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(19) **United States**(12) **Patent Application Publication****Lin et al.**(10) **Pub. No.: US 2006/0048607 A1**(43) **Pub. Date: Mar. 9, 2006**(54) **REAGENT FOR RAPIDLY ATTAINING
THERMAL EQUILIBRIUM IN A
BIOLOGICAL AND/OR CHEMICAL
REACTION**(30) **Foreign Application Priority Data**

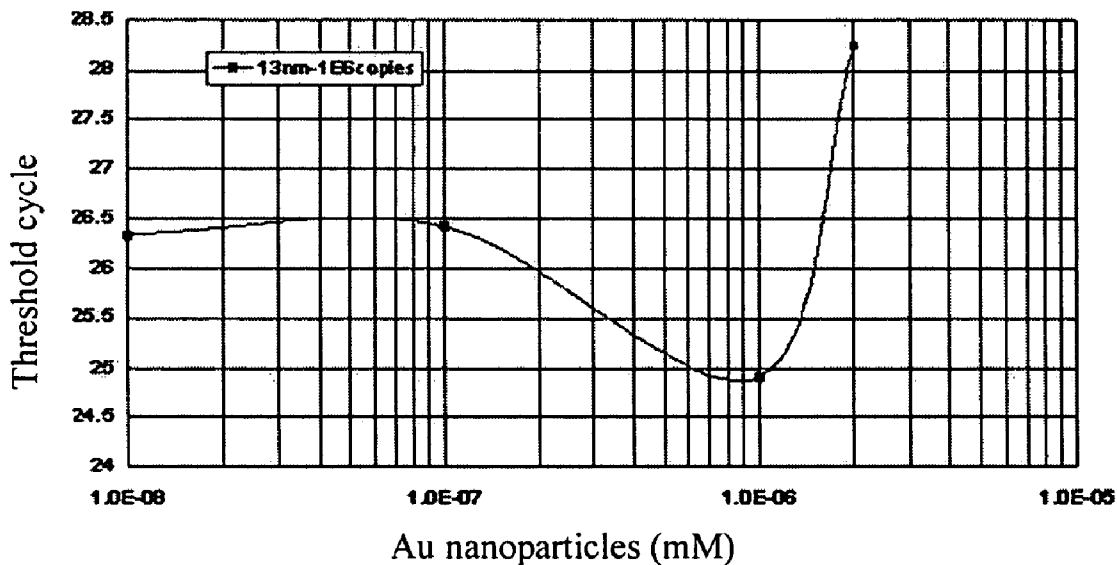
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HUSCH & EPPENBERGER, LLC**190 CARONDELET PLAZA****SUITE 600****ST. LOUIS, MO 63105-3441 (US)**(57) **ABSTRACT**

The present invention provides a reagent for rapidly attaining thermal equilibrium in a biological and/or chemical reaction, which comprises Au nanoparticles; wherein the Au nanoparticles have a Au metal core covalently bonding to a weak acid functional group, and the Au nanoparticles are aqueous. A method for rapidly attaining thermal equilibrium in a biological and/or chemical reaction and a method for producing the reagent are also provided.

(73) Assignee: **National Cheng Kung University**,
Tainan City (TW)(21) Appl. No.: **11/129,930**(22) Filed: **May 16, 2005**

