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Protocol status: Working
We use this protocol and it's working

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Transformation Protocol ATMT Trichoderma atroviride

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ABSTRACT

This is the protocol for the transformation of conidia by Agrobacterium to perform the BarSeq technique in Trichoderma atroviride, which is reported by Villalobos-Escobedo et al., 2023.

DAY 1:

1 Inoculate 3 plates of Trichoderma @ 27 °C Grow them for 4 days

> **Note**
>
> Sterilize 6 clean flasks
> 5 bottles
> 2 100 mL GC
> 2 250 ml GC

2 Prepare LB + Kan (500 mL)

> **Note**
>
> 50 ug/mL is working concentration for Kan, amount added depends on stock concentration
> Ex: 50 mg/mL stock concentration
> 1 L of LB would require 1 mL of 50 mg/ml Kan
> -Usually, I put 1 ml in 500 ml of LB

DAY 3

3 Make liquid agro induction media (ABI)

For 100 transformations 5 mL of ABI are needed, water is autoclaved and all others are filtered and sterilized
200 mL to wash, 200 mL to resuspend (grow)

MIX AND FILTER INSIDE HOOD

> **Note**
>
> -- CASamino acids (10% CASamino acids) (Heat up and stir)
> -- Glucose (36% Glucose) (Heat up and stir)
> --Thiamine (10% Thiamine)

Note

Sterilized bottles

Dataset

ABI Media Spread Sheet

NAME

LINK

https://docs.google.com/spreadsheets/d/18ni9RM73H-b3UyHxGNHadtLOwJFGj_JT/edit?usp=sharing&ouid=113575406325457072750&rtpof=true&sd=true

Dataset

Chemical Mixes for ABI

NAME

LINK

https://docs.google.com/spreadsheets/d/1ezsYgXvGxfA8iqkbBXbE3URkroUd5RCiTNU_u6hufVA/edit?usp=sharing

For AB Buffer check pH, then filter

4 Prepare: 1 mL 3,5-dimethoxy-4-hydroxy-acetophenone (acetosyringone) *dissolved in dimethyl sulfoxide (DMSO).* **(make this fresh every time)**

-- Acetosyringone is filtered through syringe and 0.22 um filter top in DMSO (0.098 g/ mL)

5

1. Autoclave 437ml ddH2O with 20g Bacto Agar
2. Add MIXED induction liquid to agar.
3. Mix together inside hood for a few minutes
4. Pour plates.

2d

Pour solid agro induction media plates and leave on bench to dry for 2 days.

6 Prepare medium for Agrobacterium LB + Kan.

7 Inoculate *A. tumefaciens* strain harboring the plasmid for Trichoderma transformation onto LB + Kan. 250 µL in 50 mL

8 Shake at 250rpm at 30C overnight

(Place around 7 pm- 8 pm)

Day 4

9 Measure the OD600 of the agro cell culture. (It will likely be around OD600 1.)

(Around 9 am)

9.1 In order to get an accurate measurement, dilute the sample 1:10 in H2O in the cuvette.

(100 uL agro cell culture, 900 uL H2O)

10 Back dilute the agro culture to OD600 0.5 in LB+Kan with twice the number of ml of media to the number of transformations you plan to do (i.e., if you are doing 100 transformations back dilute into 200ml LB).

100 mL from stock and 100 mL new LB+Kan

11 Shake at 250rpm at 30C for 2h 2h

12 Measure the OD600 of the agro cell culture after 2 hrs

> **Note**
>
> It should be around OD600 1. If it is not at least OD600 0.95, put it back on the 30C shaker for another 30min.

13 Centrifuge the agro culture at . 4000 rpm, 00:15:00 15m

14 Pour supernatant into waste container.

15 Wash cells in agro induction media

Note

The number of mL should correspond to twice the number of transformations that you are doing
ex: 20 ml for 10 transformations
New flasks are used

16 Centrifuge the agro cells at 4000 rpm, 00:15:00 15m

17 Pour supernatant into waste container.

18 Resuspend cells in agro induction media

Note

The number of mL should correspond to twice the number of transformations that you are doing
For 100 transformations 200 ml of induction media, and each transformation has 4*10^7 conidia,
it's 2*10^7 per ml. Concentration of agro.
We can use tubes with caps for roller.

18.1 Put cultures on shaker @ 25C and rpm 250, for 24 hrs

18.2

Note

Materials for next day:
-Filter top
- 0.45 um Filter
- Vacum

DAY 5

6d 0h 5m

19

Note

(using plates of T. atroviride that are 4 days old)
NOTE: Make sure the big square plates are at room temperature.

