

(19) World Intellectual Property Organization
International Bureau



(43) International Publication Date
22 November 2007 (22.11.2007)

PCT

(10) International Publication Number
WO 2007/134254 A2

(51) International Patent Classification:
H01M 8/24 (2006.01)

(21) International Application Number:
PCT/US2007/068811

(22) International Filing Date: 11 May 2007 (11.05.2007)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/799,759 12 May 2006 (12.05.2006) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— without international search report and to be republished upon receipt of that report

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

(54) Title: SURVEYING AND MONITORING RESPIRATORY TRACT VIRAL COMMUNITIES USING UNCULTURED HUMAN SPUTUM SAMPLES

(57) Abstract: The invention provides compositions (e.g., kits) and methods for surveying and characterizing the presence of viral, bacterial and/or phage nucleic acid, including the complete viral and/or phage nucleic acid (e.g., DNA and/or RNA) population found in an individual, e.g., a human or other animal, including the complete viral, bacterial and/or phage pulmonary population, using the individual's sputum, for example, by using a human sputum sample.



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Surveying and monitoring respiratory tract viral communities using uncultured human sputum samples

FIELD OF THE INVENTION

5 **[0001]** This invention related generally to infectious disease detection and medicine. In particular, the invention provides compositions (e.g., kits) and methods for surveying and characterizing the presence of viral, bacterial and/or phage nucleic acid, including the complete viral, bacterial and/or phage nucleic acid (e.g., DNA and/or RNA) population found in an individual, e.g., in a human or other animal, including its complete viral, bacterial and/or phage
10 pulmonary population, using the individual's sputum, for example, by using a human sputum sample.

SUMMARY OF THE INVENTION

[0002] This invention provides methods for isolating a virus nucleic acid-comprising sample from a sputum sample, the method comprising the following steps: (a) providing a sputum
15 sample; (b) treating the sputum sample with a reducing agent; (c) adding a buffer to the treated sample of (b); (d) mechanically homogenizing (or equivalent, e.g., macerating) the sample of (c); (e) centrifuging the sample of (d) to generate a sample pellet; (f) suspending the pellet of step (e) in a buffer and preserving an aliquot with an equal amount of para-formaldehyde or equivalent; (g) centrifuging the resuspended pellet of (f) in density gradient comprising at least a
20 1.5 g/ml density band (or equivalent); and, (h) isolating a virus nucleic acid-comprising sample from the 1.5 g/ml density band.

[0003] In one aspect, wherein the sputum sample is a human sputum sample. In one aspect, wherein the reducing agent comprises dithiothreitol (DDT), or equivalent. The sputum sample can be mixed in a solution of about 5%, 6%, 7%, 8%, 9% or 10% or more dithiothreitol (DDT).
25 In one aspect, full strength dithiothreitol (DDT) is mixed in a magnesium-based buffer to generate the DTT solution. In one aspect, the sputum sample has a volume of about 10 mls, or from between about 1 to 25 mls, or from about 3 to 20 mls, or from about 6 to 15 mls, or from about 8 to 12 mls.

[0004] In one aspect, the buffer comprises a magnesium-based buffer, e.g., a TRIS™ buffer
30 with magnesium salt, or equivalent.

[0005] In one aspect, the suspended pellet sample of step (f) is partially purified of (cleared of) bacterial and other cellular debris from the viral component of the suspended pellet sample before the centrifugation step. The suspended pellet sample of step (f) can be partially purified of (cleared of) bacterial and other cellular debris by using one or more filters, and/or one or more chromatographs. The chromatograph can comprise affinity chromatography, lectin chromatography, antibody chromatography, diethylaminoethyl cellulose chromatography, ion exchange chromatography and/or high performance liquid chromatography (HPLC). The one or more filters can be anywhere between about 0.1 to 2.0 micrometer filters, e.g., a 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and/or 1.0 micrometer filter; any combination or order of filters can be used, in combination with or separate from use of one or more chromatographs. In one aspect, the density gradient comprises a CsCl gradient.

[0006] In one aspect, the method further comprises isolating a bacterial or a phage nucleic acid from the nucleic acid isolated in step (h). In one aspect, the method further comprises sequencing the isolated nucleic acid. In one aspect, the method further comprises comparing the sequenced viral, phage and/or bacterial nucleic acid to databases of viral nucleic acids to identify the family or species of the isolated viral, phage and/or bacterial nucleic acid.

