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EASY AND INEXPENSIVE NUCLEIC ACID EXTRACTION PROTOCOL FOR RECALCITRANT SPECIES

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ABSTRACT

Background: Some of the major contributions to the rapid genetic improvement of commercial plant species and crops come through the identification of genetic markers linked to specific traits with a marketable value that have been difficult or impossible to identify and improve with conventional methods. Next-Generation Sequencing (NGS) is a powerful platform that has enabled the exploration of the genome in different plant species. The success of NGS is dependent on the quality of DNA or RNA extracted. Methods and commercial kits for extraction of intact nucleic acids for many model plant species are available but the need exists still for high-throughput methods suitable for most non-model plants, in particular, recalcitrant plant species.

Findings:

- Our group has developed a detailed method that allows the isolation of genomic DNA or total RNA from different tissues from different plant species that yield enough material suitable for high-throughput SNP genotyping, sequencing applications and traditional downstream genetic analysis.

- One single operator can extract nucleic acids from 192 samples in about 3.5 hours but the protocol is completely or partially-compatible with automation depending on available equipment.

- We provide tips and comments that will help to improve the quality of the isolated material and solutions for everyday laboratory problems that will save time and money. - Little variations in the protocol allow the application of the method to plant tissues from fresh, dry or frozen samples.

- The protocol is easy to set up in any molecular research laboratory, uses inexpensive reagents and handmade solutions that can be stored for several months, does not require the use of liquid nitrogen, and works best with 15 - 30 mg of fresh, frozen or dry plant material.

- DNA and RNA have been isolated from at least 30 species, including especially recalcitrant forest trees from angiosperm and gymnosperm families. DNA has been also extracted from stored (2 to 6 years) silica-dried samples of species of Pinaceae and Nymphaeaceae families.

- The protocol is a home-made adapatation of different published protocols that can be used in combination with different nucleic acids extraction kits. For reference consult: Ivanova, N.V. et al. 2006, Ivanova, N.V. et al 2008, and Whitlock R. et al. 2008.

GUIDELINES

General notes

This section provides general guidelines and instructions before the isolation of genomic DNA or RNA from frozen, fresh and dry samples from different types of tissues and plant species in particular recalcitrant species (**Table 1**).

Table1_List of species.xlsx

- The protocol is written for users with and without previous experience in the isolation of nucleic acids. Therefore, descriptions are detail-oriented.

- The protocol was designed and adjusted to isolate genomic material from 192 samples (2 plates of 96 samples) but can be scaled up by automation.

- Depending on the tissue, yield and quality of extracted nucleic acids will vary but we have been successful at extracting enough material for sequencing library preparation from a very small amount of tissue (e.g. from a minimum of 7-10 mg tissue from one single megagametophyte).

- For scientific work, room temperature (RT) is taken to be about 20 to 25 degrees Celsius with an average of 23 °C (about 73.4 Fahrenheit (°F)).

- We use extra-large 1200 ul tips without filters to add solutions and filtered ones to transfer samples. We reused strip caps during the protocol (as much as possible) by placing them on clean paper towels following the same order as on the plates. We also provide simple protocols to reuse the silica gel and to clean the steel beads for grinding.

- All dilutions that require water are made using DNase/RNase-free molecular grade water.

- Users need to wear gloves during the collection of material and extraction of DNA and RNA.

1.1. Centrifugation:

- All centrifugation steps will be performed at 5.000 rpm if not specified. We used Sigma 4-15C and Sigma 4K-15 (Qiagen), rotor Nr. 09100 Sigma.

- Although most of the centrifugation steps are short and can be done at RT, we recommend spinning the samples at 4 C during the RNA isolation. **Tip**: To keep the centrifuge at 4 C during RNA isolation while minimizing damage to the centrifuge motor, users can spin the rotor at very low speed (500 rpm) between steps.

Tip: We have observed that depending on the microplates, the buckets used to hold the plates during centrifugation, deep-well plates, and PCR plates could be damaged or broken at the base, specifically at the corners. To avoid the damage, we place two thin styrofoam covers or two tip trays, under the deep-well plates or the PCR plates respectively (**Figure 1**). These materials can be used several times and they are easy to find. In our case, the styrofoam covers come as part of the PALL plate packaging and the tip trays are part of a refill tip system.