Approximately 23h after putting agro cells into induction media, collect *Trichoderma* conidia from plates into 5-10ml of H2O.

20 Shake and vortex vigorously to suspend conidia

21 Measure conidial concentration

Count four corner squares, and middle square
Avg spores: Sum of both sides / 10 squares
Math: (Avg. spores)(1x10^4)(25)(100)

22 Aliquot $2e^7$ conidia into 2ml Eppendorf tubes

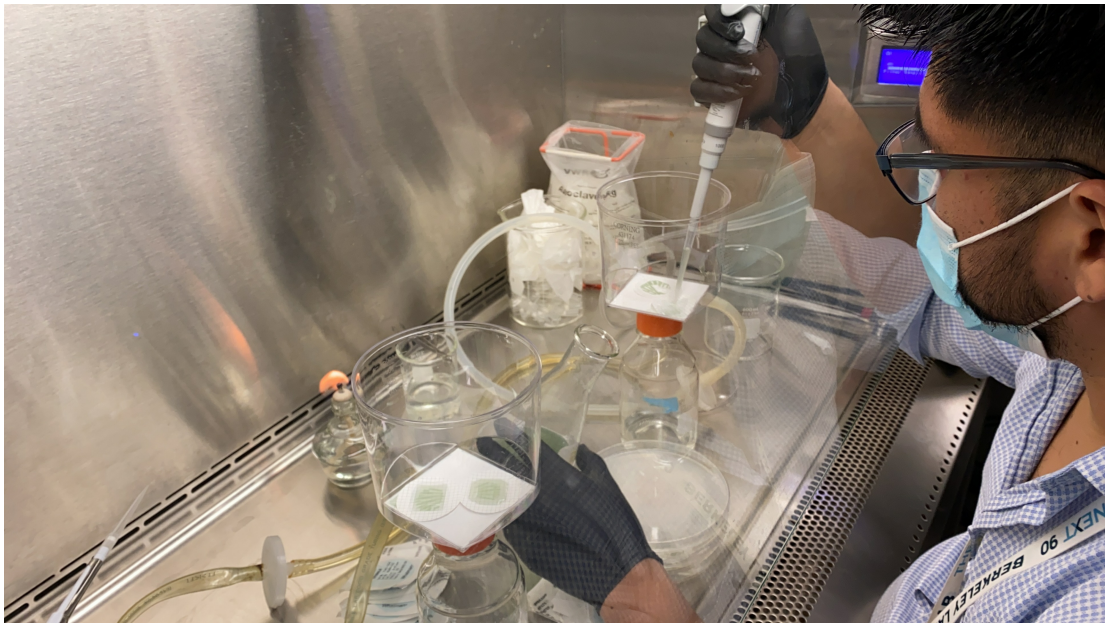
23 Spin cells at 10000 rpm, 00:05:00 5m
or 4000 rpm for 20 min

