

Purification of mitochondrial and plastid DNA

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The size, structure and conformation of mitochondrial and plastid genomes differ dramatically among eukaryotes. Similarly, the yield and purity of extracted organelle DNA also vary, and are crucial factors for the success of restriction mapping and sequencing experiments. We describe here procedures for the purification of organelle DNA from a broad range of eukaryotes. By emphasizing the underlying principles, these procedures will facilitate the development of new species-specific protocols. The presented purification schemes involve either isolation of organelles and subsequent extraction of DNA from this subcellular fraction, or processing of whole-cell lysates followed by CsCl gradient centrifugation to separate nuclear and organelle DNAs according to their A + T content. We have successfully used the described procedures for organelle genome sequencing from diverse eukaryotes, including non-axenic protists. Procedures can be completed in 3–5 days, typically yielding a few micrograms of DNA—ample for sequencing complete genomes.

INTRODUCTION

Owing to their small size, mitochondrial and plastid (chloroplast) DNAs (mtDNA, cpDNA) have been among the very first genome sequencing projects^{1,2}. Currently, several thousand complete mtDNA sequences are known, covering nearly all major eukaryotic lineages^{3–5}, but also including numerous sequences from single species such as human (>2,000) and *Plasmodium* (>200). The number of complete cpDNA sequences is much smaller (>80), yet currently increasing at an accelerated pace. Complete mtDNA sequencing has become a gold standard for molecular taxonomy of animals and for population studies, and organelle phylogenomics has shown its value in resolving the early evolutionary history of eukaryotes^{6–8}.

Initially, purification of mtDNA and cpDNA (orgDNA) seemed straightforward, but this was only due to the limited number of model organisms studied at first. Increasing taxonomic breadth of organelle genomics research not only uncovered an astounding variation in orgDNA size, shape, conformation, A+T content and gene order, but it also unveiled organism-specific difficulties of obtaining orgDNA for molecular biology studies. In fact, the yield of purified orgDNA is often deceiving when applying established procedures to poorly studied species, and the varying nature of DNA contaminants may render cloning and sequencing inefficient if not impossible.

This report does not deliver a single, universal recipe for orgDNA isolation, but rather two principal protocol variants (whole-cell lysate and purified mitochondria protocols; Fig. 1) that we have successfully applied to a wide range of eukaryotes. In addition, we describe an alternative procedure that accounts for the massive amounts of polysaccharides in many algal and plant species. For all protocols, we explain details of the underlying biochemical principles and supply advice on troubleshooting. This will facilitate the development of protocols that are tailored to species for which no procedure is available yet. In the following, we will provide general guidelines for the choice of protocol variants.

Purification of DNA from isolated organelles

Conceptually, this is the ideal method as the isolated DNA will be of proven organelle origin. Thus, genuine orgDNA can be distin-

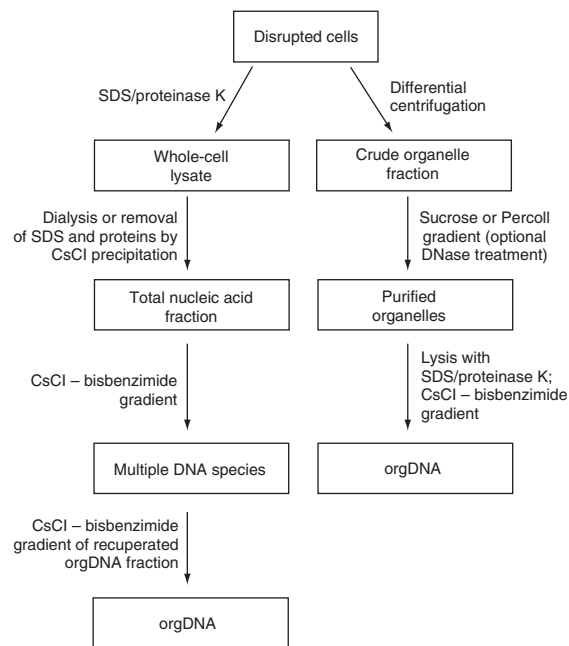


Figure 1 | Schematic representation of the work flow for the whole-cell lysate (left) and the organelle purification procedure (right).

guished from large orgDNA insertions located in nuclear DNA (nucDNA) as documented for human and certain land plants^{9,10}. The extraction of DNA from purified organelles may also be required in cases of unorthodox gene content or genome organization to formally demonstrate this genome's subcellular localization^{11,12}. Finally, organelle purification permits an efficient protection against highly active endonucleases in species that degrade their own DNA upon cell disruption. Examples are the fungi *Smittium culisetae*¹³ and *Basidiobolus ranarum*, whose mtDNAs are only spared when protected within the intact organelle. In other species, this principle can be used to remove



nucDNAs, by intentional addition of DNases that are subsequently inactivated or removed (e.g., sucrose gradients) before organelle lysis (for examples of detailed instructions see refs. 14,15).