- We recommended having a scale nearby to balance the plates before the centrifugation steps. Minimizing the variation between samples and plates is particularly helpful for the first centrifugation steps and to increase the reproducibility of the method.

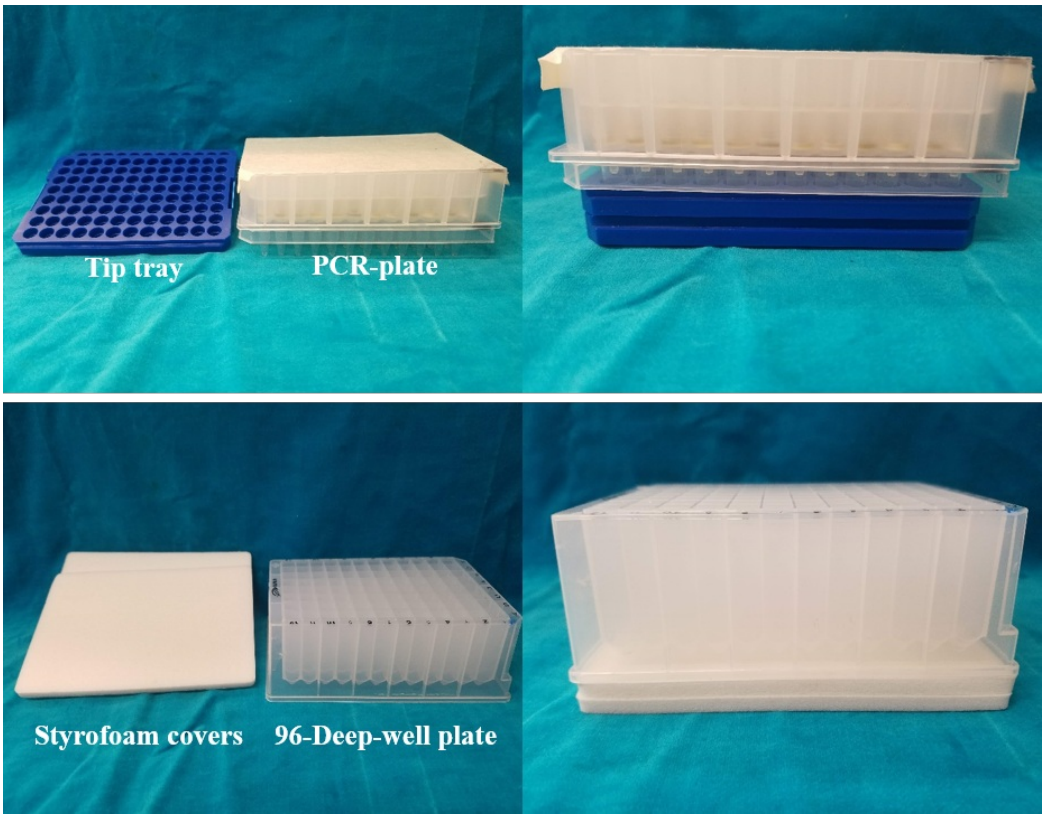


Figure 1. Add protection under plates to avoid damage during the centrifugation steps.

1.2. Plate set-up and labeling:

This section provides guidance to prepare two 1.1-ml strip microtube plates for sample collection and grinding. This section is intended to avoid the risk of reversing the orientation of the strip tubes and/or the plates and therefore mixing up the sample IDs during the extraction. Our general recommendations will guide you to make the labeling process more efficient while saving time and increasing accuracy. This method was used for frozen, dry and fresh tissues and can be adjusted depending on the user's priorities.

Using a wide-point sharpie, mark the upper left corner of each plate. The use of two different colors, as well as numbers, help to track each plate. **Tip**: marks can be done in any corner of the plate but we selected the left corner for visualization purposes.

Using a fine-point sharpie, make two lines across the upper part of the 1.1 ml strip tubes in the first row and one line on the tubes in the last row in each plate. It is not necessary to add numbers to every single tube but it is helpful to label the upper and the bottom tubes in each column. **Tip**: We recommend to draw the lines when tubes are seated on the plate; in this way, the lines will be done on a visible location without moving the tubes out of the plates during the extraction (**Figure 2.A-B**). **Tip**: always check to make sure the mark on the plate is always on the left upper corner and the marks at the end and top of the strips go back into the rack in the same position.

