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(54) Title: PRIMER EXTENSION REACTION UTILIZING A COSUBSTRATE-ENZYME PAIR FOR CONSUMING PYROPHOSPHATE

(57) Abstract

An improved method for performing a primer extension reaction is disclosed, such method including the steps of annealing an oligonucleotide primer to a portion of a template nucleic acid thereby forming a primer-template hybrid; adding primer-extension reagents to the primer-template hybrid for extending the primer; and adding a cosubstrate-enzyme pair to the primer-template hybrid for conducting a pyrophosphate-utilizing reaction, thereby reducing the amount of pyrophosphate present in the reaction. In a particularly preferred embodiment, the cosubstrate-enzyme pair comprises pyrophosphate dependent phosphofructose kinase and fructose-6-phosphate, or UDP glucose pyrophosphorylase and UDP glucose. The invention further includes kits and solutions useful for carrying out the methods of the invention.

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PRIMER EXTENSION REACTION UTILIZING A COSUBSTRATE-ENZYME PAIR FOR CONSUMING PYROPHOSPHATE

FIELD OF THE INVENTION

This invention relates to methods and kits for conducting a template-mediated primer extension reaction, e.g., Sanger-type DNA sequencing or a polymerase chain reaction.

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BACKGROUND

Template-mediated primer extension reactions play an important role in several important biochemical methods, e.g., the polymerase chain reaction (PCR) and Sanger-type DNA sequencing.

In a template-mediated primer extension reaction, an oligonucleotide primer having homology to a single-stranded template nucleic acid is caused to anneal to a template nucleic acid, the annealed mixture is then provided with a DNA polymerase in the presence of nucleoside triphosphates under conditions in which the DNA polymerase extends the primer to form a complementary strand to the template nucleic acid. In a Sanger-type DNA sequencing reaction, the primer is extended in the presence f a chain-terminating agent, e.g., a dideoxynucleoside triphosphate, to cause base-specific termination of the primer extension (Sanger). In a polymerase chain reaction, two primers are provided, each having homology to opposite strands of a double-stranded DNA molecule. After the primers are extended, they are separated from their templates, and additional primers caused to anneal to the templates and the extended primers. The additional primers are then extended. The steps of separating, annealing, and extending are repeated in order to geometrically amplify the number of copies of the template nucleic acid (Saiki).

In both DNA sequencing and PCR, it is critically important that the primer extension product accurately replicate the nucleotide sequence of the template nucleic acid. However, under certain conditions, peak "dropout" has been observed wherein certain nucleotides are not represented in the primer extension product. This problem is believed to be caused by pyrophosphorolysis of the primer extension product by a reverse nucleotide addition reaction promoted by the accumulation of pyrophosphates in the reaction mixture (Mullis; Tabor 1990; Tabor 1996).

The present invention provides a solution to the problem of pyrophosphorolysis of primer extension products.

SUMMARY

The present invention is directed towards the discovery of a primer extension method

in which the extent of pyrophosphorolysis of a primer extension product is reduced, and solutions and kits useful for practicing the method.

It is an object of the present invention to provide a primer extension method wherein "peak drop-out" is reduced and the fidelity of template-sequence reproduction is maximized.

In a first aspect, the foregoing and other objects of the invention are achieved by an improved method for performing a primer extension reaction including the steps of annealing an oligonucleotide primer to a portion of a template nucleic acid thereby forming a primer-template hybrid; adding primer-extension reagents to the primer-template hybrid for extending the primer; and adding a cosubstrate-enzyme pair to the primer-template hybrid for conducting a pyrophosphate-utilizing reaction, thereby reducing the amount of pyrophosphate present in the reaction. In a first preferred embodiment, the cosubstrate-enzyme pair comprises pyrophosphate dependent phosphofructose kinase and fructose-6-phosphate. In a second preferred embodiment, the cosubstrate-enzyme pair comprises UDP glucose pyrophosphorylase and UDP glucose.

In a second aspect, the invention includes a kit for performing the primer extension reaction of the invention comprising primer extension reagents and a cosubstrate-enzyme pair.

In a third aspect, the invention includes a primer extension solution for the extension of a primer annealed to a template nucleic acid comprising primer extension reagents and a cosubstrate-enzyme pair.

These and other objects, features, and advantages of the present invention will become better understood with reference to the following description, drawings, and appended claims.

BRIEF DESCRIPTION OF THE DRAWINGS

FIGS. 1 and 3 show an electropherogram of the products of a primer extension reaction in the absence of the cosubstrate-enzyme pair method of the invention.

FIGS. 2 and 4 show an electropherogram of the products of a primer extension reaction utilizing the cosubstrate-enzyme pair method of the invention.

DETAILED DESCRIPTION OF THE PREFERRED EMBODIMENTS

Reference will now be made in detail to the preferred embodiments of the invention, examples of which are illustrated in the accompanying drawings. While the invention will be described in conjunction with the preferred embodiments, it will be understood that they are

not intended to limit the invention to those embodiments. On the contrary, the invention is intended to cover alternatives, modifications, and equivalents, which may be included within the invention as defined by the appended claims.