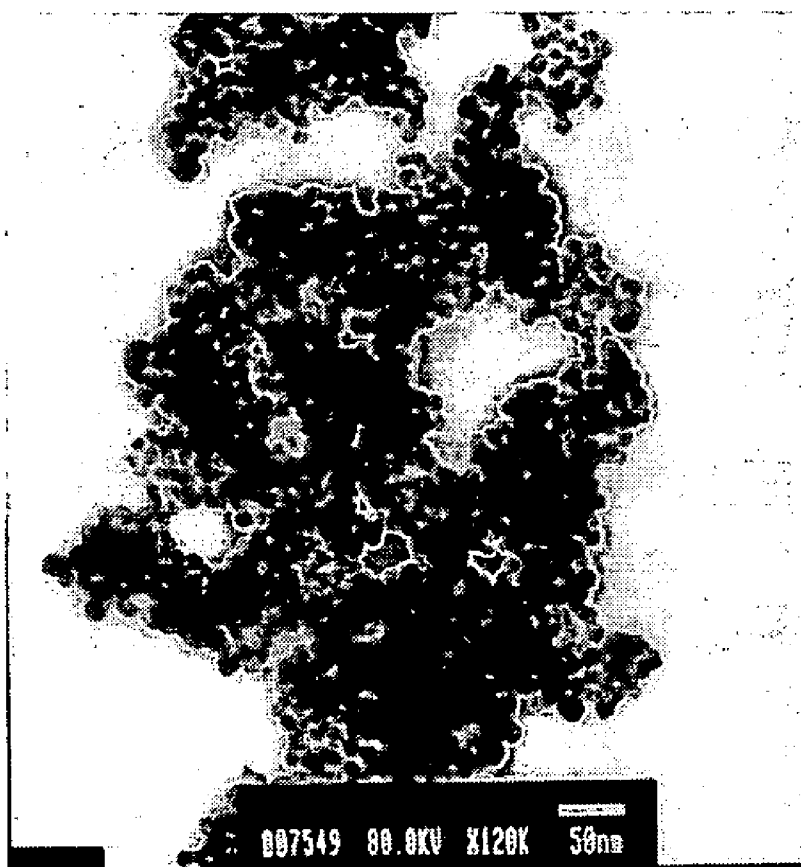


FIG. 1

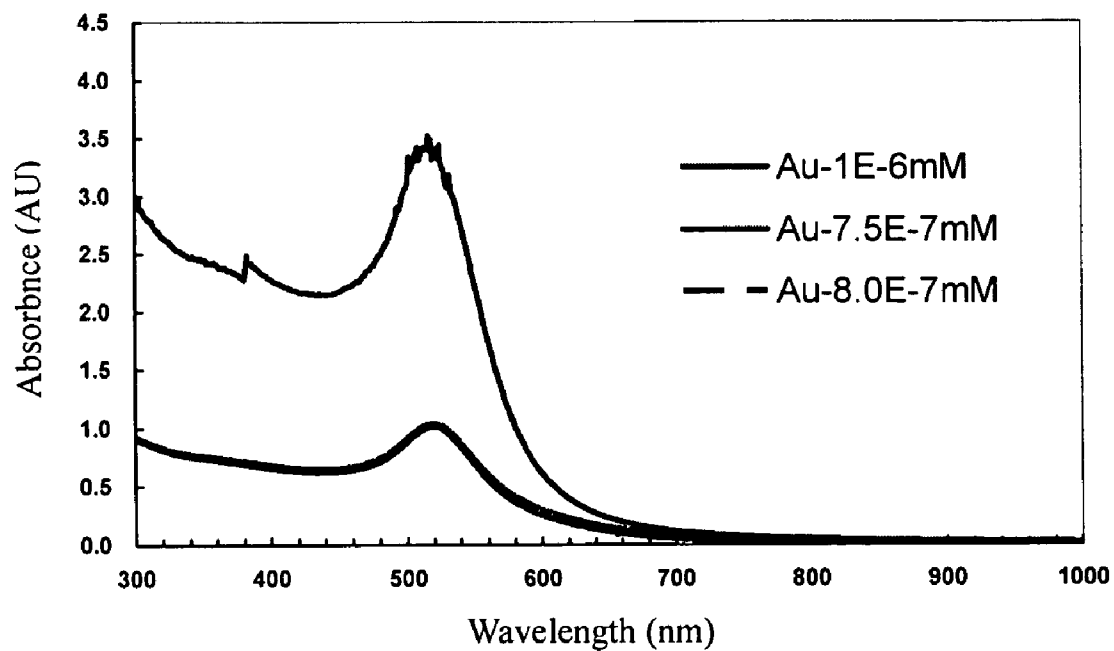


FIG. 2

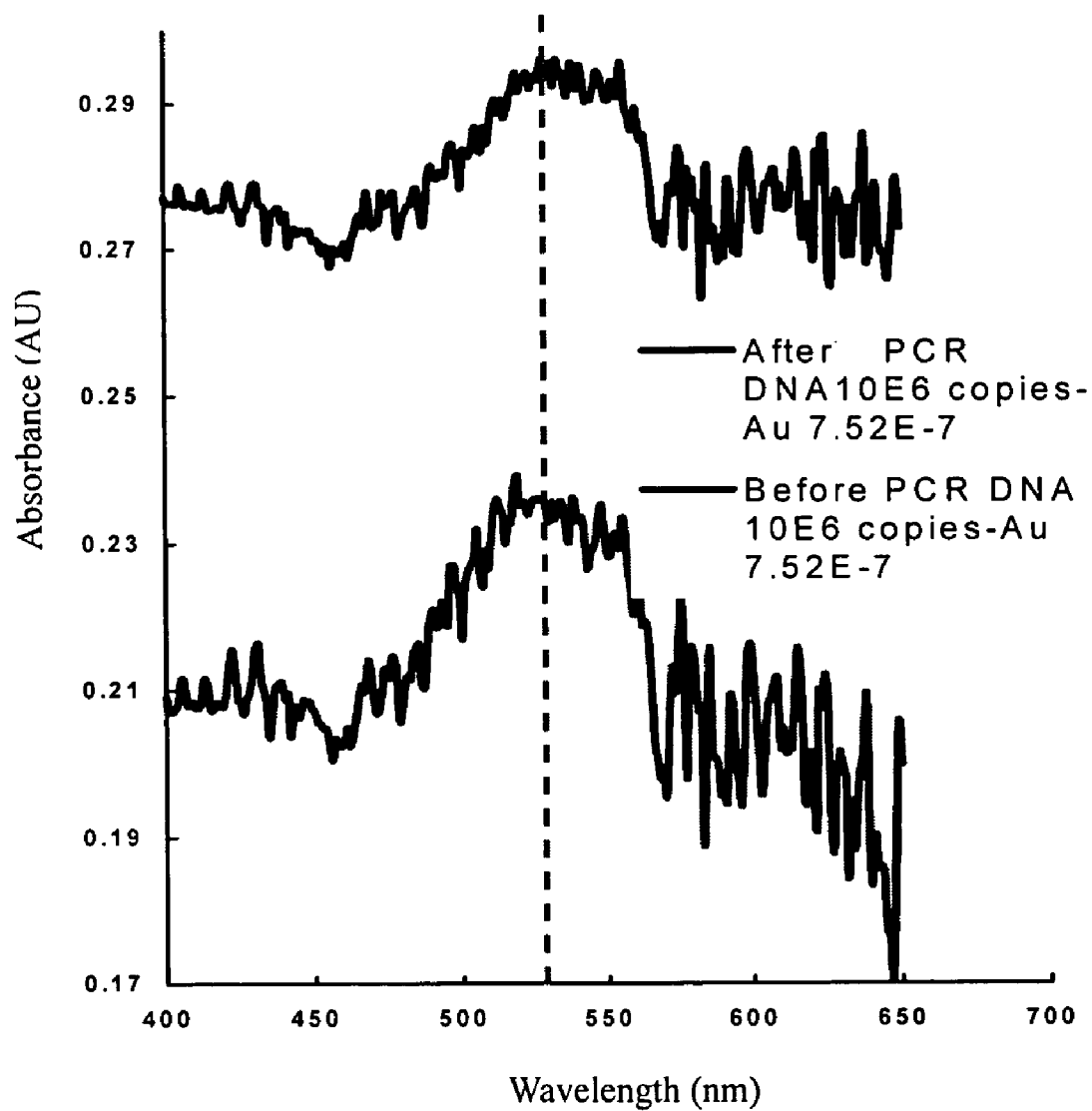


FIG. 3

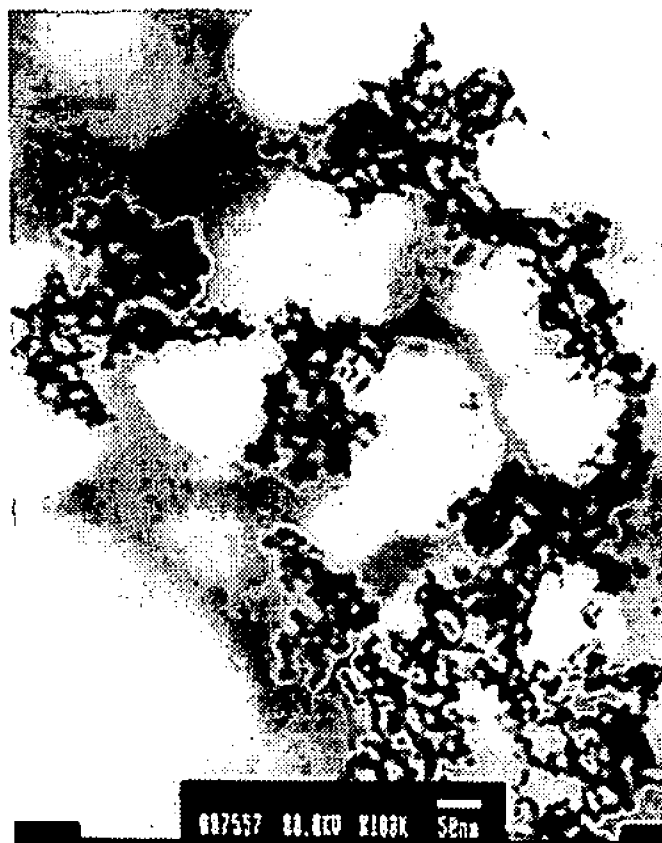


FIG. 4

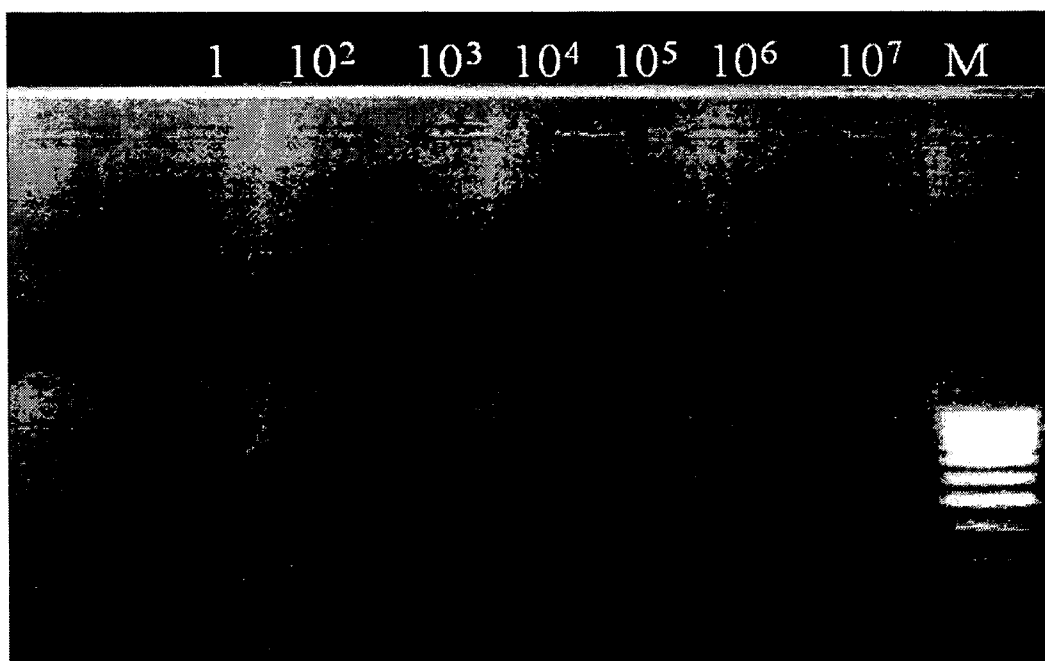


FIG. 5

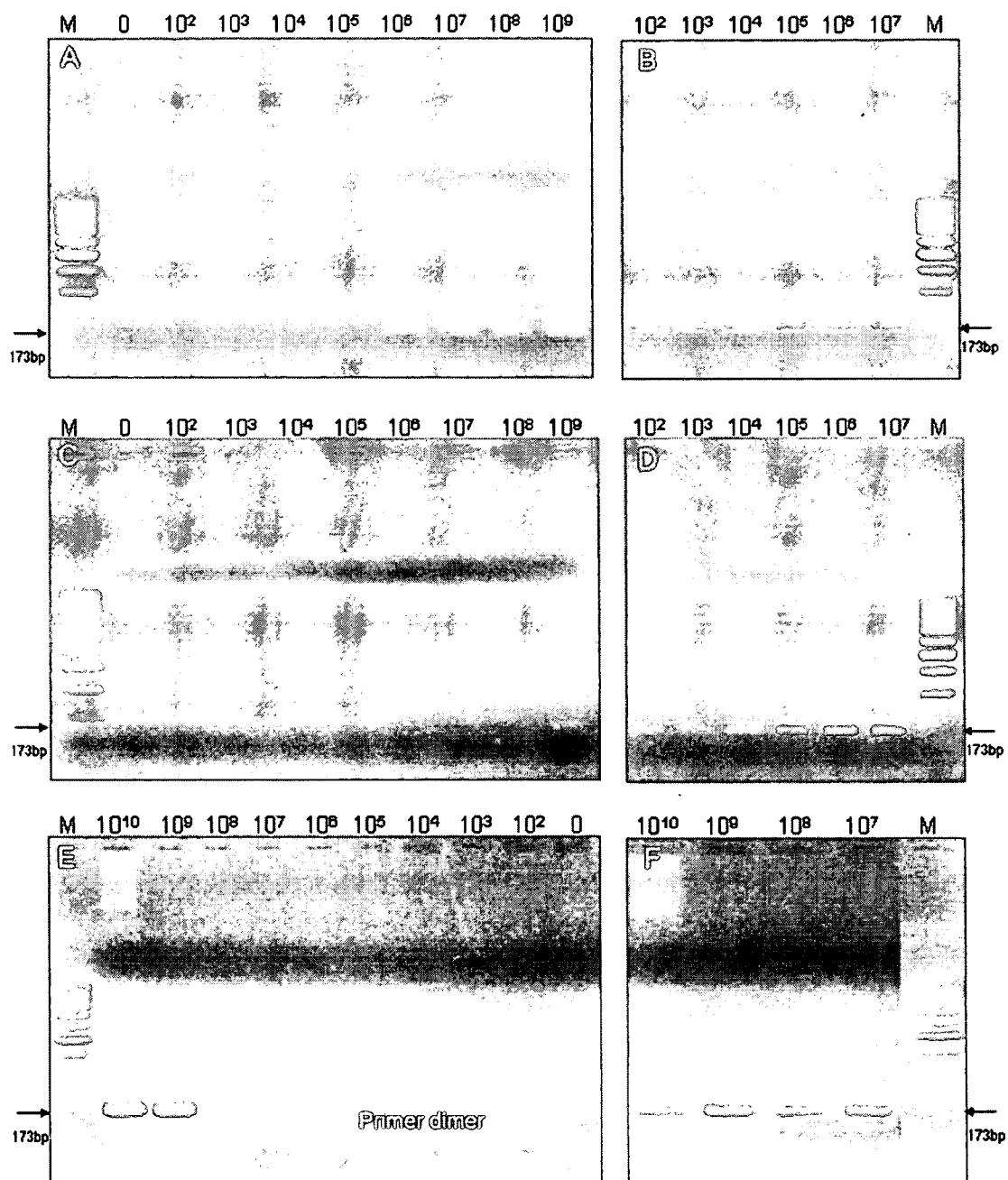


FIG. 6

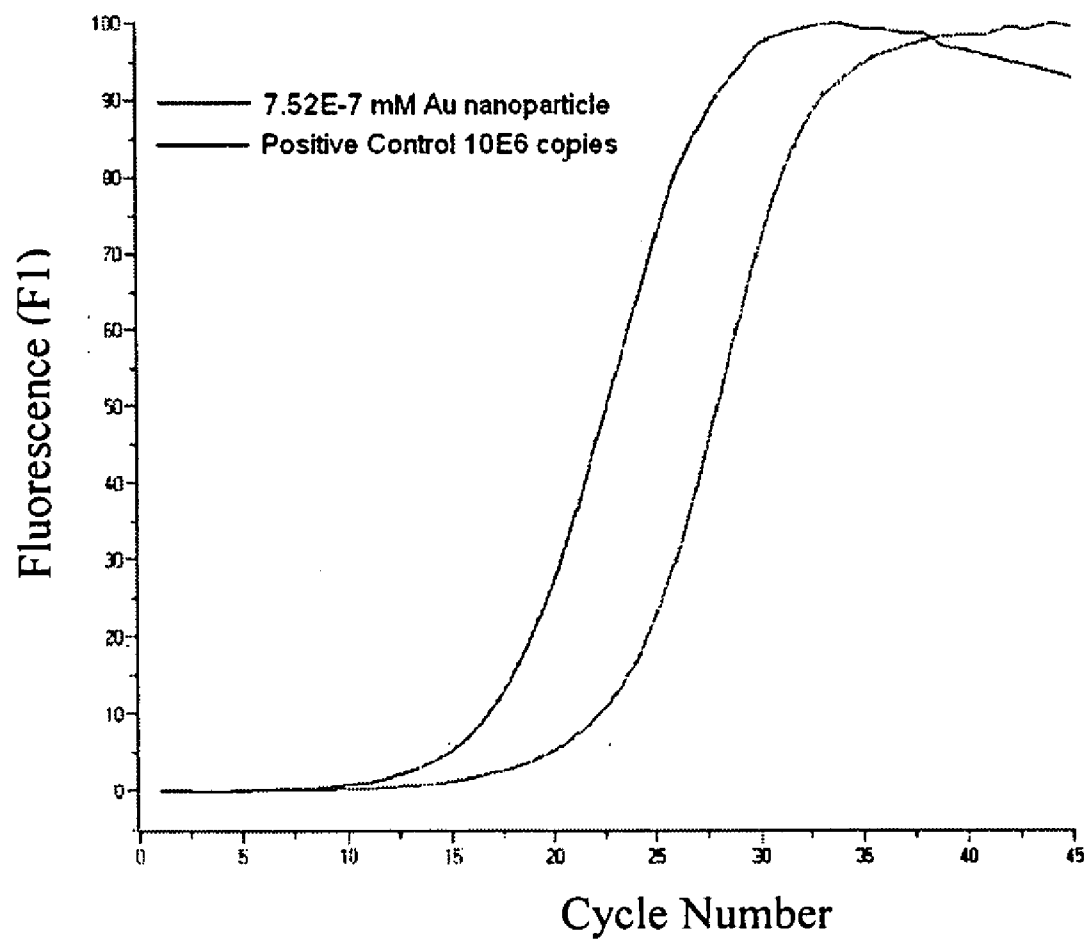


FIG. 7

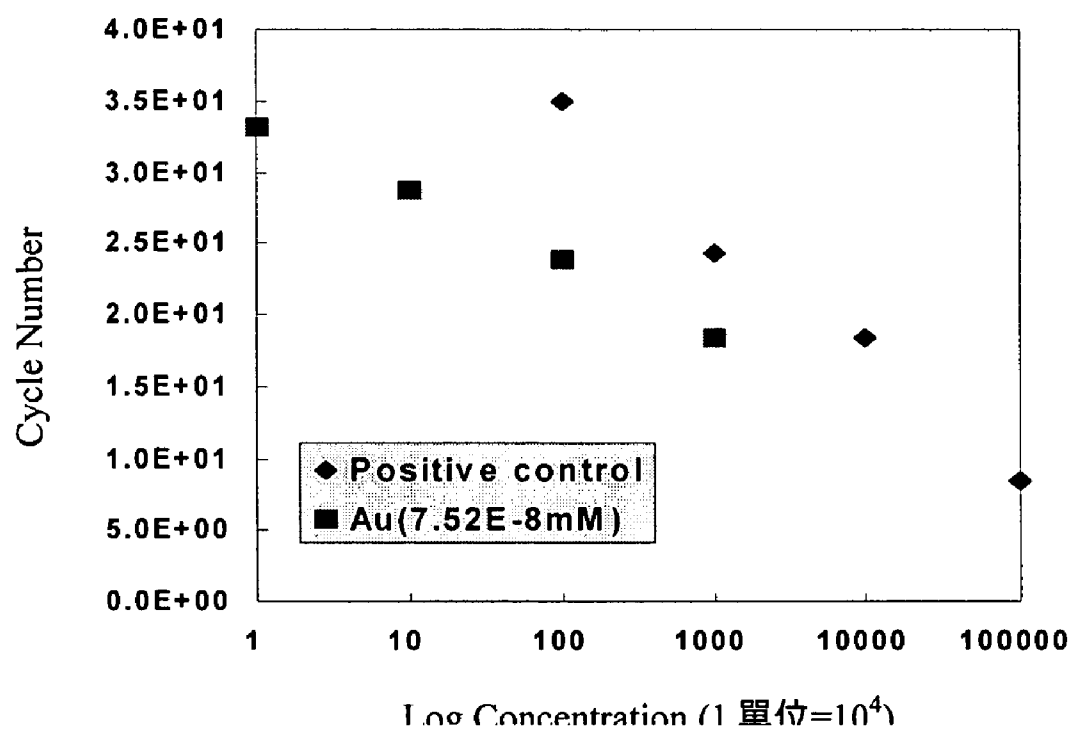


FIG. 8

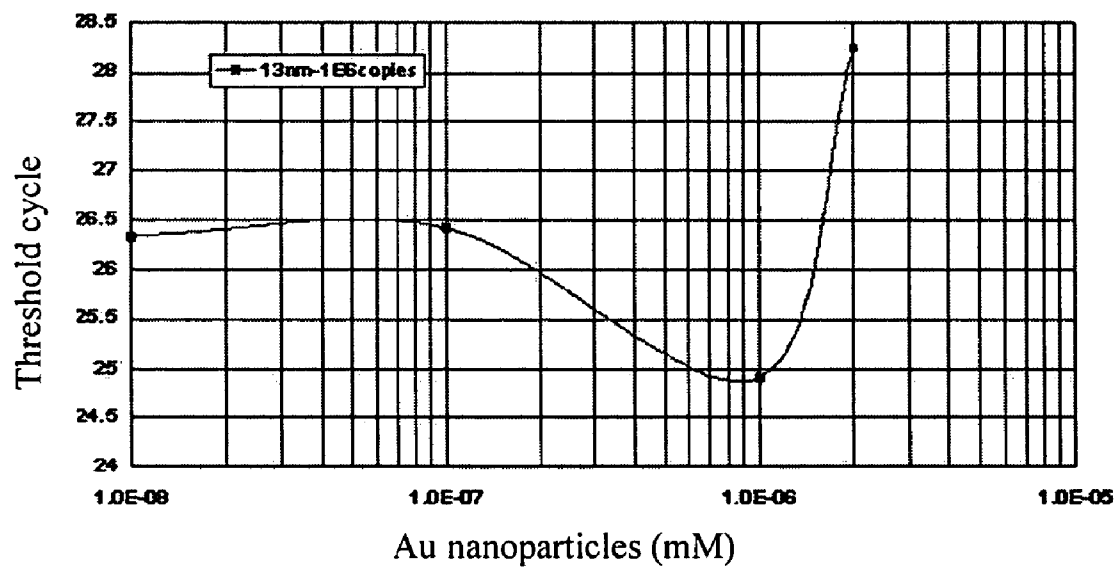


FIG. 9

REAGENT FOR RAPIDLY ATTAINING THERMAL EQUILIBRIUM IN A BIOLOGICAL AND/OR CHEMICAL REACTION

[0001] This applications claims the benefit of Taiwan application No. 093113876, filed May 17, 2004.

BACKGROUND OF THE INVENTION

[0002] 1. Field of the Invention

[0003] The invention relates to a reagent for a biological and/or chemical reaction; more particularly, to a reagent for rapidly attaining thermal equilibrium in a biological and/or chemical reaction.

[0004] 2. Description of the Related Art