[0007] The invention provides methods for isolating a viral, phage and/or bacterial nucleic acid from a sputum sample, the method comprising the following steps: (a) providing a sputum sample; (b) treating the sputum sample with a reducing agent; (c) adding a buffer to the treated sample of (b); (d) mechanically homogenizing or macerating the sample of (c); (e) generating a virus-comprising pellet or equivalent from the sample of (d); (f) suspending the virus-comprising pellet or equivalent of step (e) in a buffer; (g) centrifuging the suspended pellet of (f) in a density gradient comprising at least a 1.5 g/ml density band; and, (h) isolating a viral, phage and/or bacterial nucleic acid sample from the 1.5 g/ml density band.

[0008] The invention provides methods for isolating a viral, phage and/or bacterial nucleic acid from a sputum sample, the method comprising the following steps: (a) providing a sputum sample; (b) treating the sputum sample with a reducing agent; (c) adding a buffer to the treated sample of (b); (d) mechanically homogenizing or macerating the sample of (c); (e) generating a virus-comprising, bacterial-comprising and/or phage-comprising pellet or equivalent from the sample of (d); (f) suspending the virus-comprising, bacterial-comprising and/or phage-comprising pellet or equivalent of step (e) in a buffer; and, in one aspect, (g) isolating a viral, phage and/or bacterial nucleic acid from the sample.

[0009] In one aspect, viral, phage and/or bacterial nucleic acid is isolated by a density gradient, a filter and/or a chromatograph, or any combination thereof, and the density gradient, a filter and/or a chromatograph can be used in any order. The chromatograph can comprise an affinity chromatography, lectin chromatography, antibody chromatography, diethylaminoethyl
5 cellulose chromatography, ion exchange chromatography and/or high performance liquid chromatography (HPLC). The density gradient can comprise a CsCl gradient.

[0010] In one aspect, the method comprises isolating a phage and/or bacterial nucleic acid from an isolated virus nucleic acid sample. In one aspect, the method further comprises sequencing the isolated nucleic acid. In one aspect, the method further comprises comparing the
10 sequenced viral nucleic acid to databases of viral or phage nucleic acids to identify the family or species of the isolated virus and/or phage.

[0011] In one aspect, the method also comprises isolating phage and/or bacterial nucleic acid from the sputum sample.

[0012] In one aspect of any method of the invention, the sputum is isolated from a human or
15 an animal, e.g., an individual or patient with and acute or a chronic pulmonary infection, disease and/or condition, including an individual with the acute or chronic infection, disease; in one aspect, the condition is the flu, or is cystic fibrosis, or is a viral bronchial or lung infection.

[0013] In one aspect of any method of the invention, the methods further comprise culturing the isolated virus. In one aspect of any method of the invention, the methods further comprise
20 culturing the isolated phage. In one aspect of any method of the invention, the methods further comprise culturing the isolated bacteria.

[0014] In one aspect of any method of the invention, the methods further comprise cloning the isolated virus nucleic acid. In one aspect of any method of the invention, the methods further comprise cloning the isolated phage nucleic acid. In one aspect of any method of the
25 invention, the methods further comprise cloning the isolated bacterial nucleic acid.

[0015] The invention provides a kit comprising the appropriate solutions and instructions for practicing the invention, including a kit comprising the appropriate solutions, as set forth herein, for collecting a sample of sputum from a patient and isolating a viral sample.

[0016] The details of one or more embodiments of the invention are set forth in the
30 accompanying drawings and the description below. Other features, objects, and advantages of the invention will be apparent from the description and from the claims.

[0017] All publications, patents, patent applications cited herein are hereby expressly incorporated by reference for all purposes.

DETAILED DESCRIPTION

[0018] The invention provides compositions (e.g., kits) and methods for surveying and characterizing the presence of viral, bacterial and/or phage nucleic acid, including the complete viral, bacterial and/or phage nucleic acid (e.g., DNA and/or RNA) population found in an individual, e.g., a human or other animal, using the individual's sputum, for example, by using a human sputum sample.

