METHODS AND DEVICES FOR AMPLIFYING NUCLEIC ACIDS

Abstract: Described herein are methods and devices for amplifying nucleic acids. The methods generally involve exposing the nucleic acid to a mini-current electrical field while performing the steps of PCR. The methods provide numerous advantages over current PCR techniques such as reduced reaction times, no heating requirements, and reduced amounts of reagents (e.g., reduced amounts of polymerase). Additionally, the methods described herein amplify more DNA compared to conventional PCR techniques. In summary, the methods and devices described herein provide a more efficient and cost-effective way to perform PCR when compared to current PCR techniques.

Figure 1
METHODS AND DEVICES FOR AMPLIFYING NUCLEIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS

This application claims priority upon U.S. provisional application Serial No. 61/478,258, filed April 22, 2011. This application is hereby incorporated by reference in its entirety for all of its teachings.

BACKGROUND

Rapid and cost-effective detection and amplification of nucleic acid is vital for the identification of pathogenic and non-pathogenic agents of biomedical, industrial and research applications as well as other uses in molecular biology and biotechnology. Existing PCR technology, which is typically used in the amplification of DNA, is based on the polymerase chain reaction. Here, a test tube system for DNA replication allows a "target" DNA sequence to be selectively amplified or enriched several fold in several hours. During PCR, high temperature is used to separate the DNA molecules into single strands (one to several minutes at 94-96 °C), and synthetic sequences of single-stranded DNA (20-30 nucleotides) serve as primers. Two different primer sequences are used to bracket the target region to be amplified. The primer anneals to the DNA by way of hydrogen bonds. The annealing step occurs from one to several minutes at 50-65 °C. After primer hybridization, the DNA is subsequently heated from one to several minutes at 72 °C in the presence of a polymerase, during which time the polymerase binds and extends a complementary DNA strand from each primer.

Since it was first conceived, PCR techniques require temperature controls to treat DNA in each step of the process. Although optimization of the heating steps has been investigated, developing a PCR system that reduces time and heating temperatures is desirable in order to avoid DNA loss or damage as well potential increases of error. The methods and devices described herein address these needs.
SUMMARY

Described herein are methods and devices for amplifying nucleic acids. The methods generally involve exposing the nucleic acid to a mini-current electrical field while performing the steps of PCR. The methods provide numerous advantages over current PCR techniques such as reduced reaction times, no heating requirements, and reduced amounts of reagents (e.g., reduced amounts of polymerase). Additionally, the methods described herein require significantly shorter reaction times (e.g., 35 minutes) compared to conventional PCR techniques (minimum 2 hours). Finally, as shown in the Examples below, the methods described herein amplify more DNA compared to conventional PCR techniques. In summary, the methods and devices described herein provide a more efficient and cost-effective way to perform PCR when compared to current PCR techniques.

The advantages described below will be realized and attained by means of the elements and combinations particularly pointed out in the appended claims. It is to be understood that both the foregoing general description and the following detailed description are exemplary and explanatory only and are not restrictive.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings, which are incorporated in and constitute a part of this specification, illustrate several aspects described below.

Figure 1 shows an example of an electrolysis cell for performing the methods described herein.

Figure 2 shows electrophoresis gel bands of PCR samples from pYES vector after being run by electrolytic micro current PCR system (EMPS) described herein compared to conventional temperature-based PCR (CTP).

Figure 3 shows electrophoresis gel bands from DNA amplifications of the pBSK coiled plasmids at different concentrations by EMPS and CTP.

Figure 4 shows electrophoresis gel bands of linearized plasmids amplified by EMPS as compared to CTP.
Figure 5 shows electrophoresis gel bands of genomic DNA amplified by EMPS as compared to CTP.

Figure 6 shows electrophoresis gel bands of cDNA amplification of RNA by EMPS as compared to CTP.

Figure 7 shows *E.coli* colonies grown in SOB plus ampicillin media after transformed with plasmids amplified by EMPS holding different metal sequences.

Figure 8 shows electrophoresis gel bands of digested plasmids that have been previously amplified by EMPS.

Figure 9 shows electrophoresis gel bands of calcium and sodium inserted in their respective vectors after run by both EMPS and CTP sequentially.

Figure 10 shows an example of a device for performing real time PCR using the methods described herein.

**DETAILED DESCRIPTION**

Before the present compounds, compositions, articles, devices, and/or methods are disclosed and described, it is to be understood that the aspects described below are not limited to specific compounds, synthetic methods, or uses as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular aspects only and is not intended to be limiting.

In this specification and in the claims that follow, reference will be made to a number of terms that shall be defined to have the following meanings:

It must be noted that, as used in the specification and the appended claims, the singular forms "a," "an" and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "a bioactive agent" includes mixtures of two or more such agents, and the like.

Ranges may be expressed herein as from "about" one particular value, and/or to "about" another particular value. When such a range is expressed, another aspect includes from the one particular value and/or to the other particular value. Similarly, when values are expressed as
approximations, by use of the antecedent "about," it will be understood that the particular value forms another aspect. It will be further understood that the endpoints of each of the ranges are significant both in relation to the other endpoint, and independently of the other endpoint.

References in the specification and concluding claims to parts by weight, of a particular element or component in a composition or article, denotes the weight relationship between the element or component and any other elements or components in the composition or article for which a part by weight is expressed. Thus, in a compound containing 2 parts by weight of component X and 5 parts by weight component Y, X and Y are present at a weight ratio of 2:5, and are present in such ratio regardless of whether additional components are contained in the compound.

A weight percent of a component, unless specifically stated to the contrary, is based on the total weight of the formulation or composition in which the component is included.

Described herein are methods and devices for amplifying nucleic acids. The methods generally involve exposing the nucleic acid to a mini-current electrical field while performing the steps of PCR. In one aspect, the method involves:

a. contacting the nucleic acid with a primer and exposing the nucleic acid to a mini-current electrical field for a sufficient time and amount to anneal the primer to the nucleic acid to produce an annealed nucleic acid; and

b. contacting the annealed nucleic acid with a polymerase and exposing the annealed nucleic acid to a mini-current electrical field for a sufficient time and amount to amplify the nucleic acid.