[0005] "Temperature" is an important factor to a biological and/or chemical reaction, which has ability to control if a reaction occurs. Temperature regulation is more important to the biological reaction involving biological macromolecules such as nucleic acids or proteins that are highly sensitive to temperature. Only at the specific temperature, the biological macromolecules can maintain specific conformations and then carry out a specific reaction.

[0006] Polymerase chain reaction has a more critical temperature requirement among various biological and/or chemical reactions. The material of conventional thermal conduction interface for polymerase chain reaction comprises silicon or aluminum. ABI PRISM® 7000 Sequence Detection Systems (Appliedbiosystems.com) having a thermal conduction rate of 1 ° C/sec controls the temperature of a reaction solution by adjusting the temperature of a silicon or aluminum bulk with an intermediary of a solution container. However, such apparatus takes a lot of time for performing a reaction. Furthermore, the duration of thermal conduction outside the container and the duration of attaining thermal equilibrium inside the container are different, so that the sensitivity of the reaction is not satisfied.

[0007] There are two ways for improving the conventional systems. One directs to the reaction equipment and the other directs to the reaction formulae. For improving the reaction equipment function, several strategies are established: (1) changing the container volume for accelerating the rates of thermal conduction and thermal convection, but the effect is limitary. (2) Utilizing rapid hot air exchanger and utilizing quartz tube for increasing thermal transfer efficiency and decreasing reaction time. An Example is LightCycler™ instrument established by Roche®, but the thermal equilibrium attaining rate is still not qualified. For improving the reaction formulae function, several strategies are also established: (1) genetic modifying DNA polymerase for improving synthesis rate and sensitivity. However, it usually takes two to three years in research and development and leading a great cost. Taking FastStart Taq™ (Roche®) as an example, the price of the enzyme goes up fold after genetic modification, and it spends five years for research and development. (2) Raising the purity of the reagents. For example, when the concentration of nucleic acid is adjusted from 95% to 99%, the price is double, but the efficiency is not raised significantly. (3) Adding additive enzymes or proteins that are not involved in polymerase chain reaction for purposes of improving reaction rate and some specific uses, such as adding hot-start for eliminating DNA synthesis that is not tended to be performed. However, the cost goes

up two-to-three-fold, and it also spends a long period the same as that for genetic modification. (4) Changing the buffer contents for increasing stability and decreasing mistake of DNA polymerase. But the formulae is complicated and the effect is not significant either.

[0008] The strategies for improving attaining thermal equilibrium mentioned above are not effective and/or the costs are high.

SUMMARY OF THE INVENTION

[0009] The present invention provides an effective and economic method for rapidly attaining thermal equilibrium in a biological and/or chemical reaction.

