**Title:** THE KIT FOR DIAGNOSING MIXED MALARIA INFECTION OF PLASMODIUM VIVAX AND PLASMODIUM FALCIPARUM COMPRISING SPECIFIC ANTIBODIES AGAINST LACTATE DEHYDROGENASE OF PLASMODIUM VIVAX AND PLASMODIUM FALCIPARUM

**Abstract:** The present invention relates to a kit for diagnosing malaria including a monoclonal antibody specific to Plasmodium vivax LDH, a monoclonal antibody specific to Plasmodium falciparum LDH, and a monoclonal antibody simultaneously specific to Plasmodium vivax LDH and Plasmodium falciparum LDH and a method of diagnosing mixed malaria infection using the same. The kit is the first rapid and convenient diagnosis kit in the world, which can discriminate the kinds of infected malaria and diagnose mixed infection of P. vivax and P. falciparum with high specificity and sensitivity. Further, the present invention discloses monoclonal antibodies specifically binding to P. vivax and P. falciparum LDHs used in the kit, and hybridoma producing the same. Finally, the present invention also discloses a kit for diagnosing malaria including a monoclonal antibody specific to P. vivax.

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[DESCRIPTION]

[Invention Title]

THE KIT FOR DIAGNOSING MIXED MALARIA INFECTION OF PLASMODIUM VIVAX AND PLASMODIUM FALCIPARUM COMPRISING SPECIFIC ANTIBODIES AGAINST LACTATE DEHYDROGENASE OF PLASMODIUM VIVAX AND PLASMODIUM FALCIPARUM

[Technical Field]
The present invention relates to monoclonal antibodies specific to Plasmodium vivax lactate dehydrogenase and Plasmodium falciparum lactate dehydrogenase, respectively, and a kit for diagnosing malaria comprising both of them, and a method of diagnosing malaria using the same. More particularly, the present invention relates to the world's first technique for diagnosis of mixed malaria infection. Further, the present invention also relates to a monoclonal antibody used for the kit and hybridoma for producing the same.

[Background Art]
Malaria is an infectious disease carried by a mosquito. Annually, three to five hundred million people are infected with malaria throughout the world, of which about 2.7 million people die due to infection. There are four types of the Plasmodium parasite that can infect humans: Plasmodium falciparum (P. falciparum), Plasmodium vivax (P. vivax), Plasmodium ovale (P. ovale), and Plasmodium malariae (P. malariae), in which P. falciparum is the most serious and causes high fatality, and P. vivax is the most widely spread species. In Korea, malaria, also called miasmatic fever, was reported to have been completely exterminated in the early 1980s by the efforts of the Korean government and WHO. However, since malaria re-emerged in 1993 by mosquitoes migrating from North Korea to South Korea across the DMZ, the number of malaria infections is continuously increasing. In recent years, owing to increasing overseas travel, it has been reported that travelers infected with Plasmodium falciparum in addition to indigenous Plasmodium vivax were treated in Korea.
The life cycle of malaria parasites is categorized into two stages: asexual reproduction in which malaria parasites develop in humans and sexual reproduction in which the malaria parasites develop in mosquitoes. When a mosquito bites a person, sporozoites in the mosquito's saliva enter the bloodstream and migrate to the liver. Then, the sporozoites infect the liver cells and multiply into merozoites therein. The merozoites escape from the liver and enter red blood cells, where they multiply several hundred folds, and then rupture the red blood cells to escape back into the bloodstream. Some of the merozoites turn into gametocytes. If a mosquito pierces the skin of an infected person, it potentially picks up gametocytes within the blood. These gametocytes multiply in the mosquito's mid gut and transfer to the mosquito's salivary glands to turn into sporozoites, which are latent until the mosquito bites a person.

Among techniques for diagnosing malarial infection, a blood smear analysis under a microscope is the typical and most reliable technique. The blood smear analysis is a process in which thin blood smears prepared on a slide glass are stained and examined under a microscope to locate red blood cells infected with malaria. This technique has advantages in that the cost of each test is considerably low; it is possible to diagnose malaria infection even if the blood includes only 5 to 10 malaria Plasmodia per ml; and it can distinguish four types of malaria Plasmodia. However, there are disadvantages in that: an expensive device, such as a microscope, is required; only an expert can correctly distinguish malaria infection; and it is not appropriate for mass tests. There is also a method of diagnosing malaria infection by examining blood stained with acridine orange under a fluorescent microscope. However, this method has a high priced test and requires devices such as a centrifugal separator, a fluorescent microscope, and a filter unit. Polymerase chain reaction (PCR) is a much more sensitive and specific diagnosis method than said method employing a microscope. However, PCR also requires expensive devices, such as a PCR device and a centrifugal separator, and an expert for DNA extraction, and entails a high priced test.
In diagnosis of malaria, Plasmodium lactate dehydrogenase, aldolase, and histidine rich protein-2 (HRP-2) are mainly used as target assays. Plasmodium lactate dehydrogenase was first separated from blood infected with malaria by D. L. Vander Jagt et al. in 1981. Makler reported in 1992 that malaria infection could be diagnosed using Plasmodium lactate dehydrogenase. Thereafter, in 1992, Piper et al. reported a kit in which *P. falciparum* lactate dehydrogenase genes are cloned to prepare monoclonal antibodies, which are used to distinguish between *P. falciparum* and *P. vivax* (Piper, R. C., et al., *Am. J. Trop. Med. Hyg.* 60 (1): 109-118, 1999). However, as *P. vivax* lactate dehydrogenase gene, generally found in Asia, was only cloned recently, monoclonal antibodies binding thereto have yet to be developed. The development of monoclonal antibodies specific to *P. vivax* lactate dehydrogenase is achieved by the present invention.

The biochemical properties of Plasmodium aldolase were first discovered by H. Dobeli et al. by preparing a recombinant protein from blood of a patient infected with *P. falciparum* and purifying the same. In 1999, Tjitra et al. prepared a kit consisting of monoclonal antibodies against Plasmodium aldolase and HRP-2 based on a rapid immunochromatography, and employed the kit to diagnosis malaria infection (Tjitra et al. (1999) *J. Clin. Microbiol.* 37, 2412-2417). They used monoclonal antibodies against HRP-2 for diagnosis of *P. falciparum*, and monoclonal antibodies against aldolase for diagnosis of *P. vivax*. The kit has specificity and sensitivity of 94% and 98%, respectively, but was reported to remarkably decrease in positivity rate if malaria Plasmodia density was 500 or less per µL of blood. HRP-2 is detected only in the case of infection with *P. falciparum*, so that infection with *P. vivax* or other malaria parasites cannot be determined. Also, since HRP-2 remains in blood of a patient whose treatment has been completed for several weeks, false positive reaction may occur (Hanscheitd (1999) *Clin. Lab. Haem.* 21 235). False positive reaction causes unnecessary treatment for malaria, exposing patients to medication having a number of side effects.