Each step is discussed in detail below.

The nucleic acid can be any molecule where it is possible and desirable to amplify (i.e., generating multiple copies of a specific nucleic acid sequence). In one aspect, the nucleic acid can be an oligonucleotide, deoxyribonucleic acid (DNA), ribonucleic acid (RNA), or peptide nucleic acid (PNA). The nucleic acid of interest introduced by the present method can be a nucleic acid from any source, such as a nucleic acid obtained from cells in which it occurs in nature, recombinantly produced nucleic acid, or chemically synthesized nucleic acid. For example, the nucleic acid can be cDNA or genomic DNA or DNA synthesized to have the nucleotide sequence corresponding to that of naturally-occurring DNA. The nucleic acid can also be a mutated or
altered form of nucleic acid (e.g., DNA that differs from a naturally occurring DNA by an
alteration, deletion, substitution or addition of at least one nucleic acid residue) or nucleic acid that
does not occur in nature. In one aspect, the DNA can be genomic DNA. In other aspects, the
DNA can be double-stranded DNA including, but not limited to, a plasmid (linear or coiled, etc.),
cosmid, phage, viral, YACS, BACS, other artificial chromosomes, and the like). Alternatively, the
DNA can be single stranded.

In one aspect, the nucleic acid can be a functional nucleic acid. Functional nucleic acids
are nucleic acid molecules that have a specific function, such as binding a target molecule or
catalyzing a specific reaction. Functional nucleic acid molecules can be divided into the following
categories, which are not meant to be limiting. For example, functional nucleic acids include
antisense molecules, aptamers, ribozymes, triplex forming molecules, siRNA, miRNA, shRNA
and external guide sequences. The functional nucleic acid molecules can act as affectors,
inhibitors, modulators, and stimulators of a specific activity possessed by a target molecule, or the
functional nucleic acid molecules can possess a de novo activity independent of any other
molecules.

Functional nucleic acids can be a small gene fragment that encodes dominant-acting
synthetic genetic elements (SGEs), e.g., molecules that interfere with the function of genes from
which they are derived (antagonists) or that are dominant constitutively active fragments (agonists)
of such genes. SGEs can include, but are not limited to, polypeptides, inhibitory antisense RNA
molecules, ribozymes, nucleic acid decoys, and small peptides. The small gene fragments and SGE
libraries disclosed in U.S. Patent Publication No. 2003/0228601, which is incorporated by
reference, can be used herein.

In general, the methods described herein involve the application of a mini-current electrical
field to a sample containing the nucleic acid and primer in the absence of heat in order to amplify a
nucleic acid sequence of interest. Thus, the reaction is performed less than or equal to 30 °C,
preferably less than or equal to 25 °C. The amount and duration of the electrical field applied to
the nucleic acid can vary. For example, the amount of electrical field applied in steps (a) and (b)
above can be the same or different value. Similarly, the duration of exposure to the electrical field
can be the same or different in steps (a) and (b). Different voltages can be used including different
sets of micro currents. In one aspect, the current is 50 mV/25 mA, 100 mV/50 mA, 150 mV/80 mA, 200 mV/100 mA, 250 mV/100 mA, 300 mV/150 mA, 500 mV/250 mA, 800 mV/400 mA, 1000 mV/500 mA, 1500 mV/800 mA, or 2000 mV/1000 mA. In another aspect, the amount of the electrical field in steps (a) and (b) is from 50 mV to 2,000 mV and from 25 mA to 1,000 mA. In another aspect, the amount of the electrical field in steps (a) and (b) is from 800 mV to 1,000 mV and from 400 mA to 500 mA. In a further aspect, the duration of exposure in steps (a) and (b) can range from 5 minutes to 60 minutes, 5 minutes to 50 minutes, 5 minutes to 40 minutes, 5 minutes to 30 minutes, or 5 minutes to 20 minutes. Devices for generating the mini-current electrical field and exposing the sample of nucleic acid to the electrical field are described in detail below.

The sample of nucleic acid can be prepared using techniques known in the art for preparing samples used in conventional PCR techniques. For example, the nucleic acid can be dissolved in water and buffer, where the buffered solution contains a divalent cation such as Ca\(^{2+}\) or Mg\(^{2+}\). One significant difference regarding sample preparation is that in the methods described herein, the primer can be added directly to the sample containing the nucleic acid. This is not the case with conventional PCR. For example, if the nucleic acid is double-stranded DNA, the first step in conventional PCR involves the denaturing of the DNA by heat in the absence of the primer. It is only after the DNA is denatured that the primer is added to the sample followed by a second heating cycle. Thus, the methods described herein are more efficient compared to conventional PCR, where an additional step is required prior to annealing the primer to the DNA. Thus, in one aspect, the method involves:

a. exposing the DNA to a mini-current electrical field in the presence of a primer for a sufficient time and amount to denature the double-stranded DNA and anneal the primer to the single-stranded DNA to produce annealed single-stranded DNA; and

b. contacting the annealed single-stranded DNA with a DNA polymerase and exposing the annealed single-stranded DNA to a mini-current electrical field for a sufficient time and amount to amplify the annealed single-stranded DNA.

The primers useful herein can be an oligonucleotide, whether occurring naturally as in a purified restriction digest or produced synthetically, which is capable of acting as a point of initiation of synthesis when placed under conditions in which synthesis of a primer extension
product which is complementary to a nucleic acid strand is induced. The primer is preferably single stranded for maximum efficiency in amplification, but may alternatively be double stranded. If double stranded, the primer is first treated to separate its strands before being used to prepare extension products. The primer should be sufficiently long to prime the synthesis of extension products in the presence of the polymerase. For example, depending on the complexity of the target sequence, the oligonucleotide primer typically contains 15-25 or more nucleotides, although it may contain more or fewer nucleotides.