Purification of orgDNA from whole-cell lysates

The procedure involves the direct lysis of disintegrated cells (at temperatures up to 50 °C) with a detergent (SDS) plus a proteinase (proteinase K), and without prior enrichment of organelles. It is the most effective way of orgDNA purification both in terms of yield and ease of manipulation, in particular for species that are difficult to grow *en masse*. In addition, these conditions will inactivate most cellular endonucleases. Both SDS and proteinase K can be easily removed in subsequent steps, without the need of solvent extractions, followed by CsCl–bisbenzimidazole gradient centrifugation that separates DNA species according to their A + T content^{16–18}. In most instances, mtDNAs and cpDNAs are more A + T-rich than nucDNA, forming bands in the gradient that can be easily separated. Yet, this procedure fails when other genomic DNAs (nucDNA, DNA from bacterial endosymbionts or food bacteria, other orgDNAs) have a similar A + T content as the orgDNA in question^{19–21}. Milder detergents like Triton X-100, Sarkosyl or cetyl trimethyl ammonium bromide (CTAB) should be used when SDS-solubilized polysaccharides interfere with DNA purification. We provide a specialized CTAB protocol for DNA extraction from polysaccharide-rich algae.

Usually, microgram quantities of orgDNA can be obtained from ~1 to 20 g wet-weight cell material, which are sufficient for complete genome random sequencing. With some experience, much smaller quantities suffice. Yet, the identity of DNAs isolated by whole-cell extraction needs to be validated. Usually, this is demonstrated by a small genome size (e.g., restriction analysis), followed by identification of organelle-specific genes by sequencing. In case of uncertainty (e.g., localization of DNAs encoding dinoflagellate plastid genes in either plastids or in the cytoplasm^{22,23}, or significant hybridization of mitochondrial probes with both orgDNA and nucDNA fractions as in some plants), *in situ* hybridization experiments and/or DNA extraction from purified organelles will be mandatory.

Alternatives to CsCl gradient-based orgDNA purification from whole-cell lysates take advantage of the small size of orgDNAs (using regular or pulsed-field agarose gel electrophoresis), the presence of a large fraction of supercoiled molecules²⁴ or a linear configuration of the orgDNA²⁵. Note that (electrophoretic) size separation is of limited use, because orgDNAs are frequently organized in large linear (circular-mapping) concatemers of variable size^{26–28}. In addition, orgDNA purified from agarose gels clones inefficiently.

When and why avoid phenol extraction and alcohol precipitation of DNAs?

Phenol extraction and ethanol precipitation of DNAs belong to the classic tools for bacterial DNA purification, yet we suggest minimizing their use in eukaryotes during the initial phases of purification for the following reasons: (i) phenol extraction and alcohol precipitation tend to aggregate DNA with a variety of other components, including basic proteins and polysaccharides. This can lead to low yield and DNA cross-contamination, (ii) Phenol, when oxidized, damages and degrades DNA. Never use phenol that has turned pink, which indicates strong oxidation. Be vigilant with

commercial phenol preparations that are similarly pink (such as Trizol); rather prepare your own stabilized solution (for a recipe see ref. 29) and minimize exposure to oxygen.

When to apply dialysis of DNA solutions?

We recommend dialysis of DNA solutions at an early stage of DNA purification with the whole-cell lysis procedure. Dialysis allows efficient removal of detergents, salts and other low-molecular-weight impurities such as the peptides produced by proteinase treatment of cell lysates. In addition, evaporation of lysates enclosed in dialysis tubes (exposure to air for several hours at 20–37 °C) is an efficient means of volume reduction before CsCl gradient centrifugation. Yet, purified DNA may be lost if quantities are low, as it tends to bind to the dialysis tube. An alternative for working with highly diluted sub-microgram DNA quantities will be described.

Purification of mtDNA from Metazoa

Traditional protocols for animal mtDNA purification rely on the presence of circular DNA^{24,30–35}; these are not described in the following. The currently preferred method is amplification by long-PCR, because animal mtDNAs are mostly small, and primer design is facilitated by the knowledge of complete sequences from a variety of species, and high conservation of gene order. In addition, neither DNA purity nor yield is an issue in PCR experiments. Simple DNA extraction and long-PCR procedures are therefore described in the organelle genome sequencing protocol³⁶.

Purification of mtDNA from Fungi and axenic protists

The whole-cell lysis protocol is successful in most instances, but sometimes, nucDNA and orgDNA do not separate by CsCl–bisbenzimidazole gradient centrifugation. Specialized protocols^{32,37–42} should be consulted in difficult cases.

Purification of mtDNA from non-axenic protists

Protists growing in the presence of bacteria (as a food source, or present as culture contaminants) are most difficult to handle. We recommend purification of protist strains (if possible), that is, removal of all bacterial contaminants from the culture, and the use of a single, known food bacterium whose DNA separates well in CsCl gradients from the orgDNA in question. Because most non-axenic protist species grow slowly, and because it is difficult to purify intact organelles from little cell material that is contaminated with bacteria, the whole-cell lysis protocol is preferred. If unsuccessful, the use of a related species may be considered.

