Preparation of megabase-sized DNA from a variety of organisms using the nuclei method for advanced genomics research

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Megabase-sized DNA is crucial to modern genomics research of all organisms. Among the preparation methods developed, the nuclei method is the simplest and most widely used for preparing high-quality megabase-sized DNA from divergent organisms. In this method, nuclei are first isolated by physically grinding the source tissues. The nontarget cytoplast organellar genomes and metabolites are removed by centrifugation and washing, thus maximizing the utility of the method and substantially improving the digestibility and clonability of the resultant DNA. The nuclei are then embedded in an agarose matrix containing numerous pores, allowing the access of restriction enzymes while preventing the DNA from physical shearing. DNA is extracted from the nuclei, purified and subsequently manipulated in the agarose matrix. Here we describe the nuclei method that we have successfully used to prepare high-quality megabase-sized DNA from hundreds of plant, animal, fish, insect, algal and microbial species. The entire protocol takes ~3 d.

INTRODUCTION

Megabase-sized or high-molecular-weight DNA refers to DNA fragments >1,000 kilobase pairs (kb). Conventional DNA isolation methods, however, commonly produce DNA fragments only as large as ~100 kb[1]. Using the megabase-sized DNA isolation protocol described below, chromosome-sized DNA or DNA fragments of up to 30 megabase pairs (Mb) can be isolated[2–8]. Megabase-sized DNA is essential for many aspects of modern genomics research, particularly for the isolation, characterization and analysis of genes spanning large regions, construction of large-insert bacterial artificial chromosome (BAC)[9–13], plant-transformation-competent binary BAC (BIBAC)[12,14,15] and yeast artificial chromosome (YAC)[16] libraries, and long-range analysis of complex genomes[17].

Two methods have been developed and used for the preparation of megabase-sized DNA from different organisms[2–7,18,19]. One is the protoplast method, which uses cell wall hydrolases such as cellulase and pectinase to remove the cell walls of plants, algae, fungi, bacteria and yeast before isolating DNA[2–6,18,19]. For organisms that have no cell walls, the protoplast method directly uses entire cells to isolate DNA. The other method is the nuclei method, which is used to isolate DNA from the nuclei of various organisms regardless of whether or not they have cell walls[7]. Unlike conventional DNA isolation methods[20,21], preparation of megabase-sized DNA by either the protoplast or the nuclei method must ensure that the DNA is protected from physical shearing so that the isolated DNA fragments are either intact or of megabase size. One of the widely used approaches for doing so is to isolate protoplasts (from plants, algae, yeast, fungi and bacteria), cells (from animals, fish and insects) or nuclei (from plants, animals, fish, insects, alga, yeast and fungi), and then embed them in low-melting-point (LMP) agarose in the form of plugs or microbeads. DNA extraction, purification and subsequent manipulation are performed in the LMP agarose plugs or microbeads. **Figure 1** shows the procedures that are widely used for preparation of megabase-sized DNA from cells, protoplasts or nuclei.

The nuclei method has several advantages over the protoplast method for the isolation of megabase-sized DNA. As entire cells or protoplasts are used in the protoplast method, there must be a procedure available to remove the cell walls and to isolate a sufficient amount of protoplasts for the preparation of megabase-sized DNA[2–6,18,19]. This process is not only time consuming and costly, but also limits the isolation of megabase-sized DNA to plant, algal, fungal, bacterial and yeast species for which a specific protocol is available for protoplast isolation. Moreover, the use of entire cells or protoplasts for megabase-sized DNA isolation by the protoplast method also embeds the cytoplasm of the cells in the agarose along with the nuclei containing the target DNA. This leads to the contamination of the target nuclear DNA by the cytoplast organellar DNA, by cellular enzymes such as nucleases and proteinase K-resistance alkaline phosphatases, and by metabolic substances such as polyphenols and polysaccharides. For example, entire cells of some crustacean species, including shrimp, are rich in alkaline phosphatases; similarly, soft fruit–bearing or woody plant species, including berry, grape, rose, cotton and many other trees, are abundant in polyphenolic substances and/or polysaccharides. These substances often interact with DNA, making it no longer digestible and/or clonable. By comparison, the nuclei method for the isolation of megabase-sized DNA is simple and economical, results in DNA with little or no contamination due to cytoplast DNA and metabolites, and is applicable for the preparation of megabase-sized DNA from a wide variety of species[7]. Therefore, the nuclei method has rapidly become the method of choice for the preparation of megabase-sized DNA from various species for many research goals, including the construction of large-insert BAC and BIBAC libraries[22,23], map-based cloning of genes and quantitative trait loci[24], genome physical mapping[25–31], genome sequencing[32–40] and long-range genome analysis[17,41,42].

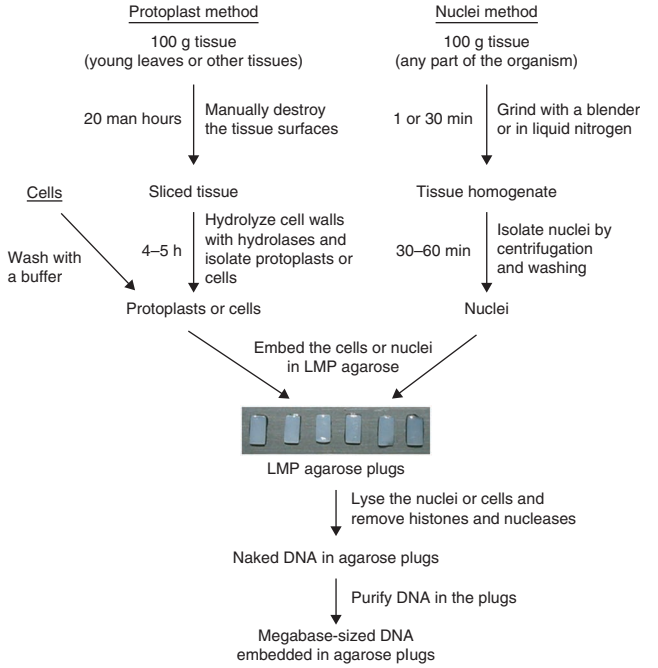

Figure 1 | An overview of the methods for megabase-sized DNA isolation from cells, protoplasts or nuclei. Each step for the preparation of megabase-sized DNA from different species using the nuclei method is described specifically in the PROCEDURE.

Applications and limitations of the protocol

The development of the nuclei method has markedly promoted genome research in many species, especially plant species, as the original protocol was developed in plants[7]. During the process of megabase-sized DNA isolation from different species, we extensively improved the procedure and further developed approaches to deal with numerous intractable problems, including the isolation of high-quality megabase-sized DNA from plants and animals that contain abundant polyphenols, polysaccharides and/or alkaline phosphatases that interact with DNA or inhibit DNA cloning enzymes, thereby making it no longer digestible and clonable. Using the nuclei method and its improved version, we successfully prepared high-quality megabase-sized DNA from hundreds of divergent species, including not only plants[7,22] but also animals[23,31], insects[43,44], marine animals[45–47], algae (H.-B.Z. and C.F. Scheuring, unpublished data) and microbes[8,48]. From the DNA isolated with the above methods, we successfully constructed high-quality BAC or BIBAC libraries, which have been widely used for genome physical mapping[8,29–31,49–53], genome sequencing[39,40] and map-based cloning[53].

Here we describe our streamlined procedure for the preparation of megabase-sized DNA from plants as well as the procedures for preparation of megabase-sized DNA from animals, insects, marine animals, algae and microbes; we also provide suggestions for overcoming the intractable problems frequently encountered in megabase-sized DNA preparation.