The primers herein are selected to be "substantially" complementary to the different strands of each specific sequence to be amplified. This means that the primers should be sufficiently complementary to hybridize with their respective strands. Therefore, the primer sequence need not reflect the exact sequence of the template. For example, a non-complementary nucleotide fragment may be attached to the 5’ end of the primer, with the remainder of the primer sequence being complementary to the strand. Alternatively, non-complementary bases or longer sequences can be interspersed into the primer, provided that the primer sequence has sufficient complementarity with the sequence of the strand to be amplified to hybridize therewith and thereby form a template for synthesis of the extension product of the other primer. However, for detection purposes, particularly using labeled sequence-specific probes, the primers typically have exact complementarity to obtain the best results.

After the primer has been annealed to the nucleic acid of interest, a polymerase is added to the sample containing the annealed nucleic acid, and the sample is exposed to the mini-current electrical field for a sufficient time and duration in order to extend or elongate the sequence of interest. Polymerases are enzymes that assist in the polymerization of new DNA or RNA against an existing DNA or RNA template in the PCR process. When the nucleic acid is DNA, a DNA polymerase of the types I-V can be used herein. Alternatively, when RNA is the nucleic acid, RNA polymerases such as type I-III and T7 RNA polymerase can be used. Another advantage of the methods described herein is that lower amounts of polymerase are required compared to conventional PCR techniques. For example, the methods described can use 0.3 μL or 0.5 μL of polymerase. Thus, in one aspect, the amount of polymerase used herein is up to 50% or 70% less than the amount needed in conventional PCR.
techniques.

The methods described herein can be used in any application where conventional PCR is used. For example, the methods described herein permit the isolation of DNA fragments from genomic DNA by selective amplification of a specific region of DNA. Thus, hybridization probes for Southern or northern hybridization and DNA cloning, which require larger amounts of DNA, can be produced by the methods described herein.

The methods described herein can be used to analyze extremely small amounts of sample. This is often critical for forensic analysis, when only a trace amount of DNA is available as evidence. Any type of organism can be identified by examination of DNA sequences unique to that species. Applications of the methods described herein with respect to forensics include, but are not limited to, identifying potential suspects whose DNA may match evidence left at crime scenes, exonerating persons wrongly accused of crimes, identifying crime and catastrophe victims, establish paternity and other family relationships, identifying endangered and protected species as an aid to wildlife officials (could be used for prosecuting poachers), detecting bacteria and other organisms that may pollute air, water, soil, and food, match organ donors with recipients in transplant programs, determine pedigree for seed or livestock breeds, authenticate consumables such as caviar and wine, and analyze ancient DNA that is tens of thousands of years old.

PCR permits early diagnosis of malignant diseases such as leukemia and lymphomas, which is currently the highest-developed in cancer research and is already being used routinely. PCR assays can be performed directly on genomic DNA samples to detect translocation-specific malignant cells at a very high sensitivity.

The methods described herein are useful in the discovery and design of new pharmaceuticals. The fact that the methods can amplify higher amounts of nucleic acid compared to conventional PCR techniques, it is possible to produce greater amounts of potential pharmaceuticals that can be evaluated as potential drug candidates. Additionally, the fact that the methods require no heating step and can be performed in much shorter times compared to conventional PCR, it is possible to generate more potential drug candidates in a cost-effective manner. Moreover, the methods described herein can be used as an analytical tool to evaluate the content and purity of nucleic acids when deigning and synthesizing new drugs. In summary, the methods described herein can substitute conventional PCR in any application related to drug
design and analytical evaluation thereof.

In certain aspects, the methods described herein can be used to amplify and simultaneously quantify a target nucleic acid in real time (i.e., quantitative real time polymerase chain reaction or qPCR). The detection of target DNA can be performed by the use of (1) non-specific fluorescent dyes that intercalate with any double-stranded DNA, and (2) sequence-specific DNA probes consisting of oligonucleotides that are labeled with a fluorescent reporter which permits detection only after hybridization of the probe with its complementary DNA target.

In general, the methods described herein are performed in an electrolysis cell (20 in Figure 1). The electrical field is produced inside the electrolysis cell, where the sample of nucleic acid is placed in a PGR tube 22 (Axygen, Inc.). The volume of the sample tube can vary from 0.2 mL, 0.6 mL, 1.0 mL, 1.5 mL and 2.0 mL. The thickness of the tube wall can also vary from 0.5 mm to 1.5 mm, preferably 1.15 mm to 1.20 mm, more preferably, 1.17 mm. The thickness of the sample tube can be varied in order to maximize electrical current distribution.

The electrolysis cell can have a number of different dimensions. In one aspect, the cell is 20 x 20 x 10 cm, 15 x 15 x 10 cm, 10 x 10 x 10 cm, 5 x 5 x 5 cm, 10 x 10 x 10 cm; 20 x 15 x 10 cm, 15 x 13 x 12 cm, or 10 x 5 x 5 cm. Additionally, the cells can be different shapes including, but not limited to, round, rectangular, and square. The housing of the cell can be composed of different materials including, but not limited to, plastic, rubber, pure glass, mixed glass or Plexiglas, acrylic and polycarbonate. The cell can also be used with an optional lid 23 so that the cell is a closed system. In certain aspects, it is desirable to use a lid in order to avoid sample evaporation or contamination. The cell can be designed to hold as many sample tubes as needed.

