Description

This invention relates to a method for the quantitation of a DNA polymerase in an aqueous specimen, such as a reaction, cloning or culturing medium or diagnostic specimen. It also relates to a test kit containing reagents for carrying out this method.

Within the field of biological diagnostics, more research and analytical procedures require the investigation of nucleic acids from biological specimens, such as whole blood, serum, semen, biological tissues and other human or animal sources. For diagnostic applications in particular, a targeted nucleic acid sequence may be only a small portion of the DNA or RNA being considered, so that detection of its presence may be very difficult. The well known DNA probe technology is steadily being improved to address this problem, providing more sensitive probes.

However, a significant development in providing highly detectable quantities of nucleic acids is described in US-A-4,683,195 and US-A-4,683,202. These references describe the amplification of a targeted nucleic acid sequence using DNA primers and agents for adding bases to DNA strands complementary to the targeted sequence. After a multiplicity of cycles of priming, extending DNA strands and denaturation, the targeted nucleic acid can be more readily detected using labeled primers, probes or other means.

Primer extension is accomplished in the presence of suitable amounts of deoxyribonucleoside triphosphates and an agent for inducing the extension (also called a polymerization agent). Generally, such agent is a DNA polymerase such as an E. coli DNA polymerase I, Klenow polymerase, T4 DNA polymerase and others which will facilitate combination of the deoxyribonucleoside triphosphates in the proper manner to form the primer extension products. Particularly useful enzymes are thermally stable enzymes, cloned or naturally occurring, such as those obtained from various bacterial species, such as the Thermus species. Some useful polymerases are commercially available. Generally, the synthesis of extension products will be initiated at the 3' end of each primer and proceed in the 5' to 3' direction along the template until synthesis is terminated.

In various instances, it is important to know how much DNA polymerase is present in a medium. It is quite costly to isolate or clone a polymerase, and its efficient use is highly desirable. The amount of polymerase present in a sequencing process should be determinable. In the isolation or cloning of polymerases, it would be desirable to know when the desired concentration of enzyme has been obtained. In addition, it may be necessary to understand the enzymatic capabilities of a given polymerase in a given amplification medium.

Various isolation and purification methods for DNA polymerases are known in the art, such as EP-A-0 258 017. The thermostable enzymes described in that reference are assayed at various stages during purification procedures involving chromatography, fractionation and other time-consuming techniques.

Another procedure for measuring the amount of a DNA polymerase involves measuring the rate of incorporation of radioactively labeled deoxyribonucleoside triphosphates into a primer extension product (see generally Kornberg, DNA Replication, W. H. Freeman & Co., San Francisco, 1980, Chapters 4-6). However, this procedure likewise has a number of disadvantages, including imprecision and awkwardness. Moreover, the use of radioactive labels is not desired due to the handling and safety hazards involved.

It has been known for some time that certain fluorescent compounds (such as one known in the art as Hoechst 33258 Dye) bind preferentially to double-stranded DNA with a shift in signal as opposed to binding with the single-stranded form. See, for example, Labarca and others, Anal. Biochem., 102, pp. 344-352 (1980), Downs and others, Anal. Biochem., 131, pp. 538-547 (1983), Sterzel and others, Anal. Biochem., 147, pp. 462-467 (1985), Perkin Elmer Technical Bulletin L-913A, Sept., 1986 and Perkin Elmer Cetus bulletin "Amplifications", pp. 8-10, February, 1989. Moreover, some researchers have used the Hoechst 33259 Dye in temperature optimization studies involving polymerase chain reaction. Such studies involved measuring the fluorescent signal obtained at certain time intervals for several polymerization temperatures.

However, such studies were not used for, and indeed were incapable of, quantitatively determining the amount of polymerase in a specimen. In such instances, the rate of polymerase reaction was not measurable with accuracy, nor was a correlation established between the measured rate and polymerase activity.

Thus, while many researchers have considered the use of various dyes for detection of DNA, none of them suggests how they can be used to quantitatively determine the amount of a DNA polymerase in a solution. Yet, there is a need in the art for a quantitative, safe and convenient method for assaying for DNA polymerase in an aqueous medium.

The problems described above for known procedures are overcome using a method for the quantitative determination of DNA polymerase activity, comprising:

A. bringing into contact:

an aqueous test specimen believed to contain a DNA polymerase having activity A_x,

a single-stranded DNA template which is present in a concentration of at least 10^{-8} molar nucleotides,

a DNA primer complementary to the single-stranded template,

a DNA polymerase metal ion cofactor,

sufficient deoxyribonucleoside triphosphates to generate a double-stranded DNA molecule from the single-stranded DNA template in the presence of the DNA polymerase, and

a colorimetric or fluorescent dye which, when bound to the double-stranded DNA formed from the single-stranded DNA template, exhibits a change in signal as opposed to when the dye is bound to the single-stranded DNA template, and

B. determining, with a precision having a covariance of less than 10%, the activity A_x of the DNA polymerase in the test specimen corresponding to the rate of colorimetric or fluorometric signal generated by the binding of the dye to the double-stranded DNA formed by the action of the test specimen DNA polymerase,

the DNA polymerase activity A_x being determined as follows:

1) generating DNA polymerase rates of reaction from the colorimetric or fluorometric signals generated over time by each of a series of samples containing DNA polymerase having known polymerase activities, A_1, A_2,...A_i...A_n wherein n is the number of samples evaluated and is at least two, and generating the DNA polymerase rate of reaction from the colorimetric or fluorometric signal generated over time from the test specimen,

2) determining a calibration using the rates of step 1) and the known DNA polymerase activities A_i, and

3) predicting A_x using the calibration of step 2) and the DNA polymerase reaction rate of the test specimen.

Moreover, this invention also provides a test kit useful for the quantitative determination of DNA polymerase activity comprising:

the four deoxyribonucleoside triphosphates, dATP, dCTP, dGTP and dTTP,

a source of a metal ion DNA polymerase cofactor, and

a DNA primer complementary to a single-stranded DNA template suitable for forming a double-stranded DNA therefrom,

the kit characterized wherein it further comprises

a single-stranded phage DNA as the single-stranded template, and

a colorimetric or fluorescent dye which, when bound to the double-stranded DNA molecule formed from the single-stranded template, exhibits a change in signal as opposed to when the dye is bound to the single-stranded DNA template.

The present invention provides a rapid, quantitative and relatively simple means for measuring the amount of DNA polymerase in a specimen such as an extraction, sequencing or culture medium or other aqueous specimen suspected or known to contain a DNA polymerase. This analytical tool is also very useful in research and development studies of DNA polymerases where there is an interest in finding enzymes having improved polymerization efficiencies or thermostability. Moreover, the method can be used to study various parameters and reagents which are used in polymerase chain reactions. The incorporation of radioisotopes in order to determine the level of DNA polymerase activity, and the tedious, complicated analytical methods previously known, are avoided with the present invention. Further, unlike previous analytical attempts, this invention is highly quantitative and convenient.

The advantages of this invention are achieved by using certain dyes which exhibit a measurable difference in signal when bound to a double-stranded DNA molecule, as opposed to when they are bound to the corresponding single-stranded template. We have found a number of dyes which exhibit such changes. These changes can be used to monitor the rate of generation of double-stranded DNA from the corresponding single-stranded template, and accordingly quantitation of DNA polymerase activity. The rate of change of signal resulting from dye binding to double-stranded molecules is correlated to polymerase concentration quantitatively using a series of graphical or analytical steps, or combinations thereof, which are described in detail below. Such steps have not heretofore been used in combination with the dyes described herein for quantitative DNA polymerase determinations.

FIG. 1A is a graphical plot of relative change in fluorescence versus time at three different concentrations of DNA polymerase. These data are described in more detail in Example 1 below.

FIG. 1B is a graphical plot of rate of change in fluorescence over time versus DNA polymerase concentration (units/ml). These data are described in more detail in Example 1 below.

FIG. 2 is a graphical plot of fluorescence versus time at three different magnesium ion concentrations. These data are described in more detail in Example 2 below.

FIG. 3 is a graphical plot of the rate of change in fluorescent versus magnesium ion concentrations for the data presented in FIG. 2.

FIG. 4 is a graphical plot of fluorescence generated over time for several known concentrations of DNA polymerase. It is discussed in more detail in Examples 4 and 5 below.

FIG. 5 is a calibration curve generated in Examples 4 and 5 below of rate of fluorescence change versus DNA polymerase concentration.

FIG. 6 is a graphical plot of the fluorescence observed over time for time samples of unknown DNA polymerase concentration as described in Examples 4 and 5 below.

FIGS. 7 and 8 are graphical plots of rate of fluorescence change versus DNA polymerase concentration for unknown DNA polymerase samples of Examples 4 and 5, respectively.

As noted above, there are instances where it is desired to measure the amount of a DNA polymerase in an aqueous medium. Known procedures are complex and tedious, and the present invention is much easier and convenient. The present invention can be used during procedures for isolation or cloning of DNA polymerases, during DNA sequencing procedures, research studies of various reaction parameters and reagents in polymerase chain reaction, as well as quality control procedures and other tests used in the manufacture of in vitro diagnostic test kits. Thus, the present invention can be used: (1) in the assay of specimen samples in which the amount of DNA polymerase is already known, but which is to be monitored in the study of other parameters, (2) in the assay of specimen samples wherein the presence of DNA polymerase is unknown, and (3) in the assay of specimen samples which are known to contain a DNA polymerase, but its concentration is unknown.

The DNA polymerases detectable by the present invention include enzymes which are naturally occurring or clones prepared from the appropriate genome using standard genetic engineering techniques. Many such enzymes are described in the art, and include, but are not limited to E. coli DNA polymerase I, T4 DNA polymerase, the Klenow subfragment and other fragments of other polymerases. The invention is particularly useful to detect thermally stable enzymes, cloned or naturally occurring, fragments or full-length molecules, such as those obtained from various Thermus bacterial species, including Thermus thermophilus. Another thermally stable enzyme detectable with this invention is isolated from Thermus aquaticus as described in EP-A-0 258 017. Still other polymerases are described by Rossi and others, Syst. Appl. Microbiol. 7(2-3), pp. 337-341, 1986.