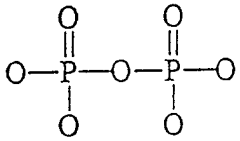
I. DEFINITIONS

Unless stated otherwise, the following terms and phrases as used herein are intended to have the following meanings:

"Primer" refers to a nucleic acid, e.g., synthetic oligonucleotide, which is capable of annealing to a complementary template nucleic acid and serving as a point of initiation for template-directed nucleic acid synthesis. Typically, a primer will include a free hydroxyl group at the 3'-end.

As used herein the term "primer-extension reagent" means a reagent including components necessary to effect the enzymatic template-mediated extension of a primer. Primer extension reagents include: (i) a polymerase enzyme, e.g., a thermostable polymerase enzyme such as Taq DNA polymerase, T7 DNA polymerase, and the like; (ii) a buffer to stabilize pH; (iii) deoxynucleotide triphosphates, e.g., deoxyguanosine 5'-triphosphate, 7-deazadeoxyguanosine 5'-triphosphate, deoxyadenosine 5'-triphosphate, deoxythymidine 5'-triphosphate, deoxycytidine 5'-triphosphate; and, optionally in the case of a Sanger-type DNA sequencing reaction, (iv) dideoxynucleotide triphosphates, e.g., dideoxyguanosine 5'-triphosphate, 7-deazadideoxyguanosine 5'-triphosphate, dideoxyadenosine 5'-triphosphate, dideoxythymidine 5'-triphosphate, dideoxycytidine 5'-triphosphate, and the like.

As used herein, the term "pyrophosphate" refers to two phosphate molecules bound together by an ester linkage, e.g., the structure



As used herein, one "unit" of T7 Sequenase V2 DNA polymerase is the amount of enzyme required to catalyze the incorporation of 10 nmol of total nucleotide into acid insoluble form in 30 min at 37 °C under the following standard assay conditions: 300 µl reaction mixture containing 88 mM potassium phosphate, pH 7.5, 6.7 mM MgCl2, 5 mM 2-mercaptoethanol,

0.15 mM each dCTP, dATP, dGTP, and [3H]dTTP, and 0.5 mM heat-denatured salmon sperm DNA.

II. METHOD OF THE INVENTION

Generally, the primer extension reaction of the present invention comprises the following steps: (i) providing a template nucleic acid; (ii) annealing an oligonucleotide primer to a portion of the template nucleic acid thereby forming a primer-template hybrid; (iii) adding primer-extension reagents to the primer-template hybrid for extending the primer; and (iv) adding a cosubstrate-enzyme pair to the primer-template hybrid for conducting a pyrophosphate-utilizing reaction.

Any source of nucleic acid can be used as a template nucleic acid provided it can be presented in a single stranded form and is capable of annealing with a primer oligonucleotide. Exemplary template nucleic acids include DNA, RNA, which DNA or RNA may be single stranded or double stranded. More particularly, template nucleic acid may be genomic DNA, messenger RNA, cDNA, DNA amplification products from a PCR reaction, and the like. Methods for preparation of template DNA may be found elsewhere (ABI PRISM™ Dye Primer Cycle Sequencing Core Kit).

Standard protocols for primer-template annealing and primer extension in the context of PCR or Sanger-type sequencing may be found elsewhere (Innis; Deiffenbach; ABI PRISM™ Dye Primer Protocol; ABI PRISM™ Dye Terminator Protocol). Generally, to perform a primer extension reaction in the context of PCR, template nucleic acid is mixed with a pair of PCR primers and primer-extension reagents comprising a buffer, $MgCl_2$, deoxynucleotide triphosphates, and a DNA polymerase. For example, a typical PCR reaction includes 20 pmol of each primer, 20 mM buffer at pH 8, 1.5 mM $MgCl_2$, 50 mM of each deoxynucleotide triphosphate (dNTP), and 2 units of Taq polymerase or other suitable thermostable polymerase. The reaction mixture is then thermocycled, a typical thermocycle profile comprising a denaturation step (e.g. 96 °C, 15 s), a primer annealing step (e.g., 55°C, 30 s), and a primer extension step (e.g., 72 °C, 90 s). Typically, the thermocycle is repeated from about 10 to 40 cycles.

To perform a primer extension reaction in the context of primer-labeled DNA sequencing, template nucleic acid is mixed with a labeled sequencing primer and primer-extension reagents comprising a buffer, $MgCl_2$, deoxynucleotide triphosphates, one or more

dideoxynucleotide triphosphates, and a DNA polymerase. For example, a typical sequencing reaction includes 1 µl of a template solution (e.g., 1 ml of a PCR reaction product diluted with 5 ml water) and 2 µl of labeled sequencing primer (e.g., 0.4 pmol/µl) are mixed with primer extension reagents comprising 2 µl buffer (e.g., 400 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), 2 µl of a deoxynucleotide / dideoxynucleotide triphosphate mixture (e.g., G-termination reaction, 1250 µM ddGTP, 250 µM dATP, 250 µM dCTP, 180 µM7-deaza-dGTP, and 250 µM dTTP), 2 µl of a labeled primer (e.g., 0.4 pmol/µl), and 2 µl of a DNA polymerase enzyme (e.g., 5 Units/µl where one unit is defined as in Lawyer). The reaction is then thermocycled using the following exemplary program: denaturation at 98 °C for 5 s followed by repeated cycles of 96 °C for 5 s; 55 °C for 40 s; 68 °C for 1 min, where the cycle is repeated 15 times.