In order to establish an electrical field in the cell, the cell is divided into two compartments (see Figure 1), one representing a cationic field 24 and the other representing an anionic field 25. In one aspect, a synthetic membrane 26 was used to divide the electrolysis cell in two compartments, thus allowing the flow of current through the electrolysis cell. Examples of polymers useful herein as the membrane include, but are not limited to, IONAC MA 3475 and IONAC MA 3470 manufactured by SYBRON CHEMICALS, INC. The electrolysis cell is connected to a power supply 27 (e.g., EC-105 Electrophoresis Power Supply by Thermo EC. (Inv #8298)) in the external end and to the membrane in the electrolysis cell. Next, the two
compartments are filled with electrolyte solution for current conduction. Different types of electrolytes can be used herein, including sulphuric acid, hydrochloric acid, iron solution, potassium chloride, and Tris-Running buffer TAE. Additionally, different concentrations of the electrolyte can be used (e.g., 1X, 2X, 5X, 10X and 20X).

Each compartment of the cell has a different electrolyte concentration. The working compartment, where the samples of nucleic acid are placed, has the higher concentration of electrolyte (cationic compartment in Figure 1), while the reference compartment (anionic compartment in Figure 1) had the lower concentration of electrolyte. Various concentration ratios of the buffer between the cells can be used. In one aspect, the ratio of buffer concentration in the working compartment relative to the reference compartment is 1:1 2:1 3:1 1.5:1 2.5:1 and 4:1.

After electrolytes are loaded in the electrolysis cell, the electrolysis cell can be closed with a lid, hermetically, thus preventing outside penetration of air. The lid covering the electrolysis cell has two openings where the working electrode (e.g., cationic 28) and the reference electrode 29 (e.g., Ag/AgCl reference electrode, double junction, SGJ, Metrohm plug-in head B) were passed through and connected to the power supply in order to establish the electrical field. Samples containing the nucleic acid are next placed inside the electrolysis cell and submerged into the buffer inside electrolysis cell until sample volume inside the vial reached electrolyte solution inside the cell box. The vials 22 can be held by a wooden or plastic support 21 attached to one of the box walls in order to keep them upright. Alternatively, the vials can be submerged completely in the buffer because they are completely sealed. Finally, voltage and amperage are applied in order to expose the samples to an electrical field. The electrical field generated in the cell can be measured by the use of voltmeter 30, which is attached to the mesh electrode 31 via wires 32. The mesh electrode 31 is attached to the synthetic membrane 26 in Figure 1.

Figure 10 provides another exemplary device for performing the methods described herein, particularly real time PCR. Referring to Figure 10, many of the components described in the cell of Figure 1 are present in the cell of Figure 10. The sample support 1 has a dark bottomless area for qPCR and RealTime analysis. The electrodes 2 (working and reference) generate and maintain the electrical field in solution. A programmable electrical and digital system 3 applies voltage and mini currents to the system and is connected to a power supply. An adaptable optical system 4 reads sample fluorescence for qPCR and RealTime analysis. Components 1-4 are integrated parts
of the system attached to the lid 11 that fits and matches the bottom holder-area 12 supporting the samples vials and seals the cell. The electrolysis cell compartment is divided into two compartments (10 in Figure 10 is one compartment) by a middle divider 5 composed of a silver mesh 13 and an ionic membrane 9. Silver wires 7 are connected to the power supply and divider in order to produce a mini current through the electrical cell system. A multi-meter 8 measures the voltage and current applied.

In one aspect, the optical device useful for qPCR and RealTime analysis includes a light source of emission, a specific filter within 450 and 520 nm of wave length, and a detection system designed to couple to the system to read fluorescence produced by probes such as, for example, SYBRgreen or taqman probes present in the sample vials prior to exposing the sample to the electrical field. The system can be adapted to read each sample independently during the during amplification process over time. The bottom of the cell can be configured with dark walls in order to avoid interference of media light and from side samples. Measurements can be taken during the amplification process, and the detector transducer can change the signal into a fluorescence value that is correlated with a standard curve to determine number of copies produced during the amplification process.

The methods described herein provide numerous advantages over current PCR techniques such as the use of lower amounts of reagents (e.g., reduced amounts of polymerase). Additionally, the methods described herein require significantly shorter reaction times (e.g., 35 minutes) compared to conventional PCR techniques (minimum 2 hours). The fact that the methods require no heat and shorter reaction times means there is less opportunity for degradation and formation of side-products. Indeed, as shown in the Examples below, the methods described herein amplify more DNA compared to conventional PCR techniques. As discussed above, conventional PCR requires controlled heating steps at every stage, which adds to the overall cost and inefficiency of the amplification process. Finally, the methods described herein produce nucleic acids that are more pure than conventional PCR. This feature is particularly important in applications where the nucleic acid is used in a comparative study (e.g., forensics where DNA at a crime site is compared to a suspect’s DNA). In summary, the methods and devices described herein provide a more efficient and cost-effective way to perform PCR when compared to current PCR techniques.
EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how the compounds, compositions, and methods described and claimed herein are made and evaluated, and are intended to be purely exemplary and are not intended to limit the scope of what the inventors regard as their invention. Efforts have been made to ensure accuracy with respect to numbers (e.g., amounts, temperature, etc.) but some errors and deviations should be accounted for. Unless indicated otherwise, parts are parts by weight, temperature is in °C or is at ambient temperature, and pressure is at or near atmospheric.

There are numerous variations and combinations of reaction conditions, e.g., component concentrations, desired solvents, solvent mixtures, temperatures, pressures and other reaction ranges and conditions that can be used to optimize the product purity and yield obtained from the described process. Only reasonable and routine experimentation will be required to optimize such process conditions.

MATERIALS AND METHODS

Sample Treatment

All plasmid sequences used during analysis were provided and synthesized by Clonetex Systems Inc. Austin, TX 78756, USA. Genomic DNA was isolated in laboratory with QIAGEN standard purification kits. Polyadenylated 1.2kb RNA transcript used as a control template for the cDNA synthesis reaction was purchased from Promega (Reverse Transcription System A3500 Promega). PCR thin wall tubes were used during all experimentation and inside the system to ensure that potential energy have an effect on the sample. Different types of DNA were tested to demonstrate the methods described herein can perform the same if not better than standard PCR. All DNA concentrations were measured with a standard nano-view spectrophotometer GE nanospectrophotometer (GE Healthcare Biosciences, P.O. Box 643065 Pittsburgh, PA 15264-3065), and also with regular standard UV/visual spectrophotometer within a 260/280 wave length (GE Healthcare Biosciences P.O. Box 643065 Pittsburgh, PA 15264-3065).
Plasmid DNA

Coiled plasmid. A pYES plasmid vector holding a sequence of YDL194W gene of 2660 bp (Clontex) was amplified with designed primers to amplify the YDL194W gene sequence and backbone vector. The sample had an initial concentration of 0.1 µg/µl (Figure 2).

