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Extraction of plant RNA:

Method: Plant RNA kit,E.Z.N.A.® Plant RNA Kit Standard Protocol

1. Collect tissue in a 1.5 mL microcentrifuge tube and freeze by dipping in liquid nitrogen. Grind the tissue with disposable pestles.
2. Transfer up to 100mg frozen ground plant tissue to a new 1.5 mL microcentrifuge tube
3. Immediately add 500µL RB Buffer. Vortex at maximum speed to mix thoroughly.
4. Insert a Homogenizer Column into a 2 mL Collection Tube.
5. Transfer the lysate to a Homogenizer Column.
6. Centrifuge at 14,000 x g for 5 minutes at room temperature.
7. Transfer cleared lysate to a new 1.5 mL microcentrifuge tube. Do not disturb or transfer any of the insoluble pellet. Measure the volume of the lysate.
8. Add 1 volume 70% ethanol. Vortex at maximum speed for 20 seconds. A precipitate may form at this point; it will not interfere with DNA isolation. Passing the mixture through a needle using a syringe or by pipetting up and down 10-15 times may break up the precipitates.
9. Insert a HiBind® RNA Mini Column into a 2 mL Collection Tube.
10. Transfer 700µL sample, including any precipitates that may have formed, to the HiBind® RNA Mini Column.
11. Centrifuge at 12,000 x g for 1 minute at room temperature.
12. Discard filtrate and reuse the collection tube.
13. Repeat Steps 10-12 until all of the sample has been transferred to the column.
14. Add 500µL RNA Wash Buffer I.
15. Centrifuge at 10,000 x g for 30 seconds.
16. Discard the filtrate and the collection tube.
17. Transfer the HiBind® RNA Mini Column to a new 2 mL Collection Tube.
18. Add 700µL RNA Wash Buffer II.
19. Centrifuge at 10,000 x g for 30 seconds.
20. Discard the filtrate and reuse collection tube.
21. Add 500µL RNA Wash Buffer II.
22. Centrifuge at 10,000 x g for 30 seconds.
23. Discard the filtrate and reuse collection tube.
24. Centrifuge the empty HiBind® RNA Mini Column at maximum speed for 2 minutes.
25. Transfer the HiBind® RNA Mini Column to a clean 1.5 mL microcentrifuge tube.
26. Add 50-100µL DEPC Water.
27. Centrifuge at maximum speed for 1 minute and store eluted RNA at -70°C.

Notice: steps 14-23 are expected to be done if you want to remove the genome gene.

Standard PCR:

1. Add DNA template up to 50ng into PCR tube,(x ul).
2. Add reagents as follow:
    - dNTP: 4ul
    - 5x PSBuffer: 10ul
    - Primer: 1ul (after diluted)
    - Enzyme: 0.5ul
    - ddH2O: (33.5-x)ul

Total: 50ul

Mix gently by vortex and briefly centrifuge to collect all components to the bottom of the tube.

3. PCR procedure:
   98℃(2min)→ 98℃(10s)→55℃(5s)→72℃(1Kb/min)→72℃(5min)→4℃(∞)
4. The amplification parameters will vary depending on the primers and the thermal cycler used. It may be necessary to optimize the system for individual primers, template, and thermal cycler.

PCR purification：

Method:Cycle-Pure Kit(200)

1. Perform agarose gel/ethidium bromide electrophoresis to analyze PCR product.
2. Determine the volume of the PCR reaction. Transfer the sample into a clean 1.5ml microcentrifuge tube and add 4-5 volumes of Buffer CP. For PCR products smaller than 200bp, add 6 volumes of Buffer CP.
3. Vortex thoroughly to mix. Briefly spin the tube to collect any drops from the inside of the lid.
4. Place a HiBind DNA Mini Column into a provided 2 ml collection tube.
5. Add the mixed sample from step 3 to the HiBind DNA Mini Column and centrifuge at 13,000 x g for 1 minute at room temperature. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.
6. Add 700µl of DNA Wash Buffer and centrifuge at 13,000 x g for 1 minute. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.
7. Add 500µl of DNA Wash Buffer and centrifuge at 13,000 x g for 1 minute. Discard the flow-through liquid and place the HiBind DNA Mini Column back into the same collection tube.
8. Centrifuge the empty HiBind DNA Mini column for 2 min at maximal speed (⩾13,000 x g) to dry the column matrix.
9. Place the HiBind DNA Mini column into a clean 1.5ml microcentrifuge tube. Depending on the desire concentration of the final product, add 30-50 µl of Elution Buffer (10mM Tris, pH8.5) or water directly onto the center of column matrix.