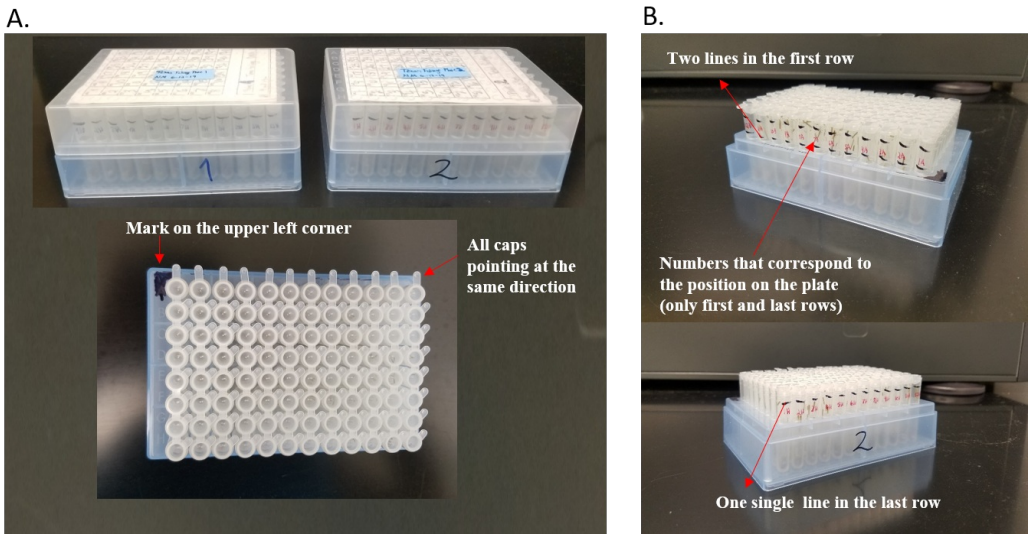


Figure 2. A-B. Instructions to label 1.1 ml tubes plates before the extraction.

Add one (4 mm) stainless steel bead into each of the empty strip tubes. **Tip**: For grinding, users will use two steel beads. We recommend adding the second steel bead after adding the plant material though. This helps to keep the plant material in between the beads and facilitates the grinding, therefore decreasing the chances to get the material stuck at the bottom of the tubes. (**Figure 3**).

Figure 3. Grinding set-up for dry material.

1.2.1. For RNA or DNA isolation from frozen material:

Users label the plate (s), add a steel bead to each tube and place them on dry ice or in a freezer at -80 °C prior to the collection of plant material for at least 30 minutes. This will keep materials frozen before adding the plant material. It is important to keep the plates frozen during the collection of the samples and grinding as well.

1.2.2. For dry or fresh material used during DNA isolation:

Users label the plate (s), add a steel bead to each tube before adding the material. All steps are done at RT.

1.3. Sample collection:

The time needed to collect plant material can vary greatly depending on the nature of the tissue, the condition and location of the plant material, and the preparation of the samples (e.g. if tissue needs to be weighed).

In our case, we used a hard transparent plastic laminate to cover the empty tubes by locating the single hole in the position A1 on the plate. This laminate moves freely on top of the plate avoiding cross-contamination of the samples while working on each individual sample tube (**Figure 4.A**).

Using a small plastic funnel, add 7 - 30 mg of plant tissue to each tube on the plate. We used disposable otoscope (an instrument doctors use to examine ears) covers or paper funnels that can be easily reused. **Tip**: to dissipate the static and to minimize the material adhered to the walls of the funnels, we covered the interior with a piece of aluminum foliage or lab tape.

We recommend cutting the plant material into little pieces by using small scissors (**Figure 4.B**). **Tip**: Users can use an estimation of the plant material instead of weighing every sample (e.g. 4 needles of ~3 cm length = ~25 mg dried tissue or 2 lab spoons of ground or semi-ground material = 15 mg).