[0019] In one aspect, the presence of viral, bacterial and/or phage nucleic acid in the individual, including the complete viral, bacterial and/or phage nucleic acid (e.g., DNA and/or RNA) population (e.g., pulmonary population) in that individual, is determined by extraction of the nucleic acid, shotgun sequencing of the nucleic acid and subsequent comparison of the resultant sequences against existing nucleic acid (e.g., DNA) databases to identify known and, if present, novel viruses and/or phage, by their nucleic acid (DNA and/or RNA) sequences. This exemplary protocol of the invention uses a combination of established techniques and novel techniques (including established techniques used in novel ways) to extract the viral, bacterial and/or phage nucleic acid (e.g., DNA and/or RNA) from the sample.

[0020] In one aspect of this embodiment: first, the sputum sample, e.g., human sputum, is mixed in a solution of 5%, 6%, 7%, 8%, 9% or 10% or more dithiothreitol (DDT, such as SPUTOLYSIN™, Calbiochem, San Diego, CA). In one aspect the sputum sample, e.g., human sputum sample, comprises a minimum of about 10 ml human sputum; or from about 1 to 25 mls, or from about 3 to 20 mls, or from about 6 to 15 mls, or from about 8 to 12 mls.

[0021] In one aspect, the 10% DTT solution is made by diluting full strength DTT with an appropriate volume of a buffer, e.g., a magnesium-based buffer (such as SM buffer, or TRIS™ buffer with magnesium salt). DDT is designed to extract large cells from human sputum, exposing the viral cells to isolation. SM is a typical buffer used for phage extraction, but because of the chemical base for the buffer (i.e., magnesium), this buffer can counteract the effects of the DDT. A 10% solution can be effective for isolating viruses.

[0022] In one aspect, the sample is subsequently mechanically homogenized. This is necessary because although DDT is supposed to extract cells at room temperature, it was found that because of the high viscosity of human sputum samples (particularly those of patients with cystic fibrosis), it was ineffective without homogenization.

[0023] Next, the sample is incubated at about 37°C, or between about 35°C to 39°C, or between about 33°C to 41°C, for about 15 minutes, or from between about 10 to 20 minutes. This allows the DDT opportunity to act on the large sputum cells. In one aspect of the invention, where the sample is incubated for about 15 minutes at about 37°C, the temperature and time can optimize the breakdown of the sputum while preserving the viral community.

[0024] After incubation, a sample pellet, or equivalent, is generated; for example, in one aspect, the sample is centrifuged to generate a sample pellet according to normal laboratory procedures.

[0025] The pellet (or equivalent) is resuspended in a buffer, e.g., a magnesium-based buffer (such as SM buffer, or TRIS™ buffer with magnesium salt), and an aliquot is preserved with an equal amount of para-formaldehyde or equivalent. The suspension is necessary to enable the sample to be loaded into a density gradient, e.g., a CsCl gradient or equivalent, that clears bacterial and other cellular debris from the viral component.

[0026] In one aspect, before loading into the density gradient, e.g., a CsCl gradient or equivalent, the sample must be filtered – or equivalent (e.g., columns having the same effect), and in one aspect, through a range of filters (or columns) that are experimentally determined based on the types of viruses found in sputum samples.

[0027] The viruses are subsequently isolated using standard density gradient techniques, e.g., CsCl gradient techniques, or equivalent, and the viruses extracted from the 1.5 g/ml density band. Equivalents include any chromatograph protocol(s) that can remove bacterial and other cellular debris from the viral component, including affinity chromatography, such as lectin chromatography or antibody chromatography; or diethylaminoethyl cellulose chromatography, or ion exchange chromatography, or any form of high performance liquid chromatography (HPLC). These equivalents can be used in place of, or adjunct to, using standard density gradient techniques.

