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Acquisition and processing of nonhuman primate samples for genetic and phylogenetic analyses

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Abstract

Primates have long been a favorite subject of evolutionary biologists, and in recent decades, have come to play an increasingly important role in biomedical research, including comparative genetics and phylogenetics. The growing list of annotated genome databases from nonhuman primate species are expected to aid in these endeavors, allowing many analyses to be performed partially or even entirely *in silico*. However, whole genome sequence data are typically derived from only one, or at best a few, individuals. As a consequence, information in the databases does not capture variation within species or populations, nor can the sequence of one individual be taken as representative across all loci. Furthermore, the vast majority of primate species have not been sequenced, and only a small percentage of species are currently slated for whole-genome sequencing efforts. Finally, for many species data on patterns and levels of RNA expression will be lacking. Thus, there will continue to be a demand for samples from nonhuman primates as raw material for genetic and phylogenetic analyses. Gathering such samples can be complicated, with many legal and practical barriers to obtaining samples in the field or transporting samples between research centers and across borders. Here, we provide basic but critical advice for those initiating studies requiring genetic material from nonhuman primates, including some guidance on how to locate and obtain samples, brief overviews of common protocols for handling and processing samples, and a table of useful links for locating resources related to the acquisition of samples. We also advocate for the creation of curated banks of nonhuman primate samples, particularly renewable sources of genetic material such as immortalized cell lines or fibroblasts, to reduce the need for repeated or redundant sampling from living animals.

INTRODUCTION

Because of their close phylogenetic relationship to humans, nonhuman primate species have long been a favorite subject of naturalists, anthropologists and evolutionary biologists. A wide variety of nonhuman primates also serve as biomedical models of human

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physiological, neuropsychiatric, behavioral, genetic and infectious diseases. Primates are also important for comparative approaches to understanding human behavior and disease, and for reconstructing the molecular evolution of genes and genomes. Whole genome sequences (WGS) of several nonhuman primate species are now available, and several more are in the pipeline. While these will serve an important role in genetics and genomics research and molecular evolutionary studies, there are important limitations. Other than humans, most primate WGS projects are derived from a single individual or type specimen, and thus fail to capture population level data (polymorphism). Thus, for any given locus, the WGS data may by chance consist of a rare or non-representative allele, particularly if the target sequence is polymorphic. As a consequence of approaches to assembling genome sequence data, there is always the possibility that a particular gene or region is missing or incomplete. For all of these reasons, many studies will require collection and analysis of additional samples, either from multiple individuals and different populations within a species, or from individuals representing diverse primate lineages, or both.

Many nonhuman primate species are endangered, and there are often additional practical barriers to the acquisition of appropriate samples, and samples once obtained may be precious and limited. Thus, it is critical that samples be handled appropriately, and that measures are taken to ensure the quality and efficiency of extraction of nucleic acid. In addition, all investigators should be encouraged to use renewable or semi-renewable sources of genetic material, such as fibroblasts or immortalized cell lines, or the use of non-invasive sources of material, such as feces and hair, whenever reasonable with regard to experimental objectives.

Recent legal restrictions on the distribution of continuous cell lines seems counterproductive, potentially favoring the collection of primary samples from individual animals over the non-invasive distribution of existing cells and cell-lines. If large banks of renewable material from nonhuman primates can be assembled, in much the same way that human geneticists bank and curate cohorts of immortalized B-cell lines, then for many studies the need to disturb primates in the wild (or in captivity) could potentially be reduced. Ideally, such a bank would consist of multiple lines representing a diverse array of primate lineages, including both male and female lines, and whenever possible, would also include samples from confirmed dam/sire/offspring trios.

OBTAINING NONHUMAN PRIMATE SAMPLES

For studies in which geographical or population level information is crucial, and for which samples collected in the field are required, investigators will necessarily have to pursue appropriate collaborative arrangements. However, a surprising diversity of primate species can also be found in captivity, in primate research centers, sanctuaries and zoological gardens around the world. Keep in mind that the primary function of many of these institutions is not necessarily to provide samples to biomedical researchers on demand, and preliminary inquiries should be made well in advance of any perceived need.