Purification of mtDNAs and cpDNAs from plants and algae

Plants and algae are particularly challenging in that they tend to contain phenolic compounds, oxidation products and polysaccharides that copurify with orgDNA. In addition, the mtDNA and cpDNA of a given species may have the same A + T content and therefore cannot be separated by CsCl–bisbenzimidazole gradients. Complete sequencing of both orgDNAs from a mixed DNA fraction is most cost-effective, as long as they are present at similar concentrations. Otherwise, mitochondria and plastids may be separated by sucrose or Percoll gradients before DNA extraction (see refs. 39,40,43–46 and protocols supplied by the manufacturer of Percoll).

If the above orgDNA purification protocols fail, specialized procedures may be attempted such as cloning of large inserts in

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fosmid or BAC vectors and rolling circle amplification (RCA) (for details, see refs. 32,41,42,44,47). Note that cloning in fosmid or BAC vectors is tuned for genomes with large (> 100 kbp) chromosomes that can be isolated intact (e.g., by pulsed-field electrophoresis), and that this approach is technically demanding and time consuming. RCA (like long-PCR) is best suited for organelle genomes that are organized as a single circular-mapping DNA. Yet, in multichromosome situations, amplification methods are

unreliable. In such instances, the completeness of genome sequencing and inferences about the gene complement (in particular, lack of otherwise expected genes) need to be corroborated by additional experimental evidence. Finally, RCA is prone to generate multiple bands when orgDNA is contaminated with DNA from other sources, and all amplification procedures are fraught with potential errors including nucleotide mis-incorporations and template switching.

MATERIALS

REAGENTS

- Culture media according to the species
- Glass beads
- Sand
- Liquid nitrogen
- Dry-ice pellets (CO₂)
- SDS
- Proteinase K, 10 mg ml⁻¹ (store frozen)
- CTAB
- CsCl
- Percoll (Amersham)
- Sucrose
- DNA molecular weight markers
- TE buffer: 10 mM Tris, 1 mM EDTA, pH 8
- Ethidium bromide, 10 mg ml⁻¹ in TE (store frozen aliquots)
! CAUTION Ethidium bromide is mutagenic; wear gloves when handling and dispose according to the safety regulations. Alternative dyes such as SYBR Green are claimed to be safer, but are more expensive.
- Bisbenzimidazole, 1 mg ml⁻¹ in TE (Hoechst dye 33258; Sigma; store frozen aliquots) **! CAUTION** bisbenzimidazole is a suspected mutagen; handle according to the safety regulations.
- ST buffer: 0.6 M sorbitol (or mannitol), 50 mM Tris-HCl, pH 7.4
- STE buffer: ST buffer containing 5 mM EDTA, which serves to inactivate certain types of DNases
- EtOH/AmAc: dissolve 0.5 M ammonium acetate in absolute alcohol
- 80% (v/v) EtOH (store at -20 °C)
- Chloroform (stabilize with a pinch of sodium bicarbonate)

- Isopropanol (store at -20 °C)
- DNA purification kit for genomic DNA (Qiagen)

EQUIPMENT

- Rotary shakers for cell cultures
- Refrigerated centrifuge (Sorvall or Beckman; SS34- and GSA-type rotors plus corresponding centrifuge tubes)
- Ultracentrifuge with fixed angle, swingout-bucket, and near-vertical or vertical rotors (e.g., Sorvall or Beckman; 55TT-, AH629-, SW50- and NVT65-type rotors, plus corresponding centrifuge tubes and tube sealing equipment)
- Apparatus for mixing of linear sucrose gradients
- Horizontal agarose gel electrophoresis apparatus UV detection/documentation equipment for ethidium-bromide-stained agarose gels and for detection of bisbenzimidazole-stained DNA bands in CsCl gradients
- Dry bath and/or water incubators for temperatures up to 65 °C
- Fine-mesh nylon (coffee) filters
- Disposable syringes (Becton Dickinson; various volumes from 1 to 20 ml) plus needles (18G1 and 22G1)

REAGENT SETUP

CsCl solution for balancing of tubes, before ultracentrifugation: Add 1.1 g CsCl per 1 ml of TE buffer (determine precise volume by weighing).

CTAB extraction buffer 3% Cetyl trimethyl ammonium bromide, 1.4 M NaCl, 0.2% β-mercaptoethanol, 20 mM EDTA, 100 mM Tris, pH 8.

Dialysis tubing (SpectraPor, 25 mm wide, MWCO 12–14,000; rehydrate and wash in 1 liter of TE buffer for at least 60 min (exchange the buffer at least once) and store refrigerated in TE.

TAE electrophoresis buffer for agarose gels 40 mM Tris, 20 mM acetic acid, 1 mM EDTA, pH 7.4–8.2 (prepared from 10× stock solution).

PROCEDURE

Culturing and harvesting cells ● TIMING 2 days to several weeks

For tissues from animals, plants and other multicellular species, collect only clean and fresh tissue, chill on ice and process immediately.

