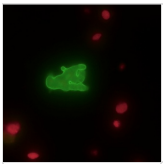

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Isolation of haustoria from stem rust *Pgt* 21-0 infected wheat seedlings

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1 *Works for me* This protocol is published without a DOI.

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ABSTRACT

Optimising haustoria isolation from infected wheat by reducing chloroplast density and increasing haustoria stability. Isolated haustoria will be used for high-molecular DNA extraction followed by long-read DNA sequencing.

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GUIDELINES

This protocol is modified and optimised from the following publications. When citing, please also note the original publications below.

Garnica, D. and Rathjen, J. (2014). Purification of fungal haustoria from infected plant tissue by flow cytometry. Methods in Molecular Biology **1127**, 103-110

MATERIALS TEXT

Reagents required for haustorial isolation from infected plant Tissue

Materials and equipment

1. 20-25 g Infected plant leaves
2. Sterile paper towel.
3. Blender.
4. Three schott Beakers (500mL)
5. Falcon Tubes 50 mL
6. one nylon meshes (~20 cm × 30 cm) pore size 100 µm
7. 3 layers of Miracloth (25 -30 µm)
8. Rotary mixer.
9. Refrigerated benchtop centrifuge with swingout rotor.
10. two 250ml Nalgene Tubes.
11. Hemocytometer

Reagents

1. 1 L of 70 % ethanol
2. 1 L of chilled MilliQ water
3. 1 L of Sodium hypochlorite 2% (v/v) (prepare fresh)
4. Concanavalin A, Alexa Fluor ® 488 Conjugate (Molecular Probes ® C11252)
5. Percoll (GE healthcare life sciences, 17-0891-01)

10x Master Mix (MM) (100ml)

Reagents	Molecular weight (g/mol)	Stock	Quantity needed for 100 mL	Quantity needed for 400 mL
2 M Sucrose	**342.30**	Powder	68.46 g	273.84 g
0.2 M MOPS pH 7.2	**209.26**	Powder	4.19 g	16.76 g

Isolation Buffer (IB) (100mL 10x MM + 100 mL 0.5 M EDTA + 800 mL MQW) = 1 L

Homogenization Buffer (50 mL of 10x MM + 0.08 g (80 mg) DTT+ 50 mL 0.5 M EDTA + 400 mL MQW) = 500 mL

Preparation of Hypotonic lysis buffer (100 mL)

Reagents	Molecular weight	Stock	Amount needed for 100 mL	Amount needed for 200 mL
50 mM HEPEES-KOH pH 7.5	238.30	1 M	5 mL	10 mL
2 mM EDTA		0.5 M	400 uL	800 uL
MQW	-	liquid	94.6 mL	189.2 mL

Preparation of Concavalin A

Sodium Bicarbonate

Chemical Compounds	Molecular weight (MW)	Stock	Amount
0.1 M Sodium Bicarbonate (NaHCo3)	**84.007**	powder	0.42 g
MillQ Water	-	liquid	50 mL

*Concanavalin A (Con A) is one of the most widely used lectins in cell biology. The Alexa Fluor® 488 conjugate of Con A exhibits the bright, green fluorescence of the Alexa Fluor® 488 dye (absorption/emission maxima ~495/519 nm). It selectively binds to a-mannopyranosyl and a-glucopyranosyl residues.

Add 1mg of Con A to 200 uL of sodium bicarbonate.

Stock Solutions can be made at 1-5 mg/ml in 0.1 M sodium bicarbonate (approx pH 8.3). Store solution at 2-6°C with the addition of 2 mM of sodium azide (if you want to store the solution for longer, divide the solution intoaliquots and freez at -20°C. AVOID REPEATED FREEZING AND THAWING. PROTECT FROM LIGHT.

Purification of Haustoria using flow cytometry

Materials and Equipment

1. Dickinson BD FACSARIA II cell sorter (BD Biosciences), or similar.
2. 5 ml glass assay tubes.

Reagents

Preparation of Phosphate Buffered saline (1x PBS)

Chemical Compounds	**Molecular Weight (MW)**	Stock	**Quantity**
Sodium Chloride (NaCl)	**58.44**	powder	8 g
Potassium Chloride (KCl)	**74.55**	powder	0.2 g
Disodium Hydrogen Phosphate (Na2 HPO4)	**141.96**	powder	1.44 g
Potassium Dihydrogen Phosphate (KH2 PO4)	**136.09**	powder	0.24 g
Dissolve them all in 1 L of MilliQ water Final pH 7.4 and sterilized by autoclaving			

ABSTRACT

Optimising haustoria isolation from infected wheat by reducing chloroplast density and increasing haustoria stability. Isolated haustoria will be used for high-molecular DNA extraction followed by long-read DNA sequencing.

Blender homogenisation

1 Harvest 20–25 g of heavily infected tissue (leaf pieces of ~6 cm length), 6 days after infection (or 1 day before sporulation).

