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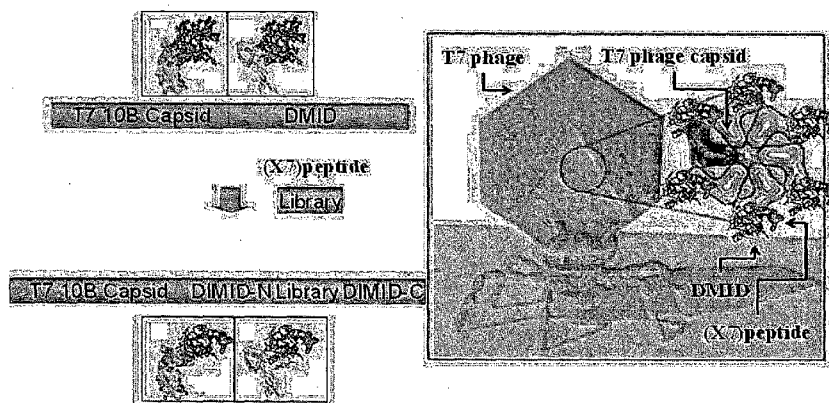
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(54) Title: CARDIAC TROPONIN I-TARGETING PEPTIDE AND USE THEREOF



(57) Abstract: As described above, the present invention provides a cardiac troponin I (cTnI)-targeting peptide and the use thereof. More specifically, the present invention provides a cardiac troponin I (cTnI)-targeting peptide comprising an amino acid sequence represented by SEQ ID NO: 17 or 24, and marker or composition for detecting and diagnosing cardiac troponin I (cTnI). The polypeptide of the present invention is effective in specifically detecting cardiac troponin I. Therefore, the peptide of the present invention can be useful for the diagnosis of cardiac troponin I (cTnI)-related condition/disease.



[DESCRIPTION]

[Invention Title]

CARDIAC TROPONIN I-TARGETING PEPTIDE AND USE THEREOF

[Technical Field]

<1> This application claims priority from and the benefit of Korean Patent Application No. 10-2011-0131973 filed on December 09, 2011, which is hereby incorporated by reference for all purposes as if fully set forth herein.

<2>

<3> The present invention relates to cardiac troponin I-targeting peptide and use thereof and more particularly, it relates to a cardiac troponin I (cTnI)-targeting peptide comprising the amino acid sequence represented the General Formula 1 as follows and use thereof.

<4> [General Formula 1]

<5> Arg-X₁-X₂-X₃-Arg-X₄-X₅

<6>

[Background Art]

<7> As indicated by the analysis of the amino acid sequences, secondary and tertiary structures of proteins, many proteins are composed of independent domains or modules. Domains are structurally and functionally independent units. One or more domains can be distributed in various proteins, and one protein can be composed of various domains. Specific information about domains can be found on websites for bioinformatics, including Prosite (Hulo N et al., Nucleic Acids Res, 36:D245-249, 2008; Website: <http://kr.expasy.org/prosite/>), SMART (Letunic I et al., Nucleic Acids Res, 34:D257-D260, 2006; Website: <http://smart.embl-heidelberg.de/>) and the like.

<8>

<9> Biopolymer substances, such as proteins, peptides or RNAs, exhibit their functions by interaction with target substances in vivo. Particularly, the basic units of these interactions are mediated by protein-protein interactions, and thus identification of a specific protein that binds to another protein is important as an index of the functional relationship between these proteins. Accordingly, identification of an unknown protein(s)

that binds to a specific protein can provide direct opportunities for studies on cell signaling mechanisms and determination of new drug targets and can provide base technology for discovery of a new drug candidate in a specific signaling system.

<io>

<ii> Interactions between biomolecules (for example, protein-protein interactions, protein-nucleic acid interactions, etc.) play important roles in various life phenomena such as the growth, differentiation and development of cells, intercellular/intracellular signaling, and mass transport. As known molecules that specifically bind to target molecules to control the biological activities of the target molecules, antibodies (full-length antibodies or their fragments) have been developed. However, the antibodies have various problems in that they are less expressed and low in solubility, require the use of animal cell-expressing cell lines, are purified in an expensive manner, and have low stability in a reducing intracellular environment. Accordingly, it is urgently required to develop non-antibody proteins that overcome the problems of antibodies and, at the same time, bind specifically to target molecules, like antibodies.

<12>

<13> Peptides for diagnosis and treatment of cancer or arteriosclerosis, discovered by methods such as phage display, have limitations, such as low binding affinity, non-stability and high immunogenicity. It is required to develop new technology, which can overcome such problems and increase the binding affinity and stability of active peptides in the human body while reducing the immunogenicity of the active peptides.

<14>

<15> As a method for screening peptides that bind specifically to target proteins, a T7 phage library has been effectively used. However, synthetic peptides screened by this method have a significantly low binding affinity for target proteins. In an attempt to increase the binding affinity between peptides and proteins, studies on increasing the affinity of a peptide by inserting the peptide into a specific protein scaffold so as to mimic

protein-protein interactions have been conducted. However, in this case, the affinity of the peptide inserted into the specific protein scaffold is not always increased. This is believed to be because either the structure of a peptide of a T7 phage library or the structure of a peptide synthesized from the amino acid sequence of the peptide differs from the tertiary structure of the peptide inserted into the specific protein scaffold.

<16>

<17> Myocardial infarction (MIF) is medically risky and has been the major cause of death and cardiovascular disease in the world. In all cases, introduced therapies aim to protect myocardial tissue from irreversible necrosis. These days, new therapies such as thrombolytic or primary angioplasty can rapidly restore coronary circulation to limit the size of infarcted sites and to reduce mortality and morbidity.

<18>

<19> The therapeutic means that are used after the expression of clinical symptoms is more effective when they are conducted as soon as possible. Clinicians who encounter the above pathology may have a more effective diagnostic device.

<20>

<21> Biochemical markers which have recently been used to diagnose heart disease patients include troponin I (Tnl), troponin T (TnT), lactate dehydrogenase (LDH), creatine kinase (CK), creatine kinase isoenzyme (CK-MB), and myoglobin.

<22>

<23> Lactate dehydrogenase exists in all the muscles of the human body, and the level thereof rises in the late stage of myocardial infarction. Thus, it has low specificity and sensitivity, and thus is not of great help for diagnosis.

<24>

<25> Creatine kinase is found in all muscles, and thus has no specificity for cardiac muscles, and the level thereof is increased by various factors, including muscular injury, trauma and brain injury.

<26>

<27> Myoglobin has very low specificity for cardiac muscles, because the level thereof increases even when other muscles are injured.

<28>

<29> Creatine kinase isoenzyme has most commonly been used as a biochemical marker of myocardial infarction. However, the level of creatine kinase isoenzyme changes fast in the acute stage of myocardial infarction, and thus should be measured several times, and the measured CK-MB levels should be obtained by complex mathematical equations. In addition, when a myocardial infarction patient visits a hospital after 24 hours of myocardial infarction, the level of creatine kinase isoenzyme has low correlation with the size of infarction and significantly changes depending on reperfusion.

<30>

<31> The above biochemical markers of heart diseases commonly show severe variations in measurements depending on the disease, have low correlation with the degree of myocardial infarction, and have low sensitivity and low diagnostic values in the case of fine myocardial injury such as unstable angina at high risk, which is not infarction.

<32>

<33> Meanwhile, in recent years, troponin I or T has been used as a biochemical marker of heart disease in some cases. Particularly, troponin_I is not found in general skeletal muscles and exists only in adult cardiac muscles, and thus it is not detected in blood in conditions in which creatine kinase isoenzyme levels can be increased, other than myocardial injury. Thus, troponin I is highly specific to myocardial injury and also shows correlation with the size of myocardial infarction.

<34>

In addition, in the case of atypical angina that is not acute myocardial infarction, troponin is released from myocardial cells, and thus is useful as an index for determining the prognosis of atypical angina.

<35>

<36> Measurement of blood troponin is also helpful in evaluating the therapeutic effect of reperfusion therapy. After thrombolysis has been

initiated, a rapid increase in troponin levels can be observed. This is because washout of cytoplasmic constituents occurs due to reperfusion.

<37>

<38>

As described above, troponin is specific to cardiac muscles, an increase in the blood level thereof is continued for a long period of time upon myocardial injury, and it does not appear in normal persons and non-cardiac diseases. Thus, troponin makes it possible to diagnosis even a slight degree of myocardial infarction, and therefore is a very useful index for diagnosing ischemic cardiac disease and acute myocardial infarction and determining the prognosis thereof.

<39>

[Disclosure]

[Technical Problem]

<40>

Accordingly, the present inventors have thought that performing screening in a state wherein a peptide was inserted and expressed in a specific protein scaffold will be advantageous for maintaining the high affinity of the peptide inserted in the protein scaffold for a target peptide. Based on this thinking, the present inventors have constructed a peptide library by inserting into a T7 phage a protein scaffold which increases the binding affinity and specificity of an active peptide, and have completed the present invention by using the constructed peptide library.

<41>

<42>

It is an object of the present invention to provide an isolated cardiac troponin I (cTnI)-targeting peptide comprising an amino acid sequence represented by the following general formula 1:

<43>

[General Formula 1]

<44>

Arg- X_1 - X_2 - X_3 -Arg- X_4 - X_5

<45>

wherein X_1 is Cys or Ala, X_2 or X_3 is Ala or Ser, X_4 is Arg or Leu, and X_5 is Ser or Trp.

<46>

<47>

Another another object of the present invention is to provide an isolated polynucleotide having nucleotide sequence that encodes the above

cardiac troponin I (cTnI)-targeting peptide, and a vector and a transformant comprising the polynucleotide.

<48>

<49> Still another object of the present invention is to provide a marker and a composition for detecting cardiac troponin I (cTnI) comprising the polypeptide as an active ingredient.

<50>

<51> Still another object of the present invention is to provide a composition for diagnosing cardiac troponin I (cTnI)-related condition/disease comprising the polypeptide as an active ingredient.