A pYES plasmid holding Pichia stipitis CBS 6054 High-affinity glucose transporter (SNF3) gene sequence was amplified with random and designed primers. The DNA had an initial concentration of 0.1 µg/µl (Figure 2).

Figure 2 shows electrophoresis gel bands of PCR samples from pYES vector after being run by electrolytic micro current PCR system (EMPS) described herein compared to conventional temperature-based PCR (CTP). The bands from the EMPS are more pronounced (lane 1 and lane 5) while CTP are less pronounced (lane 2 and 4). All wells were sampled with the same exact sample volume. The bands in the gel indicate the following: Lane 1. Sample one (YDL194W gene) amplified by EMPS. Lane 2. Sample one amplification trough standard method CTP. Lane 3. Molecular marker (1 Kb). Lane 4. Sample 2 (Pichia stipitis CBS 6054 (SNF3) gene) amplified trough standard CTP method. Lane 5. Sample 2 amplification trough new method EMPS.

Three different coiled pBSK plasmids holding calcium biding protein gene sequence (842 bp), sodium biding protein gene sequence (1934 bp) and vanadium biding protein gene sequence (433 bp), respectively, were amplified with pUC/ml3 forward and reverse primers from Promega. (pUC/ml3 sequencing primers Q5401 Promega). Plasmid stocks were diluted until plasmid DNA concentrations of 0.005, 0.01, 0.015 and 0.02 µg/µl were obtained.

Figure 3 shows electrophoresis gel bands from DNA amplifications of the pBSK coiled plasmids at different concentrations, after run by EMPS, compared to CTP. The samples run by EMPS show higher amplification than the run by CTP even at lower concentration of DNA. All wells were sampled with the same exact sample volume. The bands in the gel indicate the following: Lane 1. Molecular marker (1 Kb). Lane 2. Sample one (calcium plasmid with 0.005 µg/µl starting concentration) amplified by new method EMPS. Lane 3. Sample one (calcium plasmid with 0.005 µg/µl starting concentration) amplified by standard CTP. Lane 4. Sample two (calcium plasmid with 0.01 µg/µl starting concentration) amplified by EMPS. Lane 5. Sample two,
(calcium plasmid with 0.01 µg/µl starting concentration) amplification by CTP. Lane 6. Sample three (sodium plasmid with 0.015 µg/µl starting concentration) amplified by new method EMPS. Lane 7. Sample three, (sodium plasmid with 0.015 µg/µl starting concentration) amplified by CTP. Lane 8. Sample four, (Vanadium plasmid with 0.02 µg/µl starting concentration) amplified by EMPS. Lane 9. Sample four (Vanadium plasmid with 0.02 µg/µl starting concentration) amplified by CTP.

**Linear plasmid:** A pBSK plasmid containing sodium biding protein gene sequence (1934 bp) was linearized with a standard digestion protocol with Psil enzyme purchased from NEB, (New England Biolabs inc. R0657L) for 2.5 hours at 37 °C with alkaline phosphatase treatment. The final linearized plasmid concentration was 0.025 µg/µl. Sodium biding protein gene sequence was amplified with pUC/ml3 forward and reverse primers from Promega, which amplified the sequences between the vector cloning site. The target of approximate 2,000 bp was amplified.

Figure 4 shows electrophoresis gel bands of linearized plasmids amplified by EMPS as compared to CTP. The linearized amplification shows that the EMPS maintains intact the nature of the DNA. No denaturing of DNA occurred. All wells were sampled with the same exact sample volume. The bands in the gel indicate the following: Lane 1. Molecular marker (1 Kb) Lane 2 and Lane 4. Linearized plasmid holding sodium biding protein gene sequence amplified by EMPS. Lane 3 and Lane 5. Linearized plasmid holding sodium biding protein gene sequence amplified by CTP.

**Genomic DNA**

**Genomic DNA of a Geobacillus spp.** 18 hours pure culture was extracted and purified with QIAGEN DNeasy Blood & Tissue Total DNA purification kit. Extracted genomic DNA resulted in 0.072 µg/µl of DNA concentration. Sample Amplification with random hexamers, from Promega CI 181 was carried out.

Figure 5 shows electrophoresis gel bands of genomic DNA amplified by EMPS as compared to CTP. The bands from the EMPS are more pronounced than the CTP. All wells were filled with the same exact sample volume. The bands in the gel indicate the following: Lane 1. Molecular marker (1 Kb). Lanes 2, 3, 6 and 7. Genomic Geobacillus spp. amplified DNA by
EMPS. Lanes 4, 5, 8 and 9. Genomic *Geobacillus* spp. amplified DNA by CTP.

**RNA for RT and cDNA**

Polyadenylated RNA 0.1 µg/µl (Promega cat# A3500) was initially reverse transcribed with QIAGEN Omniscript Reverse Transcription kit cat# 205113 for one hour at 37 °C with reverse transcription reaction buffer. cDNA was amplified with oligodT primers from the same kit. A 1.2 KB fragment was amplified as expected.

Figure 6 shows electrophoresis gel bands of cDNA amplification of RNA by EMPS as compared to CTP. Intensified bands were observed by EMPS. All wells were filled with the same exact sample volume. The cDNA amplification shows that EMPS does not affect the nature of the DNA, thus no denaturation of DNA occurred. The bands in the gel indicate the following: Lane 1. Molecular marker (1 Kb). Lane 2. cDNA amplified by CTP. An unexpected band of approximate 2,000 bp was observed. Line 3. cDNA amplified by EMPS. An expected band of approximate 1,200 bp produced.