Experimental design

Source tissue. It is impossible to obtain high-quality megabase-sized, readily digestible and clonable DNA from poor-quality tissues with any DNA isolation protocol. Therefore, choosing and preparing the DNA source tissues carefully as described below is always the most important tip for the successful preparation of megabase-sized DNA from a species. If it is not possible to use the most desirable tissues, removing or minimizing the metabolites contained in cells that potentially affect the digestion and cloning of the resultant DNA is the best way to proceed. However, although CTAB (cetyl trimethylammonium bromide) and PVP (polyvinyl pyrolidone) were previously used (in combination with additional nuclei washes) to minimize the polyphenols that are abundant in some species and that inhibit DNA digestion and cloning[54], their effect on the quality of DNA prepared was not found to be as significant as the use of desirable tissues (Lee, M.-K. *et al.*, unpublished data).

Another crucial consideration on the preparation of megabase-sized DNA is the use of nuclei rather than cells or protoplasts, if metabolites are likely to cause problems for digestion and cloning of the resultant DNA. This is because the contents of the cytoplasm can be washed away before the target nuclei are embedded into the agarose matrix, thus substantially minimizing the contamination not only due to metabolites that may inhibit DNA digestion and/or cloning, but also as a result of organelles (e.g., chloroplast and/or mitochondria) in the targeted nuclear DNA.

The selection of proper tissues and sampling at the appropriate developmental stages and status is described below:

- *Plants.* Plant leaves or whole plants of grasses, legumes, vegetables or trees are used as materials for the preparation of megabase-sized DNA using the protocol presented below. However, the use of the following tissues is recommended: (i) whole plants or parts of plants at the one- or two-leaf seedling stage, preferably cotyledons for the species that are abundant in polyphenolics and/or polysaccharides, such as cotton, rose and trees (the abundance of polyphenolics and/or polysaccharides is often indicated by sticky nuclei pellets during preparation and/or the brown color of the resulting agarose plugs after nuclei lysis); (ii) very young leaves or meristems from more mature plants; and/or (iii) tissues collected from plants that are pretreated in the dark for 2–3 days. Furthermore, whenever facilities are available, plants for megabase-sized DNA preparation are grown in an environment-controllable growth chamber as the first option, then in a greenhouse and in the field as the last option. The tissues can be either frozen in liquid nitrogen and stored in a freezer at −80 °C, or they can be collected and transported on ice immediately before use.
- *Animals.* Blood cells or sperm are used as the DNA source. As animal cells have no cell walls, they can be directly embedded into LMP agarose for the preparation of megabase-sized DNA after brief washing. For mammals that lack nuclei in red blood cells, the red cells should be removed before they are used for megabase-sized DNA preparation. Sperm are specially recommended for the preparation of megabase-sized DNA from animals if the isolated DNA is difficult to digest and/or clone, because sperm often have a lower level of metabolic substances such as polysaccharides.

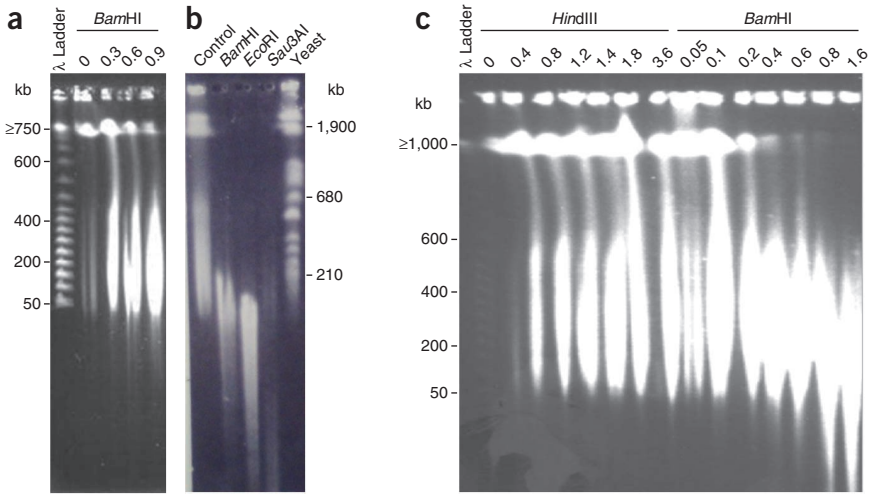

Figure 2 | Megabase-sized DNA isolated using the protocols presented in this article. (**a**–**c**) The figure illustrates the assessment of three criteria for megabase-sized DNA quality: integrity or size (see the control lanes, or those with 0 units of enzyme used), digestibility (partial digestion for DNA cloning shown in **a** and **c**; complete digestion for genome analysis shown in **b**) and concentration of DNA (see the control lanes or those with 0 units of enzyme used). (**a**) Megabase-sized DNA isolated from Pacific white shrimp. DNA was partially digested with *Bam*HI at restriction enzyme amounts of 0, 0.3, 0.6 and 0.9 units at 37 °C for 8 min, and then subjected to pulsed-field gel electrophoresis on a 1% (wt/vol) agarose gel in 0.5× TBE; this was done with an initial switch time of 50 s and a final switch time of 50 s, with a linear ramp, at 12.5 °C for 18 h (see ref. 55). The gel was stained in ethidium bromide solution (0.5 μg ml^{-1} in water) for 30 min and photographed by a gel documentation system. (**b**) Megabase-sized DNA isolated from common wheat. DNA was completely digested with *Bam*HI, *Eco*RI and *Sau*3AI at 10 units at 37 °C for 2 h and then subjected to pulsed-field gel electrophoresis on a 1% (wt/vol) agarose gel in 0.5× TBE; this was done with an initial switch time of 90 s and a final switch time of 90 s, with a linear ramp, at 12.5 °C for 20 h (see ref. 55). The gel was stained in ethidium bromide solution for 30 min and photographed by a gel documentation system. (**c**) Megabase-sized DNA isolated from wheat. DNA was partially digested with *Hind*III at 0, 0.4, 0.8, 1.2, 1.4, 1.8 and 3.6 units, and with *Bam*HI at 0.05, 0.1, 0.2, 0.4, 0.6, 0.8 and 1.6 units, respectively, at 37 °C for 8 min, and then subjected to pulsed-field gel electrophoresis on a 1% (wt/vol) agarose gel in 0.5× TBE; electrophoresis conditions included an initial switch time of 90 s and a final switch time of 90 s, with a linear ramp, at 12.5 °C for 18 h (see ref. 55). The gel was stained in ethidium bromide solution for 30 min and photographed by a gel documentation system. **Figure 2c** was reprinted from ref. 56 with permission.

- *Insects.* A major concern about the tissues used for the preparation of megabase-sized DNA from insects is the potential for contamination with their food. Therefore, the developmental stages of insects for megabase-sized DNA preparation in the preferred order are embryos, newly hatched larvae, early pupae and adults. For the preparation of megabase-sized DNA from mosquito, L1 larvae are used as the source tissue. For moths and butterflies, early pupae are preferred. Either fresh or liquid nitrogen–frozen larvae or pupae are suitable for the preparation of megabase-sized DNA. Moreover, newly hatched larvae are recommended as the starting material if the DNA is difficult to digest and/or clone.
- *Marine animals.* The preparation of high-quality megabase-sized DNA from marine animals, including fish, oysters, scallops and shrimps, is more difficult than that from other animals because they often contain high concentrations of several metabolic compounds and enzymes (such as mucopolysaccharides and alkaline phosphatase) that may affect the digestion and/or cloning of the resultant megabase-sized DNA embedded in LMP agarose. Newly hatched larvae, sperm, muscles or blood can be used as the materials for the preparation of megabase-sized DNA from these species. Sperm or blood cells can be directly embedded into LMP agarose plugs or microbeads after a couple of washes. However, for species from which it is difficult to obtain blood or sperm, nuclei can be isolated from muscles or newly hatched larvae and used for megabase-sized DNA preparation. Newly hatched larvae are recommended as the material if the DNA is difficult to digest and/or clone.
- *Algae.* Algal cells are harvested, washed twice in Tris-EDTA (TE) buffer and pelleted. The pellets can be frozen in liquid nitrogen and stored at −80 °C before use, or freshly used.
- *Fungi.* Use fresh fungi or fungi frozen in liquid nitrogen.
- *Bacteria.* Bacteria have cell walls, but do not have a nuclear membrane. Therefore, high-quality megabase-sized DNA has to be isolated from their protoplasts. Use fresh bacterial culture.

