Upload image

Jun 15, 2020

96-well CTAB-chloroform DNA extraction

Lila Fishman[1]

[1]University of Montana

1 Works for me dx.doi.org/10.17504/protocols.io.bgv6jw9e

Mimulus

Lila Fishman
University of Montana

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). We have also used it successfully for downstream applications in other plant taxa, including Asclepias, Larix, Cynoglossum, and Populus.

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 (1000-1250ml) + 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

DOI

dx.doi.org/10.17504/protocols.io.bgv6jw9e

PROTOCOL CITATION

Lila Fishman 2020. 96-well CTAB-chloroform DNA extraction. **protocols.io** dx.doi.org/10.17504/protocols.io.bgv6jw9e

CREATED

May 27, 2020

LAST MODIFIED

Jun 15, 2020

PROTOCOL INTEGER ID

37534

STEPS MATERIALS

NAME	CATALOG #	VENDOR
CTAB DNA Extraction buffer		

Tissue collection

1 Pre-label the FRONT of each plate with projectname_plate#, your initials, and the date. Pre-label the FRONT of each tube-strip with its number 1-12 (l-r), and mark the BACKs of the tube-strips with a line.

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.

2 Pre-load cleaned and autoclaved beads into plate of tubes, using the bead dispenser. Lid the plate, but do not cap the tubes.

The bead dispenser operates by aligning 2 perforated plexiglass sheets, and ONLY works with the correct bead size (3mm). Please do not put beads of other sizes or damaged/corroded beads into the dispenser, and do force it if jammed.

3 Wearing gloves and using forceps, collect a 2-pea-sized amount of fresh (e.g., 2-3 flower buds, 1 dense apical bud cluster, 2 folded young leaves or corollas, several un-extended side shoots) tissue into each tube. DO NOT OVERSTUFF. Push the tissue down to leave some airspace below cap (e.g., to lines), but do not jam the wad of tissue down close to the bead.

If collecting many rows, use a small styro w/ ice (or dry ice, if doing >1 plate) and move 1 strip at a time into it while leaving the empties and already-collected samples nestled into ice in a bigger styro. This reduces the chances of cross-contamination and keeps samples cool/frozen.

In the Fishman Lab, we generally load samples in sets of 8, front to back, left to right, row by row. That is: plate1_a1 = 1, plate1_b1 = 2, plate1_a2 = 9...plate1_a12 = 89, plate1_h12 = 96, etc.) . For large, contiguously-numbered mapping populations, it is simplest to load individuals so that well number perfectly matches individual numbers. If a plant is dead/missing, simply leave the well empty as a negative control and/or a place to insert positive controls later (e.g. parental line DNA). In this case, your phenotype spreadsheet is

Citation: Lila Fishman (06/15/2020). 96-well CTAB-chloroform DNA extraction. https://dx.doi.org/10.17504/protocols.io.bgv6jw9e

also your plate map.

It can be helpful to pre-arrange plants in flats to match their positions (i.e., in rows of 8, ordered back to front, l-r). For fine-mapping, grow plants in 96-cell flats and maintain order/orientation of flats.

If sample IDs do not exactly match plate+cell position numbers, make a plate map or spreadsheet explicitly matching position and individual ID at the time of collection.

To reduce the possibility of cross-contamination between adjacent tubes, load samples into every other position (i.e., in a checkerboard and/or every other row) and make a plate map. For small numbers of samples (<24), transfer the DNA to individually labeled tubes post-extraction (or do the single-tube version from start).

We've also used this protocol for individual seedlings, silica-dried leaf tissue, herbarium specimens, senesced (brown) wild plants, and fungus-containing roots. You may need to adjust the amount of tissue and the grinding conditions (e.g. add sand or additional small beads) for other tissues.

4 Cap tubes strip by strip as you go, lid plate when full, and transfer (on ice/dry ice as collected) to -80 (or -20, in a pinch). Can store for years.