[0010] One object of the invention is to provide a reagent for rapidly attaining thermal equilibrium in a biological and/or chemical reaction, which comprises Au nanoparticles;

[0011] wherein the Au nanoparticles have a Au metal core covalently bonding to a weak acid functional group, and the Au nanoparticles are aqueous.

[0012] Another object of the invention is to provide a method for rapidly attaining thermal equilibrium in a biological and/or chemical reaction, which is characterized in adding the reagent as mentioned above.

[0013] The other object of the invention is to provide a method for producing the reagent as mentioned above, wherein the Au nanoparticles are produced by a method comprising steps of:

[0014] (a) nucleating gold salt with the weak acid functional group; and

[0015] (b) removing the unreacted weak acid functional group in the step (a) and obtaining the Au nanoparticles.

BRIEF DESCRIPTION OF THE DRAWINGS

[0016] FIG. 1 illustrates the electromicroscopic view of the Au nanoparticles prepared according to the reduction-oxidation reaction.

[0017] FIG. 2 illustrates the absorbance peaks of the Au nanoparticles prepared according to the reduction-oxidation reaction and monitored by the spectrometer.

[0018] FIG. 3 illustrates the particle sizes of the Au nanoparticles in the polymerase chain reaction and monitored by the spectrometer.

[0019] FIG. 4 illustrates the particle sizes of the Au nanoparticles in the polymerase chain reaction and monitored by the electromicroscope.

[0020] FIG. 5 illustrates the electrophoresis results of the products obtained from the polymerase chain reaction with different concentrations of Au nanoparticles.

[0021] FIG. 6 illustrates the results of polymerase chain reaction with different reaction time in the presence of Au nanoparticles; A, C and E: reactions in the absence of Au nanoparticles; B, D and F: reactions in the presence of Au nanoparticles; A and B: reaction time, 60 sec; C and D: reaction time, 40 sec; E and F: reaction time, 20 sec.

[0022] FIG. 7 illustrates the efficiency of real-time polymerase chain reaction in the presence of 10^6 copies per unit of DNA template.

[0023] FIG. 8 illustrates the general efficiency of real-time polymerase chain reaction in the presence of 10^6 copies per unit of DNA template.

[0024] FIG. 9 illustrates the threshold cycle number of real-time polymerase chain reaction in the presence of 10^6 copies per unit of DNA template.

DETAILED DESCRIPTION OF THE INVENTION

[0025] The present invention provides a reagent for rapidly attaining thermal equilibrium in a biological and/or chemical reaction, which comprises Au nanoparticles;

[0026] wherein the Au nanoparticles have a Au metal core covalently bonding to a weak acid functional group, and the Au nanoparticles are aqueous.

[0027] As used herein, the term "biological reaction" refers to a reaction occurred inside an organism or a reaction carried out by molecules derived from an organism. The molecules involved in the biological reaction comprise but are not limited to macromolecules such as nucleotide, peptides, proteins and carbohydrates. The biological reaction according to the invention preferably comprises but is not limited to polymerase chain reaction, enzyme digestion, protein hybridization, and nucleotide-protein hybridization; more preferably, comprises polymerase chain reaction; most preferably comprises, real-time polymerase chain reaction.

[0028] As used herein, the term "chemical reaction" refers to a reaction carried out by chemical molecules. The chemical reaction according to the invention preferably comprises thermal sensitive reaction.

[0029] As used herein, the term "Au nanoparticle" refers to a particle composed by gold atom and having a nano degree particle size. The term particularly refers to a nanoparticles having an Au metal core covalently bonding to a weak acid functional group, and the Au nanoparticle is aqueous. Preferably, the weak acid functional group is a citric acid group or a tannic acid group; more preferably, the weak acid functional group is a citric acid group. In another aspect, the Au nanoparticles preferably have an average particle size in a range of 1 nm to 100 nm; wherein more preferably, the Au nanoparticles have a average particle size in a range of 1 nm to 40 nm. In one embodiment of the invention, the Au nanoparticles are in a colloid solution. In order to apply in various reactions, the colloid solution is preferably neutral in pH and does not change the pH values of the reactions.

[0030] According to the invention, the Au nanoparticles are free particles and suitable for various kinds of reactions.

[0031] For increasing the rate of attaining thermal equilibrium in the reaction solution, the Au nanoparticles according to the invention preferably have a concentration in a range of 10^{-5} mM to 10^{-8} mM in the reaction solution; more preferably, the Au nanoparticles have a concentration in a range of 10^{-6} mM to 10^{-7} mM in the reaction solution.

[0032] In view of metal nanoparticles having a good thermal conduction property, they are broadly used in lubricants.

However, common metal nanoparticles cannot be utilized in a biological and/or chemical reaction because they cannot be dissolved in water and often form aggregates. Conventionally synthesized colloid metal particles that covalently bind to protectors are aqueous and do not form aggregates, but the metal particles such as Fe and Ag colloid particles cannot improving thermal equilibrium. Furthermore, the biological reaction with enzyme involved, such as polymerase chain reaction, needs good correlation between thermal conduction and enzyme dynamics. Only the Au nanoparticles according to the invention can be dissolved in water without aggregation and are proven to improving thermal conduction when added into the reaction solutions.

[0033] Because the aqueous (i.e. without aggregation) Au nanoparticles according to the invention have properties of high thermal conduction and energy cohesion, they are able to improve thermal conduction when added into a biological and/or chemical reaction. For example, the Au nanoparticles improve the efficiency and sensitivity of polymerase chain reaction dramatically. With the assistance of the Au nanoparticles according to the invention, normal polymerase such as Taq DNA polymerase can catalyze real-time polymerase chain reaction instead of high quality DNA polymerase. The Au nanoparticles according to the invention having a large surface area and excellent thermal conduction help to attain thermal equilibrium in 10 to 100 ps. The Au nanoparticles sized 10 to 15 nm with a concentration of 10^{-8} mM are shown to improve the sensitivity of polymerase chain reaction by 10,000-fold, and shorten amplification time by thousands-fold. The concentration used is much lower than that in the previous thermal conduction study (0.3 to 5%), (J. Z. Zhang, "Ultrafast studies of electron dynamics in semiconductor and metal colloidal nanoparticles: effects of size and surface," *Acc.Chem.Res.*, Vol. 30, pp. 423-429, 1997; S. Link, C. Burda, Z. L. Wang, and M. A. El-Sayed, "Electron dynamics in gold and gold-silver alloy nanoparticles: the influence of a nonequilibrium electron distribution and the size dependence of the electron-phonon relaxation," *J.Chem.Phys.*, Vol. 111, pp. 1255-1264, 1999). Besides, when adding the Au nanoparticles according to the invention, 1 copy per unit of DNA template is efficient to real-time polymerase chain reaction. On the other hand, 10^6 to 10^{10} copies per unit of DNA template are needed in the same reaction. As a result, the reagent according to the invention has advantages of lowering reagent amount, shortening reaction time, and raising efficiency and sensitivity.