As used herein, the terms "single-stranded DNA" and "double-stranded DNA" refer to the entire DNA double helix molecule as well as a denatured single strand thereof, and to fragments of either the single-stranded or double-stranded molecule. In other words, an oligonucleotide alone or hybridized with a complementary oligonucleotide (of the same or different length) is included in the definition of these terms. When referring to a "template", what is meant is a single-stranded DNA which will become double-stranded DNA upon action by a DNA polymerase in the presence of the appropriate deoxyribonucleosides and suitable polymerization conditions.

In practicing the present invention, the aqueous test specimen having a polymerase which may contain one or more DNA polymerases of activity (A_x) is brought into contact with a number of materials which promote the formation of double-stranded DNA molecules from the single-stranded DNA template. These materials include the template (described below), a DNA primer substantially complementary to at least one sequence of the template, a polymerase metal cofactor which enhances polymerase activity and sufficient deoxyribonucleoside triphosphates which can be added to the template by the primer in forming the complementary strand.

The DNA template is a single-stranded oligonucleotide which is sufficiently long for a DNA primer to hybridize thereto and form a double-stranded DNA molecule to which the dye (defined below) can associate. Generally, the template is present in the reaction mixture in a concentration of at least 10^{-8} molar nucleotides, and preferably from 10^{-5} to 10^{-3} molar nucleotides. Another way of stating this is that the template should be present at a concentration, depending upon its base length, that would allow sensitive detection in the method using the colorimetric or fluorometric dye. For shorter molecules, such as down to as low as 100-mer, the number of molecules present must be larger relative to the concentration of a 5000-mer DNA molecule. Thus, the molecular concentration would vary depending upon the length of the template. In most instances, this means the template has at least 100 nucleotides, and more likely from 1000 to 10,000 nucleotides. Preferably, the template has from 5000 to 8000 nucleotides. The template can be synthetic or naturally occurring, and some can be obtained commercially from a number of sources.

Various single-stranded phage DNA molecules can be used as templates, and a number of them are available commercially. A particularly useful template is M 13 phage DNA which is available commercially.

Its use is advantageous because it is readily available in quantities needed for the assay, and in general, the initial polymerase activity using it is the maximum rate of polymerase activity. This provides a linear correlation of rate of signal change and polymerase activity, and hence polymerase concentration. Thus, this template provides a highly sensitive assay as well as a quantitative one.

As used herein, primers are oligonucleotides comprising ten or more deoxyribonucleotides. The exact size is not critical but depends upon many physical and chemical factors including the hybridization temperature and the degree of complementarity with the DNA template. The oligonucleotide is generally obtained synthetically. The primer is capable of acting as a point of initiation of synthesis of connected nucleotides when placed under conditions in which synthesis of a primer extension product complementary to the DNA template is induced. Such conditions include the presence of suitable concentrations of the four standard deoxyribonucleoside triphosphates, an appropriate cofactor, the DNA polymerase, and suitable temperature and pH conditions.

The primer is substantially complementary to the DNA template. By "substantially complementary" is meant that there are a sufficient number of bases on the primer that match with the corresponding bases of the template that the primer will hybridize with that sequence. It does not mean, however, that every base pair must match.

In the practice of this invention, generally the primers are entirely single-stranded. But there are primers known in the art which have a single-stranded region which is adjacent to a double-stranded region. Such primers can also be used if desired. The exact size of the primer will vary depending the length and complexity of the DNA template, reaction temperature and the source of the primer. Generally, the primers used in this invention will have from 10 to 50 nucleotides, and preferably, they have from 15 to 30 nucleotides. The amount of primer used will vary depending upon the the template concentration, the amount of polymerase suspected, its likely activity and the length of time the assay is to be run. Generally, one or more primers are present in an amount of at least 10^{-6} molar.

Primers useful herein can be obtained from a number of sources or prepared using known techniques and equipment, including for example, an ABI DNA Synthesizer (available from Applied Biosystems) or a Biosearch 8600 Series or 8800 Series Synthesizer (available from Milligen-Biosearch, Inc.) and known methods for their use. Naturally occurring primers isolated from biological sources are also useful (such as restriction endonuclease digests).

The formation of a primer extension product will occur with some DNA polymerases in the presence of the primer, DNA template and deoxyribonucleoside triphosphates (that is, dATP, dCTP, dGTP and dTTP) without cofactors present, but efficient polymerase activity for most enzymes generally requires the presence of a metal cofactor, such as a source of magnesium or manganese ion in amounts which are known in the art (generally, in molar excess of the triphosphates, for example at least 1 mmolar). Magnesium ion is preferred.

Each of the deoxyribonucleoside triphosphates noted above are generally present in an amount of at least 0.1 mmolar, and preferably from 0.2 to 2 mmolar.

The aqueous test specimen suspected of containing a predetermined (or targeted) polymerase is mixed with the noted materials in a medium which is generally buffered at a pH of from 7 to 9. Preferably, a molar excess of the primers is added to the buffered solution, and specific amounts are taught in the art. The deoxyribonucleoside triphosphates are also added in adequate amounts and the resulting solution is heated to between 30-98°C for up to 30 minutes, and preferably from 1 to 15 minutes to form the extension product.

The test specimen, colorimetric or fluorometric dye and other reagents needed for the assay can be contacted in a number of ways and in various sequences. For example, the test specimen and dye can be mixed prior to contact with the other reagents. Alternatively, the colorimetric or fluorescent dye (defined below) can be mixed with the reagents prior to or simultaneously with the mixing of the reagents with the test specimen. The dye can be added all at once, or added in portions over a time period. There may be some advantages in sensitivity achieved by mixing test specimen and dye prior to contact with the other reagents. Preferably, the dye is mixed with aliquots of a reaction solution comprising the test specimen and the other reagents, selected at various time intervals during conversion of the template to double-stranded DNA, that is, after the extension reaction has been initiated.

Once all reagents (including the dye) have been mixed with the test specimen, the resulting detectable change in signal from dye binding to double-stranded DNA is monitored. Because the signal changes as the result of enzymatic reaction, the rate of change in signal is monitored. Thus, the signal can be an increase or decrease in fluorescence from a fluorescent dye, or it can be the shifting of the λ_{max} of a colorimetric dye. With respect to colorimetric dyes, the shift in λ_{max} can be from a colorless species (outside the visible region) to a colored species (within the visible region), from a colored species to a

colorless species, or from one hue to another hue in the visible region of the electromagnetic spectrum. The rate of the measured change, or lack thereof, is then a measure of the activity of polymerase in the test specimen. The correlation of rate of change of the measured signal and the polymerase activity is described in more detail below. As one skilled in the art would readily understand, this invention could measure the activity of a mixture of polymerases in the specimen, but the individual activities of the polymerases would not be detectable.

Suitable equipment exists for measuring the change in detectable signal, including fluorometers and spectrophotometers. Suitable equipment for fluorescence measurements are available commercially as LS-2B or LS-5B fluorometers from Perkin Elmer.

Dyes which can be used in the practice of this invention are grouped as either colorimetric dyes (those providing a signal in the visible region of the spectrum), or fluorescent dyes (which are excited at one wavelength, and detected at an emission wavelength).

Preferably, the assay of this invention is carried out using fluorescent dyes, including but not limited to, bibenzimidazole, ethidium, methidium and acridine dyes. The bibenzimidazole dyes are preferred. Useful fluorescent dyes are selected from the group consisting of 2-[2-(4-hydroxyphenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)benzimidazole trihydrochloride, acridine orange, methidium bromide, propidium bromide, ethidium bromide and 4',6'-diamidino-2-phenylindole. The first dye in this list is most preferred as it exhibits a 50 nm shift upon binding to double-stranded DNA.

The amount of dye used in the assay will vary depending on the type and strength of signal it will provide when binding to DNA, and other factors known to one skilled in the art. Generally, it is present in an amount of at least 1, and preferably from 2 to 5, μmolar.

The assay can be carried out using all of the necessary reagents and materials obtained from separate sources, but advantageously, they are supplied as part of a diagnostic test kit. The critical components of such a kit include a suitable dye, a single-stranded phage DNA template (preferably, M 13 phage DNA), a polymerase metal cofactor, a DNA primer complementary to the template and the four deoxyribonucleoside triphosphates. Other materials, such as buffers, containers and instructions can also be included in the kit. The materials can be appropriately packaged (dry or wet) for needed stability, safety and ease of handling.

Polymerase activity (A_x) is determined with the present invention with a precision having a covariance of less than 10%. Precision is a known statistical parameter in diagnositic and clinical assays for measuring reproducibility. Covariance is commonly defined as equal to the standard deviation (σ) for a number of replicate measurements divided by the mean $\bar{x}$, the result then multiplied by 100%. The smaller the covariance, the more precise the assay, indicating the highly quantitative nature of the assay.

The method of this invention can be carried out using a number of graphical or mathematical functions which correlate the detectable signals obtained in the reaction to the activity of any DNA polymerase present in the test specimen. If no signals can be detectable, no polymerase is present. Generally, however, a test specimen is believed to have at least some polymerase present. While one skilled in the art would be able to use the teaching herein to find a number of ways to carried out the quantitative determination, we have considered four specific embodiments which are outlined as follows:

Embodiment A:

In this embodiment, determination of A_x in step B of the method is carried out as follows:

step B1) is carried out by

(a) generating curves from the colorimetric or fluorometric signals with time for each of the known polymerase activities, and generating a similar curve for the polymerase activity of the test specimen, and

(b) measuring the slope of each curve generated in (a) above at time t* to provide polymerase reaction rates C_i^* for each known polymerase activity A_i and a polymerase reaction rate C_i^* for the test specimen polymerase activity A_x, where t* is any given time,

step B2) is carried out by plotting the rates C_i^* determined in B1) as a function of the known polymerase activities A_i to generate a calibration curve, and

step B3) is carried out by projecting the test specimen rate C_i^* generated in step B1) off the calibration curve generated in step B2) to determine A_x.

