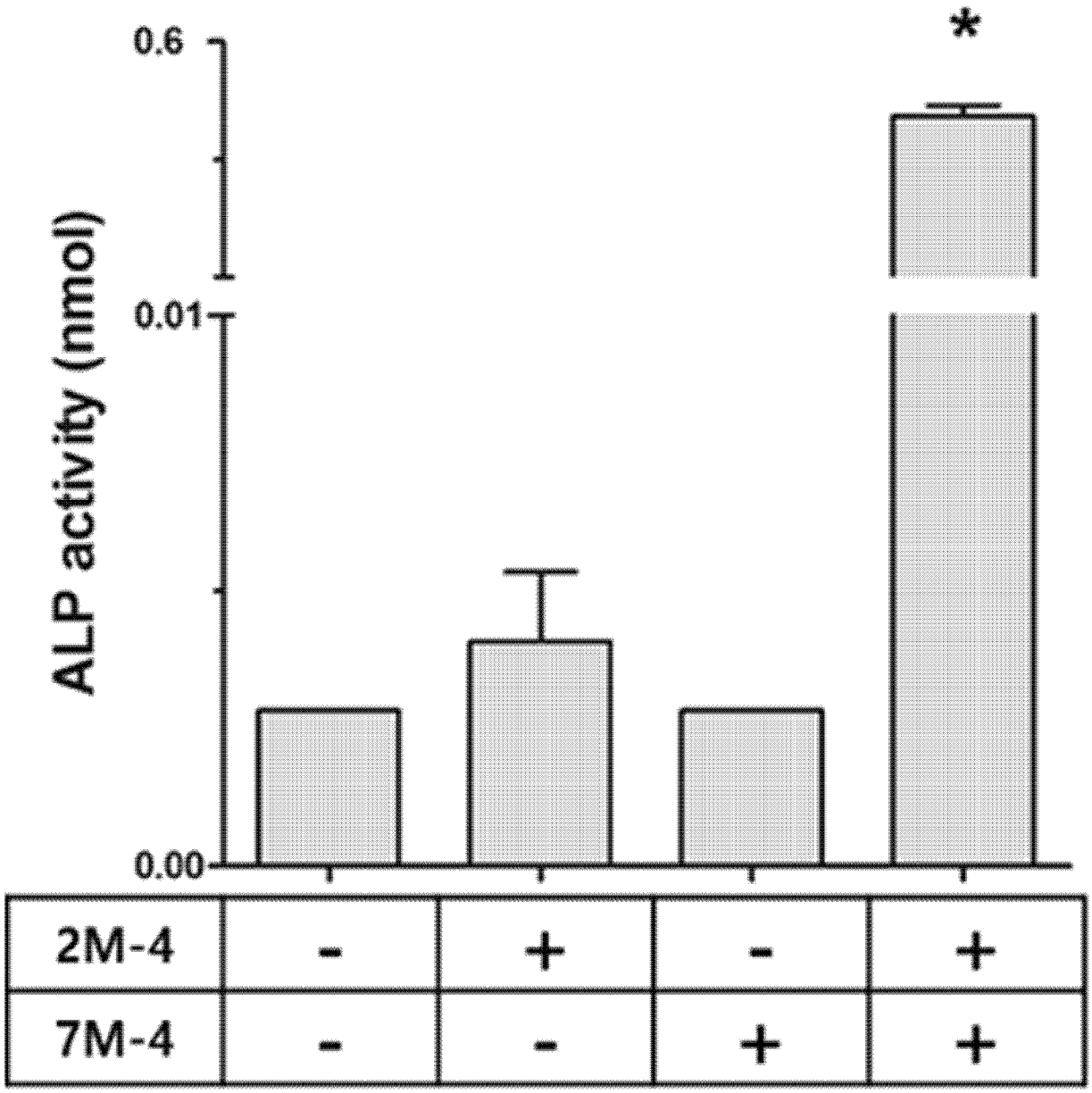

ALP Activity (nmol)
10
8
6
4
2
0
Control
2M-3C
2M-3
2M-4C
2M-4


ALP Activity (nmol)
8
6
4
2
0
Control
7M-3C
7M-3
7M-4C
7M-4

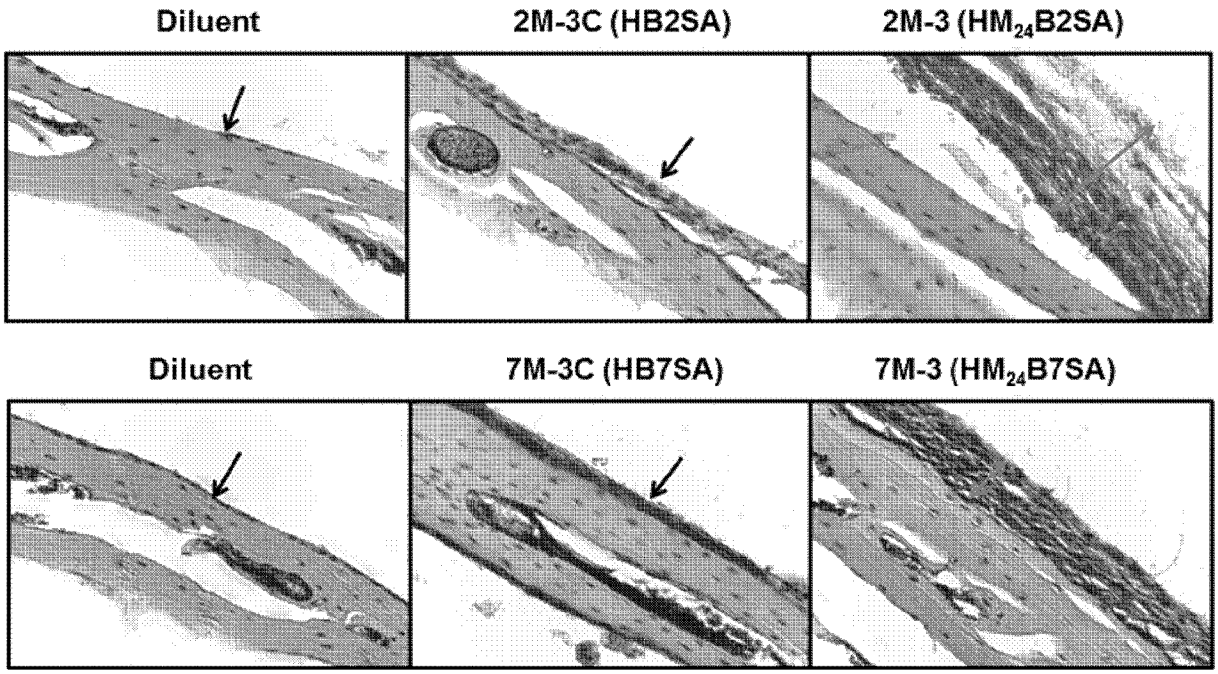
[Fig. 30]


Control
2M-4
7M-4
2M-4 + 7M-4
Control
2M-4
7M-4
2M-4 + 7M-4

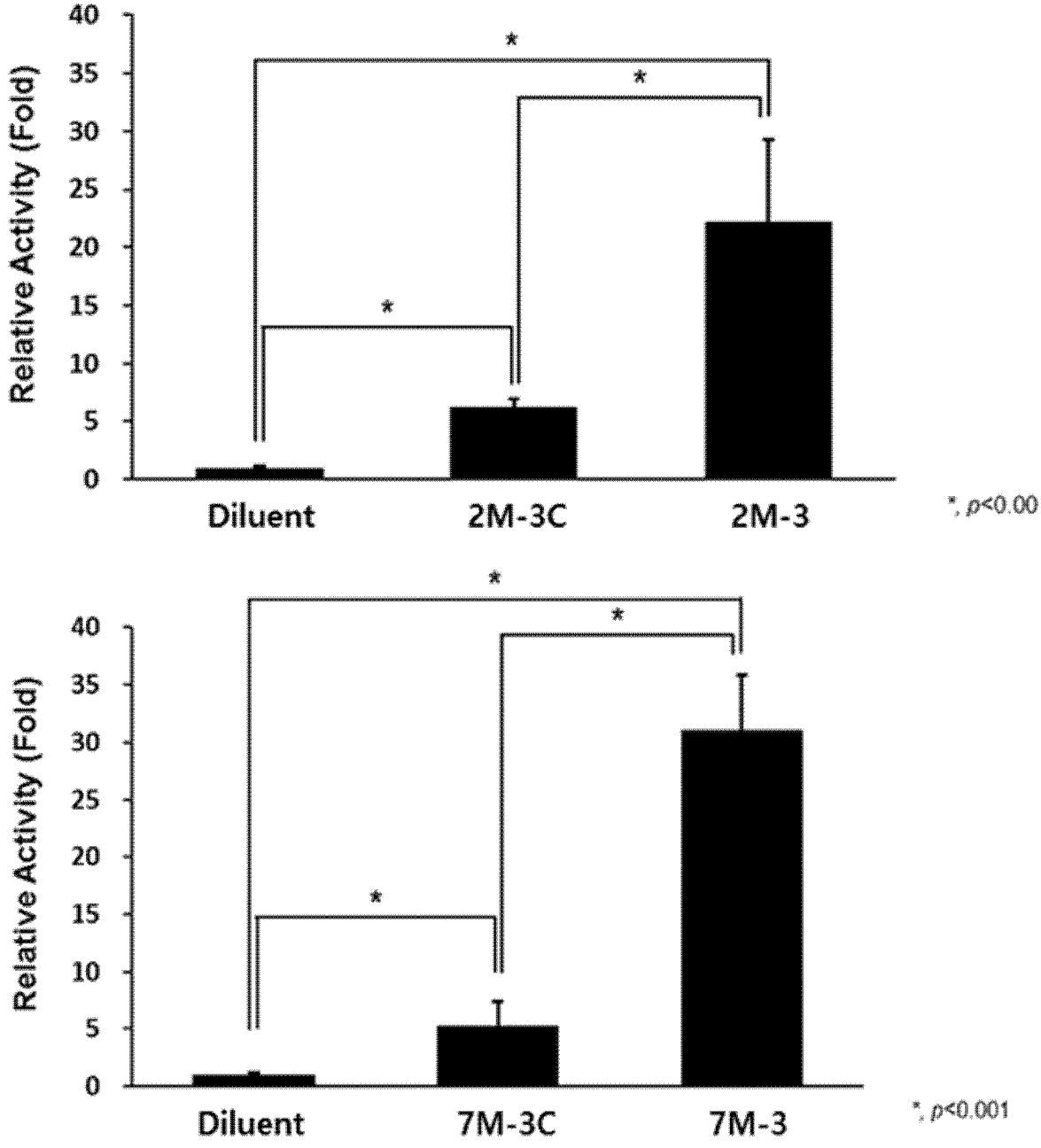
[Fig. 31]


ALP activity (nmol)
0.6
0.01
0.00
*
2M-4 - + - +
7M-4 - - + +

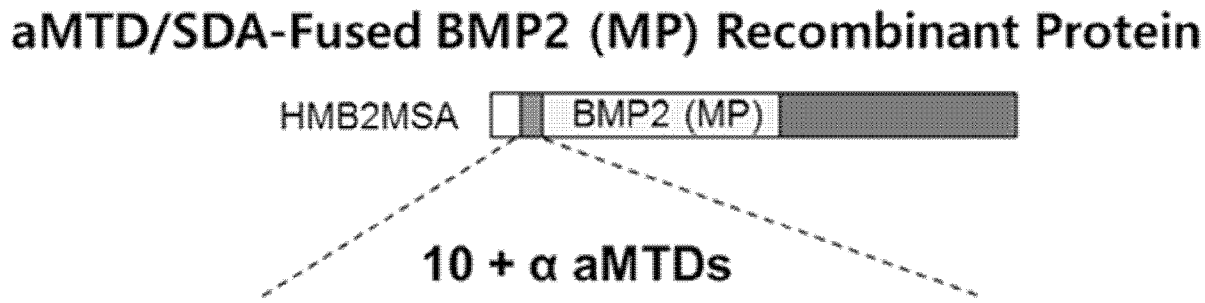
[Fig. 32]


Diluent
2M-3C (HB2SA)
2M-3 (HM24B2SA)
Diluent
7M-3C (HB7SA)
7M-3 (HM24B7SA)

[Fig. 33]

