PART 1 – LEAF COLLECTION AND PREPARATION

1. Collect 2 leaves of various plants per student. Collect 1 Garlic Mustard leaf (per group) as a positive control.
2. Acquire 2 (8) strip tubes per group.
3. Label one strip tube ITS2 and the other rbcl. Label the side of each tube of the strip tube with the numbers 1-8 as well as the group # following the notation below:

   Group#_ITS2_Tube#
   e.g. 3_ITS2_5

   The top of the tube is domed and the tubes are small so using 'I' for ITS2 and 'r' for rbcl will suffice.
4. Using the micro-punch, press 2 leaf discs 1-2mm^2 from the 1st leaf and place 1 leaf disc into tube 1 of strip tube labelled ITS2 and the other disc into tube 1 of the strip tube labelled rbcl.
5. Wipe the micro-punch with 70% ethanol to sterilize in between samples.
6. Repeat for all leaf samples.
7. Include garlic mustard in tube 7 of each strip tube as a positive control.
8. Leave tube 8 of each strip tube empty as a negative control.
9. Add 200uL FTA Purification Buffer to each PCR tube containing leaf disc and incubate at room temperature for 5 mins.
10. Remove and discard FTA Purification Buffer.
11. Repeat steps 2 and 3.
12. Add 200uL 0.1X TE Buffer to each PCR tube containing leaf disc and incubate at room temperature for 5 mins.
13. Remove and discard 0.1X TE Buffer.
14. Repeat steps 2 and 3.
15. Remove all remaining buffer and allow the leaf disc to dry for 30s - 1 minute. There should be no visible droplet in the tube.
16. Close tubes and set aside at room temperature.

Each group should now have 2 (8) strip tubes with 7 leaf discs each (6 collected and 1 garlic mustard control) and 1 empty tube to be used as a negative control.

PART 2 – PCR

1. Prepare a ITS2 Mastermix for all 8 samples by filling in the table below:

Component	Volume (ul) for 1 reaction	Volume (ul) for 8.5 reactions
2x PCR Buffer	12.5	
ITS2 Fw primer	1.25	
ITS Rv primer	1.25	
Nuclease-free water	10.0	

2. Calculate the volumes of each component required for 8.5 reactions.
3. Add the volumes of each component required for 8.5 reactions together in a 1.5mL tube. Pipette up and down several times to mix well.
4. Label the 1.5mL tube 'ITS2'.
5. Aliquot 25uL of mastermix into each of the tubes on the strip tube labelled 'ITS2' containing the washed leaf discs.
6. Centrifuge tubes briefly and place on ice until the 2nd mastermix for rbcl is ready.
7. Prepare a rbcl Mastermix for all 8 samples by filling in the table below:

Component	Volume (ul) for 1 reaction	Volume (ul) for 8.5 reactions
2x PCR Buffer	12.5	
rbcl Fw primer	1.25	
rbcl Rv primer	1.25	
Nuclease-free water	10.0	

8. Calculate the volumes of each component required for 8.5 reactions.
9. Add the volumes of each component required for 8.5 reactions together in a 1.5mL tube. Pipette up and down several times to mix well.
10. Label the 1.5mL tube 'rbcl'.
11. Aliquot 25uL of mastermix into each of the tubes on the strip tube labelled 'rbcl' containing the washed leaf discs.
12. Centrifuge all tubes briefly and place in PCR machine programmed with the following parameters:

Temperature	Time	Cycles
94°C	3 mins	1
94°C	30 secs	35
52°C	30 secs	
72°C	1 min	
72°C	10 min	1
4°C	∞	1

PART 3 – AGAROSE GEL ELECTROPHORESIS

1. Weigh 1.5g agarose and add to a 250mL Erlenmeyer flask.
2. Measure 150mL of 1X TBE Buffer and add to flask containing agarose.
3. Swirl gently to mix and microwave for 1 minute. Remove from microwave and swirl again. Microwave for another 30 – 60 seconds until agarose is fully dissolved and no longer visible. Mixture should be clear (no bubbles).
4. Allow to cool until flask can be held without heat resistant gloves (approximately 5 minutes).
5. Place gel tray/mold into gel electrophoresis unit so that the open rubber sides fit snugly against the sides of the unit. Ensure the tray is set all the way down to the bottom of the unit to ensure an even gel. Check the rubber sides and confirm they form a tight seal so that no liquid can leak.

   Alternatively, the open sides of the gel tray can be taped to form a box. Check carefully that it is taped well so that no spillage occurs. The tape should sit above halfway up the gel tray and fold down underneath the bottom. It is only necessary to tape the tray if not employing the method above.

6. Add 7.5uL of RedSafe to flask and swirl gently to mix. Mixture should be a slightly yellow colour throughout.
7. Insert a comb into each of the two slots on the gel tray.
8. Pour the gel into the gel tray slowly, starting at one corner (furthest from the top comb).
9. Inspect the gel for bubbles. If bubbles are present, take a 10ul tip and pierce to burst or drag them to the bottom corner of the gel. Ensure there are no bubbles around the combs as this can lead to breakage of the wells.
10. Allow gel to set until solid.
11. Place set gel (and gel tray) into electrophoresis.
12. Fill electrophoresis unit with 1X TBE Buffer until gel is covered by buffer.
13. Remove combs.
14. Designate 1 well per comb to load the ladder.
15. Load 5uL of each sample onto gel. Ensure pipette is under the buffer when dispensing sample. Do not allow the pipette tip to pierce the gel. 19 samples per comb can fit on gel.
16. Load 7ul of ladder into each designated ladder well.
17. Fill the following table to aid in sample identification later.

Name	Group#	Primer Set (ITS2/rbcl)	Tube#	Well#	Amplification?

18. Cover the top of the electrophoresis unit with the lid and plug into the power pack.
19. Turn the power pack on. Set voltage to ~110V. Look for bubbles.
20. Allow gel to run for ~45 mins- 1 hour or until the dye runs ¾ way down the top half of the gel. Do not allow the samples in the top half of the gel to run into the 2nd half of the gel.

Visualize the gel under UV light and take note of the samples that showed amplification. Take a picture of the gel if possible.

21. Store remainder of samples in fridge overnight to be sent for Sanger Sequencing.