[0028] In one aspect, standard nucleic acid extraction and cleanup protocols are used, e.g., formamide/CTAB buffer DNA extraction and cleanup. Techniques for the manipulation of nucleic acids, such as, e.g., subcloning, labeling probes (e.g., random-primer labeling using Klenow polymerase, nick translation, amplification), sequencing, hybridization and the like are well described in the scientific and patent literature, see, e.g., Sambrook, ed., MOLECULAR CLONING: A LABORATORY MANUAL (2ND ED.), Vols. 1-3, Cold Spring Harbor Laboratory, (1989); CURRENT PROTOCOLS IN MOLECULAR BIOLOGY, Ausubel, ed. John Wiley & Sons, Inc.,

New York (1997); LABORATORY TECHNIQUES IN BIOCHEMISTRY AND MOLECULAR BIOLOGY: HYBRIDIZATION WITH NUCLEIC ACID PROBES, Part I. Theory and Nucleic Acid Preparation, Tijssen, ed. Elsevier, N.Y. (1993).

[0029] In one aspect, the samples are sequenced according to standard methodology (e.g., PYROSEQUENCING™ by 454 Life Sciences, Branford, CT). In one aspect, the recovered DNA sequences are subsequently compared with any sequence database to characterize and/or identify the organisms, including the viruses, isolated from the sputum. In one aspect, the SEED database of the Fellowship for Interpretation of Genomes (FIG) (Burr Ridge, IL) is used to identify known and novel viral DNA sequences.

[0030] Amplification reactions can be used to quantify the amount of nucleic acid in a sample (such as the amount of viral nucleic acid isolated from a sputum sample), label the nucleic acid (e.g., to apply it to an array or a blot), detect the nucleic acid, or quantify the amount of a specific nucleic acid in a sample. In one aspect of the invention, message isolated from a cell or a cDNA library are amplified.

[0031] The skilled artisan can select and design suitable oligonucleotide amplification primers. Amplification methods are also well known in the art, and include, e.g., polymerase chain reaction, PCR (see, e.g., *PCR Protocols, A Guide to Methods and Applications*, ed. Innis, Academic Press, N.Y. (1990) and *PCR Strategies* (1995), ed. Innis, Academic Press, Inc., N.Y., ligase chain reaction (LCR) (see, e.g., Wu (1989) *Genomics* 4:560; Landegren (1988) *Science* 241:1077; Barringer (1990) *Gene* 89:117); transcription amplification (see, e.g., Kwoh (1989) *Proc. Natl. Acad. Sci. USA* 86:1173); and, self-sustained sequence replication (see, e.g., Guatelli (1990) *Proc. Natl. Acad. Sci. USA* 87:1874); Q Beta replicase amplification (see, e.g., Smith (1997) *J. Clin. Microbiol.* 35:1477-1491), automated Q-beta replicase amplification assay (see, e.g., Burg (1996) *Mol. Cell. Probes* 10:257-271) and other RNA polymerase mediated techniques (e.g., NASBA, Cangene, Mississauga, Ontario); see also Berger (1987) *Methods Enzymol.* 152:307-316; Sambrook; Ausubel; U.S. Patent Nos. 4,683,195 and 4,683,202; and Sooknanan (1995) *Biotechnology* 13:563-564.

[0032] In summary, the invention provides methods and formulations for isolating viral nucleic acid, e.g., DNA or RNA, from sputum, e.g., human sputum. In one aspect, the associated utility uses existing methodology to identify and monitor the viral communities.

[0033] The invention will be further described with reference to the following examples; however, it is to be understood that the invention is not limited to such examples.

EXAMPLES

5 Example 1: Exemplary sequencing protocols

[0034] The following example describes an exemplary nucleic acid sequencing protocol that can be used to practice this invention.

[0035] In this example, the sequencing was done using PYROSEQUENCING™ by 454 Life Sciences, Branford, CT. This sequencing can be done with as little as five micrograms of DNA.
10 This protocol can yield thirty million base-pairs that can be used to characterize the (isolated) viral community and search against various databases for similar sequence matches. The databases include the NCBI Non-Redundant (NR) database. This particular database contains the collection of most publicly available sequences for most organisms and environments. Searches against the NR will allow us to know if the sequences are similar to known sequences
15 or completely novel.

[0036] The other database can be queried is SEED, which is part of the Fellowship for Interpretation of Genomes (FIG) (Burr Ridge, IL). FIG uses subsystems to annotate both complete and partial genome sequences. Subsystems are biochemical pathway, fragments of pathways, clusters of genes that function together, or any group of genes that any annotator
20 considers to be related. The subsystems are annotated across genomes by the annotators, providing the most reliable and consistent annotations within and between genomes. The subsystems-based annotations are ongoing and at a given point in time the subsystems represent the snapshot of the best available annotation of the SEED database.