Investigators must be aware of national and international laws governing transport of primate samples, regardless of source, and acquire any necessary permits. A useful starting point is the website of CITES (Convention on International Trade in Endangered Species of Wild Fauna and Flora) at <http://www.cites.org/index.html>. CITES member states utilize a system of permits to govern the import and export of animals and samples derived from animals. International permits are often necessary for export (state of origin) and in many cases an import permit is also required. In addition, it is important to determine whether national laws are in place in the country of origin or the destination that are stricter or supercede CITES guidelines. For example, while CITES Appendix II specimens may only

require an export permit, the destination country may, for a variety of reasons, still have its own laws regarding import of samples from specific sources.

The National Center for Research Resources of the U.S. National Institutes of Health (NIH) maintains a Primate Resources listing, largely dedicated to the extensive network of primate research centers and resources within the U.S.

(www.ncrr.nih.gov/comparative_medicine/resource_directory/primates.asp). Table 1 contains a partial list including the National Primate Research Centers (NPRC). For a more exhaustive list of academic and commercial primate centers in the U.S. and around the world, the University of Wisconsin maintains a very useful website (Primate Info Net), which includes an extensive international directory of primatology (<http://pin.primate.wisc.edu/idp/>), as well as a variety of useful resources including species-specific datasheets and extensive audiovisual materials.

The newly formed National NHP DNA Bank is described elsewhere in this issue (xxx). The collection contains DNA samples from several species housed at the various U.S. National Primate Research Centers (Table 1), and information can be found via the Primate Info Net (see above). The contents of the National NHP bank are designed to provide a limited diversity of samples across the NPRCs, but are currently not sufficient to meet all research needs (for example, extended family studies, samples unique to particular studies, alternative species not found within the NPRC or field studies). Further, the current contents of the National NHP Bank are limited to DNA only. Thus there remains a need to collect and process other types of biological samples, including RNA or cell types for use in genomic studies.

When tissue source is not an essential criterion, samples can be obtained most readily from blood. It should be kept in mind, however, that even obtaining a blood sample can be complicated if it requires removing an animal from a peer group, excessive handling of the animal, and/or the use of anesthesia, or if obtaining the sample involves significant risk on the part of the human handlers (e.g., risk of physical injury or exposure to infectious pathogens). For these sorts of reasons, requests for samples are sometimes filled only at specific times - for example, when an animal requires medical attention or is scheduled for a routine veterinary examination. Although technically more difficult, archived solid tissue samples or shed material, such as feces or hair, can also be used.

Before making inquiries for samples from any source (NPRC, zoo, etc.), the investigator should also obtain information regarding the phylogeny and basic physiology of the species of interest, making sure that the requested species is relevant to the study design, and to determine whether there are additional issues that may confound analysis. For example, many aspects of primate phylogeny remain unresolved, and it is not unusual for the nomenclature to change as species are reclassified. In addition, primates range in size from diminutive 50 g mouse lemurs to large, 200 kg gorillas, and the amount of blood that can be obtained in a single draw varies accordingly. Sometimes, unique aspects of physiology can also affect genetic studies. New world monkeys in the Callitrichidae family (e.g., tamarins and marmosets) are usually born as fraternal twins, and placental fusion during development results in bone-marrow chimerism (1,2). As a result, genomic DNA samples from one sibling will contain alleles from the other sibling (for example, samples obtained from a female with a male twin will contain Y-chromosomes). For these kinds of reasons, and because many primate samples are rare or difficult to obtain, efforts to determine the necessity of the specimen and its usefulness to the study should be made prior to making a formal request.

