Abstract: Provided are methods and kits for extracting DNA from samples which contain humic acids, and which do so in a straightforward and cost-effective manner. In some aspects, two inhibitor removal steps (by precipitation of humic acids and size exclusion chromatography) and one DNA precipitation step are used to prepare purified DNA in high yields. The samples which contain humic acids include, for example, soil samples.

Figure 4

- Combine soil sample and extraction buffer
- Disrupt cells in sample
- Precipitate humic acids
- Precipitate DNA
- Purify DNA

Title: SYSTEM AND METHOD FOR DNA EXTRACTION FROM SAMPLES CONTAINING HUMIC ACIDS

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(72) Inventor: ANDERSON, Michael, Paul: 133 S. Monticello Drive, Stillwater, OK 74074 (US).


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SYSTEM AND METHOD FOR DNA EXTRACTION FROM SAMPLES CONTAINING HUMIC ACIDS

CROSS REFERENCE TO RELATED APPLICATIONS
This application claims the benefit of U.S. Provisional Patent Application serial number 61/974,634 filed on April 3, 2014, and incorporates said provisional application by reference into this document as if fully set out at this point.

TECHNICAL FIELD
This disclosure relates to the general subject of DNA extraction and, more particularly, to systems and methods for extracting DNA from soils.

BACKGROUND
DNA extraction is one of the most important steps in characterizing the microbiological, metagenomic and biochemical properties of soils. Soil is an exceedingly complex medium containing an enormous variation in chemical, environmental and microbiological properties. Many of these properties impact the ability to extract soil DNA.

Humic acid content is especially critical given that some of the humic acid tends to co-purify with DNA and interfere with downstream processes such as PCR amplification. DNA purification techniques also vary with respect to extraction yield. Yield is important given that DNA extraction from soils is often incomplete. Incomplete yield means some DNA in inaccessible sites may be bypassed and therefore not accounted for in subsequent analysis.

Fragmentation size is also important for some DNA extraction methodologies, especially for metagenomic gene analysis. Larger fragments are better because they may contain many more genes for metagenomic gene analysis on a given piece of DNA which would present much less difficulty in aligning and assembling large genome segments.

DNA extraction methodologies have not been thoroughly cross evaluated for effectiveness despite the fact that DNA extraction is known to be one of the most important variables in any soil DNA analysis. There are many commercial kits on the market for extracting soil DNA. These kits use a series of steps beginning with the
extraction of DNA from the organisms and the soil matrix, followed by several subsequent purification steps that may include a precipitation and a purification step by adhesion to silica with subsequent elution. Commercial kit effectiveness is highly variable due to the differences in fundamental methods and likely the order in which the method steps are employed.

Powerful DNA sequencing technologies provide scientists the opportunity to develop information concerning the biotic portion of the soil ecosystem which supports basic growth processes and agricultural productivity. DNA extraction technology is currently a limiting factor in this analysis. A procedure that results in acceptable purity and high yield would have a significant advantage over current methods and would be widely used.

As is well known in the DNA extraction industry, there has been a need for systems and methods that provide more effective ways to obtain this information from soil. Accordingly, it should now be recognized, as it was recognized by the present inventors, that there exists, and has existed for some time, a very real need for an invention that would address and solve the above-described problems.

Before proceeding to a description of the present invention, however, it should be noted and remembered that the description of the invention which follows, together with the accompanying drawings, should not be construed as limiting the invention to the examples or embodiments shown and described. This is so because those skilled in the art to which the invention pertains will be able to devise other forms of this invention within the ambit of the appended claims.

SUMMARY OF THE INVENTION

The invention provides novel methods and kits for extracting and purifying DNA from soil or other sources that are otherwise difficult to analyze for microbial content. The methods and kits are relatively straightforward to implement and involve the use of reagents that are readily obtained and inexpensive, and the present invention uses them in a novel and advantageous combination. The result is a low-cost and readily implemented method that results in DNA yields that are significantly higher than currently known techniques. For example, one approach described herein extracts DNA from soils using a combination of extraction and purification procedures at approximately one-fifth the cost of current commercial kits. Yet, as shown in the
Examples section below, the yield of DNA was significantly higher than yields obtained using current commercial kits, and comparable DNA purification levels were maintained. For example, 2.6-fold more highly purified DNA was extracted from the same soils compared the best and most popular commercial kits currently on the market.

A unique aspect is the reduced complexity of the purification protocol, which generally utilizes only three major steps: ammonium acetate precipitation to remove humic acids (and other contaminants) from a solution that contains the DNA of interest; alcohol precipitation to concentrate and further purify the DNA; and calibrated gel filtration chromatography to effect further purification of the DNA. The steps are carried out using commonly available chemicals and laboratory equipment, without specialized equipment or techniques. The methods are described in detail below.

There is provided herein a method of extracting DNA from cells in a sample that contains or is likely to contain humic acids, comprising i) combining said sample and an extraction medium; ii) disrupting said cells to release DNA from said cells into said extraction medium; iii) precipitating humic acids from said extraction medium by adding ammonium acetate in a quantity sufficient to precipitate humic acids but insufficient to precipitate said DNA, thereby retaining said DNA in solution in said extraction medium; iv) separating extraction medium comprising said DNA from precipitated humic acids; v) precipitating said DNA from said extraction medium by adding organic alcohol and salt in a quantity sufficient to precipitate said DNA; vi) resuspending DNA precipitated in step v) in a resuspension medium; and vii) purifying resuspended DNA.

There is also provided herein a method of conducting a metagenomic analysis, comprising obtaining a sample comprising cells from at least one organism or microorganism, wherein said sample contains or is likely to contain humic acids; extracting DNA from said cells in said sample by i) combining said sample and an extraction medium; ii) mechanically disrupting said cells to release DNA from said cells into said extraction medium; iii) precipitating humic acids from said extraction medium by adding ammonium acetate to said extraction medium in a quantity sufficient to precipitate humic acids but insufficient to precipitate said DNA from said extraction medium, thereby retaining said DNA in solution in said extraction medium; iv) separating extraction medium comprising said DNA from precipitated humic acids; v)
precipitating said DNA from said extraction medium by adding organic alcohol and salt to said extraction medium in a quantity sufficient to precipitate said DNA; vi) resuspending DNA precipitated in step v) in a low-salt resuspension medium; and vii) purifying resuspended DNA by size exclusion gel chromatography; analyzing a plurality of nucleic acid sequences of the purified DNA; and based on results obtained in said step of analyzing, establishing the taxonomic identity of said at least one organism or microorganism.