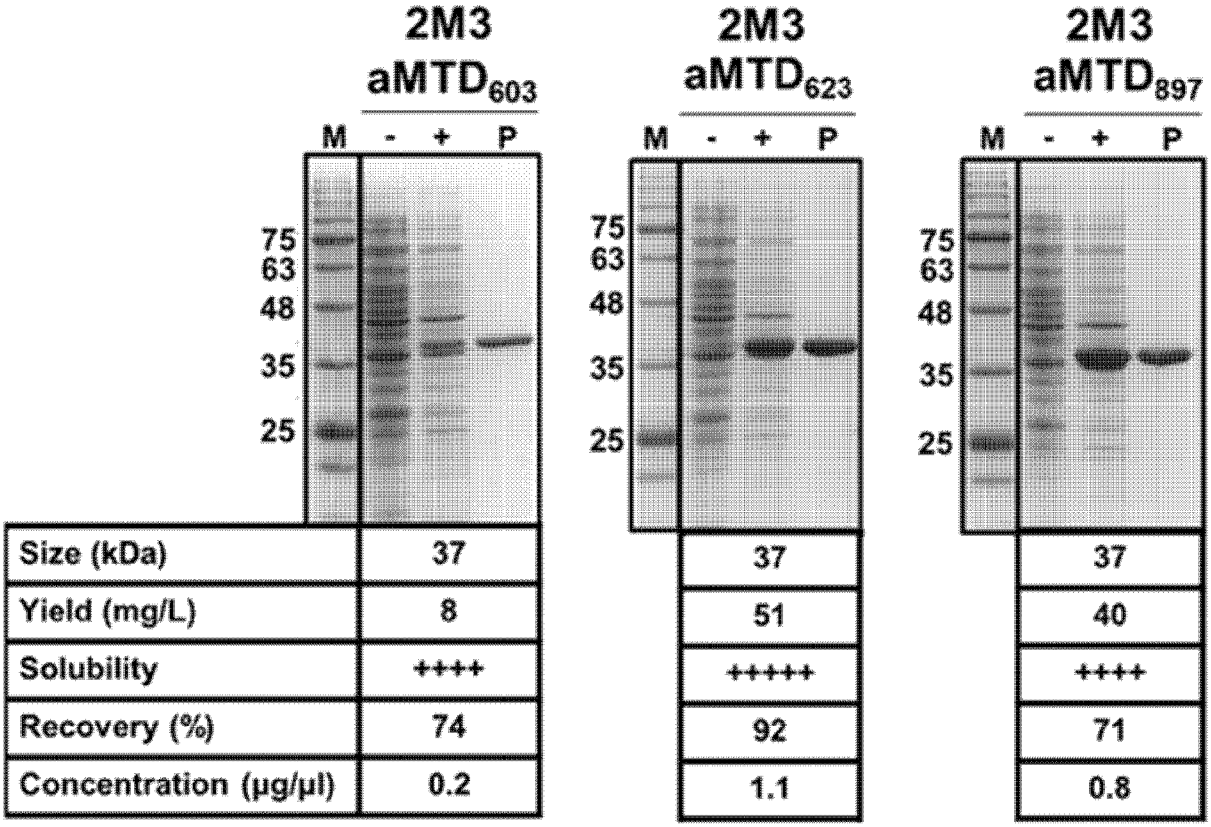

Relative Activity (Fold)
40
35
30
25
20
15
10
5
0
*
*
*
Diluent
2M-3C
2M-3
*, p<0.00
Relative Activity (Fold)
40
35
30
25
20
15
10
5
0
*
*
*
Diluent
7M-3C
7M-3
*, p<0.001

[Fig. 34]

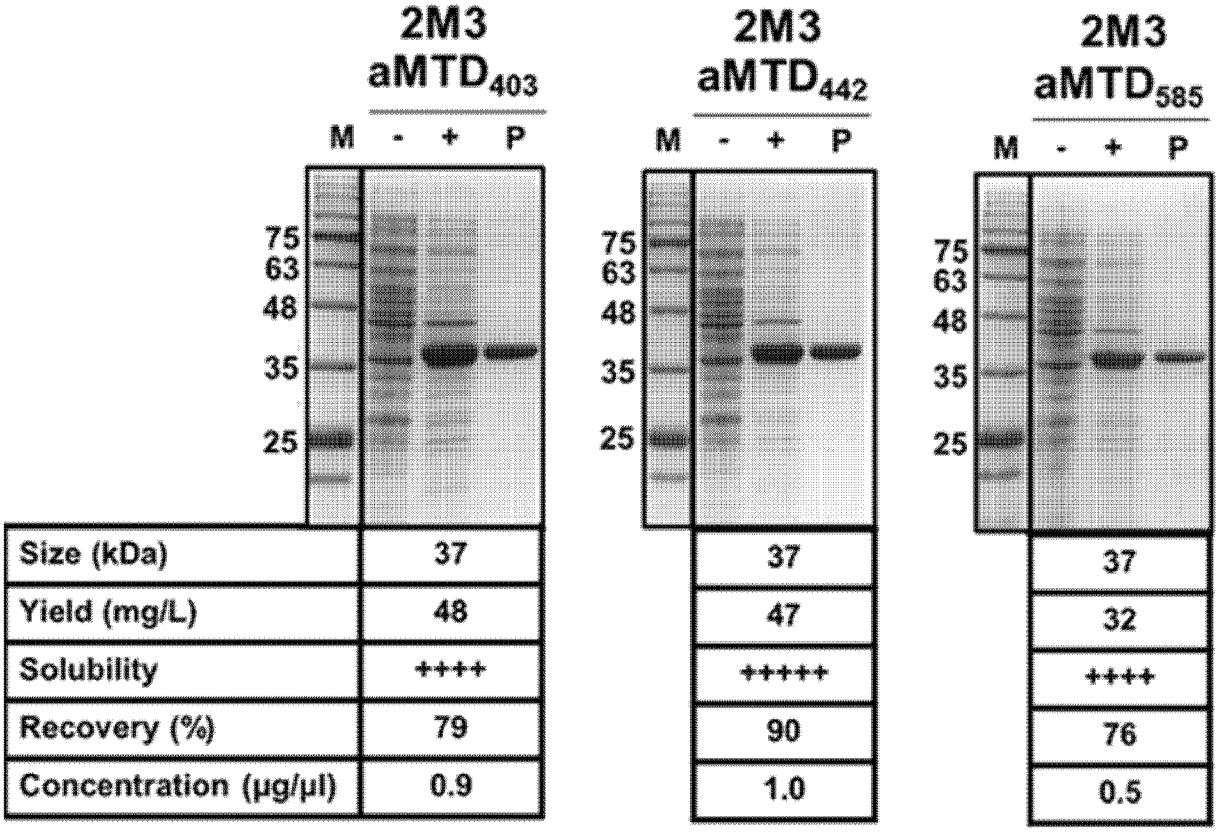


aMTD ID	Sequences	Rigidity/ Flexibility (II)	Structural Feature (AI)	Hydropathy (GRAVY)
1	AAALAPVVLALP	57.3	187.5	2.1
3	AALLVPAAVLAP	57.3	187.5	2.1
61	VAALPVLLAALP	57.3	211.7	2.3
124	IAVALPALIAAP	50.3	195.8	2.2
241	AAAVVPVLLVAP	57.3	195.0	2.4
321	IVAVALPALAVP	50.2	203.3	2.3
385	IVAIAVPALVAP	50.2	203.3	2.4
403	AAALVIPAAILP	54.9	195.8	2.2
442	ALAALVPAVLVP	57.3	203.3	2.3
603	VLVALAAPVIAP	57.3	203.3	2.4
563	ALAVIVVPALAP	50.2	203.3	2.4
481	AIAIAIVPVALP	50.2	211.6	2.4
585	ALIVAIAPALVP	50.2	211.6	2.4
623	VAAAIALPAIVP	50.2	187.5	2.3
847	LVAIVVLPAVAP	50.2	219.2	2.6
897	AVIVPVAIIAAP	50.2	203.3	2.5
899	AVVIALPAVVAP	57.3	195.0	2.4

[Fig. 35a]

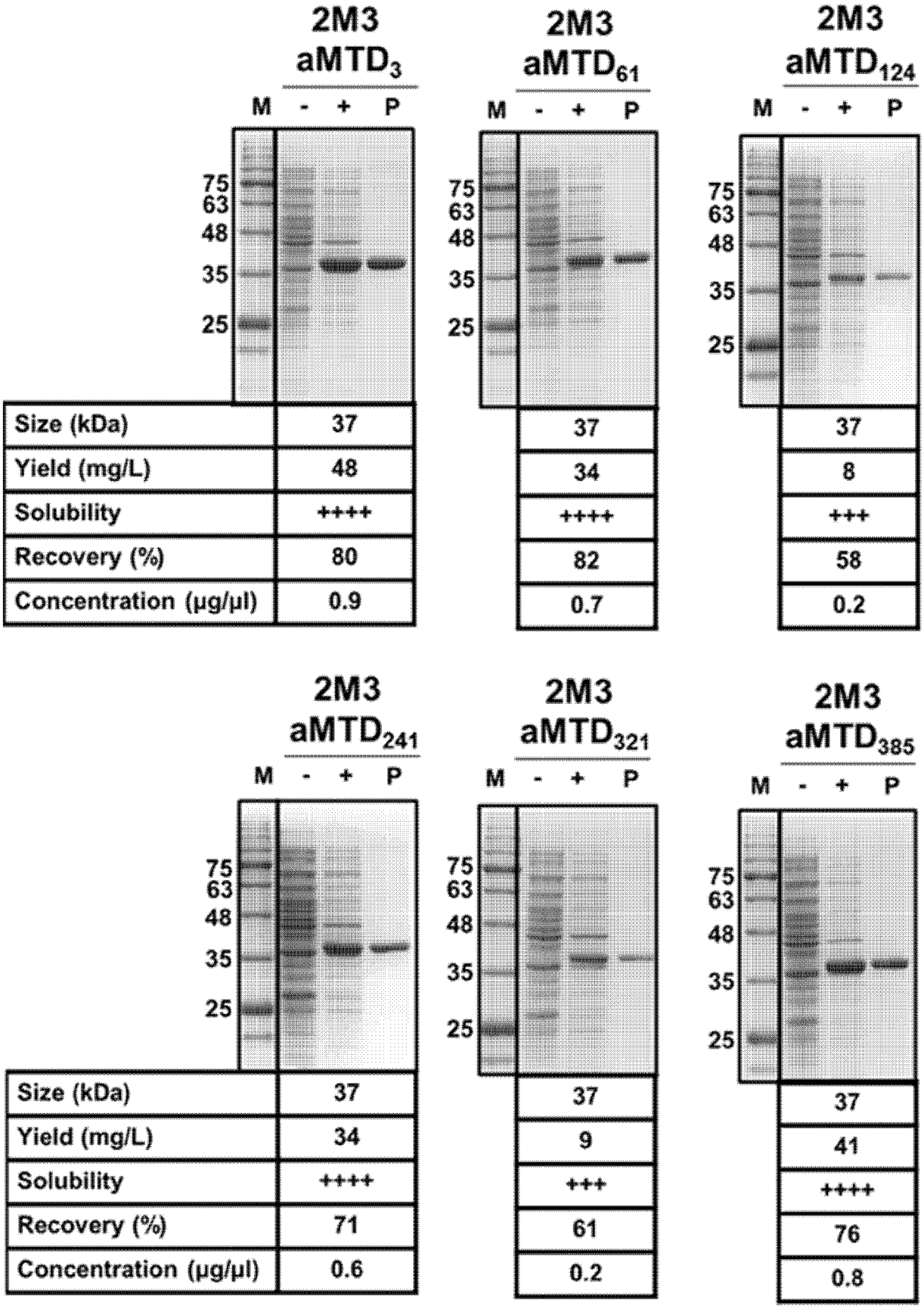


	2M3 aMTD603	2M3 aMTD623	2M3 aMTD897
Size (kDa)	37	37	37
Yield (mg/L)	8	51	40
Solubility	++++	+++++	++++
Recovery (%)	74	92	71
Concentration (μg/μl)	0.2	1.1	0.8