Example 2: Exemplary protocols

25 [0037] The following example describes an exemplary protocol of the invention. This exemplary protocol is designed for a minimum of 10 milliliters of Human sputum sample.

1. Take sample and add equal volumes of 10% SPUTOLYSIN™ (Calbiochem, San Diego, CA), or dithiothreitol (DTT). Make the 10% SPUTOLYSIN™ by adding SM (magnesium based buffer) buffer to the SPUTOLYSIN™ concentrate to the needed
30 volume.

- a. The SM buffer is the typical buffer that is used for phage work. Although the invention is not limited by any particular mechanism of action, the buffer (which is magnesium based) should counter act the SPUTOLYSIN™ reaction.
2. Mechanically homogenize (using an electric motor homogenizer) between 3500-6500
5 R.P.M. until mixture is uniform in color and no visible particles are present. When re-lid the sample container and you should be able to so no solid masses when shaking the solution. The uniform color will be a yellow to dark yellow color. There should be no areas where only SPUTOLYSIN™/buffer is observed. If so re-homogenize until the sample meets the criteria above.
- 10 a. Homogenation may be particularly important with viscous sputum samples, e.g., as samples from patients with cystic fibrosis. Agitation with a homogenizer can work best to allow full interaction of the sputum sample with the SPUTOLYSIN™ solution.
3. Transfer homogenized sample into a 50 ml Falcon tube and seal to prepare for
15 centrifuging the sample to generate a pellet.
4. Incubate sample in 50 ml Falcon tube in a 37°Celsius water bath for 15 minutes.
 - a. Although the invention is not limited by any particular mechanism of action, this was done to effect interaction of sample and SPUTOLYSIN™, and allowing the reaction will happen in a shorter time frame. The temperature choice was chosen
20 for it would be less likely to destroy the phages and allow a faster reaction time.
5. Centrifuge at 1500 R.P.M. for a minimum of ten minutes. Add five minutes if no pellet is visible in the bottom of the tube (most likely sputum sample was extremely viscous). There should now be a pellet clearly visible (the pellet is somewhat solid, so take care to decant supernatant so that the pellet does not end up in the transferred supernatant, then
25 re-lid the supernatant container. Once the pellet has been removed from the supernatant, discard the supernatant in an appropriate biohazard container.
 - a. Adding time, e.g., five or ten minutes or more, may be necessary depending on the sample's viscosity.
6. Transfer the pellet into a 15 ml Falcon tube and re-suspend with SM buffer to 10
30 milliliters. Pipette out 100 µl into a 1.5 ml. Microtube and to this sample add an equal volume of 4% Para-Formaldehyde to it; label it as pre-filter homogenate sample. Store at -20 degrees Celsius.

- a. A re-suspension of the pellet is needed to allow filtering and loading onto a CsCl gradient.
7. Take re-suspended sample and pass through a micrometer filter (size can either be 0.4 to 0.8 micrometers depending on what bacteria you are trying to remove from your pelleted sample). Pipette out 100ul into a 1.5 ml. Microtube and to this sample add an equal volume of 4% Para-Formaldehyde to it; label it as post-filter homogenate sample. Store at -20 degrees Celsius.
 - a. Various sized filters may need to be used due to the fact that bacteria can vary in size.
8. Take the now-filtered pellet sample and add CsCl until the density is 1.15 grams per milliliter and load onto a CsCl gradient.
 - a. This was done to allow like salt to dissolve and inter mix. Failure to do so will result in the phages and the bacteria being isolated in the upper layer of the gradient.
9. Each of the following fractions is 1 milliliter in volume and their densities are as follows: lower fraction is 1.7 grams per milliliter, middle fraction is 1.5 grams per milliliter, and the upper fraction is 1.35 grams per milliliter. Draw a visible line at the upper portion border of each layer poured at the end there should a total of three lines on the Ultra-Centrifuge disposable tubes). Before loading in to the centrifuge, ensure tubes are paired and have the same weight. The weights should be identical out to the thousandths decimal place (e.g., .001).
 - a. This is a standard protocol that is used in the lab for phage isolation. The densities of the layers may vary from project to project. The densities chosen can be determined through experimentation and verification with microscopy.
10. Centrifuge for two hours at twenty two thousand R.P.M. and a temperature of 4 degrees Celsius.
 - a. This is a standard protocol time and temperature when using CsCl gradients.
11. After the centrifuge has stopped take an 18-gauge needle and place it onto a 3-milliliter syringe. Take the assembled needle and puncture the tube just slightly below the bottom most line (this should be at the upper edge of the 1.7 grams per milliliter boundary) extract 1.5 milliliters of solution. Transfer the 1.5 ml. from each tube into a 15 ml. Falcon tube. Also take a 50 ml Falcon tube and collect the supernatant from each tube

