# Contents

# Soltis Lab CTAB DNA Extraction Protocol

**(Reference: Doyle & Doyle, 1987; and Cullings 1992)**

Revised for fresh tissue, November 27, 2013

1. Prepare CTAB buffer, use within 2-3 days, store capped: Add polyvinylpyrrolidone (Fisher Cat#: BP431-500) and β-mercaptoethanol (Fisher Cat#: BP176-100) and stir to dissolve right before starting extractions:

| CTAB | PVP | b-merc |
|---|---|---|
| 5 ml | 0.2 g | 25 µl |
| 20 ml | 0.8 g | 100 µl |

2. Weigh out **10-20 mg** of fresh plant tissue.
3. Place tissue with 5 Zirconia Beads (2.0 mm, Biospec) in a 2 ml eppendorf and close tube, grind:
    1. Flash freeze containing beads and tissue in liquid nitrogen.
    2. Cool down the plastic bead mill eppendorf rack in liquid nitrogen.
    3. Transfer the frozen tubes to the cold rack and immediately grind the tissue in the bead mill for 30 sec.
4. Add **850 µl of CTAB buffer** and grind samples a bit more.
5. Incubate samples at **55℃ for 1 hr**.
6. Add **850 µl of 24:1 Chloroform:Iso Amyl Alcohol** and mix well by shaking tubes.
7. Centrifuge for **10 minutes** at maximum speed.
    1. Following centrifugation, you should have three layers: top: aqueous phase, middle: debris and proteins, bottom: chloroform.
    2. Go on to the next step quickly so the phases do not remix
8. Pipette off the **aqueous phase** taking care not to suck up any of the middle or chloroform phases. Pipetting slowly helps with this.
9. Place the aqueous phase into a new labelled eppendorf tube.
10. **Estimate the volume** of the aqueous phase.
11. Add 0.08 volumes of cold **7.5 M ammonium acetate**—see attached table.
12. Add 0.54 volumes (using the combined volume of aqueous phase and added AmAc) of cold **isopropanol** (=2-propanol)—see conversion table.
13. Mix well by inverting.
14. Let sit in freezer overnight. (Longer times will tend to yield more DNA, but also more contaminants.)
15. Centrifuge for **3 min** at maximum speed.
16. Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.
17. Add **700µl of cold 70% Ethanol** and mix
18. Centrifuge for **3 min** at maximum speed.
19. Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.
20. Add **700µl of cold 95% Ethanol** and mix
21. Centrifuge for **3 min** at maximum speed.
22. Pour or pipette off the liquid, being careful not to lose the pellet with your DNA.
23. **Dry** the pellet by letting samples stand for 1 hr or until dry.
24. **Resuspend** samples with **200 µl of TE buffer**. Allow to resuspend overnight in refrigerator before running a test gel using 4 µl of the DNA.

## DNA Extraction Stocks

**CTAB**: for 1L of CTAB buffer

100 ml of 1 M Tris, pH 8.0
280 ml of 5 M NaCl
40 ml of 0.5 M EDTA
20 g of CTAB (Cetyltrimethyl ammonium bromide, Amresco cat#:0833-1Kg)

**TE buffer**:

| [Final] | for 1L use: |
|---|---|
| 10 mM | 10 ml of 1 M Tris, pH 8.0 |
| 1 mM | 2 ml of 0.5 M EDTA |

**1 M Tris, pH 8.0:** for 1 L

121.1 g Tris (Fisher Cat#: BP152-5)
700 ml ddHO
Dissolve tris and bring to 900 ml.
pH to 8.0 with concentrated HCl (will need ~50ml)
Bring to 1 L.

**0.5 M EDTA pH 8.0**: for 1 L

186.12 g of EDTA (Fisher Cat#: BP120-1)
750 ml dd$H_2O$
Add about 20 g of NaOH pellets
Slowly add more NaOH until pH is 8.0, EDTA will not dissolve until the pH is near 8.0.

**5 M NaCl:** for 1 L

292.2 g of NaCl (Fisher Cat#: BP358-10)
700 ml dd$H_2O$
Dissolve and bring to 1 L.

## References

Cullings, K.W. 1992. Design and testing of a plant-specific PCR primer for ecological and evolutionary studies. *Molecular Ecology* **1:**233-240.