Tissue homogenization. Both liquid nitrogen grinding and mechanical blending methods are used in the protocol presented here to homogenize the DNA source tissues physically to isolate cells or nuclei. These methods allow broad applications of the protocol for the preparation of megabase-sized DNA from different organisms. For instance, we successfully isolated megabase-sized DNA from 'rock-eating' microbes from rocks collected from the bottom of the ocean at a depth of 7,000 m using the liquid nitrogen grinding method (H.-B.Z., unpublished data). Although the liquid nitrogen grinding method always yields high-quality megabase-sized DNA from different species, it is possible for those with little experience to obtain poor-yield and/or poor-quality DNA using the mechanical blending method. Therefore, we suggest that, for inexperienced researchers, the liquid nitrogen grinding method should always be the first choice of method for preparation of cells or nuclei from tissues.

Cell or nuclei isolation buffer (NIB). Homogenization buffers or NIB buffers are used for cell or nuclei isolation in the protocol. However, they could be modified or replaced with those widely used in conventional-sized DNA isolation for some species (e.g., insects) to obtain better-quality megabase-sized DNA, even though they have been proven to be efficient for the isolation of cells or nuclei from the tissues of different species.

Cell or nuclei embedding. Two methods have been used to embed the nuclei or cells into the LMP agarose matrix. One method is to embed the nuclei in LMP agarose plugs (**Fig. 1**) and the other is to embed the nuclei or cells in LMP agarose microbeads[4,7,18]. Ever since an improved chemical method was developed to digest the megabase-sized DNA embedded in LMP agarose[55], LMP agarose plugs have become preferable because they are readily prepared with commercially available plug molds (see EQUIPMENT) and

are easier to manipulate. In comparison, the microbeads usually yield more uniform restriction enzyme digestion results because of the larger ratio of surface area to volume for microbeads than for plugs. However, they are more difficult and time consuming both to prepare and to manipulate during the digestion steps[55].

Controls. If a control is needed to test the protocol and prepared buffers, common wheat can be used as a control species for plants (**Fig. 2**), chicken or mouse for animals[23], scallop for marine animals[45], *Manduca sexta* or mosquito for insects[43,44] and *Penicillium chrysogenum* for fungi[8]. From these species, we have successfully isolated high-quality megabase-sized DNA using the protocol. **Figure 2** shows examples of the megabase-sized DNA of wheat isolated with the protocol, thus providing a visual reference to the quality of the megabase-sized DNA isolated.

MATERIALS

REAGENTS

- 2-Mercaptoethanol (14 M; Sigma-Aldrich, cat. no. M-6250)
  ! CAUTION 2-Mercaptoethanol is considered to be a toxin that can cause irritation to the skin upon contact, and to nasal passageways and the respiratory tract upon inhalation. Therefore, when 2-mercaptoethanol or a solution containing it is used, always wear gloves and use it inside a fume hood.
- Agarose, DNase and RNase free (Invitrogen, cat. no. 15510-027)
- *Bam*HI (Invitrogen, cat. no. 15201)
- Boric acid (Sigma-Aldrich, cat. no. B6768)
- ddH_2O
- *Eco*RI (Invitrogen, cat. no. 15202)
- Ethidium bromide (EtBr; Fluka, cat. no. 46067) **! CAUTION** Ethidium bromide may be a mutagen; therefore, it should be handled with care and gloves should be worn during use. The working concentration for gel staining is 0.5 μg ml^{-1} in water.
- HCl (EM, cat. no. HX0603-3) **! CAUTION** Concentrated HCl forms acidic aerosols. The HCl aerosols and solutions are highly corrosive to human tissues, with a potential to damage the respiratory organs, eyes, skin and intestines if ingested. Therefore, it should be handled inside a fume hood; wear rubber gloves, protective eye goggles, chemical-resistant clothing and shoes.
- *Hin*dIII (Invitrogen, cat. no. 15207)
- Isopropanol (Burdick & Jackson, cat. no. 323-4) **! CAUTION** Isopropanol is toxic and can cause flushing, headache, dizziness and vomiting if ingested. It should be handled with gloves inside a fume hood.
- KCl (Sigma-Aldrich, cat. no. P-4504)
- Lambda ladder PFG marker (New England Biolabs, cat. no. N0340S)
- Light mineral oil (Fisher, cat. no. 0121-1)
- Lysozyme (Sigma-Aldrich, cat. no. 12650-88-3)
- Sodium lauryl sarcosine (Sigma-Aldrich, cat. no. L9150)
- Na_2 EDTA (Sigma-Aldrich, cat. no. E5134)
- Na_2HPO_4 (EM, cat. no. SX0720-5)
- NaCl (Fisher, cat. no. BP358-10)
- NaH_2PO_4 (EM, cat. no. SX0710-3)
- NaOH (Sigma-Aldrich, cat. no. S8045)
- Nitrogen, liquid
- Phenylmethylsulfonyl fluoride (PMSF; Sigma-Aldrich, cat. no. P7626)
  ! CAUTION PMSF is toxic to humans. It should be handled with gloves inside a fume hood when you use it directly or in solution.
- Proteinase K (fungal) (Invitrogen, cat. no. 25530-015)
- SeaPlaque agarose, low-melting-point, DNase and RNase free (LMP agarose; BMA, cat. no. 50101)
- Spermidine trihydrochloride (Sigma-Aldrich, cat. no. S2501)
- Spermine tetrahydrochloride (Sigma-Aldrich, cat. no. S1141)
- Sucrose, molecular biology grade (Sigma-Aldrich, cat. no. S0389)
- Triton X-100 (Sigma-Aldrich, cat. no. T8787)
- Trizma base (Sigma-Aldrich, cat. no. T6066)
- Yeast chromosome PFG marker (New England Biolabs, cat. no. N0345S)

EQUIPMENT

- Agarose plug molds, 100 μl per well (Bio-Rad, cat. no. 1703706)
- Centrifuge bottles, polypropylene, 250 ml (VWR)
- Centrifuge fixed-angle rotors, JA-14 and JA-17 (Beckman)
- Centrifuge tubes, polypropylene, 40 ml (VWR)
- Cheesecloth (VWR, cat. no. 21910-105)
- CHEF Mapper or CHEF-DRIII system (Bio-Rad)
- Environ shaker (Lab-Line)
- Falcon tubes, polypropylene, 50 ml (Becton Dickinson, cat. no. 352070)
- Funnels, 15 cm in diameter (VWR)
- Glass beaker, 100 and 1,000 ml (VWR)
- Gloves (MicroFlex)
- Graduated cylinder, 100 and 1,000 ml (VWR)
- Hemocytometer (VWR, cat. no. 15170-089)
- Heparin tubes (BD Vacutainer, yellow/c, cat. no. 364816)
- High-speed centrifuge (Beckman)
- Kitchen blender (Osterizer 10 Speed Blender, Wal-Mart)
- Large orifice pipette tips, 200 and 1,000 μl (USA Scientific, cat. no. 1011-9510)
- Magnetic stir bar (Corning)
- Microscope, phase-contrast
- Miracloth (Calbiochem, cat. no. 475855)
- Mortar and pestle, 2–3 liters in volume (VWR)
- Paint brushes, child's
- Pipette tips, 200 and 1,000 μl (Phenix)
- Rotary hybridization oven (Robbins Scientific)
- Stirrer/hot plate (Corning)
- Swinging bucket centrifuge (Beckman)
- Water bath (Precision)

