DEC 27, 2022

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.n92ldpp5xl5b/v1

Protocol Citation: Sarah M Prostak, Edgar M Medina, Erik Kalinka, Lillian Fritz-Laylin 2022. Protocol 5: Agrobacterium-mediated transformation of Spizellomyces punctatus (Sp). **protocols.io** https://dx.doi.org/10.17504/protocols.io.n92ldpp5xl5b/v1

Protocol status: Working

Created: Nov 03, 2022

Last Modified: Dec 27, 2022

PROTOCOL integer ID: 72235

Keywords: Agrobacterium, Spizellomyces punctatus, transformation

Protocol 5: Agrobacterium-mediated transformation of Spizellomyces punctatus (Sp)

In 1 collection

Sarah M Prostak[1], Edgar M Medina[1], Erik Kalinka[1], Lillian Fritz-Laylin[1]

[1]University of Massachusetts at Amherst

fritzlaylinlab.umass

ABSTRACT

Once you have completed protocols 1-4, you are ready for transformation. This protocol is lengthy, requires accurate timing, and takes a minimum of 4 days to see results. Zoospores will need to be harvested from the plates prepared in Protocol 2; the process of harvesting is described below. Once Agro is at the appropriate OD, transformation is carried out by co-culturing Agro and Sp on induction media ("IM") to induce the expression of Agro's virulence genes and transformation of Sp cells. Co-culturing must be done on IM plates with premade depressions in the agar (see Protocol 4: Creating depressions in induction media plates).

ATTACHMENTS

Spizellomyces_transformation_steps.pdf

GUIDELINES

This section outlines the transformation process from growing Agro to the end of co-culturing. For harvesting the co-culture and selecting transformants, see *Protocol 6: Selecting for Spizellomyces punctatus transformants* and *Protocol 7: Picking colonies of transformed Spizellomyces punctatus (Sp)*. All steps of this protocol, except necessary centrifugation, must be carried out in a sterile environment, either in a laminar flow hood or in the sterile area around an open flame.

MATERIALS TEXT

Materials

Overnight liquid cultures of desired *Agrobacterium tumefaciens* strains (see Protocol 3: Growing liquid cultures of *Agrobacterium* prior to transformation day for transforming *Agro*)
Active plates of wild-type Spizellomyces punctatus, grown without antibiotics (see Protocol 2: Culturing Spizellomyces punctatus (Sp) prior to transformation day)
IM liquid (see recipe)
IM plates with depressions (see Protocol 4: Creating depressions in induction media plates)
DS solution (see recipe)
Culture tubes, sterile (such as
VWR® Culture Tubes Plastic with Dual-Position Caps **VWR Avantor Catalog #60818-703**
)
50 mL conical tubes, sterile (such as CellTreat
Centrifuge Tubes-Bag 50mL Centrifuge Tube - Bag Sterile **Cell Treat Scientific Products Catalog #229421**
)
1.5 mL centrifuge tubes, sterile (such as
Fisherbrand™ Low-Retention Microcentrifuge Tubes **Fisher Scientific Catalog #02-681-331**
)

Pipette aid
Serological pipettes
100-1,000 µL micropipette such as
Eppendorf Research Plus Single Channel pipette 100-1000 uL blue operating button for use wit 100 **pipette.com Catalog #3123000063 ES-1000**
20-200 µL micropipette such as
Eppendorf Research Plus single channel pipette 2-20 uL yellow operating button for use with 2 uL **pipette.com Catalog #3123000039**
Filter tips for the micropipettes, sterile such as
TIPONE® FILTER TIPS **USA Scientific Catalog #1122-1830**
M4 repeat pipettor, such as Repeater® M4 Starter Kit **Eppendorf Catalog #4982000322**
Combitips for the repeat pipettor, sterile such as
Combitips® advanced - Positive Displacement Pipette Tips **Eppendorf Catalog #0030089804**
or similar 40 µm mesh filters (
40 µm cell strainer **Fisher Scientific Catalog #22-363-547**
)
25 mm syringe filter holders (
Cole-Parmer Advantec 43303010 Polypropylene Filter Holder for 25 mm Membranes **Fisher Scientific Catalog #NC9972954**
), preloaded with Grade 1 Whatman paper (
Cytiva Whatman™ Qualitative Filter Paper: Grade 1 Circles **Fisher Scientific Catalog #09-927-223**
), sterile
Plastic storage containers, or equivalent (e.x. plastic shoe box)
Centrifuge capable of holding 50 mL conicals