It has been reported that 25% of malarial patients are dually infected with both *P. falciparum* and *P. vivax*. However, different
medications are used to treat or cure *P. falciparum* and *P. vivax*, respectively. Moreover, as many strains of *P. falciparum* have recently become drug resistant, there are difficulties in treating or curing patients. On the other hand, *P. vivax* does not show serious drug resistance yet as compared with *P. falciparum*. However, unnecessary administration of drugs may cause *P. vivax* to develop drug resistance as well, so national regulation of treatment is required.

Although genes of *Plasmodium falciparum* and *Plasmodium vivax* have already been revealed, unfortunately, there is no method to specifically diagnose only *P. vivax* or to identify the dual infection of *P. falciparum* and *P. vivax* throughout the world except for genetic testing. This is because it is hard to cultivate the recombinant protein of lactate dehydrogenase (LDH) of *P. vivax* to be water-soluble and enzyme-active in *E. coli* and genetic homogeneity between lactate dehydrogenase of *P. vivax* and lactate dehydrogenase of *P. falciparum* is high, so that antibodies specific only to *P. vivax* cannot be produced. However, since genetic testing involves a lot of time, high cost, special equipment, and a highly skilled person, it has difficulties to be used in areas where malaria actually occurs a lot.

Accordingly, there is a need to develop a convenient and inexpensive kit for diagnosis of malaria, which is excellent in specificity and sensitivity and capable of discriminating the infection of only *P. vivax*, or distinguishing the kinds of Plasmodia, and specially identifying the dual infection of malaria.

[Disclosure]

The present invention has been conceived to solve the problems of the conventional techniques as described above, and the inventors of the present invention have developed a kit for diagnosis of malaria, which exhibits excellent specificity and sensitivity and is capable of discriminating infection of *Plasmodium vivax*, distinguishing the kinds of Plasmodia, and identifying mixed malaria infection with ease and economically. Further, the inventors developed a diagnosis kit including monoclonal and polyclonal
antibodies to lactate dehydrogenase of *P. vivax* and monoclonal antibodies to lactate dehydrogenase of *Plasmodium falciparum*.

To solve the foregoing problems, the present invention provides a method of detecting dual infection of malaria and a kit for diagnosing the same by preparing a specific monoclonal antibody to *P. vivax* lactate dehydrogenase (LDH) and simultaneously using a specific monoclonal antibody to *P. falciparum* LDH.

An aspect of the present invention provides a kit for diagnosing malaria, including specific monoclonal and polyclonal antibodies to *P. vivax* lactate dehydrogenase, a specific monoclonal antibody to *P. falciparum* lactate dehydrogenase, and a simultaneously specific monoclonal antibody to *P. vivax* lactate dehydrogenase and *P. falciparum* lactate dehydrogenase.

Another aspect of the present invention provides a method of diagnosing malaria using the kit.

A further aspect of the present invention provides hybridoma producing the monoclonal antibodies.

Yet another aspect of the present invention provides novel monoclonal antibodies specific to the lactate dehydrogenases used in the kit.

To solve the problems of the conventional techniques, the present invention provides a kit for diagnosing malaria including specific monoclonal and polyclonal antibodies to lactate dehydrogenase of *P. vivax*, a specific monoclonal antibody to lactate dehydrogenase of *P. falciparum*, and a simultaneously specific monoclonal antibody to *P. vivax* and *P. falciparum* lactate dehydrogenases. The diagnosis kit of the invention is a rapid and convenient kit which has excellent specificity and sensitivity over conventional diagnosis kits and is capable of distinguishing the kinds of malaria, in particular determining dual infection of malaria.

The monoclonal antibodies used in the kit for diagnosing malaria of the invention are ones specific to lactate dehydrogenase of *P. vivax* or to lactate dehydrogenase of *P. falciparum*, or both lactate dehydrogenase of *P. vivax* and lactate dehydrogenase of *P. falciparum*. In more detail, the monoclonal antibodies used in the
kit of the invention include a monoclonal antibody 1H3C10 specific to lactate dehydrogenase of *P. vivax* produced by hybridoma of Accession No. KCTC11239BP, a monoclonal antibody T12E specific to lactate dehydrogenase of *P. falciparum* produced by hybridoma of Accession No. KCTC11240BP, and a monoclonal antibody T28C simultaneously specific to lactate dehydrogenase of *P. vivax* and the lactate dehydrogenase of *P. falciparum* produced by hybridoma of Accession No. KCTC11241BP.

The inventors of the present invention extracted DNA from malaria Plasmodia in the blood of a Korean patient infected with *Plasmodium vivax* and in the blood a Vietnamese patient infected with *Plasmodium falciparum*, respectively, and amplified the lactate dehydrogenase genes of *P. vivax* and *P. falciparum* by polymerase chain reaction (PCR), respectively, which were used to prepare two kinds of genetically recombinant antigens (Figs. 4 and 5).

The genetically recombinant antigens were expressed in E coli, followed by refining (Fig. 6), after which they were injected into mice as immunogens to separate hybridoma secreting monoclonal antibodies specifically reactive only to lactate dehydrogenase of *P. vivax*, hybridoma secreting monoclonal antibodies specifically reactive only to lactate dehydrogenase of *P. falciparum*, and hybridoma secreting monoclonal antibodies specifically reactive to both lactate dehydrogenases of *P. falciparum* and *P. vivax*, thereby preparing monoclonal antibodies. Among the prepared monoclonal antibodies, antibodies exhibiting the highest reactivity, i.e., the specific antibody 1H3C10 to *P. vivax* lactate dehydrogenase, the specific antibody T12E to *P. falciparum* lactate dehydrogenase, and the specific antibody T28C to both *P. falciparum* and *P. vivax* lactate dehydrogenases, were sorted and used for the diagnosis kit of the invention. The kit may further include instructions, usually provided with a general diagnosis kit, diagnostic reagents, etc. in addition to the antibodies.

Assay systems used for the kit of the present invention, not limited to the following, includes Enzyme-Linked Immunosorbent Assay (ELISA), dipstick immunoassay, immunochromatographic assay, separate radioimmunoassay, flow-through immunoassay, etc. Preferably, the kit
of the present invention is a diagnosis kit in a strip form or a device form using immunochromatographic assay.

Immunochromatographic assay (ICA), also referred to as a rapid test due to its convenient and rapid features, is based on a principle in which a malaria antigen in the serum of a blood sample reacts with a tracer antibody bound to a colloidal gold particle and then combines with a capture antibody settled on the inner surface of pores of a nitrocellulose membrane to form a colorized band while transferring through the pores by a capillary phenomenon, thereby identifying positivity or negativity with the naked eye. Because of its simple procedures and rapid results, the immunochromatographic assay is widely used in detecting hormones, antigens, antibodies, and pharmaceutical components.

In the diagnosis kit of the invention, an antigen-antibody complex is detected by a color particle coupling method, in which examples of color particles include a colloidal gold particle, colored glass, or plastic (e.g., polystyrene, polypropylene, latex, etc.) beads. In this invention, the colloidal gold particle is used.