PRIMATE TISSUES ARE POTENTIAL SOURCES OF MICROBIAL PATHOGENS

Captive animals, just like their wild-roaming counterparts, are frequently infected with a variety of microbial pathogens and thus pose a potential health risk to anyone working with nonhuman primate samples. Because of their close phylogenetic affinity and ecological similarities to humans, nonhuman primates play hosts to a variety of microbial agents with a particularly high potential for zoonotic transmission. This is true of both wild-caught and captive-bred animals. While some captive colonies are bred and handled to be free of specific pathogens, it is extremely unlikely that the full spectrum of microbial fauna associated with nonhuman primates is known, and samples from any source should be treated as potentially biohazardous and through the use of universal precautions. Many homologues of human pathogens have been detected in nonhuman primates. Importantly, the lack of overt pathogenesis in a nonhuman primate does not predict outcome if a zoonosis occurs. For example, an alpha herpesvirus that is prevalent among macaque species (Herpesvirus B or xxx) is essentially apathogenic in its host, but causes a rapid and often fatal infection in humans. In summary, the handling of nonhuman primate material requires application of universal precautions, including the wearing of non-porous protective garments and gloves, eye/face protection, and the use of puncture-resistant containers for the disposal of sharps.

SOURCES OF GENETIC MATERIAL

Blood

Blood is a good source for cells, DNA and RNA. It is relatively easy to obtain, and as with human subjects, can be drawn repeatedly from the same individual(s). Although not formally considered an invasive procedure, blood sampling from nonhuman primates is not nearly as straightforward as drawing blood from human volunteers or small laboratory animals. Due to the potential presence of blood-borne pathogens, caution is advisable when handling blood samples. Unless a blood sample can be processed immediately after it is drawn, it will also have to be chemically treated to prevent coagulation. Ideally, one should consult with the veterinary staff responsible for obtaining the sample(s), or from whom the samples are being requested, to determine which methods are available or recommended. In many cases, blood is collected directly into tubes that have been pre-treated with anticoagulant, such as color-coded Vacutainer Tubes (Becton, Dickinson and Company, Franklin Lakes, NJ), and can be shipped overnight at room temperature. Several chemical compounds are commonly used as anticoagulants, including EDTA, citrate, heparin and oxalate. It is also important to ascertain whether the chosen anticoagulant will interfere with downstream applications; for example, heparin can inhibit downstream enzymatic assays, and should be avoided for PCR-based assays on plasma/serum or on DNA directly extracted from whole blood (3). Blood vials should be stored at room temperature, since storage at 4°C accelerates blood clotting. Keeping vials gently mixing on a platform rocker is also a good idea, particularly if samples cannot be processed right away.

Peripheral Blood Mononucleated Cells (PBMC)

Separating the lymphocytic cell fraction of blood from the plasma and erythrocyte fraction is necessary for the cultivation of any type of peripheral blood mononucleated cells (PBMC) or specific cell types like B or T lymphocytes. This can be done through density gradient centrifugation in the presence of a separation matrix, such as Ficoll™ (GE Healthcare Bio-Sciences). This neutral, high mass, hydrophilic polysaccharide allows for an efficient separation of three fractions: blood plasma, buffy coat and erythrocytes/granulocytes. The

buffy coat fraction contains lymphocytes, monocytes, macrophages and platelets, and is therefore enriched for cells containing genomic DNA.

Immortalized cell lines

Generating banks of renewable sources of DNA or RNA, such as immortalized cell lines or expanded fibroblasts, should be a priority for the community of researchers interested in the genetics or evolution of nonhuman primates. Ultimately, the goal should be to establish a large collection of renewable nonhuman primate resources accessible to the scientific community. Such an effort would minimize the need for repeated invasive procedures or handling of captive animals for those studies where all that is required is a source of genetic material, and would assist researchers in cases where legal restrictions or other practical barriers prevent the collection of primary samples. In addition to serving as a source of genetic material, in some cases cell lines may be useful for additional biological or biochemical assays to complement or test hypotheses born out of genetic or phylogenetic analyses. A potential caveat to the use of cell-line derived genetic material is always the possibility that genetic alterations accumulating in the lines themselves are misinterpreted as genetic variation present in the individual or species from which the lines were obtained. The two methods described here, B-cell immortalization and expansion of skin-punch fibroblasts, are generated by bulk methods (no “bottleneck”) and are generally oligoclonal (unless they have gone through a cloning step or have been passaged extensively), and may therefore be preferable to other methods in which spontaneous changes can become over-represented. In addition, it would be advisable whenever possible to confirm findings with samples from additional individuals and by reference to existing genomic/sequence data.