1| Prepare preculture of the selected species (~1/10 volume of the anticipated main culture, or agar plate(s) if applicable) according to the information given in **Box 1**.

2| Main liquid culture. Inoculate from a fresh preculture and proceed as above. The time point of cell harvest should be in the late logarithmic to early stationary phase, when mitochondrial mass is high.

▲ CRITICAL STEP For protists that are fed with life bacteria or eukaryotes, cells should be harvested when most of the food organism has been eaten (to minimize contamination with its DNA).

BOX 1 | CULTURE CONDITIONS

If there is a choice, use nonfermentable carbon sources such as glycerol or ethanol, rather than glucose or other sugars, to increase the yield of mitochondria and mtDNA. In the case of photosynthetic organisms, an appropriate light source is required. In addition, optimal aeration (enriched with CO₂ for algae) is often essential for high yield. In standing cultures (fragile species), the relative volume of liquid in the culture vessel should be small (< 1/10) and the cover of the opening should allow sufficient air exchange (e.g., loosely attached alumina foil). When agitation of the culture is possible, volumes of up to 600 ml per 3-liter Erlenmeyer flasks can be used (e.g., on a rotary shaker at up to 300 r.p.m., depending on the robustness of cells). Alternatively, aeration with a pressurized sterile air supply that is connected to an aquarium stone is highly effective. Obviously, a dedicated fermenter may be used, although such equipment requires substantial care for sterilization and operation, and might be inefficient for fragile species that do not tolerate agitation of the culture (most flagellated protists and amoeba).

Cell harvest ● TIMING 10–40 min

3| Collect filamentous organisms by filtration through a fine-mesh nylon filter, wash with a small amount of SE or STE (see note below on the choice of buffer) and weigh. Collect small unicellular organisms by centrifugation in a refrigerated centrifuge for 5 min at ~1,400–13,000*g* (3,000–9,000 r.p.m. in a GSA-type rotor, speed depends on the species). Resuspend cell pellets in preparation buffer, centrifuge at the same condition and weigh (preweigh empty centrifuge tubes to permit calculation of the cell wet-weight). For whole-cell lysis, continue with Step 7 of the protocol.

▲ **CRITICAL STEP** (i) EDTA in the preparation buffer destabilizes nuclei, and the released nucDNA may interfere with the purification of organelles; therefore, we recommend ST preparation buffer for organelle purification. For whole-cell lysates, STE preparation buffer is preferred, because EDTA will inactivate a variety of endonucleases. Depending on the protocol, some authors not only avoid EDTA but recommend adding 3 mM CaCl₂ to the preparation buffer for stabilization of DNA–protein complexes and organelles in animals³⁰, whereas others recommend addition of high salt and EDTA to destabilize nuclei and thereby remove nucDNA^{37,43}. (ii) Fragile protists (e.g., flagellates) will be damaged by centrifugation and will partially lyse when resuspended in preparation buffer. In that case, a second centrifugation should be avoided.

Cell disintegration ● TIMING 1–4 h

4| Fungal and algal cells are most difficult to break because of their highly resistant cell wall. Unfortunately, commercially available lytic enzymes that digest cell walls of yeast⁴⁸, fission yeast⁴⁹ or the moss *Physcomitrella*⁴⁰ are ineffective in most eukaryotes. Soft animal and plant tissues can be disintegrated by homogenization^{30,31,34}. For small, unicellular eukaryotes, we propose three variants of cell disintegration: (i) vigorous shaking of cell solutions (in preparation buffer SE or STE) in the presence of glass beads⁵⁰ (this is efficient for all cell sizes, but may damage organelles); (ii) manual grinding or rapid stirring of cells in the presence of sand or glass beads⁵¹ (ideal for filamentous fungi and other large cells); or (iii) pulverization of frozen cells in a mortar, in the presence or absence of dry ice^{20,21} (ideal for tissues, large cells and hyphae). Amoeboid protists and most types of animal cells may be directly lysed in the presence of SDS and proteinase K, and further processed by the whole-cell lysate protocol (see below).

▲ **CRITICAL STEP** Cell disintegration and the following isolation of mitochondria should be performed within the shortest time frame possible, using buffers and materials chilled on ice and refrigerated centrifugation to minimize enzymatic degradation of DNA.

Purification of a crude mitochondrial fraction ● TIMING 30–40 min

5| If applicable, separate the disintegrated cells from glass beads or sand by decanting and repeated washing with small volumes of preparation buffer. In addition to organelles, the disintegrated cell suspension also contains intact cells, cell walls, nuclei and other debris that are removed either by filtration through a fine-mesh nylon filter (e.g., filamentous fungi) and/or by low-speed centrifugation (1 min at ~100–6,000*g*; 1,000–7,000 r.p.m. in an SS34-type rotor; speed depends on the size of organelles to be isolated). A crude mitochondrial fraction is obtained by centrifuging the supernatant from the low spin for 15 min at ~48,000*g* (20,000 r.p.m.) The crude mitochondrial pellet is carefully suspended in preparation buffer to obtain a highly turbid solution (corresponding to ~1–10 mg ml⁻¹ protein). Organelles can be further purified either by removal of nucDNA contaminants by DNase treatment^{14,15} or by gradient centrifugation as described in the following.