REAGENT SETUP

HB (homogenization buffer) stock (10×) For 10× stock (100 mM Trizma base, 800 mM KCl, 100 mM EDTA, 10 mM spermidine trihydrochloride, 10 mM spermine tetrahydrochloride), add 12.1 g of Trizma base, 59.6 g of KCl, 37.2 g of Na2 EDTA, 2.55 g of spermidine trihydrochloride and 3.48 g of spermine tetrahydrochloride to ~800 ml of ddH_2O in a 1-liter beaker. Stir until dissolved. Adjust the pH of the solution to 9.0–9.4 with NaOH and bring to a final volume of 1,000 ml in a 1,000-ml graduated cylinder. Transfer the solution to a glass stock bottle and store it at 4 °C. The 10× HB stock may be stored at 4 °C for up to 1 year. **▲ CRITICAL** The pH value of the HB stock is crucial to the success of high-quality megabase-sized DNA isolation. We found that when a HB stock with pH <8.0 was used for megabase-sized DNA isolation from monocot plants, the isolated DNA was only 100–200 kb in size.

HB solution (1×) To prepare stock solution without 2-mercaptoethanol (1× HB, 0.5 M sucrose), add 100 ml of 10× HB stock and 171.2 g of sucrose to ~700 ml of ddH_2O in a 1-liter beaker. Stir until dissolved. Bring to a final volume of 1,000 ml in a 1,000-ml graduated cylinder. Transfer the solution to a glass stock bottle and store it at 4 °C for up to 3 months. **▲ CRITICAL** Immediately before use, add 1.5 ml 2-mercaptoethanol per liter (0.15%, vol/vol).

Triton X-100 (20% (vol/vol)) For 20% (vol/vol) Triton X-100 buffer, (1× HB, 0.5 M sucrose, 20% (vol/vol) Triton X-100), add 20 ml of Triton X-100, 10 ml of 10× HB stock and 17.15 g of sucrose to ~60 ml of ddH_2O in a 100-ml beaker. Stir until dissolved. Bring to a final volume of 100 ml in a 100-ml graduated cylinder. Transfer the solution to a glass stock bottle and store it at 4 °C. The 20% Triton X-100 stock may be stored at 4 °C for 1 year or longer without major problems.

NIB Make the buffer (1× HB solution, 0.5% Triton X-100, 0.15% (vol/vol) 2-mercaptoethanol) just before use. The volume of the buffer needed is 10–15 ml per gram weight of the sample, including nuclei isolation and subsequent washes. For 1 liter of the buffer, mix 975 ml of 1× HB with 25 ml

of 20% (vol/vol) Triton X-100 and keep it at 4 °C or on ice. Immediately before use, add 1.5 ml of 2-mercaptoethanol to the buffer.
▲ CRITICAL Always use freshly made buffer for megabase-sized DNA isolation. Use of the NIB stored at 4 °C for more than 5 h may lead to partial degradation of the DNA.
EDTA (1.0 M, pH 9.0–9.4) For 100 ml of the solution, add 36.22 g of Na_2 EDTA and 4 g of NaOH to ~80 ml of ddH_2O in a 100-ml beaker. Stir until dissolved. Adjust the pH of the solution to 9.0–9.4 with NaOH and bring to a final volume of 100 ml in a 100-ml graduated cylinder. Transfer the solution to a glass stock bottle, autoclave it for 20 min and store it at room temperature (RT, 25 °C). The solution may be stored at RT for 1 year or longer without major problems. ▲ CRITICAL A pH lower than 9.0 may lead to partial degradation of the DNA.
Sodium lauryl sarcosine (2% (wt/vol)) For 100 ml of the solution, add 2 g of sodium lauryl sarcosine to ~80 ml of ddH_2O in a 100-ml beaker. Stir until dissolved. Bring to a final volume of 100 ml in a 100-ml graduated cylinder. Transfer the solution to a glass stock bottle, autoclave it for 20 min and store it at RT. The solution may be stored at RT for up to 1 year.
Lysis buffer Make the buffer (0.5 M EDTA (pH 9.0–9.4), 1% (wt/vol) sodium lauryl sarcosine, 0.3 mg ml^{-1} proteinase K) just before use. The volume of the buffer needed is 0.5–1.0 ml per 100-µl agarose plug. To prepare 100 ml of lysis buffer, mix 50 ml of 1.0 M EDTA (pH 9.0–9.4) with 50 ml of 2% (wt/vol) sodium lauryl sarcosine. Add 30 mg of proteinase K powder just before use. ▲ CRITICAL We have previously tested concentrations of proteinase K in the lysis buffer ranging from 0.1 to 1.0 mg ml^{-1}. However, no major difference in terms of DNA fragment size, digestibility and clonability was observed among the concentrations of proteinase K ranging from 0.2 to 1.0 mg ml^{-1}. Therefore, we often use 0.3 mg ml^{-1} of proteinase K in the lysis buffer.
EDTA (0.5 M, pH 8.0) For 1 liter of the solution, add 186.1 g of Na_2 EDTA to ~800 ml of ddH_2O in a 1,000-ml beaker. Stir until dissolved. Adjust the pH of the solution to 8.0 with NaOH (~20 g pellets) and bring to a final volume of 1,000 ml in a 1,000-ml graduated cylinder. Transfer the solution to a glass stock bottle, autoclave it for 20 min and store it at RT. The solution may be stored at RT for 1 year or longer.
Tris-HCl (1.0 M, pH 8.0) For 1 liter of the solution, add 121.1 g of Trizma base to ~800 ml of ddH_2O in a 1,000-ml beaker. Stir until dissolved. Adjust the pH of the solution to 8.0 with concentrated HCl (~42 ml) and bring it to a final volume of 1,000 ml in a 1,000-ml graduated cylinder. Transfer the solution to a glass stock bottle, autoclave it for 20 min and store it at RT. The solution may be stored at RT for 1 year or longer.
TE (1×; pH 8.0) For 1 liter of the buffer (10 mM Tris-HCl (pH 8.0), 1.0 mM EDTA), add 10 ml of 1.0 M Tris-HCl, (pH 8.0), 2 ml of 0.5 M EDTA (pH 8.0) and 988 ml of ddH_2O in a 1,000-ml graduated cylinder. Transfer the buffer to a glass stock bottle, autoclave it for 15 min and store it at RT. The buffer may be stored at RT for 1 year or longer. Before use, cool the buffer in ice water.
PMSF (100 mM) We usually purchase 250-mg vials of PMSF. To avoid possible laboratory contamination, add 14.35 ml of isopropanol to each 250-mg vial to make up a final concentration of 100 mM. The solution can be stored in the vials at 4 °C for at least 2 years without major problems. At this temperature, PMSF crystallizes. Therefore, warm the solution at 37 °C for 10–20 min to dissolve the PMSF crystals before use. We have found that repeatedly cooling and warming the PMSF solution did not seem to markedly affect our results.
PBS buffer For 1 liter of the buffer (138 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM NaH_2PO_4 (pH 7.4)), add 8.00 g of NaCl, 0.20 g of KCl, 1.44 g of Na_2HPO_4 and 0.24 g of NaH_2PO_4 to ~800 ml of ddH_2O in a 1,000-ml beaker. Stir until dissolved. Adjust the pH of the solution to 7.4 with HCl and bring it to a final volume of 1,000 ml in a 1,000-ml graduated cylinder. Transfer the solution to a glass stock bottle, autoclave it for 20 min and store it at RT for up to 1 year.
Moth and butterfly DNA isolation buffer For 100 ml of the buffer (100 mM NaCl, 10 mM Tris-HCl (pH 8.0), 10 mM EDTA (pH 9.4)), add 0.5844 g of NaCl, 1.0 ml of 1.0 M Tris-HCl (pH 8.0) and 1.0 ml of 1.0 M EDTA (pH 9.4) to ~80 ml of ddH_2O in a 100-ml beaker. Stir until dissolved. Adjust the pH of the solution to 9.0–9.4 with NaOH and bring it to a final volume of 100 ml in a 100-ml graduated cylinder. Transfer the solution to a glass stock bottle, autoclave it for 20 min and store it at RT. This solution can be stored for 6 months. ▲ CRITICAL Immediately before use, add 150 µl of 2-mercaptoethanol (0.15% (vol/vol)) per 100 ml of the solution.
Mosquito DNA isolation buffer For 100 ml of the buffer (100 mM NaCl, 200 mM sucrose, 10 mM EDTA (pH 9.4)), add 0.5844 g of NaCl, 6.8 g of sucrose and 1.0 ml of 1.0 M EDTA (pH 9.4) to ~80 ml of ddH_2O in a 100-ml beaker. Stir until dissolved. Adjust the pH of the solution to 9.0–9.4 with NaOH and bring to a final volume of 100 ml in a 100-ml graduated cylinder. Transfer the solution to a glass stock bottle, autoclave it for 20 min and store it at 4 °C. This solution can be stored for 6 months. ▲ CRITICAL Immediately before use, add 150 µl of 2-mercaptoethanol (0.15% (vol/vol)) per 100 ml of the solution.
TBE (5×) For 1 liter of the buffer (450 mM Trizma base, 450 mM boric acid, 10 mM EDTA, (pH 8.3)), add 54.0 g of Trizma base, 27.5 g of boric acid and 20 ml of 0.5 M EDTA (pH 8.0) to ~800 ml of ddH_2O in a 1,000-ml beaker. Stir until dissolved. The pH of the solution should be ~8.3. Bring to a final volume of 1,000 ml in a 1,000-ml graduated cylinder. Transfer the solution to a glass stock bottle, autoclave it for 20 min and store it at RT. This solution can be stored for 12 months. During storage, precipitation may occur; however, this does not markedly affect its use for pulsed-field gel electrophoresis.