A common method for rapid and fairly straightforward production of continuous human cell lines relies on virally induced transformation of B-cells using Epstein-Barr Virus (EBV), a human herpesvirus. Several related viruses are available for transformation of B-cells from other primate species (see below). The approach is desirable, because target cells are abundant in blood and readily obtainable by phlebotomy, because the resulting cells are nonadherent and therefore easy to expand in tissue culture, and because of the relatively high efficiency of transformation compared to other approaches.

To produce B lymphoblastoid cell lines (BLCL), purified peripheral blood lymphocytes are subjected to infection with a suitable virus of the *Lymphocryptoviridae* (LCV; family: *Herpesviridae*, subfamily: *Gammaherpesviridae*) to establish a permanent, latent infection of B lymphocytes in the cell mixture. While all other cell types die, an emergence of immortalized B lymphocytes is achieved due to the expression of latent viral genes (4,5). Generally, cells do not produce infectious virions. Outgrowth is typically oligoclonal, although extensive passage is likely to eventually result in a clonal line. It is important to mention that in many cases LCVs only establish infection and growth transformation in cells of the autologous and related host species (4). EBV, for example, can immortalize human and chimpanzee B cells, whereas rhesus LCV can transform rhesus and cynomolgus macaque B cells (4). Thus, depending on the species of interest, a suitable transforming virus must be obtained, and for many primates, useful viruses may still need to be isolated and characterized. T lymphocytes can be immortalized as well, using herpesvirus saimiri (HVS), but the procedure is less efficient than LCV immortalization of B-cells (6).

When working with EBV and related viruses, strict safety procedures are advisable. Almost 95% of the US population 35 years of age or older are EBV positive and control the infection, which usually originates in childhood. Contraction of EBV at a post-childhood age can be problematic, though, since it can cause infectious mononucleosis. This disease can have a quite severe course in adolescents and adults (7). It is not known whether and to what degree LCVs of other primate species are transmissible to humans, but virus-producer

cell lines and viral stocks should be handled using universal precautions and the appropriate biosafety-level procedures.

Fibroblasts

An alternate source of continuous cells can be obtained by establishing fibroblast cultures from small punch-biopsies taken from skin. Cells obtained in this way can be rapidly expanded and multiple aliquots stored viably for further expansion. While fibroblasts are not immortalized (and cannot be passaged indefinitely), it is possible to transform skin fibroblasts through the ectopic expression of transduced telomerase (8) or introduction of viral oncogenes (e.g., the E6-E7 proteins of papillomavirus (9)).

Feces

Owing to the remote location of wild populations and national and international regulations governing transportation of products derived from endangered species, there are situations where it is impossible to obtain tissue samples normally utilized for genetic analysis. In such instances it may be possible to isolate genetic material from fecal or other sample types, such as hair, that can be collected using non-invasive methods. While fecal material is a non-ideal starting source for genetic analyses, it has the advantages of being relatively plentiful and easy to identify, collect, and store. The benefits of using fecal samples as a starting material were recognized early, however fecal material is a rather complex starting material with nucleic acids present from commensal bacteria and undigested food, in addition to shed intestinal cells. Furthermore, feces are rich in nucleases and proteases, as well as plant polysaccharides whose structure mimics that of DNA and can inhibit further enzymatic reactions (10,11). Due to these limitations, it was not until the early 1990's that a protocol was developed yielding DNA suitably pure for subsequent molecular manipulation (12). This advance initially allowed for analysis of host mitochondrial DNA as well as ingested plant material (13), and was followed by analyses of nuclear DNA from shed intestinal cells (14). Subsequently, commercial products such as RNAlater (Qiagen) and QIAGEN's Stool Mini Kit® (Qiagen) have become available easing preservation and isolation of DNA from stool samples. Even with such advances, DNA yields are low, and contamination with enzymatic inhibitors remains a problem. In spite of these limitations, these advances have facilitated, among other things, complex studies surveying wild Great Ape populations for SIV infection, which utilized fecal samples as a source of genomic DNA, viral RNA, and anti-SIV antibodies (15-18). In these studies, whole viral genomes were analyzed, which was possible due to protection of the viral RNA from nucleases. Because many of the fecal samples that were collected were of unidentified origin, microsatellite analysis was also successfully performed on DNA purified from the feces to confirm the source. Utilization of DNA isolated from fecal samples has, to our knowledge, been limited to amplification of small segments of chromosomal DNA. Obviously, DNA of a quality where only small segments of DNA can be amplified is of limited applicability. With that in mind, using similar methods as Santiago et al. (17) we have successfully been able to amplify multi-exonal segments of genomic DNA approximately 2.5 kb in size from *Ateles geoffroyi* fecal samples, suggesting broader applicability (unpublished data). With additional manipulations of the purified fecal DNA, such as treatment with DNA "repair" enzymes (e.g., PreCR® from New England Biolabs, Beverly, MA) followed by total DNA amplification, DNA with near universal applicability can be obtained from fecal samples.