Further, in the diagnosis kit, the capture antibody binding to the antigen-antibody complex employs a monoclonal antibody specific to lactate dehydrogenase of *P. vivax* and a monoclonal antibody specific to lactate dehydrogenase of *P. falciparum*, preferably the monoclonal antibody 1H3C10 produced by the hybridoma of Accession No. KCTC11239BP and the monoclonal antibody T12E produced by the hybridoma of Accession No. KCTC11240BP. Another example of the capture antibody is rabbit polyclonal antibodies specific to lactate dehydrogenase of *P. vivax* and the lactate dehydrogenase of *P. falciparum*, respectively. As the tracer antibody binding to the colloidal gold particle, a monoclonal antibody simultaneously specific to both *P. vivax* and *P. falciparum* lactate dehydrogenases, specifically, T28C produced by the hybridoma of Accession No. KCTC11241BP is used.

In more detail, the kit for diagnosing malaria using the immunochromatographic assay comprises two main parts, that is, a nitrocellulose membrane with three invisible lines formed on the surface thereof and a glass fiber pad (or a plastic well) configured
to hold an antibody-gold conjugate in a dry state. Three different antibodies are fixed in the invisible lines of the nitrocellulose membrane. In detail, the lowermost test line 1(T1) holds the monoclonal antibody T12E specific to lactate dehydrogenase of *P. falciparum*, test line 2 (T2) holds the monoclonal antibody 1H3C10 specific to lactate dehydrogenase of *P. vivax*, and the uppermost control line (C) holds a goat anti-mouse immunoglobulin (IgG) antibody. The antibody-gold conjugate obtained by conjugation of the monoclonal antibody T28C simultaneously specific to both *P. vivax* and *P. falciparum* lactate dehydrogenases and colloidal gold particles is injected into a glass fiber pad (or a plastic well) and dried. If a sample is put in a sample depositary part of the kit thus constituted, the antibody-gold conjugate maintained in a dry state is hydrated and combines with an antigen in the sample while transferring through pores in the nitrocellulose membrane by a capillary phenomenon. Subsequently, the antigen binding to the antibody-gold conjugate reacts with the antibodies held in the invisible lines so that a corresponding invisible line appears red due to the antibody-gold conjugate with red. Further, the goat anti-mouse IgG antibody held in the upper line is capable of reacting with the antibody-gold conjugate even without an antigen, the control line appears red in every test, and thus, it can be verified that the test is carried out properly. That is, if an antigen is present, the antigen will react with a corresponding antibody in a test line of the kit, so that the test line and the control line appear at the same time. If there is no antigen, the test line will not appear but only the control line will turn into red.

Further, the diagnosis kit of the invention simultaneously employs the monoclonal antibody specific to lactate dehydrogenase of *P. vivax* and the monoclonal antibody specific to lactate dehydrogenase of *P. falciparum*, thereby distinguishing the kinds of malarial Plasmodia and the mixed malaria infection. If the diagnosis kit with the aforementioned element is brought into contact with a *P. falciparum* positive sample, a color band appears on the test line 1 (T1); and if the kit is brought into contact with a *P. vivax* positive sample, the color band appears on the test line 2 (T2). If
the kit is brought into contact with a sample infected with both, the color band appears on the test lines 1 and 2 (T1 and T2) at the same time. Hence, the kit of the invention can identify the mixed malaria infection and discriminate the kinds of Plasmodia with convenience and rapidity.

Yet another aspect of the present invention provides a method for diagnosing the mixed malaria infection and discriminating the kinds of Plasmodia using rabbit polyclonal antibodies specific to P. vivax lactate dehydrogenase and P. falciparum lactate dehydrogenase, which are prepared by immuno-affinity chromatography which employs the recombinant P. vivax lactate dehydrogenase and the recombinant P. falciparum lactate dehydrogenase according to the present invention. That is, as capture antibodies, a specific rabbit polyclonal antibody to P. falciparum lactate dehydrogenase, a specific rabbit polyclonal antibody to P. vivax lactate dehydrogenase, and a goat anti-mouse InG antibody are fixed to test line 1 (T1), test line 2 (T2), and control line (C), respectively. Colloidal gold particles are conjugated with a mouse monoclonal antibody T28C simultaneously specific to P. vivax and P. falciparum lactate dehydrogenases and disposed in a conjugation pad, thereby discriminatively diagnosing malaria.

Yet another aspect of the present invention provides a method of diagnosing malaria comprising performing an antigen-antibody reaction using the kit of the present invention. In the diagnostic method of the invention, collected whole blood is brought into contact with a sample depositary part of the kit and absorbed to determine the presence of P. falciparum or P. vivax in accordance with indications of test lines of the kit.

Yet another aspect of the present invention provides novel monoclonal antibodies and polyclonal antibodies specific to P. vivax and P. falciparum lactate dehydrogenases used in the kit of the invention.

Yet another aspect of the present invention provides a monoclonal antibody specific to P. vivax lactate dehydrogenase and a kit for diagnosing P. vivax comprising the same.

Yet another aspect of the present invention provides a method
of detecting the mixed malaria infection and a kit for diagnosing the same using a specific monoclonal antibody to lactate dehydrogenase of *P. vivax* and a specific monoclonal antibody to lactate dehydrogenase of *P. falciparum*, the kit further employing a monoclonal antibody simultaneously specific to *P. vivax* and *P. falciparum* lactate dehydrogenases.

