Oct 05, 2022

DNA extraction | CTAB-chloroform | 96 wells plate

Forked from 96-well CTAB-chloroform DNA extraction

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dx.doi.org/10.17504/protocols.io.5qpvor5jdv4o/v1

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Citation: Lila Fishman, Simon Joly, Andrea Corkal, JÃÂ©rÃÂ´me Burkiewicz DNA extraction | CTAB-chloroform | 96 wells plate https://dx.doi.org/10.17504/protocols.io.5qpvor5jdv4o/v1

ABSTRACT

This is a 96-well version of the classic CTAB-chloroform plant DNA extraction (Doyle & Doyle 1987), developed by John Willis and Lila Fishman in ~2000 and since optimized by multiple lab groups working on Mimulus (monkeyflowers). It has been used successfully for downstream applications in other plant taxa, including Asclepias, Larix, Cynoglossum, and Populus. In the Joly lab, we used it on Impatiens so far.

Link to the original protocol: dx.doi.org/10.17504/protocols.io.bgv6jw9e

The protocol requires more specialized equipment (see below) than the single-tube version, but allows for inexpensive high-throughput DNA extraction. The yields are sufficient in quantity and quality for next-generation sequencing applications (e.g., ddrads, whole-genome Illumina sequencing), as well as PCR marker genotyping.

Equipment:
Genogrinder or similar bead-beater
Benchtop centrifuge w/ deep-bucket plate rotor (e.g., Qiagen Sigma 4-15K)
8-channel pipettor + tips
Hood w/ waterbath
96-well bead dispenser

Materials and supplies:
dry ice (optional)
liquid N_2
3mm grinding balls or "beads"
chloroform
CTAB DNA Extraction Buffer
>95% isopropanol
70% ethanol
96-well “plates” of Costar 8-tube strips (Corning#4412, #4418) and strip-caps (Corning#4408)

CTAB DNA Extraction buffer **Contributed by users**

DOI

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EXTERNAL LINK

https://doi.org/10.1371/journal.pgen.1009095

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PROTOCOL CITATION

Lila Fishman, Simon Joly, Andrea Corkal, Jérôme Burkiewicz 2022. DNA extraction | CTAB-chloroform | 96 wells plate . **protocols.io** https://dx.doi.org/10.17504/protocols.io.5qpvor5jdv4o/v1

FORK NOTE

FORK FROM

Forked from 96-well CTAB-chloroform DNA extraction, Lila Fishman

CREATED

Oct 05, 2022

LAST MODIFIED

Oct 05, 2022

PROTOCOL INTEGER ID

70840

MATERIALS TEXT

STEP MATERIALS

CTAB DNA Extraction buffer **Contributed by users**

Sample drying

1 Put the samples to be freeze-dried at -80 and let them sit for at least one hour.

2 30m Turn on the freeze dryer and the blue pump attached to the side. The freeze dryer will take 30 minutes to reach the proper conditions.

3 2d When the machine is ready, it should say "Load/Unload" in the top left corner, load the samples that have been stored at -80C. Click "Start" and the machine. Let the machine run for 48 hours.

Do not stack samples as they may not dry properly. The best formation we found is staggering the samples in the box to minimize how much they touch each other.

4 After 48 hours, click "Aerate" to stop the machine and remove the red cap from the tube on the left side to drain the moisture collected. Unload the samples.

Tissue collection

5 Pre-label the tubes

Use this labeling scheme throughout. Please verify with PI or project lead that a projectname is unique, concise, and informative for long-term storage, and that numbers correspond exactly to individual phenotypic data.

6 Pre-load cleaned and autoclaved beads into plate of tubes (could use a bead dispenser if available).

7 Add 10mg of leaves (max. 30 mg) to each tube. Try to be consistent to have a similar yield for each extraction.

The dried leaves can be a bit tricky to add to the tubes. Rolling and folding the leaves to fit them into the tube mouth works well. Be sure to cap the tubes that you are not currently loading to avoid cross-contamination and clean off the sides of the tubes after loading.

Keep all tubes capped that you are not currently loading, you can use a second strip to do this to have tubes on both sides of the current tube you are loading capped.

Try to add a similar standard amount of leaf to each sample.

This protocol has been used with individual seedlings, silica-dried leaf tissue, herbarium specimens, senesced (brown) wild plants, and fungus-containing roots. You may need to

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adjust the amount of tissue and the grinding conditions (e.g. add sand or additional small beads) for other tissues.