Thus, in this embodiment, one uses the detectable signals generated from the formation of a double-stranded DNA molecule to provide graphical plots of activity versus time for the known samples as well as the test specimen. The slopes of these curves at a given time are determined and plotted to provide a calibration curve from which A_x is determined at the rate of reaction of the test specimen.

Embodiment B:

This embodiment is like Embodiment A in step B1), but steps B2) and B3) involve mathematical manipulations using standard calculus and algebraic operations. Thus, the slopes of the curves generated in B1) are manipulated mathematically, rather than graphically, to obtain A_x.

More specifically, step B1) is carried out by

(a) generating curves from the colorimetric or fluorometric signals with time for each of the known polymerase activities, and generating a similar curve for the polymerase activity of the test specimen, and

(b) measuring the slope of each curve generated in (a) above at time t* to provide polymerase reaction rates C_1^i for each known polymerase activity A_i and a polymerase reaction rate C_1^x for the test specimen polymerase activity A_x, where t* is any given time,

step B2 is carried out by regressing each polymerase rate C_1^i generated in B1) versus the respective known polymerase activity A_i using equation (I)

$$C_1 = \gamma_o + \gamma_1 A + \gamma_2 A^2 + \gamma_3 A^3 \qquad (I)$$

to obtain the coefficients γ_o, γ_1, γ_2, and γ_3, and

step B3) is carried out by using the rate of test specimen polymerase activity C_1^x and equation (II)

$$(II) \quad C_1^x = \gamma_o + \gamma_1 A_x + \gamma_2 A_x^2 + \gamma_3 A_x^3$$

to solve for A_x.

Embodiment C:

A further embodiment of this invention can be practiced by a method comprising the following sequence of steps. Obtaining the rates of reaction is accomplished mathematically rather than graphically as in Embodiments A and B. More specifically:

step B1) is carried out by

(a) regressing the signal vs. time data for each known polymerase activity A_i and for the unknown polymerase activity A_x to obtain the regression coefficients of equation (III)

$$(III) \quad S^i = C_0^i + C_1^i t^* + C_2^i (t^*)^2$$

wherein S^i represents the colorimetric or fluorometric signal for a given time t*and known polymerase activity A_i, or unknown polymerase activity A_x, and C_0^i, C_1^i and C_2^i represent the regression coefficients,

(b) taking the time derivative of equation (III) to obtain the rate according to equation (IV)

$$(IV) \quad \left(\frac{dS}{dt}\right)_{t^*} = C_1^i + 2C_2^i t^*$$

thereby providing the coefficient C_1^i for each known polymerase activity A_i, the coefficient C_1^i being the rate of polymerase activity A_i,

step B2) is carried out by plotting the rates C_1^i for the known polymerase activities determined in B1) as a function of the known polymerase activities A_i to generate a calibration curve, and

step B3) is carried out by protecting the test specimen rate C_1^x generated in step B1) off the calibration curve generated in step B2) to determine A_x.

Embodiment D:

Still another way to practice the present invention includes the following mathematical manipulations:
step B1) is carried out by
(a) regressing the signal vs. time data for each known polymerase activity A_l and for the unknown polymerase activity A_x to obtain the regression coefficients of equation (III)

$$(III) \quad S^i = C_0^i + C_1^i t^* + C_2^i (t^*)^2$$

wherein S^l represents the colorimetric or fluorometric signal for a given time t*and known polymerase activity A_l, or unknown polymerase activity A_x, and C_0^l , C_1^l and C_2^l represent the regression coefficients,
(b) taking the time derivative of equation (III) to obtain the rate according to equation (IV)

$$(IV) \quad \left(\frac{dS}{dt}\right)_{t^*} = C_1^i + 2C_2^i t^*$$

thereby providing the coefficient C_1^l for each known polymerase activity A_l, the coefficient C_1^l being the rate of polymerase activity A_l,
step B2 is carried out by regressing each polymerase rate C_1^l generated in B1) versus the respective known polymerase activity A_l using equation (I)

$$C_1 = \gamma_o + \gamma_1 A + \gamma_2 A^2 + \gamma_3 A^3 \qquad (I)$$

to obtain the coefficients γ_o, γ_1, γ_2, and γ_3, and
step B3) is carried out by using the rate of test specimen polymerase activity C_1^x and equation (II)

$$(II) \quad C_1^x = \gamma_o + \gamma_1 A_x + \gamma_2 A_x^2 + \gamma_3 A_x^3$$

to solve for A_x.

The mathematical calculations used in the various embodiments described above are standard and can be found in modern algebra and calculus textbooks. Their application to the present invention would be readily apparent to one skilled in the art in view of the teaching herein, such person having modest computational skills, or having readily available any of a number of commercial computer programs which would perform the needed computations.

The following examples illustrate the practice of the present invention, but are not meant to be limiting in any way. All percentages are by weight unless otherwise noted.

Example 1: Assay for DNA Polymerase Using a Bibenzimidazole Fluorescent Dye and a Synthetic Oligonucleotide Template

This example demonstrates the practice of this invention for the detection of a DNA polymerase isolated from Thermus aquaticus using a fluorescent dye.

Materials:

The fluorometer used was a Perkin-Elmer LS-2 fluorometer with an autosampler. It was set for excitation of the dye at 366 nm and emission at 474 nm.

The fluorescent dye 2-[2-(4-hydroxyphenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)benzimidazole trihydrochloride was obtained from CalBiochem. It has a molar extinction coefficient in water of 4.2×10^4 $molar^{-1}cm^{-1}$.

A primary dye stock solution was prepared in deionized water (1 mg/ml) and stored in the dark at 4°C. It is believed to be stable for up to 6 months in this environment. A secondary dye stock solution was prepared using a 1:50 dilution of the primary stock solution. This second solution was calibrated using an absorbance reading at 338 nm and the known molar extinction coefficient. It was then adjusted to 3.5 x 10^{-5} molar (O.D. = 1.45 at 338 nm) with either deionized water or the primary stock solution. The resulting solution was kept in the dark at 4°C. On the day of use, the stored solution was diluted 1:10 and kept in a 50 ml tube wrapped in aluminum foil to protect it from light. This working solution is stable at room temperature for at least a day.

The synthetic single-stranded oligonucleotide template was 125 nucleotides in length and was prepared and purified on an automated synthesizer using standard procedures. The template sequence was as follows (using standard abbreviations for the nucleotides, spacing used for convenience in reading the sequence):

SEQ ID NO 1:

5'-AACCTCTGGG TCCAAGCCGT GGCCAGCGGC AGACATGGTT-
GATACCAACC TGCACATTCT TACTATTTTA TTTAATCCCA-
GGATTGGGAT AGGTGGATTA TTTGTGCAGA CTTCTCCTCA GGAGT-3'.

The template stock solution consisted of the template (2.5 µmolar) and dNTPs (3 mmolar, 0.75 mmolar of each) in assay buffer solution (described below).

The primer used had the following sequence:

SEQ ID NO 2: 5'-CTCCTGAGGA GAAGTCTGCA CAA-3'

The primer stock solution consisted of primer (1.8 µmolar) and dNTPs (3 mmolar, 0.75 mmolar of each) in assay buffer solution.

The assay buffer solution consisted of potassium chloride (50 mmolar), tris(hydroxymethyl)-aminomethane buffer (10 mmolar, pH 8.3), magnesium chloride (1.5 mmolar) and gelatin (0.01%).

A dilution buffer for the DNA polymerase isolated from Thermus aquaticus strain YT-1 (available as ATCC 25,104 from the American Type Culture Collection, Rockville, Maryland) consisted of tris-(hydroxymethyl)aminomethane buffer (25 mmolar, pH 8), potassium chloride (50 mmolar), gelatin (500 µg/ml), β-mercaptoethanol (1 mmolar), Nonidet™ NP-40 nonionic surfactant (0.5%) and Tween™ 20 nonionic surfactant (0.5%).

Assay:

The primer and template were hybridized by mixing the template stock solution (300 µl) and primer stock solution (700 µl) and incubating at 42°C for 4 hours. The resulting mixture of hybridized product was then cooled in ice (0°C), and mixed with the DNA polymerase (4 units/µl, diluted to 0.4 units/µl according to following procedure to form reactions mixtures:

Tube	Hybridization Mixture Volume (µl)	DNA Polymerase Solution Volume (µl)	Final DNA Polymerase Concentration
1	855	45	20 units/ml
2	889	11	4.9 units/ml
3	900	1	0.49 units/ml

A unit of DNA polymerase corresponds to the amount of enzyme which can incorporate 10 mmoles of total nucleotides into an acid-precipitable product using activated DNA as a template in 30 seconds at 70°C, in a solution comprising potassium chloride (50 mmolar), tris(hydromethyl)aminomethane buffer (10 mmolar, pH 8.3) magnesium chloride (1.5 mmolar) and dNTPs (0.75 mmolar total).

Each reaction mixture (100 µl) was put into separate tubes and stored at 0°C. The extension reactions were then initiated by removing the reaction mixture samples from ice and placing them in a water bath heated to 60°C. At selected time intervals (0, 3, 6, g and 12 minutes), duplicate tubes containing reaction

mixture (100 µl) having different DNA polymerase concentrations were taken from the water bath and placed in ice (0 °C). From each tube, three separate aliquots (25 µl) were mixed with the dye solution (1 ml) and the relative fluorescence was measured.

The results of these tests are shown in FIG. 1A as a graphical plot of the change in relative fluorescence versus the time for duplicate tests at the three different DNA polymerase concentrations.