In the present invention, the monoclonal antibodies and polyclonal antibodies can be prepared by any known method in the art. In more detail, the monoclonal antibodies are prepared as follows: a mouse is immunized with *P. falciparum* and *P. vivax* lactate dehydrogenases as immunogens; its splenocyte is fused with myeloma to reproduce hybridoma; and hybridoma are selected based on the specificity to the respective lactate dehydrogenases. Specifically, DNA is extracted from malarial *Plasmodium* in patient's blood infected with *P. falciparum* and from one infected with *P. vivax*, respectively, as illustrated in Fig. 1, and subjected PCR using primers BL-PvLDH-F and BL-PvLDH-R given in Fig. 3 to amplify *P. vivax* lactate dehydrogenase genes (Fig. 4), and using primers BL-PfLDH-F and BL-PfLDH-R to amplify *P. falciparum* genes (Fig. 5), thereby preparing recombinant antigens. The antigens are purified and injected as immunogens into a mouse to selectively clone hybridoma secreting anti-*Plasmodium* lactate dehydrogenase monoclonal antibodies specifically reactive only to *P. vivax* and *P. falciparum*, respectively, and hybridoma secreting an anti-*Plasmodium* lactate dehydrogenase monoclonal antibody reactive to both *P. falciparum* and *P. vivax*. In more detail, to select hybridoma secreting anti-malaria lactate dehydrogenase monoclonal antibodies specifically reactive to each of *P. vivax* and *P. falciparum*, respectively, an ELISA plate binding to recombinant *P. vivax* lactate dehydrogenase and an ELISA plate binding to recombinant *P. falciparum* lactate dehydrogenase are prepared and screened in reactivity. Hybridoma are selected which secrete a monoclonal antibody reactive in the plate binding to the recombinant *P. vivax* lactate dehydrogenase but not reactive in the plate binding to the recombinant *P. falciparum* lactate dehydrogenase. The monoclonal antibody thus secreted was named 1H3C10. Hybridoma are selected which secrete a monoclonal antibody reactive in the
plate binding to the recombinant *P. falciparum* lactate dehydrogenase but not reactive in the plate binding to the recombinant *P. vivax* lactate dehydrogenase. The monoclonal antibody thus secreted was named T12E. Hybridoma are selected which secrete a monoclonal antibody reactive in both plates. The monoclonal antibody thus secreted was named T28C. These monoclonal antibodies are employed in preparing the kit of the present invention. The hybridoma thus selected are abdominally injected into mice, respectively, and, after a predetermined period of time, the ascitic fluid is extracted, followed by separation of monoclonal antibodies. Hybridoma which produce the monoclonal antibody 1H3C10 selectively identifying only lactate dehydrogenase of *P. vivax* were accepted as KCTC11239BP (Acceptance Date: Nov. 15, 2007), hybridoma which produce the monoclonal antibody T12E selectively identifying only lactate dehydrogenase of *P. falciparum* were accepted as KCTC11240BP (Acceptance Date: Nov. 15, 2007), and hybridoma which produce the monoclonal antibody T28C selectively identifying *P. falciparum* and *P. vivax* lactate dehydrogenases were accepted as KCTC11241BP (Acceptance Date: Nov. 15, 2007).

 Accordingly, still another aspect of the present invention relates to the hybridoma KCTC11239BP, KCTC11240BP, and KCTC11241BP which produce the monoclonal antibodies 1H3C10, T12E, and T28C, respectively.

[Description of Drawings]

Fig. 1 is a flowchart illustrating a method of producing a mouse monoclonal antibody against lactate dehydrogenase of *Plasmodium vivax* according to the present invention;" Fig. 2 is a flowchart illustrating a method of producing a rabbit polyclonal antibody against lactate dehydrogenase of *P. vivax* according to the present invention;  

Fig. 3 is a DNA sequence of a primer used for gene amplification of *P. vivax* and *P. falciparum* lactate dehydrogenases according to the present invention;  

Figs. 4 and 5 are a DNA sequence of lactate dehydrogenase of *P. vivax* and a DNA sequence of lactate dehydrogenase of *P. 
falciparum which are used to produce recombinant proteins according to the present invention, respectively!

Fig. 6 illustrates results of genetic amplification of lactate dehydrogenase of P. vivax and lactate dehydrogenase of P. falciparum according to the present invention!

Fig. 7 illustrates the results obtained by purifying a recombinant protein, a gene of which has been expressed in E. coli. In Fig. 7, M is standard size markers for the analysis of protein on the basis of molecular weight, lane 1 is a cell effluent where P. vivax lactate dehydrogenase is expressed, lane 2 is purified P. vivax lactate dehydrogenase, lane 3 is a cell effluent where P. falciparum lactate dehydrogenase is expressed, and lane 4 is purified P. falciparum lactate dehydrogenase!

Figs. 8 and 9 are graphs depicting reactivities of monoclonal antibodies on P. vivax lactate dehydrogenase and P. falciparum lactate dehydrogenase according to the present invention. The reactivities were measured by treating the monoclonal antibodies of the invention having each different concentration with plates containing P. falciparum lactate dehydrogenase (Fig. 8) and P. vivax lactate dehydrogenase (Fig. 9), respectively!

Fig. 10 is graphs depicting dissociation constants of the monoclonal antibodies on P. vivax lactate dehydrogenase and P. falciparum lactate dehydrogenase according to the present invention!

Fig. 11 is a diagram illustrating a strip for an immunochromatographic assay that can rapidly diagnose infection of P. falciparum, P. vivax, or the mixed infection thereof according to the present invention!

Fig. 12 is pictures showing test results obtained by the strip in the rapid immunochromatographic assay diagnosing a malarial specific antigen according to the present invention!

Fig. 13 is a diagram illustrating a device for an immunochromatographic assay that can rapidly diagnose infection of P. falciparum, P. vivax, or mixed infection thereof according to the present invention! and

Fig. 14 is pictures showing test results obtained by the device in the rapid immunochromatographic assay diagnosing a
malarial specific antigen according to the present invention.

[Embodiments]
Hereinafter, the present invention will be described in detail with reference to exemplary embodiments. However, it should be noted that these embodiments are given by way of illustration, and do not limit the scope of the present invention.

Example 1: Cloning, Massive Expression, and Refining of Plasmodium Vivax and Plasmodium Falciparum Lactate Dehydrogenase Genes

1) Genetic amplification of P. vivax and P. falciparum lactate dehydrogenases through purification and polymerase chain reaction of malaria parasite DNA

DNAs of P. vivax and P. falciparum were extracted from the blood of a patient carrying P. vivax provided by Sungkyunkwan University of Medicine (Suwon, Korea) and from the blood of a patient carrying P. falciparum provided by Ho Chi Minh City University of Medicine (Ho Chi Minh City, Vietnam), respectively, by Dr. GenTLE Kit (TAKARA BIO, Ltd. Japan) according to instructions provided therewith. With each of the DNAs given as a template, a portion corresponding to a Plasmodium lactate dehydrogenase gene was amplified using opposite end-primers, and then cloned to an expression vector pET-28a (Novagen Ltd., USA), capable of expression in E. CoII. The primer has a sequence given in Fig. 3. That is, in the amplification and cloning, primers BL-PvLDH-F (Sequence Number 1) and BL-PvLDH-R (Sequence Number 2) were used for P. vivax lactate dehydrogenase, and primers BL-PfLDH-F (Sequence Number 3) and BL-PfLDH-R (Sequence Number 4) were used for P. falciparum lactate dehydrogenase. The cloned Plasmodium lactate dehydrogenase genes were analyzed by a DNA sequence analyzer, ABI 377 (PerkinElmer Ltd., USA), results of which are shown in Fig. 4 (P. vivax lactate dehydrogenase: Sequence Number 5) and Fig. 5 (P. falciparum lactate dehydrogenase: Sequence Number 6).

2) Purification of recombinant P. vivax and P. falciparum lactate dehydrogenases
The recombinant Plasmodium lactate dehydrogenases were purified using histidine-affinity columns (Invitrogen Ltd., USA) and treated with thrombin (Novagen Ltd., USA) to separate a histidine tag. The product was passed through the histidine-affinity column again to eliminate the histidine tag, and passed through a HiTrap Benzamidine column (Amersham Bioscience, Sweden) to eliminate the thrombin, thereby producing pure recombinant Plasmodium lactate dehydrogenases.