The foregoing has outlined in broad terms some of the more important features of the invention disclosed herein so that the detailed description that follows may be more clearly understood, and so that the contribution of the instant inventors to the art may be better appreciated. The instant invention is not to be limited in its application to the details of the construction and to the arrangements of the components set forth in the following description or illustrated in the drawings. Rather, the invention is capable of other embodiments and of being practiced and carried out in various other ways not specifically enumerated herein. Finally, it should be understood that the phraseology and terminology employed herein are for the purpose of description and should not be regarded as limiting, unless the specification specifically so limits the invention.

**BRIEF DESCRIPTION OF THE DRAWINGS**

These and further aspects of the invention are described in detail in the following examples and accompanying drawings.

Figure 1 is a table that shows DNA yield for different measurement types;

Figure 2 contains an image of soil DNA extracted from Perkins soils loaded onto a 0.9% agarose gel that had been post stained with ethidium bromide and illuminated with ultraviolet radiation;

Figure 3 contains estimates of maximum and average size of the fragments generated by the DNA soil extraction procedures.

Figure 4 is a flow chart illustrating steps of the method.

**DETAILED DESCRIPTION**

While this invention is susceptible of embodiment in many different forms, there is shown in the drawings, and will herein be described hereinafter in detail, some specific embodiments of the instant invention. It should be understood, however, that the present
disclosure is to be considered an exemplification of the principles of the invention and is not intended to limit the invention to the specific embodiments or algorithms so described.

Humic acid is a principal component of humic substances, which are the major organic constituents of soil (humus), peat, coal, many upland streams, dystrophic lakes, and ocean water. It is produced by biodegradation of dead organic matter. Humic acid is a complex mixture of many different acids containing carboxyl and phenolate groups so that the mixture behaves functionally as a dibasic acid or, occasionally, as a tribasic acid. Humic acids can form complexes with ions that are commonly found in the environment creating humic colloids. Humic and fulvic acids (fulvic acids are humic acids of lower molecular weight and higher oxygen content than other humic acids) are commonly used as a soil supplement in agriculture, and less commonly as a human nutritional supplement. While beneficial in these circumstances, humic acids are detrimental when they occur in samples from which it is desired to isolate DNA, as they interfere with yield and purity. The present invention provides methods and kits for obtaining DNA from samples comprising humic acids (including fulvic acids), e.g. from soil or other samples which, for example, comprise microorganisms whose DNA is of interest, and which are otherwise difficult to analyze for DNA content. Similar to prior art methods, when the DNA of interest is within cells of a microorganism, the sample is subjected to mechanical disruption of the cells by using non-reactive particles such as beads. However, in contrast to some prior art methods, the present method tailors the selection of beads to the microorganism to increase effectiveness. While prior art methods may employ one or more precipitation steps, the present technology uniquely combines two precipitation steps, one of which precipitates unwanted contaminants (such as humic acids) and the other of which precipitates the DNA itself. This latter step not only concentrates the DNA quantitatively, but also serves to increase purification since additional contaminants are left in solution. In addition, a step of size exclusion chromatography is used to effect DNA purification, rather than conventional silica binding technology, thereby increasing purity and yield. Size exclusion has the advantage of keeping the DNA in the liquid phase without solid phase interactions such as binding to silica membranes which may theoretically bias DNA recovery. The details of the method steps are described below.
The methods of the invention may be used to extract DNA from a wide range of samples. While not a necessary prerequisite, in one aspect, the samples contain or are likely to contain humic acids, and are known or suspected of containing one or more DNAs of interest. Exemplary samples which contain humic acids include but are not limited to: a variety of soil types, including without limitation humus, sandy soil, silty soil, clay soil, peaty soil, saline soil, and mixtures of these such as loamy soil (which contains a balance of silt, sand and clay), clay-loam, silt-loam, sediment, volcanic ash, soils high in organic matter, etc. The invention is particularly useful for analyzing DNA located in soils with relatively high humic acid content. Further, the sample may be, without limitation, a rock sample (e.g. pulverized or otherwise treated) such as a fossilized rock which is known or suspected to contain DNA of interest; or a sample from a crime scene which is known or suspected to contain DNA of interest; or a material such as clothing which may be contaminated with soil containing humic acids; permafrost samples; samples from bogs, swamps and tar pits; samples from hot springs; samples from underwater ecological niches; submerged soil samples (e.g. from under bodies of water); saline soils, soils from within rhizosphere soils (soils near the root surface) etc. In addition, manures and other fecal matter may be analyzed. Also, contaminated soils may be analyzed, e.g. soils that have been contaminated with hydrocarbon waste, radioactive waste, chemical waste, etc. Aqueous samples that contain humic acids may also be analyzed, e.g. waste water, dystrophic (humic) lake water, ocean water, ice or snow encased samples, etc. Depending on the origin of the sample, additional steps may be required prior to carrying out the steps of the method, e.g. soaking of fabric, crushing or pulverizing of stone, dehydration or drying, etc.

In some aspects, the samples are soil samples and for ease of description, the discussion below generally refers to the samples as "soils". However, it should be understood that the method may be carried out on samples that are not soils, as described above.

The samples may be collected by known techniques and stored, e.g. aerated soil collection bags or other suitable containers. Any amount of sample may be collected but a minimum of e.g. at least about 1 gram of soil or 1 ml of liquid is collected per sample. Samples may optionally be dried, dessicated or dehydrated (e.g. overnight or longer) as needed to permit ease of handling. However, moist or liquid samples may also be
assessed. Typically, soil samples are pulverized, e.g. by grinding (for example, using a high speed grinder or a mortar and pestle), to mix and homogenize the sample, and to break up aggregates. Treated soils may be assayed immediately, or may be stored, e.g. in the cold in a refrigerator or freezer, in suitable containers (such as plastic 50 ml tubes).