The data depicted in FIG. 1A was regressed in time according to the following equation:

$$S^i = C_0^i + C_1^i t + C_2^i t^2$$

with the results provided in the following Table:

TABLE I

i	A_i (Units/ml)	C_1^i (f/min.)*
1	0.5	1.48
2	5	9.45
3	20	24.5

*f = relative fluorescence

The initial rates (C^i) were then plotted versus polymerase activities, as shown in FIG. 1B. These data (that is, the C^i) were in turn regressed versus polymerase activities (A_i) according to the following equation (which represents the solid line in FIG. 1B):

$$C_1^i = Y_0 + Y_1 A + Y_2 A^2$$

yielding the following regression coefficients:

$\gamma_0 = 0.50$
$\gamma_1 = 1.99$ and
$\gamma_2 = -0.0394$

As an example as to how the immediately preceeding equation could be used to determine the amount of polymerase in a test sample in view of a known enzymatic rate, one could consider a rate of 15 (f/min). Using FIG. 1B, this rate corresponds to a polymerase concentration of about 8.7 units/ml.

Alternatively, this same result can be obtained using standard mathematical calculations and solving the following equation for A_x (activity):

$$A_x = \frac{-Y_1 - \sqrt{Y_1^2 - 4(Y_2)(Y_0 - C_1)}}{2Y_2}$$

wherein for a C_1 of 15 f/min (initial rate), A is determined to be about 8.8 units/ml.

This example shows that DNA polymerase concentrations can be effectively determined using the method of the present invention and the bibenzimidazole fluorescent dye described herein. This determination can be carried out using either a plotted calibration curve, or standard mathematical calculations.

Example 2: Assay for DNA Polymerase Using a M13 Template and Fluorescent Dye

This example is similar to Example 1 except a naturally occuring phage DNA was used as a template. A number of other minor changes were also made.

Materials:

The fluorometer used was a Perkin Elmer LS-2 fluorometer which was modified to handle sample that were loaded automatically using a Perkin Elmer Multi-Sampler. The flow cell and the tubing in the equipment held about 1 ml of solution. Triplicate readings were taken at each point in time.

The dye solution was prepared as described in Example 1.

The template used was single-strand M13mp0/TSYC 657-8 obtained from Cetus Corp.

The primer had the following nucleotide sequence (using standard base identification):

SEQ ID NO 3: 5'-CCCGGGCGGC GCCGCAGCGG CGGG-3'

The primer extension reaction mixture comprised the following:

primer (0.26 μmolar), template (13 nmolar), DNA polymerase isolated from Thermus aquaticus (20 units/ml, with 1 unit defined above), dNTPs (1.5 mmolar of each) which are added last to begin extension (see below), gelatin (1 mg/ml), potassium chloride (50 mmolar), ethylenediaminetetraacetic acid (1 mmolar), tris(hydroxymethyl)aminomethane buffer (10 mmolar, pH 8) and magnesium chloride (8, 10 and 13 mmolar).

Assay:

The primer and template were hybridized, after which the dNTP's (60 μl, 100 mmolar total triphosphates) were added to the reaction mixture (1 ml, yielding a final dNTP concentration of 6 mmolar) at 75 °C. As noted above, this addition of the dNTP's initiated the reaction. The temperature was maintained at 75 °C for 12 minutes. At selected times (1, 2, 3, 10, 11 and 12 minutes), aliquots (150 μl) were removed and added to vials which had been precooled in a brine mixture (0 °C) to quench the reaction. The dye solution (1 ml of solution containing 0.1 μg/ml) was placed in glass tubes, the cold reaction solution (10 μl) was added and the tubes were vortexed thoroughly to mix the solutions. Triplicate solutions (about 1 ml) were placed in the fluorometer. This procedure was carried out at three different magnesium ion concentrations in the reaction mixture (8, 10 and 13 mmolar).

The results are shown in the graphical plots of FIG. 2 as plots of fluorescence versus time. The slopes (reaction rates) of these curves were then plotted versus magnesium ion concentration in FIG. 3. These data indicate that a 8 mmolar concentration of magnesium ion gives the fastest rate of reaction.

Example 3: Assay for DNA Polymerase Using Bibenzimidazole and Ethidium Bromide Fluorescent Dyes and M13 Phage DNA Template

This example illustrates the use of two different dyes in the method of this invention.

Materials:

The fluorometer used was a Perkin Elmer LS-5B, equipped with a Perkin Elmer Multi-Sampler. The fluorometer was set at an excitation of 342 nm and emission of 474 nm for the bibenzimidazole, and at an excitation of 312 nm and emission of 580 nm for ethidium bromide

Assay reagents were prepared or obtained as described in Example 1, except that the DNA polymerase concentration was 25 units/ml.

The primer had the following sequence

SEQ ID No 4: 5'-GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT-3'.

Assay:

Each dye solution was put into separate sets of test tubes and evaluated separately.

The primer and template were hybridized as described in Example 1. The tubes containing 100 μl of reaction mixture were stored at 0 °C. Primer extension reactions were then initiated by removing the reaction mixtures from the low temperature environment and placing them into water maintained at 60 °C. At selected times (4, 4.5, 5, 13, 13.5 and 14 minutes), tubes were taken from the water bath and placed in ice (0 °C). From each tube, three separate aliquots (25 μl) were mixed with each dye solution (1 ml)

separately and the relative fluorescence measured.

The results of these tests are shown in the Table below. They indicate that, while the bibenzimidazole is more sensitive, ethidium bromide is also useful in the practice of this invention.

	Relative Fluorescence	
Time (minutes)	Bibenzimidazole	Ethidium Bromide
4	599	172
4.5	632	182
5	663	183
13	913	227
13.5	912	230
14	930	228

Examples 4 and 5: Determination of Polymerase activity in Two Samples

These examples are similar to Example 1 except M 13 phage DNA was used as a template and a number of other changes were made.

Materials:

The fluorometer used was a Perkin Elmer LS-5B fluorometer which was modified to handle samples that were loaded automatically using a Perkin Elmer Multi-Sampler. The flow cell and the tubing in the equipment held about 1 ml of solution. Triplicate readings were taken at each point in time.

The dye solution was prepared as described in Example 1.

The template used was single-strand M13mp18 available from International Biotechnologies, Inc. (New Haven, Connecticut, catalog #77242).

The primer had the following nucleotide sequence (using standard base identification):

SEQ ID NO 5: 5'-GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT-3'

The primer extension reaction mixture comprised the following:

primer (0.07 µmolar), template (16 nmolar), DNA polymerase isolated from Thermus aquaticus (having activities stated below), dNTPs (0.5 mmolar of each), gelatin (1 mg/ml), ethylenediaminetetraacetic acid (1 mmolar), tris(hydroxymethyl)aminomethane buffer (10 mmolar, pH 8) and magnesium chloride (6 mmolar).

Assay:

The primer and template were hybridized, after which the dNTP's and DNA polymerase were added to the reaction mixture. An sharp increase in temperature (from 0 °C to 75 °C) was used to initiate the reaction. The temperature was maintained at 75 °C for 14 minutes. At selected times (3, 3.5, 4, 13.5 and 14 minutes), aliquots (150 µl) were removed and added to vials which had been precooled in a brine mixture (0 °C) to quench the reaction. The dye solution (1 ml of solution containing 0.1 µg/ml) was placed in glass tubes, the cold reaction solution (10 µl) was added and the tubes were vortexed thoroughly to mix the solutions. Triplicate solutions (about 1 ml) were placed in the fluorometer. This procedure was carried out at five different DNA polymerase concentrations (0, 5, 7.5, 15 and 25 units/ml) in the reaction mixture.

The results are shown in the graphical plots of FIG. 4 as plots of fluorescence versus time at various polymerase concentrations. The slopes of these curves were then plotted versus DNA polymerase concentration as a calibration curve in FIG. 5. The slopes of the plots were calculated by linear regression according to Equation (I). The regression coefficients (C^l) are listed in the following Table II:

T A B L E II

i	A_i (Units/ml)	C_1^i (f/min.)*
1	0	−0.21
2	5	4.62
3	7.5	5.60
4	15	8.32
5	25	11.55

*f = relative fluorescence

When these initial rates were regressed against the known activities (A_i) according to Equation (III), the following regression coefficients were obtained:) according to Equation (III), the following regression coefficients were obtained:

$$\gamma_0 = 0.261$$
$$\gamma_1 = 0.790$$
$$\gamma_2 = -0.0138 \text{ and}$$
$$\gamma_3 \neq 0,$$

that is,

$$C_1 = 0.261 + 0.790\,A - 0.0138\,A^2$$

The foregoing equation can be inverted to permit determination of unknown activities A_x from known rates (C_i) as follows:

$$A_x = \frac{0.790 - \sqrt{(0.790^2 - 4(0.0138)(C_1 - 0.261)}}{2(0.0138)}$$

Two different samples containing unknown amounts of DNA polymerase were also tested according to the same procedure described above. One sample was purchased from International Biotechnologies, Inc. (Example 4, identified commercially by catalog #19010). A second sample (Example 5) was a recombinant form of the enzyme commercially available from Cetus Corp. Graphical plots of the resulting fluorescence versus time are shown in FIG. 6.

Relative fluorescence readings for Examples 4 and 5 were regressed versus time, leading to the following two equations:

Example 4: f = 140 + 9.11t (t = time)
Example 5: f = 187 + 9.86t (t = time)
The initial rates were computed using Equation (III), as follows:
Example 4: $(\frac{df}{dt})_{t=0} = C_1 = 9.11$ (f/min.)
Example 5: $(\frac{df}{dt})_{t=0} = C_1 = 9.86$ (f/min.)

These two rates were then projected graphically off the calibration curve (FIG. 5), as shown on FIGS. 7 and 8, respectively for examples 4 and 5. The estimated DNA polymerase concentrations for each sample were: 15 units/ml for Example 4 and 17.6 units/ml for Example 5.