[0034] The present invention also provides a method for rapidly attaining thermal equilibrium in a biological and/or chemical reaction, which is characterized in adding the reagent as mentioned above.

[0035] The present invention further provides a method for producing the reagent as mentioned above, wherein the Au nanoparticles are produced by a method comprising steps of:

[0036] (a) nucleating gold salt with the weak acid functional group; and

[0037] (b) removing the unreacted weak acid functional group in the step (a) and obtaining the Au nanoparticles.

[0038] According to the invention, the manner in the step (a) for nucleating gold salt with the weak acid functional

group is well known to persons skilled in this field. Au has ability to form colloid, and then produce aqueous nanoparticles by a reduction-oxidation reaction. Preferably, the weak acid functional group is a citric acid group or a tannic acid group; more preferably, the weak acid functional group is a citric acid group. In one embodiment of the invention, gold salt and citric acid group is used to synthesize aqueous and acidic Au nanoparticles, which is published by M. A. Hayat (M. A. Hayat, Colloidal Gold, Principle, Methods and Applications). Preferably, the gold salt is HAuCl_4 , and in another aspect, the citric acid group is preferably $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$. In the step, Au ion is reduced to Au atom as a crystal seed in the nucleation reaction and then Au nanoparticles grows. When the three-charged ions are exhausted, Au particles stop growth. Different sizes of Au nanoparticle can be prepared by changing the ratio of gold salt to citric acid group. For example, when the ratio of gold salt to citric acid group (gold salt:citric acid group) is 5:1, the Au nanoparticles having particle sizes of about 10 to 15 nm are prepared.

[0039] According to the invention, the unreacted weak acid functional group is removed and the Au nanoparticles are obtained in the step (b). The conventional method for preparing Au nanoparticles is acidic because the detergent used is acidic. Additionally, the unreacted weak acid functional group is not removed in the conventional methods, so that the conventional Au nanoparticles are not suitable in neutral or basic reactions. Furthermore, the reagents for Au nanoparticles preparation would interfere the reaction. For example, citric acid group is a strong chelator that chelates metal ions (e.g. Mg^{2+}) in the reaction solution. The Au nanoparticles can be applied in the biological and/or chemical reaction after the step (b) treatment. In one embodiment of the invention, the method for removing in the step (b) is centrifuge at a high speed. The centrifuge condition is well known to artisans skilled in this field. For example, the particles are centrifuged at 10,000G for 7 minutes. Because the Au nanoparticles with the particle size smaller than about 15 nm can not be recovered completely by centrifuge, the method according to the invention preferably further comprises a vacuum suction step for concentrating the Au nanoparticles to achieve a desired concentration and pH value.

[0040] The following Examples are given for the purpose of illustration only and are not intended to limit the scope of the present invention.

EXAMPLE 1

Au Nanoparticles Preparation

[0041] According to the method described by Hayat, HAuCl_4 and $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$ in the ratio of 5:1 were used for aqueous nanoparticle preparation.

[0042] The particle size of nanoparticles were monitored by transmission electromicroscope and shown in FIG. 1. It shows that the particle size of the nanoparticles is 13.7 ± 0.8 nm. The nanoparticles have a strong absorption peak at 520 nm when detected by UV-Vis spectrum (as shown in FIG. 2). It also proves that the particle size is about 13 nm.

[0043] The aqueous nanoparticles were subjected to centrifuge at 10,000G for 7 minutes, and then concentrated by vacuum suction to obtain concentrations of 10^{-4} , 10^{-5} , 10^{-6} and 10^{-7} mM.

EXAMPLE 2

Polymerase Chain Reaction Using Au Nanoparticles

[0044] Real-Time PCR system, Lightcycler (Roche®) was utilized for assaying the thermal conduction property of Au nanoparticles. The volume of a single capillary tube is about 20 to 25 μL , and 32 tests can be carried out at the same time. The reaction solution contained 20 μL of 10 $\times$ PCR buffer, two primers, 200 μM dNTPs, 0.5 $\mu\text{g}/\mu\text{L}$ BSA, 1/30, 000 SYBR® Green I (Roche®), 1 U of Supertherm® Taq DNA polymerase, 2 μL DNA template, and 2 μL Au nanoparticles prepared in Example 1.

[0045] The fragments to be amplified were enhanced green fluorescent protein (EGFP) and Bcl-2 and nineteen kda interacting protein-3 (BNIP3) cDNA. The primers for EGFP were EGFP-N-S: 5'-TGC AGT GCT TCA GCC GCT AC-3' (SEQ ID NO. 1) and EGFP-N-AS: 5'-CAG CTC GAT GCG GTT CAC CA-3' (SEQ ID NO. 2) which amplify a 173-bp fragment from a template pEGFP-N1 (CLONTECH®). The ratio of GC content is more than 60%. The primers for BNIP3 cDNA are 5'-CGC AGC TGA AGC ACA TCC-3' (SEQ ID NO. 3) and 5'-AAC GAA CCA AGT TAG ACT CCA-3' (SEQ ID NO. 4) which amplify a 238-bp fragment. The ratio of GC content is 50.48%. The templates for amplifying EGFP and BNIP3 cDNA are 10^2 to 10^9 copies per unit.

[0046] SYBR® Green I dye was used for quantification and F1 mode was chosen for detecting the absorbance at 520 nm wavelength. The standard thermal condition of polymerase chain reaction was denaturing at 95° C. for 15 sec, annealing at 58° C. for 15 sec, and extending at 72° C. for 30 sec (total reaction time: 60 sec). The dye was detected at 84° C. for 1 sec. Such condition was common for use in a common polymerase chain reaction. The reaction time of each stage was reduced in proportion in the example.