The cosubstrate-enzyme pair of the invention may be any combination of one or more cosubstrates and an enzyme capable of performing a pyrophosphate-consuming phosphorylation reaction. Typically, such reactions will cause the phosphorylation of the cosubstrate and result in the consumption of one or both of the orthophosphates making up the pyrophosphate according to the equation

$$\text{Cosubstrate(s)} + \text{PPi} \xrightarrow{\text{Enzyme}} \text{P-Cosubstrate} + \text{nP}$$

where PPi is pyrophosphate, P-Cosubstrate is the phosphorylated cosubstrate, and nP is a monophosphate (n=1) or nothing (n=0). Preferably, the enzyme is a member of E.C. Class 2, 3, 4 or 6 (Enzyme Nomenclature). More preferably, the enzyme is thermostable, i.e., retains significant activity at temperatures between 55 °C and 95 °C.

In a variation of the cosubstrate reaction shown above, a second-step reaction is utilized to regenerate the unphosphoylated cosubstrate according to the equation

$$\text{Cosubstrate(s)} + \text{PPi} \xrightarrow{(\text{Enzyme})_1} [\text{P-Cosubstrate} + \text{P}] \xrightarrow{(\text{Enzyme})_2} \text{Cosubstrate(s)} + 2$$

In the multistep system, Enzyme 1 and Enzyme 2 can be the same or different enzymes. An advantage of this type of system is that the cosubstrate is not used up, therefore less cosubstrate need be added to the primer extension reaction. An exemplary multistep cosubstrate-enzyme

reaction is the reaction of pyrophosphate and glucose in the presence of the enzyme glucose-6-phosphatase according to the equation

$$PPi + Glucose \xrightarrow{\text{Glucose-6-phosphatase}} [Glucose\text{-}6\text{-}phosphate] + P \xrightarrow{\text{Glucose-6-phosphatase}} Glucose + P$$

Several exemplary cosubstrate-enzyme pairs are provided in Table 1 immediately below.

Table 1
Exemplary Cosubstrate-Enzyme Pairs

Cosubstrate(s)	Enzyme	Phosphorylated Cosubstrate (P-Cosubstrate)	Reference
Phospho-enolpyruvate + Adenosinemono-phosphate (AMP)	Pyruvate Phosphatedikinase	Adenosinetri-phosphate (ATP)	(Cagen)
L-Serine	Pyrophosphate Dependent L-Serine Phosphotransferase	O-phosphoserine	(Cagen)
Nicotinamide Adenine Dinucleotide (NAD)	NMN Adenylyltransferase	Nicotinamide Ribonucleotide	(Kornberg)
Oxaloacetate	Phosphopyruvate Carboxylase	Phospho-enolpyruvate	(Siu)
Pyrimidine - Ribonucleotide	Pyrimidine - Ribonucleotide Pyrophosphorylase	5-phospho-a-D-ribosyl-pyrophosphate	(Hatfield)
Nicotinate Ribonucleotide	Nicotinate Phosphoribosyl-transferase	5-phospho-a-D-ribosyl-pyrophosphate	(Nakamura)
L-tyrosyl-RNA + AMP	Tyrosyl-RNA Synthetase	ATP	
UDP Glucose	UDP Glucose Phosphorylase	UTP andGlucose-1-Phosphate	

Glycerol	Pyrophosphate-Glycerol Phosphotransferase	Glycerol-1-Phosphate	
Glucose	Glucose-6-Phosphatase	Glucose-6-Phosphate[a]	
Fructose-6-Phosphate	Pyrophosphate-Dependent Phosphofrucose-Kinase	Fructose-1,6-Diphosphate	

a. The glucose-6-phosphate is then further processed by the glucose-6-phosphatase to yield glucose.

Particularly preferred cosubstrate-enzyme pairs include pyrophosphate dependent L-serine phosphotransferase / L-serine, UDP glucose pyrophosphorylase / UDP glucose, and pyrophosphate dependent phosphofructose kinase / fructose-6-phosphate.

In the method of the invention, an enzyme-cosubstrate pair is added to a primer extension reaction. The concentration of the enzyme-cosubstrate pair is chosen to be sufficient to result in essentially no peak "dropout" in the primer extension product, i.e., each nucleotide of the template nucleic acid is represented by its complementary nucleotide in the primer extension product to an essentially equivalent degree.

III. THERMOSTABLE ENZYME

Preferably, the enzyme component of the enzyme-cosubstrate pair is a thermostable enzyme, i.e., an enzyme which maintains its integrity and activity at temperatures at or above about 45 °C, e.g., Taq polymerase. Thermostability is preferred so that the enzyme is active and remains intact at the elevated temperatures used in a PCR reaction or in a cycle sequencing reaction.