In some aspects, the DNA of interest is located within one or more microorganisms in the sample. However, this is not necessarily the case; the invention may be practiced to isolate DNA that is not contained within a cell. For example, the DNA may be viral, or eukaryotic DNA, or DNA that has already been released from a cell and is resident in the soil solution or bound to soil particles. In this case, a step of breaking open cells may not be required. However, if the DNA of interest is present within cells, they may be either eukaryotic or prokaryotic cells and may be multi- or monocellular, such as, without limitation: bacterial cells, plant cells, animal cells (e.g. from mammals, reptiles, insects, birds, fish, crustaceans, etc. whether living or extinct), spores, yeasts, algae, moss, arthropod, etc.

The conditions of carrying out the cell-breaking step are selected according to what is suitable for the cell type. For example, mammalian or plant cells may be disrupted by physical disruption such as grinding in a mortar and pestle assisted by a freezing step, blended using a high speed blender, by sonication using a high frequency sound wave disruption or by chemical disruption using solvents such as chloroform or phenol, etc.

In the case of bacteria-containing samples, the methods involve selecting appropriate reagents and conditions for breaking open (lysing) the bacterial cells e.g. by a combination of mechanical and chemical lysis, and releasing the DNA e.g. into an aqueous buffer. Generally, soil is initially mixed with an aqueous extraction buffer. Exemplary extraction buffer (EBs) comprise, for example at least one buffering agent at a concentration of from about 50 to 200 mM, e.g. about 50 mM TRIS (base or HCl); about 10 to about 100 mM of at least one chelating agent, e.g. about 25 mM EDTA, or EGTA; about 100 to about 1000 mM (or about 250 to about 750 mM) of at least one salt, e.g. about 500 mM NaCl, or other suitable salt; and at least one detergent at a final concentration of from about 0.5 to about 10%, or from about 1 to about 7.5%, or from about 1 to about 4%. Those of ordinary skill in the art will recognize that a variety of cationic, anionic or zwiterionic detergents may be utilized, examples of which include
but are not limited to: SDS, CTAB (cetyltrimethylammonium bromide), sodium laureth
sulfate, sodium myreth sulfate, alkylbenzene sulfonate, sodium stearate, sodium lauryl
sarcosinate, CHAPS, and sultaines. The use of CTAB may be advantageous in that is
may help purify the DNA by precipitating some of the humic acids. In some aspects, the
buffers employed for the lysis and extraction steps include guanidine isothiocyanate and
hexadecyltrimethylammonium bromide (CTAB) to preserve the integrity of the high
molecular weight genomic DNA. Generally, the extraction buffer solution is made to a
desired volume e.g. with highly purified water. Other components that may be included
in the extraction buffer include but are not limited to: nuclease inhibitors such as
auintricarboxyhc acid (See Nucleic Acids Research 4: 3055-3064; or antioxidants such
as β-mercaptoethanol, dithiothreitol, etc. The pH of the buffer is generally in the range of
from about 6.5 to 8, i.e. it is generally about 6.5, 7.0, 7.5 or 8.0. In some aspects, the pH
is adjusted to pH 8.0, e.g. with HCl or NaOH.

The soil is mixed or combined with extraction buffer e.g. in a ratio of buffensoil
(v/wt) in the range of from about 1:1 to about 10:1, and generally about 4:1. The amount
of soil used per assay is generally in the range of from about 100 mg to about 300 mg,
and is typically about 200-250 mg. In such a mixture, about 1 ml of extraction buffer
may be employed. Exact proportions of these and other components listed herein may
vary, depending, for example, on the type of sample, the amount of sample to be treated,
the number of organisms and/or the amount of DNA in the sample, etc.

The mixture is then subjected to mechanical disruption of the cell walls and/or
membranes, usually in combination with chemical disruption with detergents. In an
exemplary aspect, this step involves the use of very strong agitation (e.g. shaking or
vortexing) in the presence of one or more agents that cause or facilitate disruption such
as beads ("beadbeating") or other similar objects. Exemplary bead types include but are
not limited to glass, ceramic, metal (e.g. steel, zirconium, etc.), and stone such as agate.
The beads generally have average diameters in the range of from about 0.1 to about 6
mm, and usually in the range of from about 0.1 mm to about 4mm, and most frequently
from about 0.1 mm to 2.5 mm, especially when bacterial cells are being treated. A
mixture of beads with average diameters in these ranges may be employed, as may a
mixture of bead types. For example, for a 2 ml total reaction volume, a total of about 750
µl of beads ranging in diameter from about 0.1 to about 2.5 mm may be employed. In an
exemplary embodiment, a bead mixture comprises about 150 µl each of 0.1 and 0.5 zirconium beads and 2.5 mm glass beads. Together, the soil, extraction buffer and beads make up a total final volume of about 2 ml.

The combination of sample, extraction buffer and suitably sized beads is then subjected to strong agitation, e.g. by violent shaking for a period of time sufficient to release break open the cells and release the DNA. For example, the mixture is subjected to a rapid back and forth motion cycling shaking to 5000 times per minute for a period of time of from about 5 to about 30 minutes, e.g. for about 5, 10, 15, 20, 25 or 30 minutes. Generally, about 10-20 minutes suffices. In an exemplary embodiment, tubes containing the mixture are horizontally affixed to the platform of a vortex and vortexed for 10 to 20 min at the maximum setting (8+). While the usual disruption method is beadbeating, other methods may be used as well, including but not limited to: sonication, cryopulverization, nitrogen decompression, etc.

After cell walls are disrupted, unwanted soil particles and microbial debris are removed from the sample, e.g. by centrifugation. However, other suitable means such filtration may also be employed.

If centrifugation is employed, about 5 minutes at 14Kg is usually sufficient. The supernatant is decanted into a fresh container (e.g. typically about 700 µl to 850 µl of supernatant is recovered) and contains the released DNA, but also contains many unwanted contaminants such as humic acids. The supernatants are generally brown if there is significant humic acid in the sample. Humic acids can be removed, or largely removed, by ammonium acetate precipitation. For example, a desirable ammonium acetate concentration to be attained is at least about 3 M. Thus, for a supernatant of 700-850 µl, 450 µl of 7.5 M stock solution of ammonium acetate is added. The mixture is mixed thoroughly (e.g. by vortex briefly such as for 5 seconds), and then placed on ice for 5 minutes to allow precipitation to occur. Thereafter, the precipitate is removed, e.g. by centrifugation (such as at 14Kg for 5 minutes), and the supernatant is retained.

