Low-cost DNA extraction for use in TILLING and Ecotilling assays

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1. MATERIALS

	Company
MATERIALS FOR LOW-COST DNA EXTRACTIONS	
Celite 545 silica powder (Celite 545-AW reagent grade)	Supelco 20199-U
SDS (Sodium dodecyl sulfate) for mol biol approx 99%	Sigma L-4390-250G
Sodium acetate anhydrous	Sigma S-2889 (MW=82.03g/mol)
NaCl (Sodium chloride)	Sigma S-1314-1KG (MW=58.44g/mol)
RNase A	10 microgram per ml.
Ethanol	Ethanol absolute for analysis (Merck 1.00983.2500)
Nuclease-free H2O	Gibco ultrapure distilled water (DNase, RNase-free)
Guanidine thiocyanate	Sigma G9277 (MW=118.2g/mol)
Microcentrifuge tubes (1.5mL, 2.0mL)	Any general laboratory supplier
Micropipettes (1000uL, 200uL, 20uL)	Any general laboratory supplier
Microcentrifuge	Eppendorf Centrifuge 5415D
Optional: Shaker for tubes	Eppendorf Thermomixer comfort for 1.5mL tubes
MATERIALS FOR GRINDING OF LEAF MATERIAL (depending on grinding method)	
Liquid nitrogen	
Mortar and pestle or, TissueLyser, …	e.g. Qiagen TissueLyser II
Metal beads (tungsten carbide beads, 3mm)	Qiagen Cat.No. 69997 (for TissueLyser)
EVALUATION OF DNA YIELD AND QUALITY	
DNA concentration	ND-NanoDrop 1000 Spectrophotometer (optional)
Agarose gel equipment	Any supplier providing horizontal mini-gels
TILLING-PCR	
Thermocycler	Biorad C1000 Thermal cycler, or equivalent
PCR tubes	Life Science No 781340
TaKaRa Ex Taq™ Polymerase (5U/ul)	TaKaRA
10X Ex Taq™ Reaction Buffer	TaKaRa
dNTP Mixture (2.5mM of each dNTP)	TaKaRa
Agarose gel equipment	Any supplier providing horizontal mini-gels

SOLUTIONS TO PREPARE:

BUFFER	Receipt		Comments
STOCK SOLUTIONS			
5M NaCl stock solution	MW=58.44g/mol 29.22g / 100mL		If keeping stocks for a long period, check to make sure high molarity stocks stay in solution. If percipitate forms, warm solution until back in solution, or discrar d and make fresh.
3M Sodium acetate (pH = 5.2)	MW=82.03g/mol 24.61g / 100mL		Adjust pH value with glacial acetic acid
95% (v/v) Ethanol	95 mL ethanol abs + 5 mL H_2O		
Tris-EDTA (TE) buffer (10x)	Composition: 100mM Tris-Cl, pH=8.0 10 mM EDTA	For 100mL (10x): 10mL of 1M Tris-Cl stock 2mL of 0.5M EDTA stock	Tris and EDTA can be prepared from powder. Note that the pH of tris changes with temperature.
LYSIS BUFFER (standard)	0.5% SDS (w/v) in 10x TE 0.5g SDS /100mL		PBGL has developed a range of lysis buffers for different crops. If performace is poor, contact PBGL for modified buffers.
DNA BINDING BUFFER	6M Guanidine thiocyanat MW = 118.2 g/mol 70.92 g / 100mL (6M)		**!!!** it takes several hours until dissolved (leave it approx. 4-5 hours)
WASH BUFFER	1mL of 5M NaCl + 99mL of 95% EtOH		**!!!** PREPARE FRESH, because the salt precipitates during storage
DNA ELUTION BUFFER	depending on application (e.g. TE-buffer; Tris-HCl buffer)		