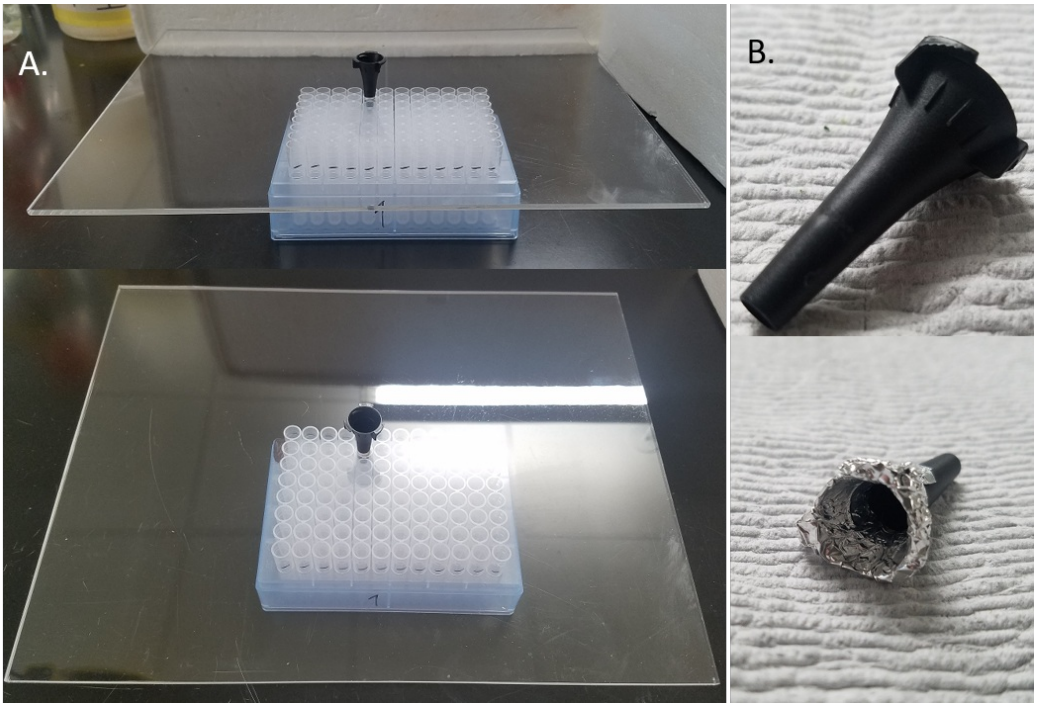


Figure 4. A. Plastic cover for 1.1 ml plate to avoid cross-contamination while placing the plant material into each tube. B. Disposable otoscope cover used as a funnel, covered with aluminum foil to minimize static.

After placing the material, add a second steel bead to each sample and close the tubes. **Tip**: if users are working with frozen material, we recommend to keep the second steel bead that will be added to the tubes on dry ice before adding them to samples. This will reduce the chances that the tissue gets attached to the balls due to the change in the temperature of the surfaces and will have a positive effect during the grinding.

Use 8-strip caps to close tubes. **Tip**: Strip caps have a flange at one extreme. It would be easier to close and open the tubes if all flanges are pointing at the upper part of the plates because one hand can hold the plate while the second hand is pulling the caps out of the tubes in the opposite direction (**Figure 2.A**).

1.4. Tissue grinding:

This section provides guidelines and instructions to grind plant tissue before the isolation of DNA or RNA. Tissue grinding influences the yield and quality of the extracted nucleic acids. We use a Qiagen Tissuelyser Retsch mixer Cat. No. 85210 and two sets of TissueLyser adapters to homogenize the tissue. **Tip**: During storing at - 80°C keep tubes sealed with proper caps. Samples can be ground days or even months before the isolation of nucleic acids but special care must be taken for frozen or dry samples (see below).

1.4.1. For frozen samples (before isolation of DNA and RNA):

Be sure each tube has two steel beads before grinding. Have dry ice on hand. Liquid nitrogen can be used as well but we tried to minimize

its use because dry ice presents more advantages. Manipulation and transportation of dry ice are easier; this is particularly important when samples need to be collected in the field and far away from a laboratory. Dry ice can also be transported in coolers and lasts for longer periods of time.

If your grinding equipment does not control temperature we recommend to keep tissue lyser adapters on dry ice or in a freezer at - 80°C for at least one hour prior to grinding.