Example 2: Preparation of Monoclonal Antibodies against P. vivax and P. falciparum lactate dehydrogenases

1) Immunization with recombinant Plasmodium lactate dehydrogenases

A suspension was prepared by mixing a phosphate buffered saline (PBS) solution containing a certain amount of the recombinant Plasmodium lactate dehydrogenases with the same volume of a complete Freund's adjuvant and subcutaneously injected into a goat, a rabbit, or other animals prone to produce a polyclonal antibody. After three weeks, the subcutaneous injection was carried out again in the same manner with an incomplete Freund's adjuvant instead, and after another three weeks the injection was repeated in the same manner. Three to five days after the last immunization, blood was collected to obtain a serum, which was diluted with a PBS solution (1/1000) to determine the titer of an antibody by enzyme-linked immunosorbent assay (ELISA). If the titer was low, immunization was carried out one week later.

Blood was collected from thoroughly immunized subject and centrifuged to obtain a serum. The serum was injected into a column containing a Sepharose filler binding to the recombinant lactate dehydrogenase via a covalent bond. Five times or more of physiological saline solution as much volume as fillers in the column was used to wash away proteins that were not bound to the column. Then, antibodies combined to the column were eluted with a 100mM glycine solution (pH: 2.8). Here, a 1M Tris solution (pH: 9.0) was added at a volume of 1/10 to adjust pH. The eluted antibody solution was concentrated and dialyzed in a 150mM phosphate buffer.
Then, the amount of the antibodies was determined using a Bradford assay and the antibodies were stored in a freezer until use.

Example 3: Preparation of Monoclonal Antibodies against *P. vivax* and *P. falciparum* lactate dehydrogenases

1) Immunization of antigen

A suspension was prepared by mixing a PBS solution containing 100/μg of each of the recombinant Plasmodium lactate dehydrogenases with the same volume of a complete Freund's adjuvant and injected into a six to eight-week-old female mouse's peritoneal (BALA/C, Dae Han Biolink Co., Ltd, Korea). After two weeks, the peritoneal injection was carried out again in the same manner with an incomplete Freund's adjuvant instead, and after another two weeks the injection was repeated in the same manner. Two days later after the last immunization, blood was collected from the tail to obtain a serum, which was diluted with a PBS solution (1/1000) to determine the titer of an antibody by ELISA. If the titer was low, immunization was carried out two weeks later.

2) Fusion of splenocyte cells and myeloma cells

The spleen of the immunized mouse was crushed in a tissue homogenizer, and the cell suspension was collected in a container and centrifuged to collect cells. Myelomas were collected from a cell culture flask and suspended in RPMI1640 to calculate the number of cells. The number of splenocyte cells was also calculated. IX10⁷ myeloma cells and 1X10⁸ splenocyte cells were put in a 50ml container and mixed with an adequate amount of RPMI1640, followed by centrifugal separation at 200Xg for five minutes to collect cells. The cells were suspended and gently shaken, adding ImI of 50% polyethylene glycol (PEG) over one minute. Five minutes later, ImI of RPMI1640 was added over 30 seconds, and then another 3ml was added over 30 seconds. Subsequently, 17ml of RPMI1640 was added over one minute, and another 20ml more to react for five minutes. After centrifugal separation at 200Xg for five minutes, the medium was removed. The product was suspended carefully in 50ml of RPMI1640 containing 1% Hypoxanthine Aminopterin Thymidine medium (HAT), after which fused cells were aliquoted at 100/^/well in a 96-well cell
culture plate containing feeder cells and cultured at 37\(^\circ\)C in a 5% CO2 incubator.

3) Production of antibody using cloning and ascitic fluid of hybridoma

About ten days later, when identifying the fused cell colonies, some medium was removed from the 96-well cell culture plate to determine if the well produced antibodies to the Plasmodium lactate dehydrogenases by ELISA. Then, cells collected from the well were moved to a 24-well cell culture plate and cultured, followed by continuous cloning by limited dilution until stable monoclonal cells were obtained. When the cloning was completed, the product was put in a T flask and bulk cultured. Then, the product was frozen and stored in liquid nitrogen.

0.5ml of an incomplete Freund's adjuvant was injected into the abdominal cavity of a six to eight-week-old mouse (BALA/C). Suspending hybridoma in a PBS solution and cell counting on day seven, 1.5X10\(^6\) cells per mouse were suspended in 0.5ml of a PBS solution and injected into a mouse's peritoneal. When being produced sufficiently after one or two weeks, the ascitic fluid was collected with an injector and kept in a freezer.

4) Isolation of monoclonal antibodies

Ammonium sulfate was added to the collected ascitic fluid at a concentration of 10% and mixed for 30 minutes. Then, the product was centrifuged at 15,000 rpm for 30 minutes to collect a supernatant. Ammonium sulfate was added to the supernatant at a concentration of 50% and left at 4\(^\circ\)C for 30 minutes. Centrifuging the product at 15,000 rpm for 30 minutes, a supernatant was discarded and a precipitant was suspended in a 20mM phosphate buffer (pH: 7.0). The suspension was dialyzed in a 20mM phosphate buffer for over 18 hours. Then, the dialyzed solution was injected to a protein G-coupled column and equilibrated by a 20mM phosphate buffer (pH: 7.0), after which unabsorbed materials were removed using a phosphate buffer. Antibodies attached to the column were eluted with a 100mM glycine solution (pH: 2.8). Here, a 0.1M Tris solution (pH: 9.0) was added at a volume of 1/10 of the eluted solution to adjust pH. The eluted antibody solution was concentrated and dialyzed in a
150mM phosphate buffer. Then, the amount of the antibodies was determined using a Bradford assay and the antibodies were stored in a freezer before use.

5) Selection of monoclonal antibody and calculation of dissociation constant (Kd)

The refined Plasmodium lactate dehydrogenase-specific monoclonal antibodies were measured by ELISA to identify reactivity with respect to Plasmodium lactate dehydrogenases. For this purpose, 96-well ELISA plates were coated with two kinds of Plasmodium lactate dehydrogenases at a concentration of 5 mg/ml, respectively. The refined Plasmodium lactate dehydrogenase-specific monoclonal antibodies were diluted in each step to react with a plate coated with *P. vivax* lactate dehydrogenase and a plate coated with *P. falciparum* lactate dehydrogenase, respectively, and uncombined antibodies were removed by washing. A goat anti-mouse immunoglobulin (IgG) antibody was reacted with horseradish peroxidase (HRP), and unreacted peroxidase-IgG was removed by washing. Tetramethyl benzidine (TMB), serving as a substrate of peroxidase, was added to give color, and absorbance (A450) was measured according to reactivity. As shown in Figs. 8 and 9, 1H3C10 exhibited good reactivity with respect to *P. vivax* lactate dehydrogenase, T12E exhibited good reactivity with respect to *P. falciparum* lactate dehydrogenase, and T28C exhibited good reactivity with respect to both.

