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Supplementary Material

Methodology for single-cell genetic analysis of planktonic foraminifera for studies of protist diversity and evolution

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Detailed methods for single-cell genetic analysis of planktonic foraminifera, including printable versions as laboratory protocols

In the practical execution of the different steps for genetic analysis of planktonic foraminifera, many details vary between different laboratories and different researchers (in terms of e.g. technical specification of laboratory consumables, brands of equipment), which may potentially affect the results (e.g. Gaillard & Strauss 2001). We therefore have tried to standardize the descriptions of all methods as far as possible, yet we believe that these particular factors have limited influence on amplification success.

1 Isolation and preservation of foraminifera

1.1 Isolation of individual specimens

After plankton sampling, the bulk sample is immediately transferred into an open-top beaker of at least 250 ml volume and diluted with freshly filtered seawater from the sampling station (filtered through a 0.2 µm filter) to an overall volume of 200 ml. If the amount of phytoplankton in the sample is exceedingly high, it is diluted with filtered seawater to about 1 L in a larger container. Dilution of the sample with fresh, well-oxygenated seawater is essential in order to prevent deoxygenation of the sample, which would lead to the deterioration of the foraminiferal specimens. In order to remove large metazoans, which may impede the isolation of foraminifera, the sample can be pre-filtered over a sieve with about 2 mm mesh size before dilution. When sampling in cold regions or from great depths, beakers with the diluted plankton sample are kept refrigerated (4°C) until the isolation of foraminifera commences. When sampling in temperate regions the beakers are kept at ambient temperature in the laboratory (~20°C). In both cases, beakers are kept open to avoid the loss of oxygen. In the case of sampling on board a small boat close to the coast, the beakers are closed and kept in a cooler at about water temperature until they are transported back to the laboratory.

Isolation of individual planktonic foraminifera commences as soon as possible after the collection, typically starting within less than 1 hour. When sampling with a multiple-closing net, which provides multiple samples at the same time, some of the plankton material may have to be kept unprocessed until the next day. Bulk plankton samples tend to become quickly deoxygenated and planktonic foraminifera less viable if the isolation cannot be finished on the day of sampling. If this is unavoidable, we recommend a further dilution with fresh filtered seawater with additional bubble aeration to prevent their deoxygenation.

The isolation of foraminifera is carried out by transferring a subsample of the concentrate that settled at the bottom of the main beaker into a petri dish using a plastic pipette. The microplankton is concentrated in the middle of the dish by swirling its content and the foraminifera are separated from the remaining plankton under a stereomicroscope using needles and/or fine brushes (**Figure 2 main text**). Foraminifera are then transferred individually into small watch glasses or petri dishes containing filtered seawater (about 2-10 ml) using fine brushes or a glass pipette. Once in these smaller dishes the picked specimens are individually cleaned with brushes to remove attached contaminating organic material (**Figure 2 main text**). The handling of planktonic foraminifera with brushes naturally results in some degree of damage to the spines of spinose species. However, in most cases spines are damaged already in the plankton net and for most genetic and morphological analyses, they are of minor importance. The isolation of foraminifera from the bulk plankton sample can be conducted either by representative or exhaustive picking. Using the first approach, only selected specimens, which seem most promising for successful DNA amplification, are taken from the sample, whereas with the second approach every single individual, including empty shells, are picked for quantitative analysis of the sample.

After finishing the isolation of foraminifera, the dishes are washed in tap water or if available in distilled water and dried. The brushes used for picking may be sterilized with boiling distilled water between picking of each specimen or may be simply washed with distilled water. The plankton net and filter mesh cloth are hosed down or washed with fresh water and dried between collections.

1.2. Preservation of specimens

After isolation, foraminiferal specimens are preserved for the transport to the home laboratory or even for long-term storage until DNA extraction is carried out. The preservation of specimens can be conducted according to two different approaches.