Methods for the screening for and isolation of thermostable enzymes are well known in the art of enzymology (Lawyer; Engelke; O'Brien). For example, the screening and isolation of a thermostable pyrophosphate dependent phosphofructose kinase may proceed as follows. To screen for enzyme activity, thermophilic bacteria are grown up in a culture under conditions suggested by the American Type Culture Collection (ATCC) manual for the particular organism to be screened, e.g., a 100 ml culture. Cultured cells are harvested by centrifugation, e.g., at 4 °C at 10,000xg for 10 min. The pelleted cells are washed with a buffered aqueous solution, e.g., 100 mM Tris-HCl pH 7.4, and

collected by centrifugation, e.g., at 4°C at 10,000xg for 10 min. The pelleted cells are resuspended in a buffered aqueous solution, e.g., 100 mM Tris-HCl pH 7.4, to a desired density, e.g., 1g/ml wet cell mass. An aliquot of the cell suspension, e.g., 0.2 ml, is sonicated, e.g., for 7 seconds, and the resulting cell debris is removed by centrifugation, e.g., at 4°C at 3,000xg for 15 min, leaving a cell free extract as the supernatant. An aliquot of the cell free extract, e.g., 1μl, is added to a volume of a PPiPFK assay solution, e.g., 49μl. An exemplary PPiPFK assay solution contains 115 nmol NADH, 230 nmol fructose-6-phosphate, 78 nmol imidazol, 32.2 nmol $MgCl_2$, 78 nmol BSA, 151 munit aldolase, 78 munit glycerol-3-phosphate dehydrogenase, 780 munit triose-phosphate isomerase, 100 nmol pyrophosphate, 5 munit glycerol-3-phosphate oxidase, 100 munit horseradish peroxidase, and 20 nmol o-dianisidine, adjusted to pH 7.4. The cell free extract from thermophilic bacteria that expresses PPiPFK will turn the assay solution to a burnt sienna color within approximately 1 hour.

Once a thermophilic species that produces PPiPFK has been identified, a genomic DNA library is generated according to the method described by Lawyer. The genomic library is then introduced into a suitable host, e.g., *E. coli* strain DF 1020. The transformed cells are then plated on minimal medium, e.g., with hexoses as a sole carbon source. The colonies of transformed host cells are then screened for PPiPFK activity according the screening procedure described above. The colony that expresses the PPiPFK activity is than grown up, e.g., in a 100 ml culture with YPD medium (10 g yeast extract, 20 g peptone, and 20 g dextrose dissolved in 1 liter) . After the cells have been grown up, they are disrupted and PPiPFK is isolated according to well known procedures, e.g., the procedure described by Engelke. The PPiPFK may then be further purified by cellulose phosphate chromatography, e.g., according to the method described by O'Brien.

IV. KITS AND SOLUTIONS OF THE INVENTION

In another aspect, the present invention includes kits and solutions for performing the primer extension methods of the invention. The kits and solutions of the invention include primer extension reagents and a cosubstrate-enzyme pair. Optionally, the kits may also include primers. The elements of the kits may be packaged in a single container or multiple containers. In one preferred configuration, a polymerase enzyme and a cosubstrate-enzyme pair are packaged in the same container.

V. EXAMPLES

The invention will be further clarified by a consideration of the following examples, which are intended to be purely exemplary of the invention and not to in any way limit its scope.

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EXAMPLE 1

Comparison of DNA Sequencing Performance With and Without Addition of Pyrophosphate Dependent Phosphofructokinase and Fructose-6-Phosphate

10 A. Reagents

Dye Primer ssDNA Sequencing Core Kit with Sequenase DNA Polymerase (PN# 402081), dye-labeled M13 (-21) primer (PN# 401131), and nucleoside phosphoramidites were obtained from PE Applied Biosystems Division of the Perkin-Elmer Corporation (PEABD). Single-stranded M13mp18 template DNA (PN# P2113) was purchased from 15 Penvera Corp. T7 DNA Polymerase Sequenase V2 (PN# US70775) was purchased from Amersham International, PLC. Strepavidin-coated silica beads (PN# C0010000RN) were purchased from Bangs Lab, Inc. Biotin-ON™ Phosphoramidite and Spacer™ Phosphoramidite (PN#s 5191 and 5260, respectively) were purchased from Clontech Laboratories, Inc. Pyrophosphate dependent phosphofructokinase (PPiPFK) and fructose-20 6-phosphate (F6P) (PN#s F4384 and F1520, respectively) were purchased from Sigma Chemical Co.

B. Primer Extension Reactions

The T7 Sequenase primer extension reactions were prepared essentially according 25 to the Sequenase Core Kit User's Manual (ABI PRISM™ Sequenase Protocol) except for enzyme dilutions. Generally, the primer extension reactions were prepared as follows.

A reaction buffer was prepared by combining equal volumes of a 3-[N-morpholino]propanesulfonic acid (MOPS) buffer (400mM MOPS, pH 7.5, 500mM NaCl, and 100mM $MgCl_2$) and a Mn^{2+} solution (50mM $MnCl_2$, and 100mM sodium isocitrate).