24 Pipet off supernatant

25 Resuspend cells (pellet) in 2ml of agro induction media cell culture (from yesterday)

26 Incubate at room temperature for at least 5 min

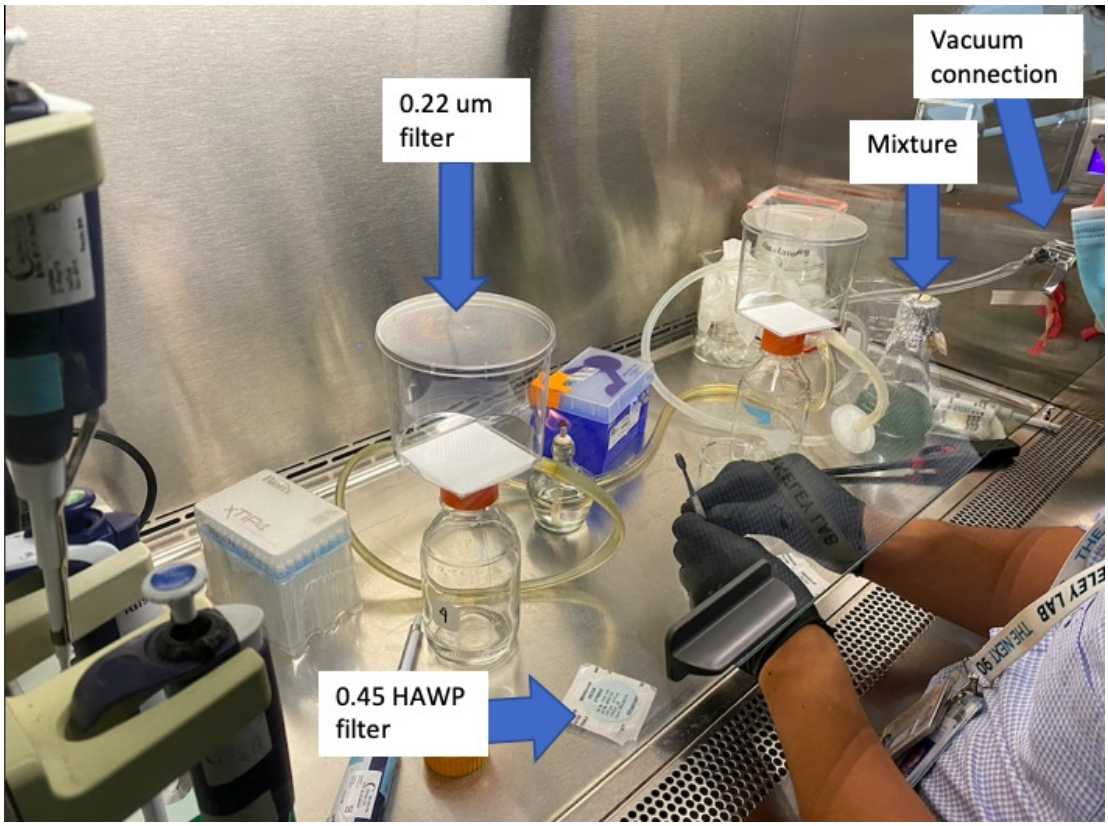
27 Put a 0.2um sterile PES bottle top filter onto a bottle and set up the vacuum



Equipment

Thermo Scientific™ Nalgene™ Rapid-Flow™ Sterile Disposable Bottle Top Filters with PES Membrane	NAME
0.2 µm Bottle Top Filter or equivalent	TYPE
Nalgene	BRAND
09-741-07	SKU
https://www.fishersci.com/shop/products/nalgene-rapid-flow-sterile-disposable-bottle-top-filters-pes-membrane/0974107	LINK
500 mL, 0.2 µm PES Bottle Top Filter	SPECIFICATIONS

28 Place a 0.45um HAWP filter onto the bottle top filter using sterile forceps



Equipment

HAWP MF-Millipore Membrane Filter, 0.45 µm pore size	NAME
Membrane filter	TYPE
Millipore	BRAND
HAWP04700	SKU
https://www.sigmaaldrich.com/catalog/product/mm/hawp04700?lang=en&region=US&gclid=CjwKCAjw8pH3BRAXEiwA1pvMsdoaQbbYstapLy8iGgQMuPbpUIubisFSK9v3zg7Ab-Uv1HEHZmOhSBoCPx8QAvD_BwE	LINK
0.45 um 47 mm	SPECIFICATIONS