When the rates (9.11 and 9.86 f/min. for Examples 4 and 5, respectively) were inserted into the terms of Equation (IV), the following results were obtained:

Example 4: about 15.3 units/ml, and
Example 5: about 17.5 units/ml.

The results correspond well to those obtained from the calibration curve (FIG. 5). These examples demonstrate that unknown concentrations of DNA polymerase can be determined using the method of this invention, either by using a calibration curve, or by strict mathematical treatment of experimental data.

Example 6: Assay Carried Out with Premixed Flourescent Dye

This example illustrates the practice of the present invention wherein the fluorescent dye was mixed with the template and other polymerization reagents prior to contact with the DNA polymerase to be detected. The polymerase chain reaction was then initiated by the addition of aliquots of DNA polymerase, and fluorescent signals were measured continously over time. This procedure has some advantages in that it allows more samples to be tested in a given time, and more dye signal measurements can be made. Thus there is more potential for higher precision as sampling and handling errors are minimized.

Materials:

The template was a single-stranded M13 DNA phage obtained from IBI (New Haven, Connecticut).

The fluorescent dye solution used was that shown in Example 1.

The polymerase chain reaction reagent solution contained: bovine serum albumin (30 µl, 2%), buffer solution (30 µl of 100 mmolar, pH 8.5), magnesium chloride (36 µl, 100 µmolar), template (37.9 µl, 0.25 µmolar), primer (1.9 µl), dNTPs (100 µmolar each, 12 µl) and water (149 µl). The primer had the sequence:
SEQ ID NO 6: 5'-GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT-3'

The buffer solution noted above contained tris(hydroxymethyl)aminomethane buffer (10 mmolar, pH 8.3), magnesium chloride (1.5 mmolar) and gelatin (0.01%).

Assay:

The fluorescent dye solution (300 µl) was mixed with the polymerase chain reaction reagent solution (297 µl). The resulting mixture was placed in a LS-5B fluorometer after the mixture was equilibrated at 62 °C.

Reaction was initiated by adding a solution of DNA polymerase (isolated from Thermus aquaticus as identified above in Example 1) at various concentrations (3.75 µl having 25 units/µl, 1.88 µl having 12.5 units/µl and 0.94 µl having 6.25 units/µl). Fluorescence was measured for each polymerase concentration at various times. No fluorescence change was observed with a Control solution (buffer only, no DNA polymerase). The resulting data was plotted as shown in FIG. 9. The initial rates were obtained from the graphical curves of FIG. 9 by graphical measurement, and plotted as shown in FIG. 10. Good correlation of the initial rates and DNA polymerase activity can be seen from these data.

SEQUENCE LISTING

SEQ ID NO: 1
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 125 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: Template for PCR
IMMEDIATE SOURCE: Synthetically prepared

5'-AACCTCTGGG TCCAAGCCGT GGCCAGCGGC AGACATGGTT-
GATACCAACC TGCACATTCT TACTATTTTA TTTAATCCCA-
GGATTGGGAT AGGTGGATTA TTTGTGCAGA CTTCTCCTCA GGAGT-3'.

SEQ ID NO: 2
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 23 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: PCR primer
IMMEDIATE SOURCE: Synthetically prepared

5'-CTCCTGAGGA GAAGTCTGCA CAA-3'

SEQ ID NO: 3
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 24 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: PCR primer
IMMEDIATE SOURCE: Synthetically prepared

5'-CCCGGGCGGC GCCGCAGCGG CGGG-3'

SEQ ID NO: 4
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 40 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: PCR primer
IMMEDIATE SOURCE: Synthetically prepared

5'—GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT—3'.

SEQ ID NO 5:
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 40 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: PCR primer
IMMEDIATE SOURCE: Synthetically prepared

5'—GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT—3'

SEQ ID NO 6:
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 40 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: PCR primer
IMMEDIATE SOURCE: Synthetically prepared

5'—GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT—3'

Claims

1. A method for the quantitative determination of DNA polymerase activity, comprising:

A. bringing into contact:

an aqueous test specimen believed to contain a DNA polymerase having activity A_x,

a single-stranded DNA template which is present in a concentration of at least 10^{-8} molar nucleotides,

a DNA primer complementary to the single-stranded template,

a DNA polymerase metal ion cofactor,

sufficient deoxyribonucleoside triphosphates to generate a double-stranded DNA molecule from the single-stranded DNA template in the presence of the DNA polymerase, and

a colorimetric or fluorescent dye which, when bound to the double-stranded DNA formed from the single-stranded DNA, exhibits a change in signal as opposed to when the dye is bound to the single-stranded DNA template, and

B. determining, with a precision having a covariance of less than 10%, the activity A_x of the DNA polymerase in the test specimen corresponding to the rate of colorimetric or fluorometric signal generated by the binding of the dye to the double-stranded DNA formed by the action of the test specimen DNA polymerase,

the DNA polymerase activity A_x being determined as follows:

1) generating DNA polymerase rates of reaction from the colorimetric or fluorometric signals generated over time by each of a series of samples containing DNA polymerase having known polymerase activities, A_1, A_2,...A_i...A_n wherein n is the number of samples evaluated and is at

SEQ ID NO: 4
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 40 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: PCR primer
IMMEDIATE SOURCE: Synthetically prepared

5'-GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT-3'.

SEQ ID NO 5:
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 40 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: PCR primer
IMMEDIATE SOURCE: Synthetically prepared

5'-GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT-3'

SEQ ID NO 6:
SEQUENCE TYPE: Nucleotide
SEQUENCE LENGTH: 40 bases
STRANDEDNESS: Single
TOPOLOGY: Linear
MOLECULAR TYPE: PCR primer
IMMEDIATE SOURCE: Synthetically prepared

5'-GAGCCACCAC CGGAACCGCC TCCCTCAGAG CCGCCACCCT-3'

Claims

1. A method for the quantitative determination of DNA polymerase activity, comprising:

A. bringing into contact:

an aqueous test specimen believed to contain a DNA polymerase having activity A_x,

a single-stranded DNA template which is present in a concentration of at least 10^{-8} molar nucleotides,

a DNA primer complementary to the single-stranded template,

a DNA polymerase metal ion cofactor,

sufficient deoxyribonucleoside triphosphates to generate a double-stranded DNA molecule from the single-stranded DNA template in the presence of the DNA polymerase, and

a colorimetric or fluorescent dye which, when bound to the double-stranded DNA formed from the single-stranded DNA, exhibits a change in signal as opposed to when the dye is bound to the single-stranded DNA template, and

B. determining, with a precision having a covariance of less than 10%, the activity A_x of the DNA polymerase in the test specimen corresponding to the rate of colorimetric or fluorometric signal generated by the binding of the dye to the double-stranded DNA formed by the action of the test specimen DNA polymerase,

the DNA polymerase activity A_x being determined as follows:

1) generating DNA polymerase rates of reaction from the colorimetric or fluorometric signals generated over time by each of a series of samples containing DNA polymerase having known polymerase activities, A_1, A_2,...A_i...A_n wherein n is the number of samples evaluated and is at

least two, and generating the DNA polymerase rate of reaction from the colorimetric or fluorometric signal generated over time from the test specimen,

2) determining a calibration using the rates of step 1) and the known DNA polymerase activities A_l, and

3) predicting A_x using the calibration of step 2) and the DNA polymerase reaction rate of the test specimen.

2. The method as claimed in claim 1 wherein:

step B1) is carried out by

(a) generating curves from the colorimetric or fluorometric signals with time for each of the known DNA polymerase activities, and generating a similar curve for the DNA polymerase activity of the test specimen, and

(b) measuring the slope of each curve generated in (a) above at time t* to provide DNA polymerase reaction rates C_1^l for each known DNA polymerase activity A_l and a DNA polymerase reaction rate C_1^x for the test specimen DNA polymerase activity A_x, where t* is any given time,

step B2) is carried out by plotting the rates C_1^l determined in B1) as a function of the known DNA polymerase activities A_l to generate a calibration curve, and

step B3) is carried out by projecting the test specimen rate C_1^x generated in step B1) off the calibration curve generated in step B2) to determine A_x.

3. The method as claimed in claim 1 wherein:

step B1) is carried out by

(a) generating curves from the colorimetric or fluorometric signals with time for each of the known DNA polymerase activities, and generating a similar curve for the DNA polymerase activity of the test specimen, and

(b) measuring the slope of each curve generated in (a) above at time t* to provide DNA polymerase reaction rates C_1^l for each known DNA polymerase activity A_l and a DNA polymerase reaction rate C_1^x for the test specimen DNA polymerase activity A_x, where t* is any given time,

step B2 is carried out by regressing each DNA polymerase rate C_1^l generated in B1) versus the respective known DNA polymerase activity A_l using equation (I)

$$C_1 = \gamma_o + \gamma_1 A + \gamma_2 A^2 + \gamma_3 A^3 \qquad (I)$$

to obtain the coefficients $\gamma_o, \gamma_1, \gamma_2$, and γ_3, and test specimen DNA polymerase activity C_1^x and equation (II)

$$(II) \quad C_1^x = \gamma_o + \gamma_1 A_x + \gamma_2 A_x^2 + \gamma_3 A_x^3$$

to solve for A_x.