DNA in the supernatant is then concentrated by precipitation using an organic solvent, which is generally an alcohol such as isopropanol, ethanol, butanol, chloroform-isoamyl alcohol, or mixtures thereof, and which is usually carried out in the cold and/or on ice. For precipitation of DNA from solution, the solution also may require a high salt concentration. Salts such as LiCl (0.8M), NaCl (0.3-0.5M), NaOAc (pH 5.2, 0.3M)
NH₄Ac (2.0 - 2.5 M) may be used with an appropriate volume of alcohol (30%-50% final percentage isopropanol; 60%-80% final percentage ethanol). Or DNA may be precipitated using twice the volume of isopropanol alcohol without salts. Other additives such as, for example, nuclease inhibitors, ion chelators such as EDTA, etc., may be present as necessary or desired. In an exemplary embodiment, ice cold isopropanol alcohol is added to an equal volume of supernatant (e.g. about 1000 µl of each) in a suitable container such as a plastic or glass tube, and mixed e.g. by inverting the tube at least three times. The tube is then placed on ice for at least 10 minutes, and may be refrigerated or frozen. Precipitated DNA is separated from the alcohol e.g. using centrifugation (e.g. 14 Kg for 5 min, where "g" is the force of gravity), after which the supernatant is discarded (e.g. by upending the tube and allowing the remaining liquid to drain onto a paper towel for 5 minutes). The DNA-containing pellets are generally brown to dark brown in color and are plainly visible, and a final rinse with e.g. ice-cold 70% ethanol may be used to remove additional salts (desalting).

As a final step, the DNA pellet is resuspended in a desired volume of running buffer (e.g. 50 µl) with a low salt concentration and purified using gel filtration. Suitable low salt running buffers include but are not limited to, for example: TE buffer (10 mM Tris, pH 8.0, 1 mM EDTA); and buffer formulated from 100 mM TRIS, pH 8.0, 100 mM NaCl. Thorough mixing, e.g. by vortexing for 20 seconds, waiting 5 minutes and then vortexing again for 20 seconds, is carried out. The DNA is then purified using a size exclusion centrifugal gel filtration column capable of separating high molecular weight DNA from contaminants using, e.g. a centrifuge set at low speeds. Size-exclusion chromatography (SEC) is a chromatographic method in which molecules in solution are separated by their size, and in some cases molecular weight. Typically, when an aqueous solution is used as the mobile phase to transport the sample through the column, the technique is known as gel-filtration chromatography. SEC is a widely used polymer characterization method because of its ability to provide good molar mass distribution (Mw) results for polymers based on size exclusion and also, for genomic DNA. Exemplary column matrices (Sephacryl 300 HR) retain molecules with a molecular weight of less than about 1.5 x 10⁶ daltons so that they pass slowly through the matrix, but allow DNA molecules (e.g. with a Mr of greater than about 1.5 x 10⁶ daltons to pass through rapidly. The bead size of the particles is generally in the range of from about 10-
100 µm, or from about 25-75 µm, when wet. Suitable matrices include but are not limited to various high resolution (HR) chromatography media such as those based on Sephadex, Superose, SEPHACRYL®, Bio-Gel, or Toyopearl. Exemplary media include but are not limited to: SEPHACRYL® 300 S HR, Toyopearl HW-65, Superose 6, Sepharose 6B. The matrices are positioned in a support such as an open tube capable of receiving a DNA sample, and capable of withstanding a force of 100 g. The tube contains a chromatography frit at the bottom which holds the matrix in the tube but permits the DNA solution to pass through. In an exemplary aspect, the column dimensions allow for a 6 mm bed diameter and a 20 mm bed height resulting in an overall bed volume of 565 µl. The bed volume can be increased so long as the procedure is calibrated accordingly, e.g. by increasing the chase and elution volumes. Alternative matrix supports may be designed in a multi-well format for parallel processing of samples. A suitable column is prepared by adding a hydrated size exclusion matrix suspended in a solution of running buffer e.g., 100 mM TRIS pH 8.0 to the column and centrifuging it at 100 g for 2 minutes to drain away the excess liquid, and then repeating the process until the desired bed volume is achieved.

In an exemplary aspect, about 50 µl of sample is loaded onto an approximately 550 µl bed volume spin column positioned on top of a new 2 ml tube and centrifuged at 0.1 Kg for 2 minutes, followed by chasing via addition of e.g. about 100 to 150 µl of 100 mM TRIS 100 mM NaCl pH 8.0 to the top of the column and re-centrifuging at 0.1 Kg for 2 minutes. The resulting DNA solution is purified sufficiently to undergo further analysis such as sequencing, PCR reaction, etc. It is noted that loading 100-150 µl of the initial resuspended DNA will result in a slightly higher yield of DNA but in lower purity.

The practice of the present invention does not necessarily exclude the inclusion of an optional silica or ion exchange matrix (such as DEAE-cellulose) purification step before or after gel filtration. Introduction of such a step might increase purity but might do so at the expense of yield.

An exemplary aspect of the invention is illustrated schematically in Figure 4. Figure 4 shows the steps of combining a sample (in this exemplary embodiment, a soil sample) with a buffer that is suitable for extracting DNA. Next, cells within the sample are disrupted, i.e. the cell walls and membranes are broken in order to release the DNA contained therein. One or both of chemical and mechanical disruption may be used, e.g.
mechanical disruption using the technique of beadbeating (strong or violent agitation of the cells in the presence of beads). Next, humic acids are precipitated from the sample (e.g. by adding ammonium acetate). The DNA remains in the supernatant, which is recovered, and the DNA is precipitated from the supernatant, typically by adding one or more salts and one or more organic alcohols. The precipitated "pellet" of DNA is recovered and the DNA is resuspended in a suitable buffer, e.g. a low salt buffer. The DNA is then further purified, e.g. by size exclusion chromatography, e.g. using a spin chromatography protocol.

DNA isolated and purified as described herein may be used for any purpose. Exemplary further processes include but are not limited to: for various sequencing reactions, including PCR amplification; shotgun sequencing; various high throughput sequence analyses, next generation sequencing platforms such as Illumina Hiseq and Miseq platforms, etc.