29 Pipet the 2ml of Trichoderma/agro cell mixture onto 0.45um HAWP filter

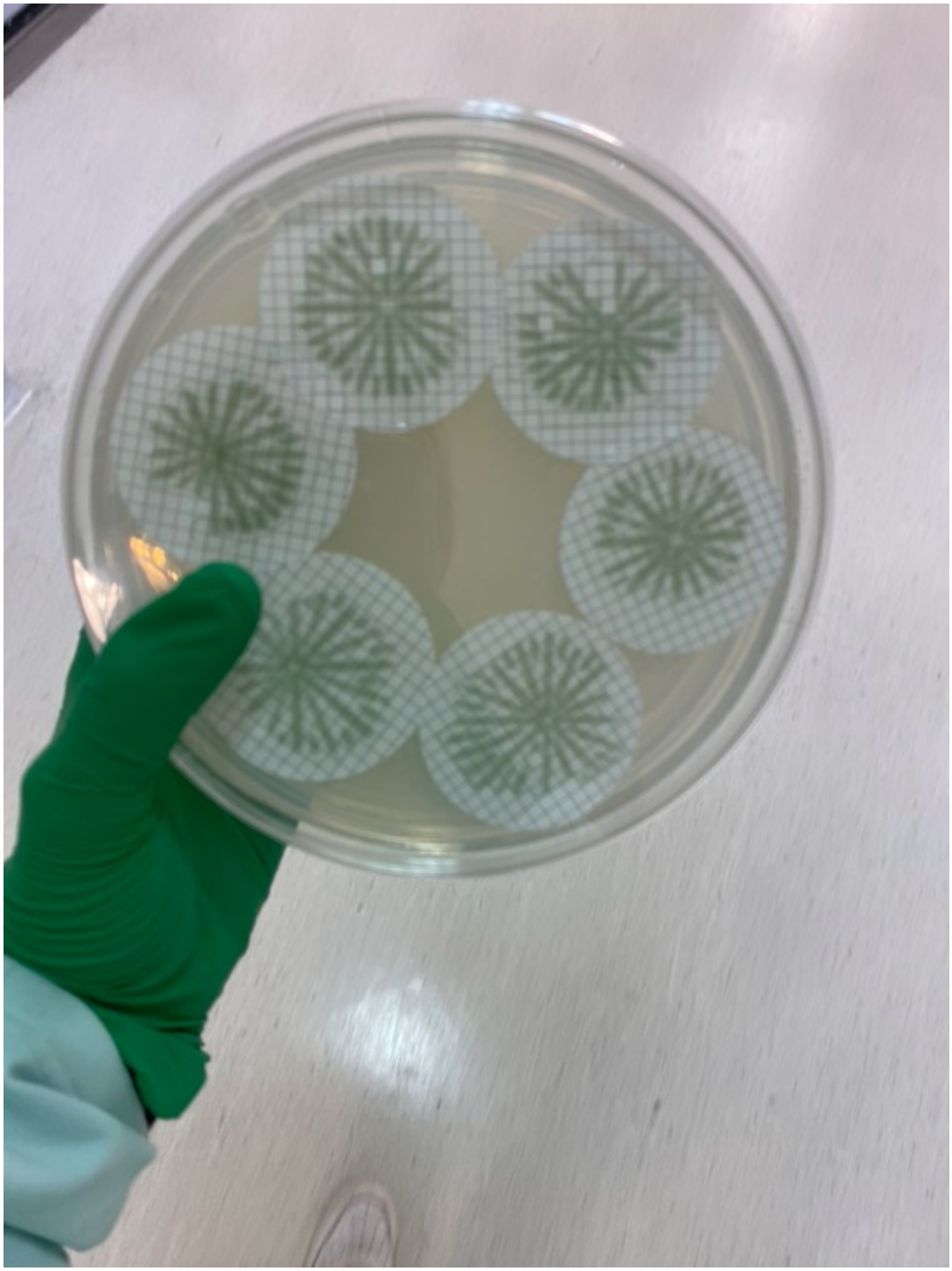
Note

Being careful to only get cell mixture on the HAWP filter.

30 Wait for the liquid induction media (ABI) to filter through the bottle top filter

31 Using sterile forceps, carefully transfer the filter with cells on it to the solid induction media petri dish, making sure to keep the side with the cells on it facing up!

32 Incubate induction media plates with filters at 20C in the dark for 6 days. 6d



Dried filters ready for incubation

32.1

Note

Materials for next part:
- 50 mL or 500 mL tubes with sterile beads and sterile water
- Layered PDA plates
- MM plates for quantifying
- Sterile water

Day 11

33 Label 2 falcon tubes for each HAWP filter

Note

25 - 50 filters fit into 500 mL tubes

34 Add 4ml sterile distilled H2O to a 50ml conical tube

Note

500 mL conical tube

35 Using sterile forceps, carefully transfer the HAWP filter with cells on it to the 50 mL conical

36

Note

Use beads and VORTEX until it has all fallen off

37 Vortex vigorously to get the rest of the cells off of the HAWP filter

38 Transfer 2ml of cell suspension to sterile 2 mL Eppendorf tube.

38.1 Spin at 10000 rpm, 00:05:00 . 5m

39 Pipet off supernatant into a waste bottle

40 Transfer the rest of the cell suspension to the 2 mL Eppendorf tube and Spin at 10000 rpm, 00:05:00 . 5m
Pipet off supernatant into a waste bottle

41 Resuspend cell mixture into 100 µL sterile distilled H2O

42 To spread in large plates (150 mm) PDA.

1.- PDA with hyg and Carb (4 ml Hyg and 1 ml Carb per L): dispense 50 ml.
2.- PDA with Carb (1 ml per Liter): dispense 13.5 ml.
3.- Conidia transformants: 1 ml
4.- Low-melt PDA with Carb (1 ml per L). 13.5 ml. 37 C. we can mix with conidia.
5.- PDA layer carb and hyg. 13.5 ml, kept at 55 C.