OVERLAP PCR:

1. order two primers which are complements of one another.
2. These primers will each have a 60°C Tm with one part and a 60°C Tm with the other part.
3. The "end primers" will not have any complements and will likely only have restriction sites.
4. PCR amplify the necessary fragments separately. Use a proofreading polymerase enzyme. Use an annealing temp of 60°C.
5. Clean up the product using a DNA column.
6. "Overlap PCR" Use cleaned up fragments as template in a PCR reaction:
7. About 1/2 to 3/4 volume of the Overlap PCR reaction should be equimolar amounts of purified fragments. Do not use Phusion polymerase. Try Pfu Turbo. Do not add any primers; the templates will prime each-other. 3.Run 15 PCR cycles without primers.Use an annealing temp of 60°C.
8. Add end primers to the Overlap PCR reaction: Continue cycling for another 15-20 rounds. Use an annealing temp of 72°C
9. Gel extract the correct size fragment.

Gel Extraction:

Method:QIAquick Gel Extraction Kit I(250)

1. Excise the gel slice containing the DNA band with a clean, sharp scalpel. Minimize the size of the gel slice by removing excess polyacrylamide.
2. Weigh the gel slice. Add 1–2 volumes of diffusion buffer to 1 volume of gel(i.e., 100–200 µl for each 100 mg of gel).
3. Incubate at 50°C for 30 min.
4. Centrifuge the sample for 1 min.
5. Carefully remove the supernatant using a pipet or a drawn-out Pasteur pipet. Pass thesupernatant through a disposable plastic column or a syringe containing either aWhatman GF/C filter or packed, siliconized glass wool to remove any residualpolyacrylamide.
6. Determine the volume of the recovered supernatant.
7. Add 3 volumes of Buffer QG to 1 volume of supernatant and mix. Check that the col or of the mixture is yellow.
8. Place a QIAquick Spin Column in a provided 2 ml collection tube.
9. To bind DNA, apply the sample to the QIAquick Spin Column and centrifuge for30–60 s.
10. Discard flow-
    through and place QIAquick Spin Column back into the same collection tube.
11. To wash, add 0.75 ml Buffer PE to column and centrifuge for 30–60 s.
12. Discard flow-through and place QIAquick Spin Column back in the same tube. Centrifuge column for an additional 1 min at maximum speed.
13. Place QIAquick Spin Column into a clean 1.5 ml microcentrifuge tube.

14. To elute DNA, add 50 µl Buffer EB (10 mM Tris·Cl, pH 8.5) or water to the center of t he QIAquick Spin Column and centrifuge for 1 min.

Plasmid Extraction:

Method:Plasmid Mini Kit I(200)

1. Isolate a single colony from a freshly streaked selective plate, and inoculate a culture of 1- 5 mL LB medium containing the appropriate selective antibiotic. Incubate for 12-16 hours at 37°C with vigorous shaking (300 rpm). Use a 10-20 mL culture tube or a flask with a volume of at least 4 times the volume of the culture.
2. Centrifuge at 10,000 x g for 1 minute at room temperature. Decant or aspirate and discard the culture media.
3. Add 250 µL Solution I/RNase A. Vortex or pipet up and down to mix thoroughly. Complete resuspension of cell pellet is vital for obtaining good yields.
4. Add 350 µL Solution III. Immediately invert several times until a flocculent white precipitate forms.
5. Centrifuge at maximum speed (≥13,000 x g) for 10 minutes. A compact white pellet will form. Promptly proceed to the next step.
6. Insert a HiBind® DNA Mini Column into a 2 mL Collection Tube.
7. Transfer the cleared supernatant from Step 5 by CAREFULLY aspirating it into the HiBind® DNA Mini Column. Be careful not to disturb the pellet and that no cellular debris is transferred to the HiBind® DNA Mini Column.
8. Centrifuge at 10000g speed for 1 minute.
9. Discard the filtrate and reuse the collection tube.
10. Add 500 µL HB Buffer.
11. Centrifuge at 10000g speed for 1 min.
12. Discard the filtrate and reuse collection tube.
13. Add 700 µL DNA Wash Buffer.
14. Centrifuge at 10000g for 1 minute.
15. Discard the filtrate and reuse the collection tube.
16. Centrifuge the empty HiBind® DNA Mini Column for2 min at 13000g to dry the column matrix.
17. Transfer the HiBind® DNA Mini Column to a clean 1.5 mL microcentrifuge tube.
18. Add 30-50 µL Elution Buffer or sterile deionized water directly to the center of the column membrane.
19. Let sit at room temperature for 5 minute.
20. Centrifuge at 13000g for 1 minute.
21. Store DNA at -20°