**Amplification Process**

The amplification process was performed using the device as shown in Figure 1 and the standard PCR thermocycler (Maxigene gradient thermo-1000 from Axygene). Enzymes and reagents used for master mix and reaction were from Promega. Magnesium Chloride Solution cat # A3511, dNTPs mix cat # C114B, Magnesium free buffer cat# M190G, Nuclease free water part# P19E and taq polymerase cat #M166B and DNA polymerase 1 cat # M205A, except for RT and cDNA, where the Quiagen Omniscript reverse transcription kit and TopTaqPCR master mix kit reagents were used for amplification.

**Sample Preparation for Conventional Temperature-Based PCR (CTP)**

- Nuclease free water 35 µl
- dNTPs mix 2 µl
- Mg free Buffer 5 µl
- MgCl 25 mM 1.5 µl
DNA 5 µl
Forward primer 0.5 µl
Reverse primer 0.5 µl
Taq polymerase 0.5 µl
TOTAL VOLUME 50 µl

Temperature Profile for CTP PCR Samples
1. 94 °C x 5 minutes - 1 cycle
2. 94 °C x 0.5 minutes
   56 °C x 0.5 minutes - 30 cycles
3. 72 °C x 1 minute
4. 4 °C

Sample Preparation for Electrolytic Micro-current PCR system (EMPS)
Nuclease free water 35 µl
Mg free Buffer 5 µl
MgCl 25 mM 1.5 µl
DNA 5 µl

Current and Time Schedule for EMPS Samples

Several times with respect to exposing the samples to the electrical field until ideal results were obtained. Time sets for the 3 step process included 20/10/20 minutes, 5/5/10 minutes, 15/10/20 minutes and 10/5/20 minutes, were tested, with 10/5/20 the preferred time schedule.

Denaturation. Samples were placed in the holder in the device of Figure 1, ensuring the tubes are in contact with the buffer within the system. The sample was exposed to an electrical filed for 10 minutes at 900 mV of voltage and 450 mA.

Annealing. After 10 minute at above conditions, the DNA strands separated from each other and
forward and reverse specific or universal primers, depending on the DNA sample, were added. Samples were exposed to an electrical field for 900 mV and 450 mA for 5 minutes.

**Elongation or extension.** After primer annealing, DNA polymerase and dNTPs mix were added to the samples to complete the amplification process. The volume of DNA polymerase and dNTPs mix was less than or equal to the volume used in CTP (0.5 μl or 0.3 μl of polymerase and 2 or 1.5 μl of dNTPs). Samples were exposed to an electric field of 900 mV and 450 mA for 20 minutes.

**Results visualization of both procedures.** Amplification results were visualized in a 1.2% agarose gel run in IX TAE buffer with standard Thermo EC (EC-150) electrophoresis equipment and power supply for 40 minutes at 70 V. Gels were revealed in a UVP 3UV™ Benchtop Transilluminator.

**DNA Concentration**

After the amplification process, either by standard PCR (CTP) or the methods described herein (EMPS), DNA from the samples were quantified using standard nano view spectrophotometer GE nanospectrophotometer. Tables 1-4 demonstrate that EMPS is able to amplify DNA as effectively if not better than standard PCR (CTP), which resulted in higher DNA concentrations.

**Table 1.** DNA concentrations of two different samples, (samples 1 (YDL194W gene) and 2 (Pichia stipitis CBS 6054 (SNF3) gene) amplified by EMPS as compared to CTP.

| SAMPLE 1- EMPS | 973.4 ± 12.1 |
| SAMPLE 1- CTP | 477 ± 11.2 |
| SAMPLE 2- EMPS | 1193.5 ± 10.4 |
| SAMPLE 2- CTP | 623.4 ± 1 |

**Table 2.** DNA concentrations of samples 1 (calcium plasmid with 0.005 μg/μl starting concentration), sample 2 (calcium plasmid with 0.01 μg/μl starting concentration), sample 3 (sodium plasmid with 0.015 μg/μl starting concentration) and sample 4 (Vanadium plasmid with
0.02 µg/µl starting concentration) after amplification by EMPS, as compared to CTP. Even at lower concentrations of DNA the amplifications were higher with EMPS than the CTP.

**DNA concentration**

<table>
<thead>
<tr>
<th>Sample</th>
<th>EMPS</th>
<th>CTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sample 1-EMPS</td>
<td>2.563 µg/µl ± 0.008</td>
<td>1.01 µg/µl ± 0.02</td>
</tr>
<tr>
<td>Sample 2-EMPS</td>
<td>2.046 µg/µl ± 0.036</td>
<td>0.896 µg/µl ± 0.04</td>
</tr>
<tr>
<td>Sample 3-EMPS</td>
<td>3.071 µg/µl ± 0.03</td>
<td>0.894 µg/µl ± 0.04</td>
</tr>
<tr>
<td>Sample 4-EMPS</td>
<td>1.256 µg/µl ± 0.009</td>
<td>1.009 µg/µl ± 0.02</td>
</tr>
</tbody>
</table>

**Table 3.** DNA concentration of Sodium Na protein gene from linearized plasmid and *Geobacillus* spp. genomic DNA after amplification by EMPS as compared to CTP.

**DNA concentration**

| Na gene from Linearized plasmid - BY EMPS | 2.114 µg/µl ± 0.012 |
| Na gene from Linearized plasmid - BY CTP  | 0.875 µg/µl ± 0.02  |
| Genomic DNA -BY EMPS                      | 1.461 µg/µl ± 0.03  |
Genomic DNA - BY CTP 0.585 µg/µl ± 0.011

**Table 4.** DNA concentrations of cDNA produced after reverse transcription and amplification by EMPS as compared to CTP.