Tissue grinding

8 Put the samples in the tissue lyser for 2 min at a frequency of 30 (1/s).

Make sure the samples are finely ground. If not, do another round of grinding.

9 Centrifuge the samples for 2 min @2000rpm to get powder off caps and down into bottom of the tubes.

It is very important that tissue powder not spray all over the place when you open the tubes in next step.

DNA extraction

10

This protocol works best (less time spent balancing the centrifuge...) if done on 2 entire 96-well plates OR an even number of 8-tube strips spread across 2 plates. If you are just doing 1 plate (recommended first time), spread the 12 tube-strips across 2 plates before starting.

For all reagent-dispensing steps, you can re-use pipet tips if they absolutely do not touch sample tubes (if in doubt, get fresh ones).

For all sample-transfer steps, use a fresh box of (filtered) tips for each plate. If you need to use extra tips, pull from a separate box so that the tip row ALWAYS corresponds to the tube-strip number.

Turn on water bath in fume hood (60°C) and clear workspace in hood.
Make sure you have sufficient CTAB buffer and reagents/supplies for all steps before starting.

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11 Glove up.
In hood, measure out 600 µl of CTAB extraction buffer per sample (+8-10%) into a wide trough (or clean tipbox lid)
Add 1 µl b-mercaptoethanol per 600 µl of CTAB extraction buffer, and stir to mix.
Add 4µl of RNAseA per sample.

β-mercaptoethanol is nasty, so we keep a dedicated L200 in the hood to get it from the bottle (stored in hood) rather than contaminating one of the main lab ones.

12 Uncap the tubes with the ground samples VERY CAREFULLY/SLOWLY and discard cap strips immediately into waste bin (they are a contamination risk).
Add 600 µl of CTAB buffer (including β-mercaptoethanol and RNAseA) to each sample using multichannel pipettor.
Re-cap tightly with FRESH caps.

13 Use the vortex or the tissue lyser to homogenize the sample in the buffer. Make sure there is no powder left at the bottom of the tube.

14 I**ncubate tubes for ~20 min. in 60°C water bath.**
Meanwhile, label next set(s) of plates/tube-strips to match your collection plates.

Place brick + paper towel on top of samples to keep caps from popping off in waterbath. Invert at least once if trying to maximize yield -- put caps back on very tightly before inverting…

15 Post-incubation, remove plates from the water bath and blot them off. Re-tighten caps.
Centrifuge plates for 2 min to pellet solids @4000 rpm.

16 Transfer all the supernatant possible to a new labelled 96-well Costar plate using multi-channel pipettor.

Use FILTERED tips.
If the tips clog, bounce gently on the beads to dislodge. We use filtered tips since the multichanel pipettor can spew grindate upward into shaft if tips clog and then unclog.

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Reserve tube-strips with beads out of the way in hood for later bead recovery.

17 Decant chloroform into large dedicated trough (tipbox lid or other HARD plastic).
Add 500 µl of chloroform (equal volume) to each sample tube.
Re-cap samples (tightly!) with fresh caps.

Do not MEASURE chloroform into the trough unless you have a plan to clean the graduated cylinder. Decant a reasonable amount and then get more if you need it.

Chloroform melts standard troughs. Use a hard-plastic trough dedicated to it.

Rinse fresh pipette tips 1-2x with chloroform (back into trough) before attempting to dispense (prevents dripping).

If you have not double-dipped tips, return excess chloroform to bottle carefully. If you have, put it into chloroform waste bottle immediately and re-start 16.

Try not to drip any chloroform outside of the tubes. If you do, wipe it off before step 18!

18 Hand-invert for 5 minutes using a paper towel over the lids to catch leaking from the caps.

19 Centrifuge for 15 min @ 4000 rpm to separate aqueous (upper) and chloroform (lower) layers.

20 *While you are waiting on the spin:*
Fully label a fresh plate of strip tubes -- these will be final home of samples.
Arrange the handy-dandy-cut-down-rack for interface-viewing/pipetting (center front) and your freshly labeled Costar plate of tubes (center-back). Put an empty Costar base for waste tubes (to the left, if right-handed).

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Set pipettor to 300 mL and SLOW speed (if automated), and get a fresh box of tips for each plate.