E.coli Competent cells:

1. Innoculate a single colony of E. coli into 5 ml LB and grow O/N at 37
2. Innoculate 1 ml into 100ml and grow to an O.D. 600 of 0.4
3. Aliquot the culture into 2x 50ml pre-chilled Sorvall tubes and leave on ice for 5 -10

mins

4. Centrifuge cells for 7 mins at 3000rpm, 4℃ without brakes
5. Pour off supernatant and resuspend each pellet in 10 ml of ice-cold CaCl2 soln
6. Centrifuge cells for 5 mins at 2500rpm, 4 degree. Discard supernatant and resuspend each pellet in 10 ml of cold CaCl2 solution.
7. Keep resuspended cells on ice for 30min.
8. Centrifuge cells for 5 mins at 2500rpm, 4℃. Discard supernatant and resuspend each pellet in 2ml of ice-cold CaCl2.
9. Dispense cells into pre-chilled sterile eppendorfs.
10. Freeze immediately @ -70 degresss

Notice: CaCl2 Soln: 60mM CaCl2, 15% Glycerol

Protein Extraction and expression:

a. Bacterial transformation
   1. 80ul competent cells (BL21) + DNA samples (≤ 10ng; first add the plasmid and then add to small volume)
   2. Place on ice for 30 min → 42 ° C for 45 s → place on ice for 1~2 min → add 1 ml of LB-Ep tube → shake at 37 ° C for 1 h
   3. Take 100ul on LB resistant plate, overnight at 37 ° C

b. IPTG induced expression
   1. Pick colonies on the plate and draw 8 (for example) small squares.
   2. Pick up the bacteria with LB resistance solution, overnight at 37 ° C
      (OD test method: (Cuvette in A312, tube photometer in A317) Click cell growth---general test---smart test---cell growth (read-only) (wavelength is 600nm)---measure Blanket (what is used as a blank for dilution, such a test should be blank with LB)---measure sample)
   3. Cuvette liquid OD value, OD = A, diluted into Bml
      (B * 0.2 / A = C, Bml LBK added Cml bacteria), OD = 0.2 (recommended to be diluted to 5ml or 10ml at the beginning)
      Shake for 2h at 37℃, generally to OD = 0.5;
   4. Add IPTG induction generally optimally 0.5mM
   5. Initially induced expression requires exploration conditions: 1 IPTG amount: 0.1 ~ 1 mM gradient (0, 0.25, 0.5, 1)
   6. 4 hours(depend on the type of the protein) of culture at 37 degrees
   7. Centrifuge the cells to precipitate and wash them twice with PBS (make sure to wash) (add 3ml PBS) (move into the Ep tube when washing with PBS for the first time)

c. Protein extraction
   1. lysate with mix: PMSF (1:40). protein inhibitor (1:100), DTT (500 mM → 5 mM);
      Add ulsate 500ul per tube;
      Ultrasonic disruption.
   2. Centrifuge at 44 ° C, 13000 rpm for 20 min, take the supernatant.

Coomassie Blue Staining:

Materials and Reagents:

1. Coomassie Brilliant Blue R250 (EM Science)

Equipment:

1. Shaker

Procedure:

1. Incubate the gel in staining solution with shaking for 30 min or longer (can leave it overnight).
2. Remove the dye solution (it can be reused for many times) and rinse the gel with water 1-2 times to remove the dye.
3. Add destaining solution to the gel and incubate for 30-60 min.
4. Transfer the gel to water (can keep it in water for several days)

Recipes:

1. 100 ml Staining solution
    - Coomassie Brilliant Blue R250 0.25 g
    - Glacial acetic acid 10 ml
    - MetOH:H2O (1:1 v/v) 90 ml
2. Destaining solution

Destaining solution is the same as staining solution, but not containing the Coomassie R250 dye Powder.