30 Four annealing reaction mixtures were prepared, one reaction corresponding to each of the four dideoxynucleotide triphosphate (ddNTP) terminators. Annealing reactions to be combined with ddATP or ddCTP terminators were prepared by mixing 1μl reaction buffer, 1μl template (0.25 μg/μl M13mp18) and 1μl fluorescently labeled primer (JOE-

labeled primer for A-terminated reactions and FAM-labeled primer for C-terminated reactions, each at 0.4 pM/µl). Annealing reactions to be combined with ddGTP or ddTTP terminators were prepared by mixing 3µl reaction buffer, 3µl template (0.25 µg/µl M13mp18) and 2µl primer (TAMRA-labeled primer for G and ROX-labeled primer for T, each at 0.4 pM/µl). The annealing reaction mixtures were heated to 65 °C for 5 min then incubated at room temperature for 10 min. To each annealing reaction mixture was added a nucleotide mixture including each of the four deoxynucleotide triphosphates and one of the four dideoxy nucleotide triphosphates (deoxynucleotides 1mM each, dideoxynucleotides 5 µM each).

One µl of the ddA or ddC nucleotide mixture was added to the A or C terminated reactions and 3µl of the ddG or ddT nucleotide mixture was added to the G or T terminated reactions. In addition, 2µl of distilled water was added to the G or T terminated reactions. All four reaction tubes were then incubated at 40°C for 5 min prior to addition of polymerase enzyme solutions.

For primer extension reactions not including a cosubstrate-enzyme pair, 1 µl of T7 Sequenase V2 DNA polymerase (26 unit/µl), 2 µl of reaction buffer, and 11 µl deionized (DI) water was added to each annealing reaction. For extension reactions including the PPiPFK / F6P cosubstrate-enzyme pair, 1 µl of T7 Sequenase V2 DNA polymerase (26 unit/µl), 2 µl of reaction buffer, and 11 µl of a 0.1 unit/µl PPiPFK solution was added to each annealing reaction. The PPiPFK solution was prepared by reconstituting 10 units of PPiPFK with 100µl of a 120 mM F6P solution, where a unit of PPiPFK is defined such 1 unit will convert 1 µmol fructose-6-phosphate to fructose-1,6-diphosphate in 1 minute at pH 7.4 at 30°C.

The primer extension reactions were incubated at 40 °C for 30 min then heated to 95°C for 5 min to inactivate the enzymes. Separate reactions including A, C, G, and T ddNTP terminators were pooled and the extension products were purified by hybridization or by ethanol precipitation as described below.

C. Extension Product Purification

Extension product purification was accomplished by one of two alternative methods: hybridization or ethanol precipitation.

C.1. Extension Product Purification by Hybridization

To separate the primer extension products from the enzymes, cosubstrate, and nucleotide triphosphate reactants using the hybridization protocol, the primer extension reaction mixture was contacted with hybridization beads functionalized with an oligonucleotide hybridization probe having a sequence complementary to the M13 (-21) sequencing primer (Fry).

Preparation of Hybridization Beads: The method used to prepare the hybridization beads was as follows. A 3'-biotinylated M13 (-21)-complementary oligonucleotide was synthesized using an ABI 394 DNA Synthesizer using standard phosphoramidite chemistry (PEABD; chemicals and protocols were used as recommended by the manufacture). The biotin was incorporated into the complementary oligonucleotide during synthesis by direct coupling of Biotin-ON™ Phosphoramidite (PN#5191-1) and Spacer™ Phosphoramidite (PN#5260-1) prior to the first nucleotide addition step following the supplier's recommended protocols (Clontech Laboratories, Inc.). The biotinylated oligonucleotide was then purified by reverse phase HPLC as follows:

Column:	PLRP-S reverse phase, 100 Å pore diameter, 8 mm particle diameter.	
Solvents:	Solvent A--acetonitrile	
	Solvent B--0.05 M triethylammonium acetate TEAA	
Gradient:	0 to 5 min	5%A: 95% B
	5 to 45 min	5%A: 95% B → 45%A: 55%B
	45 to 50 min	45%A: 55%B
	50 to 55 min	45%A: 55%B → 5%A: 95% B

The biotin-oligonucleotide (500 pmol) was coupled to 2 mg of strepavidin coated silica beads and incubated for 15 min at room temperature in TTL buffer (100mM Tris-HCl, pH 8.0, 0.1% Tween 20, 1M LiCl). The beads were centrifuged briefly, the supernatant was discarded, and the beads were resuspended in 200 μl TTL buffer.

Isolation of Extension Products: The isolation of the extension products was performed as follows. 50 µl of the hybridization bead suspension from above was pipetted into a 0.5 ml tube and the TTL buffer was removed by centrifugation followed by aspiration of the supernatant. The bead pellet was washed with binding buffer (2M NaCl, 10 mM Tris, 1 mM EDTA, pH 7.5) and the buffer was removed by centrifugation and aspiration. Next, the pelleted beads were resuspended in 40 µl of the primer extension reaction thereby forming a hybridization mix. The hybridization mix was heated to 80 °C for 2 min then incubated at 32 °C for 30 min. At the end of the incubation step, the tube was centrifuged briefly and the supernatant was discarded. The pelleted beads were then washed twice with TET buffer (10 mM Tris-HCl pH 8, 40 mM EDTA (TE) buffer in 0.1 % Tween 20) where the buffer was removed after each wash by centrifugation and aspiration. The pelleted beads were then rinsed with 70 % ethanol and dried under vacuum. The captured extension products were released from the hybridization beads by addition of 2.5 µl loading buffer (10 mM EDTA in 80% di-formamide) and heating to 60 °C for 4 min. The hybridization beads were removed by centrifugation and the supernatant containing the extension products was transferred to a fresh tube for subsequent electrophoresis and analysis.