Storage in DNA extraction buffer: If specimens are to be stored directly in DNA extraction buffer, a thorough documentation of the taxonomic identity of every single specimen is recommended, especially if the DNA extraction is destructive for the calcite shells (see section 2). Therefore, once they are isolated and cleaned in small petri dishes, each specimen is individually described in the sample collection sheet, assigned a specimen ID and digitally photographed. For imaging, specimens are oriented in the standard umbilical view (compare e.g. **Figure 2 main text**, **image B5**) and confined on a watch glass to help reduce the effect of ship motion. Imaging each individual is highly recommended for future morphological reference in case the shell is lost or dissolved during DNA extraction. After documentation, each individual is transferred with a brush into a small microtube containing extraction buffer (see section 2 for details). Microtubes are autoclaved and transported to the ship in sealed bags. They are opened on board and fresh buffer is pipetted into them immediately before picking.

If DOC buffer (section 2.1.) is chosen for extraction, the entire extraction procedure is carried out immediately after sampling. The extracted samples are then stored at 4°C until they are transported to the home laboratory, stowed in a cooling bag on ice. Back in the laboratory, samples are kept again at 4°C and may be stored in this way for several years. Freezing samples in DOC buffer seems detrimental for DNA extraction and should thus be avoided.

If GITC* buffer (section 2.2.) is chosen, the tubes containing the buffer and the specimen are stored at -20°C. At the end of sampling, the tubes are shipped frozen on dry ice to the home laboratory, where they can be stored at -20°C for several years. It is recommended to protect the tubes from light. The remaining steps of the extraction procedure for GITC* buffer are carried out in the home laboratory and are described in section 2.2.

If specimens are preserved in urea buffer (section 2.3.), samples are stored and transported to the home laboratory at room temperature. Both transport and storage at room temperature is very important, since crystals precipitate in the urea buffer if frozen, which may break the shell. The extraction procedure for this buffer is continued in the home laboratory as described in section 2.3.

Drying and freezing of isolated specimens: For this approach, specimens are cleaned in petri dishes and transferred immediately to cardboard faunal slides (**Figure 2 main text**). The specimens are individually arranged on the slide, air dried and frozen at temperatures between -20°C to -80°C before transport to the home laboratory on dry ice. The slides are then stored at -80°C until processed. Prior to DNA extraction, specimens are individually picked off the slides, photographed under a stereomicroscope, taxonomically identified and catalogued and then transferred to microtubes containing the chosen extraction buffer (section 2). Photographic documentation of the shells can also take place after DNA extraction if the non-destructive buffers are used (see section 2). Since all specimens on a faunal slide defrost while processing individual specimens, we recommend a maximum of 100 specimens per slide to allow for fast processing while avoiding degradation of DNA.

1.3. Culture maintenance

Only healthy looking individuals collected by either diving or plankton tow are chosen for gametogenic culture. Care is taken to minimize physical damage to spines and shells during handling and a plastic Pasteur pipette with cut off tip has proved a good handling device for transfer of cultured individuals. After sampling, individual foraminifera are carefully cleaned of obvious contaminants using a brush and transferred with a Pasteur pipette to 50 ml cell culture jars. These jars are fitted with an air permeable lid and filled with filtered seawater (0.2 µm filter) to avoid additional contamination. It is of advantage to use culturing vials with a flat bottom, since it allows the observation of the specimen in culture under an inverted microscope. All culturing jars are kept at a constant temperature corresponding to the water temperature at the sampling stations and under daylight lamps programmed to a day-night cycle to simulate conditions in the water column. Culturing vials can be kept either in a temperature controlled incubator or in water tanks in which the temperature is kept constant via a flow-through system (**Figure 2 main text**). Usually, planktonic foraminifera recover from sampling stress within a day and the spinose species rebuild their spines (**Figure 2 main text**). Spinose species tend to float in the culturing vial, whereas non-spinose species sink to the bottom of the vial. Each foraminifera is fed a fresh day-old *Artemia salina* nauplius every other day by carefully placing or ejecting the brine-shrimp from a Pasteur pipette onto the foraminiferal rhizopodial network (**Figure 2 main text**). Spinose specimens are vigilantly monitored until they exhibit signs of approaching gametogenesis, which include sinking following spine shedding and expulsion of symbiont debris (Bé *et al.* 1983). At this stage they are transferred to smaller vials containing only 5 ml of freshly filtered sea water and observed even more frequently. When granular cytoplasm or a gametogenic bulge forms close to the aperture, signifying imminent release of gametes (Bé *et al.* 1983), the shell containing the