PROTOCOLS

Here we present a brief selection of methods for obtaining and generating nonhuman primate samples as a source of nucleic acid for genetics, genomics and molecular evolutionary studies. The protocols as given are intended only as guidelines for planning

experiments, and the reader is strongly encouraged to consult the bibliography for more detailed protocols and more comprehensive sources of technical information, as well as relevant background material.

Extraction of fibroblasts from skin patches

Materials

1. GIBCO® DMEM with 20% fetal bovine serum (FBS) supplemented with 10mM HEPES, 2mM L-glutamine, Pen/Strep (penicillin 50 IU/ml and streptomycin 50µg/ml), Vancomycin (30µg/ml), and Gentamicin (10µg/ml).
2. Sterile forceps, microscope slides, scalpel, Petri dishes
3. trypsin/EDTA (0.05% trypsin, 0.53 mM EDTA)
4. Sterile Phosphate-Buffered Saline (PBS)

Procedure

1. 4-6 mm skin punch is acceptable. Working in laminar flow hood, remove skin biopsy from conical by using 10 ml pipette and suction. Place biopsy in Petri dish with approximately 5 ml of DMEM. Using scalpel and forceps, carefully dissect off subcutaneous fat and any hair present on biopsy.
2. Using scalpel, score bottom of second Petri dish with cross-hatched lines at 2-3 mm intervals. Wash off any plastic chips with medium.
3. Using forceps, transfer skin biopsy to scored Petri dish with around 5 ml of medium. Using forceps and scalpel, carefully dissect biopsy in to 1-2 mm pieces. Group pieces together over scored area.
4. After dissection is completed, use sterile forceps to place microscope slide over pieces. Gently add 5 ml additional medium.
5. Place Petri dish in humidified 37°C incubator
6. Examine under inverted scope on day 4 for fibroblast growth. Perform partial medium exchange with approximately 5 ml medium every 3-4 days.
7. When growth is confluent (10-14 days) wash plate with 5 ml sterile PBS.
8. Add 5 ml of trypsin-EDTA and place plate in incubator for 2 min
9. Check under inverted scope for cells dissociating from the plate. If necessary incubate longer checking every 2 minutes.
10. Using sterile forceps, lift slide and turn over, incubate 1-2 minutes longer.
11. Add 5-6 ml of 20% DMEM, resuspend cells using a pipet and place in 15 ml conical centrifuge tube.
12. Pellet cells at 1,500 rpm for 10 min, resuspend in 5.0ml of 20% DMEM and place in a T25 flask.
13. Feed twice weekly with 20% DMEM
14. When cells are confluent, wash once with sterile PBS, add 5.0 ml trypsin, incubate for 2-3 min until cells are coming off the plate. Add 5-6 ml of 20% DMEM to stop trypsinization, then hit bottom of flask to loosen cells. Count and plate.
15. T25 - 0.5-1.0 million cells
T75 - 2.0 million cells

T225 - 5.0 million cells

24 well - 0.06 million/well

Immortalization of macaque B-lymphocytes

The following basic procedure is used to generate immortalized macaque BLCL using rhesus LCV (sometimes called rhEBV) or *herpesvirus papio*, and is essentially the same protocol for using EBV to immortalize human cells. LCV with tropism for other species can potentially be applied in the same way (5).