C2. Extension product Purification by Ethanol Precipitation

The primer extension reactions were precipitated by addition of 95% ethanol to final ethanol concentration of 55%. The solution was centrifuged at 16,000 rpm in an Eppendorf centrifuge Model 5415C for 15 min at room temperature. The supernatant was discarded and the pellet was dried in a vacuum centrifuge for 2 min. The dried pellet was redissolved in 5 µl loading buffer and heated to 95 °C for 2 min before subsequent electrophoresis and analysis.

D. Electrophoresis and Analysis

The purified DNA sequencing extension products (2.5 µl) were electrophoretically separated and detected using an ABI PRISM™ 377 DNA Sequencer (PEABD) using a 36 cm well-to-read length and a 5% Long-Ranger gel (FMC PN# 50717) (chemicals and protocols were used as recommended by the manufactures). Data was analyzed with ABI PRISM™ Sequencing Analysis Software v 2.1.2 (PEABD).

The analyzed data are shown in FIGS. 1 and 2. FIG. 1 shows the DNA sequence of the M13 mp18 template sequenced using a M13 (-21) dye-labeled primer and T7 DNA Polymerase Sequenase V2. Pyrophosphorolysis during the primer extension reaction is evidenced by the loss of signal at base 60 and the 'N' base called at that position. FIG. 2 shows the same DNA sequence as FIG 1 where the sequencing reaction was performed using the PPiPFK and F6P protocol. Pyrophosphorolysis was prevented due to addition of the enzyme-cosubstrate pair, and consequently there was no loss of signal at base 60, a 'T' being clearly identified as expected from the known sequence.

EXAMPLE 2

Comparison of DNA Sequencing Performance With and Without Addition of UDP Glucose Pyrophosphorylase and UDP Glucose

A. Reagents

Standard sequencing reagents were provided as described in Example 1. UDP glucose pyrophosphorylase (UDPGPPase) and UDP glucose (UDPG) were purchased from Sigma Chemical Co. (PN#'s U4625 and U8501, respectively).

B. Primer Extension Reactions

Primer extension reactions were assembled as described above in Example 1. For primer extension reactions not including a cosubstrate-enzyme pair, 1 μl of T7 Sequenase V2 DNA polymerase (26 unit/µl), 2 μl of reaction buffer, and 11 μl deionized (DI) water was added to each annealing reaction. For extension reactions including the UDPGPPase / UDPG cosubstrate-enzyme pair, 1 μl of T7 Sequenase V2 DNA polymerase (26 unit/µl), 2 μl of reaction buffer, 1 μl of a 1.0 unit/μl UDPGPPase solution, and 10 µl DI water was added to each annealing reaction. The UDPGPPase solution was prepared by reconstituting 100 units of UDPGPPase with 100μl of a 40 mM UDPG solution, where a unit of UDPGPPase is defined such that 1 unit will convert 1 μmol UDP glucose to glucose-1-phosphate in 1 minute at pH 7.6 at 25 °C.

The primer extension reactions were incubated at 40 °C for 30 min then heated to 95°C for 5 min to inactivate the enzymes. Separate reactions including A, C, G, and T ddNTP terminators were pooled and the extension products were purified by ethanol precipitation as described below.

C. Extension Product Purification by Ethanol Precipitation

The sequencing extension products were purified by ethanol precipitation as described above in Example 1.

D. Electrophoresis and Analysis

The purified DNA sequencing extension products (2.5 μl) were electrophoretically separated and detected as described above in Example 1.

The analyzed data are shown in FIGS. 3 and 4. FIG. 3 shows the DNA sequence of the M13 mp18 template sequenced using a M13 (-21) dye-labeled primer and T7 DNA Polymerase Sequenase V2. Pyrophosphorolysis during the primer extension reaction is evidenced by the reduced signal at base 69. (Note that the base indicated at position 69 in this Example is the same base as base number 60 in Example 1, the difference in numbering being due to variability in base-calling software used to analyze the data.) FIG. 4 shows the same DNA sequence as FIG 3 where the sequencing reaction was performed using the UDPGPPase and UDPG protocol. Pyrophosphorolysis was prevented due to addition of the enzyme-cosubstrate pair, and consequently there was no reduction of signal at base 69, a 'T' being clearly identified as expected from the known sequence.

All publications and patent applications referred to herein are hereby incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated to be incorporated by reference.

Although only a few embodiments have been described in detail above, those having ordinary skill in the molecular biology art will clearly understand that many modifications are possible in the preferred embodiment without departing from the teachings thereof. All such modifications are intended to be encompassed within the following claims.

WE CLAIM:

1. A method for performing a primer extension reaction comprising the steps of:

annealing an oligonucleotide primer to a portion of a template nucleic acid thereby forming a primer-template hybrid;

adding primer-extension reagents to the primer-template hybrid for extending the primer; and

adding a cosubstrate-enzyme pair to the primer-template hybrid for conducting a pyrophosphate-utilizing reaction in an amount sufficient to reduce peak dropout.