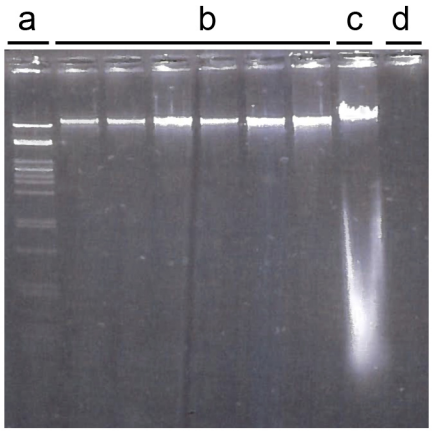
Doyle, J.J. and J.L. Doyle. 1987. A rapid DNA isolation procedure for small quantities of fresh leaf tissue. *Phytochemistry Bulletin* **19:**11-15.

# Genomic DNA Purification Protocol (using a DNeasy Kit)

Following CTAB extraction of high molecular weight DNA from fresh tissue, RNA digestion, protein removal and DNA shearing needs to be carried out.

1. Add 2 µl RNase A (10 mg/ml, Fermentas #EN0531), **incubate 55℃ for 10 min**.
2. Starting at **step #9** of the DNeasy Plant Mini Kit (Qiagen, #69104) protocol **add 67µl AP2 Buffer** to the 202µl CTAB-extracted genomic DNA (including RNase A). Incubate on ice 5 min.
3. Continue through to end of protocol, eluting only once with 100 µl AE Buffer (allow AE to soak membrane for 5 min before centrifugation).
4. Run 4 µl of purified genomic DNA on a agarose gel with size marker.
5. Check DNA concentration on spectrophotometer.

**Example Genomic DNA Samples**




a) 0.5 µg lambda DNA/PstI marker
b) Genomic DNA samples (following CTAB extraction, RNase A-treatment and DNeasy purification). Note that there is no obvious smear (i.e. low molecular weight fragments) and that DNA is forming a single prominent band. Concentrations for samples shown are between 30 to 80 ng/µl. 4 µl of gDNA loaded per lane.
c) Genomic DNA sample (following CTAB extraction only). Note that RNA is still present (low mol. weight smear) and the molecular weight of the gDNA is too high to migrate properly through the gel.
d) empty lane

Alongside running an agarose gel, the DNA concentrations of gDNA should be measured using the Nanodrop spectrophotometer. If Buffer AE was used to elute DNA this should be used to blank the spectrophotometer.

# Probes for Fluorescence *in situ* Hybridisation

All probes are made by labelling DNA template using the nick translation protocol for 'Large chromosomal targets' in Kato et al. (2011), with minor modifications (see below).

DNA-based probes for fluorescence *in situ* hybridisation (FISH) to *Tragopogon* chromosomes have been made from three different types of DNA template. These are:

- Total genomic DNA (i.e., for "genomic *in situ* hybridisation" - GISH)
- Double-stranded oligonucleotides (dsDNA oligo probes)(e.g., for FISH localisation of tandem repeats)
- PCR-amplified sequences (e.g., for FISH localisation of 18S rDNA)

## Notes on Probe Template Synthesis and Labelling

**Total Genomic DNA**
Input for nick translation is gDNA that has been extracted, RNase A-treated and purified using the DNeasy Kit following the protocol in the previous section. Genomic DNA should be stored at -20°C. The amount of gDNA template that should be labeled in the nick translation reaction is 500 ng (instead of 5000 ng).
The final concentration of the probe, after labelling, will therefore be 10 ng/µl.

**dsDNA Oligo Probes**
We purchase our unmodified DNA oligonucleotides from Eurofins MWG Operon (Huntsville, AL, USA) at the 10 nM scale, which is limited to a maximum length of 60 bp. This is the same service that we use for standard PCR primers.

Oligonucleotides should be made double-stranded in the following way:
- Dry oligonucleotides should be suspended in water to give a final concentration of 100 µM (following the manufacturer's guidance).
- Combine equal volumes of complementary oligonucleotides in a 0.2 ml PCR tube and heat to 95°C for 5 min in a PCR machine.
- Tubes should then immediately be transferred to a beaker containing ~400 ml of >90°C water (heated in a microwave) and left to cool slowly until the water temperature reaches approximately 35°C. This gradual cooling allows dsDNA to form.
-The resulting dsDNA can be quantified using a spectrophotometer. Once made, dsDNA oligos can be stored at -20°C. 5 µg of dsDNA oligo (typically 4 µl) should be used as input for the nick translation reaction.