Material

1. Hank's Buffered Saline Solution (HBSS)
2. GIBCO® RPMI 1640 medium supplemented with 10% FBS and 0.2% Primocin™ as an antibiotic
3. R20 = GIBCO® RPMI 1640 medium supplemented with 20% FBS, 1% Penicillin/Streptomycin (Pen: 10.000U/ml, Strep: 10.000 µg/ml) and 1% L-glutamine (200µM)
4. Optional: Cyclosporine A (CsA), 1 mg/ml. CsA eliminates cross-reactive T-cells that may be present in purified PBMC because LCV infection is widespread in primates. The presence of anti-LCV T-cells can reduce the efficiency of transformation and outgrowth of B-cell lines.
5. Optional: Azidothymidine (AZT), 1mM. Foamyviruses (retroviruses in the *Spumavirus* genus) are a common contaminant in blood samples, and although they are not known to be pathogenic in humans or other primates, they readily infect cells in culture, leading to vacuolation, formation of syncytia and cell death (19). Addition of the nucleotide analogue AZT inhibits replication of simian foamy viruses. AZT is a reverse-transcriptase inhibitor, and can be included during transformation and expansion of lines to prevent foamyvirus replication and preserve cell lines.

Procedure

6. Isolate PBMC as usual or thaw frozen PBMC, split 4×10^6 cells in two aliquots and spin cells down at 1000 rpm for 10 min at room temperature
7. Resuspend pellet in 0.5 ml R20 and transfer to a 24-well plate, add 1 µl CsA and 0.5 ml filtered rhesus LCV (rhLCV) supernatant, incubate at 37°C. NOTE: Alternatively, instead of rh LCV, *Herpesvirus papio* can be used. This virus is produced by S594 cells (NIH Nonhuman Primate Reagent Resource).
8. Once medium yellows (after 3-8 days), add 1 ml R20.
9. Continue feeding with R20 twice a week until transformation occurs (evidenced by cell clumping).
10. When cells are growing well, split each well into two (you now have four wells per animal) and switch medium to R20/AZT.
11. At the next feeding time point, combine all four wells of one animal, mix well and transfer cells to T25 tissue culture flask.

Production of rhLCV in H254 Cells

12. Thaw H254 cells and place in 30 ml HBSS. Spin cells down at 1000 rpm for 10 min at room temperature.
13. Resuspend pellet in 10 ml RPMI/FBS/Primocin. Add cells to a TC25 flask and place in incubator standing up.
14. When media yellows (about every 5 days), remove 3/4 culture for expansion at 1:4 or discard. Replace media to 10 ml total volume.
15. rhLCV can be isolated by removing culture supernatant without capturing too many cells and filtering it through a 0.45 μ m filter.
16. Freeze 1 ml aliquots at -80°C.

Titration of rhLCV

17. Thaw and spin down frozen rhesus PBMC as before. Remove all but ~ 5 ml HBSS and resuspend cells in remainder by smacking against hood. Add 12 ml RPMI/FBS and plate at 1 drop per well in 96-well plates containing macrophage feeder cells.
18. For each virus, 4 columns and 4 rows plus one negative control well for the whole titer plate will be needed. Dilute each virus in quadruplicate to 10^{-2} , 10^{-3} , 10^{-4} , 10^{-5} and add 100 μ l to each well. Incubate 10 days in incubator.
19. After 10 days, check wells for clumping (indicative of viral infection).

DNA Isolation from RNAlater Preserved Stool Samples

Material

1. QIAGEN RNAlater
2. QIAamp DNA stool kit
3. Proteinase K

Procedure—Stool should be collected fresh mixed 1:1 with RNAlater, stored cold until it can be aliquotted in 1ml aliquots and frozen at -80°C

1. Thaw stool/RNAlater sample on ice and split 1ml aliquot into $5 \times 200 \mu$ l aliquots (in 2 ml microcentrifuge tubes). For best results it is recommended that 2 or fewer stool samples be processed at a time (i.e. 5 or 10 minipreps).
2. Add 1.6 ml Buffer ASL to each sample and vortex continuously for 1 min.
3. Centrifuge sample at full speed for 1 minute.
4. Transfer 1.4 ml of the supernatant into a new 2 ml microcentrifuge tube (discard pellet).
5. Add 1 InhibitEX tablet to each sample and immediately vortex for 1 min, or until tablet fully dissolves.
6. Incubate for 1 min at room temperature, then centrifuge at maximum speed for 5 min.
7. During spin, add 25 μ l Proteinase K into new 2 ml microcentrifuge tubes.
8. After spin, transfer 600 μ l supernatant into Proteinase K-containing tubes.
9. Add 600 μ l Buffer AL and vortex for 15 sec.