<52>

<53> Still another object of the present invention is to provide the use of the polypeptide for preparation of an agent for diagnosing cardiac troponin I (cTnI)-related condition/disease.

<54> Still another object of the present invention is to provide a method for diagnosing cardiac troponin I (cTnI)-related condition /disease comprising a step of: (a) contacting the polypeptide of claim 1 with a sample and a label bound thereto, wherein the label is selected from the group consisting of chromogenic enzymes, radioisotopes, chromophores, light emitting materials, fluorescers, super paramagnetic particles, and ultrasuper paramagnetic particles,' and (b) detecting cardiac troponin I (cTnI) from the sample.

<55>

[Technical Solution]

<56> In order to accomplish the object, the present invention provides an isolated cardiac troponin I (cTnI)-targeting peptide comprising an amino acid sequence represented by the following general formula 1:

<57> [General Formula 1]

<58> Arg-Xi-X₂-X₃-Arg-X₄-X₅

<59> wherein Xi is Cys or Ala, X₂ or X₃ is Ala or Ser, X₄ is Arg or Leu, and X₅ is Ser or Trp.

<60>

<61> In order to accomplish another object, the present invention provides an isolated polynucleotide having nucleotide sequence that encodes the above cardiac troponin I (cTnI)-targeting peptide, and a vector and a transformant comprising the polynucleotide.

<62> In order to accomplish still another object, the present invention provides a marker and a composition for detecting cardiac troponin I (cTnI) comprising the polypeptide as an active ingredient.

<63> In order to accomplish still another object, the present invention provides the use of the polypeptide for preparation of an agent for diagnosing cardiac troponin I (cTnI)-related condition/disease.

<64> In order to accomplish still another object, the present invention provides a method for diagnosing cardiac troponin I (cTnI)-related condition/disease comprising a step of: (a) contacting the polypeptide of claim 1 with a sample and a label bound thereto, wherein the label is selected from the group consisting of chromogenic enzymes, radioisotopes, chromophores, light emitting materials, fluorescers, super paramagnetic particles, and ultrasuper paramagnetic particles; and (b) detecting cardiac troponin I (cTnI) from the sample.

<65>

<66>

<67> Hereinafter, the present invention will be described in more detail.

<68>

<69> The present invention provides an isolated cardiac troponin I (cTnI)-targeting peptide comprising an amino acid sequence represented by the following general formula 1:

<70> [General Formula 1]

<71> **Arg-X₁-X₂-X₃-Arg-X₄-X₅**

<72> wherein X₁ is Cys or Ala, X₂ or X₃ is Ala or Ser, X₄ is Arg or Leu, and X₅ is Ser or Trp.

<73>

<74> In one Example of the present invention, a plaque assay was performed, and as a result, it was found that a phage comprising the polypeptide of the

present invention has an excellent ability to bind to cardiac troponin I (cTnI) and does not bind to other marker substances for diagnosing heart attack. In another Example of the present invention, it was found by ELISA that a phage clone comprising the polypeptide of the present invention binds specifically to cardiac troponin I (cTnI).

<75> Meanwhile, the present invention provides a polypeptide consisting of an amino acid sequence represented by SEQ ID NO: 17 or 24.

<76> As used herein, the term "polypeptide fragment", "peptide" or "polypeptide" is meant to include all kinds of peptides, proteins, peptide mimetics, compounds and biological agents, which have activity capable of binding specifically to cardiac troponin I (cTnI). The polypeptide of the present invention may be derived from natural sources and may also be synthesized using known peptide synthesis methods.

<77>

<78> In addition, the scope of the polypeptide of the present invention includes not only peptides having native amino acid sequences, but also amino acid sequence variants thereof. As used herein, the expression "variant of the peptide of the present invention" refers to a peptide having a sequence that is different from the amino acid sequence of SEQ ID NO: 17 or 24 due to a deletion, an insertion, a non-conservative or conservative substitution or combinations thereof of one or more amino acid residues. Amino acid exchanges which do not generally alter the activity of molecules are known in the art (H. Neurath, R. L. Hill, The Proteins, Academic Press, New York, 1979).

<79> According to circumstances, the peptide of the present invention may be phosphorylated, sulfated, acrylated, glycosylated, methylated, farnesylated.

<80>

<81> In addition, the present invention provides a polypeptide having nucleotide sequence encoding the polypeptide of the present invention. The polynucleotide finally could be any combination of nucleotide sequence as long as it encodes the polypeptide of the present invention.

<82>

<83> Further, the present invention provides a polynucleotide sequence

having a nucleotide sequence that encodes the above polypeptide, and a vector and a transformant comprising the polynucleotide.

<84>

<85> The vector of the present invention may comprise, but not limited thereto, plasmid vector, cosmid vector, bacteriophage vector and viral vector and etc. The vector of the present invention may be a conventional cloning vector or expression vector and the expression vector may comprise membrane targeting sequence or signal sequence for secretion as well as expression regulating sequences such as promotor, operator, initiation codon, termination codon, polyadenylated signal and enhance (enhancing gene) and it may be prepared in accordance with purpose. In addition, the vector comprises a selection marker for selecting the host cell having the vector and in case if replicable vector, it comprises the replication origin.

<86>

<87> The transformation with the vector can be performed with well known skills in the art. Preferably, microprojectile bombardment, electroporation, CaPO_4 precipitation, CaCl_2 precipitation, PEG-mediated fusion, microinjection and liposome-mediated method and the transfectant may be, but not limited thereto, *Escherichia coli*, *Bacillus subtilis*, *Streptomyces*, *Pseudomonas*, *Proteus mirabilis*, *Staphylococcus*, *Agrobacterium tumefaciens*.

<88>

<89> The present inventors conducted an experiment in order to examine the functions of screened polypeptides that bind specifically to cardiac troponin I (cTnI). As a result, it was found that the polypeptide of the present invention shows binding specificity to cardiac troponin I (cTnI) stronger than to BSA or CKB protein.

<90>

Thus, it could be seen that the polypeptide of the present invention can be used as a marker or a composition for detecting cardiac troponin I (cTnI), and furthermore, can be used as a diagnostic composition for detecting cardiac troponin I (cTnI) in sample, specifically, blood or urine.

<91>

<92> In one Example of the present invention, phages that bind specifically

to cardiac troponin I (cTnI) were screened using a general peptide library. As a result, phages that bind specifically to cardiac troponin I (cTnI) could be screened by a total of 5 rounds of screening, and the results of sequencing of the screened phages indicated that polypeptides having amino acid sequences of PAAAMRV represented by SEQ ID NO: 15, SSR^TGSQ represented by SEQ ID NO: 16, RASSRLW represented by SEQ ID NO: 17, and RCAARRS represented by SEQ ID NO: 24 were mainly screened.

<93> In one Example of the present invention, whether the screened polypeptides of the present invention bind specifically to cardiac troponin I (cTnI) was examined. As a result, it could be seen that the polypeptide of the present invention, which has an amino acid sequence of RASSRLW represented by SEQ ID NO: 17, binds to cardiac troponin I (cTnI) more strongly than to BSA or CKB protein.

<94> In conclusion, it could be seen that the polypeptide of the present invention can bind specifically to cardiac troponin I (cTnI).

<95> The polypeptide of the present invention enabled the detection of cTnI in a phosphate solution condition (5% BSA, 0.05% Tween, pH 7.4) which coincides with the blood albumin level of normal persons (3.4-5.4 g/dl (-5%)). This shows that cTnI can be sufficiently detected in blood by the polypeptide of the present invention, although data are not shown. Detection of cTnI in urine was reported to have no great significance in myocardial infarction patients, but detection of cTnI in urine is also significant in that cTnI is detected in urine samples from renal failure/myocardial infarction patients. For reference, cTnI is not detected in the urine of myocardial infarction patients having normal renal function. Although data are not shown, it can be predicted that cTnI can be sufficiently detected in urine by the polypeptide of the present invention, when considering the fact that the urine of normal persons has a pH of 4.6-8.0, particularly 7.0 [Nephrol Dial Transplant (2008) 23: 231-238].

<96>

<97> The above polypeptide means cardiac troponin I (cTnI)-specific polypeptide screened in the present invention, and a sample may be, for

example, blood or urine. When the composition of the present invention is mixed with a sample, cardiac troponin I (cTnI) in the sample can be detected.

<98>

<99> The present invention provides a marker for detecting cardiac troponin I (cTnI) comprising the polypeptide.

<i00>

<i01> The present invention provides a composition for detecting cardiac troponin I (cTnI) comprising the polypeptide as an active ingredient.

<102>

<i03> The present invention provides a composition for detecting cardiac troponin I (cTnI), wherein the polypeptide is labeled with one selected from the group consisting of biotin, color-developing enzymes, radioactive isotopes, chromophores, light-emitting substances, fluorescers, superparamagnetic particles and ultrasuper paramagnetic particles.

<104>

<105> The above polypeptide means cardiac troponin I (cTnI)-specific polypeptide screened in the present invention, and a sample may be, for example, blood or urine. When the composition of the present invention is mixed with a sample, cardiac troponin I (cTnI) in the sample can be detected.

<106>

<i07> To make confirmation of binding of the polypeptide to cardiac troponin I (cTnI), detection and quantification easy, the peptide of the present invention may be prepared with label. That is, it may be linked (for example, covalent bond or crosslinking) with detectable labels. The detectable labels may be chromozyme (for example, peroxidase, alkaline phosphatase), radioisotope (for example, ¹⁸F, ¹²⁴I, ¹²⁵I, ³²P, ³⁵S), chromophore, luminous material or fluorescent material (for example, FITC, RITC, fluorescent protein (GFP (Green Fluorescent Protein); EGFP (Enhanced Green Fluorescent Protein), RFP (Red Fluorescent Protein); DsRed (Discosoma sp. red fluorescent protein); CFP (Cyan Fluorescent Protein), CGFP (Cyan Green Fluorescent Protein), YFP (Yellow Fluorescent Protein), Cy3, Cy5, Cy7.5), superparamagnetic particles or ultrasuper paramagnetic particles.