[0047] The particle size of the Au nanoparticles in the polymerase chain reaction was monitored by the spectrometer (as shown in FIG. 3) and electromicroscope (as shown in FIG. 4). It shows that the particle size of the Au nanoparticles does not change and the Au nanoparticles do not form aggregates.

[0048] The effect of the Au nanoparticles at a single temperature was assayed. It shows that the Au nanoparticles do not only improve primer extension involving enzymes but also improve denaturing and annealing involving thermal equilibrium.

[0049] The results of real-time polymerase chain reaction in the presence of using different amount of Au nanoparticles are shown in FIG. 5. When the concentration reached 10^{-8} mM, the reaction was catalyzed. The concentration was much lower than that in the previous thermal conduction study. Besides, when adding the Au nanoparticles, 1 copy per unit of DNA template was efficient to real-time polymerase chain reaction. On the other hand, 10^6 to 10^{10} copies per unit of DNA template were needed in the same reaction.

[0050] The efficiency of Au nanoparticles in real-time polymerase chain reaction was also observed. 7.52×10^{-7} mM of Au nanoparticles with different concentrations of templates were subjected to the reactions having reaction time of 60 sec, 40 sec and 20 sec. The result is shown in FIG. 6. According to FIG. 6, the products were detected when adding 10^6 to 10^9 copies of template per unit in the reaction in the absence of Au nanoparticles. On the other hand, the products were detected when adding 10^2 to 10^9 copies of template per unit in the reaction in the presence of the Au nanoparticles. When the reaction time was reduced to 40 sec, the products were detected in the reaction with 10^3 to 10^9 copies of template per unit in the presence of the Au nanoparticles. When the reaction time was reduced to 20 sec, the products were detected in the reaction with 10^9 copies of template per unit in the presence of the Au nanoparticles. On the other hand, the products were detected in the reaction with 10^7 to 10^9 copies of template per unit in the absence of the Au nanoparticles. The results show that the Au nanoparticles have ability to enhance the formula for normal polymerase chain reaction with Supertherm® Taq DNA polymerase to be suitable for real-time polymerase chain reaction.

[0051] The amplification curve of the reaction in the presence of 7.52×10^{-7} mM of Au nanoparticles and 10^6 copies of template is shown in FIGS. 7 and 8. In the presence of the Au nanoparticles, the threshold cycle was

accelerated by 11 cycles. According to the instrument protocol of LightCycler™, the general efficiency of polymerase chain reaction is defined as:

$$\text{Slope} = -1/\log E;$$

wherein E is the efficiency of polymerase chain reaction.

[0052] In the presence of the Au nanoparticles, the slopes were changed from -8.566 to -4.918 . In other words, the efficiency was increased twice (from 0.9017 to 1.59). It proves that the Au nanoparticles improve the efficiency of polymerase chain reaction significantly.

[0053] The effect of the Au nanoparticles concentration on threshold cycle number was assayed with 10^6 copies per unit of template. The result is shown in FIG. 9. It shows that when the Au nanoparticles concentration are reduced from 10^{-6} mM to 10^{-7} mM, the threshold cycle number is eliminated dramatically; i.e. the efficiency is improved and the reaction is accelerated. The optimum concentrations of the Au nanoparticles of 10^{-6} mM to 10^{-7} mM were obtained thereby.

[0054] While embodiments of the present invention have been illustrated and described, various modifications and improvements can be made by persons skilled in the art. It is intended that the present invention is not limited to the particular forms as illustrated, and that all the modifications not departing from the spirit and scope of the present invention are within the scope as defined in the appended claims.

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What is claimed is:

1. A reagent for rapidly attaining thermal equilibrium in a biological and/or chemical reaction, which comprises Au nanoparticles;

wherein the Au nanoparticles have a Au metal core covalently bonding to a weak acid functional group, and the Au nanoparticles are aqueous.

2. The reagent according to claim 1, wherein the reaction is a thermal sensitive reaction.

3. The reagent according to claim 1, wherein the reaction is polymerase chain reaction.

4. The reagent according to claim 3, wherein the reaction is real-time polymerase chain reaction.

5. The reagent according to claim 1, wherein the weak acid functional group is a citric acid group or a tannic acid group.

6. The reagent according to claim 1, wherein the weak acid functional group is a citric acid group.

7. The reagent according to claim 1, wherein the Au nanoparticles have an average particle size in a range of 1 nm to 100 nm.

8. The reagent according to claim 1, wherein the Au nanoparticles have an average particle size in a range of 1 nm to 40 nm.

9. The reagent according to claim 1, wherein the Au nanoparticles are in a colloid solution.

10. The reagent according to claim 9, wherein the colloid solution is neutral in pH.

11. The reagent according to claim 1, wherein the Au nanoparticles have a

concentration in a range of 10^{-5} mM to 10^{-8} mM in the reaction solution.

12. The reagent according to claim 11, wherein the Au nanoparticles have a concentration in a range of 10^{-6} mM to 10^{-7} mM in the reaction solution.

13. A method for rapidly attaining thermal equilibrium in a biological and/or chemical reaction, which is characterized in adding the reagent according to claim 1.

14. A method for producing the reagent according to claim 1, wherein the Au nanoparticles are produced by a method comprising steps of:

(a) nucleating gold salt with the weak acid functional group; and

(b) removing the unreacted weak acid functional group in the step (a) and obtaining the Au nanoparticles.

15. The method according to claim 14, wherein the gold salt is HAuCl_4 .

16. The method according to claim 14, wherein the weak acid functional group is a citric acid group or a tannic acid group.

17. The method according to claim 16, wherein the weak acid functional group is a citric acid group.

18. The method according to claim 17, wherein citric acid group is $\text{Na}_3\text{C}_6\text{H}_5\text{O}_7 \cdot 2\text{H}_2\text{O}$.

19. The method according to claim 16, wherein the method for removing in the step (b) is centrifuge at a high speed.

20. The method according to claim 19 further comprising a vacuum suction step for concentrating the Au nanoparticles.

* * * * *