2. METHODS (method for centrifuge tubes):

PREPARATION OF SILICA POWDER-DNA BINDING-SOLUTION

- Fill silica powder (Celite 545 silica) into 50 mL-Falcon-tube (to about 2.5-mL = approx. 800mg)
- Add 30 mL dH2O
- Shake vigorously (vortex and invert)
- Let slurry settle for approx. 15 min
- Remove (pipette off) the liquid
- Repeat 2 times (a total of 3 washes)
- After last washing step: resuspend the silica powder in about the same amout of water (up to about 5 mL)
- STORE the silica solution at RT until further use (silica : H2O = 1 : 1)

Before use:

- suspend stored silica solution (silica : H2O = 1 : 1) by vortexing
- Transfer ~50 uL of silica solution to 2mL-tubes (prepare 1 tube per sample)
  !! try to keep the silica suspended during pipetting to ensure an equal distribution
- Add 1mL H2o (a final wash step)
- Mix by vortexing
- Centrifuge: full speed (13.200) for 10-20 sec
- Pipette off liquid
- Add 700 uL DNA binding buffer (6M Guanidine thiocyanate)

- Suspend the silica powder in DNA binding buffer
- The silica binding solution is now ready for further use in the protocol (see Methods)

PREPARATIONS

- For TissueLyser: Prepare 2 mL-tubes (1 per sample): add 3 metal beads (tungsten carbide beads, 3mm) per tube
- Harvest leaf material (starting amount of material: about 100 mg fresh weight)

GRINDING

Use appropriate / available grinding protocol (Mortar&Pestel, Qiagen TissueLyser,)

For the TissueLyser:

- Freeze 2-mL tubes containing leaf material and 3 metal beads in liquid nitrogen
- Grind in TissueLyser by shaking (10 sec at 1/30 speed)
- Re-freeze in liquid nitrogen (>30 sec)
- Grind again in TissueLyser by shaking (10 sec at1/ 30 speed)
- Re-freeze in liquid nitrogen (>30 sec)
- Store in liquid nitrogen until lysis buffer is added

LYSIS

- Add 800 uL **Lysis buffer**
- Add 4 uL **RNaseA** (10 ug/ml)
- Vortex (~2 min until the powder is dissolved in the buffer)
- Incubate: 10min at room temperatur
- Add 200 uL **3M Sodium Acetate (pH = 5.2)**
- Mix by inversion of tubes
- Incubate on ice for 5 min
- Centrifuge 13.200 rpm / 5 min / RT (pellet the leaf material)

DNA BINDING

- prepare **700 uL silica binding solution** (see above)
- transfer 800 uL of the supernatant to the tubes containing silica binding solution)
  !! Do not transfer leaf material!
- Completely resuspend the silica powder by vortexing and inversion of tubes (approx. 20 sec)
- incubate 15 min at RT (on a shaker at 400 rpm and/or invert tubes from time to time)
- Centrifuge 13.200 rpm / 3 min / RT (pellet the silica)
- Remove the supernatant (with pipette)

WASHING (2 times washing)

- Add 500 mL **wash buffer**
  !! Prepared fresh (see above)!
- Completely resuspend the silica powder by vortexing and inversion of tubes (approx. 20 sec)
- Centrifuge 13.200 rpm / 3 min / RT (pellet the silica)
- Repeat the washing step (optional: a third washing step)
- Remove the supernatant with pipette (as complete as possible)
- optional: short spin and remove residual liquid
- After last washing step: dry the silica in the hood up to 1 hour at RT (make sure there is no wash buffer left)

RESUSPENSION

- Add 200uL **TE buffer** or **10mM Tris buffer**
- Completely resuspend the silica powder by vortexing and inversion of tubes (approx. 20 sec)
- Incubate: 20 min / RT / with gentle agitation (on a shaker at 400 rpm and/or invert tubes from time to time)
- Centrifuge (for tubes): 13.200 rpm / 5 min / RT (pellet the silica)

- transfer 180 uL supernatant to new tube (avoid transferring silica powder!)
- optional: if there is still silica powder in the preps – repeat the centrifugation
- check for concentration and integrity of DNA
- store the genomic DNA at -20^{0}C for long-term storage or 4^{0}C for short-term storage

3. VALIDATION OF LOW-COST DNA PREPARATIONS FOR TILLING APPROACHES

Follow the protocol contained in "Positive control for mutation discovery using agarose gels, version 2.4"available at http://mvgs.iaea.org/LaboratoryProtocols.aspx , to test that your DNA is suitable for TILLING and Ecotilling applications.