Grind the samples one time at 25 Hz for **00:02:00** . Place the adapters and plates on dry ice for **00:02:00** . Repeat this step 2-3 times. For <10-25 mg of frozen tissue, a total of **00:04:00** , should be enough to get a nice powder. **Tip**: Reverse orientations of the plates and switching shaker arms between grinds will improve the homogenization among the samples. Repeat this step if plant material does not look homogenous. We do not recommend more than 8 minutes of total grinding to avoid possible damage at the base of the tubes by friction with the steel beads under frozen conditions. **Tip**: Never let the samples be thawed without adding buffer and maintain plates and samples on dry ice until the extraction.

Although samples can be ground weeks before the extraction, we suggest to not grind samples more than three months prior to the extraction.

1.4.2. For dried samples (isolation of DNA):

Grind the samples one time at 25 Hz for 2-3 minutes. Repeat this step 2-3 times. For 10-25 mg of dried tissue, a total of nine minutes grinding should be enough to get a nice powder (**Figure 5**). Reverse orientations of the mini tube plates and switching shaker arms between grinds will improve the homogenization among the samples. Repeat this step if plant material does not look homogenous. **Tip**: We do not recommend more than nine minutes of total grinding to avoid possible damage at the base of the tubes by friction with the steel beads.

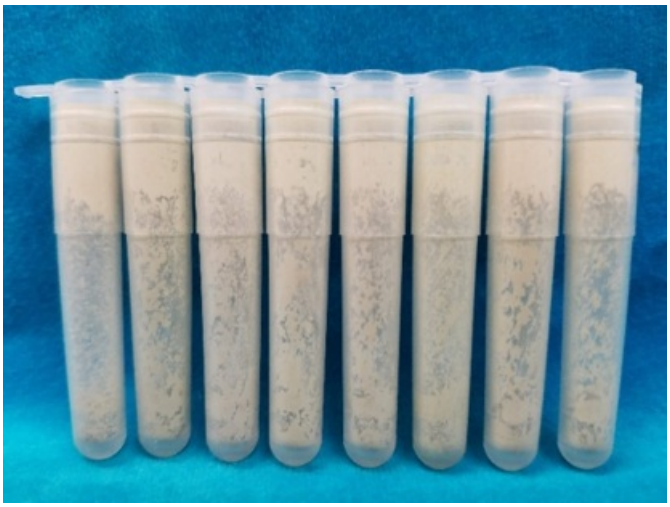


Figure 5. Dry sample after grinding using two 4 mm steel beads.

Although samples can be ground weeks before the extraction, we suggest keeping tubes sealed and plates in a ziplock bag with silica gel. Samples can be stored for at least 3 years on silica gel. **Tip**: Longer periods on silica gel decrease yield and quality of the DNA but could be enough for sequencing and downstream analysis depending on the user's purpose.

1.4.3. For fresh samples (Isolation of DNA and RNA):

Labeling and collection of material can be done at RT or keeping samples and plates on ice. Before grinding add half of the total lysis buffer needed for the extraction (see Lysate preparation in Steps section). **Tip**: Never grind fresh tissue without adding lysis buffer first, this will degrade both DNA and RNA.

1.5. Reusing silica gel beads and steel beads:

Our group tries to reuse as many materials as possible while taking care to avoid future cross-contamination. Here, users can find two

simple protocols to clean and reuse silica gel beads and steel beads. Dry silica gel protocol.pdf

Steel beads cleaning method for RNA and DNA extraction protocol.pdf

MATERIALS TEXT

Table 2. Materials_protocolsio.xlsx

SAFETY WARNINGS

- Guanidine and chloroform residues must be sent out as hazardous chemical waste due to hazardous chemicals in the lysis buffer (guanidine thiocyanate, beta-mercaptoethanol.) and the high alcohol content of the wash buffers (~70-90% alcohol concentration); this waste cannot be disposed of in the sink. **Tip**: be sure all waste from the protocol will be disposed of by following the appropriate waste management procedure in your institution.