2. The method of **claim 1** wherein the cosubstrate-enzyme pair comprises pyrophosphate dependent phosphofructose kinase and fructose-6-phosphate.

3. The method of **claim 1** wherein the cosubstrate-enzyme pair comprises UDP Glucose Pyrophosphorylase and UDP Glucose.

4. A kit for performing a primer extension reaction comprising:

primer extension reagents; and

a cosubstrate-enzyme pair present in an amount sufficient to reduce peak dropout.

5. The kit of **claim 4** wherein the cosubstrate-enzyme pair comprises pyrophosphate dependent phosphofructose kinase and fructose-6-phosphate.

6. The kit of **claim 4** wherein the cosubstrate-enzyme pair comprises UDP Glucose Pyrophosphorylase and UDP Glucose.

7. The kit of **claim 4** further including an oligonucleotide primer.

8. A primer extension solution for the extension of a primer member of a primer-template hybrid comprising:

primer extension reagents; and

a cosubstrate-enzyme pair present in an amount sufficient to reduce peak dropout.

9. The solution of **claim 8** wherein the cosubstrate-enzyme pair comprises pyrophosphate dependent phosphofructose kinase and fructose-6-phosphate.

10. The solution of **claim 8** wherein the cosubstrate-enzyme pair comprises UDP Glucose Pyrophosphorylase and UDP Glucose.