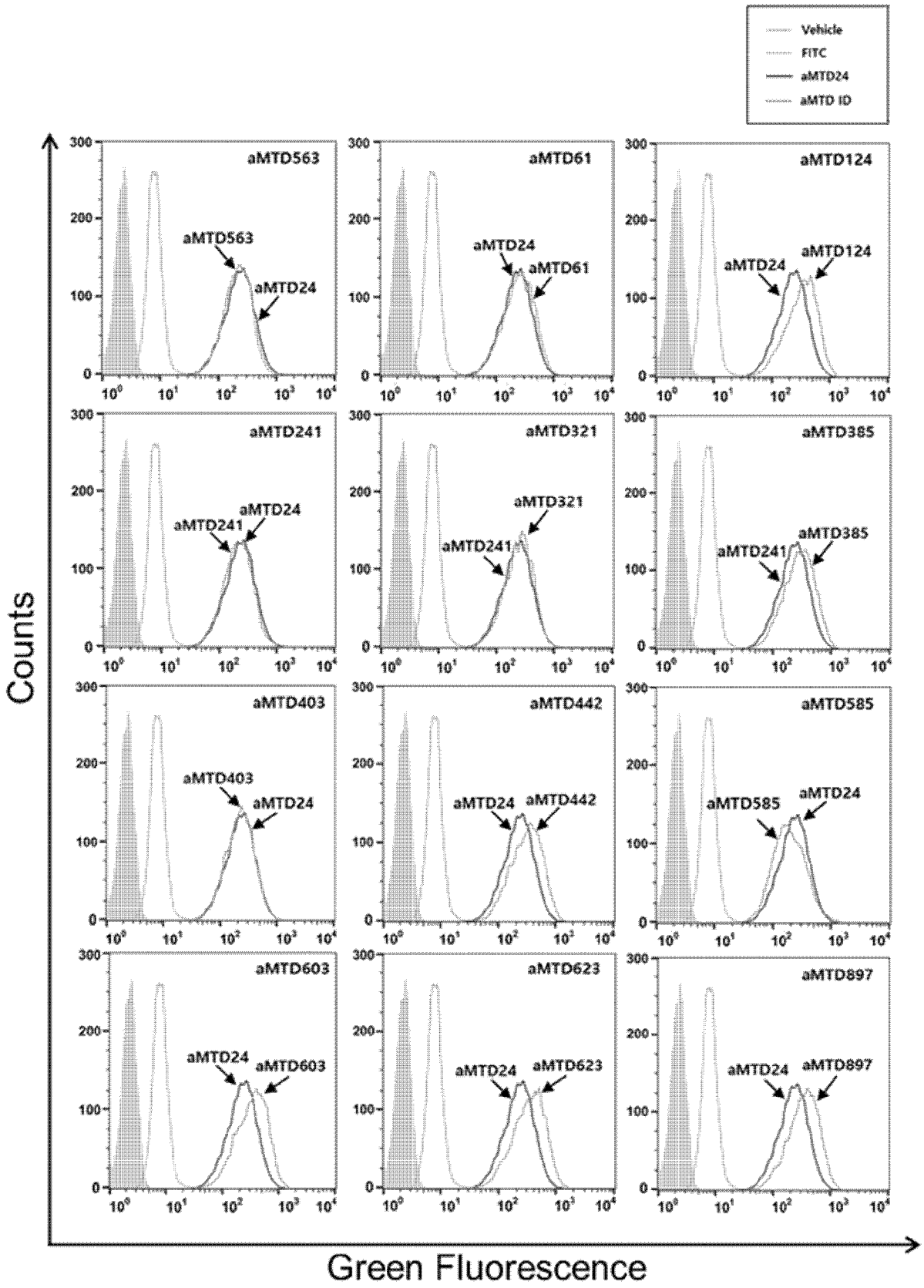


	2M3 aMTD403	2M3 aMTD442	2M3 aMTD585
Size (kDa)	37	37	37
Yield (mg/L)	48	47	32
Solubility	++++	+++++	++++
Recovery (%)	79	90	76
Concentration (μg/μl)	0.9	1.0	0.5

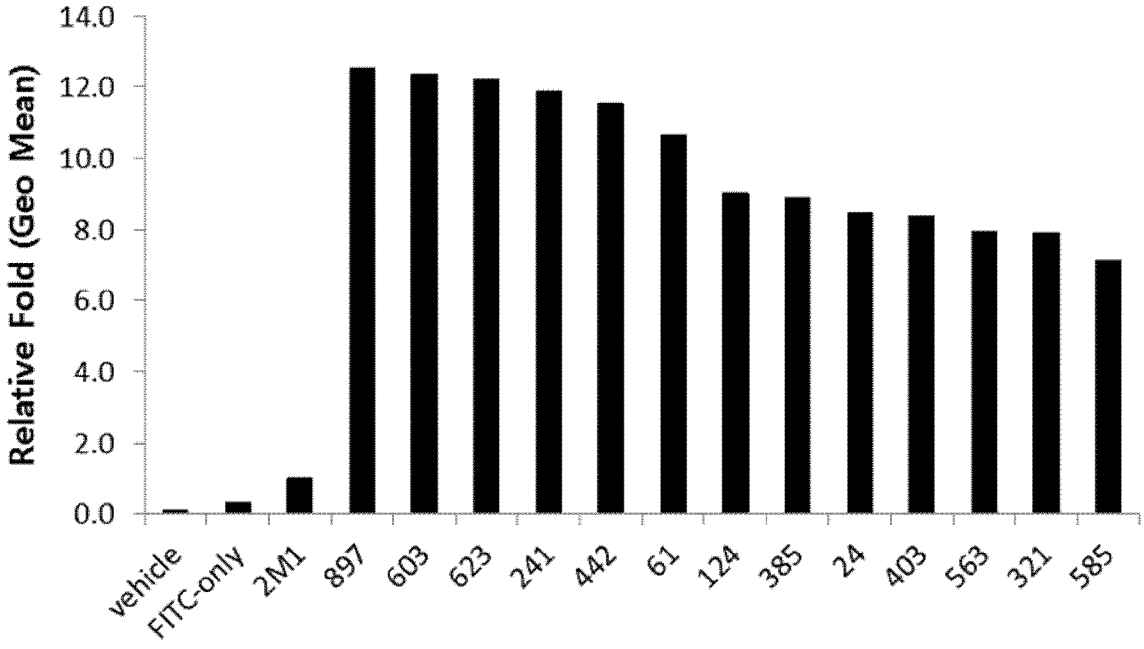
[Fig. 35b]


2M3 aMTD3
M - + P
75
63
48
35
25
Size (kDa) 37
Yield (mg/L) 48
Solubility ++++
Recovery (%) 80
Concentration (μg/μl) 0.9
2M3 aMTD61
M - + P
75
63
48
35
25
37
34
++++
82
0.7
2M3 aMTD124
M - + P
75
63
48
35
25
37
8
+++
58
0.2
2M3 aMTD241
M - + P
75
63
48
35
25
Size (kDa) 37
Yield (mg/L) 34
Solubility ++++
Recovery (%) 71
Concentration (μg/μl) 0.6
2M3 aMTD321
M - + P
75
63
48
35
25
37
9
+++
61
0.2
2M3 aMTD385
M - + P
75
63
48
35
25
37
41
++++
76
0.8

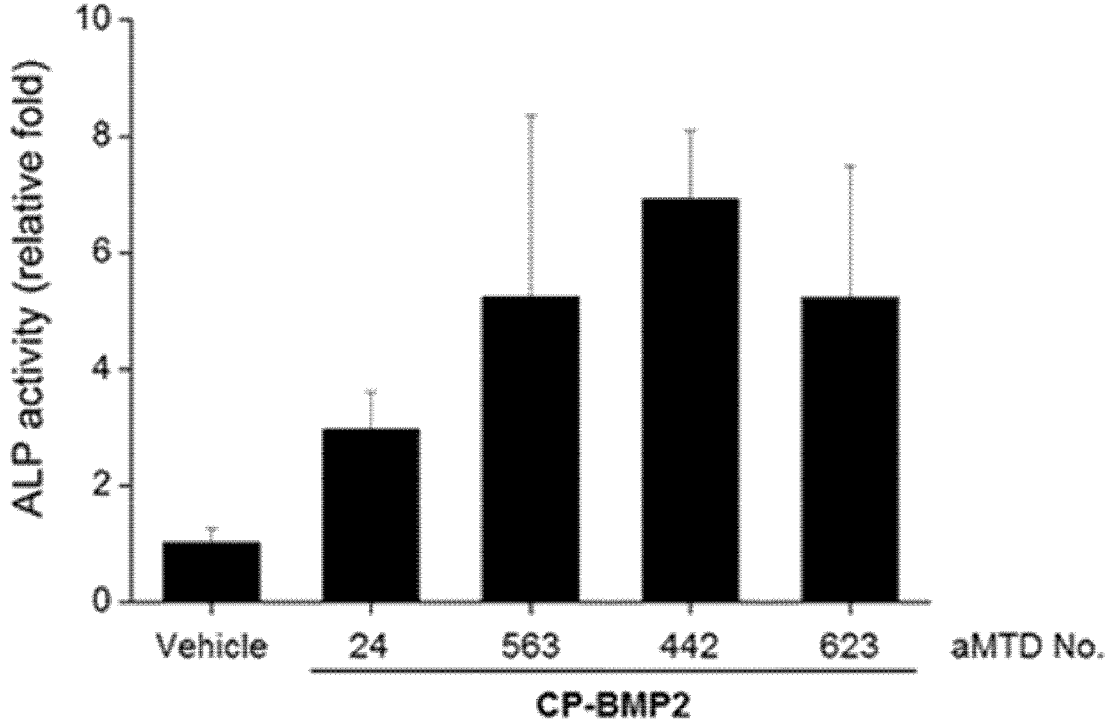
[Fig. 36a]


Vehicle
FITC
aMTD24
aMTD ID
Counts
Green Fluorescence
aMTD563
aMTD61
aMTD124
aMTD241
aMTD321
aMTD385
aMTD403
aMTD442
aMTD585
aMTD603
aMTD623
aMTD897

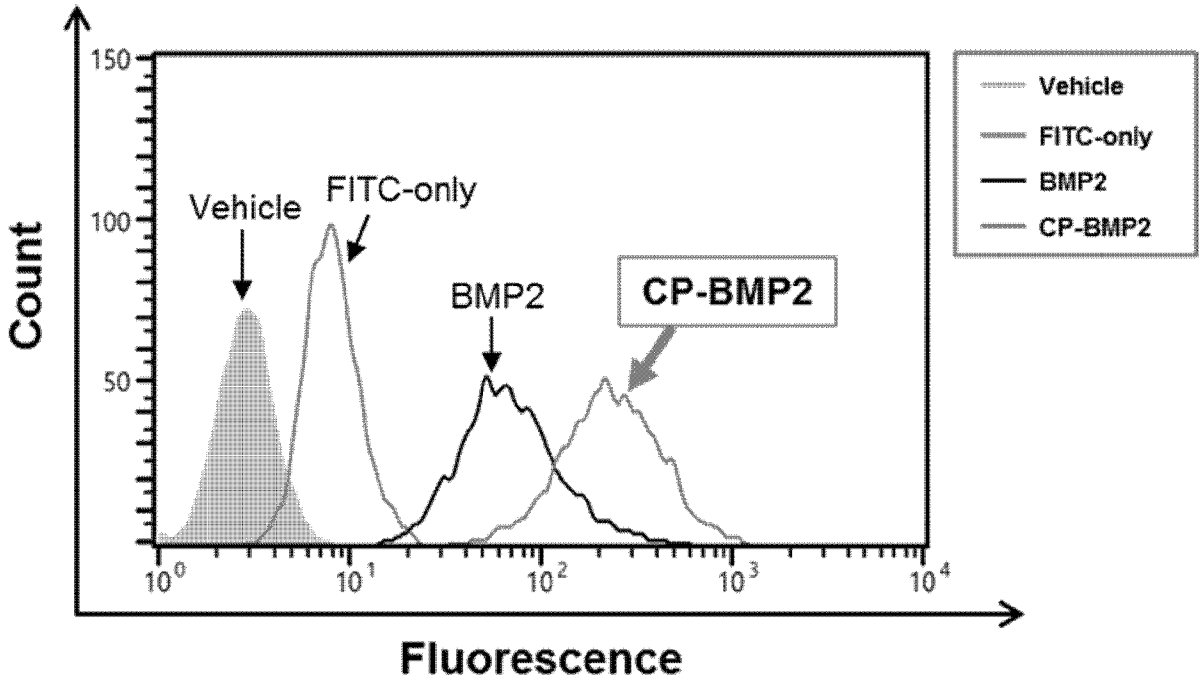
[Fig. 36b]



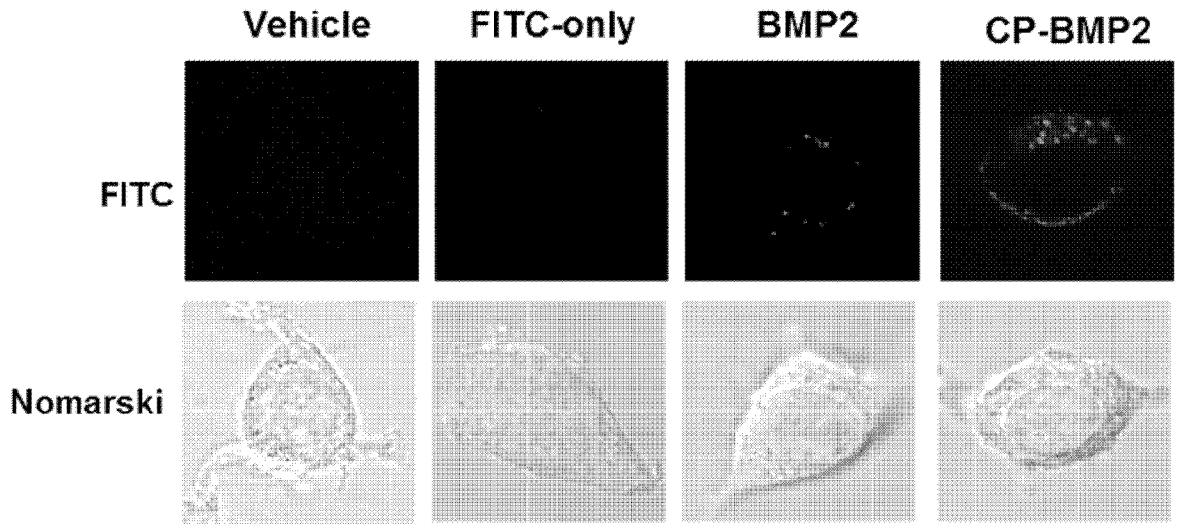
[Fig. 37]