<i08>

Detection methods according to the label are well known in the art, but it may be performed as follows. In case of fluorescent material, immunofluorescent method may be performed. For example, the peptide of the present invention labeled with fluorescent material is reacted with a sample and remove non-binding or non-specific product. Then, fluorescence of the peptide can be observed under fluorescent microscope. In case of enzyme, color reaction of substrate is measured by optical density and in case of radioisotope, it may be performed by measuring the radiation.

<109>

<iio>

Based on the property of the polypeptide of the present invention that binds specifically to cardiac troponin I (cTnl), the present invention provides a diagnostic composition for detecting cardiac troponin I (cTnl) and a method for detecting cardiac troponin I (cTnl) in blood or urine.

<iu>

<ii2>

In the present invention, cardiac troponin I (cTnl)-related condition/disease may be one or more selected from the group consisting of myocardial infarction, angina, hypertension, infectious disease, sepsis, arrhythmias, pulmonary embolism, increased intracranial pressure, renal insufficiency, UGI bleeding, acute respiratory distress syndrome, end-stage renal disease, amyloidosis, diabetes and cardiac toxicity [Clin Chem. 2009 Dec; 55(12):2098-112, Am Heart J 2011;162:64-73]. Preferably, cardiac troponin I (cTnl)-related condition/disease may be one or more selected from the group consisting of myocardial infarction and angina.

<li3>

When the muscular tissue of cardiac muscles and skeletal muscles in the body is injured or muscular cells are necrotized, cardiac troponin I (cTnl), a myocardial marker, will be released in blood and will also be excreted in urine. The level of cardiac troponin I (cTnl) in urine is influenced by the amount of cardiac troponin I (cTnl) released from muscles, the level of cardiac troponin I (cTnl) in blood, GFR (glomerular filtration rate), and the amount of urine. Thus, the polypeptide of the present invention can be used for the diagnosis of cardiac troponin I (cTnl)-related condition/disease which is accompanied by muscle tissue injury. Accordingly, the present

invention provides a composition for diagnosing cardiac troponin I (cTnl)-related condition/disease, which contains the polypeptide of the present invention as an active ingredient, and a method for detecting cardiac troponin I (cTnl) sample, specifically, blood or urine.

<114>

<i15> In addition, the present invention provides the use of the above polypeptide for preparation of an agent for diagnosing cardiac troponin I (cTnl)-related condition/disease.

<116>

Further more, the present invention provides a method for diagnosing cardiac troponin I (cTnl)-related condition /disease comprising a step of- (a) contacting the polypeptide of claim 1 with a sample and a label bound thereto, wherein the label is selected from the group consisting of chromogenic enzymes, radioisotopes, chromophores, light emitting materials, fluorescers, super paramagnetic particles, and ultrasuper paramagnetic particles; and (b) detecting cardiac troponin I (cTnl) from the sample.

<117>

As used herein, the "sample" refers to specimen isolated from subject in need thereof, specifically isolated from blood or urine. And as used herein, the "effective amount" refers to the amount of a compound or extract which makes possible to trace diagnosis or effect of treatment when it is administered to a patient, and the "subject" refers to animals, particularly, mammals comprising human. The subject may be cells, tissue, or organ. The subject also may be patient in need of treatment.

<118>

The detection of cardiac troponin I (cTnl) is indicative that the subject has cardiac troponin I (cTnl)-related condition/disease. Therefore, the diagnosis could be accomplished.

<119>

An agent for detecting Cardiac troponin (cTnl) of the present invention may be prepared in the form of the polypeptide as it is or with pharmaceutically acceptable carrier. As used herein, the phrase "pharmaceutically acceptable" means that the components present in the composition are physiologically acceptable and usually do not invoke allergic or similar reactions when administered to humans. The carrier comprises all kinds of solvents, dispersing media, oil-in-water or water-in-oil emulsions,

water soluble compositions, liposomes, microbeads and microsomes, and biodegradable nanoparticle.

<i20> Meanwhile, an agent for detecting Cardiac troponin (cTnl) of the present invention may be formulated with a proper carrier according to administration routes. The administration routes of the pharmaceutical composition of the present invention comprise, but not limited thereto, oral or parenteral routes. The parenteral routes comprise, for example, subcutaneous, intranasal, peritoneal, intramuscular, intracutaneous, or intravenous administration.

<i2i> In case of oral administration of an agent for detecting Cardiac troponin (cTnl) of the present invention, a pharmaceutical composition of the present invention may be formulated into powder, granule, tablets, pills, sugar coated pills, capsules, liquid, gel, syrup, suspension, wafers by well known method in the art. Examples of the proper carriers, it may be comprise sugars such as lactose, dextrose, sucrose, sorbitol, mannitol, xylitol, erythritol and maltitol, starches such as corn starch, wheat starch, rice starch and potato starch, celluloses such as cellulose, methyl cellulose, sodium carboxymethyl cellulose and hydroxy propyl methylcellulose, and fillers such as gelatin and polyvinyl pyrrolidone. And, if desired, it may comprise cross-linked polyvinyl pyrrolidone, agar, alginic acid or sodium alginate as a solutionizer. Further, the pharmaceutical composition of the present invention may comprise anti-coagulating agent, lubricant, wetting agents, flavors, emulsifying agents and antiseptics.

<i22> Also, in case of parenteral administration, a pharmaceutical composition of the present invention may be formulated with a proper carrier for parenteral administration into injections, transdermal preparations, and nasal inhalers by using the method known in the art. The injection must be sterilized and protected from microorganisms such as bacteria and fungi. Proper carriers for injection may be, but not limited thereto, water, ethanol, polyol (e.g. glycerol, propylene glycol and liquid polyethylene glycol) or mixture thereof and/or solvent or dispersing media comprising plant oil. More preferably, proper carriers may be Hank's solution, Ringer's

solution, PBS (Phosphate buffered saline) containing triethanol amine, or a isotonic solution such as distilled water for injection, 10% ethanol, 40% ethanol, 40% propylene glycol and 5% dextrose. To protect the injection from contamination of microorganisms, it may further comprise various antibiotics or antifungal reagent such as paraben, chlorobutanol, phenol, sorbic acid, thimerosal. In addition, in most cases, the injection may further comprise an isotonic reagent such as sugars or sodium chloride. The formulation of the above-mentioned is well described in Remington's Pharmaceutical Science, 15th Edition, 1975, Mark Publishing Company, Easton, Pennsylvania.

<123>

<i24>

In case of nasal inhalers, a compound of the present invention may be delivered with a form of aerosol spray from pressure pack or spray by using proper propellants such as dichloro fluoromethane, trichloro fluoromethane, dichloro tetrafluoroethane, carbon dioxide or other proper gas. In case of pressure aerosols, dose may be determined by providing valve which delivers the measured amount of a compound. For example, a gelatin capsule and cartridge for inhaler or insufflator may be formulated to contain compound, and proper powder compound such as lactose or starch.

<125>

<126>

Other pharmaceutically acceptable carriers are well described in Remington's Pharmaceutical Sciences, 19th Edition, Mack Publishing Company, Easton, PA, 1995.

<127>

<i28>

Also, an agent for detecting Cardiac troponin (cTnl) of the present invention may further comprise one or more buffers (e.g. saline or PBS), carbohydrates (e.g. glucose, mannose, sucrose or dextran), antioxidant, bacteriostat, chelating agent (e.g. EDTA or glutathione), adjuvant (e.g. aluminium hydroxide), suspension agent, thickening agent and/or preservative (benzalkonium chloride, methyl- or propyl paraben and chlorobutanol).

<129>

Also, a pharmaceutical composition of the present invention may be formulated using a method well known in the art to provide quick, durable,

delayed release of an active ingredient after administration.

<i30>

As seen foregoing, formulated agent for detecting Cardiac troponin (cTnl) may be administered with effective amount into oral, subcutaneous, intracutaneous, intravenous or intramuscular routes. The pharmaceutically effective amount of the agent for detecting Cardiac troponin (cTnl) of the present invention suitably determined by considering various factors, such as administering route, administering subject, disease, severity thereof, age of patient, sex, body weight, individual variation, and status of disease. Preferably, the agent for detecting Cardiac troponin (cTnl) comprising the polypeptide of the present invention may be comprised with effective amount according to severality of disease. However, in generally, it may be administered in effective amount of 10 ug to 10 Mg per adult at once and it may be also administered multiple times.

<131>

<i32>

Meanwhile, in one Example of the present invention, whether the screened polypeptides of the present_invention bind specifically to cardiac troponin I (cTnl) was examined. As a result, it could be seen that a polypeptide comprising an amino acid sequence represented by SEQ ID NO: 17 or 24 binds to cardiac troponin I (cTnl) more strongly than to BSA or CKB protein. As described above, the polypeptide of the present invention can detect cardiac troponin I (cTnl) by binding to cardiac troponin I (cTnl) in blood or urine, and thus can be used for effective diagnosis of diseases myocardial infarction or angina, which are accompanied by muscle tissue injury.

<133>

[Advantageous Effects]

<134>

As described above, the present invention provides an isolated cardiac troponin I (cTnl)-targeting peptide and the use thereof. More specifically, the present invention provides a cardiac troponin I (cTnl)-targeting peptide, and marker or composition for detecting and diagnosing cardiac troponin I (cTnl). The polypeptide of the present invention is effective in specifically detecting cardiac troponin I. Therefore, the peptide of the present invention can be useful for the diagnosis of cardiac troponin I (cTnl)-related

condition/disease .

<135>

[Description of Drawings]

<i36>

FIG. 1 shows the structure of a cassette comprising a DMID-encoding nucleotide in a T7 phage 10B capsid (DMID: protein scaffold module disclosed in Korean Patent Application No. 10-2011-0035613 filed by the present inventors) .

<137>

FIG. 2 shows a structure comprising a DMID-encoding nucleotide and a peptide library-encoding nucleotide in a T7 phage 10B capsid (DMID-' protein scaffold module disclosed in Korean Patent Application No. 10-2011-0035613 filed by the present inventors).

<i38>

FIG. 3 shows various nucleotide sequences that encode the peptide library of the present invention.