21 Bring plate of centrifuged samples to hood. Place on right (if right-handed).
One strip at a time, put a sample strip and the corresponding empty strip in the handy-dandy-cut-down-rack.
Un-cap sample (can do before, during, or after moving, depending on preference).

Carefully pipet off the aqueous layer (up to 300mL) of sample strip and transfer it the matched empty strip.
Move chloroform waste to left waste plate and return filled strip to new base.
Repeat until all samples are transfered.

The handy-dandy-cut-down rack is a costar plate that has had the bottom several cm removed (or cut away from 1/2 of it, so a tipbox or costar lid can be slid beneath). When the tube-strips are placed in this contraption, the aqueous-chloroform interface should be completely visible, but the tube-strips should be stable enough to stay upright without being held.

You can buy/improvise a different striptube-holder -- the key is that you can see the interface and pipet efficiently.

Tips: Brace the pipette tips against the inner wall of the strip-tubes above the interface, while tilting the strip somewhat in the opposite direction (to create a deeper pool on the pipette side). Don't be greedy.

Try to find an arrangement of plates/hood height (if adjustable)/arm position that lets you stably and uni-directionally transfer the samples in a series of relaxed-but-robotic motions, without crossing sample tips over either sample or recipient plates (to avoid cross-contamination/mix-ups)

If this step is super-difficult or physically uncomfortable, experiment with the ergonomics until it is not (it doesn't have to be).

IMPORTANT: There is generally a solid/chalky/slimy interface between the chloroform (dark, green or orange-y, oily) and aqueous (clear, very light green or yellow depending on tissue) layers. Avoid drawing up interface debris or chloroform. Check the tips as you pipet.

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If you did transfer something other than the aqueous, don't panic. Wait until all strips are transfered, and re-centrifuge the plate(s) as in step 19, then re-transfer any rows with gunky samples to fresh (labeled) tube-strips and sub them into their correct positions.

Because the next step is the precipitation of DNA, anything denser than water will stay with your DNA and potentially inhibit downstream applications. So check one last time that all is clear before proceeding.

22 [Out of hood if you like.]
Add 1.5 volumes of cold 100% isopropanol to each aqueous sample, cap well with fresh strip-caps, and mix by hand- inverting plate once or twice.
(e.g. for a sample of 200uL add 300uL of isopropanol)

Place in -20 freezer for at least 30 minutes or as long as overnight (we generally do the latter).

23 Centrifuge for 15 min. at 4000 rpm.
A greyish gelatinous pellet is often visible in the bottom of the tube (but may not be).

24 Un-cap. To re-use caps (optional): lay them in order on a trifold towel labelled with plate #, NOT touching.
One strip at a time, gently tilt to pour off isopropanol supernatant (into a dedicated waste tipbox lid).
While each strip is still tilted but mostly empty, blot the open ends with a fresh bit of trifold towel to wick out as much liquid as possible.

25 Add 200 µl cold 70% ethanol (mol. grade) to each sample.
Flick to rinse pellet. It should float up off the bottom of the tube.
(OR can invert to really get rid of isopropanol on walls. Cap if so)

Centrifuge at 4500rpm for 5 min (no need to re-cap yet, but you can)

Pour off ethanol as with isopropanol, wicking out as much as possible with paper towel at the end of the pour.

The pellets are MUCH more slippery during the ethanol pour than the isopropanol step. Keep an eye on them and pour slowly but continuously (i.e., don't tilt back 1/2 way

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through and then try to pour again).

26 Air-dry the pellet for 1-2 hours at room temp. with a Kimwipe placed over the open tubes. If you have to leave for longer, re-cap loosely and dry later. Don't overdry.

27 Once pellets are dry (no ethanol smell), add 50µL TE buffer to each sample.
Cap with retained or new caps.
Flick tube-strips to mix well and/or leave out at room temperature for at least an hour to fully re-suspend.
Refrigerate if diluting/using wihtin a few weeks, otherwise store in -20 or -80. (Do not freeze before pellet has fully resuspended!)

If you know that your yields will be unusually high or low, re-suspend pellet in more or less water (e.g. 20µL for dead field samples of *M. guttatus* or seedlings, or 100µL for silica dried buds).

The fluorometer that we use to roughly quantify DNA is optimized for concentrations 10-100 ng/µL and 50µL generally gets values on the high end of that range for good fresh bud tissue.

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