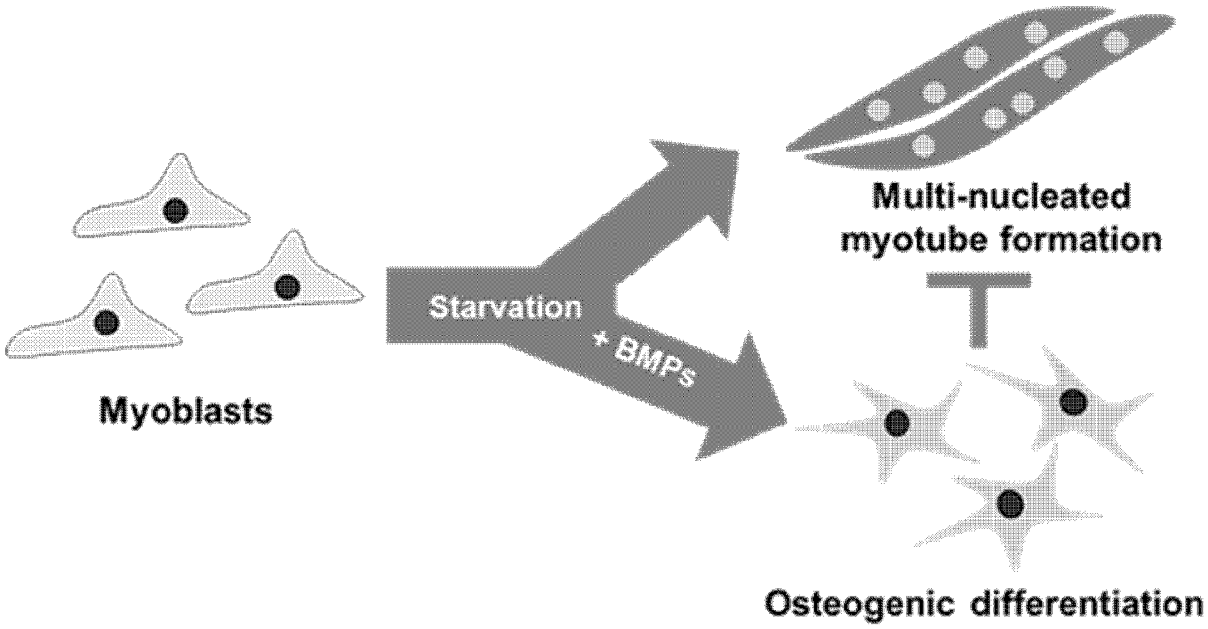
[Fig. 38]


Count
150
100
50
Vehicle
FITC-only
BMP2
CP-BMP2
10^0
10^1
10^2
10^3
10^4
Fluorescence
Vehicle
FITC-only
BMP2
CP-BMP2

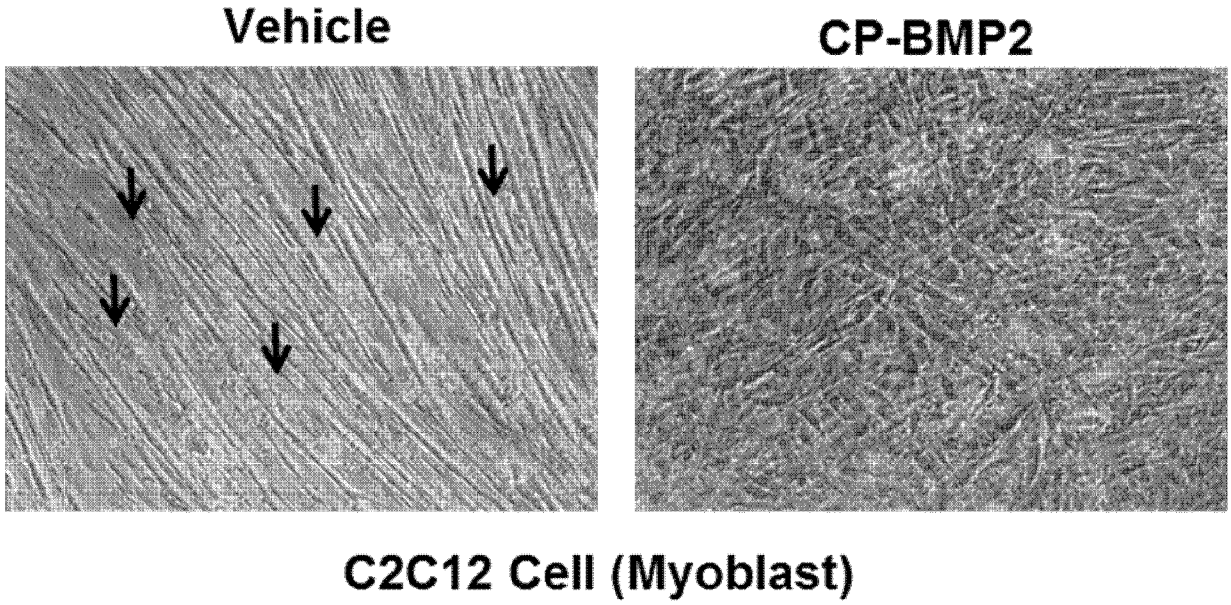
[Fig. 39]


Vehicle
FITC-only
BMP2
CP-BMP2
FITC
Nomarski

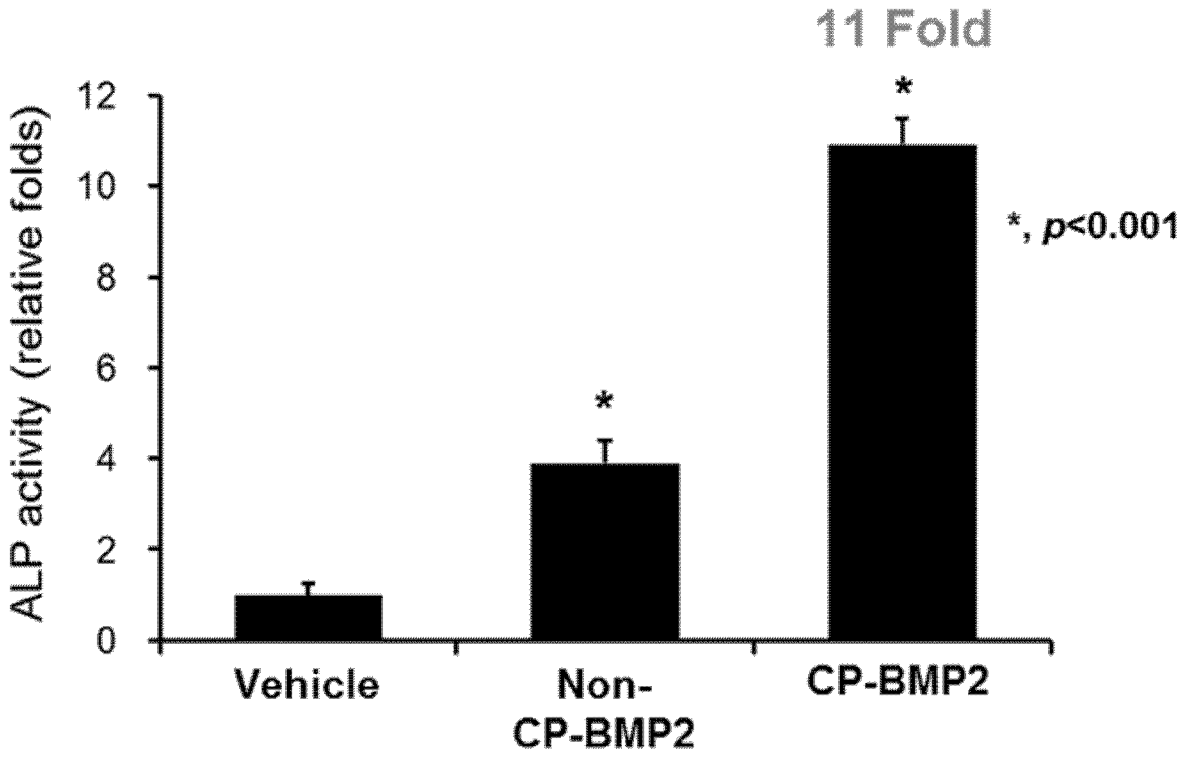
[Fig. 40a]



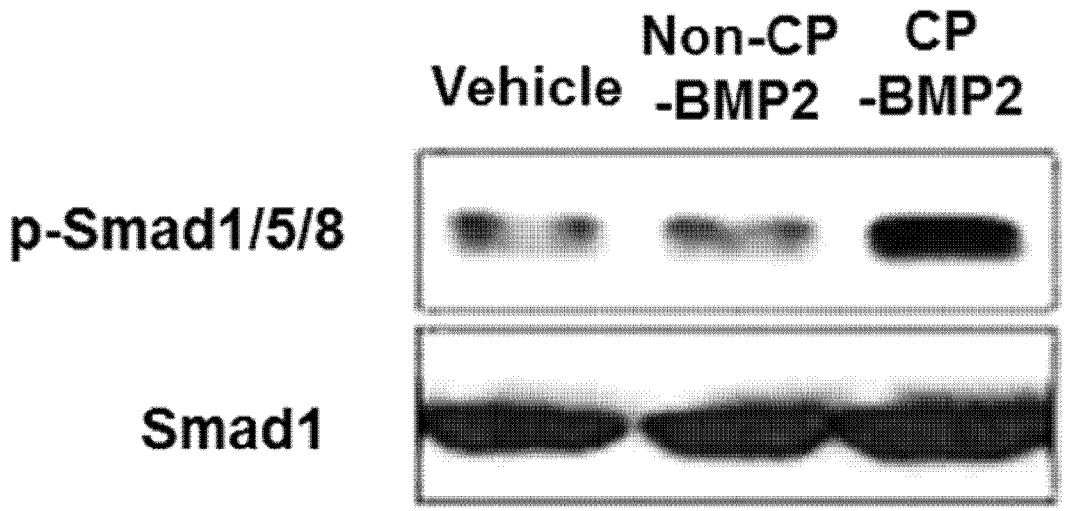
[Fig. 40b]



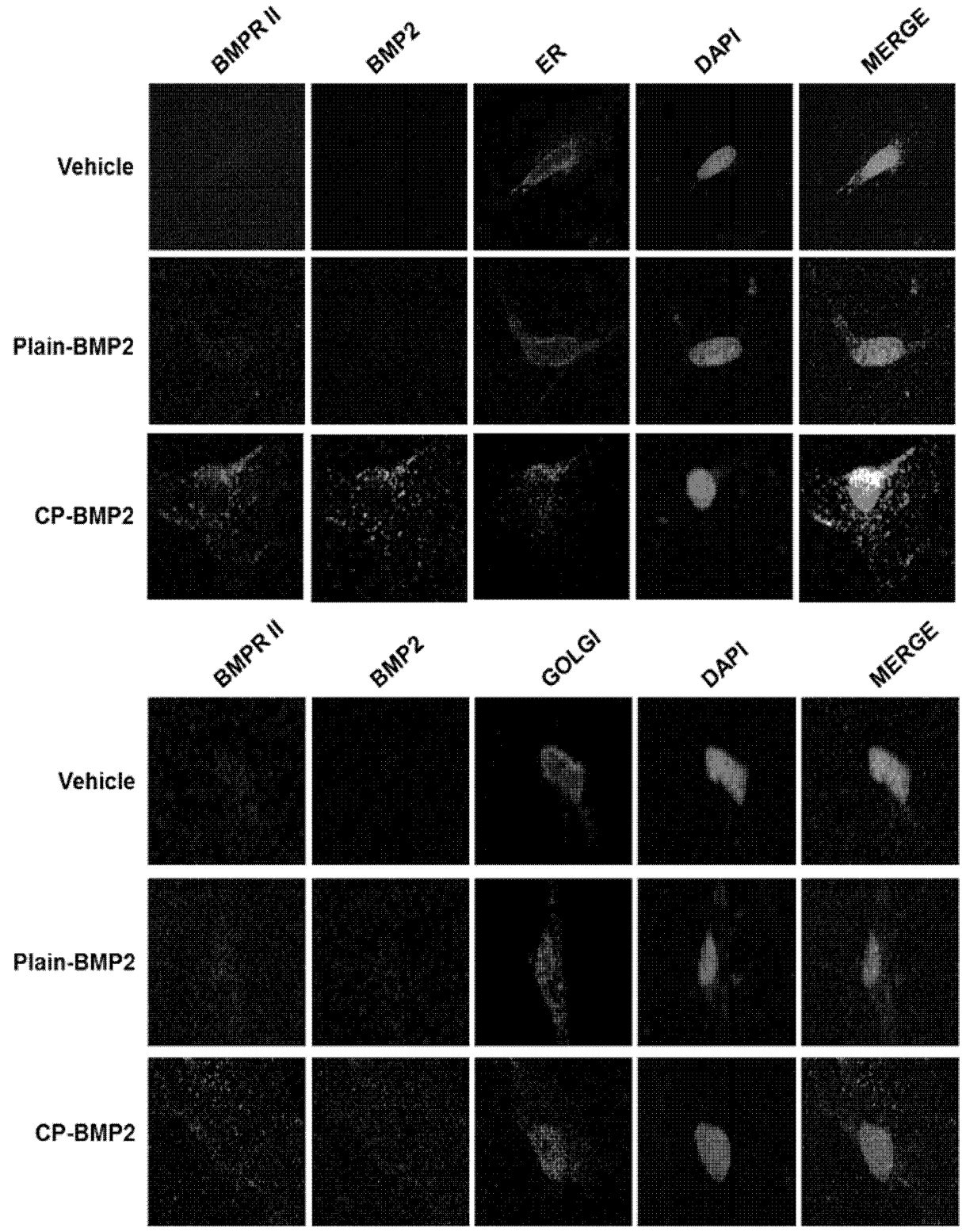
[Fig. 41]