<i39>

FIG. 4 shows the results of performing a total of 5 rounds of screening for a cardiac troponin I (cTnl)-targeting phage.

<i40>

FIG. 5A shows the results of a plaque assay conducted to examine the abilities of screened candidate phage clones to bind to troponin I (cTnl).

<i41>

FIG. 5B shows the results of an ELISA assay conducted to examine the abilities of screened candidate phage clones to bind to troponin I (cTnl).

<i42>

FIG. 6 shows the results of examining the ability of a 5R19 clone (RASSRLW) to detect suspended cTnl (5R19: candidate clone having the highest frequency among phage clones screened in the present invention).

<i43>

FIG. 7A shows the results of examining the ability of a DMID recombinant protein loaded with an amino acid sequence of RASSRLW to detect cTnl attached to a plate (cTnl-specific binding).

<i44>

FIG. 7B shows the results of examining the ability of a DMID recombinant protein loaded with an amino acid sequence of RASSRLW to detect suspended cTnl (cTnl-specific binding and detection).

<i45>

FIG. 7C shows the results of examining the ability of a DMID recombinant protein loaded with an amino acid sequence of RASSRLW to detect suspended CKB (cTnl-specific binding and detection).

<i46>

FIG. 8 shows the results of ELISA conducted to examine the abilities of

screened candidate 5R19 phage clone and 4R5 phage clone to bind to cardiac troponin I (cTnl).

<147>

[Mode for Invention]

<148>

Hereinafter, a preferred embodiment of the present invention will be described with reference to the accompanying drawings.

<149>

<i50>

In the following description and drawings, the same reference numerals are used to designate the same or similar components, and so repetition of the description on the same or similar components will be omitted.

<151>

<i52>

<Example 1>

<i53>

Construction of T7 phage DMID (designed modular immunodiagnostics)-(X7) peptide library

<154>

<155>

In this Example, a protein scaffold(designed modular immunodiagnostics (DMID)) was fused to the 10B capsid of T7 phage, and a peptide library was constructed in the protein scaffold. For this purpose, a T7 phage-DMID cassette was prepared, and a T7 phage DMID-(X7) peptide library that expresses various peptide libraries was constructed in the cassette.

<156>

<157>

<1-1> Preparation of T7 phage-DMID cassette

<i58>

For the preparation of a T7 phage DMID cassette, a DNA fragment corresponding to amino acids T1-L86 of DMID (DNA fragment corresponding to amino acids T1470-L1556 of EGF) was amplified by PCR and then cloned into the BamHI and XhoI sites of the vector pET-32a(+) (Novagen; Madison, WI), thereby preparing a pET-32a(+)-DMID-BX vector.

<159>

In order to make restriction enzyme sites for inserting nucleotide sequences encoding a peptide library, SacI and Sail restriction enzyme sites were inserted by PCR between R21 and T22 and between R26 and R27, respectively, and the resulting DNA was cloned into the EcoRI and HindIII site of pET-32a(+). Specifically, primary PCR was performed using pET-32a(+)-

DMID-BX as a template, a primer pair of DMIDEFwd and DMIDSSRvs and a primer pair of DMIDSSFwd and DMIDHRvs. The primary PCR products were mixed with each other, and secondary PCR was performed using the mixture as a template and a primer pair of DMIDEFwd and DMIDHRvs, thereby obtaining a recombinant DNA in which SacI and SalI restriction enzyme sites were inserted between R21 and T22 and between R26 and R27 of DMID, respectively. The recombinant DNA was treated with EcoRI and HindIII restriction enzyme sites and cloned into a pET-32a(+) vector treated with the same restriction enzymes. The resulting vector was named "pET-32a(+)-DMID-ESSH" .

<160> pET-32a(+)-DMID-ESSH was treated with the restriction enzymes EcoRI and HindIII and subjected to agarose gel electrophoresis to separate DMID-ESSH cassette DNA. The separated DMID-ESSH cassette DNA was ligated to the T7 vector arm of T7select10-3b (Novagen) by ligase. The resulting structure was mixed with a packaging extract of T7select10-3b and subjected to *in vitro* packaging, thereby constructing recombinant T7 phage-DMID-ESSH cassette virus. FIG. 1 shows the constructed recombinant cassette virus. The packaging of the T7 phage was performed according to the experimental method provided by Novagen.

<161>

<i62> <1-2> Preparation of T7 phage DMID-(X7) peptide library

<i63> T7 phage-DMID-ESSH cassette DNA was separated from the T7 phage-DMID-ESSH cassette virus and treated with SacI and SalI restriction enzymes. As nucleotide sequences encoding a peptide library, the forward oligonucleotide sequence gatcgagctc[^]M (NNKNNKNNKNNKNNKNNK)gtcgaccatcatcaccaccatcac (N=any nucleotide, K=G or T) (SEQ ID NO: 5) having SacI and SalI restriction enzyme sites and the reverse oligonucleotide sequence gtgatggtggtgatgatggtcgac (SEQ ID NO: 6) complementary to the forward oligonucleotide sequence at the 3' region were used. The two oligonucleotide sequences were mixed with each other at the same molar ratio, heated at 95 °C for 5 minutes and cooled to room temperature to form a complementary hydrogen bond. Then, DNA complementary to the forward oligonucleotide sequence gatcgagctcNNKNNKNNKNNKNNKNNKgtcgaccatcatcaccaccatcac is synthesized with

the DNA polymerase Klenow fragment, thereby making complete double-stranded library DNA.

<164> The library DNA was treated with SacI and SalI. A nucleotide sequence encoding the peptide library treated with the restriction enzymes was ligated by ligase with the T7 phage-DMID-ESSH cassette DNA treated with the same restriction enzymes. The resulting structure was mixed with a packaging extract of T7select10-3b and subjected to *in vitro* packaging, thereby constructing recombinant T7 phage-DMID-(X7) peptide library virus. FIG. 2 shows the constructed peptide library virus.

<165> For verification of the recombinant T7 phage-DMID-(X7) peptide library virus, sequencing was performed. Specifically, the region having the peptide library inserted therein was amplified by PCR using a T7 UP FWD-100 primer (agcgcgctcgcgctgctaac (SEQ ID NO: 22)), which binds to a nucleotide sequence located 107-bp upstream from the EcoRI site of the capsid DNA of the T7 phage-DMID-(X7) peptide library virus, and a T7 Down Rvs-100primer (ctgataactcagcggcagtc (SEQ ID NO: 23)) which binds to a nucleotide sequence located 108-bp downstream from the HindIII site. The PCR amplification product was purified using a DNA purification column, and then the peptide library region was sequenced using the T7 UP FWD-100 primer and the T7 Down Rvs-100 primer. The sequencing was performed by Genotech (Korea) using the Sanger's method which is generally used. As a result, various nucleotide sequences encoding the peptide library were identified, and some aligned nucleotide sequences of the identified nucleotide sequences are shown in FIG. 3.

<166> In FIG. 3, among the sequences of conserved sequence areas showing sequence homology indicated by black color in the nucleotide sequence alignment, the sequence tctgccaaggctgactgtaagagagagctc expresses the sequence SAKADCKRGS which is encoded by the amino terminal end of DMID and the SacI region, which have the library inserted therein, and the sequence gtcgaccgagtggtgcacgtgcaaagcaggctac expresses the sequence LERVCTCKAGY which is encoded by the SalI region and the carboxy-terminal end of DMID, which have the library inserted therein. Meanwhile, the sequences of sequence diversity

areas showing sequence heterogeneity indicated by black and red, which were not aligned in the nucleotide sequence alignment, were derived from the sequence NNKNNKNNKNNKNNKNNK (N=any nucleotide, K=G or T) inserted between the SacI and Sail regions and actually show collections of various nucleotide sequences. It can be seen that various peptides are made from such collections of various nucleotide sequences. The portions indicated by "-" are blanks indicated because libraries were not aligned with each other, and these portions do not mean deletions in the nucleotide sequences.

<i67> The identified recombinant T7 phage-DMID-(X7) peptide library virus was amplified in large amounts and used for biopanning of a target peptide. To amplify the virus in large amounts, the *E. coli* strain BLT5403 was seed-cultured overnight in LB media containing 100 ug/ml of ampicillin, and then on the next day, the culture broth was diluted in fresh LB/Amp (100 ug/ml) media at a volume of 1:100 and cultured until the absorbance at 600 nm reached 0.5-1.0 ($OD_{600} = 0.5-1.0$). The T7 phage-DMID-(X7) peptide library virus was transfected the *E. coli* strain BLT5403 until MOI (multiplicity of infection) reached 0.001-0.01, and the strain was shake-cultured at 37 °C for 2-3 hours until the cells were dissolved to become clear, thereby amplifying the virus. The amplified T7 phage-DMID-(X7) peptide virus was analyzed by a plaque assay to determine the titer, and 97 ml of T7 phage-DMID-(X7) peptide library virus having a titer of 8.67×10^{10} pfu/ml was prepared.

<i68>

<i69> <Example 2>

<i70> Screening and selection of phage clones that specifically target cardiac troponin I (cTnl)

<i71>

<i72> <2-1> Cloning and expression of disease-targeted recombinant protein

<i73> For the preparation of a human cardiac troponin I recombinant protein, a DNA fragment corresponding to the whole ORF of human troponin I was amplified by PCR using TroB5Fwd and Trn3Rvrs primers, and then cloned into the BamHI and NotI sites of the vector pET-29a(+) (Novagen; Madison, WI), thereby preparing a recombinant human troponin I expression vector which was

named "pET29a-hTroponin I". The expression vector comprises amino acid residues 1-210 of the amino acid sequence of human Troponin I, and the sequence MKETAAAKFERQHMDSPDLGTLVPRGSMAD IGS derived from the vector is fused to the amino-terminal end of human troponin I, and the sequence AAALHHHHHH derived from the vector is fused to the carboxy-terminal end.