2 To remove external contaminating organisms, wash with tap water several times.

3 Incubate for 3 min in 2% sodium hypochlorite.

4 Wash with tap water three times or until sodium hypochlorite is completely removed.

5 Incubate the tissue for 1 min in chilled 70% ethanol. Wash tissue with chilled MilliQ water several times to remove the alcohol. Dry the tissue as much as possible with Kimwipes (Kimtech).

6 Using a blender, homogenise the infected plant material in 250 mL of homogenisation buffer at maximum speed for 25 s.

Nutribullet Select 1000 W: speed 2-3 for duration of 30 s-1 min.

Filtration

7 Filter the homogenate through a 100 µm nylon mesh by gravity flow into Schott bottle using funnel. Forcibly squeeze out as much residual homogenate from the leaf debris as possible, maximising cell capture.

8 Recover the solid particles retained on the mesh and return them to the blender, add the remaining homogenisation buffer and blend for 15 s.

9 Filter the remaining homogenate through the 100 µm nylon mesh, combining with the first homogenate.

10 Filter homogenate through 1 layer of Miracloth using a funnel and 1 L Schott bottle. Gently squeeze residual homogenate through the Miracloth.

> Miracloth 22-25 um. The pore size of the meshes is critical for reducing contaminating particles before FACS. Different fungal species display a diverse range of haustoria size, thus small-scale isolations to test different pore sizes and microscopic analyses are necessary to optimize the filtration step.

11 Repeat Filtration through 2 layers of Miracloth (using a new Schott bottle).

12 Distribute the filtrate evenly across eight chilled 50 mL Falcon tubes.

13 Centrifuge at 1,080 rcf and 4°C for 15 min, using a centrifuge with swing out rotor.

14 Carefully discard the supernatant, being careful not to dislodge the pellet.

Hypotonic lysis 1 (optional)

15 Resuspend each pellet in 20 mL pre-chilled hypotonic lysis buffer by gentle mixing or pipetting with a transfer pipette.

16 Incubate at 4°C for 1 h, with gentle rotation, ~20 rpm. Can place a rotator in a cold room.

17 Centrifuge at 1,080 rcf and 4°C for 15 min, using a centrifuge with swing out rotor.

18 Carefully discard the supernatant, being careful not to dislodge the pellet.

Percoll 1: cell fractionation by density

19 Bring each pellet to 5 mL with 1x IB, carefully resuspending with a transfer pipette.

20 Prepare a 40% Percoll solution (15 mL per sample).

Reagent	Per sample (15 mL)	For 13 (includes 1 dead volume) (195 mL)
Percoll	6 mL	78 mL
10x MM	1.5 mL	19.5 mL
MQW	7.5 mL	97.5 mL

- Later, 15 mL of Percoll solution will be added to 5 mL of sample (20 mL total)
- After combining with the sample, final Percoll concentration will be 30%.
- Will have approximately 12 tubes. Make one extra sample solution for dead volume.
- This protocol increases the Percoll to sample ratio, providing better separation of layers.

21 Add 15 mL of the 40% Percoll solution to each sample, bring the final volume to 20 mL. Mix gently by inverting and swirling.

22 Centrifuge at 25,000 rcf and 4°C for 30 min without braking.

11824 rpm on Avanti J-E (Beckman Coulter) Centrifuge.

23 Carefully remove the tubes from the centrifuge, and draw off the top two layers (approx 7-10 ml) from each tube very slowly using a transfer pipette.

Haustoria from different fungal species exhibit slightly different density characteristics. It is highly recommended to establish the density level at which the majority of haustoria resolve. Small-scale density tests can be performed by following the protocol above until step 8. Once the tubes are removed from the centrifuge, aliquots of 1 ml are taken carefully with a wide-bore pipette tip, and the number of haustoria can be counted in each aliquot using a hemocytometer.

24 Dilute the pooled haustorial fraction 1:10 with 1× IB (for 70 mL, add 630 mL 1x IB). Mix well and distribute the mixture into four chilled Nalgene tubes (250 mL)

The volumes may differ depending on how much grams of infected leaves were used.

25 Centrifuge at 1,080 rcf and 4°C for 15 min.

26 Carefully discard the supernatant, being careful not to dislodge the pellet.

Hypotonic lysis 2 (optional)

27 Resuspend each pellet in 20 mL pre-chilled hypotonic lysis buffer by gentle mixing or pipetting with a transfer pipette.

28 Incubate at 4°C for 1 h, with gentle rotation, ~20 rpm. Can place a rotator in a cold room.

29 Centrifuge at 1,080 rcf and 4°C for 15 min, using a centrifuge with swing out rotor.

30 Carefully discard the supernatant, being careful not to dislodge the pellet.

Percoll 2: cell fractionation by density (optional)

31 Bring each pellet to 5 mL with 1x IB.

32 Prepare a 33% Percoll solution (15 mL per sample).

Reagent	Per sample (15 mL)	For 9 (includes 1 dead volume) (135 mL)
Percoll	5 mL	45 mL
10x MM	1.5 mL	13.5 mL
MQW	8.5 mL	76.5 mL

- Later, 15 mL of Percoll solution will be added to 5 mL of sample (20 mL total)
- After combining with the sample, final Percoll concentration will be 25%.
- Will have approximately 12 tubes. Make one extra sample solution for dead volume.
- This protocol increases the Percoll to sample ratio, providing better separation of layers.