<table>
<thead>
<tr>
<th>DNA concentration</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>cDNA - BY EMPS</td>
<td>3.146 µg/µl ± 0.021</td>
</tr>
<tr>
<td>cDNA - BY CTP</td>
<td>0.854 µg/µl ± 0.092</td>
</tr>
</tbody>
</table>

5 **Transformation**

Invitrogen *Escherichia coli* Top10 Chemically competent cells were transformed with coiled pBSK vanadium, sodium and calcium amplified plasmids produced by EMPS. *E.coli* was transformed according to manufactures indications. Cells were selected in SOB (Luria Brethani plus salts) with ampicillin media. No degeneration of DNA was evident due to the presence of transformed colonies, which was even higher than positive control.

Figure 7 shows *E.coli* colonies grown in SOB plus ampicillin media after transformed with plasmids amplified by EMPS holding different metal sequences. Positive control was taken as same *E. coli* competent cells transformed with CTP amplified product. Higher number *E. coli* colonies transformed with plasmid amplified by EMPS as compare to the lower number of colonies transformed with plasmid amplified by CTP (positive control).

**Digestion**

Linearized and amplified plasmids were used for digestion to demonstrate the DNA did not suffer any damage during amplification by EMPS. A standard digestion protocol with Spel and EcoRI enzymes, for sodium gene containing plasmid and with BamHI and Clal enzymes for calcium gene containing plasmid bought from NEB, (New England Biolabs Inc. R0657L) for 2.5 hours at 37 °C with alkaline phosphatase treatment was carried out. Digestion was successful and bands were present in the gel as expected.

Figure 8 shows electrophoresis gel bands of digested plasmids that have been previously
amplified by EMPS. The plasmid is still intact and ready to be used after previously being amplified by EMPS. The bands in the gel indicate the following: Lane 1. Molecular marker (1 Kb). Lane 2. Sodium Na amplified plasmid by EMPS digested with Spel and EcoRI enzymes, sequences was extracted from plasmid. Lane 3. Sodium Na complete and undigested amplified plasmid by EMPS. Lane 4. Calcium Ca amplified plasmid by EMPS digested with BamHI and Clal enzymes. Lane 5. Calcium Ca amplified plasmid by EMPS digested with BamHI and Apal enzymes. Lane 6. Calcium Ca complete and undigested amplified by EMPS.

Further standard PCR amplification

The amplified samples obtained by EMPS were compared to conventional standard PCR (CTP) to ensure DNA sample integrity. Promega GOTaq Green master mix reagents were used. The same temperature cycles used to amplify initial samples were used to amplify these last samples already amplified by EMPS. The presence of same size DNA from this amplification process as well as for EMPS indicates DNA is being correctly amplified and is not being degraded.

Figure 9 shows electrophoresis gel bands of calcium and sodium inserted in their respective vectors after run by both EMPS and CTP sequentially. The DNA remains intact, as proven by CTP, which were run after EMPS. The bands in the gel indicate the following: Lane 1. Molecular marker (1 Kb). Lane 2. No sample served, not used. Lane 3. Calcium plasmid amplified by EMPS Lane 4. Calcium plasmid, CTP amplification from EMPS product. Lane 5. Sodium plasmid amplified by EMPS. Lane 6. Sodium plasmid, CTP amplification from EMPS product. The results are also consistent with the previous results in which the samples run by the EMPS are more pronounced when compared to CTP.

SUMMARY

The methods described herein are able to amplify DNA up to 4.5 fold more than conventional PCR methods. These results were obtained within very short time (35 minutes), after several trials in which exposure to specific current during different experiments showed the exact time DNA samples needed to denature, anneal and elongate. Different currents were used in each step of the amplification process ranging from 250 mV and 125 ma, to 500 mV and 250 mA, 800 mV and 400 mA, 900 mV and 450 mA, 1000 mV 500 mA and 1250 mV and 650 mA showing consistent results. The best amplification results were obtained with the current of 900 mV and
450 mA. The present invention showed effective amplification results for different kinds of samples, including linear and coiled plasmids with a starting DNA concentration of 5 ng/µl, genomic DNA amplification with random primers, and with cDNA amplification processes. Targets of different sizes were tested, all showing consistent amplification results. Successful transformation cells with amplified DNA produced by the methods described herein were achieved as well as effective digestion and further standard PCR amplification of the amplified product, which showed there was no DNA damage during the new amplification process.

Throughout this application, various publications are referenced. The disclosures of these publications in their entireties are hereby incorporated by reference into this application in order to more fully describe the compounds, compositions and methods described herein.

Various modifications and variations can be made to the compounds, compositions and methods described herein. Other aspects of the compounds, compositions and methods described herein will be apparent from consideration of the specification and practice of the compounds, compositions and methods disclosed herein. It is intended that the specification and examples be considered as exemplary.
What is claimed:

1. A method for amplifying a nucleic acid, the method comprising:
   a. contacting the nucleic acid with a primer and exposing the nucleic acid to a mini-current electrical field for a sufficient time and amount to anneal the primer to the nucleic acid to produce an annealed nucleic acid; and
   b. contacting the annealed nucleic acid with a polymerase and exposing the annealed nucleic acid to a mini-current electrical field for a sufficient time and amount to amplify the nucleic acid.

2. The method of claim 1, wherein the nucleic acid comprises, a natural or synthetic oligonucleotide, a natural or modified/blocked nucleotide/nucleoside, a DNA or fragment thereof, or an RNA or fragment thereof.

3. The method of claim 1, wherein the nucleic acid comprises cDNA, a plasmid DNA, or genomic DNA.

4. The method of claim 1, wherein nucleic acid comprises double-stranded DNA, the method comprising:
   a. exposing the DNA to a mini-current electrical field in the presence of a primer for a sufficient time and amount to denature the double-stranded DNA and anneal the primer to the single-stranded DNA to produce annealed single-stranded DNA; and
   b. contacting the annealed single-stranded DNA with a DNA polymerase and exposing the annealed single-stranded DNA to a mini-current electrical field for a sufficient time and amount to amplify the annealed single-stranded DNA.