<i74> Each of the vectors was transformed into an *E. coli* cell line {*E. coli* BL21) which was then cultured in LB media containing 50 ug/ml of kanamycin at 37 °C and when absorbance at 595 nm reached 0.5-0.6, the cells were treated with 1 mM IPTG (isopropyl-β-D-(-)-thiogalactopyranoside) and incubated at 20 °C for 24 hours to induce the expression of the recombinant protein. The incubated *E. coli* strain was re-suspended in lysis buffer (50 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM PMSF, 0.5 mM DTT), after which it was disrupted by sonication and then purified using Ni-NTA resin (Qiagen). The expressed and purified recombinant protein was subjected to SDS-PAGE to determine the purity of the protein, before use in subsequent experiments.

<i75>

<i76> <2-2> Phage plaque assay

<i77> BLT5403 *E. coli* was cultured in M9LB media until the absorbance at 600 nm (OD_{600}) reached 1.0, and then 250 µl of the culture broth was dispensed into a 10-ml tube and stored at 4 °C. The phage to be analyzed for concentration was diluted at various dilution factors and stored at 4 °C until use. Top agar was dissolved at 100 °C and maintained at 37 °C. 100 µl of a phage solution was mixed with 250 µl of the prepared host BLT5403 *E. coli*, and then 4 ml of the dissolved top agar was added thereto, after which the resulting mixture was transferred to an agar plate which was then hardened. The hardened agar plate was incubated at 37 °C for 3-4 hours, and then the number of plaques produced was measured.

<i78>

<i79> <2-3> Screening of cTnl-targeted phage library

<i80> Using the T7 phage DMID-(X7) peptide library, phage clones that bind specifically to cTnl were screened. Specifically, each of cTnl (Abeam,

Cambridge, UK) and BSA (Gibco, Auckland, New Zealand) was prepared at a concentration of 10 ug/ml, and then 100 ul of each of the prepared solutions was injected into each well of a 96-well plate and incubated at 4 °C for 16 hours to coat each well. Each coated well of the plate was blocked with 200 ul of 5% skim milk, washed twice with PBS and stored at 4 °C until use.

<i81> The phage library was prepared at a concentration of 1×10^{10} pfu/ml in phosphate buffered saline (PBS, pH 7.4) containing 5% BSA and 0.1 mg/ml DMID protein. The prepared phage library was injected into each BSA-coated well at a concentration of 1×10^9 pfu and bound at room temperature for 10 minutes. Non-bound phages were transferred into fresh BSA-coated wells and bound again for 10 minutes. This procedure was repeated three times to remove non-specifically binding phage clones. The phage clones which did not bind to the BSA-coated wells in the three repeated procedures were transferred into the cTnl-coated wells and bound to the wells at room temperature for 30 minutes. Then, the wells were washed three times with PBS containing 0.05% Tween to remove non-specifically binding phage clones. Herein, each washing was performed for 5 minutes.

<i82> The phage clones bound to the cTnl-coated wells were detached by incubation with 100 ul of 1% SDS for 20 minutes, and the detached phage clones were recovered. The number of phages bound to cTnl in the recovered phages was measured by a phage plaque assay. The recovered phages were transfected into the culture broth of BLT5403 *E. coli* and amplified. The amplified phage clones were used in the next round of screening, and a total of 5 rounds of screening were performed.

<i83> As a result, as shown in FIG. 4, the phage concentration increased 2-3 times when each round was performed, and the phage concentration in round 5 was about 25 times higher than that in round 1. On the contrary, the increases in the numbers of BSA-bound phages and T7 phage-DMID(-) phages having no amino acid inserted therein were not observed.

;184>

:185> <2-4> Determination of nucleotide sequences of phages and selection of candidate phage clones

<186> Among the phage clones screened in the 5 rounds, 21 clones were selected, and nucleotide sequences in the selected clones were determined by PCR. The PCR was performed using the upstream primer 5'-AGCGGACCAGATTATCGCTA-3' and the downstream primer 5'-AACCCCTCAAGACCCGTTTA-3' for 35 cycles, each consisting of 50 sec at 94 °C, 1 min at 50 °C and 1 min at 72 °C. After determination of the nucleotide sequences, amino acids in the clones were aligned using the ClustalX program, and the repeated motifs and clones were selected as candidates. The candidates are shown in Table 1 below.

<187> [Table 1]

No.	Phage clone	Sequence	Frequency
1	5R1	PAAAMRV	4/21
2	5R5	SSRTGSQ	1/21
3	5R19	RASSRLW	9/21

<189> As can be seen in Table 1 above, the sequence RASSRLW was found in 9 clones among the 21 clones, and the sequence PAAAMRV was found in 4 clones. Particularly, the SSR motif in the sequence RASSRLW was found again in the 5R5 clone, and thus selected as a candidate for cTnI.

<190>

<191> <2-5> Examination of cTnI-binding abilities of phage clones screened by plaque assay

<192> Each of cTnI (Abeam, Cambridge, UK), BSA and CKB proteins was prepared at a concentration of 10 ug/ml, and 100 ul of each of the prepared solutions was injected into each well of a 96-well plate and incubated at 4 °C for 16 hours to coat each well. Each coated well of the plate was blocked with 200 ul of 5% skim milk, washed twice with phosphate buffered saline and stored at 4 °C until use. The selected candidate phage clones were prepared at a concentration of 1×10^{10} pfu/ml in phosphate buffered saline (pH 7.4) containing 5% BSA and 0.1 mg/ml DMID proteins. Each of the prepared phage clones was injected into each protein-coated well at a concentration of 1×10^9 pfu and bound to the wells at room temperature for 1 hour. Then, each well was washed three times with phosphate buffered saline containing 0.05% Tween to remove non-specifically binding phages. Herein, each washing was performed

for 5 minutes. The phage clones bound to the cTnl-coated wells and the protein-coated wells were detached by incubation with 100 μ l of 1% SDS, and the detached phages were recovered. The number of protein-bound phages in the recovered phages was measured by a phage plaque assay, and the results of the measurement are shown in FIG. 5A.

<193> The results of measuring the number of cTnl-bound phages by the plaque assay are shown in FIG. 5A. As can be seen therein, the 5R-19 clone (RASSRLW) had an excellent ability to bind to cTnl. In addition, it was shown that the 5R-19 clone did bind selectively to cTnl without binding to BSA or the CKB protein that is an indicator of diagnosis of heart attack. However, the specificities of the other candidates 5R-1 (PAAAMRV) and 5R-5 (SSRTGSQ) clones to cTnl could not be observed.

<194>

<195> <2-6> Examination of cTnl-binding abilities of screened candidate phage clones by ELISA

<196> Each of cTnl (Abeam, Cambridge, UK), BSA, cTnl (Example 2-1) purified from *E. coli*, and CKB protein was prepared at a concentration of 10 μ g/ml, and 100 μ l of each of the prepared protein solutions was injected into each well of a 96-well plate and incubated at 4 $^{\circ}$ C for 16 hours to coat each well. Each coated well of the plate was blocked with 200 μ l of 5% skim milk, washed twice with PBS and stored at 4 $^{\circ}$ C until use.

<197> Each of the selected candidate phage clones was prepared at a concentration of 1×10^9 pfu/ml in phosphate buffered saline (pH 7.4) containing 5% BSA and 0.1 mg/ml DMID proteins. Each of the prepared phages was injected into each well at a concentration of 1×10^9 pfu and bound to the wells at room temperature for 1 hour. Then, each well was washed three times with phosphate buffered saline containing 0.05% Tween to remove non-specifically binding phages. Herein, each washing was performed for 5 minutes.

<198> The phage clones bound to the cTnl-coated wells and to the protein-coated wells were detected with a T7 phage tail fiber antibody (Merck KGaA, Darmstadt, Germany) and was color-developed with a TMB substrate (Koma

biotech Inc., Seoul, Korea). The TMB color development was terminated by addition of 100 μ l of 2N H_2SO_4 , and the absorbance at 450 nm was measured, thereby determining the degree of binding of each candidate phage. The results of the measurement are shown in FIG. 5B.

<i99> As can be seen in FIG. 5B, the selective targetability of the 5R-19 clone to cTnl was also verified by the ELISA assay. The 5R-19 clone showed binding specificity not only to native human cTnl, but also to cTnl extracted from *E. coli*, and the excellent binding ability thereof did not appear in BSA or CKB protein.

<200>

<20i> <2-7> Examination of the ability of 5R19 clone (RASSRLW) to detect suspended cTnl

<202> cTnl purified from *E. coli* was prepared at a concentration of 10 μ g/ml, and 100 μ l of the prepared protein was injected into each well of a 96-well plate and incubated at 4 $^{\circ}C$ for 16 hours to coat each well. Each coated well of the plate was blocked with 200 μ l of 5% skim milk, washed twice with phosphate buffered saline and stored at 4 $^{\circ}C$ until use. cTnl to be used in detection was suspended in a phosphate buffer containing 5% BSA and 0.1 mg/ml of DMID protein, at concentrations of 10 μ g/ml, 5 μ g/ml, 2.5 μ g/ml, 1.25 μ g/ml, 0.625 μ g/ml and 0.3125 μ g/ml. 1×10^8 pfu (50 μ l) of the 5R19 phage clone (RASSRLW) was added to and mixed with 50 μ l of each of the cTnl samples prepared at various concentrations, followed by incubation at room temperature for 30 minutes. Then, the mixture was transferred into the cTnl-coated wells and bound to the wells at room temperature for 30 minutes. Then, each well was washed three times with phosphate buffered saline containing 0.05% Tween to remove the phages bound to the suspended cTnl. Herein, each washing was performed for 5 minutes.

<203> The remaining phage clones bound to the cTnl-coated wells were detected with a T7 phage tail fiber antibody (Merck KGaA, Darmstadt, Germany) and were color-developed with a TMB substrate (Koma Biotech Inc., Seoul, Korea). The TMB color development was terminated by addition of 100 μ l of 2N H_2SO_4 , and

the absorbance at 450 nm was measured. Based on the measurement results, the degree and pattern of detection of suspended cTnI by the 5R19 phage as a function of the concentration of cTnI were measured. The results of the measurement are shown in FIG. 6.