PROCEDURE

Preparation of cells or intact nuclei ● TIMING up to 3 h

1| Prepare the cells or nuclei from the DNA source materials of different organisms collected as described in the Experimental design section by using option A to prepare nuclei from plant, algal or fungal tissues; option B to prepare cells or nuclei from animal tissues; option C to prepare cells or nuclei from insect tissues; or option D to prepare bacterial cells.

(A) Preparation of intact nuclei from plant, algal or fungal tissues

(i) Grind ~100 g of the frozen or fresh tissue into fine powder in ~1,000 ml of liquid nitrogen with a mortar and pestle, and then immediately transfer the powder into an ice-cold 1,000-ml beaker containing 800–1,000 ml of the NIB buffer. Alternatively, follow the method described in **Box 1** to homogenize tissues with a kitchen blender.
! CAUTION The mortar must be cooled in a freezer at −80 °C for 2–3 h before use; otherwise, it will break when liquid nitrogen is added.
▲ CRITICAL STEP For researchers with less experience with this protocol, homogenizing the tissues in liquid nitrogen is strongly recommended, because it is easier to control the degree of homogenization. The amount of tissue can be as little as 5 g in fresh weight. We routinely use ~10 ml of NIB for every gram of tissue.
▲ CRITICAL STEP Depending on the amount and type of tissue, it often takes 20–30 min to grind the tissue into fine powder. It is necessary to frequently add liquid nitrogen to the mortar to keep the tissue frozen. We found that

Box 1 | Homogenization of plant tissues with a kitchen blender ● TIMING ~30 min

1. Homogenize ~20 g of tissue each time in 200 ml of ice-cold 1× HB solution in a kitchen blender (see EQUIPMENT) at speed 4 or 'puree' for 30–60 s.
▲ CRITICAL STEP Depending on the amount and type of tissues, the blending time may vary slightly, but it would not markedly affect the yield and quality of DNA if it is within the range of 30–60 s. We blend the tissues of dicot plants such as *Arabidopsis*, cotton, soybean and cucumber for 35 s, and those of monocot plants such as wheat, barley, rice, sorghum and maize for 45 s. However, a blending time longer than 60 or more seconds may lead to damage to the nuclei and, consequently, to the partial degradation of the DNA. On the contrary, a blending time less than 30 s may lead to a lower yield of nuclei and thus DNA.
2. Filter the homogenate into an ice-cold 250-ml centrifuge bottle as described in Step 1A(ii) of the PROCEDURE.
3. Repeat steps 1 and 2 above to complete the remaining tissues, if any.
4. Add 5 ml of 20% (vol/vol) Triton X-100 to each bottle (containing 200 ml 1× HB solution), making a final concentration of 0.5% (vol/vol) Triton X-100; gently mix the contents and incubate on ice for 10 min.

overgrinding may lead to substantial damage to the nuclei and, consequently, to the partial degradation of the DNA. In contrast, insufficient grinding could result in a lower yield of nuclei and thus DNA.
▲ CRITICAL STEP When the tissue has been ground into fine powder, carefully and slowly add the tissue powder to the NIB. The mixture tends to bubble vigorously and spew out the powder when the tissue is added too quickly.

(ii) Gently swirl the contents of the beaker with a magnetic stir bar for 10 min on ice on a stirrer plate; filter it directly into six 250-ml centrifuge bottles cooled on ice through one layer of Miracloth and two layers of cheesecloth supported by a funnel. Collect the remaining nuclei suspension by squeezing the cheesecloth-wrapped pellet gently with gloved hands.
! CAUTION This step should be conducted inside a fume hood because the NIB contains 2-mercaptoethanol, which is toxic (see MATERIALS).
▲ CRITICAL STEP The same result will be obtained regardless of the order of Miracloth and cheesecloth. If Miracloth is not available, 3–4 layers of cheesecloth can be used to separate the cell debris from the nuclei.
▲ CRITICAL STEP The tissue powder tends to float on the surface of the NIB in large clumps; accordingly, it is important to stir gently until the tissue is thawed completely and is thoroughly wetted by the NIB buffer.