4. The method as claimed in claim 1 wherein:

step B1) is carried out by

(a) regressing the signal vs. time data for each known DNA polymerase activity A_l and for the unknown DNA polymerase activity A_x to obtain the regression coefficients of equation (III)

$$(III) \quad S^i = C_0^i + C_1^i t^* + C_2^i (t^*)^2$$

wherein S^l represents the colorimetric or fluorometric signal for a given time t* and known DNA polymerase activity A_l, or unknown DNA polymerase activity A_x, and

C_0^l, C_1^l and C_2^l represent the regression coefficients,

(b) taking the time derivatives of equation (III) to obtain the rate according to equation (IV)

$$(IV) \quad \left(\frac{dS}{dt}\right)_{t^*} = C_1^i + 2C_2^i t^*$$

thereby providing the coefficient C_1^i for each known DNA polymerase activity A_i, the coefficient C_1^i being the rate of DNA polymerase activity A_i,

step B2) is carried out by plotting the rates C_1^i for the known DNA polymerase activities determined in B1) as a function of the known DNA polymerase activities A_i to generate a calibration curve, and

step B3) is carried out by projecting the test specimen rate C_1^x generated by step B1) off the calibration curve generated in step B2) to determine A_x.

5. The method as claimed in claim 1 wherein:

step B1) is carried out by

(a) regressing the signal vs. time data for each known DNA polymerase activity A_i and for the unknown DNA polymerase activity A_x to obtain the regression coefficients of equation (III)

$$(III) \quad S^i = C_0^i + C_1^i t^* + C_2^i (t^*)^2$$

wherein S^i represents the colorimetric or fluorometric signal for a given time t^* and known DNA polymerase activity A_i, or unknown DNA polymerase activity A_x, and

C_0^i, C_1^i and C_2^i represent the regression coefficients,

(b) taking the time derivative of equation (III) to obtain the rate according to equation (IV)

$$(IV) \quad \left(\frac{dS}{dt}\right)_{t^*} = C_1^i + 2C_2^i t^*$$

thereby providing the coefficient C_1^i for each known DNA polymerase activity A_i, the coefficient C_1^i being the rate of DNA polymerase activity A_i,

step B2 is carried out by regressing each DNA polymerase rate C_1^i generated in B1) versus the respective known DNA polymerase activity A_i using equation (I)

$$C_1 = \gamma_0 + \gamma_1 A + \gamma_2 A^2 + \gamma_3 A^3 \qquad (I)$$

to obtain the coefficients $\gamma_0, \gamma_1, \gamma_2$, and γ_3, and

step B3) is carried out by using the rate of test specimen DNA polymerase activity C_1^x and equation (II)

$$(II) \quad C_1^x = \gamma_0 + \gamma_1 A_x + \gamma_2 A_x^2 + \gamma_3 A_x^3$$

to solve for A_x.

6. The method as claimed in any of claims 1 to 5 wherein n is from 2 to 5.

7. The method as claimed in any of claims 1 to 6 wherein the test specimen is mixed with the single-stranded template, DNA primer, cofactor and deoxyribonucleoside triphosphates prior to contact with the colorimetric or fluorometric dye.

8. A test kit useful for the determination of DNA polymerase activity, comprising:

the four deoxyribonucleosides, dATP, dCTP, dGTP and dTTP,

a source of a metal ion DNA polymerase cofactor, and

a DNA primer complementary to a single-stranded DNA template suitable for forming a double-stranded DNA therefrom,

the kit characterized as further comprising:

a single-stranded phase DNA as a single-stranded template, and

a colorimetric or fluorescent dye which, when bound to the double-stranded DNA molecule formed from the single-stranded template, exhibits a change in signal, as opposed to when the dye is bound to the single-stranded DNA template.

9. The test kit as claimed in claim 8 comprising a source of magnesium or manganese ion as the cofactor.

10. The method as claimed in any of claims 1 to 7 for the detection of a DNA polymerase obtained from Thermus aquaticus or Thermus thermophilus.

11. The method as claimed in any one of claims 1 to 7 or 10 wherein the dye is a bibenzimidazole, ethidium, methidium or acridine fluorescent dye.

12. The method as claimed in any one of claims 1 to 7, 10 or 11 wherein the dye is , 2-[2-(4-hydroxyphenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)-benzimidazole trihydrochloride, acridine orange, methidium bromide, propidium iodide, ethidium bromide or 4', 6'-diamidino-2-phenylindole.

13. The method as claimed in any of claims 1 to 7 or 10 to 12 wherein the single-stranded DNA template is M 13 phage.

14. The kit as claimed in claim 8 or 9 for the detection of a DNA polymerase obtained from Thermus aquaticus or Thermus thermophilus.

15. The kit as claimed in any one of claims 8, 9 and 14 wherein the dye is a bibenzimidazole, ethidium, methidium or acridine fluorescent dye.

16. The kit as claimed in any one of claims 8, 9, 14 and 15 wherein the dye is 2-[2-(4-hydroxyphenyl)-6-benzimidazole]-6-(1-methyl-4-piperazyl)-benzimidazole trihydrochloride, acridine orange, methidium bromide, propidium iodide, ethidium bromide or 4', 6'-diamidino-2-phenylindole.

17. The kit as claimed in any one of claims 8, 9 and 14 to 16 wherein the single-stranded DNA template is M 13 phage.

18. The use of a colorimetric or fluorescent dye for the quantitative determination of a DNA polymerase activity, the colorimetric or fluorescent dye being one that, when bound to a double-stranded DNA molecule, exhibits a change in signal, as opposed to when the dye is bound to a single-stranded DNA molecule derived from the double-stranded DNA molecule.

Patentansprüche

1. Methode zur quantitativen Bestimmung von DNS-Polymeraseaktivität, bestehend aus:

A. Zusammenbringung von: einer wässrigen Testprobe, von der angenommen wird, daß sie eine DNS-Polymerase mit einer Aktivität A_x enthält,

eines einsträngigen DNS-Templates, das in einer Konzentration von mindestens 10^{-8} molar Nukleotiden vorkommt,

eines DNS-Primers ergänzend zu dem einsträngigen Template,

eines DNS-Polymerase-Metallionkofaktors,

ausreichend deoxyribonukleosider Triphosphate, um ein doppelsträngiges DNS-Molekül aus dem einsträngigen DNS-Template bei Vorhandensein der DNS-Polymerase zu gewinnen, und

eine kolorimetrische oder fluoreszierende Farbe, die, wenn sie an eine doppelsträngige, aus der einsträngigen DNS geformte DNS gebunden ist, eine Veränderung des Signals zeigt, das verschieden vom Signal ist, das die Farbe zeigt, wenn sie an ein einsträngiges DNS-Template gebunden ist, und

* C L M S *

B. Bestimmung der Aktivität A_x der DNS-Polymerase in der Testprobe, mit einer Präzisionskovarianz von weniger als 10%, entsprechend der Rate, des kolorimetrischen oder fluorometrischen Signals, das durch die Bindung der Farbe an die doppelsträngige, von der Aktion der Testproben-DNS-Polymerase gebildeten DNS hervorgerufen wird,

die DNS-Polymeraseaktivität A_x wird wie folgt bestimmt:

1) Erzeugung von DNS-Polymerase-Reaktionsraten aus den kolorimetrischen oder fluorometrischen Signalen, die über die Zeit von jeder der Serien der Muster mit einer DNS-Polymerase mit bekannten Polymeraseaktivitäten A_1, A_2, ...A_i..A_n hervorgerufen werden, wobei n die Anzahl der ausgewerteten Muster ist und mindestens zwei beträgt und Erzeugung der DNS-Polymerase-Reaktionsrate aus dem kolorimetrischen oder fluorometrischen Signal, das über die Zeit in der Testprobe gemessen wird.

2) Bestimmung einer Kalibration anhand der Raten in Schritt 1) und der bekannten DNS-Polymeraseaktivitäten A_i, und

3) Vorhersage von A_x anhand der Kalibration aus Schritt 2) und der DNS-Polymerase-Reaktionsrate der Testprobe.

2. Methode, aus Anspruch 1, wobei:
Schritt B1) durchgeführt wird durch

(a) Erzeugung von Kurven aus den kolorimetrischen oder fluorometrischen Signalen mit der Zeit für jede der bekannten DNS-Polymeraseaktivitäten und Erzeugung einer ähnlichen Kurve für die DNS-Polymeraseaktivität in der Testprobe, und

(b) Messung der Steigung jeder in (a) erzeugten Kurve mit einer Zeit t*, um die DNS-Polymerase-Reaktionsraten C_i^i für jede bekannte DNS-Polymeraseaktivität A_i und eine DNS-Polymerase-Reaktionsrate C_i^x für die DNS-Polymeraseaktivität A_x der Testprobe zu bekommen, wobei t* jede gegebene Zeit ist.

Schritt B2) wird durchgeführt, indem die in B1) bestimmten Raten C_i^i als eine Funktion der bekannten DNS-Polymeraseaktivitäten A_i graphisch dargestellt werden, um eine Kalibrationskurve zu erzeugen, und Schritt B3) wird durchgeführt, indem die in Schritt B1) erhaltene Rate C_i^x der Testprobe von der in Schritt B2) erzeugten Kalibrationskurve übertragen wird, um A_x zu bestimmen.

3. Methode, aus Anspruch 1, wobei:
Schritt B1) durchgeführt wird durch:

(a) Erzeugung von Kurven aus den kolorimetrischen oder fluorometrischen Signalen mit der Zeit für jede der bekannten DNS-Polymeraseaktivitäten und Erzeugung einer ähnlichen Kurve für die DNS-Polymeraseaktivität in der Testprobe, und

(b) Messung der Steigung jeder in (a) erzeugten Kurve mit einer Zeit t*, um die DNS-Polymerase-Reaktionsraten C_i^i für jede bekannte DNS-Polymeraseaktivität A_i und eine DNS-Polymerase-Reaktionsrate C_i^x für die DNS-Polymeraseaktivität A_x der Testprobe zu bekommen, wobei t* jede gegebene Zeit ist,

Schritt B2) wird durchgeführt, indem jede in B1) erzeugte DNS-Polymeraserate C^i_i gemäß der Gleichung (I) zu der entsprechenden bekannten DNS-Polymeraseaktivität A_i in Beziehung gesetzt wird

$$C_i = \gamma_0 + \gamma_1 A + \gamma_2 A^2 + \gamma_3 A^3 \qquad (I)$$

um die Koeffizienten γ_0, γ_1, γ_2 und γ_3 und die DNS-Polymeraseaktivität C_i^x der Testprobe zu erhalten und die Gleichung (II)

$$(II)\ C_i^x = \gamma_0 + \gamma_1 A_x + \gamma_2 A_x^2 + \gamma_3 A_x^3$$

um A_x zu lösen.