<204> As described above, whether the 5R-19 (RASSRLW) clone can detect suspended cTnI was examined, and the lowest detection concentration of suspended cTnI which can be detected by the 5R-19 (RASSRLW) clone was examined. As a result, as can be seen in FIG. 6, the selected 5R-19 clone could detect suspended cTnI in a concentration-dependent manner, and the lowest detection concentration of suspended cTnI was found to be 312 ng/ml (absolute detection level: 15.5 ng/50 μ l).

<205>

<206> <2-8> Examination of the ability of DMID protein (RASSRLW-DMID) loaded with RASSRLW to detect suspended cTnI

<207> To prepare a DMID recombinant protein loaded with RASSRLW, the template 5R-19 phage clone that binds specifically to cTnI was amplified by PCR using a T7 super UP primer (5'-AGCGGACCAGATTATCGCTA-3') and a T7 super DOWN primer (5'-AACCCCTCAAGACCCGTTTA-3'). The PCR amplification product was cloned into the BamHI and XhoI of the vector pET-29b(+) (Novagen; Madison, WI), thereby preparing a RASSRLW-loaded DMID protein expression vector which was named "RASSRLW-DMID". The expression vector comprises amino acid residues 1470-1555 of the full-length amino acid sequence of human stabilin-2 and has restriction enzyme (SaiI and SacI) recognition sequences between amino acid residues 1490 and 1496 and a sequence of RASSRLW arranged between the restriction enzyme recognition sequences. To the amino end, there is fused MKETAAAKFERQHMDSPDLGTLVPRGSMA ISDPNS derived from the vector, and to the carboxy end, there is fused LEHHHHHH amino acid. The vector was transformed into an *E. coli* cell line in the same manner as Example 2-1, and the cells were cultured, thereby preparing a recombinant protein.

<208> BSA and the cTnI and CKB proteins purified in *E. coli* were prepared at concentrations of 10 μ g/ml, 5 μ g/ml and 2.5 μ g/ml, respectively, after which 100 μ l of each of the proteins was added to each well of a 96-well

plate and coated onto each well at 4 °C for 16 hours. Each coated well of the plate was blocked with 200 μ l of 5% skim milk, washed twice with phosphate buffered saline and stored at 4 °C until use.

<209> The RASSRLW-DMID protein purified in *E. coli* was conjugated to HRP (Dojindo, Tokyo, Japan) at the amino group and prepared at a concentration of 10 μ g/ml. Herein, a phosphate buffered saline (pH 7.4) containing 5% BSA, 0.05% Tween 20 and 0.1 mg/ml of DMID protein was used as buffer. 100 μ l of the prepared RASSRLW-DMID-HRP protein was added to each coated well and bound to each well at room temperature for 1 hour. Then, each well was washed three times with 0.05% Tween-containing phosphate buffered saline to remove non-specific binding. Each washing was performed for 5 minutes.

<210> The RASSRLW-DMID-HRP protein bound to each well coated with cTnI and each well was color-developed with a TMB substrate (Koma Biotech Inc., Seoul, Korea) to comparatively analyze the degrees of binding of the proteins. Color development with the TMB substrate was stopped by addition of 100 μ l of 2N H₂SO₄, and the absorbance at 450 nm was measured. The results of the measurement are shown in FIG. 7A.

<211> cTnI purified in *E. coli* was prepared at a concentration of 10 μ g/ml, and then 100 μ l of the prepared cTnI was added to each well of a 96-well plate and coated onto each well at 4 °C for 16 hours. Each coated well of the plate was blocked with 200 μ l of 5% skim milk, washed twice with phosphate buffered saline and stored at 4 °C until use. Each of the cTnI and CKB protein to be used was suspended at concentrations of 10 μ g/ml, 5 μ g/ml, 2.5 μ g/ml, 1.25 μ g/ml, 0.625 μ g/ml, 0.312 μ g/ml, 0.156 μ g/ml, 0.078 μ g/ml and 0 μ g/ml in a phosphate buffer containing 5% BSA and 0.1 mg/ml of DMID protein. 0.5 μ g (50 μ l) of RASSRLW-DMID-HRP was added to and mixed with 50 μ l of each concentration of the prepared cTnI and CKB protein, and each of the mixtures was allowed to react at room temperature for 30 minutes. Each of the reaction mixtures was transferred into each well coated with cTnI and further allowed to react at room temperature for 30 minutes, so that free RASSRLW-DMID-HRP which was not bound to suspended cTnI was bound to the cTnI-coated well. Then, each well was washed three times with 0.05% Tween-

containing phosphate buffered saline to remove RASSRLW-DMID-HRP bound to suspended cTnI or CKB. Each washing was performed for 5 minutes.

<212> The RASSRLW-DMID-HRP protein bound to each well coated with cTnI and each well was color-developed with a TMB substrate (Koma Biotech Inc., Seoul, Korea) to comparatively analyze the degrees of binding of the proteins. Color development with the TMB substrate was stopped by addition of 100 μ l of 2N H₂SO₄, and the absorbance at 450 nm was measured. The results of the measurement are shown in FIGS. 7B and 7C.

<213> As can be seen from the results in FIG. 7, the DMID protein loaded with the amino acid sequence RASSRLW did bind specifically to cTnI, like the phage state. The ability of the protein to detect suspended cTnI was specific to cTnI, and the detection limit concentration was about 78 ng/ml (absolute detection- 3.9 ng/50 μ l).

<214>

<215> <2-9> Selection of new candidate phage clone and analysis of the ability of the phage clone to cTnI by ELISA

<216> Among the phage clones screened in the same manner as Example 1 or Example 2-3, 20 clones in round 4 were randomly selected, and a nucleotide sequence included in each of the selected clones was determined by PCR. The PCR was performed using the same primers under the same conditions as those described in Example 2-4. After determination of the nucleotide sequence, amino acids included in each of the clones were aligned using the ClustalX program, a motif and a clone, which were repeated twice or more, were selected as a candidate group. The selected candidate group is shown in Table 2 below.

<217> [Table 2]

No.	Phage clone	Sequence	Frequency
1	4R5	RCAARRS	9/20

<219> As can be seen in Table 2, 4R5 (RCAARRS; SEQ ID NO: 24) was repeated 9 times among a total of 20 clones. Particularly, 4R5 showed an amino acid sequence similar to 5R19 (RASSRLW; SEQ ID NO: 17) in Example 2-4 and was predicted to bind specifically to cTnI, and for this reason, it was selected

as a new candidate.

<220> The 5R19 phage clone shown in Table 1 above and the 4R5 phage clone shown in Table 2 were analyzed by ELISA in the same manner as Example 2-5, and the degrees of binding of the phages were compared based on the absorbance. As a result, it was found that the 5R19 phage clone and the 4R5 phage clone selectively targeted cTnI, and the binding levels for other BSA and CKB proteins were significantly low.

<22i> However, as can be seen in FIG. 8, not only for naturally occurring human cTnI, but also for human cTnI extracted from *E. coli*, the binding specificity of the newly screened 4R5 candidate was significantly higher than that of the 5R19 candidate.

<222>

<223> As described above, the present invention provides a polypeptide specific to cardiac troponin I and the use thereof. More specifically, the present invention provides a cardiac troponin I (cTnI)-specific polypeptide comprising an amino acid sequence represented by SEQ ID NO: 17 or 24, and compositions for detecting and diagnosing cardiac troponin I (cTnI), which contains the polypeptide as an active ingredient. The cardiac troponin I-specific polypeptide of the present invention comprises a protein scaffold module that increases the binding affinity and specificity of the polypeptide. Therefore, the cardiac troponin I-specific polypeptide is effective in detecting a polypeptide or protein specific to myocardial disease, and thus is effective for the diagnosis of myocardial disease.

<224>

[Industrial Applicability]

<225>

As described above, the present invention provides an isolated cardiac troponin I (cTnI)-targeting peptide and the use thereof. More specifically, the present invention provides a cardiac troponin I (cTnI)-targeting peptide, and marker or composition for detecting and diagnosing cardiac troponin I (cTnI). The polypeptide of the present invention is effective in specifically detecting cardiac troponin I. Therefore, the peptide of the present invention can be useful for the diagnosis of cardiac troponin I (cTnI)-related

condition/disease.

<226>

[CLAIMS]

[Claim 1]

<228> An isolated cardiac troponin I (cTnl)-targeting peptide comprising an amino acid sequence represented by the following general formula 1:

<229> [General Formula 1]

<230> Arg-X1-X2-X3-Arg-X4-X5:

<231> wherein X1 is Cys or Ala, X2 or X3 is Ala or Ser, X4 is Arg or Leu, and X5 is Ser or Trp.

<232>

[Claim 2]

<233> The peptide of claim 1, wherein the amino acid sequence is represented by the SEQ ID NO: 17 or 24.

<234>

[Claim 3]

<235> An isolated polynucleotide having nucleotide sequence encoding the peptide of claim 1.

<236>

[Claim 4]

<237> A vector comprising the polynucleotide of claim 3.

<238>

[Claim 5]

<239> A transformant transfected with the vector of claim 4.

<240>

[Claim 6]

<241> A marker for detecting cardiac troponin I (cTnl) comprising the peptide of claim 1.

[Claim 7]

<242> A composition for detecting cardiac troponin I (cTnl) comprising the peptide of claim 1 as an active ingredient.

<243>

[Claim 8]

<244> The composition of claim 7, wherein the polypeptide is labeled with one

selected from the group consisting of biotin, color-developing enzymes, radioactive isotopes, chromophores, light-emitting substances, fluorescers, super paramagnetic particles and ultrasuper paramagnetic particles.

<245>

[Claim 9]

<246>

A composition for diagnosing cardiac troponin I (cTnl)-related condition/disease comprising the peptide of claim 1 as an active ingredient.

<247>

[Claim 10]

<248>

The composition of claim 9, wherein the cardiac troponin I (cTnl)-related condition/disease is selected from the group consisting of myocardial infarction, angina, hypertension, infectious disease, sepsis, arrhythmias, pulmonary embolism, increased intracranial pressure, renal insufficiency, UGI bleeding, acute respiratory distress syndrome, end-stage renal disease, amyloidosis, diabetes and cardiac toxicity.