gametes is transferred into an empty 1.5 ml microtube using a Pasteur pipette and frozen at -20°C. This step has to be carried out quickly before the gametogenic bulge ruptures, releasing the gametes. For non-spinose species, approaching gametogenesis cannot be predicted as easily. It may be marked by a slight change in colour of the cytoplasm due to secondary thickening of the shell wall prior to gametogenesis (Bé 1980) and in some species it is possible to observe the movement of the flagellated gametes through the shell wall (Darling *et al.* 1996). It is therefore important to observe mature specimens very closely, to pick and freeze them just prior to gamete release. All microtubes containing gametogenic individuals are transported back to the home laboratory on dry ice and stored at -20°C (or lower) until DNA extraction.

2 DNA extraction

Extraction of DNA can be carried out using one of three different DNA extraction buffers. While GITC* and urea buffers allow preservation of the calcite shell, DOC buffer dissolves the shell. Due to the danger of contamination it is essential to include negative controls in every step of the molecular analysis.

2.1. DOC buffer

Sodium-deoxycholate, the major component of the DOC buffer (Holzmann & Pawlowski 1996; **Table 1**), acts by dissolving the cell membranes, together with the detergent Triton-X-100, which fulfils the same function. Tris pH 8.5 is used in the buffer to maintain the solution at a constant pH, preventing the denaturation of the DNA that may otherwise occur under extremes of pH (pH <3 or >10; Tsai & Olson 1991; Hurt *et al* 2001). The Ethylenediaminetetraacetic acid (EDTA) disables proteins including DNases, which would denature the DNA. However, EDTA is also a strong calcium chelator commonly used for dissolving $CaCO_3$ (Bodine & Fernalld 1973; Fredd & Fogler 1998; Wade & Garcia-Pichel 2003) and the buffer therefore dissolves the calcite shells of the analysed foraminifera. Therefore, this buffer cannot be used for DNA extraction if the shells are required for further morphometric analysis.

Table 1: Composition of 100 ml DOC buffer. The buffer can be stored at room temperature.

Substance	**Concentration of stock solution**	**Volume**
Tris buffer pH 8.5	1 M	10 ml
Ethylenediaminetetraacetic acid (EDTA), pH 8	0.5 M	0.8 ml
Sodium- deoxycholate (DOC)	10%	10 ml
Triton-X-100	10%	2 ml
H_2O_{bidest}		add up to 100 ml

The buffer is prepared by stirring all ingredients into approximately half the volume of water and then making up to the full volume when all components are dissolved. The solution can be kept at room temperature.

For DNA extraction using the DOC protocol, each specimen is transferred with a fine brush to a 1.5 ml microtube containing 50 µl DOC buffer. The tubes are checked under a stereomicroscope to confirm that the specimens are in the buffer, especially in the case of small individuals that cannot be seen by eye. Care should be taken not to transfer excess sea water. Excess seawater modifies the buffer composition, which might impede the DNA extraction. Each tube is labelled with the specimen ID and then incubated at 60°C (heating block or drying oven) for one hour, during which time the calcite shell is dissolved. Afterwards, the tubes are centrifuged at 10,000 rpm for 5 min to settle remaining particles. To ensure that the DOC buffer reaches quickly the entire cellular content of the specimen, the calcite shell of the foraminifera can be crushed prior to the heating step using a sterilized homogenizer (e.g. a small custom-made glass crusher [modified Pasteur pipette] or a commercially available plastic crusher). Glass crushers are sterilized after each use in 0.1 M HCl and plastic crushers are discarded. Care should be taken to make sure that the specimens or parts of the cytoplasm are not left sticking to the crusher. Afterwards, the extractions are stored at 4°C until required for further analysis. It is not recommended to freeze DOC extracted samples for long term storage, as repeated freezing and thawing has been found detrimental for DNA preservation.