▲ **CRITICAL STEP** DNase treatment of organelles is recommended only when the majority of organelles remain intact after cell breakage. This is usually the case when cell walls have been digested by enzyme treatments (yeasts and fission yeasts) or when large filamentous cells (e.g., filamentous fungi) have been ground in the presence of sand or glass beads. If applied on damaged organelles, DNasing will drastically reduce the DNA yield.

Organelle isolation by sucrose or Percoll gradient centrifugation ● TIMING 2–2.5 h

6| Layer the organelle suspension on top of either a continuous sucrose gradient (20–80% sucrose, which fills about 3/4 or more of the centrifuge tubes holding 15–40 ml) or a sucrose step gradient (a set of sucrose solutions that differ by 10% in concentration, layered on top of each other). Centrifugation is for 30 min at ~110,000*g* in a swing-out bucket-type rotor of an ultracentrifuge (e.g., ~25,000 r.p.m. rotor SW28, Beckman). Generally, mitochondria form an orange to brownish-colored band at about 30–45% sucrose (for separation of plastids and mitochondria in plants or algae, see special literature; for substituting sucrose with Percoll, follow the instructions of the manufacturer). The mitochondrial band is collected from the top with a syringe or Gilson pipette after aspirating most liquid above the mitochondrial fraction by a water pump. After dilution with 3 volumes of TE buffer and centrifugation for 15 min at ~84,000*g* (20,000 r.p.m. in an SS34-type rotor), mitochondria are recuperated as an intensely colored pellet (ranging from light orange to dark brown; occasionally brown–green).

■ **PAUSE POINT** The mitochondrial pellet can be stored frozen at –20 °C for prolonged periods. Note that broken and intact mitochondria tend to collect at different levels in the sucrose gradient, and that both contain mtDNA as it is attached to the mitochondrial membranes. Usually, the lower band contains intact mitochondria (which may be tested by measuring the relative activity of intra-mitochondrial marker enzymes such as TCA cycle components) and is generally less contaminated with nucDNA or broken nuclei than the upper band. As an alternative to sucrose gradients, Percoll gradients may be used, which are often superior in separation. Unfortunately, the conditions for organelle purification with Percoll are much less predictable and have to be optimized for each species. We recommend the supplier’s instructions as a starting point for experimentation with Percoll.



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▲ CRITICAL STEP As yields are variable, purification of mitochondria and plastids by gradient centrifugation requires generous amounts of starting material. This protocol has been most successful with animal, fungal and plant cells. Yet in some instances, cells contain but a single mitochondrion that is physically connected with the nucleus and other cellular membranes in a way making mitochondrial purification virtually impossible. This occurs frequently in protists and is largely depending on the growth phase. In such instances, the whole-cell lysis protocol is preferable.

Lysis of organelles or whole-cell lysates ● TIMING 1–2 h

7| Adjust the suspension of organelles (enriched or purified) or broken cells to 0.5% SDS and 100 $\mu\text{g ml}^{-1}$ proteinase K, and incubate for ~ 60 min at 50 °C. If the suspension becomes too viscous for easy transfer from one beaker to another, add more TE buffer, which is mixed under gentle swirling by hand.

Removal of SDS and cell debris ● TIMING 30 min to 1 day

8| If the lysate is turbid owing to remaining cell walls and other debris, remove this material by a “clear spin” at $\sim 27,000g$ (5 min at 15,000 r.p.m. in an SS34-type rotor). Two variants of protocol are proposed for further processing, depending on the volume of the DNA solution. (a) For small volumes (e.g., mitochondrial lysates), the detergent together with remaining proteins is precipitated by adding CsCl (see below, continue with Step 9, CsCl gradients). After centrifugation for 10 min at $\sim 84,000g$ (20,000 r.p.m. in an SS34-type rotor), the precipitate is removed as a solid, floating top layer. (b) Alternatively, the lysate is dialyzed for 16–24 h against ~ 3 liters of TE buffer, with at least one buffer exchange. The advantage of the dialysis step is that the volume of the DNA solution can subsequently be reduced *ad gusto* by evaporation. To this end, the dialysis bags are placed on a dry paper towel in an incubator at 37 °C, for a time period of a few hours to 1 day. Complete drying should be avoided.

▲ CRITICAL STEP Certain cell types, in particular brown algae, red algae and some fungi, produce prodigious amounts of polysaccharides that solubilize in the presence of SDS plus proteinase K. Whereas fungal polysaccharides can usually be removed by one CsCl gradient, the amounts of algal polysaccharides can be overwhelming. We provide a specialized protocol based on CTAB lysis (see **Box 2**) that has been applied successfully for numerous red and green algae.