42.1 1 plate with filter goes to 3 plates of top/bottom media

42.2
Plate cells on

5mL Top: MM + Carb
25 mL bottom: MM + Carb + Hyg

You don’t need to dilute the control plate but for the experimental ones:

1. 100%:
2. **1:25:** **4 ul of cells in 96 ul of water**

> Note
>
> Make up media in half the amount of water, and autoclave water separately, then combine

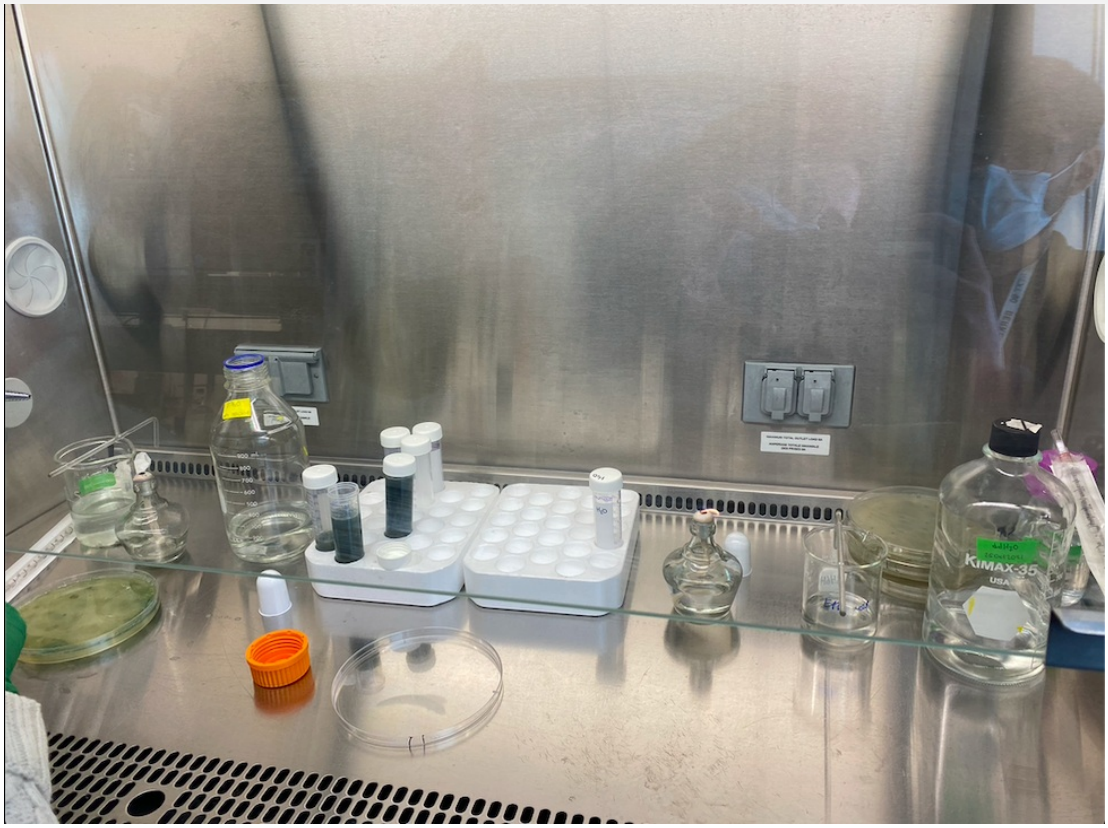
43 Place at 25C in the light for 7 days (cheking efficiency with Triton)

43.1

Final Harvest (Day 18)

20m

44



Washing of plates with water

> **Note**
>
> Materials needed:
>
> - 50% glycerol
> - Sterile water
> - Glass spreaders
> - 1 ml Pipettes
> - 25 ml pipettors and pipet tip
> - Tips 100-1000 ul
> - 15 mL Falcon tubes

Dispense 10 mL of sterile water onto the plate

45 Use the sterile glass spreader to detach as many as possible

46 Transfer into a 50 mL falcon tubes

46.1 Add another 10 mL of sterile water to plate and transfer into tube

47 Spin down 50 mL falcon tubes at 4,000 RPM for 20 minutes 20m

48 Resuspend pellets in 10 ml of 50% glycerol

48.1 Store in 1.5 mL tubes that are no more than half full, stored at an angle to avoid rupturing the tube.

> **Note**
>
> Where is it stored? What temperature?