As mentioned above, the disadvantage of DOC buffer lies in the fact that it dissolves the calcite shell of the foraminiferal specimens. However, it does have the advantage of easy preparation, transport and storage, together with an uncomplicated extraction protocol. It has therefore been used extensively in planktonic foraminiferal genetic analysis and it is the buffer of choice if shell recovery is not required.

2.2. GITC* buffer

The protocol for the GITC* buffer was initially developed by de Vargas *et al*. (unpublished, but available in Morard 2010) and extensively used in Morard *et al*. (2009; 2011; 2013), Ujiié *et al*. (2010; 2012; 2014; 2016), Quillévéré *et al*. (2013), André *et al*. (2013; 2014) and Weiner *et al*. (2015). It is based on the classic guanidium isothiocyanate (GITC) RNA extraction buffer first described by Chirgwin *et al*. (1979). GITC buffer is commonly used to isolate intact RNA from animal tissues (Maniatis *et al.* 1982). Cellular structures disintegrate and nucleoproteins dissociate quickly from nucleic acids in the presence of GITC and a reducing agent such as β-mercaptoethanol. The buffer further strongly inactivates RNases. Tris pH 7.6 is added in order to maintain a stable pH in the solution. The detergent Sodium N-Lauroyl-Sarcosine (Sarkosyl; Chakravorty & Tyagi 2001; Hurt *et al*. 2001) is added to promote cell lysis by disruption of the hydrophobic attraction between membrane phospholipids leading to denaturation. For planktonic foraminifera, in order to make the DNA extraction non-destructive for the calcareous shell, the original composition of the buffer was modified by removing EDTA, which is a strong calcium chelator, as described above (**Table 2**; Morard 2010).

Table 2: Composition of 220 ml GITC* buffer. The buffer has to be stored at 4°C in the dark. The difference between GITC and GITC* buffer lies in the addition of EDTA, which is omitted in the GITC* version.

Substance	**Concentration of stock solution**	**Volume**
Guanidinium isothiocyanate (GITC)		100 g
Tris buffer pH 7.6	1 M	10.6 ml
Sodium N-Lauroyl-Sarcosine (Sarkosyl)	20 %	21.2 ml
β-Mercaptoethanol		2.1 ml
H_2O_{bidest}		add up to 220 ml
(EDTA	0.5 M	10.6 ml)

To prepare the buffer, 100g of GITC are dissolved in 100 ml of H_2O_{bidest} by stirring for 6h at room temperature until the solution is clear. This is followed by the addition of 10.6 ml of Tris buffer pH 7.6 and the mix is stirred again for a further 1-2h. This is followed by the addition of 21.2 ml of Sarkosyl and 2.1 ml of β-Mercaptoethanol. The volume is then adjusted to 220 ml by adding H_2O_{bidest} and the buffer is stirred again for 30 min. Since GITC* buffer is sensitive to both light and high temperatures, it should be protected from light during preparation and stored in a brown glass bottle at 4°C.

For DNA extraction using the GITC* protocol for shell preservation, each individually cleaned foraminifera is placed into a 1.5 ml microtube containing 50 µl GITC* buffer. Care should be taken not to transfer excess sea water. At this stage the extraction can be interrupted by freezing the tubes at -20°C, where they can be stored for several years. To continue the extraction, tubes are vortexed followed by a short spin to ensure homogenous mixing of the buffer and that the shell is at the bottom of the tube submerged in the buffer. The tubes are then incubated for 1 hour at 70°C. Every 10–15 minutes tubes are vortexed and centrifuged to ease the digestion of the cellular material by the buffer. The vortexing steps may be omitted when working with thin-walled species to prevent mechanical damage of the shell (Quillévéré *et al*. 2013). The buffer with the extracted DNA is then gently pipetted out of the tubes leaving the shell behind and transferred into a new 1.5 ml microtube. In case of small specimens not visible by eye, this step may be carried out under a stereomicroscope.