CsCl gradient centrifugation ● TIMING One to several days, depending on centrifugation time and the number of repeated centrifugation cycles

9| (steps 9–12: We suggest that CsCl gradient purification be always applied, as it will remove remaining detergent and proteinase K without the need for solvent extractions.) First check DNA concentration and integrity by agarose electrophoresis. Weigh the DNA solution to estimate its volume and add solid CsCl at 1.1 g per g of DNA solution; dissolve under slight warming and occasional gentle swirling of the solution. If SDS was removed previously by dialysis, continue with Step 11.

? TROUBLESHOOTING

10| Centrifuge for 10 min at $\sim 84,000g$ (20,000 r.p.m. in an SS34-type rotor); the precipitated SDS together with proteins and other undissolved particles form a solid top layer. Decant the precipitate slowly or aspirate with a syringe (large needle size 18G1 to minimize DNA damage) to carefully separate it from the transparent DNA solution.

11| Add bisbenzimidazole to a final concentration of $\sim 5 \mu\text{g ml}^{-1}$ under gentle swirling and then transfer the solution to ultracentrifuge tubes of appropriate size using a syringe (large needle size 18G1 to minimize DNA shearing). Close tubes by sealing or cramping according to the instructions of the manufacturer of the equipment.

12| Centrifuge tubes according to the rotor type used. Centrifugation times vary between 8 and 16 h with vertical or near-vertical rotors such as NVT65, and up to 2 days with fixed-angle rotors such as 55TI. Stop the centrifuge with brakes off.

Collection of DNA bands from CsCl gradients ● TIMING 1–2 h

13| Mount centrifuge tube with clamps to a tripod, close to a long-wavelength UV lamp. Inspect DNA bands under UV light (bright light-blue color; visible down to ~ 100 ng DNA per band).

? TROUBLESHOOTING

14| First, pierce the tube at the very top with a needle (air inlet), and subsequently pierce the tube at the height of a DNA band with a needle (size 22G1) attached to a 2 ml syringe.

15| Slowly draw the DNA into the syringe. Collect separately all DNA bands of interest.

▲ CRITICAL STEP bisbenzimidazole is a suspected carcinogen and should be handled accordingly. The collection of DNA bands from the gradients requires patience when bands are close to one another. If band separation is imperfect, a second or third subsequent CsCl gradient centrifugation has to be performed. Note that vertical or near-vertical rotors have elevated separation power. In addition, centrifugation conditions (time and force) can be optimized for better band separation.

Recuperation of DNA from collected CsCl solution ● TIMING 8–24 h

16| The final amount of purified orgDNA may be deceptively small, varying substantially from one species to another. A safe method for DNA recuperation with virtually no loss is ultracentrifugation: dilute the DNA fraction with at least 2 volumes of TE buffer, mix and centrifuge in a swing-out bucket rotor (e.g., SW50 TI) for 6 h or overnight at ~190,000g (40,000 r.p.m.).

17| After centrifugation, the bisbenzimid-stained DNA is visible as a bright blue pellet under long-wavelength UV light and can be recuperated with a Gilson pipette in several tens of microliters of liquid.

18| Precipitate this material by adding 2 volumes of EtOH/AmAc at room temperature for 10 min and then by centrifugation for 15 min at 14,000 r.p.m. in a microcentrifuge (~14,000g).

? TROUBLESHOOTING

19| Wash pellet once with ice-cold 80% EtOH and air-dry after careful removal of the ethanol.

20| Dissolve the dry pellet in a small volume of TE buffer (5–200 µl; more volume at yields > 20 µg).

▲ CRITICAL STEP The handling of small DNA amounts is challenging, requiring close attention to avoid loss. Most losses occur during ethanol precipitation of the DNA, either because of incomplete precipitation from large volumes, or during removal of the ethanol after centrifugation (sub-microgram DNA precipitates are barely visible and might detach from the tube). Additions of carriers such as starch solution will improve the visibility of the pellet, but are normally not required. We do not recommend dialysis of highly diluted DNA solutions for removal of salts such as CsCl, as the DNA is easily lost by attachment to the dialysis tubing.

Determination of orgDNA quantity and quality ● TIMING 4–8 h

21| The concentration and intactness of the purified orgDNA is estimated by restriction analysis, through comparison of the ethidium bromide-stained pattern with a DNA standard of known concentration. Restriction patterns of most orgDNAs consist of well-separated bands (with little background smear), adding up to sizes of several to >100 kbp (see example in Fig. 2). Background smear is indicative of contamination with other DNAs, the presence of polysaccharides and/or remaining proteins

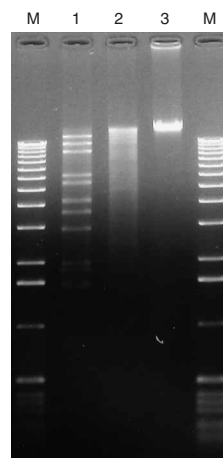


Figure 2 | Restriction analysis of DNA fractions obtained in a whole-cell lysate purification experiment with the protist *Reclinomonas americana*. A 2.5 g portion of wet weight cells was used as the starting material. Total DNA was separated by CsCl–bisbenzimid gradient centrifugation into two distinct bands (~12 mm distant; not shown). The A + T-rich (upper) band contains mtDNA (~2 µg yield) as revealed by restriction analysis. **M**, 1 kbp DNA ladder; **1**, ~100 ng of upper band, cut by *Hind*III; **2**, ~400 ng of G + C-rich (lower) band (mixture of nucDNA plus contaminating DNA from food bacterium; total yield of ~3 mg), cut by *Hind*III; **3**, ~400 ng of lower band, undigested.