Silver Staining:

1. Fix gel in 10% MeOH / 10% Acetic Acid for 30 min or overnight
2. Wash 4X in H2O for at least 5 min
3. Incubate in sodium thiosulfate (1-2 pellets [400mg] per 500 ml) for 90 sec (Save 20 ml for later)
4. Wash 3X quickly with H2O
5. Add Silver nitrate solution (0.9 g silver nitrate in 500 mL H2O) for 10 min. Gel will turn slightly yellow
6. Wash the gel 3X quickly in H2O
7. Add developer solution (10g potassium carbonate, 20 ml sodium thiosulfate, 250 µl 40% Formaldehyde in 500 ml)
8. Stop the reaction by adding destain (10% MeOH and 5% Acetic Acid)
9. Wash gel in water

Western blot:

Put blot into antibody:

1. Remove blot from fridge.
2. Prepare primary antibody:
    - 10mls TBS
    - 2% Non-fat powdered milk

- 0.05% Tween

3. Add appropriate amount of antibody for dilution (1:5,000, 1:1,000, 1:500, etc.).
4. Prepare plastic bag:
    - Cut plastic tube to size of gel.
    - Seal bag with bag sealer set on 3.
    - Put membrane blot in bag.
    - Seal three sides of the plastic bag and cut excess (leave extra room on the top for when adding the antibody).
5. Add antibody with pipette and aide.
6. Completely wet membrane.
7. Use a kimwipe to push out all the air bubbles.
8. Seal bag closed once all bubbles are removed and cut bag excess.
9. Put blot on rocker 1-2 hours at room temperature or overnight at 4 degrees Celsius.
10. Take blot out of the bag and put in TBST rinse.
11. TBST (For 1 Liter):
    - 900mls H2O
    - 100mls 10X TBS
    - 0.5mls Tween
12. Save primary antibody. Store at 4 degrees Celsius.
13. Put blot in tray with 100-200mls TBST and put on rocker for 3-5 minutes.
14. Repeat rinse in TBST.
15. Prepare secondary antibody (1:5,000):
    - 25mls TBST
    - 5ul secondary antibody
16. Pour secondary antibody onto blot and put on rocker for at least 1 hour.
17. Wash blot with 3 washes of TBST for 3-5 minutes each.
18. Prepare Western Blotting Detection Solution:
    - 3mls solution A
    - 3mls solution B
19. Lay glass plate down on bench and lay blot on glass plate.
20. Put developer drop-wise over blots surface.
21. Tilt blot to make sure the whole blot is covered.
22. Let sit 1 minute.
23. Lay out a piece of smooth seran wrap.
24. Take blot off plate and let developer drip off.
25. Lay blot protein side down onto seran wrap and fold seran wrap to seal blot in bag.
26. Tape blot into cassette.
27. Take Biomax light film, cassette, timer and scissors down to the dark room.
28. Cut film into 2 pieces (fold upper right corner).
29. Put film over blot and close cassette.
30. Expose blot for 1 minute.
31. Put film in developer.
32. Expose blot for longer or shorter times depending on first exposure.
33. Label film using blot (when it is still taped into the cassette).

34. If using another antibody strip blot.

List of some kits and reagents

List of experimental kits		List of biological reagents	
kits	**origin**	**anti-Ha, anti-Flag antibody**	**Sigma,Abcam**
Plasmid Mini Kit I(200)	Omega	anti-rabbit IgG	Proteintech
Plasmid Mini Kit(25)	Omega	anti-mouse IgG	Proteintech
Cycle-Pure Kit(200)	Omega	GoTaq Colonrless Master	Promega
Gel Extraction Kit I(200)	Omega	PrimerSTAR Max DNA Polymerase	Takara
Endo-free Plasmid Mini Kit II(50)	Omega	PrimerSTAR HS DNS Polymerase	Takara
GoScript Reverse Transcription System	Promega	DL2000 DNA Marker	Takara
QIAquick PCR Purification Kit(250)	QIAGEN	1Kb DNA Ladder(Dye Plus)	Takara
QIAquick Gel Extraction Kit I(250)	QIAGEN	6x DNA Loading Buffer	Takara
In-Fusion HD Cloning Kit	Takara	10x DNA Loading Buffer	Takara
DNA Ligation Kit Ver.2.1	Takara	Protease Inhibitor Cocktail	CWBIO
Blunting Kination Ligation Kit	Takara	2x Es Taq Master Mix	CWBIO

BCA Protein Assay Kit	CWBIO	Protease Inhibitor 8340	Sigma
cECL Western Blot Kit	CWBIO	PMSF	Sigma
eECL Western Blot Kit	CWBIO	Prestained Protein Ladder	Thermo
Plant RNA kit	OMEGA	Xma I, Restriction enzyme	NEB
		Apa I, BamH I, EcoR I, , Kpn I, Not I, Sal I, Xba I, Spe I, Pst I, Xba I	Takara