Following shell isolation, 50 µl of 100% isopropanol are added to the buffer to precipitate the nucleic acids. The tubes are gently vortexed and then kept overnight at -20°C. The next day

they are centrifuged at 15-18,000 rpm for 15 to 45 minutes (time depending on the centrifugation speed) at 4°C to pellet the DNA. Immediately after the centrifugation, the supernatant is discarded. The pellet, which usually is invisible, is washed with 50 µl ethanol (70 %), and the tubes are centrifuged for 5 to 15 minutes at 15-18,000 rpm at 4°C. The supernatant is again discarded and the tubes are then left open for 2–3 hours for the ethanol to evaporate. Complete evaporation of the ethanol is crucial, since it may otherwise impede the PCR reaction. The pellet is then resuspended in 20 µl of sterilized MilliQ water and briefly vortexed. The tubes containing the extracted foraminifera DNA are stored at -20°C until further analysis. The foraminiferal shell is recovered from the original tube with a brush, cleaned with H_2O_{bidest} to remove the remains of the buffer and kept for future morphological analysis. Cleaning the shell with H_2O_{bidest} is necessary in order to avoid the accumulation of buffer precipitates (**Figure 3 main text**), which impede detailed analysis of the surface of the shell. Shells must be handled with care in order to avoid breaking off chambers or fragile structures (**Figure 3 main text**). The advantage of using GITC* buffer clearly lies in the possibility of preserving the calcite shell. However, preparation and extraction protocols are more time-consuming compared with the DOC buffer and the storage and transport of the GITC* buffer more complex.

2.3. Urea buffer

A buffer containing high concentrations of urea (6-8 M) was originally developed for long-term storage of fish tissue at ambient temperatures and subsequent DNA extraction (Asahida *et al.* 1996). Although urea can denature DNA, it was not observed to have a strong negative effect on DNA amplification success (Asahida *et al.* 1996; Seears & Wade 2014). Urea buffer was developed for foraminifera to allow the preservation of the shells without the requirement for freezing, facilitating transport of samples collected in situations where cold storage and shipment are not available (Aurahs *et al.* 2011; Weiner *et al.* 2014). Modified urea buffers were tested on benthic foraminifera by Seears & Wade (2014).

Tris pH 8.5 is used to maintain the solution of the buffer at a constant pH. NaCl is included to break down the cell membrane by osmotic shock to release the nucleic acids (Tsai & Olson 1991; Tebbe & Vahjen 1993). The detergent Sarkosyl is added to promote cell lysis, as described above. A range of reducing agents can be used in urea buffers, which irreversibly denature the proteins of the cell membrane, leading to cell lysis (Seears & Wade 2014). They include β-mercaptoethanol (Chakravorty & Tyagi 2001), Dithiothreitol (DTT; Bienvenue *et al.* 2006) and 2-Aminethanethiol Hydrochloride. These reducing agents may have strong odours, must be handled in a fume cabinet and must be added fresh to the buffer since they degrade quickly in solution. The reducing agent used in the urea buffer described below (**Table 3**) is Tris(2-carboxyethyl)phosphine (TCEP) which has the advantages of being odourless, a more powerful reducing agent and more stable in solution.

Table 3: Composition of 100 ml urea buffer. For long term storage the buffer should be kept at room temperature.