BEFORE STARTING

- Preheat an oven at 65 °C for one hour before starting the protocol. Prepare lysis buffers according to the recipes and be sure to add Na_2SO_3 or beta-mercapthoethanol right before use. **For DNA**: Prewarm LBD for one hour before adding it to each sample. We provide an excel spreadsheet that can be easily used to calculate the amounts of solutions based on the initial volume that users require. Table 3. Buffer and recipes_protocolsio.xlsx
- Clean the area and the pipettes that you are planning to use during the extraction with 75% Ethanol.
- All samples need to be ground to a fine powder (10- 30 mg) and kept in the appropriate condition: at - 80 °C in the case of frozen samples, or into a ziplock bag with silica gel if they are dry (For tissue grinding see section 1.4 in the Guidelines & Warnings).
- Read the entire protocol before starting the extraction. Prepare and label extra tubes and plates as necessary. Avoid exposing samples to extended periods of time between steps; this will not only save time but enhance the quality of the extracted genomic material.
- Prepare ethanol at 70% with free DNase/RNase-free molecular grade water and keep it at 20 °C for at least one hour before the extraction.
- Place ethanol 95% v/v and the Wash Buffer at - 20 °C for at least one hour before the extraction.

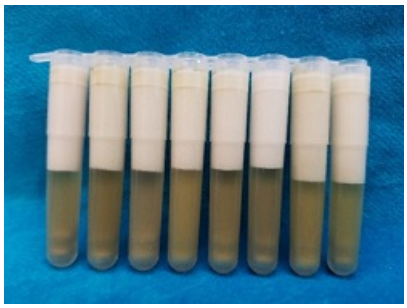
Prepare lysate

1 This section provides guidelines and instructions to isolate DNA and RNA from different types of tissues and plant species. A general protocol is described but users need to read carefully for specific instructions to improve the quality and yield of the isolated genomic material. We have tested both lysis buffers for DNA and RNA interchangeably with similar results but we encourage the users to test the buffers for their own type of tissues and downstream analyses. We provide a table with the material that has been working best for us. **Tip**: The protocol can be used for the extraction of DNA and RNA from tissues that have lots of starch, lipids and reserved material, like megagametophytes. We recommend using the protocol for RNA extraction for isolation of both DNA and RNA because this one has a shorter period of **65 °C** incubation.

1.1 After grinding, tissue can be stuck at the upper part of the tube forming a layer that will not allow the buffer to pass through. Spinning at **1000 x g 00:00:30** (thirty seconds) before removing the caps is enough to pull down all tissue. Open tubes carefully and clean your gloves with tissues and ethanol **75 % volume** while removing the strip caps or sealing mat. This will reduce cross-contamination between samples. **Tip**: toothpicks can be used to pull down the tissue instead of the centrifugation step. Use only one per sample. **For RNA**: spin samples at **1000 rpm, 4°C 00:00:15** (15 seconds).

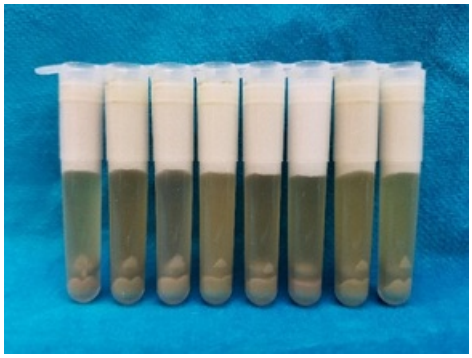
1.2 Add **600 µl** Lysis buffer. **For RNA (LBR)**: Place minitube plates with ground samples for 1-2 minutes on ice to thaw the sample right before adding the lysis buffer. Keep the LBR on ice before adding it to each sample. **Tip**: Do not proceed until the buffer in all tubes is liquid. **For DNA (LBD):** add prewarmed LBD at **60 °C** . Close tubes with strip caps.

1.3 Homogenize the lysate by shaking plates in the Tissue Lyser and repeat these steps if the lysate does not look homogenous. **For DNA:** 4-6 minutes at 25 Hz and **For RNA:** 3 minutes at 25 Hz. Keep plates and adaptors **On ice** in case you need to repeat this step. While plates are shaking, prepare a second set of 192 clean 1.1 ml tubes in two plates to transfer the clean lysate. **For RNA**: Add **10 µl** of **20 % volume** sarkosyl to each tube (**10 µl** sarkosyl/ **1 ml** lysate)) and keep plates at **4 °C** .