and label the Falcon tube as such. Pipette out 100 ul into a 1.5 ml. Microtube and to this sample add an equal volume of 4% paraformaldehyde to it. Perform this step for both the phage fraction (1.5 ml per each tube) as well as the supernatant that was collected from each tube. Label the microtubes as phage concentration sample and supernatant sample respectively. Store at -20 degrees Celsius.

- a. Phages are predominantly found in the 1.5 g/ml band.

12. Microscopy check: In order to see that you are following the protocol, make slides from the parts of the protocol where 100 ul of sample was collected. To make slides follow the following protocol:

- a. Take three 15ml Falcon tubes and label them with the same labeling that is on the microtubes you plan to transfer fixed sample from;
- b. Fill each tube with 4990 ul of Sigma water;
- c. Transfer 10 ul of sample from the appropriate Microtube to the correctly labeled Falcon tube;
- d. Vortex all tubes;
- e. Filter through a .02 um filter that is capable of being mounted on to a microscope slide;
- f. While the filtering is done prepare a 5x solution of SYBERGOLD™ (Molecular Probes, Invitrogen, Carlsbad CA) from either a 1000x or 10,000 x stock solution;
 - i. This was done experimentally to visualize the phages through microscopy. The reason for such a larger concentration of SYBERGOLD™ is that the phages seem to have a small amount of DNA being that SYBERGOLD™ binds to DNA and fluoresces under the right nanometers of light. If there were a small amount of DNA per phage then the “glow” would be hard to detect. Our initial microscopy showed that there were very dull glowing objects. We increased the normal concentration of the SYBERGOLD™ so that the small concentration of DNA will fluoresce and hence be clearly visible as well as easily counted.
- g. Label Petri dishes with the same labels as the Falcon tubes;
- h. Transfer 100 ul of the 5x SYBERGOLD™ to the bottom half of the Petri dish;

- 5
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- i. Once the filtering is complete remove the apparatus to expose the filter (do not turn the vacuum pump off until all filters have been removed. Failure to do so will result in a ruptured filter and you having to start over);
 - j. Place the filter on 100 ul of SYBERGOLD™. Recover the Petri dish and then place in a dark area for ten minutes;
 - k. Once the time is up place 100 ul of Sigma water on the opposite side of the Petri dish and transfer the filter onto the water. Be sure to physically pick up the filter. If you simply drag it from the SYBERGOLD™ to the water then you will not properly stop the SYBERGOLD™ reaction. Leave the filter in the water for two minutes.
 - l. Once the 2 minutes are done take a few kem-wipes and place them on the lab bench on top of another. Then remove the filter and place it upon the kem-wipes so that the excess water will diffuse away from the filter.
 - m. Now that the filter has been drained of excess water, make a slide with the same label as the Falcon tube from contents was placed on to the filter. Place 10 ul of mounting solution to the slide. Take the filter and place it upon the mounting solution. Then take another 10 ul of mounting solution and place it upon the filter. Finally place a cover slip on top of the filter.
 - n. You can now check the slide using a fluorescence microscope and the oil immersion objective to verify that you have phages. If your phages are dimly lit, you will have to change the concentration of the SYBERGOLD™ to 10x. If it is at the correct concentration then when you look through the eyepiece it should look similar to a clear night in the desert sky. Meaning you should see something similar to a sky full of stars.
 - o. You can also do counts to estimate the number of phages in the sample as well as to see the amount phages lost during the filtering process and also how many phages are still in the supernatant fraction of the CsCl process. This can be done through a program called SCOPE PRO VERSION 5.1™ (Media Cybernetics, Silver Spring, MD).
13. Formamide Extraction of DNA from Phage Isolate is as follows:
- a. Add 0.1 volume 2 M TrisXCl (pH 8.5)/0.2 M EDTA, 100 µl 0.5 M EDTA per 10 mls, 1 volume formamide, and 10 µl glycogen.