(iii) Pellet the homogenate by centrifuging the 250-ml bottles in a fixed-angle rotor at 3,110*g*, 4 °C for 20 min.
▲ CRITICAL STEP The centrifugation step is done to harvest and separate the nuclei from organelles (such as chloroplasts and mitochondria) as well as from the cytoplasm-containing metabolites, such as polyphenols, polysaccharides and cellular enzymes. The speed of centrifugation is crucial to the DNA quality. For most plant and animal species, centrifugation at 1,880–3,840*g* is carried out to pellet the nuclei; however, an adjustment may be needed for different species, depending on their nuclei or genome sizes. For species with a smaller genome such as *Arabidopsis thaliana*, the nuclei could be pelleted at a higher speed (such as 3,840*g*). For species with a larger genome such as wheat, the nuclei may be pelleted at a lower speed (such as 1,880*g*). However, we have found that a higher speed of centrifugation may lead to higher contamination of the target nuclear DNA with chloroplasts, mitochondria, polyphenols and polysaccharides, which reduces the DNA quality. In contrast, a lower speed of centrifugation may not pellet a sufficient number of nuclei, thus resulting in a lower DNA yield.
▲ CRITICAL STEP The nuclei pellet from grasses is often loose; therefore, the pellet should be harvested as soon as the centrifugation is completed.
▲ CRITICAL STEP Given the smaller genome sizes of algal species, harvest the nuclei by centrifugation at 3,500–4,000*g*.
▲ CRITICAL STEP As most fungal species have a genome size of 30–70 Mb, harvest the nuclei by centrifugation at 3,500–4,000*g*.
? TROUBLESHOOTING

(iv) Discard the supernatant fluid and add 1 ml of ice-cold NIB to each bottle.
! CAUTION This step should be conducted inside a fume hood because the NIB contains 2-mercaptoethanol, which is toxic (see MATERIALS).
▲ CRITICAL STEP Care should be taken when discarding the supernatant fluid from monocot species because the pellet from the species is often loose. A small amount of retained supernatant should not affect the quality of the DNA.

(v) Resuspend the pellet gently by using a small paint brush presoaked in the ice-cold NIB; add an additional 5 ml of the ice-cold NIB to each centrifuge bottle, combine the resuspended nuclei from all bottles into a 40-ml centrifuge tube and, finally, fill the tube with the ice-cold NIB. Alternatively, in the case that the nuclei pellets, such as those from monocot plants, are loose, they can be resuspended by gently pipetting with a wide-bore or cut-off pipette tip.

Box 2 | Estimation of nuclei concentration

We use several methods to estimate the nuclei concentration before embedding in LMP agarose. All of the following methods have been proven to provide useful information about the concentration of the nuclei.

1. Count the nuclei under a phase-contrast microscope, if possible. This is the most accurate method, but it requires the appropriate microscope. The suspension of nuclei or 10–100-fold dilution of the nuclei solution is applied to a hemocytometer and counted under a phase-contrast microscope. According to the volume of a large square of the hemocytometer and the number of nuclei in the square, the number of nuclei per milliliter can be calculated. As the exact concentration of DNA per 100-µl plug is not that crucial to applications of the resultant DNA, we usually use the methods described below to estimate the concentration of the nuclei.
2. The concentration of nuclei can also be estimated empirically. On the basis of our research experience, a solution of nuclei that is just transparent under light is estimated to have a concentration of 5–10 × 10^7 nuclei per ml. Nevertheless, the light-transparency of the nuclei isolated from dicot plants is usually much lower than that of the nuclei isolated from monocot plants. This is because the dicot plant nuclei are often contaminated with a larger amount of metabolites or starches that may reduce the light-transparency of the nuclei suspension. Therefore, for dicot plant nuclei suspension, the desirable degree of light transparency for the preparation of megabase-sized DNA LMP agarose plugs may be much lower than that for the monocot plant nuclei suspension.
3. If no microscope is available to count nuclei and it is not efficient to estimate the concentration of nuclei by means of its light transparency, it is advisable to make plugs with several dilutions of nuclei for selecting the best concentration for megabase-sized DNA preparation. Start by resuspending the nuclei pellet in an equal volume of 1× HB solution with no 2-mercaptoethanol. By using half of the resuspended nuclei, make several 1:1 serial dilutions in 1× HB solution with no 2-mercaptoethanol and embed these nuclei dilutions separately in agarose plugs. Keep the plugs made with different concentrations of nuclei separate in all subsequent steps. Analysis of the DNA on a pulsed-field gel will indicate the optimal concentration of nuclei required for DNA plug preparation (see PROCEDURE Step 7).
4. Alternatively, you can make the nuclei LMP agarose plugs according to the fresh weight of tissues used in Step 1A(i), 1B(i), 1C(i) or 1D(i). We routinely make one or two 100-µl plugs from the nuclei isolated from every 1 g of the starting tissues, depending on the tissue quality. From young tissues such as cotyledons we make two 100-µl plugs per g of tissue, whereas for older tissues, we make one 100-µl plug per g of tissue.

If particulate matter remains in the suspension, filter the resuspended nuclei into the 40-ml centrifuge tube through two layers of Miracloth by gravity. Alternatively, centrifuge the contents at 57*g*, 4 °C for 2 min to remove intact cells and tissue residues and transfer the supernatant fluid into a fresh centrifuge tube.

(vi) Pellet the nuclei by centrifugation at 3,110*g*, 4 °C for 15 min and discard the supernatant.

(vii) Wash the nuclei pellet an additional one to five times by repeating Steps 1A(v) and 1A(vi).

▲ **CRITICAL STEP** This step is crucial for minimizing the contamination of cytoplast organelles and metabolic compounds in the nuclei. Additional washes allow the maximal removal of the cytoplast contents, such as chloroplasts, mitochondria, metabolic substances and cellular enzymes. This is especially important for the isolation of high-quality megabase-sized DNA from plants that have abundant polyphenolic substances, such as cotton, rose and trees. The color of the pellet should become lighter with increased number of washes. Ideally, the final pellet should be white or a very pale yellow or green. Although we attempted to use CTAB and/or PVP to minimize the effects of polysaccharides and polyphenols on DNA quality[54], we found that the most efficient method is the use of young tissues as starting materials (see above), and then the centrifugation of the tissue homogenate at an appropriate speed (see Step 1A(iii)) and multiple washes of the nuclei in the NIB (this step).

? TROUBLESHOOTING

(viii) After the final wash, resuspend the pelleted nuclei in a small amount (0.5 ml) of 1× HB solution without 2-mercaptoethanol, count the nuclei (**Box 2**), bring to a concentration of ~5 × 10^7 nuclei per ml (for a species with a genome size of 1,000 Mb per haploid) with the addition of ice-cold 1× HB and store it on ice. The nuclei are stable on ice at this stage for 1 h. The nuclei concentration desirable for megabase-sized DNA preparation varies, depending on the genome sizes of different species (**Box 3**). Therefore, the concentration of nuclei in the suspension for LMP agarose plug preparation should be adjusted. In general, 5–10 µg DNA per 100-µl plug or microbead is suitable for most large-insert BAC and BIBAC cloning, as well as for long-range genome analyses.

▲ **CRITICAL STEP** Given the smaller genome sizes of algal species, use ~10^9 nuclei per ml for LMP agarose plug preparation (**Box 3**).

▲ **CRITICAL STEP** As most fungal species have a genome size of 30–70 Mb, use 2–3 × 10^9 nuclei per ml for the preparation of LMP agarose plugs (**Box 3**).

(ix) Prepare 10 ml of 1% (wt/vol) LMP agarose in 1× HB solution without 2-mercaptoethanol in a 15-ml Falcon tube by melting the LMP agarose in boiling water, cooling it down to 45 °C and maintaining it in a water bath at 45 °C before use.

Box 3 | Calculation of the number of nuclei needed for the preparation of plugs

Assuming that we want 5 µg of DNA per 100-µl plug and the organism has a genome size of 1,000 Mb/haploid or 2,000 Mb/diploid, and given that 1,000 Mb is equivalent to 1.03 pg of DNA and 2,000 Mb is equivalent to 2.06 pg of DNA, the number of nuclei needed for one 100-µl plug containing 5 µg of DNA is:

$(5\ \mu g \times 10^6\ pg\ \mu g^{-1})/2.06\ pg$ per nuclei $= 2.427 \times 10^6$ nuclei

As each 100-µl plug contains 50 µl of nuclei suspension (the remaining 50 µl is LMP agarose), the concentration of the nuclei suspension to make the plugs that each contain 5 µg of DNA is:

(1,000 µl per 50 µl) $\times 2.427 \times 10^6$ nuclei $= 4.854 \times 10^7$ nuclei per ml

Therefore, for a species with a genome size of 500 Mb/haploid, $2 \times (4.854 \times 10^7) = 9.7 \times 10^7$ nuclei per ml is needed to make plugs that each contain 5 µg of DNA.