In order to measure the dissociation constants (Kd) of the three antibodies to be used for the kit of the invention, the Klotz plot was used via indirect competitive ELISA to obtain the results given in Fig. 10. That is, the monoclonal antibody T12E has a dissociation constant of 25.24 ± 0.51×10⁻⁹; the monoclonal antibody T28C has a dissociation constant of 24.21±2.07×10⁻⁹; and the monoclonal antibody 1H3C10 has a dissociation constant of 36.52±1.12×10⁻⁹.

**Example 4:** Preparation of Strip Diagnosis Kit for Rapid Immunochromatographic Assay Using Monoclonal Antibodies against *P. vivax* and *P. falciparum* lactate dehydrogenases
1) Fixation of antibodies

As shown in Fig. 11, the specific monoclonal antibody T12E to P. falciparum lactate dehydrogenase, the specific monoclonal antibody 1H3C10 to P. vivax lactate dehydrogenase, and the goat anti-mouse immunoglobulin (IgG) antibody were placed on specific positions of a nitrocellulose membrane attached to a plastic card, i.e., on test line 1 (T1), test line 2 (T2), and control line (C), respectively, and dried in an incubator at 30°C for two days, so that they could be completely settled on the nitrocellulose membrane.

2) Preparation of gold conjugate and gold conjugate well

The monoclonal antibody T28C simultaneously specific to both P. vivax and P. falciparum lactate dehydrogenases was mixed with colloidal gold particles of about 40 nm and reacted in a 37°C water bath for one hour so that the monoclonal antibody was bound to the colloidal gold particles. Then, 3% and 1% bovine serum albumin (BSA) and sucrose were added thereto, respectively, and reacted for one hour with colloidal gold particles which had not bound to the antibody. Subsequently, the product was centrifuged at 10,000 rpm to collect gold conjugates binding to the monoclonal antibody, which were measured for absorbance at 540 nm and kept refrigerated. The gold conjugates were injected into a plastic micro-well plate and dried, thereby preparing a gold conjugate well.

3) Assembly of strip kit

As illustrated in Fig. 11, an absorbance pad capable of absorbing a sample and a buffer was attached to an upper portion of the plastic card to which the nitrocellulose membrane binding to a monoclonal antibody to Plasmodium lactate dehydrogenase was adhered, and a sticker was attached to the top thereof, thereby completing a strip kit.

Example 5: Preparation of Device Diagnosis Kit for Rapid Immunochromatographic Assay Using Monoclonal Antibodies against P. vivax and P. falciparum lactate dehydrogenases

1) Fixation of antibodies

As shown in Fig. 13, the specific monoclonal antibody T12E to P. falciparum lactate dehydrogenase, the specific monoclonal
antibody 1H3C10 to *P. vivax* lactate dehydrogenase, and the goat anti-mouse immunoglobulin (IgG) antibody were placed on specific positions of a nitrocellulose membrane attached to a plastic card, i.e., on test line 1 (T1), test line 2 (T2), and control line (C), respectively, and dried in an incubator at 30°C for two days, so that they could be completely settled on the nitrocellulose membrane.

2) Preparation of gold conjugate and gold conjugate pad

The monoclonal antibody T28C simultaneously specific to both *P. vivax* and *P. falciparum* lactate dehydrogenases was mixed with colloidal gold particles of about 40 nm and reacted in a 37°C water bath for one hour so that the monoclonal antibody was bound to the colloidal gold particles. Then, 3% and 1% bovine serum albumin (BSA) and sucrose were added thereto, respectively, and reacted for one hour with colloidal gold particles which had not bound to the antibody. Subsequently, the product was centrifuged at 10,000 rpm to collect gold conjugates binding to the monoclonal antibody, which were measured for absorbance at 540 nm and kept refrigerated. The gold conjugates were absorbed to a pad of glass fiber and dried, thereby preparing a gold conjugate pad.

3) Assembly of device kit

As illustrated in Fig. 13, an absorbance pad capable of absorbing a sample and a buffer was attached to an upper portion of the plastic card to which the nitrocellulose membrane binding to a monoclonal antibody to *Plasmodium* lactate dehydrogenase was adhered, and a sample pad having a sample deposited thereto and a conjugate pad were attached to lower portions thereof. The assembled reaction strip was placed in a plastic housing, thereby completing a device kit, as shown in Fig. 13.

Example 6: Test Process with Strip Kit

A gold conjugate well and a washing well were prepared. One drop of a sample mobile phase was dropped in the gold conjugate well, and four drops thereof were dropped in the washing well provided with the kit. By pricking a patient on the index finger sterilized by alcohol with a disposable lancet, 10ml of blood was collected with a capillary tube. The collected blood was placed in the gold
conjugate well and mixed with the sample mobile phase using a
capillary tube. Then, after placing a reaction strip in the well for
ten minutes to absorb the sample solution, the reaction strip was
moved to the washing well containing a washing solution and kept for
ten minutes. Once the strip have completely reacted, infection with
P. falciparum or P. vivax or the mixed malaria infection was
determined according to a position of a red band appearing on the
test lines, as illustrated in Fig. 12. Namely, it was determined
that the sample was positive for P. falciparum when the color band
appeared only on the test line 1 (T1); the sample was positive for P.
vivax when the color band appeared only on the line 2 (T2); and the
sample was infected with mixed malaria when the color bands appeared
on both lines.

Example 7- Test Process with Device Kit
The device was placed on a flat surface. By pricking a
patient on the index finger sterilized by alcohol with a disposable
lancet, 10µl of blood was collected with a capillary tube. The
collected whole blood was placed in a sample depositary part to be
absorbed, as shown in Fig. 13. Three drops of a washing solution
were dropped in a washing solution inlet and stored for 15 minutes.
Once the kit was completely reacted, the infection of P. falciparum
or P. vivax or the mixed malaria infection was determined according
to a position of a red band appearing on the test lines, as shown in
Fig. 14. Namely, it was determined that the sample was positive for
P. falciparum when the color band appeared only on the test line 1
(T1); the sample was positive for P. vivax when the color band
appeared only on the line 2 (T2); and the sample was infected with
mixed malaria when the color bands appeared on both lines.

Example 8: Performance Tests of Strip Kit and Device Kit for
Rapid Immunochromatographic Assay Using Plasmodium Lactate
Dehydrogenases
The diagnosis kits prepared according to Examples 3 to 5 were
used to test 129 samples positive for P. vivax, 86 samples positive
for P. falciparum, and 7 samples positive for mixed malaria. A total
of 129 P. r/raz-positive samples were tested, in which 30 samples were donated by Sungkyunkwan University of Medicine (Suwon, Korea), 47 samples by Ho Chi Minh City University of Medicine (Ho Chi Minh City, Vietnam), and 52 samples by Inje University of Medicine (Busan, Korea). A total of 86 P. falciparum positive samples were tested, in which 25 samples were donated by Ho Chi Minh City University of Medicine (Ho Chi Minh City, Vietnam), 9 samples by Inje University of Medicine, and 52 samples by National Institute of Malariology, Parasitology and Entomology (Hanoi, Vietnam). The seven samples infected with mixed malaria of P. vivax and P. falciparum, confirmed by genetic testing via PCR, were donated by Ho Chi Minh City University of Medicine to test performances of the kits. The test results are given in Table 1 (Comparative test results of samples positive for P. vivax), Table 2 (Comparative test results of samples positive for P. falciparum), and Table 3 (Comparative test results of samples positive for mixed infection of P. vivax and P. falciparum).