- b. Incubate at room temperature for 30 minutes and then add 2 volumes of room temperature 100% ethanol.
- c. Pellet in SORVALL™ (12,000 rpms for 20 min) and wash with 70% ethanol two times.

5 d. Resuspend into 567 µl TE and continue with CTAB extraction.

CTAB (DNA clean up from formide extraction)

- 1. Resuspend pellet in 567 µl TE
- 2. Add 30 µl of 10% SDS and 3 µl of 20 µg/ml proteinase K (in freezer), mix, incubate 1 hr at 37°C.
- 10 3. Add 100 µl of 5 M NaCl and mix thoroughly.
Add 80 µl CTAB/NaCl solution, mix, incubate 10 minutes at 65°C
- 4. Add equal volume of chloroform, mix, microcentrifuge 5 minutes, transfer supernatant to separate tube;
Add equal volume phenol/chloroform to the supernatant fraction,
- 15 mix, microcentrifuge 5 minutes, transfer supernatant to separate tube;
- 5. Add equal volume of chloroform to the supernatant fraction, mix, microcentrifuge 5 minutes, transfer supernatant to separate tube;
- 6. Add 0.7 volume isopropanol to the supernatant fraction, mix gently until DNA precipitates, centrifuge 15 minutes in cold;
- 20 7. Wash with 70% ethanol;
- 8. Re-suspend in 50 µl Sigma water and check O.D. using a NANODROP™ spectrophotometer (NanoDrop Technologies, Wilmington, DE).

[0038] While the invention is susceptible to various modifications and alternative forms, 25 specific examples thereof have been shown by way of example in the drawings and are herein described in detail. It should be understood, however, that the invention is not to be limited to the particular forms or methods disclosed, but to the contrary, the invention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the claims.

WHAT IS CLAIMED IS:

1. A method for isolating a virus nucleic acid-comprising sample from a sputum sample, the method comprising the following steps:
 - (a) providing a sputum sample;
 - 5 (b) treating the sputum sample with a reducing agent;
 - (c) adding a buffer to the treated sample of (b);
 - (d) mechanically homogenizing the sample of (c);
 - (e) centrifuging the sample of (d) to generate a sample pellet;
 - (f) suspending the pellet of step (e) in a buffer and preserving an aliquot with an equal
10 amount of para-formaldehyde or equivalent;
 - (g) centrifuging the resuspended pellet of (f) in density gradient comprising at least a 1.5 g/ml density band; and,
 - (h) isolating a nucleic acid-comprising sample from the 1.5 g/ml density band.
2. The method of claim 1, wherein the sputum sample is a human sputum sample.
- 15 3. The method of claim 1, wherein the reducing agent is dithiothreitol (DDT).
4. The method of claim 3, wherein the sputum sample is mixed in a solution of about 5%, 6%, 7%, 8%, 9% or 10% or more dithiothreitol (DDT).
5. The method of claim 4, wherein full strength dithiothreitol (DDT) is mixed in a magnesium-based buffer to generate the DTT solution.
- 20 6. The method of claim 1, wherein the sputum sample has a volume of about 10 mls, or from between about 1 to 25 mls, or from about 3 to 20 mls, or from about 6 to 15 mls, or from about 8 to 12 mls.
7. The method of claim 1, wherein the buffer comprises a magnesium-based buffer.
8. The method of claim 7, wherein the buffer comprises a TRIS™ buffer with
25 magnesium salt.
9. The method of claim 1, wherein the suspended pellet sample of step (f) is partially purified of (cleared of) bacterial and other cellular debris from the viral component of the suspended pellet sample before the centrifugation step.
10. The method of claim 9, wherein the suspended pellet sample of step (f) is
30 partially purified of (cleared of) bacterial and other cellular debris by using one or more filters, and/or one or more chromatographs.