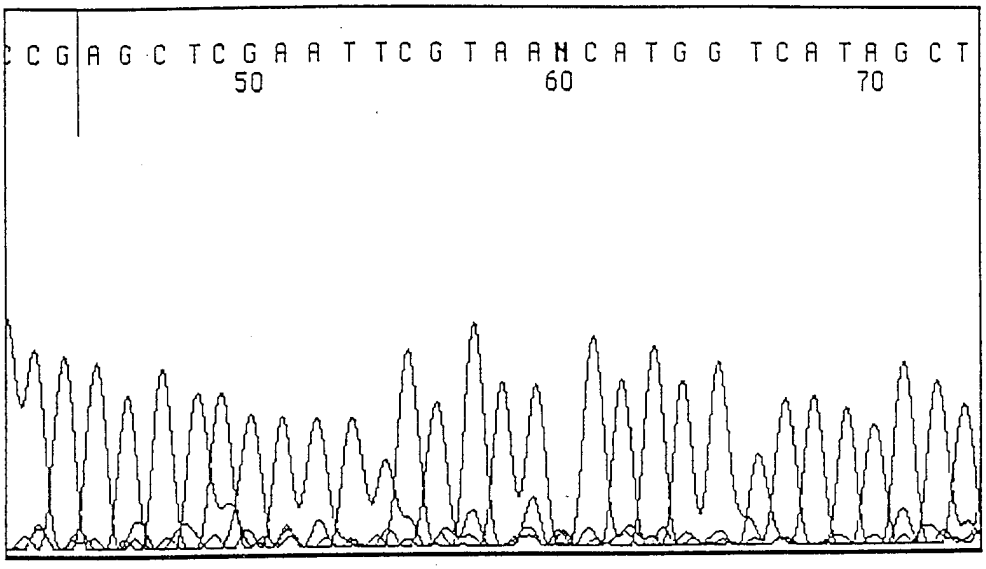

Fig. 1

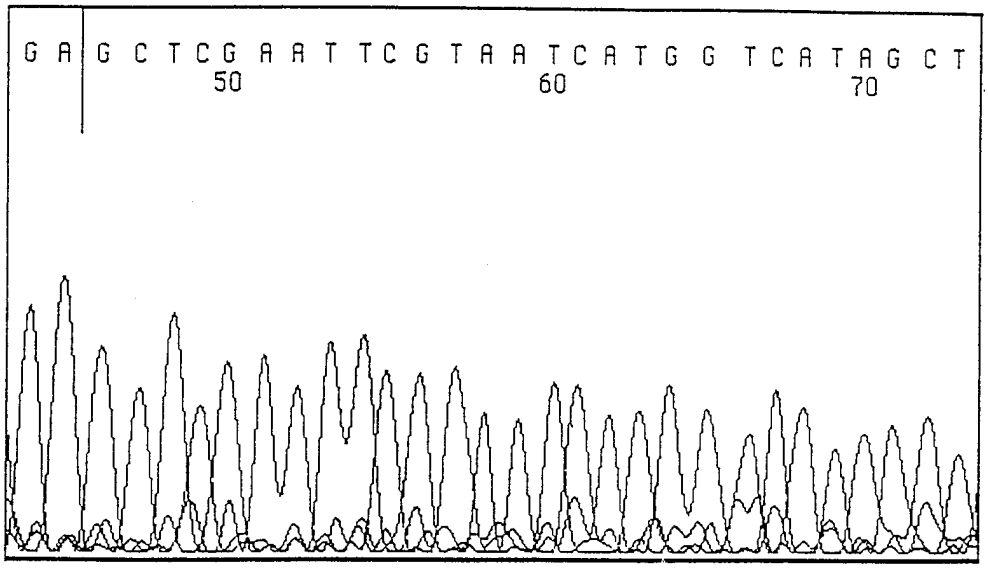

Fig. 2

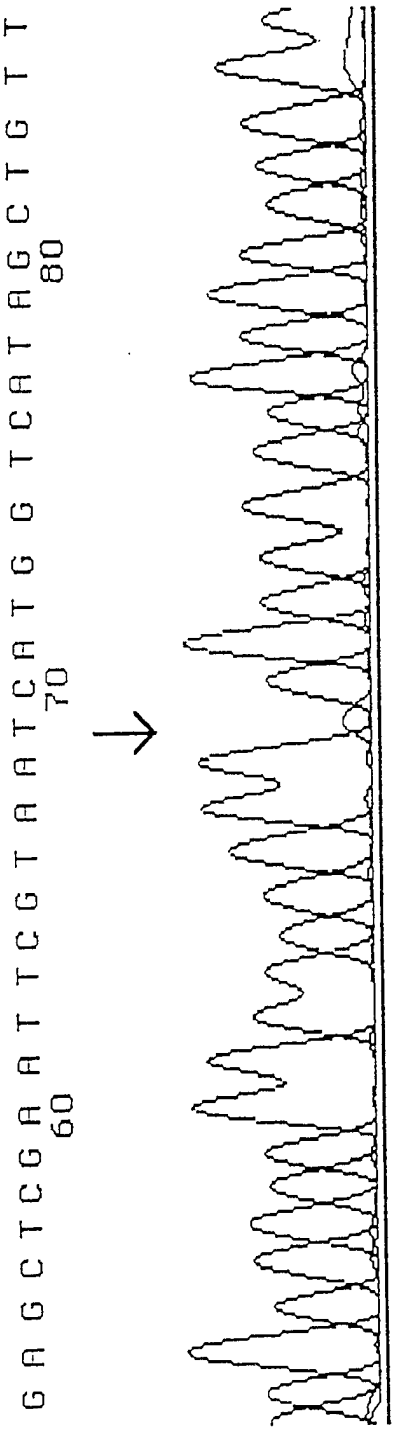

Fig. 3

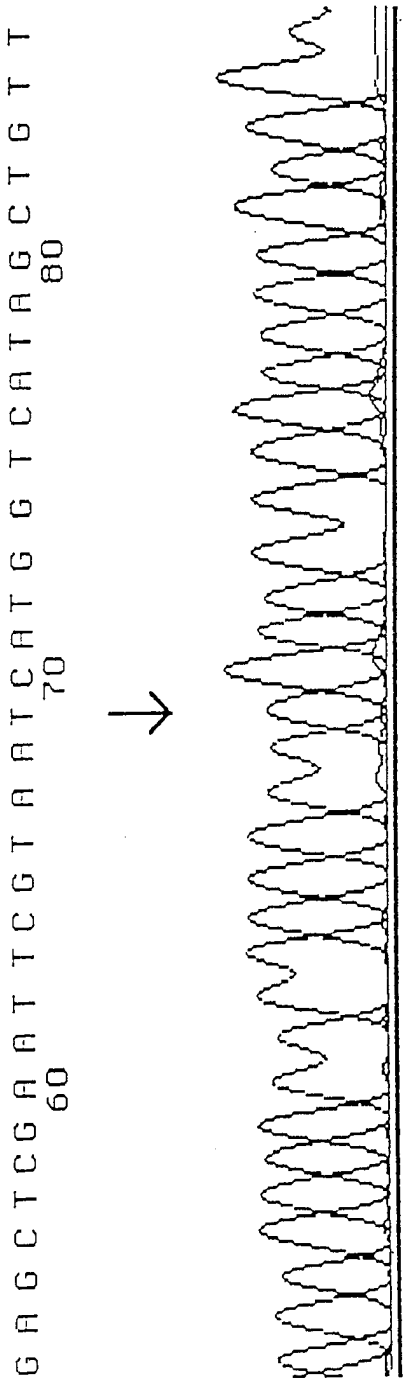

Fig. 4

INTERNATIONAL SEARCH REPORT

Ir. ational Application No
PCT/US 97/17301

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/68 C12Q1/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	WO 90 12111 A (HARVARD COLLEGE) 18 October 1990 cited in the application see the whole document	1
A	WO 94 05797 A (KISELEV VSEVOLOD ;SEVERIN EVGENII (RU); KORPELA TIMO (FI)) 17 March 1994 see claims 1-9	1
Y	WO 92 16654 A (UNIV REIMS CHAMPAGNE ARDENNE) 1 October 1992 see page 5, paragraph 3 - page 10, paragraph 5	1
Y	EP 0 351 138 A (HARVARD COLLEGE) 17 January 1990 see page 12, line 16 - line 44	1

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

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"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

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Date of the actual completion of the international search	Date of mailing of the international search report
26 January 1998	09/02/1998

Name and mailing address of the ISA	Authorized officer
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INTERNATIONAL SEARCH REPORT

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PCT/US 97/17301

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	WO 89 09283 A (HYMAN EDWARD DAVID) 5 October 1989 see claims 1-18 -----	1

2

INTERNATIONAL SEARCH REPORT

Information on patent family members

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