<249>

[Claim 11]

<250>

A method for diagnosing cardiac troponin I (cTnl)-related condition/disease comprising steps of:

<251>

(a) contacting the polypeptide of claim 1 with a sample and a label bound thereto, wherein the label is selected from the group consisting of chromogenic enzymes, radioisotopes, chromophores, light emitting materials, fluorescers, super paramagnetic particles, and ultrasuper paramagnetic particles! and

<252>

(b) detecting cardiac troponin I (cTnl) from the sample.

<253>

[Claim 12]

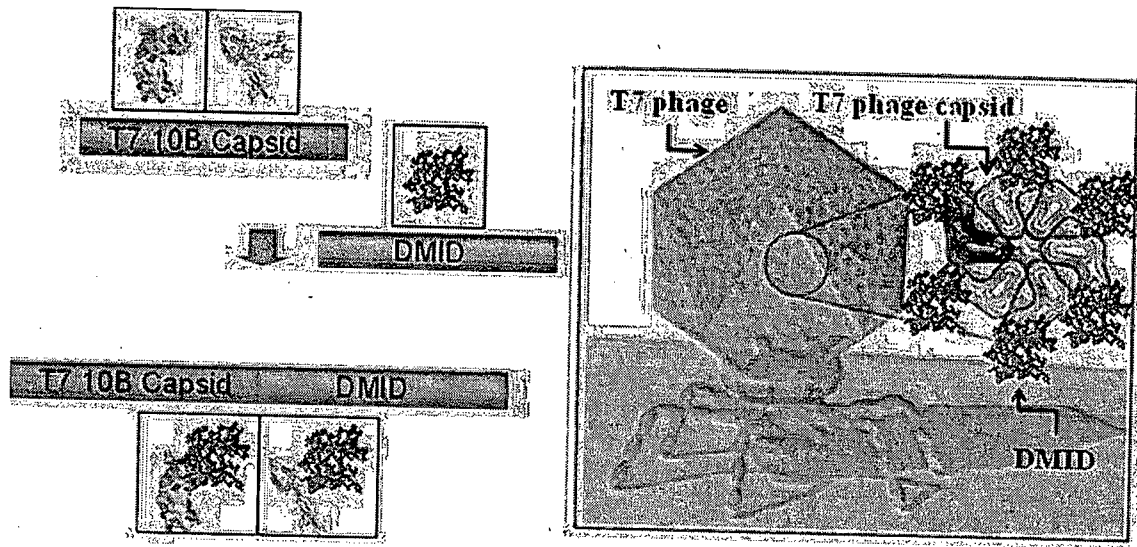
<254>

Use of the polypeptide of claim 1 for preparing an agent for diagnosing cardiac troponin I (cTnl)-related condition/disease.

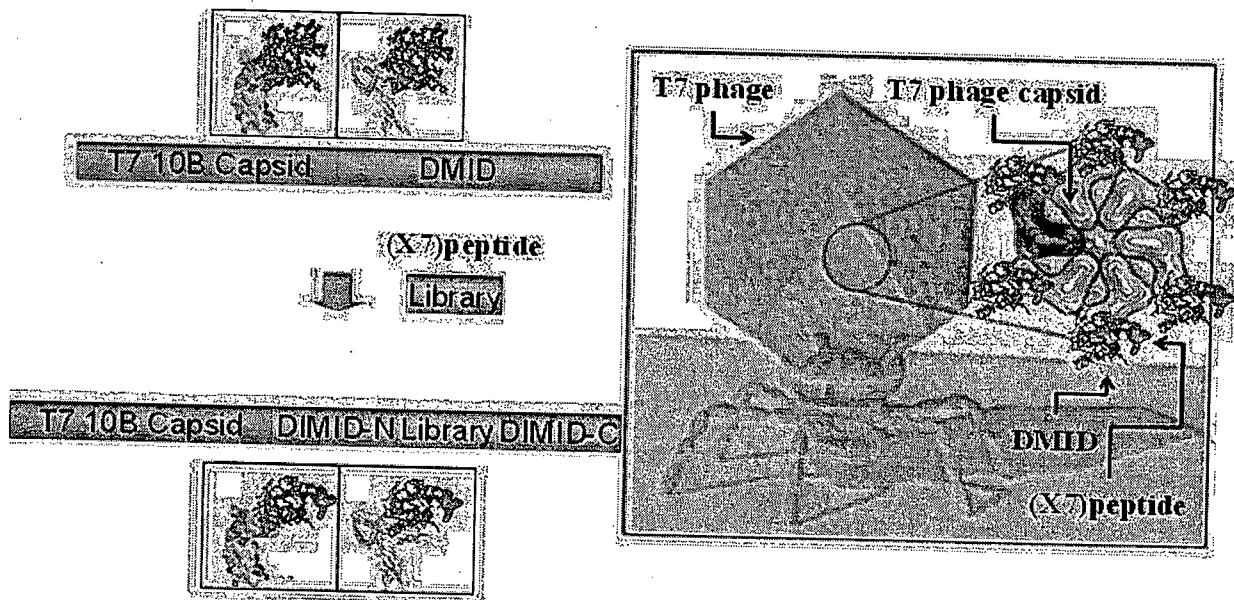
1/5

【DRAWINGS】

【1】



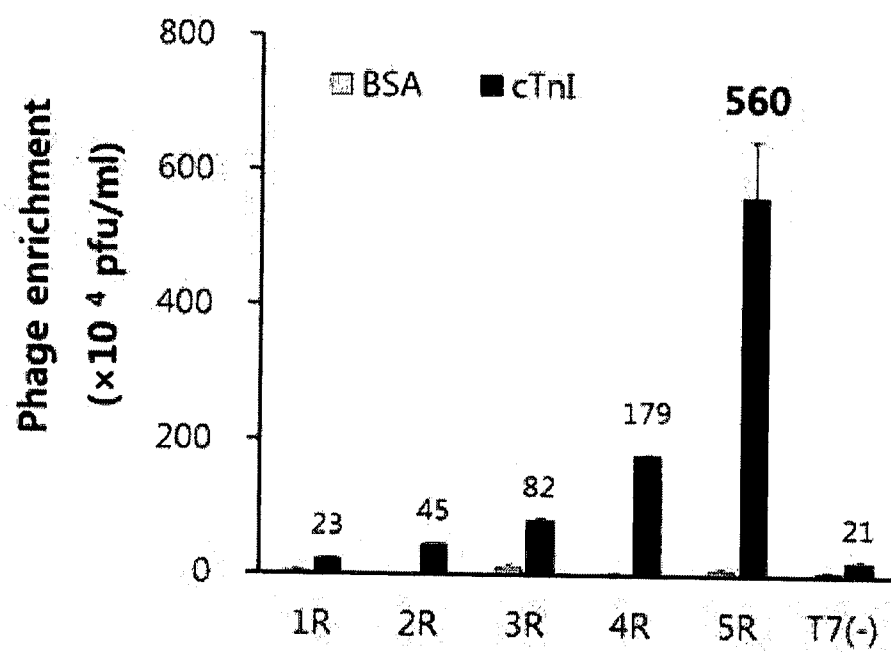
【2】



	110	120	130	140	150	160	170	180	190
Translate	GCTTCGCCAAGGCTGACTGTATAGACAGAGCTC-CTGGGTGCCTCTCGTG-IGC-G-CTCCACCGAGTGTGCTACGTGTCAAACAGGCTAC								
T7MDIX#1-10	T7UpFwd-100_B06_seq(1>538)	→							
T7MDIX#1-9	T7UpFwd-100_A06_seq(1>539)	→							
T7MDIX#1-4	T7UpFwd-100_D05_seq(1>537)	→							
T7MDIX#1-5	T7UpFwd-100_E05_seq(5>542)	→							
T7MDIX#1-2	T7UpFwd-100_R05_seq(5>541)	→							
T7MDIX#1-3	T7UpFwd-100_C05_seq(5>543)	→							
T7MDIX#1-1	T7UpFwd-100_A05_seq(1>528)	→							
T7MDIX#1-6	T7UpFwd-100_T05_seq(2>528)	→							
T7MDIX#1-12	T7UpFwd-100_D06_seq(9>543)	→							
T7MDIX#1-8	T7UpFwd-100_H05_seq(16>540)	→							
T7MDIX#1-7	T7UpFwd-100_G05_seq(16>541)	→							
T7MDIX#1-11	T7UpFwd-100_C06_seq(16>542)	→							