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<th>Results of Microscopic Examination and PCR</th>
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<tr>
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<td>P. vivax Positive</td>
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<tr>
<td>Nano Sign Malaria</td>
<td>123</td>
</tr>
<tr>
<td>Pf/Pv/Mixed</td>
<td>0</td>
</tr>
<tr>
<td>Mixed Malaria infection</td>
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<td>Negative</td>
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<tr>
<td>Total</td>
<td>129</td>
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Sensitivity 95.3%

According to results of a microscopic examination and PCR
with the nano sign malaria of the present invention, as shown in Table 1, 123 samples turned out to be positive and the remaining six proved to be negative among 129 samples positive for *P. vivax*, corresponding to a sensitivity of 95.3%.

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<td>Results of Microscopic Examination and PCR</td>
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<td></td>
<td>P. vivax Positive</td>
<td>P. falciparum Positive</td>
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<td>0</td>
</tr>
<tr>
<td>P. falciparum Positive</td>
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<td>83</td>
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<tr>
<td>Mixed Malaria Infection</td>
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<td>0</td>
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<tr>
<td>Negative</td>
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<td>3</td>
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<tr>
<td>Total</td>
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<td>86</td>
</tr>
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Sensitivity: 96.5%

According to results of a microscopic examination and PCR with the nano sign malaria of the present invention, as shown in Table 2, 83 samples turned out to be positive and the remaining three proved to be negative among 86 samples positive for *P. falciparum*, corresponding to a sensitivity of 96.5%.
According to results of a microscopic examination and PCR with the nano sign malaria of the present invention, as shown in Table 3, six samples turned out to be positive and the remaining one proved to be negative among seven samples positive for both P. vivax and P. falciparum, corresponding to a sensitivity of 85.7%.

As apparent from the examples, the diagnosis kit of the invention had a superior sensitivity to conventional diagnosis kits in view of a sensitivity of 85 to 95% with respect to P. falciparum and a sensitivity of 90% or less with respect to P. vivax. Further, the kit of the present invention could detect mixed malaria infection, which could not have been detected by the conventional kits, and thus excellent performance of the kit according to the invention was confirmed.

[Industrial Applicability]

The diagnosis kit having antibodies for P. vivax and P. falciparum lactate dehydrogenases realized by the present invention is a convenient and inexpensive kit, which can discriminate P. vivax
and *P. falciparum*, diagnose mixed infection of *P. vivax* and *P. falciparum*, which cannot be diagnosed by the conventional kits, and overcome a low sensitivity to *P. vivax*. In particular, the present invention will have a global impact on control of bacteria resistant to *P. vivax* medication.
[CLAIMS]

[Claim 1]
A kit for diagnosing malaria, comprising a monoclonal antibody specific to *P. vivax* lactate dehydrogenase (LDH), a monoclonal antibody specific to *P. falciparum* lactate dehydrogenase, and a monoclonal antibody simultaneously specific to *P. vivax* and *P. falciparum* lactate dehydrogenases.

[Claim 2]
The kit for diagnosing malaria according to claim 1, wherein the monoclonal antibody specific to *Plasmodium vivax* LDH is a monoclonal antibody 1H3C10, the monoclonal antibody specific to *Plasmodium falciparum* LDH is T12E, and the monoclonal antibody simultaneously specific to *Plasmodium vivax* and *Plasmodium falciparum* LDHs is T28C.

[Claim 3]
A kit for diagnosing malaria, comprising a polyclonal antibody specific to *Plasmodium vivax* LDH, a polyclonal antibody specific to *Plasmodium falciparum* LDH, and a monoclonal antibody simultaneously specific to *Plasmodium vivax* and *Plasmodium falciparum* LDHs.

[Claim 4]
The kit for diagnosing malaria according to any one of claims 1 to 3, wherein the kit diagnoses mixed infection of *Plasmodium vivax* and *Plasmodium falciparum*.

[Claim 5]
The kit for diagnosing malaria according to any one of claims 1 to 3, wherein the kit is a strip-shaped kit based on immunochromatographic assay.

[Claim 6]
The kit for diagnosing malaria according to any one of claims 1 to 3, wherein the kit detects an antigen-antibody complex by a color particle coupling method.

[Claim 7]
A method of diagnosing malaria comprising conducting an antigen-antibody reaction using the kit for diagnosing malaria according to any one of claims 1 to 3.
[Claim 8]
The method according to claim 7, wherein the method diagnoses mixed infection of \textit{Plasmodium} \textit{vivax} and \textit{Plasmodium falciparum}.

[Claim 9]
Hybridoma (Acceptance Number: KCTC11239BP) producing a monoclonal antibody 1H3C10 specific to \textit{Plasmodium vivax} LDH, hybridoma (Acceptance Number: KCTC11240BP) producing a monoclonal antibody T12E specific to \textit{Plasmodium falciparum} LDH, or hybridoma (Acceptance Number: KCTC11241BP) producing a monoclonal antibody T28C simultaneously specific to \textit{Plasmodium vivax} and \textit{Plasmodium falciparum} LDHs.

[Claim 10]
A monoclonal antibody specific to \textit{Plasmodium vivax} LDH.

[Claim 11]
The monoclonal antibody according to claim 10, wherein the antibody is 1H3C10 produced by hybridoma (Acceptance Number: KCTC11239BP).

[Claim 12]
A kit for diagnosing \textit{Plasmodium vivax} comprising the monoclonal antibody specific to \textit{Plasmodium vivax} LDH according to claim 10 or 11.

[Claim 13]
A method of diagnosing mixed malaria infection using a monoclonal antibody specific to \textit{Plasmodium vivax} LDH and a monoclonal antibody specific to \textit{Plasmodium falciparum} LDH.

[Claim 14]
A kit for diagnosing mixed malaria infection using a monoclonal antibody specific to \textit{Plasmodium vivax} LDH and a monoclonal antibody specific to \textit{Plasmodium falciparum} LDH.