In one aspect, the DNA is suitable for metagenomic analyses. Metagenomics is the study of genetic material recovered directly from environmental samples. The broad field may also be referred to as environmental genomics, ecogenomics or community genomics. While traditional microbiology and microbial genome sequencing and genomics rely on cultivated clonal cultures, metagenomic analyses produce a profile of diversity in a natural sample. Recent studies use, for example, either "shotgun" or PCR directed sequencing to get largely unbiased samples of all genes from all the members of the sampled communities. This technology has the ability to reveal previously hidden diversity of microscopic life, but can be limited by the availability of high quality DNA available from natural samples. The higher yields made possible by the present methods can lead to greater metagenomic coverage of the living matter contained in each sample, and can do so at a lower cost than was previously possible. For example, in a sample that contains a mixture of diverse microbes, such an analysis allows the taxonomic identification of the different microbes e.g. by genus, species, subspecies, etc.

Various PCR and/or sequencing protocols may be employed in combination with the methods described herein, including but not limited to: emulsion PCR, shotgun sequencing, bridge PCR, single-molecule real time sequencing, ion semiconductor sequencing, pyrosequencing, sequencing by synthesis, sequencing by ligation, etc. Generally, the purified DNA produced by the size exclusion chromatography step is
subjected to any of the many known techniques to ultimately determine the sequences of the individual DNA molecules in the sample. Further, the identity of the organisms (e.g. microorganisms such as bacteria) may be established thereby, and a profile of the microbiota in the sample and/or of the region or locale from which the sample was obtained, may be determined.

In another aspect, the present invention provides kits for carrying out the methods described herein. The kits typically include, for example: ammonium acetate (either dry or as a concentrated stock solution), a quantity of beads as described herein of a suitable size (e.g. in the range of from 0.1 mm to 2.5 mm if bacteria are to be disrupted); and a quantity of a suitable gel filtration size exclusion matrix, which may optionally be preloaded into suitable containers. A quantity of an alcohol suitable for precipitating DNA may be included, as well as various buffers such as high salt extraction buffer, low salt resuspension buffer, etc. The kit may include tubes to contain the sample during processing and columns for creating the gel filtration column purification. Instructions for use of the kit may also be included.

EXAMPLES

EXAMPLE 1. Isolation and Purification of DNA from three different soil samples and comparison of the present technology to known techniques

DNA extraction is one of the most important steps in characterizing the microbiological, metagenomic and biochemical properties of soils. Soil is an exceedingly complex medium containing an enormous variation in chemical, environmental and microbiological properties. Many of these properties impact our ability to extract soil DNA. Humic acid content is especially critical given that some of the humic acid tends to co-purify with DNA and interfere with downstream processes such as PCR amplification. DNA purification techniques also vary with respect to extraction yield. Yield is important given that DNA extraction from soils is often incomplete. Incomplete yield means some DNA in inaccessible sites may be bypassed and therefore not accounted for in subsequent analysis. Fragmentation size is also important for some DNA extraction methodologies, especially for metagenomic gene analysis. Larger fragments are better because they may contain many more genes for metagenomic gene analysis on a given piece of DNA which would present much less difficulty in aligning and assembling large genome segments. DNA extraction methodologies have not been
thoroughly cross evaluated for effectiveness despite the fact that DNA extraction is known to be one of the most important variables in any soil DNA analysis. There are many commercial kits on the commercial market for extracting soil DNA. These kits always use a series of step beginning at extraction of DNA from the organisms and the soil matrix, and several subsequent purification steps often including a precipitation and a purification step by adhesion to silica with subsequent elution. Commercial kit effectiveness is highly variable due to the differences in fundamental methods and likely the order in which these methods are employed.

An embodiment of the above-described technique, referred to herein as "Lab Tech", was tested against the MoBio and Epicenter soil extraction kits using three diverse soil types: "Efau", "Home", and "Perkins". Efau soil came from the Stillwater Efau field which is used extensively and is typical of wheat production soil; the Home soil came from an intensively cultivated garden and is an organic soil with high humic acid content; the "Perkins" soil came from the Perkins Experiment Station and is a sandy soil obtained from a site growing switchgrass. The Home soil had the highest DNA content followed by the Efaw and Perkins as expected. Four replicate extracts with three assays per extract were run twice.

The Lab Tech procedure was carried out as follows:

REAGENTS

**Extraction Buffer** (EB) to make 1 Liter: 50 mM TRIS Base (3.02g); 50 mM EDTA (9.3 g); 500 mM NaCl (14.6g); SDS 20 g (final concentration is 4%); E water 400 ml. Adjust to pH 8.0 with HCl and bring to total volume of 1 liter with E water.

**Ammonium Acetate**: to make 50 ml of 7.5 M: Ammonium acetate 28.8g; add highly purified water to 50 ml.

**Spin column preparation**

Cut off the cap of a 600 µ tube and place it on top of a 2 ml centrifuge tube from which the cap has also been removed. Make a hole in the bottom of the 600 µ tube using a dissecting needle. Insert a crumpled 1 cm² piece of support material or cellulose frit, preferably lint free (e.g. a KIMWIPE™) and place it at the bottom of the 600 µ tube/column. Alternative materials that may be used include but are not limited to: commercial preparation of a individual column containing a porous and nonbinding frit to retain the gel matrix in the tube. Load the tube with gel filtration matrix. Shake the
SEPHACRYL® 300S HR bottle to completely resuspend the matrix. Transfer 600 µl of resuspension mix to the 600 µl column, e.g. using a 1 ml pipette tip from which the tip has been removed and centrifuge the column at 0.1 Kg for 2 minutes to remove suspension liquid. Place the column on top of a new 2 ml tube for collection of a DNA sample.

**Soil Collection and Processing:** Soil was collected in aerated soil collection bags and dried overnight (or longer as needed). Soils were ground using a high speed coffee grinder to mix and break up aggregates, and samples were place in a labeled 50 ml tube and place in a freezer.

**Beadbeating** (beadbeating breaks open the cells by agitation with the beads in the presence of detergents such as SDS). -250 mg of soil was added to a bead beating tube of 2 ml internal volume. 1 scoop each of 0.1 and 0.5 zirconium beads and 2.5 mm glass beads was added to the tube. (The scoop is approximately 150 ul volume). 1000 ul of Extraction Buffer (EB) was added to each tube and the tubes were tightly capped. The tubes were then bead beaten using a vortexer with tubes taped horizontally on a platform for 10 to 20 min at the maximum setting (8+). Those of ordinary skill in the art will recognize that more or less time might be used. However, shorter beat times may result in less DNA yield but with possibly less DNA shear. Conversely, longer times could result in slightly greater yield but with additional shear. From 700 ul to 850 ul of supernatant was recovered and centrifuged for 14Kg for 5 minutes. Shorter or longer times could certainly be used, but times beyond 5 minutes may not improve the results. The supernatant was transferred into a new 2 ml tube. Note: the supernatants should be brown if there is significant humic acid in the sample.