11 Fold
ALP activity (relative folds)
12
10
8
6
4
2
0
*
*
*, p<0.001
Vehicle
Non-CP-BMP2
CP-BMP2

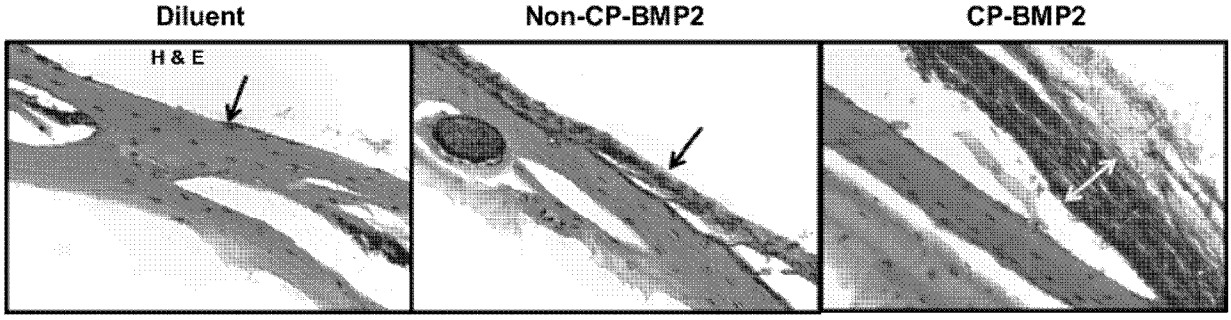
[Fig. 42]


Vehicle
Non-CP-BMP2
CP-BMP2
p-Smad1/5/8
Smad1

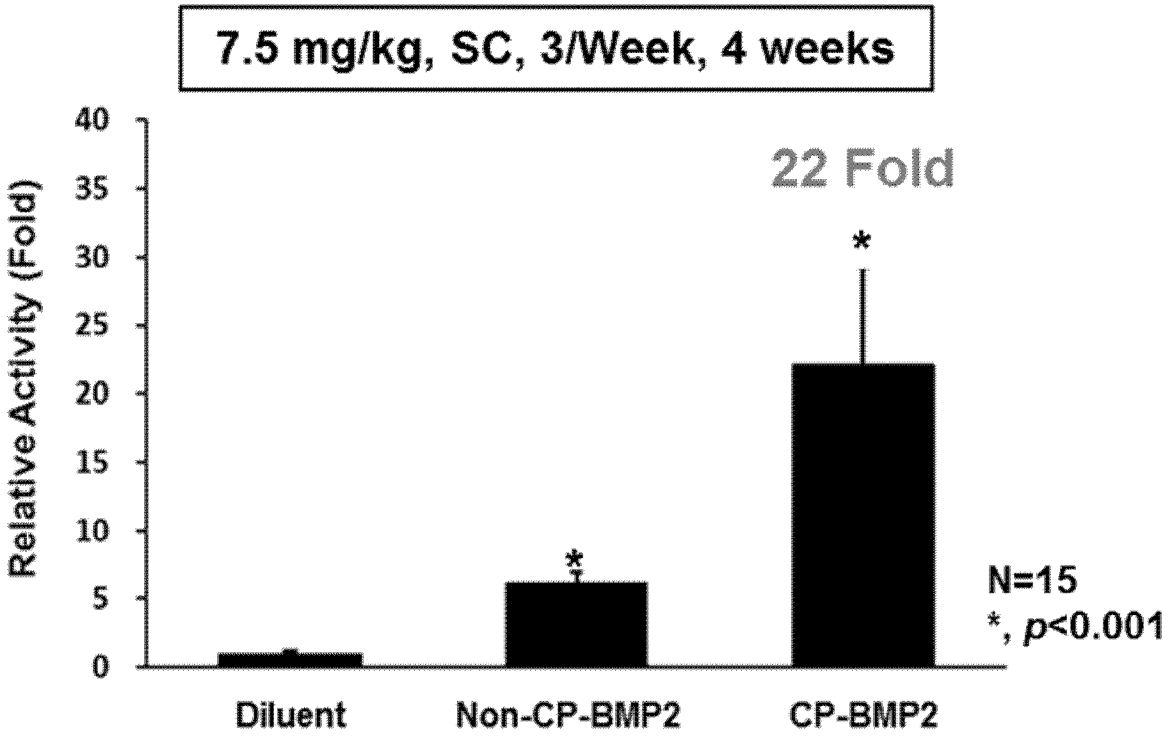
[Fig. 43]



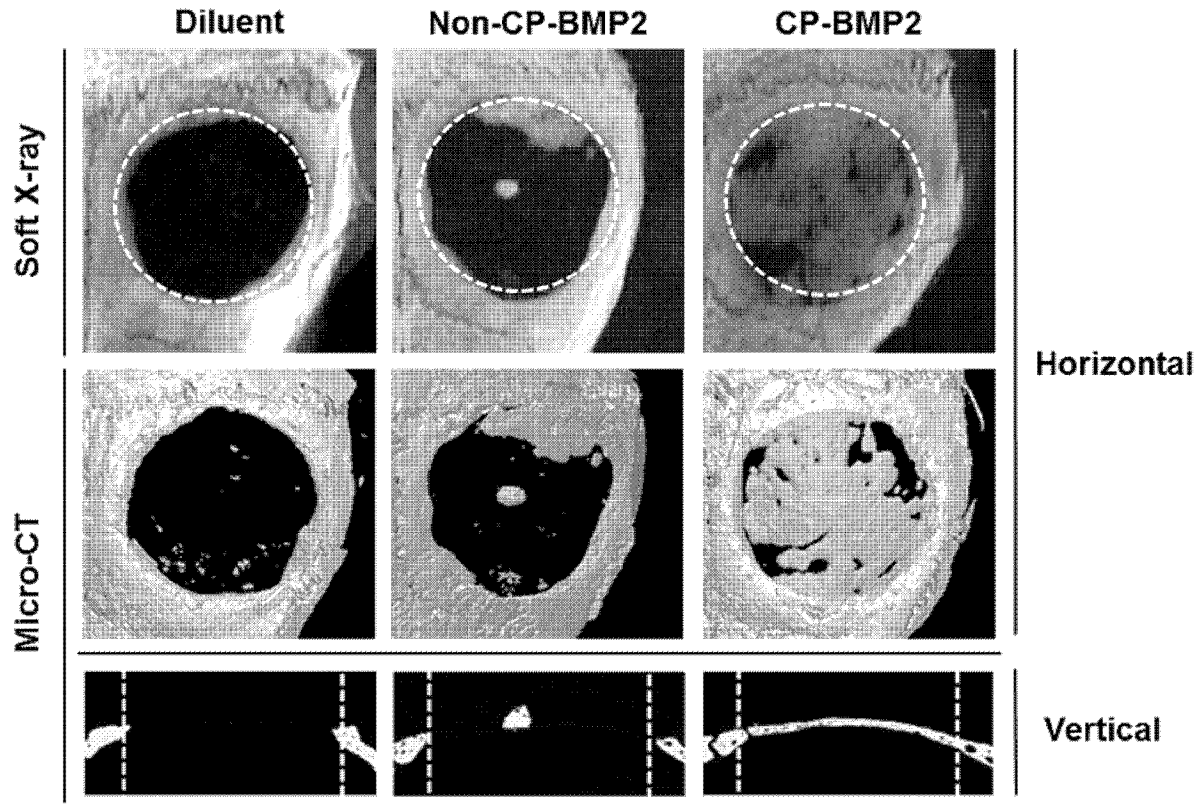
[Fig. 44a]



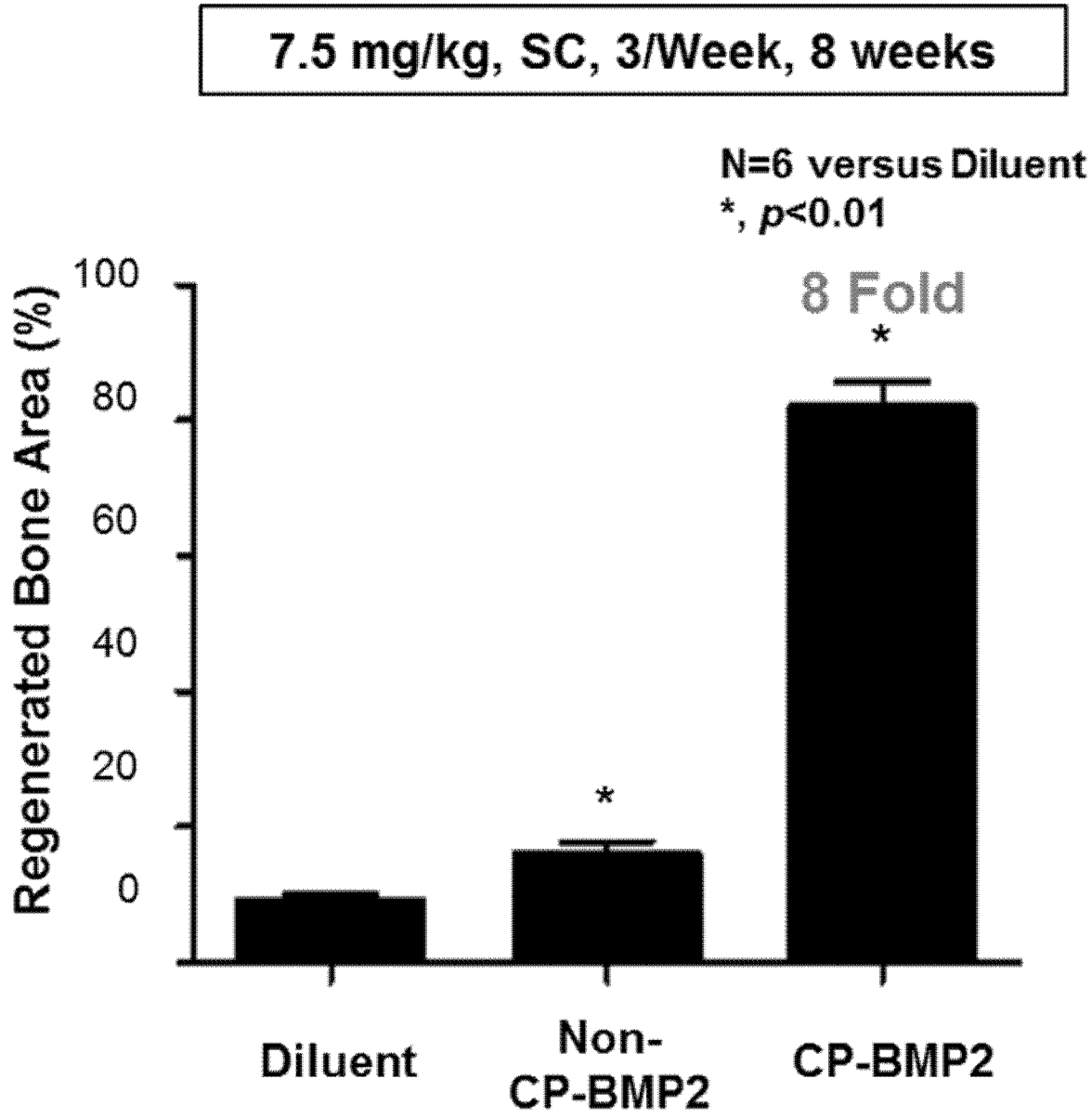
[Fig. 44b]



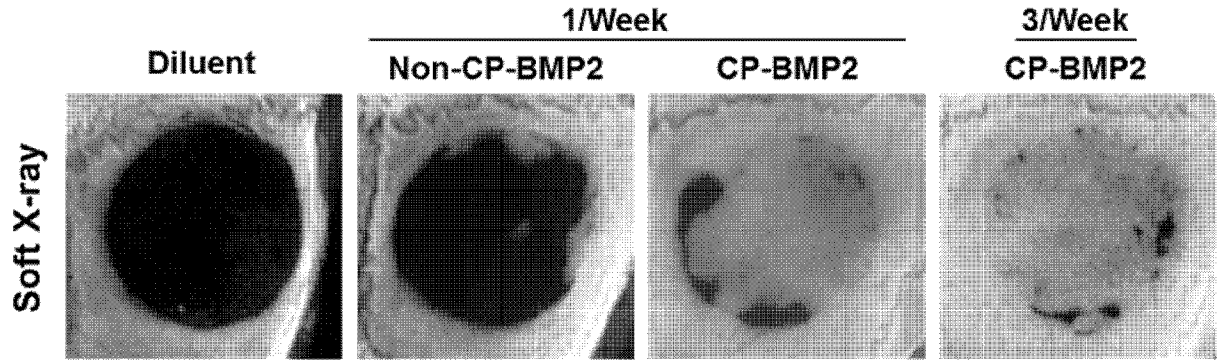
[Fig. 45a]