5. The method of claim 1, wherein the nucleic acid in step (a) comprises an aqueous solution and a divalent cation.

6. The method of claim 4, wherein the divalent cation comprises Ca$^{+2}$ or Mg$^{+2}$. 
7. The method of claim 1, wherein the polymerase comprises Type 1, Type 2, Type 3, Type 4, Type 5, or any combination thereof.

8. The method of claim 1, wherein the amount of the electrical field in steps (a) and (b) is the same.

9. The method of claim 1, wherein the amount of the electrical field in steps (a) and (b) is different.

10. The method of claim 1, wherein the amount of the electrical field in steps (a) and (b) is from 50 mV to 2,000 mV and from 25 mA to 1,000 mA.

11. The method of claim 1, wherein the amount of the electrical field in steps (a) and (b) is from 800 mV to 1,000 mV and from 400 mA to 500 mA.

12. The method of claim 3, wherein the double-stranded DNA in step (a) is exposed to an electrical field in the amount of 800 mV to 1,000 mV and from 400 mA to 500 mA for 5 minutes to 30 minutes.

13. The method of claim 3, wherein the DNA in step (b) is exposed to an electrical field in the amount of 800 mV to 1,000 mV and from 400 mA to 500 mA for 10 minutes to 30 minutes.

14. The method of claim 1, wherein the entire method is performed less than or equal to 30 °C.

15. The method of claim 1, wherein the entire method is performed less than or equal to 25 °C.

16. The method of claim 1, wherein the method is performed in real time.

17. A method for detecting the presence or absence of at least one nucleic acid, the method comprising:

   a. contacting the nucleic acid with a primer and exposing the nucleic acid to a mini-current electrical field for a sufficient time and amount to anneal the primer to the nucleic acid to produce an annealed nucleic acid; and
b. contacting the annealed nucleic acid with a polymerase and exposing the annealed nucleic acid to a mini-current electrical field for a sufficient time and amount to amplify the nucleic acid;

c. adding to the product in step (b) a labeled oligonucleotide probe for each sequence being detected capable of hybridizing to the specific nucleic acid or mutation thereof; and

d. determining if hybridization had occurred.

18. A device for amplifying a nucleic acid, the device comprising:

a. a container comprising a first compartment and a second compartment for holding an electrolye solution, wherein the first and second compartments are separated by a semi-permeable membrane that permits the flow of electrolye to and from the first and second compartments;

b. a mesh electrode attached to the semi-permeable membrane;

c. a reference electrode and a working electrode, wherein both the reference electrode and the working electrode are positioned in the second compartment;

d. a support for holding one or more sample vials containing the nucleic acid, wherein the support is affixed to the second compartment; and

e. a power supply connected to the reference electrode and a working electrode.

19. The device of claim 18, wherein the device further comprises a lid.

20. The device of claim 18, wherein the device further comprises a device for measuring the voltage and amperage of the electrical field.
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12P 19/34; C12Q 1/68; G01N 33/48 (2012.01)
USPC - 435/91.2, 435/6.1 1, 435/6.12

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

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C12P 19/34; C12Q 1/68; G01N 33/48 (2012.01)
USPC: 435/91.2, 435/6.1 1, 435/6.12

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 practicable, search terms used)
PubWEST, Google Scholar: Mini-current, electrical field, primer, polymerase, anneal, nucleic acid, DNA, amplification, semi-permeable, membrane, container, compartment, measure, voltage, lid, power, vial, reference, working, solution, RNA

C. DOCUMENTS CONSIDERED TO BE RELEVANT

<table>
<thead>
<tr>
<th>Category</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
<th>Relevant to claim No.</th>
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</thead>
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<tr>
<td>00</td>
<td>WO 2010/1 14842 A 1 (ECKER et al.) 07 October 2010 (07.10.2010); abstract; page 2, lns 3-9; page 3, lns 7-14; page 7, lns 14-30; page 19, lns 32-35; page 37, lns 9-12</td>
<td>1-4, 8-9, 16-17</td>
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<tr>
<td>00</td>
<td>US 5,646,019 A (NIELSON et al.) 08 July 1997 (08.07.1997); col 7, lns 12-14; col 11, lns 55-59; col 16, lns 10-19</td>
<td>5-7</td>
</tr>
<tr>
<td>00</td>
<td>US 6,011,349 A (MEADE) 11 January 2000 (11.01.2000); abstract; col 4, lns 25-30; col 21, lns 55-57; col 22, lns 6-24</td>
<td>10-13</td>
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<td>00</td>
<td>US 6,197,563 B1 (ERLICH et al.) 06 March 2001 (06.03.2001); col 21, lns 43-45</td>
<td>14-15</td>
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<tr>
<td>00</td>
<td>US 7,087,148 B1 (BLACKBURG et al.) 08 August 2006 (08.08.2006); col 12, lns 32-35; col 65, lns 37-52; col 84, lns 61-85, lns 6</td>
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<tr>
<td>00</td>
<td>US 5,645,801 A (BOUMA et al.) 08 July 1997 (08.07.1997); col 35, lns 55-30; col 56</td>
<td>18-20</td>
</tr>
</tbody>
</table>

Further documents are listed in the continuation of Box C. ❌

* Special categories of cited documents:
  * "A" document defining the general state of the art which is not considered to be of particular relevance
  * "E" earlier application or patent but published on or after the international filing date
  * "L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
  * "O" document referring to an oral disclosure, use, exhibition or other means
  * "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search
09 June 2012 (09.06.2012)

Date of mailing of the international search report
0 2 J U L 2012

Name and mailing address of the ISA/US
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Authorized officer: Lee W. Young
PCT Helpdesk: 571-272-4300
PCT OSP: 571-272-7778

Form PCT/ISA/210 (second sheet) (July 2009)