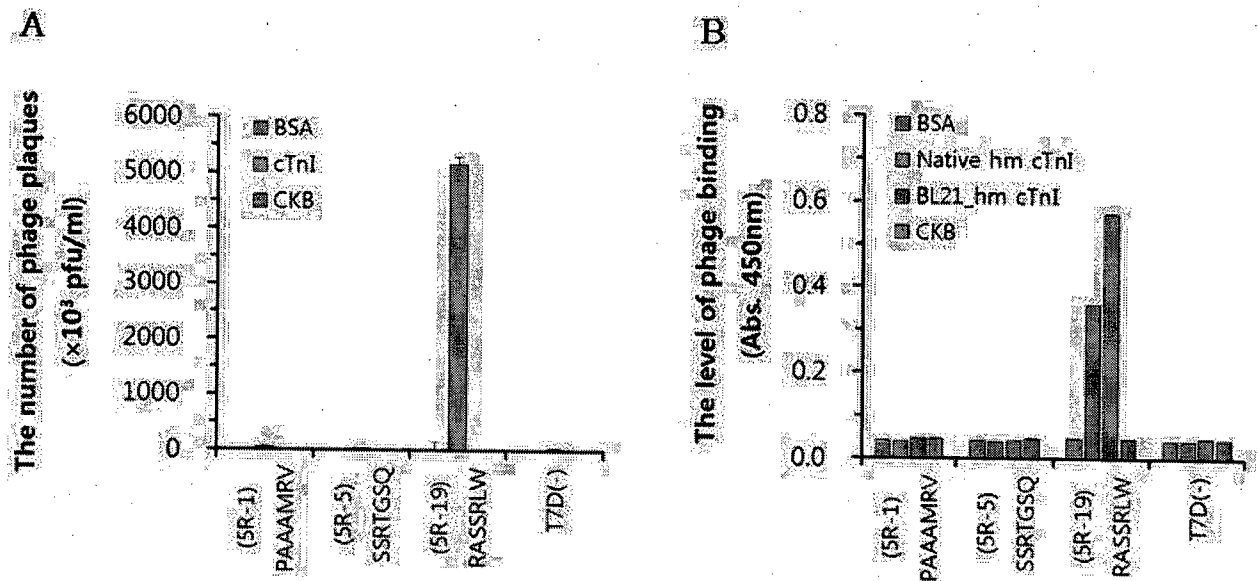
3/5

【4】



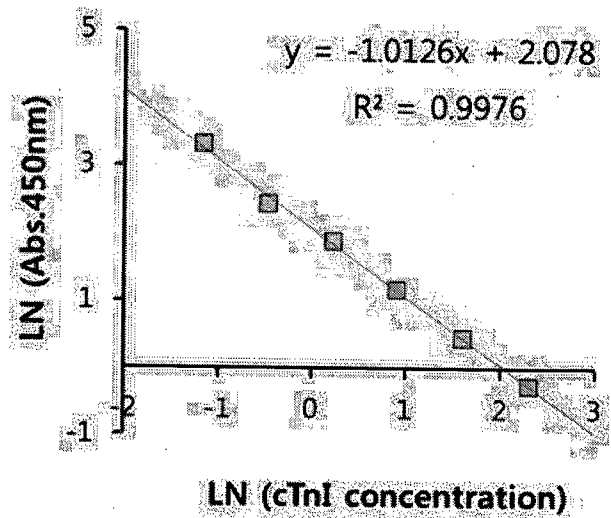
4/5

[5]



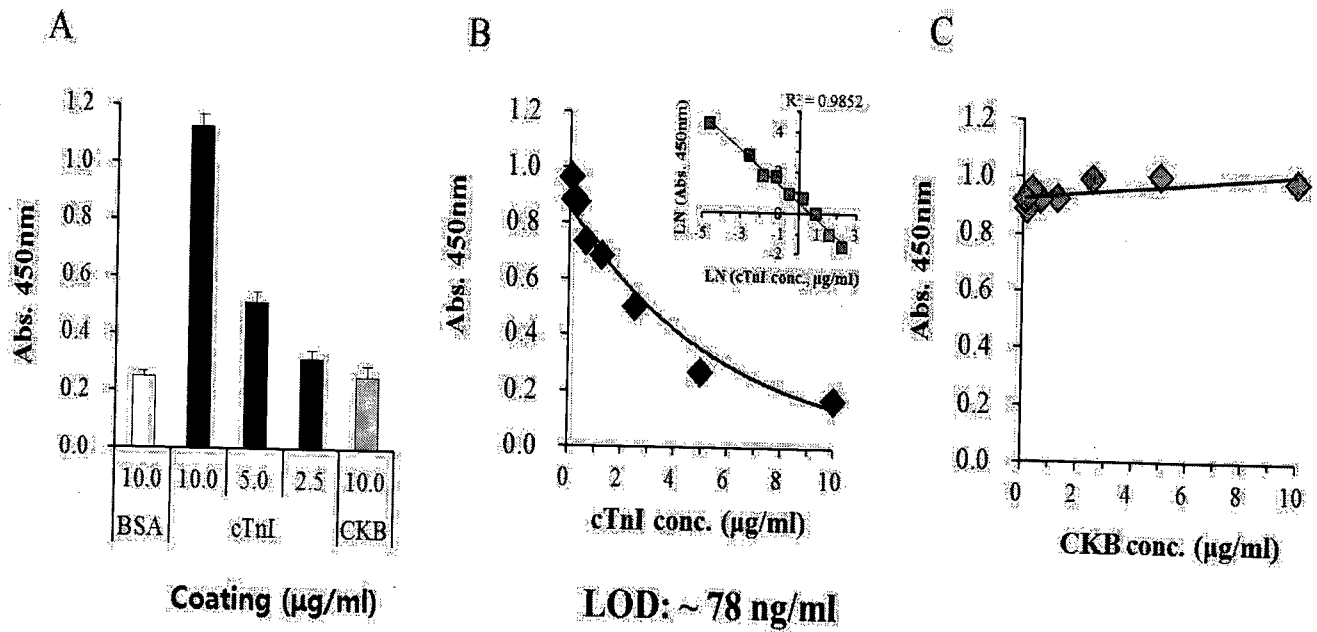
[6]

X (cTnI Conc.)		Y (Abs. 450nm)
($\mu\text{g/ml}$)	LN(x)	LN(y)
10.00	2.3	-0.257
5.00	1.6	0.446
2.50	0.9	1.171
1.25	0.2	1.893
0.62	-0.5	2.433
0.31	-1.2	3.320

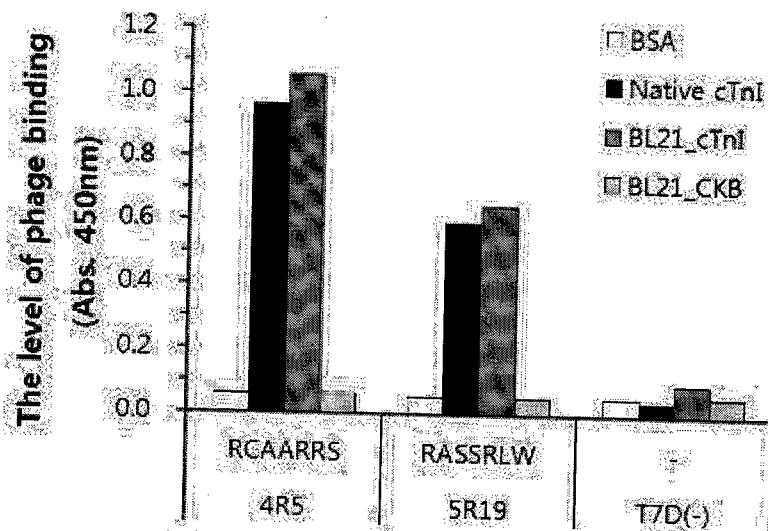


5/5

[7]



[8]



INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR2012/010705**A. CLASSIFICATION OF SUBJECT MATTER****C07K 14/47(2006.01)i, C12N 15/12(2006.01)1, GOIN 33/68(2006. 01)i, GOIN 33/52(2006.01)1**

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 14/47; GOIN 33/00; A61B 5/0452; GOIN 33/53

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: cardiac, tropoinin I, target, isolated, peptide, sequence, diagnosing, label

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 2009-0197344 A1 (VILLARD-SAUSSINE, s. et al.) 06 August 2009 See the whole document.	1-10,12
A	US 2004-0072255 A1 (VAN EYK, J. E. et al.) 15 April 2004 See the whole document.	1-10,12
A	US 2004-0175754 A1 (BAR-OR, D. et al.) 09 September 2004 See the whole document.	1-10,12
A	US 5834220 A (WICKS, R. W. et al.) 10 November 1998 See the whole document.	1-10,12



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 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

21 MARCH 2013 (21.03.2013)

Date of mailing of the international search report

22 MARCH 2013 (22.03.2013)

Name and mailing address of the ISA/KR

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City, 302-701, Republic of Korea

Facsimile No. 82-42-472-7140

Authorized officer

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Telephone No. 82-42-481-3479



INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2012/010705

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

1. With regard to any nucleotide and/or amino acid sequence disclosed in the international application, the international search was carried out on the basis of :

a. **a sequence listing filed or furnished**



on paper



in electronic form

b. time of filing or furnishing



contained in the international application as filed



filed together with the international application in electronic form



furnished subsequently to this Authority for the purposes of search

2.  In addition, in the case that more than one version or copy of a sequence listing has been filed or furnished, the required statements that the information in the subsequent or additional copies is identical to that in the application as filed or does not go beyond the application as filed, as appropriate, were furnished.

3. Additional comments:

INTERNATIONAL SEARCH REPORT

International application No.

PCT/KR2012/010705

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.: 11
because they relate to subject matter not required to be searched by this Authority, namely:
Claim 11 pertains to diagnostic method, and thus relates to a subject matter which this International Searching Authority is not required, under Article 17(2)(a)(i) of PCT and Rule 39.1(iv) of the Regulations under the PCT, 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.:
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. IAs all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. IAs all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. IAs 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. INo 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.
- I No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.

PCT/KR2012/010705

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2009-0 197344 A1	06.08.2009	CA 2660034 A1 CN 10 1568837 A EP 1887361 A1 EP 2047276 A2 JP 2010-500545 A Wo 2008-017928 A2 Wo 2008-017928 A3	14.02.2008 28.10.2009 13.02.2008 15.04.2009 07.01.2010 14.02.2008 24.04.2008
US 2004-0072255 A1	15.04.2004	AU 2003-273674 A1 CA 250 1756 A1 EP 1554587 A2 JP 2006-502203 A US 7 196 169 B2 wo 2004-034060 A2 wo 2004-034060 A3	04.05.2004 22.04.2004 20.07.2005 19.01.2006 27.03.2007 22.04.2004 03.03.2005
US 2004-0 175754 A1	09.09.2004	AU 2003-299933 A1 AU 2003-299933 B2 CA 2500739 A1 EP 1587409 A2 EP 1587409 A4 EP 2386862 A1 EP 2386863 A1 EP 2386864 A1 JP 2006-517286 A JP 2010-07 1997 A JP 2011-075579 A wo 2004-0327 11 A2 wo 2004-0327 11 A3	04.05.2004 24.06.2010 22.04.2004 26.10.2005 18.02.2009 16.11.2011 16.11.2011 16.11.2011 20.07.2006 02.04.2010 14.04.2011 22.04.2004 02.11.2006
US 5834220 A	10.11.1998	EP 0699306 A1 EP 0699306 B1 JP 09-503050 A US 5756682 A wo 94-27 156 A1	06.03.1996 08.09.1999 25.03.1997 26.05.1998 24.11.1994