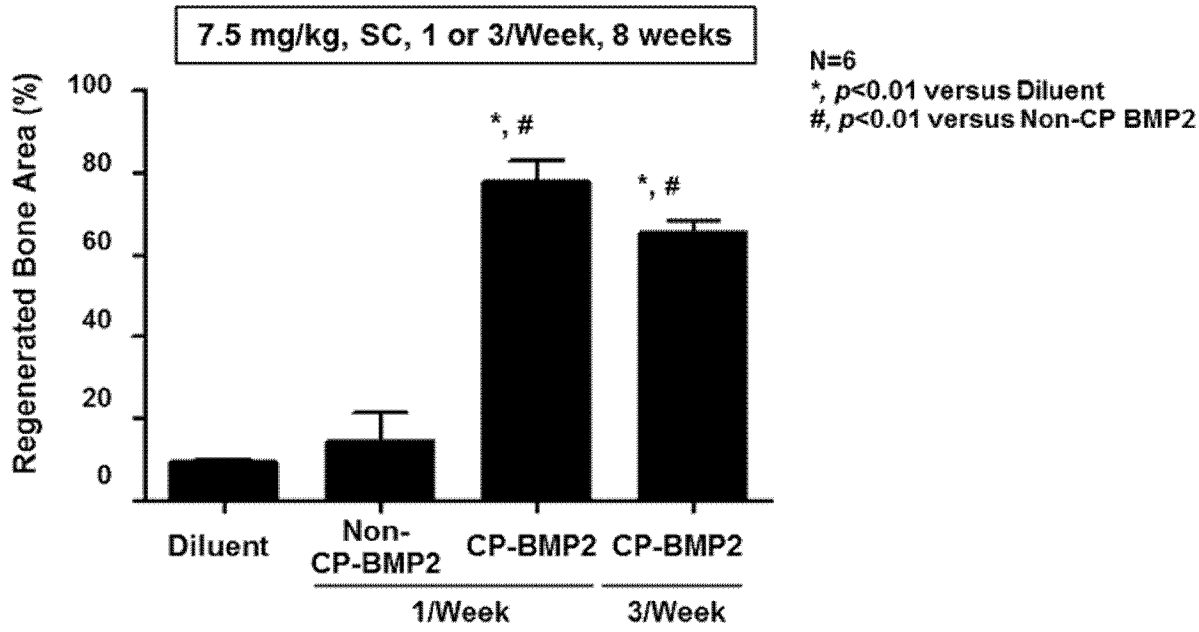
[Fig. 45b]



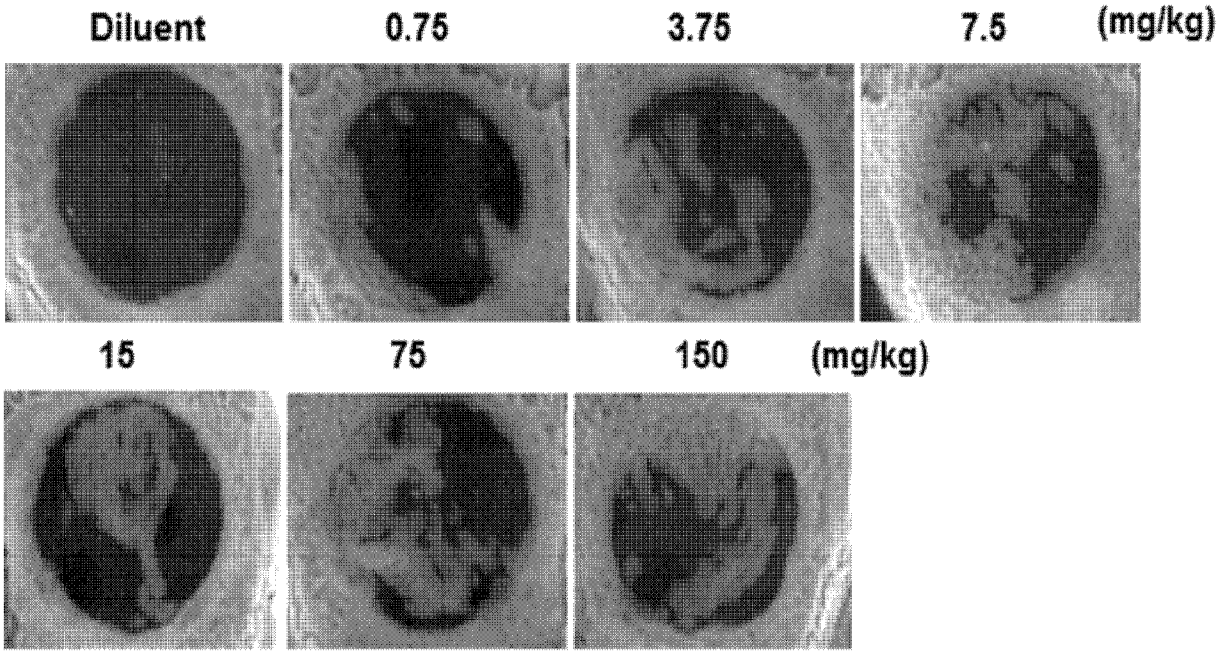
[Fig. 46a]



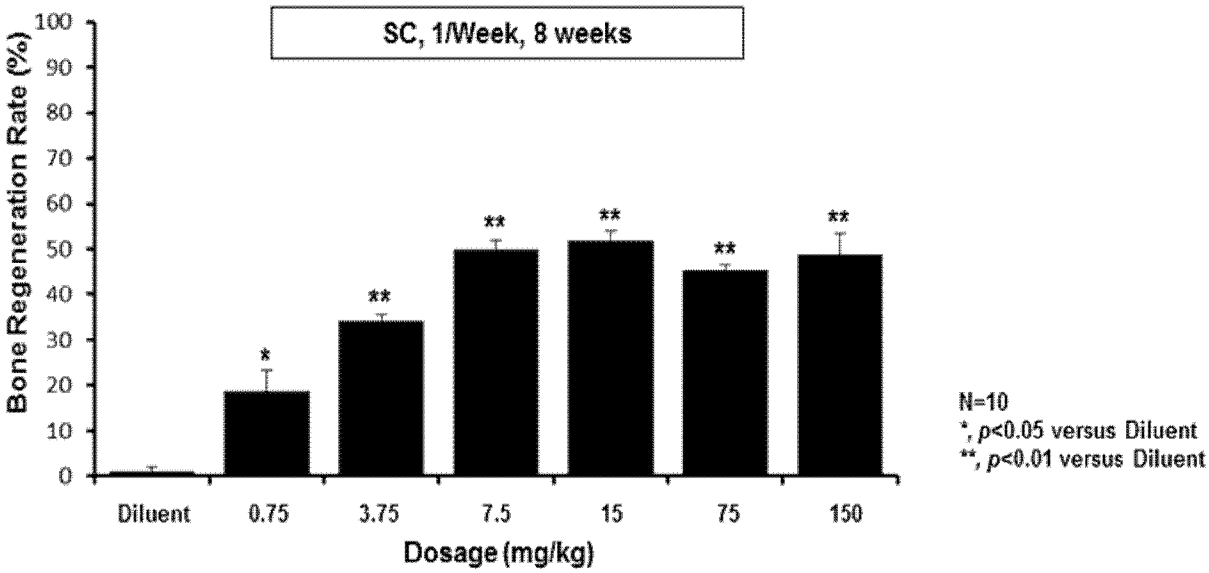
[Fig. 46b]



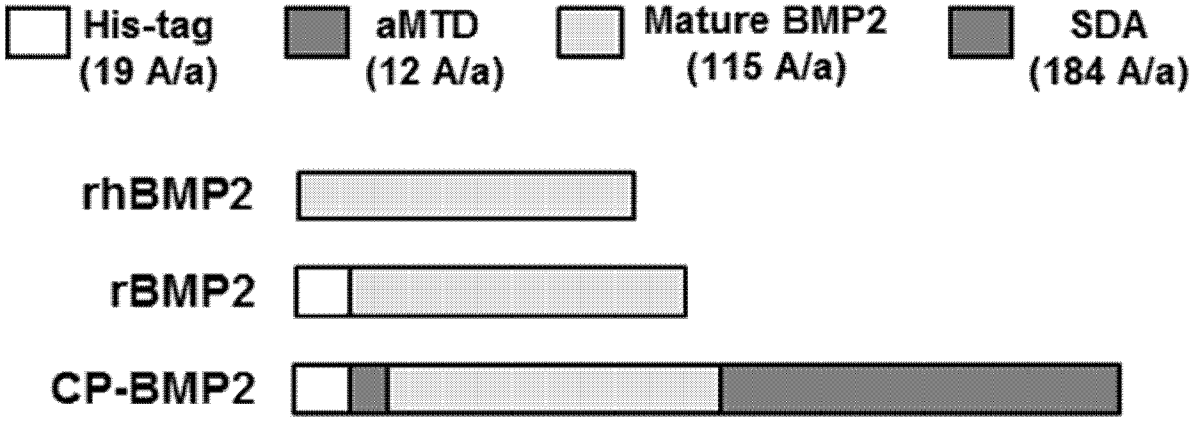
[Fig. 47a]



[Fig. 47b]



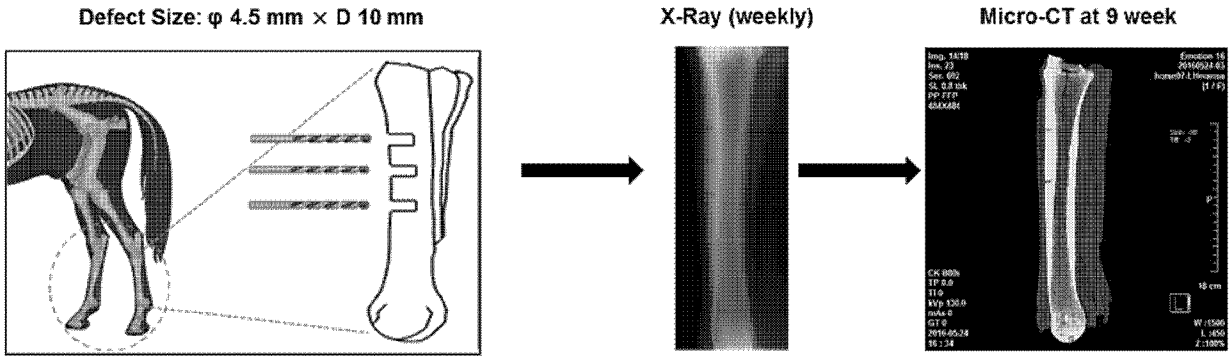
[Fig. 48]



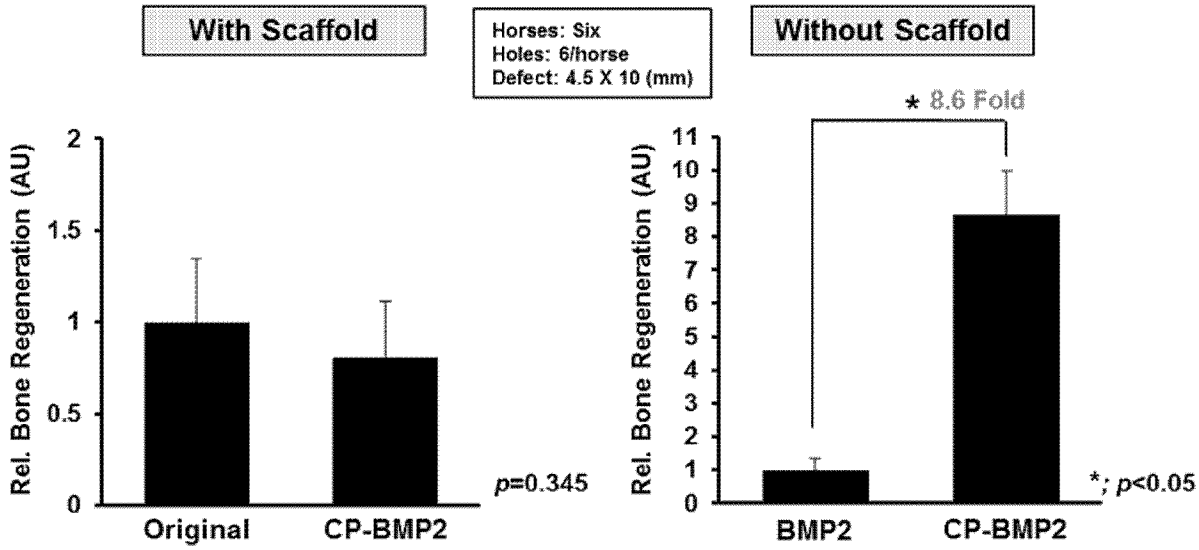
[Fig. 49a]

		Horses = 6
Age (Interquartile range)		5 (5-7.5)
Sex	F M	2 4
Weight		469 ± 48.9 kg