11. The method of claim 10, wherein the chromatograph comprises affinity chromatography, lectin chromatography, antibody chromatography, diethylaminoethyl cellulose chromatography, ion exchange chromatography and/or high performance liquid chromatography (HPLC).

5 12. The method of claim 10, wherein the one or more filters is a 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8, 0.9 and/or 1 micrometer filter.

13. The method of claim 1, wherein the density gradient comprises a CsCl gradient.

14. The method of claim 1, further comprising isolating a phage or a bacterial nucleic acid from the viral nucleic acid isolated in step (h).

10 15. The method of claim 14, further comprising sequencing the isolated nucleic acid.

16. The method of claim 15, further comprising comparing the sequenced viral nucleic acid to databases of viral nucleic acids to identify the family or species of the isolated virus.

17. A method for isolating a viral nucleic acid from a sputum sample, the method
15 comprising the following steps:

(a) providing a sputum sample;

(b) treating the sputum sample with a reducing agent;

(c) adding a buffer to the treated sample of (b);

(d) mechanically homogenizing or macerating the sample of (c);

20 (e) generating a virus-comprising pellet or equivalent from the sample of (d);

(f) suspending the virus-comprising pellet or equivalent of step (e) in a buffer;

(g) centrifuging the suspended pellet of (f) in a density gradient comprising at least a 1.5 g/ml density band; and,

(h) isolating a viral nucleic acid-comprising sample from the 1.5 g/ml density band.

25 18. A method for isolating a virus nucleic acid from a sputum sample, the method comprising the following steps:

(a) providing a sputum sample;

(b) treating the sputum sample with a reducing agent;

(c) adding a buffer to the treated sample of (b);

30 (d) mechanically homogenizing or macerating the sample of (c);

(e) generating a virus-comprising pellet or equivalent from the sample of (d);

(f) suspending the virus-comprising pellet or equivalent of step (e) in a buffer; and

(g) isolating a virus from the sample.

19. The method of claim 18, wherein virus nucleic acid is isolated by a density gradient, a filter and/or a chromatograph.

20. The method of claim 19, wherein the chromatograph comprises affinity
5 chromatography, lectin chromatography, antibody chromatography, diethylaminoethyl cellulose chromatography, ion exchange chromatography and/or high performance liquid chromatography (HPLC).

21. The method of claim 19, wherein the density gradient comprises a CsCl gradient.

22. The method of claim 17 or claim 18, further comprising isolating a bacterial or a
10 phage nucleic acid from the isolated nucleic acid.

23. The method of claim 22, further comprising sequencing the isolated nucleic acid.

24. The method of claim 23, further comprising comparing the sequenced viral
nucleic acid to databases of viral nucleic acids to identify the family or species of the isolated
virus.

25. The method of any of claims 1 to 24, wherein the sputum is isolated from a
15 human.

26. The method of any of claims 1 to 24, wherein the sputum is isolated from a
patient with and acute or a chronic pulmonary infection, disease and/or condition.

27. The method of claim 26, wherein the acute or chronic infection, disease and/or
20 condition is the flu, or is cystic fibrosis, or is a viral bronchial or lung infection.

28. The method of claim 17 or claim 18, further comprising culturing the isolated
virus.

29. A method for isolating nucleic acid from a bacteria, a phage and/or a virus from a
sputum sample, the method comprising the following steps:

25 (a) providing a sputum sample;

(b) treating the sputum sample with a reducing agent;

(c) adding a buffer to the treated sample of (b);

(d) mechanically homogenizing the sample of (c);

(e) centrifuging the sample of (d) to generate a sample pellet;

30 (f) suspending the pellet of step (e) in a buffer and preserving an aliquot with an equal
amount of para-formaldehyde or equivalent;

(g) centrifuging the resuspended pellet of (f) in a density gradient comprising a density band that isolates a viral nucleic acid, a phage nucleic acid and/or a bacterial nucleic acid; and,

(h) isolating the phage, viral and/or bacterial nucleic acid from the density band.

30. The method of claim 29, wherein the

5 31. A kit comprising the solutions for practicing any of the methods of claims 1 to 28, and optionally the kit further comprises instructions for practicing any of the methods of claims 1 to 28.

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