4. Methode aus Anspruch 1, wobei:
Schritt B1) durchgeführt wird, indem

(a) die Signaldaten zu den Zeitdaten für jede bekannte Polymeraseaktivität A_i und für die unbekannte Polymeraseaktivität A_x in Beziehung gesetzt werden um die Regressionskoeffizienten der Gleichung (III) zu erhalten

$$(III)\ S^i = C_0^i + C_1^i t^* + C_2^i (t^*)^2$$

wobei S^i das kolorimetrische oder fluorometrische Signal für eine gegebene Zeit t^* und eine bekannte Polymeraseaktivität A_i oder eine unbekannte Polymeraseaktivität A_x darstellt, und C_0^i, C_1^i und C_2^i die Regressionskoeffizienten darstellt.

(b) Bestimmung der Zeitableitung aus der Gleichung (III) errechneten Zeit, um die Rate gemäß Gleichung (IV) zu erhalten

$$(IV)\ \left[\frac{ds}{dt}\right]_{t^*} = C_1^i + 2C_2^i t^*$$

was den Koeffizienten für jede bekannte Polymeraseaktivität A_i liefert, wobei der Koeffizient C_1^i die Rate der Polymeraseaktivität A_i ist,

in Schritt B2) werden die Raten C_1^i für die bekannten, in Schritt B1) ermittelten Polymeraseaktivitäten aufgezeichnet, als Funktion der bekannten Polymeraseaktivitäten A_i, um eine Kalibrationskurve zu erzeugen und

in Schritt B3) wird die in Schritt B1) erzeugte Rate C_1^x der Testprobe aus der in Schritt B2) erzeugten Kalibrationskurve entnommen, um A_x zu bestimmen.

5. Methode, aus Anspruch 1, wobei

Schritt B1) durchgeführt wird, indem

(a) die Signaldaten zu den Zeitdaten für jede bekannte Polymeraseaktivität A_i und für die unbekannte Polymeraseaktivität A_x in Beziehung gesetzt werden, um die Regressionskoeffizienten der Gleichung (III) zu erhalten

$$(III)\ S^i = C_0^i + C_1^i t^* + C_2^i (t^*)^2$$

wobei S^i das kolorimetrische oder fluorometrische Signal für eine gegebene Zeit t^* und eine bekannte Polymeraseaktivität A_i oder eine unbekannte Polymeraseaktivität A_x darstellt, und C_0^i, C_1^i und C_2^i die Regressionskoeffizienten darstellen,

(b) Bestimmung der Zeitableitung aus der Gleichung (III) errechneten Zeit, um die Rate gemäß Gleichung (IV) zu erhalten

$$(IV)\ \left[\frac{ds}{dt}\right]_{t^*} = C_1^i + 2C_2^i t^*$$

was den Koeffizienten C_1^i für jede bekannte Polymeraseaktivität A_i liefert, wobei der Koffizient C_1^i die Rate der Polymeraseaktivität A_i ist.

Schritt B2 wird durchgeführt, indem jede in B1) erzeugte DNS-Polymeraserate C_1^i mit der entsprechenden bekannten DNS-Polymeraseaktivität A_i in Beziehung gesetzt wird anhand von Gleichung (I)

$$C_1 = \gamma_0 + \gamma_1 A + \gamma_2 A^2 + \gamma_3 A^3 \qquad (I)$$

um die Koeffizienten γ_0, γ_1, γ_2 und γ_3 zu erhalten, und

Schritt B3) wird durchgeführt anhand der Testprobe der DNS-Polymeraseaktivität C_1^x und der Gleichung (II)

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$$(II)\ C_1^x = \gamma_0 + \gamma_1 A_x + \gamma_2 A_x^2 + \gamma_3 A_x^3$$

um A_x zu lösen.

6. Methode, aus einem der Ansprüche 1 bis 5, wobei n eine Anzahl von 2 bis 5 ist.

7. Methode, aus einem der Ansprüche 1 bis 6, wobei die Testprobe mit dem einsträngigen Template, dem DNS-Primer, dem Kofaktor und den deoxyribonukleosiden Triphosphaten vor dem Kontakt mit der kolorimetrischen oder fluorometrischen Farbe gemischt wird.

8. Testset zur Bestimmung von DNS-Polymeraseaktivität, bestehend aus:
den vier Deoxyyribonukleosiden, dATP, dCTP, dGTP und dTTP,
einer Quelle für einen Metalion-DNS-Polymerasefaktor, und einem DNS-Primer ergänzend zu einem einsträngigen DNS-Template, der geeignet ist, um daraus eine doppelsträngige DNS zu bilden,
das Set ist gekennzeichnet dadurch, daß es weiterhin enthält:
eine einsträngige Phage-DNS als einsträngiges Template und
eine kolorimetrische oder fluoreszierende Farbe, die, wenn sie an eine doppelsträngige aus der einsträngigen DNS geformte DNS gebunden ist, eine Veränderung des Signales zeigt, das verschieden vom Signal ist, das die Farbe zeigt, wenn sie an ein einsträngiges DNS-Template gebunden ist.

9. Testset aus Anspruch 8 enthaltend eine Quelle für Magnesium- oder Manganionen als Kofaktor.

10. Methode aus einem der Ansprüche 1 bis 7 zur Bestimmung einer DNS-Polymerase gewonnen aus Thermus aquaticus oder Thermus thermophilus:

11. Methode, aus einem der Ansprüche 1 bis 7 oder 10, wobei die Farbe fluoreszierende Bibenzimidasol-, Äthidium-, Methidium- oder Akridin-Farbe ist.

12. Methode, aus einem der Ansprüche 1 bis 7, 10 oder 11, wobei die Farbe, 2-[2-(4-Hydroxyphenyl)-6-Benzimidasol]-6-(1-Methyl-4-Piperazyl)Benzimidasol Trihydrochlorid, Akridin-Orange, Methidium-Bromid Propidium-Iodid, Äthidium-Bromid oder 4', 6'-Diamidino-2-Phenylindol.

13. Methode, aus einem der Ansprüche 1 bis 7 oder 10 bis 12, wobei das einsträngige DNS-Template ein M13-Phage ist.

14. Set, aus einem der Ansprüche 8 oder 9, zur Bestimmung einer aus Thermus aquaticus oder Thermus thermonhilus gewonnenen DNS-Polymerase.

15. Set, aus einem der Ansrpüche 8, 9 und 14, wobei die Farbe eine fluoreszierende Bibenzimidasol-, Äthidium-, Methidium-, oder Akridin-Farbe ist.

16. Set, aus einem der Ansprüche 8, 9, 14 und 15, wobei die Farbe 2-[2-(4-Hydroxyphenyl)-6-Benzimidasol]-6-(1-Methyl-4-Piperazyl)Benzimidasol Trihydrochlorid, Akridin-Orange, Methidium-Bromid Propidium-Iodid, Äthidium-Bromid oder 4', 6'-Diamidino-2-Phenylindol ist.

17. Set, aus einem der Ansprüche 8, 9 und 14 bis 16, wobei das einsträngige DNS-Template eine M13 Phage ist.

18. Verwendung einer kolorimetrischen oder fluoreszierenden Farbe zur quantitativen Bestimmung einer DNS-Polymeraseaktivität, wobei die kolorimetrische oder fluoreszierende Farbe bei Bindung an ein doppelsträngiges DNS-Molekül einen Signalwechsel zeigt gegenüber dem Signal, das die Farbe zeigt, wenn sie an ein aus dem doppelsträngigen DNS-Molekül gewonnenes einsträngiges DNS-Molekül gebunden ist.

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Revendications

1. Procédé pour la détermination quantitative de l'activité de l'ADN polymérase, comprenant :
A. la mise en contact :
d'un échantillon d'essai aqueux susceptible de contenir une ADN polymérase possédant une activité A_x,
d'une matrice d'ADN monocaténaire, qui est présente en une concentration d'au moins 10^{-8} molaire de nucléotides,
d'une amorce d'ADN complémentaire à la matrice monocaténaire,
d'un cofacteur d'ion métallique de l'ADN polymérase,
d'une quantité suffisante de désoxyribonucléoside triphosphates pour générer une molécule d'ADN bicaténaire à partir de la matrice d'ADN monocaténaire en présence de l'ADN polymérase, et
d'un colorant colorimétrique ou fluorescent qui, lorsqu'il se lie à l'ADN bicaténaire formé à partir de l'ADN monocaténaire, manifeste un changement de signal opposé à celui que l'on obtient lorsque le colorant se lie à la matrice d'ADN monocaténaire, et
B. la détermination, avec une précision dont la covariance est inférieure à 10%, de l'activité A_x de l'ADN polymérase dans l'échantillon d'essai correspondant à la valeur du signal colorimétrique ou fluorométrique généré par la liaison du colorant à l'ADN bicaténaire formé par l'action de l'ADN polymérase de l'échantillon d'essai,
l'activité A_x de l'ADN polymérase étant déterminée comme suit :
1) en générant des taux de réaction de l'ADN polymérase à partir des signaux colorimétriques ou fluorométriques générés au cours du temps par chaque série d'échantillons contenant de l'ADN polymérase possédant des activités de polymérase connues, A_1, A_2, ... A_i ... A_n où n représente le nombre d'échantillons évalués et est au moins égal à 2, et en générant le taux de réaction de l'ADN polymérase à partir du signal colorimétrique ou fluorométrique généré au cours du temps à partir de l'échantillon d'essai,
2) en déterminant un étalonnage en utilisant les taux de l'étape 1) et les activités connues A_i de l'ADN polymérase, et
3) en prédisant A_x en utilisant l'étalonnage de l'étape 2) et le taux de réaction de l'ADN polymérase de l'échantillon d'essai.