Substance	**Concentration of stock solution**	**Volume**
Tris buffer pH 8.0	500 mM	20 ml

Sodium chloride (NaCl)	1 M	10 ml
Sodium N-Lauroyl-Sarcosine (Sarkosyl)	10%	10 ml
Urea		48.048 g
Tris(2-carboxyethyl)phosphine (TCEP)	100 mM	2 ml
H_2O_{bidest}		add up to 100 ml

To prepare the buffer, 48.048 g of urea (8 M final concentration) is weighed out and placed into a 500 ml glass beaker. All liquid components are then added to the urea (20 ml of 500 mM Tris buffer (pH 8.0), 10 ml of 1M Sodium chloride, 10 ml of 10% Sarkosyl, 2 ml of 100 mM TCEP) and the volume adjusted to 95 ml with H_2O_{bidest}. The glass beaker is warmed (60°C) on a hotplate stirrer and mixed manually with a glass mixing rod to start dissolving the urea crystals. A magnetic mixing bar is then added and the buffer magnetically stirred until all the urea crystals are dissolved which may take several hours at this level of saturation. The volume is then adjusted to 100 ml and the buffer stored at room temperature, to avoid urea precipitation at low temperatures.

For DNA extraction from the shell using the urea protocol, each live foraminifera is placed into the bottom of a 1.5 ml microtube using a fine brush and the tube is checked to ensure the presence of the shell. Care should be taken not to transfer excess sea water. 100 µl of urea buffer are then carefully placed into the bottom of the tube covering the shell while ensuring that the tip does not touch and break the shell. A further check is carried out to ensure that the shell is at the bottom of the tube and not floating on the buffer meniscus. As soon as the cytoplasm within the shells disappears (usually within a week) the shells can be separated from the buffer. This is done under a stereomicroscope to avoid breaking the empty shell. Using a long fine pipette tip, the buffer containing the DNA is taken up from around the shell and placed into a new 1.5 ml microtube. The foraminiferal shell is recovered from the original tube with a brush, cleaned with H_2O_{bidest} to remove the remains of the buffer and kept for future morphological analysis.

To extract DNA, 5 µl tRNA (0.2 µg/µl), 200 µl H_2O_{bidest} and 300 µl chloroform : isoamylalcohol (24:1) are added to the urea buffer containing the DNA and tubes are gently shaken for 10 min. The microtubes are then centrifuged for 12 min at 13,000 rpm and the supernatant is transferred to a new 1.5 ml microtube. Subsequently, 2.5 volumes (700 µl) of ice cold ethanol (95%) and 0.1 volumes (28 µl) of 3 M Sodium Acetate are added, the tubes are vortexed and left in the freezer (-20°C) overnight. The next day they are centrifuged at 13,000 rpm for 15 min to pellet the DNA. Immediately after the centrifugation, the supernatant is carefully pipetted off and discarded. The usually invisible pellet is washed by adding 500 µl of 70% ethanol. The tubes are centrifuged for 15 min at 13,000 rpm and the supernatant is again discarded. The tubes are shortly spun again to be able to remove the

remaining liquid phase and the pellet is dried for 15 min at 42°C. The pellet is then re-suspended in 40 µl sterilized MilliQ water and the tubes are briefly vortexed. The tubes containing extracted foraminifera DNA can then be stored at -20°C.

The major advantage of the urea buffer is that samples are transported from the sampling location to the home laboratory without refrigeration, while still preserving the foraminiferal shells. Specimens can be stored in the urea buffer at room temperature for long periods of time and well preserved shells have been obtained using this method (**Figure 3 main text**). However, the preparation of the buffer and especially the DNA extraction are relatively time-consuming when compared with DOC buffer extraction.

3 Analysis of single-cell DNA extracts

3.1 DNA amplification

The PCR reaction mixture is prepared according to standard protocols and is typically composed of H_2O_{bidest}, reaction buffer, $MgCl_2$, dNTPs, a primer pair, polymerase and template DNA. Since the exact composition of the reaction mixture depends on the type and brand of polymerase used, no detailed composition is given here. Throughout the years, different polymerases have been used due to their differences in PCR efficiency and proof reading capacity. However, Taq Polymerase has proved an excellent compromise between amplification speed and proof reading ability. No direct advantage concerning the amplification success of one polymerase over the others has been observed in recent years. In a few cases, PCR additives such as bovine serum albumin (BSA) or dimethyl sulfoxid (DMSO) were added to the reaction mixture in order to achieve higher amplification specificity.