4. EXAMPLE DATA

Table 1. Different combinations of self-made (low-cost) buffers and buffers from Qiagen DNeasy Plant Mini kit tested with barley tissue

Sample	1		2		3		4		5		6		7		8	
	+	-	+	-	+	-	+	-	A	B	A	B	A	B	A	B
Lysis	Dneasy kit* +Shredder columns -Shredder columns		Dneasy kit* +Shredder columns -Shredder columns		Dneasy kit* +Shredder columns -Shredder columns		Dneasy kit* +Shredder columns -Shredder columns		**Lysis buffer (PBGL)**		**Lysis buffer (PBGL)**		**Lysis buffer (PBGL)**		**Lysis buffer (PBGL)**	
DNA binding buffer	Buffer AP3/E*		Buffer AP3/E*		**6M Guanidine thiocyanat**		**6M Guanidine thiocyanat**		Buffer AP3/E*		Buffer AP3/E*		**6M Guanidine thiocyanat**		**6M Guanidine thiocyanat**	
DNA wash buffer	Buffer AW*		**Wash buffer – PBGL**		Buffer AW*		**Wash buffer-PBGL**		Buffer AW*		**Wash buffer-PBGL**		Buffer AW*		**Wash buffer-PBGL**	
DNA concentration (ng/uL)	14	13	34	41	7	8	4	10	12	11	12	20	10	16	13	17
Total yield (ug)	2.6	2.4	6.2	7.3	1.3	1.5	0.7	1.9	2.2	2.0	2.2	3.5	1.8	2.8	2.4	3.0
260/280 value	1.95	1.83	1.8	1.91	1.37	1.52	1.41	1.73	1.66	1.63	1.64	1.83	1.75	1.55	1.76	1.7

*components of Qiagen DNeasy Plant Mini kit

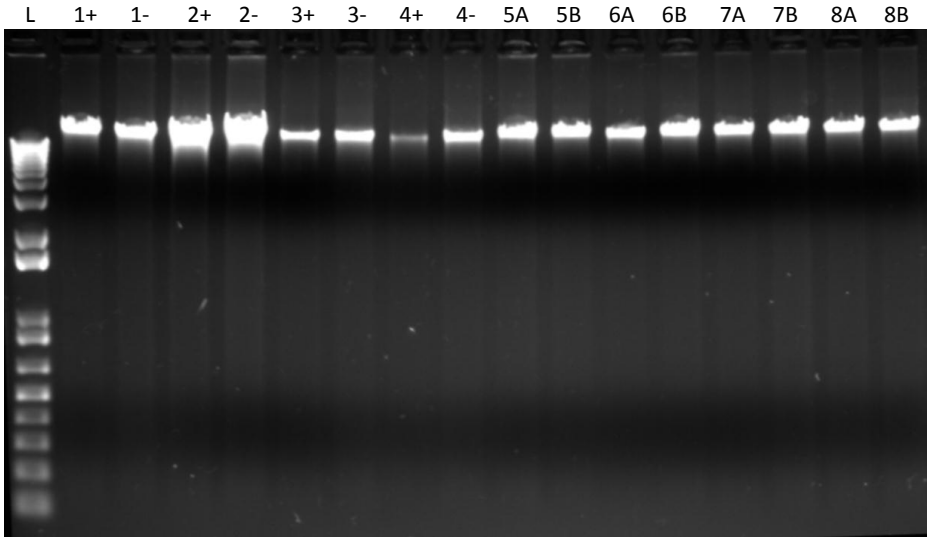

Figure 1. Quality of barley genomic DNA extractions using silica powder and different combinations of self-made (low-cost) buffers and buffers provided by Qiagen DNeasy kit. 8 uL of each genomic DNA extraction were separated on a 0.7% agarose gel.