TABLE 1 | Examples of organelle DNA purification.

Species	Organismal group	Wet weight cells (g) ^a	Culture period	Genome type	Genome organization	Purification method	Yield of orgDNA (µg)
<i>Amoebidium parasiticum</i>	Ichthyosporia	30	2 days	mt	Multiple linear	Organelle purification	14
<i>Allomyces macrogynus</i>	Chytrid fungi	50	2 days	mt	Circular mapping	Organelle purification	8
<i>Harpochytrium</i> sp 94	Chytrid fungi	1.5	10 weeks	mt	Circular mapping	Whole-cell lysate	1.7
<i>Hyaloraphidium curvatum</i>	Chytrid fungi	40	3 days	mt	Monomeric linear	Whole-cell lysate	45
<i>Klebsormidium flaccidum</i>	Charophyte algae	8	2 weeks	mt + cp ^b	Circular mapping	CTAB method for plants/algae	8
<i>Naegleria gruberi</i>	Heterolobosea	0.7 ^c	5 days	mt	Circular mapping	Whole-cell lysate	2.5
<i>Reclinomonas americana</i>	Jakobids	2.5	2 days	mt	Circular mapping	Whole-cell lysate	2
<i>Schizosaccharomyces pombe</i>	Ascomycota	30	3 days	mt	Circular mapping	Organelle purification	11
<i>Spizellomyces punctatus</i>	Chytrid fungi	35	5 days	mt	Multiple circular mapping	Organelle purification	13

^aFrom 2 liters cultures. ^bEqual mixture of mtDNA and cpDNA. ^cFrom 100 ml culture.



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(DNA pellets after ethanol precipitation are then often unexpectedly large) or partial degradation of the DNA (when smear starts with the largest restriction fragments).

? TROUBLESHOOTING

● TIMING

Steps 3–21, purified orgDNA of known concentration and quality can be obtained in 3 (when avoiding the dialysis step and using short ultracentrifugation conditions) to 5 working days. Additional time is required accordingly, when more than one cycle of CsCl gradient centrifugation is performed. The time involved in cell culturing is not included in this calculation (Steps 1 and 2), as it varies significantly with the species (for examples, see **Table 1**)

Day 1: harvest cells (10–40 min), break cells (1–4 h), (optionally) purify organelles (30 min to 3 h), lyse organelles or broken cells (1–2 h), remove SDS and debris (30 min; add an extra day when dialysis is performed) and prepare and start CsCl gradient centrifugation (1 h)

Day 2 or 3: stop gradient centrifugation and recuperate DNA bands (1–2 h), dilute DNA fractions and pellet DNAs by ultracentrifugation (6–16 h). Depending on centrifugation time, harvest DNA pellets in the evening or next morning (30 min)

Day 4: Ethanol-precipitate, wash, dry and dissolve DNAs (1 h) and perform quality control and quantification by restriction analysis (3–6 h)

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 2**.

TABLE 2 | Troubleshooting table.

Steps	Problem	Possible reason	Solution
4–9	Unexpectedly small DNA yield	(i) Insufficient cell disintegration; (ii) degradation of DNA by cellular endonucleases (common in sponges and widespread in protists and fungi)	(i) Always check results using a microscope; (ii) use the EDTA-containing preparation buffer (STE). Other options are extraction of DNA from phenol-treated cells, or treatment of the harvested cells with 8 M guanidinium chloride, 10 mM Tris and 5 mM EDTA for at least 24 h at room temperature. If cells lyse during this treatment (e.g., animal cells or amoeba), cell debris is removed by a clear-spin (~27,000g; 5 min at 15,000 r.p.m. in an SS34-type rotor). The DNA is then precipitated from the supernatant with 2 volumes EtOH/AmAc, dissolved in TE buffer plus 100 µg/ml proteinase K, incubated for 60 min at 50°C, followed by CsCl–bisbenzimidate gradient centrifugation (Step 9 of Protocol). If cells stay intact in the presence of the guanidinium buffer, they can be disintegrated by shaking with glass beads ⁵⁰ . Another option is DNA extraction from purified organelles
13	The whole-cell lysate protocol yields several well-separated fluorescent bands in the CsCl–bisbenzimidate gradient, but none contains clean mtDNA or cpDNA	The desired orgDNA does not separate from other genomic DNAs under the conditions used. In rare instances, the relative yield of orgDNAs is too low so that its bands are barely discernable	If additional minor bands are visible, a second CsCl–bisbenzimidate gradient is used to concentrate this material. Otherwise, isolation of DNA from purified organelles is recommended
13	The CsCl–bisbenzimidate gradient contains material forming one or more slightly turbid, greenish fluorescent bands, sometimes co-migrating with the blue-fluorescent DNA bands	In most fungi, plants and algae, these are polysaccharides, sometimes associated with proteins	Dilute the material collected from the gradient with at least 2 volumes of TE buffer and centrifuge for ~6 h or overnight at ~190,000g; 40,000 r.p.m. in a swinging bucket rotor (SW50 type). The high-molecular-weight polysaccharides usually form a solid translucent pellet, with a fluffy blue-fluorescent DNA layer on top that can be easily collected and directly used, or processed in a subsequent CsCl–bisbenzimidate gradient. As an alternative, try DNA