The PCR reaction is carried out under standard conditions for about 30 cycles, with the annealing temperature depending on the primer set used. PCR products are run on an agarose gel (1%) together with a calibration ladder to verify the amplification success and are then purified. This can be done in one of two ways. Firstly the entire PCR product is loaded on a gel and the band of desired length is cut from the gel and purified using a spin column or gel extraction kit. The second is to purify the entire PCR product with a PCR purification kit without gel extraction. The purified products of both methods can be either directly sequenced or cloned and sequenced. When non-specific, more general eukaryote primers are used for amplification, a second band is frequently observed on the gel, derived from symbiont or contaminant DNA. The first purification method of cutting the foraminiferal band from the gel avoids sequencing such potential contaminant DNA, which could obscure the foraminiferal signal during sequencing. Therefore, direct purification of the PCR product using a PCR purification kit should only be used if a single foraminiferal band is observed on the gel.

3.2 Nested and semi-nested PCR

In the case that no band is observed on the gel after the primary PCR run, a secondary PCR can be conducted using a different primer pair. Therefore, after the primary PCR run using foraminiferal specific or universal primers, a secondary PCR run with 20 to 40 repeating cycles is conducted, with more specific primers that anneal within the already amplified fragment. Although beneficial in greatly increasing target template number, using a secondary PCR approach may also increase contaminant DNA. However, such contamination is

bypassed by cutting out the appropriate foraminiferal band when the PCR product is run on a gel.

3.3 Cloning

For the cloning of the rRNA of planktonic foraminifera, a ~500-1000 bp fragment of the SSU rDNA is inserted into a plasmid vector (e.g. TOPO® TA Cloning® Kit, Invitrogen) and multiplied in chemically competent *E. coli* cells. All steps are carried out according to the manufacturer's protocol. The plasmids can optionally be purified using e.g. the PureLink® HQ Mini Plasmid Purification Kit (Invitrogen) or sequenced directly after dissolving the colonies in 10 to 20 µl of the sterilized Milli-Q water and conducting a post-cloning PCR. Sequencing of all PCR products and clones is done by Sanger sequencing.

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DOC DNA Extraction

Buffer composition

Substance	Concentration of stock solution	Volume
Tris buffer pH 8.5	1 M	10 ml
EDTA pH 8	0.5 M	0.8 ml
DOC	10%	10 ml
Triton-X-100	10%	2 ml
H_2O_{bidest}		add up to 100 ml

Buffer preparation

- Stir all ingredients into half the volume of H_2O_{bidest}
- Add up to 100 ml with H_2O_{bidest} when all components are dissolved
- Store buffer at room temperature

DNA Extraction

- Place single, cleaned specimen into 1.5 ml microtube containing 50 µl DOC buffer using a fine brush (avoid transfer of excess sea water)
- Check that shell is present in the tube and covered by buffer
- Label tubes with specimen ID
- Optional: Crush the calcite shell of the foraminifera using a sterilized homogenizer
- Incubate tubes at 60°C (heating block or drying oven) for 1 hour
- Centrifuge at 10,000 rpm for 5 min
- Store extracted DNA at 4°C

GITC* DNA Extraction

Buffer composition

Substance	Concentration of stock solution	Volume
GITC		100 g
Tris buffer pH 7.6	1 M	10.6 ml
Sarkosyl	20 %	21.2 ml
β-Mercaptoethanol		2.1 ml
H_2O_{bidest}		add up to 220 ml