**Ammonium Acetate precipitation** (ammonium acetate precipitates unwanted humic acids and other compound). 450 ul of 7.5 M of ammonium acetate was added to each 2 ml tube. The amount used was proportional to the amount of beadbeating extract which, in this embodiment, is about 700ul to 800ul. The mixture was vortexed to mix for 5 seconds, placed on ice for 5 minutes, and then centrifuged for 14Kg for 5 minutes.

**Isopropyl Alcohol Precipitation** (isopropyl alcohol precipitates the DNA in order to concentrate it for the next step. Some removal of contaminants and salts will also take place). About 1000 ul of supernatant was carefully removed while avoiding the pellet, and transferred to a new 2 ml tube. 1000 ul of ice cold isopropyl alcohol was
added. In some embodiments, about the same amount of isopropyl alcohol as ammonium acetate will be used, e.g., they should be approximately in a 1:1 ratio. The combined liquids were mixed thoroughly and placed on ice for at least 10 minutes, and then centrifuged at 14 Kg for 5 min. The DNA pellets were brown to dark brown and plainly visible. The supernatant was discarded and the tubes were inverted and allowed to drain onto a paper towel for 5 minutes. After 5 minutes, the pellets were resuspended in 50 µl of 100 mM TRIS 100 mM NaCl pH 8.0 by vortexing for 20 seconds, waiting 5 minutes and then vortexing again for 20 seconds. It should be noted that 50 µl was chosen based on the bed volume of the spin column which, according to this embodiment, would be in a ratio of about 11:1 of bed volume to sample. Much lower volumes could potentially decrease the purification efficiency.

**Spin column chromatography**

A SEPHACRYL® column was previously prepared as described above. 50 µl of resuspended DNA sample was loaded onto a 550 µl bed volume spin column that has been placed on top of a new 2 ml tube. The column was centrifuged at 0.1 Kg for 2 minutes and chased with 100 to 150 µl of 100 mM TRIS 100 mM NaCl pH 8.0, with the amount being chosen based on the bed volume and sample ratio. The column was again centrifuge at 0.1 Kg for 2 minutes. Column effluent contains the purified DNA.

**DISCUSSION**

The results are depicted in tabular form in Figure 1. With respect to Figure 1, the total yield is the total yield of DNA in a given soil sample expressed as µg DNA per gram of soil. This value was adjusted for sample loss due to incomplete sampling and losses during purification. Values above 20 µg/g of soil are excellent. As can be seen, the Lab Tech technique resulted in much higher yields than the MoBio and Epicenter techniques. Also in Figure 1, the assay yield is the amount of DNA extracted from the soil expressed as µg DNA per µl of extraction volume. This measurement represents the quantity of DNA that was obtained from a single soil extraction. As can be seen, the LabTech technique also significantly outperformed both MoBio and Epicenter in assay yield.

The 260/280 spectrophotometric ratio (i.e. the ratio of absorbance at 260nm and 280 nm) for each DNA sample is also shown in Figure 1. This ratio is widely understood to be a relative measure of DNA purity where values above 1.8 are considered to indicate
a sample that is acceptably pure, with maximum achievable purity being at values close to 2.0. The results showed that the average MoBio and Lab Tech purity was very good (>1.90), while that of Epicenter not acceptable (< 1.80).

Measurement 260/230 is the ratio of spectral absorbance at 260 nm (nucleic acid amount) to 230 nm (humic acid amount), with lower ratios indicating less humic acid. The data was obtained using a nanodrop spectrophotometer and the results shown are the average of eight extractions and three assay measurements for each soil sample that was tested. As can be seen, the kit provides a significant greater overall yield with similar quality of soil DNA from a diverse set of soils compared to the commercial MoBio PowerSoil.

Figure 2 shows results obtained when soil DNA extracted from Perkins soils was analyzed by electrophoresis using 0.9% agarose gel that was post stained with ethidium bromide and illuminated with ultraviolet radiation. Molecular weights are estimated based on the exponential relationship between migration distance and molecular weight as judged by the standard molecular weight markers beside each lane.

The results show a smearing of DNA in the Lab Tech and MoBio procedures but not with the Epicenter procedure. The smearing is likely due to DNA shearing during bead beating in the Lab Tech and MoBio techniques. On the other hand the Epicenter kit resulted in larger fragment size. The difference between the Epicenter kit and the others is likely due to the reliance on enzymatic and detergent extraction vs physical disruption techniques like bead beating which tend to break up and shear the DNA to a greater extent.

Figure 3 contains estimates of maximum and average size of the fragments generated by the DNA soil extraction procedures. Average fragment size is greatest in Epicenter followed by Lab Tech and then MoBio.

By way of comparison between the current embodiment and the Mo Bio Power Soil kit, it should be noted that the Lab Tech method has a higher yield. The MoBio kit uses a silica membrane to bind the DNA under high salt conditions with subsequent release under low salt conditions. Without being bound by theory, it is believed that complete binding or complete elution may be a yield limiting factor. The Lab Tech does not use silica membranes to purify DNA, but uses a size exclusion filtration technology that allows large molecules such as DNA to pass on through and traps smaller molecules.
in a gel matrix. Gel filtration technologies do not rely on binding and elution steps and gel filtration matrixes have been extensively produced to eliminate DNA or protein- gel matrix interactions, so the DNA just passes through without significantly interacting with the gel matrix. The lack of binding to a matrix with subsequent elution may make the gel filtration a much better process than silica for purification purposes, and may be a factor in the differential yield. In the Lab Technique a Sephacryl 300S gel filtration matrix was used which excludes from matrix interaction molecules the size of 1,500,000 molecular weight, which is in the range of 2500 base pairs for DNA. Other gel filtration matrixes can be used to exclude higher or lower sized fragments. Thus DNA fragments larger than 2500 base pairs pass on through while other molecules smaller than this are filtered out. The use of gel filtration also provides an advantage in that smaller fragments of DNA are also eliminated. Small fragments are suspected of producing more chimera artifacts in down-stream analysis.