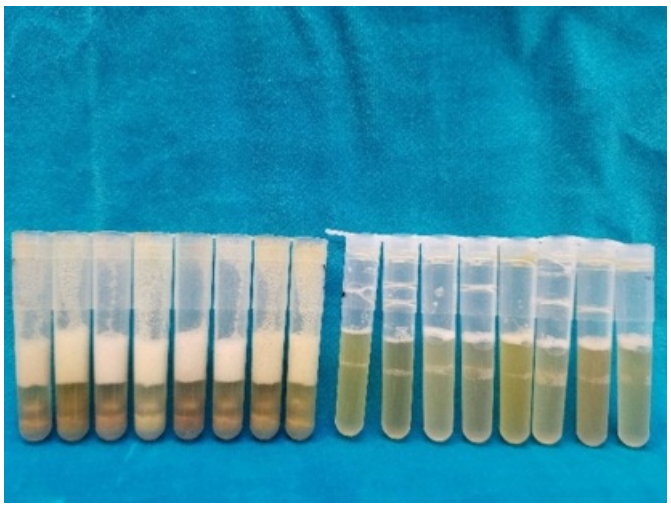


An alternative for DNA: this step can be extended if samples are too old (more than two years in silica gel) or have a high content of phenols. After the addition of LBD, shake the plates for 5 minutes at 25 Hz, then incubate at **65 °C** for **00:30:00** (30 minutes). Repeat this step 3X for a total incubation of 1.5 hours. Spin plates at **5000 rpm, 4°C 00:10:00** (10 minutes) and go to step 2.

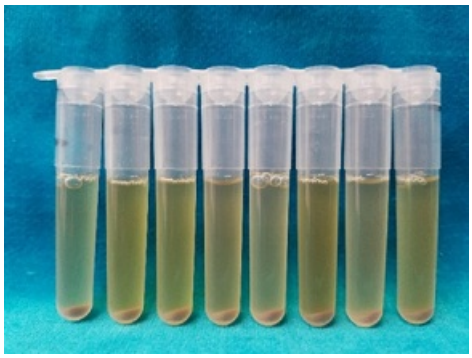
1.4 Spin the plates at **1000 x g 00:00:30** (30 seconds) to settle out of the liquid. **For RNA**: place tubes on ice. Remove the caps from all tubes and be sure to clean your gloves between rows.



1.5 Transfer 400 - 600 ul of lysate and add fresh LBD or LBR up to 1 ml to the new clean set of tubes (step 1.3). **For RNA**: tubes might contain **10 µl** of **20 % volume** Sarkosyl. **Tip:** this step can be adjusted depending on the type and nature of the tissue. Increasing or decreasing the amount of lysate and the dilution may help to improve the results.



1.6 Incubate for 10 minutes at **65 °C** , mix by slowly flipping the position of the plate by hand every 5 minutes. Cool on ice for about 10 minutes, then spin **5000 rpm, 4°C 00:01:00** (1 minute).



Remove proteins and debris

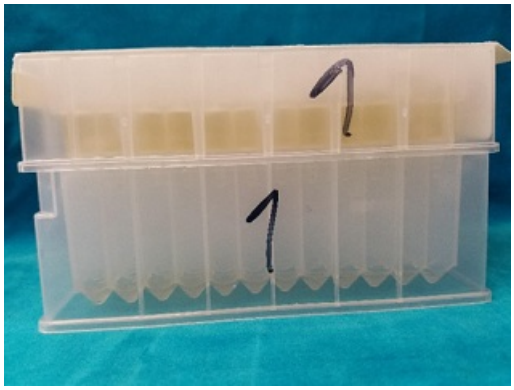
2 Prepare 96-deep well plates, airpore tape sheets covers, PALL-plates, PCR plates and sealing foils/strip caps for PCR plates. **Tip**: Keep the same labels and marks used for the minitube plates. Minimize the spinning times help to keep the quality of the filters in the PALL plates and therefore improve the quality and DNA yield. If a small amount of liquid remains in some wells, empty into waste by flipping over quickly before moving on to the next wash addition. Usually this is an indication that original lysate needs to be more diluted.

2.1 In a new empty deep-well plate, combine **900 µl** of **95 % volume** ethanol and **600 µl** lysate from step 1.6. Mix with a multichannel pipette.