10. Incubate at 70°C for 10 min.
11. Add 600 µl 100% EtOH to lysate and vortex.
12. Transfer 600 µl lysate to spin column and centrifuge at full speed for 1 min. Discard flow-through and repeat until all of the lysate has passed through column.
13. Move spin column to a fresh 2 ml collection tube, add 500 µl Buffer AW1 and spin for 1 min at full speed.
14. Move spin column to a fresh 2 ml collection tube, add 500 µl Buffer AW2 and spin for 1 min at full speed.
15. Move spin column to a fresh 2 ml collection tube and spin for 2 min at full speed.
16. Move spin column to 1.5 ml microcentrifuge tube. Pipet 200 µ Buffer AE directly onto membrane. Incubate for 1 min at room temperature and centrifuge for 1 min at maximum speed.
17. Recommended: Break 200 µl eluate into 50 µl aliquots and freeze at -20°C. If this is done, you will have 20 - 50 µl DNA aliquots from 1 ml starting stool/RNAlater sample.
18. Spec DNA. Use about 50 ng total DNA per PCR reaction in downstream applications.

Extraction of RNA from Fecal Samples in RNAlater

(Using RNAqueous-Midi kit)

Procedure

1. Add 5 ml “Lysis/Binding Solution” to (labeled) 15 ml conical tube.
2. Using a 1 ml pipette with the tip removed transfer the contents of 1 fecal sample tube to the 15 ml conical tube.
3. Wash 2 ml fecal sample tube with 1 ml fresh “Lysis/Binding Solution” and transfer remnants to 15 ml conical tube.
4. Vortex sample for 30 sec.
5. Spin for 20 min at maximum speed (3270xg) in table-top centrifuge (verify that all of the particulate matter has been pelleted - ie. the solution is no longer cloudy).
6. Using a 3 ml syringe with an 18 gauge needle, transfer solution to new 15 ml conical tube.
7. Add 6.5 ml 64% EtOH and mix by inversion (~ 3x).
8. Remove plunger from 20 ml syringe and attach a “disposable filter unit”.
9. Transfer sample to syringe and slowly (3-5 drops per second) push solution through filter.
10. Remove filter unit, retract the plunger, reattach filter and force air through the filter ~ 2-3 times until no more foamy material is expelled.
11. Remove filter unit, remove the plunger, reattach filter, add 14 ml “Wash Solution #1” and slowly pass the solution through the filter.
12. Force air through syringe as in step #10.
13. Wash with 10 ml “Wash Solution #2/3” as in step 11.

14. Repeat step 13.
15. Remove filter unit, retract the plunger, reattach filter and vigorously force air through the filter times until no droplets or fine spray comes out (~ 10x), blot liquid remaining on tip onto a clean Kimwipe.
16. Heat “Elution Solution” to ~ 100°C in a boiling water bath.
17. Label and add 0.5 ml LiCl to collection tube.
18. Remove plunger from 3 ml syringe, attach filter unit, add 0.5 ml hot “Elution Solution” and push through filter into collection tube.
19. Repeat step 18.
20. Incubate at -20°C for at least 30 min.
21. Spin at maximum speed for 15 min at 4°C.
22. Carefully decant supernatant.
23. Add 1 ml 70% EtOH, briefly vortex, and re-spin as in step 21.
24. Carefully decant supernatant.
25. Briefly spin, and use 200 µl pipette tip to remove remaining EtOH, being careful not to disturb pellet.
26. Briefly dry pellet, add 50 µl “Elution Solution”, vortex, heat at 60°C for 10 min, vortex.
27. Determine RNA concentration and freeze in aliquots.

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