The greater yield associated with the Lab Technique may also be the result of the bead beating process. The Mo Bio kit uses larger agate rocks that are agitated violently in the bead beater to break open individual bacteria cells, thus possibly resulting in higher shear and lower yield. The Lab Technique uses a 1:1 mixture of 0.1 mm and 0.5 mm zirconium beads for use in the bead beater. The literature suggests that the 0.1 mm is more appropriate for bacteria while the 0.5 mm beads are better for eukaryotic organisms. The larger MoBio agate beads may not be as effective as the smaller beads in breaking open bacteria or small eukaryotic cells.

Another area where greater yield may be realized is in the precipitation step. The MoBio kit uses two steps including what appears to be ammonium acetate and aluminum sulfate. Aluminum sulfate does differentially precipitate humic acids, but may also co-precipitate DNA, resulting in lower yield. This may be the reason why there appears to be less humic acid in the MoBio extraction compared to Lab Tech. The Lab Tech uses ammonium acetate to precipitate DNA but also uses an isopropyl alcohol precipitation designed to concentrate the DNA down to the 50 ul prior to gel filtration- an important factor based on the current gel filtration column configurations. The isopropyl alcohol precipitation also provides some purification and removal of salts (ammonium acetate) prior to gel filtration. The isopropyl alcohol precipitation step is quantitative in nature.
meaning that it is known to precipitate the vast majority of DNA which is not true for aluminum sulfate procedures.

The dramatic difference in yield between this embodiment and the Epicenter kit is most likely due to the method to break open the cells and soil matrix. The Epicenter kit uses an enzymatic procedure coupled with detergent to break open the inner and outer cell membranes of the bacteria. Enzymatic techniques are known for their incomplete extraction ability compared to bead beating. They are also very unlikely to break open the soil matrix to release the DNA for enzymatic digestion thus resulting in a much lower yield. However, the enzymatic step has an advantage in that it does not significantly shear the DNA resulting in the larger fragments associated with the Epicenter kit extraction. However, this is at the expense of a drastic decrease in yield.

EXAMPLE 2. Metagenomic analyses

Powerful DNA sequencing technologies provide scientists the opportunity to develop information concerning the biotic portion of the soil ecosystem which supports basic growth processes and agricultural productivity. DNA extraction technology is currently a limiting factor in this analysis. The Lab Tech procedure results in acceptable purity and high yields of DNA and thus has significant advantages over prior art methods. DNA obtained from a variety of soil samples is isolated using the Lab Tech methodology and is used to perform metagenomic analyses. The results show that the DNA is suitable for performing metagenomic analyses, with the advantage that the high yield provides DNA in quantities sufficient to permit detection of microorganisms that are present only in trace amounts.

For purposes of the instant disclosure, the term "at least" followed by a number is used herein to denote the start of a range beginning with that number (which may be a ranger having an upper limit or no upper limit, depending on the variable being defined). For example, "at least 1" means 1 or more than 1. The term "at most" followed by a number is used herein to denote the end of a range ending with that number (which may be a range having 1 or 0 as its lower limit, or a range having no lower limit, depending upon the variable being defined). For example, "at most 4" means 4 or less than 4, and "at most 40%" means 40% or less than 40%. Terms of approximation (e.g., "about", "substantially", "approximately", etc.) should be interpreted according to their ordinary and customary meanings as used in the associated art unless indicated otherwise. Absent
a specific definition and absent ordinary and customary usage in the associated art, such terms should be interpreted to be ± 10% of the base value.

When, in this document, a range is given as "(a first number) to (a second number)" or "(a first number) - (a second number)" , this means a range whose lower limit is the first number and whose upper limit is the second number. For example, 25 to 100 should be interpreted to mean a range whose lower limit is 25 and whose upper limit is 100. Additionally, it should be noted that where a range is given, every possible subrange or interval within that range is also specifically intended unless the context indicates to the contrary. For example, if the specification indicates a range of 25 to 100 such range is also intended to include subranges such as 26 -100, 27-100, etc., 25-99, 25-98, etc., as well as any other possible combination of lower and upper values within the stated range, e.g., 33-47, 60-97, 41-45, 28-96, etc. Note that integer range values have been used in this paragraph for purposes of illustration only and decimal and fractional values (e.g., 46.7 - 91.3) should also be understood to be intended as possible subrange endpoints unless specifically excluded.

It should be noted that where reference is made herein to a method comprising two or more defined steps, the method can also include one or more other steps which are earned out before any of the defined steps, between two of the defined steps, or after all of the defined steps (except where context excludes that possibility). As a specific example, in some embodiments a silica gel stage might be incorporated after the gel filtration to increase purity, but this might also decrease yield. This would require diluting the gel filtration purified sample with a high salt buffer, binding the sample to a silica membrane, washing the membrane with high salt and eluting with no salt buffer.

Further, it should be noted that terms of approximation (e.g., "about", "substantially", "approximately", etc.) are to be interpreted according to their ordinary and customary meanings as used in the associated art unless indicated otherwise herein. Absent a specific definition within this disclosure, and absent ordinary and customary usage in the associated art, such terms should be interpreted to be plus or minus 10% of the base value.

Still further, additional aspects of the instant invention may be found in one or more appendices attached hereto and/or filed herewith, the disclosures of which are incorporated herein by reference as if fully set out at this point.
Thus, the present invention is well adapted to carry out the objects and attain the ends and advantages mentioned above as well as those inherent therein. While the inventive device has been described and illustrated herein by reference to certain preferred embodiments in relation to the drawings attached thereto, various changes and further modifications, apart from those shown or suggested herein, may be made therein by those of ordinary skill in the art, without departing from the spirit of the inventive concept the scope of which is to be determined by the following claims.
CLAIMS

What is claimed is:

1. A method of extracting DNA from cells in a sample that contains or is likely to contain humic acids, comprising
   i) combining said sample and an extraction medium;
   ii) disrupting said cells to release DNA from said cells into said extraction medium;
   iii) precipitating humic acids from said extraction medium by adding ammonium acetate in a quantity sufficient to precipitate humic acids but insufficient to precipitate said DNA, thereby retaining said DNA in solution in said extraction medium;
   iv) separating extraction medium comprising said DNA from precipitated humic acids;
   v) precipitating said DNA from said extraction medium by adding organic alcohol and salt in a quantity sufficient to precipitate said DNA;
   vi) resuspending DNA precipitated in step v) in a resuspension medium; and
   vii) purifying resuspended DNA.