**PCR-amplified Sequences**
In most cases, a plasmid-cloned sequence is used as a template. For example, to paint the 35S rDNA regions we have used a 1333 bp 18S rDNA sequence cloned from *Tragopogon dubius* (clone number: 17.1, vector: pSC-A-amp/kan). To obtain a large amount of product, several 100 µl PCR reactions can be carried out in one go. For a 100 µl PCR reaction 3 to 5 ng of plasmid DNA will provide sufficient template (we add 3.5 µl of 1:100-diluted plasmid, original plasmid concentration is about 100 ng/µl). Standard reagent concentrations should be used together with 10 % (v/v) 5M betaine.

Primers for the amplification of 18S clone (clone: 17.1) are:

| | |
|---|---|
| dub_18S_F | TGTGCCGGCGACGCATCATT |
| dub_18S_R | GCGAGCTGATGACTCGCGCT |

PCR Program for 18S clone amplification:
94°C 2 min, then 38 cycles of: 94°C for 1 min, 60°C for 40 sec, 72°C for 40 sec and 72°C for 1 min 20 sec followed by a final extension of: 72°C for 4 min.

PCR products should be run on an agarose gel and then pooled and precipitated using: 0.1 volumes of 3 M sodium acetate pH 5.2 and 2.5 volumes of 100% ethanol, mixing and then incubating overnight at -20°C. The mixture should then be centrifuged for 25 min at max speed. The pellet can be washed with 70%, then 100% ethanol and then air dried. Pellet can be eluted in TE or Tris buffer. The resulting dsDNA should be quantified using a spectrophotometer. Stocks can be can be stored at -20°C. 5 µg of dsDNA oligo should be used as input for the nick translation reaction.

## Nick Translation

This is a 2 hr reaction carried out at 15°C. See Kato et al. (2011) for details.

Example Nick Translation Components for labeling 18S rDNA PCR product:
4.6 µl (5000 ng) 18S rDNA PCR product
5 µl of 5× Nick Translation Buffer
5 µl of dATP, dCTP and dGTP nucleotides all 2 mM
27.4 µl water
1 µl dUTP-fluorescein
6.25 µl of 10 U/µl DNA Polymerase I
1 µl of 100 mU/µl DNase I
Total: 50.25 µl

After labeling, the reaction can be terminated by freezing the samples to -20°C.

### Probe purification

All probes are cleaned using a Qiagen QIAquick nucleotide removal kit. For the final step in the cleaning procedure 54 µl of Elution Buffer should be added to the column to recover 50 µl of probe.

### Reference

Akio Kato, Jonathan C. Lamb, Patrice S. Albert, Tatiana Danilova, Fangpu Han, Zhi Gao, Seth Findley, and James A. Birchler. *Plant Chromosome Engineering: Methods and Protocols*, *Methods in Molecular Biology,* 2011, Vol. 701, Chapter 4)pp. 68-69.

# Chromosome Preparation and FISH

This method based on that described in Birchler et al. Tropical Plant Biology, 2008, 1:34–39 and Kato et al. *Plant Chromosome Engineering: Methods and Protocols*, *Methods in Molecular Biology,* 2011, Vol. 701, Chapter 4.

## Root Pretreatment and Fixation

In advance, dissolve 0.145 g of 8-hydroxyquinoline in 500 ml of distilled water at 60°C on a magnetic stirrer for 3-6 h, to give a concentration of 2 mM. Once fully dissolved, store the solution in the dark (the solution is light sensitive) at 4°C. We cover the 8HQ stock bottle with foil and store it in the refrigerator.

- Using forceps remove the final 2 cm of root and immerse in 2 mM 8-hydroxyquinoline. Using a 2 ml Eppendorf tube containing 1 ml 8HQ works well.

- Incubate at 4°C in the dark, for 5-20 hours. We have collected roots from *Tragopogon* species late in the day (4 pm - 6 pm) and pretreated them overnight until 8 am -10 am the following day. The optimal collection time may be different for other species and should be tested.

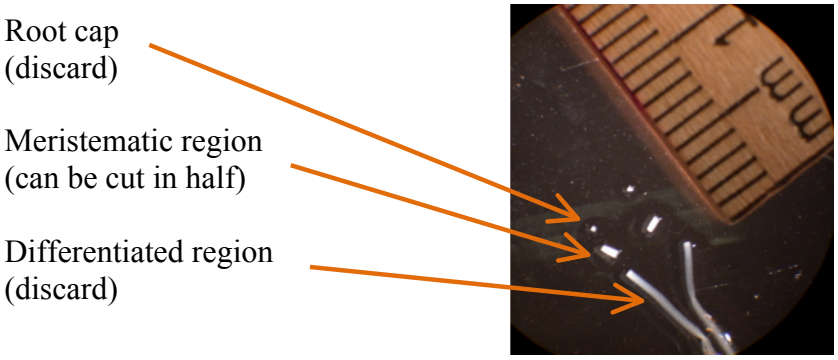
- Remove 8-hydroxyquinoline and add ice-cold 90 % acetic acid and incubate on ice for 10 minutes.