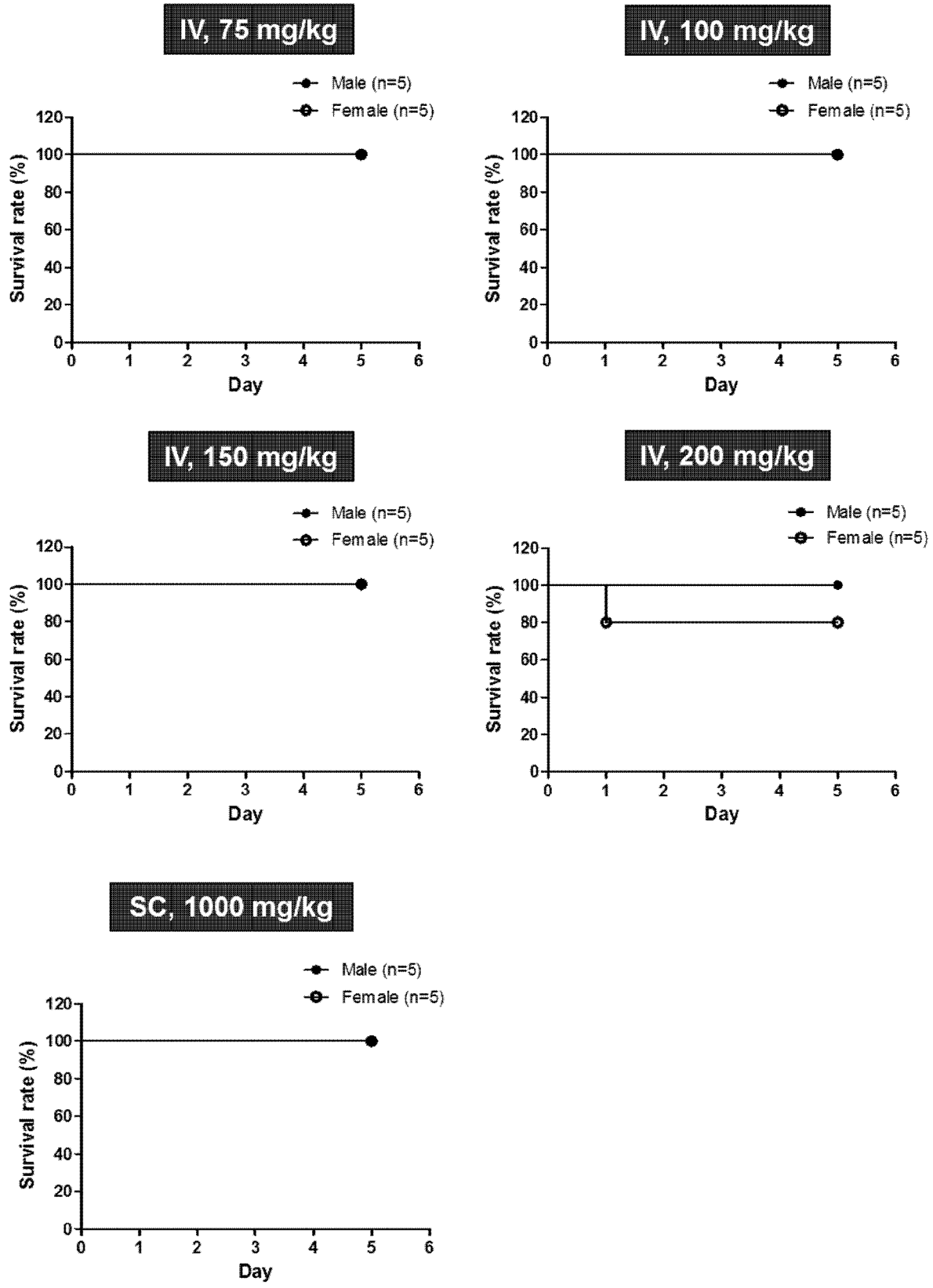
[Fig. 49b]



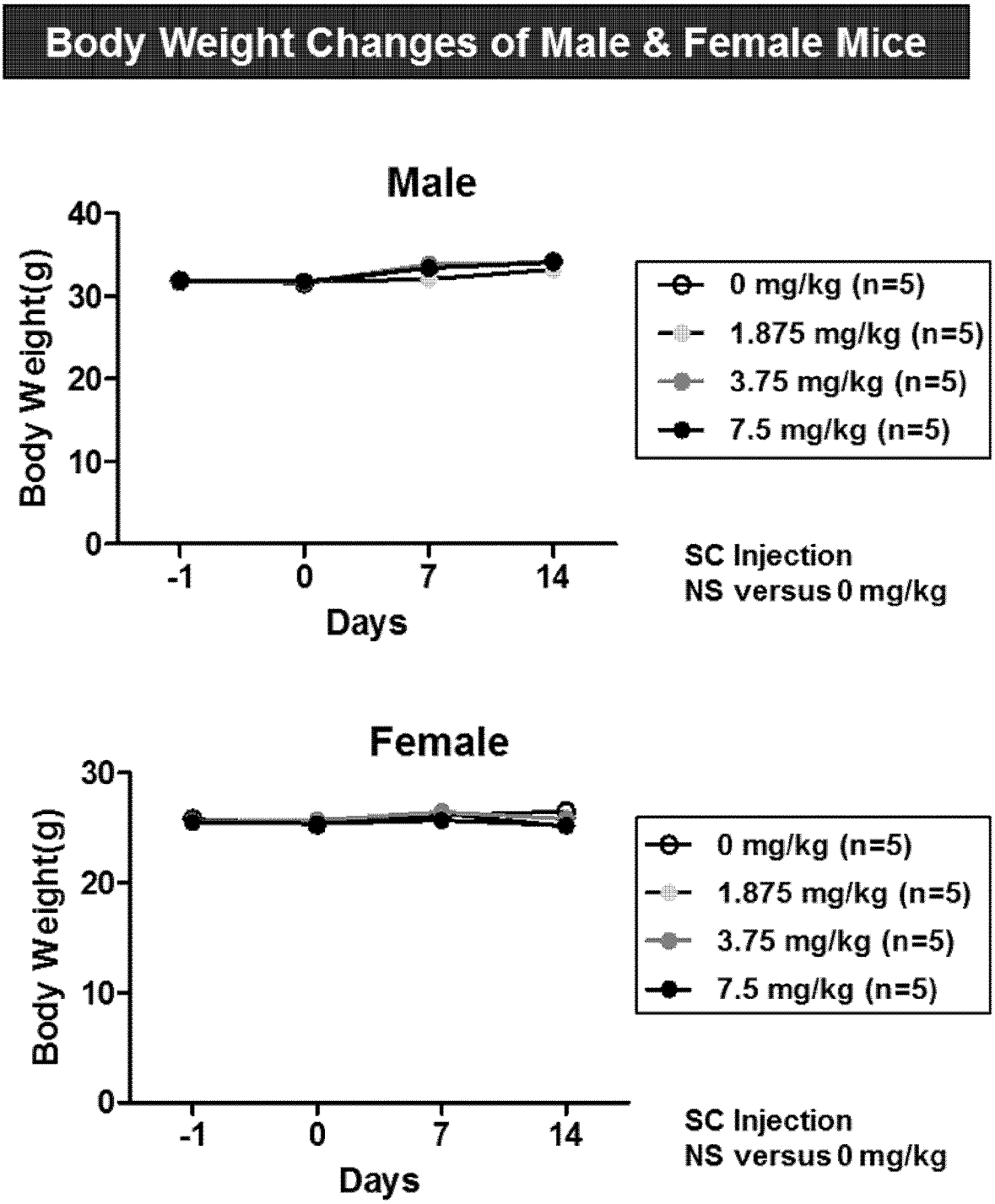
[Fig. 50]



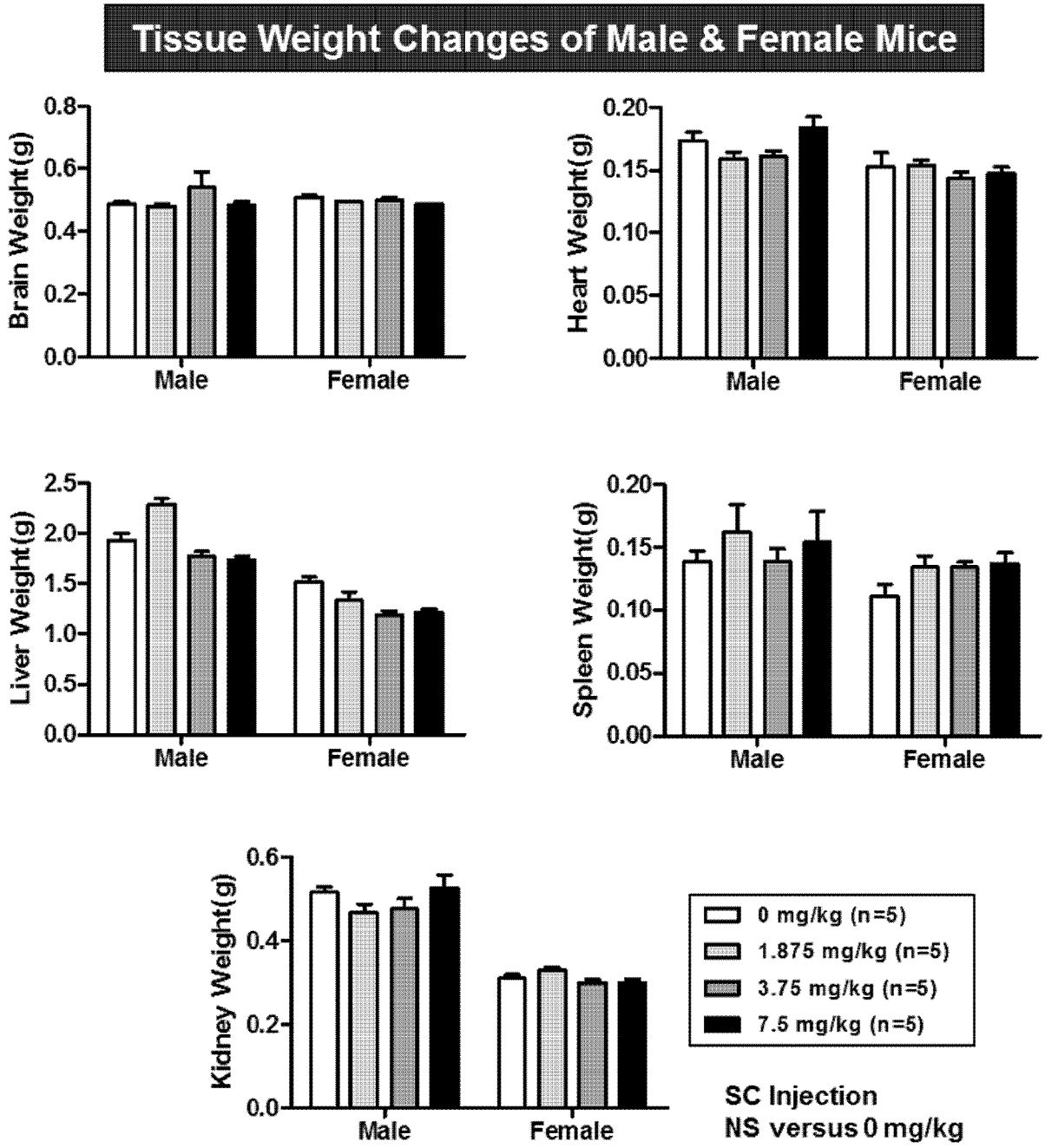
[Fig. 51]


IV, 75 mg/kg
IV, 100 mg/kg
IV, 150 mg/kg
IV, 200 mg/kg
SC, 1000 mg/kg
Male (n=5)
Female (n=5)
Survival rate (%)
Day
0
20
40
60
80
100
120
0
1
2
3
4
5
6