[Claim 15]
The kit according to claim 14, wherein the kit further employs a monoclonal antibody simultaneously specific to \textit{Plasmodium vivax} and \textit{Plasmodium falciparum} LDHs.
[Figure 1]

- Clone genes of *P. vivax* and *P. falciparum* lactate dehydrogenases
- Produce genetic recombinant antigens of *P. vivax* and *P. falciparum* lactate dehydrogenases
- Immunize mouse and prepare hybridoma
- Produce monoclonal antibodies against *P. vivax* and *P. falciparum* lactate dehydrogenases
- Refine monoclonal antibodies using protein G-coupled affinity column
[Figure 2]

1. Produce genetically recombinant proteins of *P. vivax* and *P. falciparum* lactate dehydrogenase

2. Prepare immuno-affinity column binding to *P. vivax* recombinant lactate dehydrogenase

3. Prepare immuno-affinity column binding to *P. falciparum* recombinant lactate dehydrogenase

4. Immunize rabbit using recombinant proteins of *P. vivax* and *P. falciparum* lactate dehydrogenases

5. Refine polyclonal antibodies specific to plasmodium lactate dehydrogenases via immuno-affinity chromatography
### Figure 3

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<td>P. vivax LDH forward primer</td>
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<tr>
<td>BL-PvLDH-R</td>
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<td>P. vivax LDH reverse primer</td>
</tr>
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<td></td>
<td>(Sequence No. 2)</td>
<td></td>
</tr>
<tr>
<td>BL-PfLDH-F</td>
<td>5'-caagatccatggcaaaagaacga-3'</td>
<td>P. falciparum LDH forward primer</td>
</tr>
<tr>
<td></td>
<td>(Sequence No. 3)</td>
<td></td>
</tr>
<tr>
<td>BL-PfLDH-R</td>
<td>5'-cgctctgagtaactaagtgcttcattc-3'</td>
<td>P. falciparum LDH reverse primer</td>
</tr>
<tr>
<td></td>
<td>(Sequence No. 4)</td>
<td></td>
</tr>
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</table>

### Figure 4

5'-atgacgcccag acacccaaat tgtctctgct agggcgcggc cagtcggtttg tgcagaaaacctgggggc gtaagacgcgtt tgaacatcctt gcatggcag gatcctcttagg aagccgtggtc gccggtgtcag gagaacctttg aagcgaagcagc gagcttgtagt cggagaagcc aagccgggtactacagctgcgtttcagttgtgctgtttgctgcttcgcttcgcttcgtttgtgcttcgcttcgcttcggcgttggctttttgccgctgccgttttttttgggctttgttgttttgggctttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttttt
[Figure 5]
5′-atggcaccacaaaagcaaaat cgttttagtt ggctcaggta tgattggagg agtaatggct accitaatg tgtcaagaaaaa tttaggagat gtatgtttttgt tcgatatttgt aaagaacagt ccacatttga aagcttttaga tacaactcat acctaatgtta tggcatattc aaatggcaaa gtaagttgtt caacacatata tcacgttttg gctgggacag aigtagtaat agtaacagct ggatttacca aggccccagg aaagagttgc aaagaatgga atagagatga ttattacca ttaaaacaca agatatagat tggtaattgg ggtcatatta agaaagattg tccaatgtct tttattagt tgtcaacaaaa cccagtagat gttatgttac aattttaca tcaacatcca ggtgctcctata aaaaagagat tatttggttgg ggtgtgttat tagatacatc aagattgaag tattacatat tccaagaattaatgtatggc ccaagagatg taaatgcaca catttttaggt gctcaattggaa ataaaatggt tctttttttaa agatacattta ctgtaggtgg tagccctttta caagaattta ttaataacaa gttaatctct gatgctgaat tagaagctat atttgtgata actgttaata ctgctttagaa aatttgaac tttacaactcat caccatatgt tcgcaccacat gctgctattta tggaaatgggc tgaatcttac tttaaagatt tgaaaaaatt attaatttgcc tcaacottttgtagaagggca ataaagggcaccatatttttttacgctttgctata atgggtgtgca acaagtttaic gaattcaaat taaatagttgaaagaaaggtaaaatttgatg aagccatagc tgaactaag agaatgaagg cattactaata 3′

[Figure 6]
For P. falciparum LDH

- ■ T12E
- ○ T28C
- △ 1H3C10

OD_{450} vs [anti-malarial LDH mAbs], ng/ml

< Reactivities of monoclonal antibodies for P. falciparum LDH >
Figure 9

For P. vivax LDH
- ■ T12E
- ○ T28C
- △ 1H3C10

OD₄₅₀-₆₂₀

[anti-malarial LDH mAbs], ng/ml

< Reactivities of monoclonal antibodies for P. vivax LDH >
[Figure 10]

\[
\frac{A}{(A-A)} \text{ vs. } \frac{1}{a_p, \text{ nM}^{-1}}
\]

\[K_d = 25.24 \pm 0.51 \times 10^{-9}\]

< Dissociation constant of monoclonal antibody T12E >

\[
\frac{A}{(A-A)} \text{ vs. } \frac{1}{a_p, \text{ nM}^{-1}}
\]

\[K_d = 24.21 \pm 2.07 \times 10^{-9}\]

< Dissociation constant of monoclonal antibody T28C >

\[
\frac{A}{(A-A)} \text{ vs. } \frac{1}{a_p, \text{ nM}^{-1}}
\]

\[K_d = 35.52 \pm 1.12 \times 10^{-9}\]

< Dissociation constant of monoclonal antibody 1H3C10 >
[Figure 11]

- Control line (C, goat anti-mouse IgG antibody)
- Test line 2 (T2, monoclonal antibody 1H5C10 specific to P. vivax)
- Test line 1 (T1, monoclonal antibody T12E specific to P. falciparum)

Strip:

- Nitrocellulose membrane
- Plastic card
- Absorbance pad

P. vivax and P. falciparum specific monoclonal antibody T28C-gold conjugate

Gold conjugate well

Washing well
Figure 12

example negative  example positive for P. falciparum  example positive for P. vivax  example positive for mixed infection
Figure 13]

<diagram of plastic housing>

<diagram of strip installed in lower part of plastic housing>
<example negative> <example positive for P. falciparum> <example positive for P. vivax> <example positive for mixed infection>
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This form was received with the international application: (yes or no)

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INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

GOIN 33/569(2006.01)i, GOIN 33/50(2006.01)i, C12Q 1/04(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)

IPC 8 GOIN 33/50, GOIN 33/569, C07K 16/18, C12Q 1/04

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 since 1975
- Japanese Utility models and applications for Utility models since 1975

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
eKIPASS(KIPO internal) & keywords "malaria", "antibody", "vivax", and "LDH"

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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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
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  "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
11 SEPTEMBER 2008 (11.09.2008)

Date of mailing of the international search report
11 SEPTEMBER 2008 (11.09.2008)

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Form PCT/ISA/210 (second sheet) (July 2008)
Box No. 1  Nucleotide and/or amino acid sequence(s) (Continuation of item b 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  type of material
       ☑ a sequence listing
       ☐ table(s) related to the sequence listing

   b  format of material
       ☑ on paper
       ☐ in electronic form

   c  time of filing/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 and/or table relating thereto 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
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