If you have to collect fresh frozen tissue into strip-tubes without pre-loaded beads, you can push the tissue down a bit more and leave tubes uncapped for ease of bead-loading later. Tape the plate lid on well, and transport and store very carefully. Try to not load beads onto WET tissue, as they will freeze onto it and not grind well.

DNA extraction

5

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.
Get liquid nitrogen and dry ice (Chemstores).
Plug in 8-channel pipettor to charge (if electronic) and pre-set first volume (500µl).
Make sure you have sufficient CTAB buffer and reagents/supplies for all steps before starting.

Citation: Lila Fishman (06/15/2020). 96-well CTAB-chloroform DNA extraction. https://dx.doi.org/10.17504/protocols.io.bgv6jw9e

6 Glove up.
In hood, measure out 500 µl of CTAB extraction buffer per sample (+8-10%) into a wide trough (or clean tipbox lid)
Add 1 µl b-mercaptoethanol per 500 µl of CTAB extraction buffer, and stir to mix.
For 96 samples, we use 52 mL buffer + 104 µl β-mercaptoethanol, but you may want more extra for smaller absolute volumes.

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.

7 Get samples in Costar plates from -80.
Remove bottom of Costar base(s) if still in, and place on dry ice (if not moving very fast).
Pour liquid N_2 into a small, shallow styro (need a pool deep enough to submerge and flash-freeze tissue).
Dip each Costar plate of samples into pool of liquid N_2, using the silver Qiagen rotor-racks as holders (or similar method to submerge).
Replace plate bottoms OR quickly transfer the cold tubes (in order) to labeled non-frozen costar racks with bottoms in place.

The transfer of tubes to fresh base is optional, but is prudent IF you collected into strip tubes that were bulk and re-loaded into a used costar base. Very rarely (but distastrously!) a base will crack in the centrifuge or genogrinder, and we think that has to do with brittleness of old and cold plastic bases.

8 Clamp sample plates into shaker platform in Genogrinder.

Always clamp a plate (don't have to be same weight) into both genogrinder platforms, and make sure that tube-strips are spread across each plate with a*ll corner positons occupied*.

Do not put a lid on the entire plate – you want the strip-tube caps tightly in plants and snug against the Genogrinder platforms (one can add a folded trifold paper towel for extra cushioning).

9 Genogrind at 1500-1700 rpm (500-700, 1x setting) for 20 seconds to 2 minutes in 20-30 second sets.

A good grind is the key to good yield. But don't grind so long that samples thaw. You want the samples to look like pale green wasabi powder, not cooked spinach, at the end of this step.

We don't bother changing the timer on the genogrinder. Just watch it and stop/check after 20-30 seconds, then repeat as necessary.

10 Centrifuge briefly (at whatever speed…) 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.

Citation: Lila Fishman (06/15/2020). 96-well CTAB-chloroform DNA extraction. https://dx.doi.org/10.17504/protocols.io.bgv6jw9e

11 Move to hood.
Uncap the tubes VERY CAREFULLY/SLOWLY and discard cap strips immediately into waste bin (they are a contamination risk).
Add 500 µl of CTAB buffer to each sample using multichannel pipettor.
Re-cap tightly with FRESH caps.

12 *Optional, depending on degree of thawing and grind-quality:*

Return to Genogrinder for <5 sec (hi speed) to get good mix of tissue slurry and CTAB buffer. Just turn on and then off quickly.

The key at this stage is to have a nice smooth mix of tissue and buffer going into the incubation (and to not cross-contaminate samples). If you have large chunks of unground tissue or frozen powder not mixing with buffer, it is a good idea to either briefly genogrind or gently hand mix (e.g., flicking, or inverting if you are very confident in your cap-tightness...) the tubes before incubation.

13 **Incubate 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…

14 Post-incubation, remove plates from waterbath and blot them off. Re-tighten caps.
Centrifuge plates for ~10-30 sec to pellet solids @4500 rpm.