- Remove 90% acetic acid and add 70 % ethanol, store at -20°C.

## Root Tip Digestion

- Immerse roots in 1× citric buffer, on ice, for 10 min. Repeat twice more.

- If possible, remove the root cap, then cut the meristematic region from the remainder of the root with a razor blade. (Thicker roots can be cut into 0.5 mm long pieces to improve enzyme digestion).




- Transfer the dissected root tip to 0.2 ml PCR tube containing 20 µl of enzyme mix (1%

pectolyase Y23 and 2% cellulase, in 1× citric buffer).

- Incubate for 46 min at 37°C. (Incubation time will vary depending on root structure and size.)

- After digestion, transfer tube to ice.

- Carefully wash roots with ice-cold 70% ethanol. Repeat twice more, or until the enzyme suspension is no longer visible.

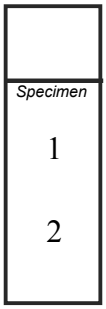
- Remove ethanol from tube, leaving about 2 µl behind. (This will help to prevent the acetic acid from freezing.)

- Add 27 µl of glacial acetic acid, return tube to ice.

- Break up tissue with a blunt dissecting needle and then rest tube on ice.

## Chromosome Spreading

- Mist the inside of the cardboard humid chamber and a half-folded Kimwipe.




- Place slides in the humid chamber and pipette two 3.5 µl droplets of cell suspension onto each slide (see number positions in slide cartoon). Breathing on each slide after pipetting the suspension helps to increase the humidity, immediately cover box with damp Kimwipe to maintain humidity.

- Incubate slides for 7-10 min in the covered humidity chamber.

- Remove slides from chamber, allow to air dry and check preparations with phase contrast microscope.

- Mark good chromosome spreads (i.e., those that are complete, have sufficiently compressed chromosomes and have no overlapping chromosomes) with a marker pen on the underside of the slide.

- Slides can be left at room temperature for a few hours or placed in a slide box and stored at -20°C for longer term storage.

- Prepared slides are best used within a few days of being made. Longer-term storage typically results in chromatin degradation observable as a loss of glossiness or shininess.

## Fluorescence *in situ* Hybridization

- Prepare metallic tray with a layer of wet Kimwipes.

- Select slides for FISH. Slides should be allowed to warm to room temperature after removal from the freezer before taking them out of their storage container. This will prevent condensation forming on the slide.

- UV cross-link slides (apply 120 mJ/cm$^2$).

- Probes are light sensitive. Work under low light.

- Pipette one drop of hybridization mix on the target area of the slide (*example mixes below).

- Place coverslip on droplet.

- Transfer slides to metal tray, cover, and denature for 2.5 min at 83°C.

- Transfer slides to a plastic lightproof humid box, leave to hybridize at 55°C overnight for FISH (16 hrs) and two days for GISH (36 hrs).

*Example FISH Mix Components for *Tragopogon species*:
3 µl (150 ng) TTR3 fluorescein-labeled probe
3.5 µl (175 ng) TPRMBO Cy5-labeled probe
1.8 µl (90 ng) TGP7 Cy3-labeled probe
0.4 µl mix of 18S rDNA Cy3-labeled and fluorescein-labeled probe (20 ng + 20 ng)
5 µl (700 ng) sheared salmon sperm DNA in 2× SSC, 1× TE buffer
2x SSC, 1x TE buffer to 10.5 µl

*Example GISH Mix Components for *Tragopogon miscellus*:
4 µl (40 ng) *T. pratensis* gDNA Cy3-labeled probe
4 ul (40 ng) *T. dubius* gDNA fluorescein-labeled probe
4 µl 560 ng sheared salmon sperm DNA

## Post-Hybridization Coverslip Removal and Slide Wash

- To remove coverslips, place slides to a Coplin jar containing 55°C 2× SSC.

- Remove slides and use a Kimwipe to blot excess 2× SSC.

- Without allowing slides to dry out, add a drop of Vectashield and mount a 22 × 40 mm coverslip (coverslip thickness needs to be: "1 ½").

- Gently blot away any excess Vectashield using Whatman chromatography paper and store slides in the refrigerator.

- Slides are best viewed at least several hours after stabilization in the refrigerator.