33 Add 15 mL of the 33% Percoll solution to each sample, bring the final volume to 20 mL. Mix gently by inverting and swirling.

34 Centrifuge at 25,000 rcf and 4°C for 30 min without braking.

35 Carefully remove the tubes from the centrifuge, and draw off the top two layers (approx 7-10 ml) from each tube very slowly using a transfer pipette.

36 Dilute the pooled haustorial fraction 1:10 with 1× IB (for 70 mL, add 630 mL 1x IB). Mix well and distribute the mixture into chilled Nalgene tubes (250 mL)

37 Centrifuge at 1,080 rcf and 4°C for 15 min.

38 Carefully discard the supernatant, being careful not to dislodge the pellet.

If extracting DNA from the raw pellet

39 Add 1.5 mL of 1x IB to the pellet and gently resuspend with a transfer pipette.

40 Transfer to a 2 mL Eppendorf tube. Save a 40 µL aliquot for microscopy confirmation of haustoria, storing in fridge at 4°C.

41 Centrifuge at 1,080 rcf and 4°C for 15 min.

42 Carefully discard the supernatant, being careful not to dislodge the pellet.

43 Store the pellet at -80°C until DNA extraction.

If proceeding to flow cytometry

44 Resuspend the pellets in 1× IB to a final volume of 4 mL, and transfer the suspension to a glass vial with plastic lid, 6 ml capacity.

45 Add 200 µl of 1 mg/ml Con A-Alexa 488 (Invitrogen), cover the container with foil, and mix gently on a rotary mixer for 20 min at room temperature (or for 45 min at 4 °C).

46 Pellet the haustoria at 4,000 × g in a benchtop centrifuge for 5 min at 4 °C. Remove the supernatant and wash the pellet twice with 1× IB. Resuspend in a final volume of 4 ml of 1× IB; keep sample on ice in the dark and proceed directly to FACS sorting.

47

This section describes the use of flow cytometry to sort haustoria based their fluorescence after labeling with Con

A-Alexa 488. The Alexa 488 fl uor is excited using a 13 mW 488 nm solid-state laser (Sapphire, Coherent Inc. Santa Clara, CA) and detected using a 502 nM longpass as well as a 530/30 nM bandpass filter. Chlorophyll autofluorescence is excited with the same 488 nm laser and detected using a 655 longpass and 695/40 bandpass filter.

48 Dilute the haustorial sample 1:10 with chilled 1× PBS to adjust the concentration of the chloroplast–fungal cell mixture to approximately $2.5–5.0 \times 10^{6}$ particles/ml.

49 Set up and optimise the cell sorter. For fungal haustoria (5–20 µm in diameter): 100 µM nozzle, 20 psi sheath pressure.

The procedure of setting up a flow cytometer varies depending on the machine and needs to be performed by appropriately trained personnel.

50 Use 1× PBS as sheath fluid, run cells using the lowest sample pressure with a resulting sample rate of 5,000–10,000 events per second. Collect sorted cells in 5 mL glass tubes containing 1× PBS, keeping them at 4 °C, and agitating them periodically at 300 rpm to prevent settling.

51 Set fluorescence and scatter parameters. Identify and exclude chlorophyll-containing particles based on chlorophyll autofluorescence. Identify and select the population of Con A-positive particles by Alexa 488 fluorescence. Use forward and side scatter information to identify and exclude doublets by comparing the forward scatter height and width and the side scatter height and width. To optimise the forward scatter signal, use a neutral density 2 filter in front of the forward scatter detector.

52 Confirm sort purity by running a small amount of the sorted cells to determine the percentage of positive events for the sort parameters (see Note).

Typically, $9 \times 10^{5} – 1.5 \times 10^{6}$ purified haustorial cells can be purified from 15 to 20 g of wheat tissue heavily infected with *P. striiformis* (in our case it is *P. graminis*).
Vital tests should be applied at this stage to verify the viability of haustoria post-sorting. A high percentage of viable cells could be very important for some subsequent applications as isolation of intact RNA or proteins. In this protocol the author used CellTracker™ Orange CMRA (Invitrogen, Catalog number C34551) successfully following the manufacturer's instructions, and obtained ≥98 % viable haustoria post-sorting.

53 Pool the samples from FACS purification and centrifuge them at 1,080 × g for 15 min at 4 °C. Discard the supernatant and snap-freeze the final pellet in liquid nitrogen, and store it at −80 °C until required.