Buffer preparation

- Dissolve 100g of GITC in 100 ml of H_2O_{bidest} by stirring for 6h at room temperature
- Add 10.6 ml of Tris buffer pH 7.6 and stir for a further 1-2h
- Add 21.2 ml of Sarkosyl and 2.1 ml of β-Mercaptoethanol
- Adjust volume to 220 ml with H_2O_{bidest} and stir for 30 min
- Store buffer at 4°C in a brown glass bottle

DNA Extraction

- Place single, cleaned specimen into 1.5 ml microtube containing 50 µl GITC* buffer using a fine brush (avoid transfer of excess sea water)
- Vortex tubes, spin shortly and check that shell is present in the tube and covered by buffer
- Incubate tubes for 1h at 70°C and vortex and spin tubes every 10–15 min in between
- Take up buffer containing the DNA from around the shell and place it into a new 1.5 ml microtube

- Recover the empty shell from the original tube with a brush, clean it with H_2O_{bidest} and keep it for morphological analysis
- Add 50 µl of 100% isopropanol to the buffer containing the DNA
- Gently vortex the tubes and keep them overnight at -20°C
- Centrifuge at 15-18,000 rpm for 15 to 45 minutes (time depending on the centrifugation speed) at 4°C
- Discard supernatant
- Wash pellet with 50 µl of 70 % ethanol
- Centrifuge for 5 to 15 minutes at 15-18,000 rpm at 4°C
- Discard supernatant
- Leave tubes open for 2–3 hours for the ethanol to evaporate
- Re-suspend the pellet in 20 µl of sterilized MilliQ water and vortex briefly
- Store extracted DNA at -20°C

Urea DNA Extraction

Buffer composition

Substance	Concentration of stock solution	Volume
Tris buffer pH 8.0	500 mM	20 ml
NaCl	1 M	10 ml
Sarkosyl	10%	10 ml
Urea		48.048 g
TCEP	100 mM	2 ml
H_2O_{bidest}		add up to 100 ml

Buffer preparation

- Place 48.048 g of urea into a 500 ml glass beaker
- Add all liquid components: 20 ml of 500 mM Tris buffer (pH 8.0), 10 ml of 1M Sodium chloride, 10 ml of 10% Sarkosyl, 2 ml of 100 mM TCEP
- Adjust volume to 95 ml with H_2O_{bidest}
- Heat glass beaker to 60°C on a hotplate stirrer and mix first manually with a glass mixing rod then add a magnetic mixing bar and stir for several hours until urea is dissolved
- Adjust volume to 100 ml
- Store buffer at room temperature

DNA Extraction

- Place single, cleaned specimen into bottom of a 1.5 ml microtube using a fine brush (avoid transfer of excess sea water)
- Add 100 µl of urea buffer into the bottom of the tube covering the shell

- Check that shell is present in the tube and covered by buffer
- When cytoplasm has disappeared from the shell (~ 1 week), take up the buffer containing the DNA from around the shell and place it into a new 1.5 ml microtube
- Recover the empty shell from the original tube with a brush, clean it with H_2O_{bidest} and keep it for morphological analysis
- Add 5 µl tRNA (0.2 µg/µl), 200 µl H_2O_{bidest} and 300 µl chloroform : isoamylalcohol (24:1) to the urea buffer containing the DNA
- Shake gently for 10 min
- Centrifuge for 12 min at 13,000 rpm
- Transfer the supernatant into a new 1.5 ml microtube.
- Add 2.5 volumes (700 µl) of ice cold ethanol (95%) and 0.1 volumes (28 µl) of 3 M Sodium Acetate
- Vortex the tubes and keep them overnight at -20°C
- Centrifuge at 13,000 rpm for 15 min
- Carefully pipet of supernatant and discard it
- Wash pellet with 500 µl of 70% ethanol
- Centrifuge for 15 min at 13,000 rpm
- Carefully pipet of supernatant and discard it
- Shortly spin the tubes and remove remaining liquid phase
- Dry pellet for 15 min at 42°C in heating block
- Re-suspend the pellet in 40 µl of sterilized MilliQ water and vortex briefly
- Store extracted DNA at -20°C