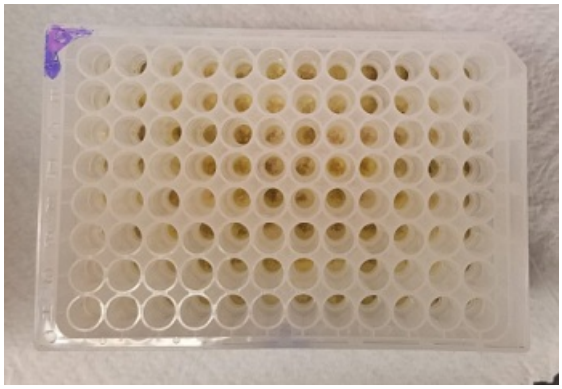
An alternative for DNA: In a new set of 8-strip minitubes, combine **400 µl** of lysate from step 1.3 and **600 µl** chloroform:isoamyl alcohol (24:1), under the fume hood. Close tubes with strip caps. Invert plates for ~20X gently by hand, then spin **5000 rpm, 4°C 00:10:00** (10 minutes). **Tip**: we strongly recommend to use a gas mask even if the work is done under a fume hood. Chloroform:isoamyl alcohol needs to be poured into a glass reservoir or petri dish; plastic containers made of polystyrene will melt and should be avoided.

2.2 Transfer **600 µl** of lysate from step 2.1 to the PALL-plate. Cover each PALL- plate with an airpore tape sheet and let them sit at least 45 minutes in **-20 °C** freezer. **Tip**: 30 -60 minutes have given the best results for most of the tested samples, longer periods of time at **-20 °C** or **-80 °C** increased yield but decreased the quality of the genomic material.

An alternative for DNA: Transfer 150 - 200 ul of the supernatant directly to a PALL-plate and add 500 - 600 ul cold **95 % volume** ethanol. **Tip**: Be careful at removing the top layer (clear supernatant). Touching lower layers will have a significant effect on the quality. Cover each PALL- plate with an airpore tape sheet and let them sit at least 45 minutes in **-20 °C** freezer. **Tip**: 30 -60 minutes have given the best results for most of the tested samples, longer periods of time at -20 or -80 C increased yield but decreased the quality of the genomic material.



2.3 Spin plates 1-2 minutes. **Tip**: Entending the centrifugation time by another two minutes can help ensure all liquid goes down through the glass-fiber filter. We do not recommend to spin plates for more than 5 minutes in total.



2.4 Add **600 µl** of PWB (Protein Wash Buffer) to each sample. Spin plates for **00:01:00** (1 minute). Discard flow-through. Repeat this step 2X. **Tip**: plates can be spun another two minutes until all liquid goes down. We do not recommend to spin the plates for more than three minutes.

2.5 Add **750 µl** of WB (wash buffer) to each sample. Spin plates for **00:01:00** (1 minute). Discard flow-through. Repeat this step 2X.

Elute DNA and RNA

3 Place the PALL plate on top of a PCR plate. **Tip:** *Label the plate properly and make sure the positions match between plates (e.g. position A1 on PALL plate matches position A1 on PALL-plate and PCR plate).*

3.1 Spin plates for 1 minute, then hold at room temperature (in the hood) for **00:05:00** (5 minutes). **For RNA:** Keep plates on ice while drying on the hood.

3.2 **For DNA**: Elute DNA with 20 - 40 uL AE buffer (at 50 C), over 5 minutes at room temperature. **For RNA**: Elute RNA with 20 - 40 uL molecular grade water (DNase and RNase-free), over 2 minutes keeping plates on ice. Spin plates for **00:01:00** (1 minute).

3.3 Cover each plate with a sealing foil or with 8-strip caps for PCR plates. **For RNA**: plates can be stored at **-80 °C** . **For DNA**: plates can be placed at **-20 °C** or at **-80 °C** for long storage.

Cleaning DNA and RNA

4 After isolation, the optional DNase or RNase treatments were performed. The nucleic acid concentrations and purity were measured using a NanoDrop 2000 spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA) and integrity was analyzed on an Agilent 2100 Bioanalyzer with Pico chips (Agilent Technologies, Waldbronn, Germany). Users can evaluate if this step is required based on the downstream analysis needed. **Tip**: Add DNases or RNases only with filtered tips. RNases and DNases need to be handled very carefully to avoid contamination of the equipment and degradation of RNA or DNA during the extraction.

4.1 **For RNA**: To clean and concentrate RNA samples we use the RNA Clean & Concentrator- 5 TM kit from Zymo Research.
RNA clean-up protocol.docx

For DNA: To clean and concentrate DNA samples a variety of kits and protocols can be used depending on downstream analysis and requirements.