15 Transfer 300-400 µl supernatant to a new labeled 96-well Costar plate using multi-channel pipettor.

We use a Matrix automatic pipettor w/ FILTERED 1250 µl cliptips for this step. Set pipettor to 400, raise tips slightly above solid glop & bead and take what you get. If the tips clog, bounce gently on the beads to dislodge. We use filtered tips since the automated pipettor can spew grindate upward into shaft if tips clog and then unclog.

IMPORTANT: The new Matrix 1250 tall clip-tips displace too much liquid to reach the bottom of the tubes without causing overflow. Depending on the amount of tissue, you may need to do each row in TWO steps, transferring 200 each time. Can reuse tips for both draws if careful.

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

Decant chloroform into large dedicated trough (tipbox lid or other HARD plastic).

Citation: Lila Fishman (06/15/2020). 96-well CTAB-chloroform DNA extraction. https://dx.doi.org/10.17504/protocols.io.bgv6jw9e

16 Add 400 µl of chloroform 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!

17 Clamp plates into the Genogrinder
Shake at 0.5x rate at 00 (LOWEST SETTING) for 5-10 min to mix chloroform and aqueous layers.
Alternatively, hand-invert for 5 minutes.

There have, on very rare occasions, been reports of tube caps exploding off at this step. This may be because the liquids are still warm from the waterbath. If you are super-fast and the tubes are still warm after all the chlorform-dispensing, let the tubes cool to room temp before re-capping and shaking.

18 Centrifuge for 5 min @ 4500 rpm to separate aqueous (upper) and chloroform (lower) layers.

19 *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).
Set pipettor to 300 mL and SLOW speed (if automated), and get a fresh box of tips for each plate.

20 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.

Citation: Lila Fishman (06/15/2020). 96-well CTAB-chloroform DNA extraction. https://dx.doi.org/10.17504/protocols.io.bgv6jw9e

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.

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.

Very rarely, gunk will floats on the surface of the aqueous layer that is hard to avoid pipetting. For us, this has generally happened at the phenol-chloroform step of the phenol-chloroform variant on this protocol (which is basically same, but has a parallel phenol-chlorofrom-isoamyl addition and aqueous transfer before steps 16-20). [We don't do that protocol anymore becuase it doesn't seem to be necessary for downsteam applications in Mimulus - but it might be useful for other taxa with different secondary compounds]. In that case, it went away at the chlorofrom step.

So one solution if it happens a lot in this protocol would be to complete 20, and then just repeat 16-20 (potentially adding more CTAB buffer to dilute whatever the gunk is). But, if it just a few tubes, we generally go in with one tip at a time and pipet as much as possible from below the gunk into a replacement strip.

21 [Out of hood if you like.]
Add 300 µl cold 100% isopropanol to each aqueous sample, cap well with fresh strip-caps, and mix by hand- inverting plate once or twice.

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

Citation: Lila Fishman (06/15/2020). 96-well CTAB-chloroform DNA extraction. https://dx.doi.org/10.17504/protocols.io.bgv6jw9e

22 Centrifuge for 5-10 min. at 4500 rpm.
A greyish gelatinous pellet is often visible in the bottom of the tube (but may not be).

23 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.

If you sandwich the tube-strip between your index and middle finger palm-up to do the pour (as if you are smoking a stack of 8 cigarettes, like some 1950s movie star), the tubes stay nicely lined up. Also, you can see the pellets and back off if they start sliding.

24 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 through and then try to pour again).

25 Air-dry the pellet for 1-2 hours (or overnight at longest) 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.

26 Once pellets are dry (no ethanol smell), add 50µL ultrapure, sterile, H_2O 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.

Citation: Lila Fishman (06/15/2020). 96-well CTAB-chloroform DNA extraction. https://dx.doi.org/10.17504/protocols.io.bgv6jw9e