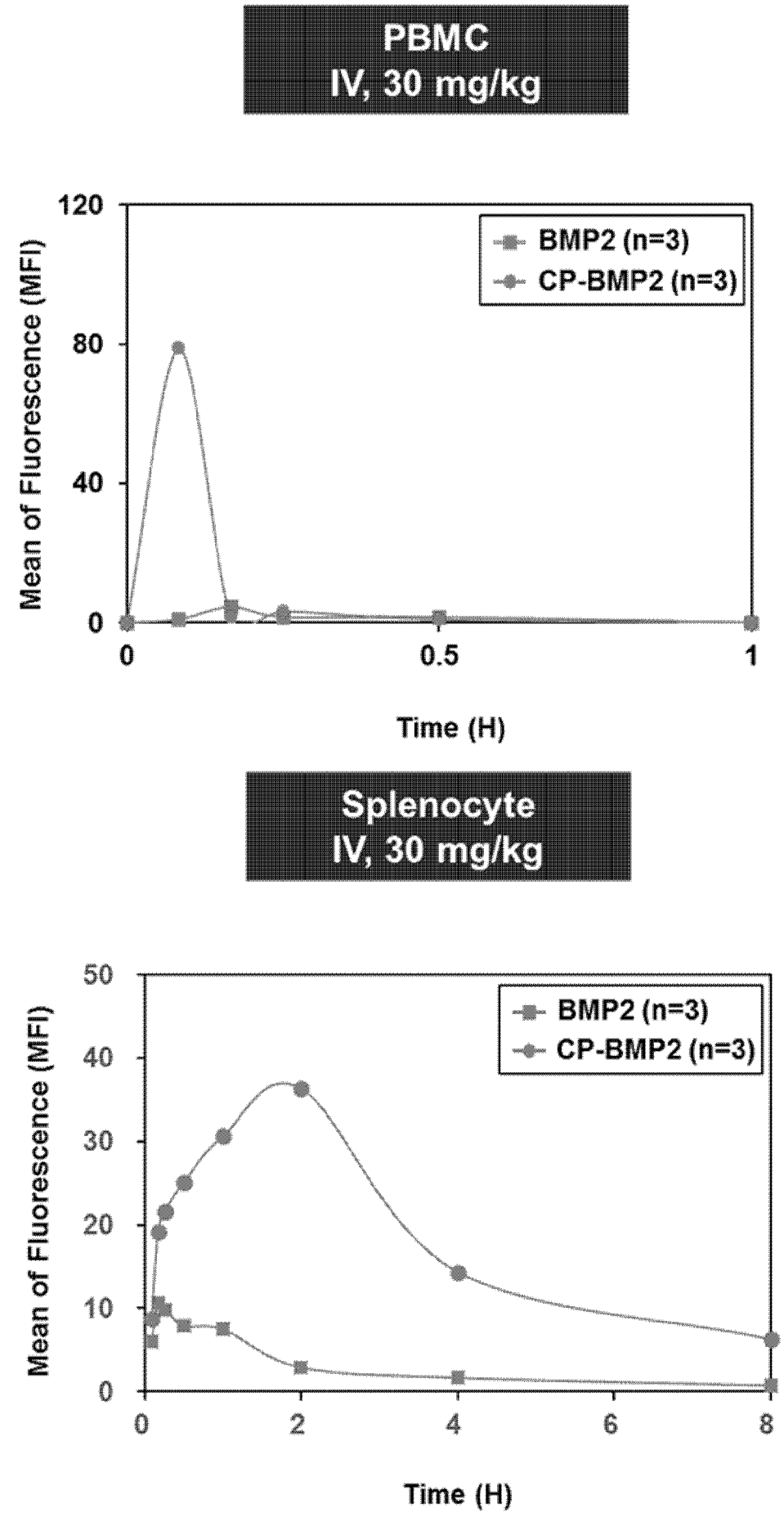
[Fig. 52a]


Body Weight Changes of Male & Female Mice
Male
Body Weight(g)
40
30
20
10
0
-1
0
7
14
Days
0 mg/kg (n=5)
1.875 mg/kg (n=5)
3.75 mg/kg (n=5)
7.5 mg/kg (n=5)
SC Injection
NS versus 0 mg/kg
Female
Body Weight(g)
30
20
10
0
-1
0
7
14
Days
0 mg/kg (n=5)
1.875 mg/kg (n=5)
3.75 mg/kg (n=5)
7.5 mg/kg (n=5)
SC Injection
NS versus 0 mg/kg

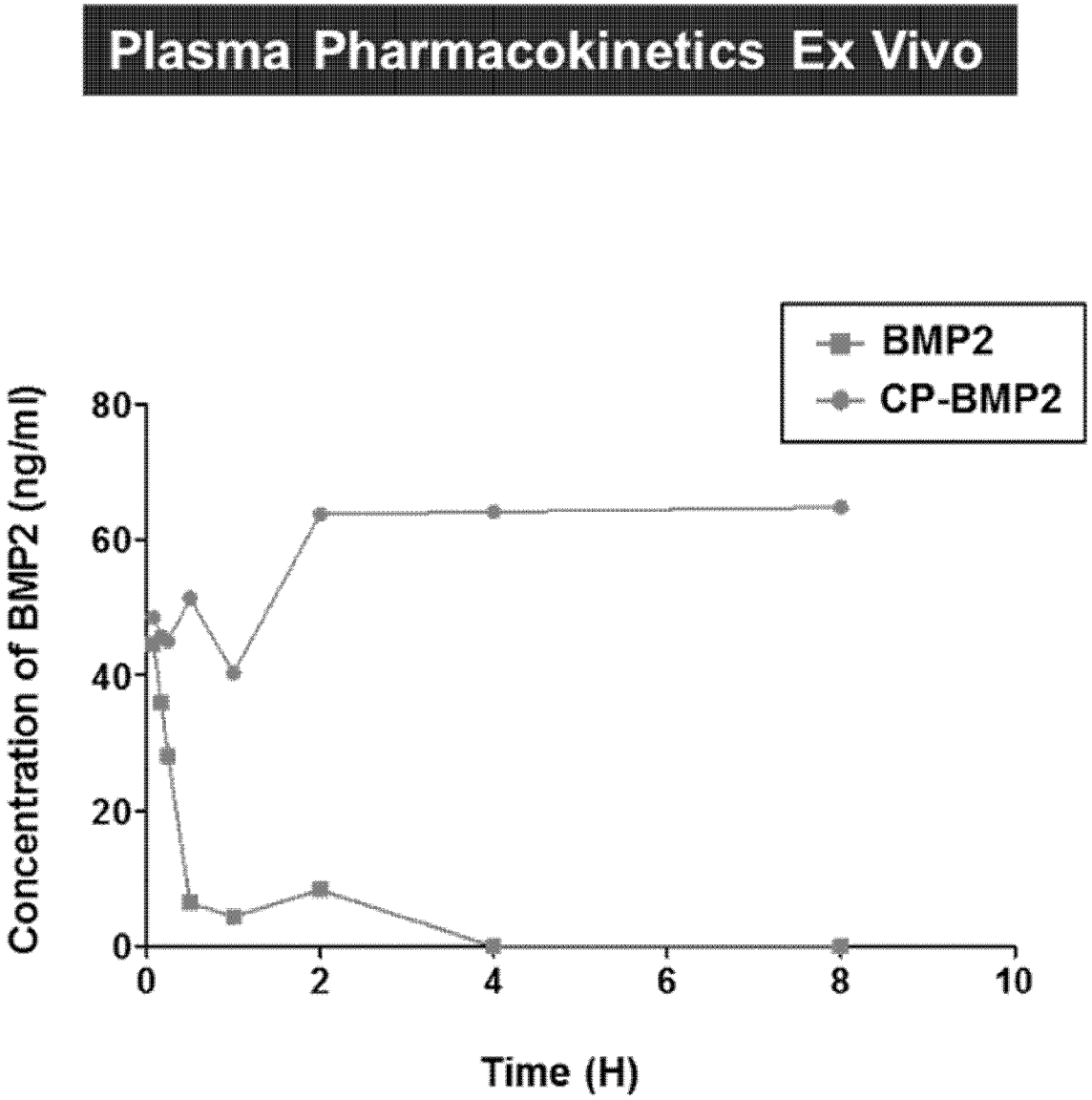
[Fig. 52b]


Tissue Weight Changes of Male & Female Mice
Brain Weight(g)
0.0
0.2
0.4
0.6
0.8
Male
Female
Heart Weight(g)
0.00
0.05
0.10
0.15
0.20
Male
Female
Liver Weight(g)
0.0
0.5
1.0
1.5
2.0
2.5
Male
Female
Spleen Weight(g)
0.00
0.05
0.10
0.15
0.20
Male
Female
Kidney Weight(g)
0.0
0.2
0.4
0.6
Male
Female
0 mg/kg (n=5)
1.875 mg/kg (n=5)
3.75 mg/kg (n=5)
7.5 mg/kg (n=5)
SC Injection
NS versus 0 mg/kg

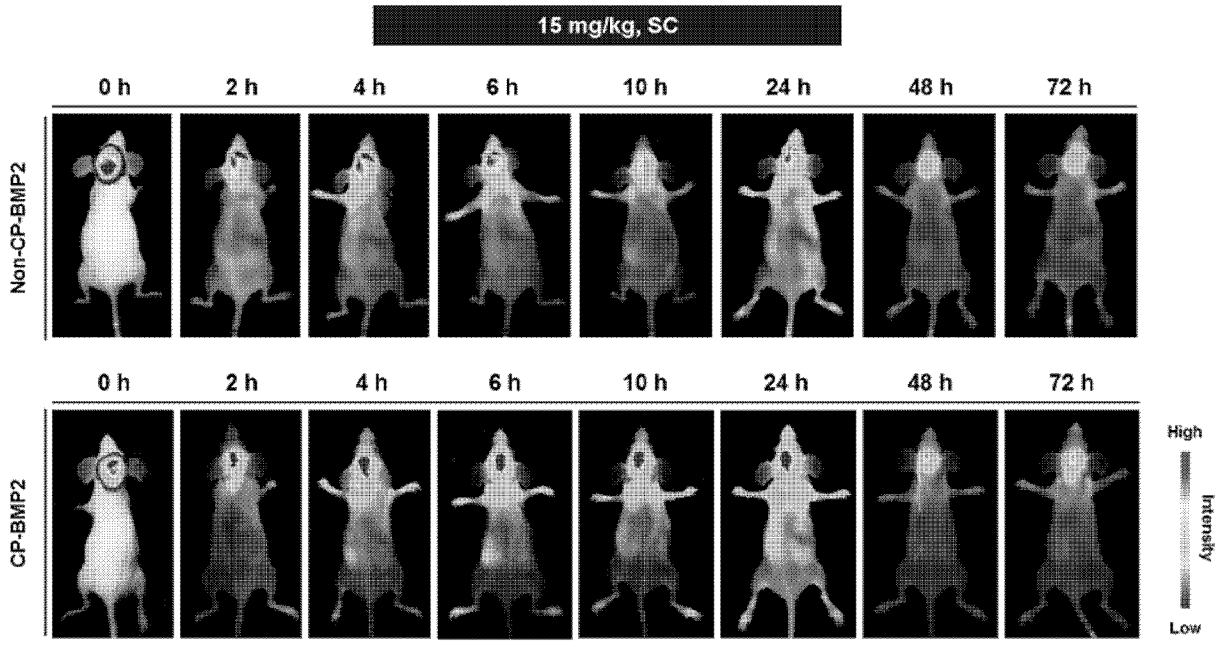
[Fig. 53]


PBMC
IV, 30 mg/kg
120
80
40
0
Mean of Fluorescence (MFI)
BMP2 (n=3)
CP-BMP2 (n=3)
0
0.5
1
Time (H)
Splenocyte
IV, 30 mg/kg
50
40
30
20
10
0
Mean of Fluorescence (MFI)
BMP2 (n=3)
CP-BMP2 (n=3)
0
2
4
6
8
Time (H)

[Fig. 54]


Plasma Pharmacokinetics Ex Vivo
BMP2
CP-BMP2
Concentration of BMP2 (ng/ml)
80
60
40
20
0
0
2
4
6
8
10
Time (H)

[Fig. 55]


15 mg/kg, SC
0 h
2 h
4 h
6 h
10 h
24 h
48 h
72 h
Non-CP-BMP2
CP-BMP2
High
Intensity
Low

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2016/009405

A. CLASSIFICATION OF SUBJECT MATTER

C07K 14/51(2006.01)i, C07K 7/08(2006.01)i, A61K 38/00(2006.01)i

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 14/51; C07H 21/04; C07K 7/08; C07K 19/00; C07K 14/475; C07K 7/06; C07K 14/00; C12P 21/02; A61K 38/10; C12N 15/62; A61K 47/48; A61K 38/00

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched
Korean utility models and applications for utility models
Japanese utility models and applications for utility models

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKOMPASS(KIPO internal) & keywords: CP-BMP, BMP2, BMP7, aMTD, permeability, proline, instability index, aliphatic index, GRAVY, regeneration, defected bone

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2010-0197598 A1 (JO et al.) 5 August 2010 See abstract; paragraphs [0051], [0087], [0090], [0133], [0149], [0150]; and claims 8-10, 17.	1-23,25-29
Y	US 2004-0197867 A1 (TITUS et al.) 7 October 2004 See abstract; claims 1-5.	1-23,25-29
A	WO 2012-050402 A2 (PROCELL THERAPEUTICS INC.) 19 April 2012 See abstract; claims 1-14.	1-23,25-29
A	US 2014-0186379 A1 (PROCELL THERAPEUTICS INC.) 3 July 2014 See abstract; claims 2-19.	1-23,25-29
A	KR 10-2008-0044710 A (KIM et al.) 21 May 2008 See abstract; claims 1-3.	1-23,25-29
PX	US 2016-0060319 A1 (JO et al.) 3 March 2016 See the whole document.	1-23,25-29

☐ 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	Date of mailing of the international search report
24 November 2016 (24.11.2016)	**25 November 2016 (25.11.2016)**

Name and mailing address of the ISA/KR
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INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2016/009405

Box No. II Observations where certain claims were found unsearchable (Continuation of item 2 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.: 30
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 30 is directed to a treatment method of the human body by therapy and thus relates to a subject matter which this International Searching Authority is not required, under PCT Article 17(2)(a)(i) and Rule 39.1(iv), to search.

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 24
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III Observations where unity of invention is lacking (Continuation of item 3 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required addtional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fees, this Authority did not invite payment of any additional fees.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.
☐ The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.
☐ No protest accompanied the payment of additional search fees.

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Information on patent family members

International application No.

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