TABLE 2 | Troubleshooting table (continued).

Steps	Problem	Possible reason	Solution
			extraction from purified mitochondria or apply the CTAB protocol (see Box 2)
18, 21	DNA pellets are more voluminous after ethanol precipitation than expected; restriction digest of the orgDNA has background smear	The purified orgDNA contains remaining contaminants (such as proteins or polysaccharides)	Add 0.2% SDS plus 1 mg ml ⁻¹ proteinase K to the DNA solution, incubate at 50 °C for ~1 h, layer the DNA solution on top of a CsCl solution (0.5 g ml ⁻¹) containing 1 mM EDTA and 5 µg ml ⁻¹ bisbenzimidazole in a small ultracentrifuge tube (e.g., SW50 type), and centrifuge for ~8 h or overnight at 40,000 r.p.m. in a swinging bucket rotor (~190,000g). Collect the fluorescent DNA pellet in a small volume, precipitate with 2 volumes of EtOH/AmAc, wash with 80% EtOH and air-dry pellet. Proceed with Step 20 of the main protocol
21	The purified orgDNA is contaminated with nucDNA, although the DNA was extracted from isolated organelles	Frequently, nuclei are damaged during cell disintegration and nuclear DNA is released, which binds to organelles	DNase treatments of the purified organelles are an option (provided that organelles are intact)
13, 21	No orgDNA obtained by any of the methods described	Reason unknown	In this case, we refer to protocol variants described in the literature, including (i) columns for purification of genomic DNA (e.g., Qiagen) followed by CsCl-bisbenzimidazole gradients, (ii) long-PCR amplification, (iii) rolling circle amplification ³² , (iv) cloning of total DNA in fosmids or BACs ^{40,41} and subsequent identification of orgDNA by hybridization or end sequencing of clones

ANTICIPATED RESULTS

A yield of ~0.1–4 µg per g wet weight cells of orgDNA can be expected, depending on the species, the quality of the cellular starting material and the variant of purification method applied (**Table 2**). In algae, the yield of mtDNA may be lower than that of cpDNA, in part, because of the lower genome size and a low copy number of mtDNA. As a general rule, orgDNA yield is high in fungi, animals and amoeba, but low in filamentous growing algae and heterotrophic flagellates. In addition, the latter species often pose problems as to DNA contamination and DNA stability. The purity of DNA fractions isolated by the described protocols is usually higher than 95% when applying two subsequent cycles of CsCl-bisbenzimidazole gradient centrifugation (e.g., **Fig. 2**). This degree of purity is largely sufficient for random genome sequencing.

BOX 2 | ALTERNATIVE CTAB PROTOCOL FOR ALGAE

● TIMING 2–4 h

- 1| Preheat CTAB extraction buffer at 60 °C (50 ml per 5 g wet weight cells)
- 2| Freeze cells in liquid nitrogen and grind to powder in a mortar that was precooled with liquid nitrogen plus dry ice (in a closed styrofoam box)
- 3| Transfer powdered cells into the preheated CTAB extraction buffer and continue lysis at 60 °C for 30 min
- ▲ CRITICAL STEP Let mortar warm up slowly at room temperature before cleaning. It may otherwise crack
- 4| Transfer lysate to SS34 tubes (~20 ml per tube) and chill on ice for 5 min
- 5| Extract lysate with ~10 ml chloroform (under hood), then spin for 10 min at 18,000 r.p.m. in an SS34-type rotor
- 6| Collect aqueous phase, determine its volume, add 2/3 volumes of cold isopropanol and precipitate DNA for 30–60 min at room temperature
- 7| Centrifuge for 10 min at 18,000 r.p.m. in an SS34-type rotor
- 8| Wash the resulting DNA pellet with ~10 ml of 80% EtOH, centrifuge briefly, remove liquid, drip-dry tube and let DNA pellet air-dry for 10–30 min
- 9| Dissolve DNA in TE plus 100 µg ml⁻¹ proteinase K (~1 ml per preparation from 5 g cells) and incubate for 60 min at 50 °C
- 10| Proceed with CsCl-bisbenzimidazole gradient centrifugation (Step 9 of the main protocol)



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