2. The method of claim 1, wherein said step of disrupting includes one or both of mechanical and chemical disruption.

3. The method of claim 2, wherein said mechanical disruption is performed by beadbeating.

4. The method of claim 1, wherein said method further comprises a step of drying the sample and mechanically dispersing said sample prior to said step of combining.

5. The method of claim 1, wherein said sample is a soil sample.

6. The method of claim 1, wherein said cells are bacterial cells.

7. The method of claim 1, wherein said resuspension medium is a low-salt medium.
8. The method of claim 1, wherein said extraction medium contains guanidine isothiocyanate and hexadecyltrimethylammonium bromide (CTAB).

9. The method of claim 1, wherein said step of purifying is performed by size exclusion gel chromatography.

10. A method of conducting a metagenomic analysis, comprising
    I) obtaining a sample comprising cells from at least one organism or microorganism, wherein said sample contains or is likely to contain humic acids
    II) extracting DNA from said cells in said sample by
        i) combining said sample and an extraction medium;
        ii) mechanically disrupting said cells to release DNA from said cells into said extraction medium;
        iii) precipitating humic acids from said extraction medium by adding ammonium acetate to said extraction medium in a quantity sufficient to precipitate humic acids but insufficient to precipitate said DNA from said extraction medium, thereby retaining said DNA in solution in said extraction medium;
        iv) separating extraction medium comprising said DNA from precipitated humic acids
        v) precipitating said DNA from said extraction medium by adding organic alcohol and salt to said extraction medium in a quantity sufficient to precipitate said DNA;
        vi) resuspending DNA precipitated in step v) in a low-salt resuspension medium; and
        vii) purifying resuspended DNA by size exclusion gel chromatography;
    III) analyzing a plurality of nucleic acid sequences of the purified DNA; and
    IV) based on results obtained in said step of analyzing, establishing the taxonomic identity of said at least one organism or microorganism.
11. The method of claim 10, wherein said step III) of analyzing includes one or more of polymerase chain reaction (PCR) amplification of said one or more nucleic acid sequences and sequencing of said one or more nucleic acid sequences.

12. The method of claim 10, wherein said sample is a soil sample.

13. The method of claim 10, wherein said at least one organism or microorganism is a bacterium.

14. A kit for carrying out the methods of any of the preceding claims, said kit comprising at least one tube to contain the sample during processing and at least one column for purifying resuspended DNA by size exclusion gel chromatography.

15. The kit according to claim 14, further comprising instructions for carrying out the method according any of the preceding claims.
Figure 1

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Figure 2

![SOIL 3](image-url)
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Figure 3

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Figure 4

- COMBINE SOIL SAMPLE AND EXTRACTION BUFFER
- DISRUPT CELLS IN SAMPLE
- PRECIPITATE HUMIC ACIDS
- PRECIPITATE DNA
- PURIFY DNA
INTERNATIONAL SEARCH REPORT

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8): C07H 21/00, 21/04; C12P 19/34; C12N 5/00, 5/02 (2015.01)
CPC: C12N 15/10, 15/003, 15/006; 15/101; C12Q 1/6806, 1/686, 1/6869; A61K 38/00, C07H 21/00; C07K 14/47

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): C07H 21/00, 21/04; C12P 19/34; C12N 5/00, 5/02; CPC: C12N 15/10, 15/003, 15/1006; 15/101; C12Q 1/6806, 1/686, 1/6869; A61K 38/00, C07H 21/00; C07K 14/47; USPC: 536/25.4, 23.1, 22.1, 18.7, 1.11; 435/325, 91.1, 89, 85, 84, 72, 41

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)

PatSeer; Google; Google Scholar; Dialog Proquest; PubMed; EBSCO; soil sample; 'bacterial taxonomy'; DNA extraction; 'humic acid peroxidation'; 'size-exclusion chromatography'; 'PCR'; 'CTAB'; 'guanidinium isothiocyanate'; kit; 'alcohol precipitation'; 'low salt resuspension'; drying; pulverizing

C. DOCUMENTS CONSIDERED TO BE RELEVANT

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<th>Category*</th>
<th>Citation of document, with indication, where appropriate, of the relevant passages</th>
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<td>Y</td>
<td>US 2005/0282202 A1 (BROLASKI, MN et al.) December 22, 2005; abstract; paragraphs [0002], [0010] [0014], [0016], [0019], [0024], [0025], [0030], [0032], [0037], [0041], [0061], [0074], [0078]; [0081], [0089], [0136]</td>
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<td>Y</td>
<td>WO 2004/027080 A2 (EMBROSIS PHARMACEUTICALS) April 01, 2004; page 4, lines 12-20; page 6, lines 19-33; page 7, lines 1-11; page 11, lines 6-7; page 14, lines 8-15</td>
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<td>Y</td>
<td>US 7255989 B1 (JEANNIN, P et al.) August 14, 2007; column 5, lines 10-20; column 10, lines 22-44; column 49, lines 45-55; column 50, lines 36-41</td>
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<td>US 6617108 B1 (WILLSON, III, PC et al.) September 09, 2003; abstract; column 4, lines 13-32; column 15, lines 13-15</td>
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Further documents are listed in the continuation of Box C. | See patent family annex.

* Special categories of cited documents: 
**A**: document defining the general state of the art which is not considered to be of paniculaT 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 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 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
22 June 2015 (22.06.2015)

Date of mailing of the international search report
13 JUL 2015

Name and mailing address of the ISA/ 
Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450
Facsimile No. 571-273-8300

Authorized officer
Shane Thomas

PCT 1999-02898
PCT O16: 571-272-4300
PCT O16: 571-272-4300
**INTERNATIONAL SEARCH REPORT**

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<td>2.</td>
<td>Claims Nos.: because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:</td>
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<td>3.</td>
<td>Claims Nos.: 15 because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).</td>
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<td>No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:</td>
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Remark on Protest

- The additional search fees were accompanied by the applicant’s protest and, where applicable, the payment of a protest fee.
- The additional search fees were accompanied by the applicant’s protest but the applicable protest fee was not paid within the time limit specified in the invitation.
- No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet (2)) (January 2015)