3% CTAB DNA extraction (96-well plate)

(modified from Zeng et al. 2002, Acta Botanica Sinica 44; 694-697)

Depending on the quality of your tissue, you might want to skip the initial wash in CTAB-free buffer. Since the protocol requires chloroform, use Greiner individual tubes (they fit well in the blue Qiagen boxes) instead of the regular Qiagen strips. Add only one metal bead to each tube. I normally use 1-2 very small leaves as starting material (around 1-1.5 cm long).

Before you start:
- pre-cool on ice CTAB-free buffer + 0.6% β-mercaptoethanol (for one plate: 100 ml buffer + 600 μl β-mercaptoethanol)
- pre-warm at 65°C in a waterbath the 3% CTAB buffer + 1% β-mercaptoethanol (for one plate: 50 ml CTAB 3% + 500 μl β-mercaptoethanol)

- Collect tissue in tubes already containing a metal bead, freeze, grind on a shredder.

- Spin briefly, add **1 ml** of CTAB-free buffer w/ β-mercapto. To resuspend the pellet close well all the lids and use the shaker you used for grinding tissue (30 Hz, two pulses of about 20 seconds). It's important that you put a paper towel on top of the lids, since there might be some spills. Check all the tubes and eventually shake some more. Some of the tubes with more tissue in the central rows might not resuspend anyway, so you'll have to do it individually by pipetting. This is more of a problem if you start with more material. For these tubes, I usually move 600 μl to a new tube, resuspend the pellet in what's left of the supernatant and then add the 600 **μl** back. When you have done this for all of the recalcitrant pellets, mix the plate on the shaker for a few more second. Keep **10 minutes on ice**.

- **Spin at 4000 g 10 minutes (IMPORTANT: check that acceleration and deceleration are set maximum to 5)**. If you remember, set the temperature of the centrifuge to 10°C, although it's not fundamental. Discard the supernatant by pipetting it out with a 300 μl multi-channel (you'll have therefore to do it 3-4 times per row/column. The last time, go all the way to the bottom of the tube – you'll remove a bit of tissue as well, but I think it's still more important to remove all of the supernatant).

- Add **500 μl** of pre-warmed 3% CTAB w/ β-mercapto. Resuspend using the shaker (as you did for the CTAB-free buffer). **Incubate for at least 60 minutes at 65°C.** You might want to put a paper towel on top of the plate, and some weight to press it down, as there might be some spills.

- **If purity is a concern (you start from "difficult" samples), do this step one plate at the time.** Let cool down before removing lids (**20 minutes on ice**). It is important that the plate is cool before moving on. When the lids are warm they don't close the tubes very well, and when you'll mix the plate after adding the chloroform there will be chloroform+your sample everywhere. Add **500 μl** of chloroform-isoamylalcohol 24:1. Chloroform will tend to drip out of the tipw at first. Pipetting it in and out a few times in the reservoir before starting to dispense it in the tubes should improve the situation. Be careful anyway not to spill chloroform on the plate. Invert the plate until the chloroform and CTAB solution are well mixed. There might be some spill, especially after you invert the first time, so keep the lids closed with a piece of tissue paper. You can use the shaker again to have all the tissue well mixed (obviously, be very careful).

- Immediately centrifuge at full speed (6000 g) for **4 minutes. Important: Reduce acceleration/deceleration to 3 or lower.** While I used the same

plates and tubes before in the very same centrifuge, our seems to be especially harsh on the blue boxes, and stronger accelerations can brake them (with very unpleasant consequences). For the same reason, try not to use the transparent lids that come with the Qiagen boxes, as they often fall to pieces. Go figure.

- Transfer **400 µl** of supernatant to a plate to which you already added **40 µl** NaCl 5M. To do that, use a 300 µl multi-channel set to 200 µl. 300 µl tips are not completely smooth but have a series of small steps. If you rest the second of these steps from the bottom to the border of the tubes, you should be able to pipet out the 400 µl without touching the organic phase or the interphase. Mix by pipetting. Add **300 µl** of cold isopropanol, mix by pipetting. Cool at **-20°C** for at **20-30 minutes (not much longer!)**, spin at full speed for **25 minutes**.

- Discard the supernatant (just invert the plate in the sink). Remove as much supernatant as you can by tapping the plate on a paper towel. Pellets should be tightly bound to the bottom of the plate as this point, but don't overdo that, just in case. Wash the pellet with in **1 ml** of cold 75% ethanol. Spin at full speed for **5 minutes** and discard the supernatant. Repeat the wash.

- Dry the pellet (about 15 minutes at 37°C should suffice), then resuspend it in **400 µl** of high salt TE + 2 µl RNAseA 20 mg/ml (For one plate: 40 ml TE plus 200 µl RNAseA. Check the concentration of the RNase stock you are using and eventually adjust accordingly). Let the plate sit at room temperature (or 37°C) for about 10 minutes, then resuspend the pellet by pipetting.

- Incubate for **1 hour** at 37°C. Add 800 µl of cold 100% ethanol, mix by pipetting and store for about **20-30 minutes** at -20°C. If you have a clear pellet, you can also leave the samples at -20°C overnight.

- Spin at full speed for **25 minutes**, discard the supernatant.

- Wash pellet twice with **1 ml** of 75% ethanol (see above).

- Dry the pellet completely and resuspend in your buffer of choice (I normally go for 50-100 µl of Tris pH 8.0 10mM).

CTAB-free buffer

Tris-HCl pH 8.0	200 mM
EDTA	50 mM
NaCl	250 mM

3% CTAB buffer

Tris-HCl pH 8.0	100 mM
EDTA	25 mM
NaCl	1.5 M
CTAB	3% (w/v)
PVP	1% (w/v)

High salt TE

1 M NaCl in TE buffer