▲ **CRITICAL STEP** The volume of 1% (wt/vol) LMP agarose depends on the volume of nuclei obtained from Step 1A(viii). We usually make 50–60 µl of 1% (wt/vol) LMP agarose per 100-µl plug. We melt LMP agarose by placing the loosely capped tube containing LMP agarose in 1× HB solution without 2-mercaptoethanol in a small volume of boiling water in a beaker on a hot plate.

(B) Preparation of cells or intact nuclei from animal tissues, including marine animals

(i) Centrifuge the animal blood or sperm sample, or blood sample from marine animals, at 1,000*g*, RT for 5 min and discard the supernatant. Alternatively, to prepare nuclei from the muscles, larvae and sperm of marine animals, follow Step 1A(i–ix).

(ii) To prepare intact cells for embedding in LMP agarose plugs, wash the cells two or more times by resuspending in ice-cold PBS buffer with a large-orifice pipette tip, followed by centrifuging at 1,000*g*, 4 °C for 5 min. Alternatively, follow the method shown in **Box 4** to prepare intact nuclei for LMP agarose embedding.

(iii) Discard the supernatant, resuspend the cells in 0.5–1.0 ml PBS buffer and bring the concentration of cells to an appropriate level (**Boxes 2** and **3**).

(iv) Prepare 10 ml of 1% (wt/vol) LMP agarose in PBS buffer as described in Step 1A(ix).

(C) Preparation of cells or intact nuclei from insect tissues

(i) Grind the fresh or liquid nitrogen–frozen larvae or pupae into fine powder in liquid nitrogen with a mortar and pestle, and then transfer the powder into the mosquito or moth and butterfly DNA isolation buffer; alternatively, add 10–20 ml of the buffer to the mortar and allow it to warm up until the frozen sample and solution just begin to melt.

(ii) Filter the cell suspension through two layers of Miracloth into a centrifuge tube on ice to remove the large pieces of cell debris; centrifuge at 3,440*g*, 4 °C for 10 min to collect the cells and discard the supernatant.

(iii) To prepare intact cells for embedding in LMP agarose plugs, resuspend the cells in 0.5–1.0 ml of the DNA isolation buffer without 2-mercaptoenthanol using a large-orifice pipette tip. For every 500 mg of fresh weight of mosquito larvae, we resuspend the cell pellet in 1.0 ml of the DNA isolation buffer and make 20 plugs of 100 µl. Alternatively, follow the method shown in **Box 4** to prepare intact nuclei for LMP agarose embedding.

(iv) Prepare 5 ml of 1% (wt/vol) LMP agarose in DNA isolation buffer without 2-mercaptoenthanol as described in Step 1A(ix).

(D) Preparation of bacterial cells

(i) Harvest the bacterial cell overnight culture by centrifugation at 4,000*g*, 4 °C for 10 min and completely resuspend the cells in 50 mM EDTA (pH 8.0) by vortexing.

(ii) Resuspend the cells in a small volume (e.g., 1.0 ml) of 50 mM EDTA (pH 8.0) and bring to a concentration of 5×10^9 cells per ml (**Box 3**).

(iii) Prepare 5 ml of 1% (wt/vol) LMP agarose in 50 mM EDTA (pH 8.0) as described in Step 1A(ix).

Embedding the nuclei or cells in LMP agarose matrix ● TIMING ~1 h

2| Embed the nuclei or cells prepared in Step 1 in LMP agarose matrix by using option A to embed in LMP agarose plugs or option B to embed in LMP agarose microbeads.

(A) Embedding the nuclei or cells in LMP agarose plugs

(i) Prewarm the cells or nuclei (from Step 1A(viii), 1B(iii), 1C(iii) or 1D(ii)) to 45 °C in a 45 °C water bath (for ~5 min).
▲ **CRITICAL STEP** The incubation time of the nuclei or cell suspension in the water bath (at 45 °C) before mixing with the 1% (wt/vol) LMP agarose depends on the volume of the nuclei or cell suspension. If the nuclei or cells are not warm enough, the agarose may solidify in the tube, thus making it difficult to pipette into plug molds to make uniform plugs; however, a little longer incubation time in the water bath would not substantially damage the cells or nuclei.

Box 4 | Preparation of intact nuclei from animal or insect tissues ● TIMING ~1.5 h

1. Resuspend the cells in ice-cold NIB with a large-orifice pipette tip; incubate them on ice for 10 min, with occasional gentle vibration, and spin them at 2,200*g*, 4 °C for 15 min for animal tissues (spin at 3,440*g* for insect tissues).
2. Wash the nuclei pellet an additional one or two times by resuspending it in ice-cold NIB as in PROCEDURE Step 1A(v); follow this by centrifugation at 2,200*g*, 4 °C for 15 min for animal tissues (3,440*g* for insect tissues).
3. Discard the supernatant, resuspend the cells in ice-cold 1× HB solution without 2-mercaptoethanol and bring the concentration of nuclei to an appropriate level (**Boxes 2** and **3**).
4. Prepare 5 ml of 1% (wt/vol) LMP agarose in 1× HB solution without 2-mercaptoethanol by melting LMP agarose contained in a loosely capped tube in boiling water, cooling it down to 45 °C and maintaining it in a water bath at 45 °C before use.

(ii) Mix the prewarmed nuclei or cells with an equal volume of the prewarmed 1% (wt/vol) LMP agarose in an appropriate buffer (from Step 1A(ix), 1B(iv), 1C(iv) or 1D(iii), as appropriate) with a cut-off pipette tip.
▲ **CRITICAL STEP** At this step, the firmness of the LMP agarose plugs can be adjusted. If firmer plugs are preferred, more (55–60%) LMP agarose could be added. The use of a slightly higher LMP agarose concentration would not markedly affect DNA digestion *in situ*.
▲ **CRITICAL STEP** The buffer used to prepare the 1% (wt/vol) LMP agarose is usually the buffer used in the suspension of the nuclei or cells to be embedded (see Step 1).

(iii) By using a wide-bore or cut-off pipette tip, aliquot 100 µl per plug of the nuclei or cell and LMP agarose mixture into ice-cold 100 µl plug molds positioned on ice. Allow the plug molds to incubate on ice for 10 min (see **Fig. 1** for megabase-sized DNA LMP agarose plugs).

(iv) When the agarose is completely solidified, transfer the plugs into 5–10 volumes of lysis buffer with 1–2 plugs per 1 ml of the lysis buffer.

(B) Embedding the nuclei or cells in LMP agarose microbeads

(i) Prewarm 20 ml of light mineral oil in a 50-ml Falcon tube to 45 °C in a water bath (for ~15 min).

(ii) Prewarm a 500-ml flask in the water bath (45 °C) for 20 min.

(iii) Pour 150 ml of ice-cold buffer (from Step 1A(ix), 1B(iv), 1C(iv) or 1D(iii), as appropriate) into a 1,000-ml beaker and place the beaker in an ice bath on the top of a magnetic stirrer plate. Vigorously swirl the buffer with a magnetic stir bar.

(iv) Prewarm the cells or nuclei and mix with LMP agarose by repeating Steps 2A(i) and 2A(ii).

(v) Pour the mixture into the prewarmed 500-ml flask (from Step 2B(ii)) and add 20 ml of the prewarmed light mineral oil (from Step 2B(i)) at 45 °C.