1-8: Barley genomic DNA preparation
+: using QIAshredder columns for the preparation of barley leaf lysates (lysis procedure following the kit instructions)
-: preparation of leaf lysates using the kit instruction (but without using QIAshredder columns
A, B: technical replicates
L: size standard (1 kB Plus DNA ladder - Invitrogen)

All of the genomic DNA preparations show similar DNA concentrations (Table 1) and a good quality of the genomic DNA on the agarose gel (Figure 1).

Only the DNA preparations “2+” and “2-” (buffer components from the kit in combination with our wash buffer) show clearly higher concentrations and yields (about 2-3 times higher) than all other DNA preparations. These results indicate that by modifications of the protocol (i.e. modifications of buffers) some improvements of the DNA yields are possible.

The DNA preparations of samples 8A and 8B were extracted exclusively with self-made (low-cost) buffers and show a comparable concentration and yield as the other extractions.

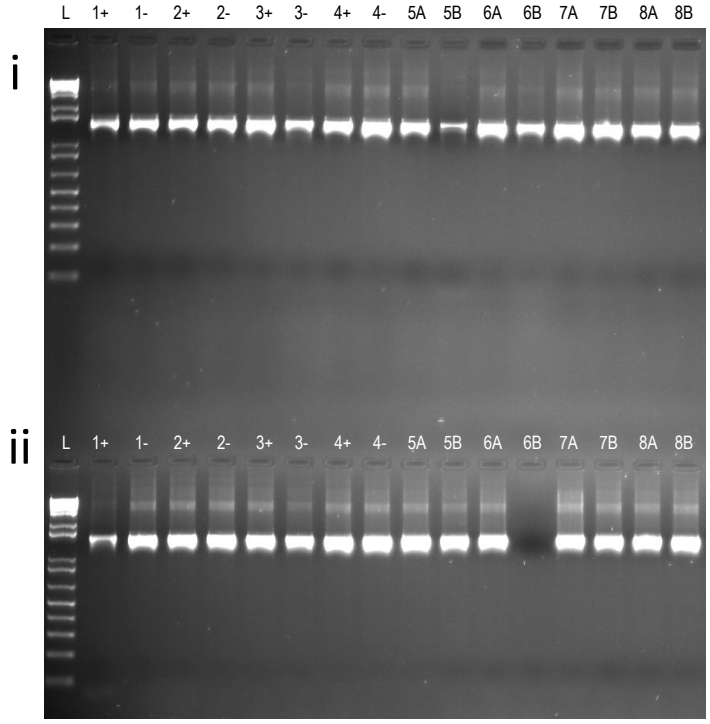

Figure 2. TILLING-PCR products amplified from genomic DNA extractions of barley (obtained by silica-based, low-cost DNA isolation method using different combinations of self-made buffers and buffers provided by Qiagen DNeasy kit). An aliquot of 5uL of each PCR reaction was separated on a 1.5% agarose gel.

i – Target gene: nb2-rdg2a (1500bp-PCR product);
ii – Target gene: nbs3-rdg2a (1491bp-PCR product)
1-8: Barley genomic DNA preparation (see Table 1)
+: using QIAshredder columns for the preparation of barley leaf lysates – Lysis procedure following the kit instructions;
-: preparation of leaf lysates using the kit instruction (but without using QIAshredder columns
A, B: technical replicates
L: size standard (1 kB Plus DNA ladder - Invitrogen)

5. CONCLUSIONS:

The DNA extractions from barley using the silica-based, low-cost method provided high-quality genomic DNA and sufficient yield suitable for standard PCR application such as molecular markers and TILLING