2. Procédé selon la revendication 1, dans lequel :
on effectue l'étape B1)
(a) en générant des courbes à partir des signaux colorimétriques ou fluorométriques obtenus au cours du temps pour chacune des activités connues de l'ADN polymérase et en générant une courbe similaire pour l'activité de l'ADN polymérase de l'échantillon d'essai, et
(b) en mesurant la pente de chaque courbe générée dans (a) ci-dessus au temps t' pour obtenir des taux de réaction C_i^t de l'ADN polymérase pour chaque activité connue A_i de l'ADN polymérase et un taux de réaction C_i^x de l'ADN polymérase pour l'activité A_x de l'ADN polymérase de l'échantillon d'essai, où t' représente n'importe quel temps donné,
on effectue l'étape B2) en reportant sur un graphique les taux C_i^t déterminés dans B1) en fonction des activités connues A_i de l'ADN polymérase pour générer une courbe d'étalonnage, et
on effectue l'étape B3) en projetant le taux C_i^x de l'échantillon d'essai généré à l'étape B1) en dehors de la courbe d'étalonnage générée à l'étape B2) pour déterminer A_x.

3. Procédé selon la revendication 1, dans lequel :
on effectue l'étape B1)
(a) en générant des courbes à partir des signaux colorimétriques ou fluorométriques obtenus au cours du temps pour chacune des activités connues de l'ADN polymérase et en générant une courbe similaire pour l'activité de l'ADN polymérase de l'échantillon d'essai, et
(b) en mesurant la pente de chaque courbe générée dans (a) ci-dessus au temps t' pour obtenir des taux de réaction C_i^t de l'ADN polymérase pour chaque activité connue A_i de l'ADN polymérase et un taux de réaction C_i^x de l'ADN polymérase pour l'activité A_x de l'ADN polymérase de l'échantillon d'essai, où t' représente n'importe quel temps donné,
on effectue l'étape B2) en procédant à la régression de chaque taux C_i^t de l'ADN polymérase généré en B1) par rapport à l'activité connue respective A_i de l'ADN polymérase en utilisant l'équation (I)

$$C_1 = \gamma_0 + \gamma_1 A + \gamma_2 A^2 + \gamma_3 A^3 \quad (I)$$

pour obtenir les coefficients γ_0, γ_1, γ_2 et γ_3 et l'activité C_1^x de l'ADN polymérase de l'échantillon d'essai, et l'équation (II)

$$(II)\ C_1^x = \gamma_0 + \gamma_1 A_x + \gamma_2 A_x^2 + \gamma_3 A_x^3$$

pour calculer A_x.

4. Procédé selon la revendication 1, dans lequel :
on effectue l'étape B1) :
(a) en procédant à la régression des données de signaux par rapport aux données de temps pour chaque activité connue A_i de l'ADN polymérase et pour l'activité inconnue A_x de l'ADN polymérase pour obtenir les coefficients de régression de l'équation (III)

$$(III)\ S^i = C_0^i + C_1^i t^* + C_2^i (t^*)^2$$

où S^i représente le signal colorimétrique ou fluorométrique pour un temps donné t^* et pour une activité connue A_i de l'ADN polymérase ou pour une activité inconnue A_x de l'ADN polymérase, et C_0^i, C_1^i et C_2^i représentent les coefficients de régression,
(b) en calculant les dérivées de temps de l'équation (III) pour obtenir le taux selon l'équation (IV)

$$(IV)\ \left[\frac{ds}{dt}\right]_{t^*} = C_1^i + 2C_2^i t^*$$

en procurant ainsi le coefficient C_1^i pour chaque activité connue A_i de l'ADN polymérase, le coefficient C_1^i représentant le taux de l'activité A_i de l'ADN polymérase,
on effectue l'étape B2) en reportant sur un graphique les taux C_1^i pour les activités connues de l'ADN polymérase déterminés dans B1) en fonction des activités connues A_i de l'ADN polymérase pour générer une courbe d'étalonnage, et
on effectue l'étape B3) en projetant le taux C_1^x de l'échantillon d'essai généré à l'étape B1) en dehors de la courbe d'étalonnage générée à l'étape B2) pour déterminer A_x.

5. Procédé selon la revendication 1, dans lequel
on effectue l'étape B1) :
(a) en procédant à la régression des données de signaux par rapport aux données de temps pour chaque activité connue A_i de l'ADN polymérase et pour l'activité inconnue A_x de l'ADN polymérase pour obtenir les coefficients de régression de l'équation (III)

$$(III)\ S^i = C_0^i + C_1^i t^* + C_2^i (t^*)^2$$

où S^i représente le signal colorimétrique ou fluorométrique pour un temps donné t^* et pour une activité connue A_i de l'ADN polymérase ou pour une activité inconnue A_x de l'ADN polymérase, et C_0^i, C_1^i et C_2^i représentent les coefficients de régression,

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(b) en calculant la dérivée de temps de l'équation (III) pour obtenir le taux selon l'équation (IV)

$$(IV)\ \left[\frac{ds}{dt}\right]_{t^*} = C_1^i + 2C_2^i t^*$$

en procurant ainsi le coefficient C_1^i pour chaque activité connue A_i de l'ADN polymérase, le coefficient C_1^i représentant le taux de l'activité A_i de l'ADN polymérase,

on effectue l'étape B2) en procédant à la régression de chaque taux C_1^i de l'ADN polymérase généré en B1) par rapport à l'activité connue respective A_i de l'ADN polymérase en utilisant l'équation (I)

$$C_1 = \gamma_0 + \gamma_1 A + \gamma_2 A^2 + \gamma_3 A^3 \qquad (I)$$

pour obtenir les coefficients γ_0, γ_1, γ_2 et γ_3 et

on effectue l'étape B3) en utilisant le taux de l'activité C_1^x de l'ADN polymérase de l'échantillon d'essai et l'équation (II)

$$(II)\ C_1^x = \gamma_0 + \gamma_1 A_x + \gamma_2 A_x^2 + \gamma_3 A_x^3$$

pour calculer A_x.

6. Procédé selon l'une quelconque des revendications 1 à 5, dans lequel n représente une valeur de 2 à 5.

7. Procédé selon l'une quelconque des revendications 1 à 6, dans lequel on mélange l'échantillon d'essai avec la matrice monocaténaire, l'amorce d'ADN, le cofacteur et les désoxyribonucléoside triphosphates avant sa mise en contact avec le colorant colorimétrique ou fluorométrique.

8. Trousse d'essai utile pour la détermination de l'activité de l'ADN polymérase, comprenant :
les quatre désoxyribonucléosides dATP, dCTP, dGTP et dTTP,
une source d'un cofacteur d'ion métallique de l'ADN polymérase, et
une amorce d'ADN complémentaire à une matrice d'ADN monocaténaire appropriée pour former un ADN bicaténaire à partir de cette dernière,
la trousse étant caractérisée en ce qu'elle comprend, en outre :
un ADN phagique monocaténaire comme matrice monocaténaire, et
un colorant colorimétrique ou fluorescent qui, lorsqu'il se lie à la molécule d'ADN bicaténaire formée à partir de la matrice monocaténaire, manifeste un changement de signal opposé à celui que l'on obtient lorsque le colorant se lie à la matrice d'ADN monocaténaire.

9. Trousse d'essai selon la revendication 8, comprenant une source d'ion magnésium ou manganèse comme cofacteur.

10. Procédé selon l'une quelconque des revendications 1 à 7, pour la détection d'une ADN polymérase obtenue à partir de Thermus aquaticus ou Thermus thermophilus.

11. Procédé selon l'une quelconque des revendications 1 à 7 ou 10, dans lequel le colorant est un colorant fluorescent de bis-benzimidazole, d'éthidium, de méthidium ou d'acridine.

12. Procédé selon l'une quelconque des revendications 1 à 7, 10 ou 11, dans lequel le colorant est le trichlorhydrate du 2-[2-(4-hydroxyphényl)-6-benzimidazole]-6-(1-méthyl-4-pipérazyl)-benzimidazole, l'orange d'acridine, le bromure de méthidium, l'iodure de propidium, le bromure d'éthidium ou encore le 4',6'-diamidino-2-phénylindole.

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13. Procédé selon l'une quelconque des revendications 1 à 7 ou 10 à 12, dans lequel la matrice d'ADN monocaténaire est le phage M 13.

14. Trousse selon la revendication 8 ou 9, pour la détection d'une ADN polymérase obtenue à partir de Thermus aquaticus ou de Thermus thermophilus.

15. Trousse selon l'une quelconque des revendications 8, 9 et 14, dans laquelle le colorant est un colorant fluorescent de bis-benzimidazole, d'éthidium, de méthidium ou d'acridine.

16. Trousse selon l'une quelconque des revendications 8, 9, 14 et 15, dans laquelle le colorant est le trichlorhydrate du 2-[2-(4-hydroxyphényl)-6-benzimidazole]-6-(1-méthyl-4-pipérazyl)-benzimidazole, l'orange d'acridine, le bromure de méthidium, l'iodure de propidium, le bromure d'éthidium ou encore le 4',6'-diamidino-2-phénylindole.

17. Trousse selon l'une quelconque des revendications 8, 9 et 14 à 16, dans laquelle la matrice d'ADN monocaténaire est le phage M 13.

18. Utilisation d'un colorant colorimétrique ou fluorescent pour la détermination quantitative d'une activité de l'ADN polymérase, le colorant colorimétrique ou fluorescent étant un colorant qui, lorsqu'il se lie à une molécule d'ADN bicaténaire, manifeste un changement de signal opposé à celui que l'on obtient lorsque le colorant se lie à une molécule d'ADN monocaténaire dérivée de la molécule d'ADN bicaténaire.