(vi) Cool the contents to about 37 °C (check by feeling with your hands); shake the contents in the flask vigorously for 2–3 s and immediately pour them into the ice-cold 1,000 ml beaker containing 150 ml of ice-cold appropriate buffer from Step 2B(iii) while vigorously swirling with a magnetic stir bar.

(vii) Continue to swirl the contents for 5–10 min on ice until uniformly sized agarose microbeads are formed.

(viii) Harvest the agarose microbeads by centrifugation at 1,200*g*, 4 °C for 20 min in a swinging bucket centrifuge.

(ix) Discard the supernatant fluid and resuspend the microbeads in 5–10 volumes of lysis buffer.

Extraction of megabase-sized DNA ● TIMING ~24 h (overnight incubation)

3| Incubate the LMP agarose plugs or microbeads in the lysis buffer for 16–24 h at 50 °C in a rotary hybridization oven or an environmental shaker with very gentle agitation.

▲ **CRITICAL STEP** 50 °C is the optimal temperature for the proteinase K reaction to digest the histones that are intercalated with DNA and nucleases that digest DNA. A temperature higher than 50 °C may melt the plugs. In our experience in the preparation of megabase-sized DNA from hundreds of diverged species, it appears that the concentrations and digestion time of proteinase K are not markedly affected by the sources of DNA.

▲ **CRITICAL STEP** As bacterial cells have walls, but do not have nuclear membranes, remove the cell walls by incubating the cells at 37 °C for 2–3 h with the lysozyme at a final concentration of 0.25 mg ml^{-1} in 50 mM Tris-HCl (pH 7.5) before the LMP agarose plugs or microbeads are incubated in the lysis buffer. After the incubation, replace the lysozyme solution with the lysis buffer.

4| Wash the plugs or beads once in 50 mM EDTA (pH 8.0) for 1 h on ice and store them in 50 mM EDTA (pH 8.0) at 4 °C.
■ **PAUSE POINT** The DNA at this step can be stored at 4 °C for 1 year without marked degradation.

Purification and quality inspection of megabase-sized DNA embedded in LMP agarose ● TIMING ~24 h

5| Wash the plugs or microbeads once in 10–20 volumes of ice-cold TE and then three times in 10–20 volumes of ice-cold TE containing 0.1 mM PMSF (1 µl 100 mM PMSF per 1 ml of TE) on ice, each wash for 1 h.

! CAUTION Always work with PMSF solution inside a fume hood because of its toxicity. It is degraded in TE within 30 min.
▲ CRITICAL STEP This step is critical to remove the proteinase K used in the lysis buffer, as it could also digest restriction enzymes used in subsequent DNA digests.

6| To remove the PMSF, further wash the plugs or microbeads three times in 10–20 volumes of ice-cold TE on ice, each wash for 1 h. The plugs or microbeads in TE can be stored at 4 °C for at least 5 months without substantial DNA degradation.
▲ CRITICAL STEP The megabase-sized DNA plugs or microbeads of high quality should look colorless or transparently pale in color at this point. In most (if not all) cases, the plugs or microbeads prepared from animals, marine animals, insects and microbes are colorless or transparently pale. Pale but nontransparent plugs or microbeads usually suggest a high level of contamination of the DNA with starch, whereas brown-colored plugs or microbeads usually suggest a high level of contamination of the DNA with polyphenolics. In either of these cases, the DNA is often difficult to clone for construction of large-insert DNA libraries.
■ PAUSE POINT At this stage, the plugs or microbeads can be stored at 4 °C for several months without substantial degradation.
? TROUBLESHOOTING

7| Assess the concentration and quality of the DNA embedded in the LMP agarose plugs or microbeads by digesting them with one or more restriction enzymes (completely and/or partially); follow this by pulsed-field gel electrophoresis of digested and undigested DNA against a DNA molecular weight marker (e.g., lambda ladder PFG marker) (for details of this step, see ref. 55).
▲ CRITICAL STEP It is necessary to check the concentration and quality of the DNA embedded in LMP agarose matrix resulting from any protocol before use. High-quality megabase-sized DNA not only is large in size but also has an appropriate concentration in its supporting plugs or microbeads (5–10 μg per 100 μl plug or microbeads). It is readily digestible with a restriction enzyme and is readily clonable into a BAC or BIBAC vector (see ref. 55). DNA that is readily digestible with a restriction enzyme may not always be readily clonable, but agarose plugs or microbeads with an appropriate concentration of DNA usually are better suited for the construction of large-insert BAC or BIBAC libraries.
? TROUBLESHOOTING

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Solution
1A(iii)	Sticky nuclei pellets	Old tissues grown in the field or greenhouse	Use cotyledons or meristems grown in a growth chamber and treated in the dark for 2–3 d
1A(vii)	Dark green or sticky nuclei pellets	Insufficient washing and/or centrifugation at excessive speed	Perform additional washes of the nuclei with NIB and centrifuge at a lower speed
6	Nontransparent, pale or brown plugs or microbeads	Old tissues grown in the field or greenhouse	Use cotyledons or meristems grown in a growth chamber and treated in dark for 2–3 d
		Insufficient washing and/or centrifugation at excessive speed	Carry out additional washes of the nuclei with NIB and centrifuge at a lower speed
7	DNA fragments <500 kb	Tissues are excessively ground	Grind the tissues properly
		NIB pH <8.5	Adjust the NIB such that pH = 9.0–9.4
		Old NIB is used or there is insufficient proteinase K digestion at Step 3	Use freshly made NIB or add more proteinase K in the lysis buffer; extend the digestion time and/or change the lysis buffer one or two times
	DNA is difficult to digest and/or clone	Old tissues	Use very young tissues such as plant cotyledons and meristems, animal sperm, newly hatched insect larvae or fresh microbe cultures
		Insufficient washing and/or centrifugation at excessive speed	Carry out additional washes of the nuclei and centrifuge at a lower speed

● TIMING

Step 1, preparation of cells or intact nuclei: up to 3 h
Step 2, embedding the nuclei or cells in LMP agarose matrix: ~1 h
Steps 3 and 4, extraction of megabase-sized DNA: ~24 h (overnight incubation)
Steps 5–7, purification and quality inspection of megabase-sized DNA embedded in LMP agarose: ~24 h
Box 1, homogenization of plant tissues with a kitchen blender: ~30 min
Box 4, preparation of intact nuclei from insect or animal tissues: ~1.5 h

ANTICIPATED RESULTS

With this protocol, we have successfully prepared high-quality megabase-sized DNA from hundreds of divergent species, including not only herbaceous and woody plants such as grasses, legumes, vegetables, trees, bushes and bamboos, but also animals, marine animals, insects, algae and microbes (fungi and bacteria). We have successfully isolated megabase-sized DNA from every organism with which this process has been attempted. The vast majority of the DNA fragments isolated with the methods were <1,000 kb (ref. 7), readily digestible and readily clonable, thus being well-suited for large-insert BAC and BIBAC library construction, long-range genome analysis and whole-fungal chromosome separation (genome karyotyping)[8]. **Figure 2** shows examples of the megabase-sized DNA of wheat and shrimp isolated with this protocol, thus providing a visual reference of the megabase-sized DNA isolated from a species of interest using the protocol.

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AUTHOR CONTRIBUTIONS
M.P.Z., Y.Z., C.F.S., C.-C.W., J.J.D. and H.-B.Z. conducted the experiments on different species with the protocol presented here, and improved and extended the original protocol through these experiments. M.P.Z. wrote the manuscript; H.-B.Z. developed the original concept of the protocol and the original protocol, designed the experiments and wrote the manuscript.

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