Equipment

Eppendorf™ 5810R Centrifuge	NAME
Centrifuge	TYPE
Eppendorf	BRAND
02-262-8187	SKU
https://www.fishersci.com/shop/products/eppendorf-5810r-centrifuge-rotor-packages-16/022628187	LINK

Laminar flow hood and/or open flame, for maintaining sterility.
70% (v/v) ethanol for maintaining sterility (if using laminar flow hood)
Shaking incubator at 28 °C

BEFORE START INSTRUCTIONS

On transformation day, Agro must be diluted to an OD_{660} of 0.15 and then grown to an OD_{660} of 0.6. Under our laboratory conditions, this takes about 4-6 hours, but this time should be empirically tested for each lab before starting to ensure proper and repeatable results. Be sure to take this growth time into account when planning all steps on and prior to transformation day.

ATTACHMENTS

Spizellomyces_transformation_steps.pdf

Growing *Agro* to the appropriate OD_{660}

1 Dilute each overnight *Agro* culture to an OD_{660} = 0.15 using IM.

Note

In our experience, this works out to be 500 µL of overnight culture that has been spun for 4500 rcf, 00:05:00 and then resuspended into 4 mL of IM. Dilution conditions should be empirically tested for each laboratory.

2 Grow the *Agro* at 28 °C and shaking at 225 rpm until OD_{660} = 0.6.

Note

Under our laboratory conditions, this takes about 4-6 hours. Growth time to reach optimal OD_{660} should be empirically tested for each laboratory.

Harvesting Sp zoospores for transformation

1h

3 Flood all wild type *Sp* plates with 1 mL of DS or IM for 01:00:00.

Note

Do this about one hour before *Agro* has reached an OD_{660} of 0.6.
The solution used should have little to no effect on transformation success.

4 Harvest *Sp* zoospores by running 1 mL of fresh DS or IM along the agar of one plate, holding the plate at a 45 degree angle.

Note

Do this 00:20:00 before Agro has reached an OD_{660} of 0.6.

5 Take the liquid from the first plate and use it to harvest the zoospores on a second plate in the same manner.

6 Do this for all plates of *Sp* that were flooded in step 3.

Note

If the volume becomes too much to hold in the serological pipette, empty the contents into a 50 mL conical tube (or other appropriately sized tube), then use 1 mL of this zoospore suspension to continue harvesting the remaining plates.

7 Pool all zoospores into a 50 mL conical (or other appropriately sized tube).

8 Pass the zoospore suspension through a 40 µm mesh filter into a new 50 mL conical tube.

Note

This removes clumps of cells that could clog a syringe filter.

9 Use a 18 gauge needle and an appropriately sized syringe to take up all of the 40 µm-filtered zoospore suspension.

Note

Be very gentle, the needle may damage the cells.

Alternatively: Directly load a 25 mL syringe with the 40 µm-filtered zoospore suspension by using a p1000 set to 1000 µL and gently pushing the cells into the syringe through the tip.

10 Use a sterile 25 mm syringe filter preloaded with grade 1 Whatman paper to further filter the spores into a new 50 mL conical tube (or other appropriately sized tube).

Note

PRESS SLOWLY, or risk damaging spores. As a rule of thumb, do not filter more than 5 mL of zoospores for one syringe filter.

This suspension is now to be used in co-culturing and should have a concentration of zoospores between 1-10 x10^6 cells/mL.

4d 1h

Co-culturing Agro and Sp in varying ratios

11 Per each control and plasmid to be transformed, prepare and label four microcentrifuge tubes according to Figure 3.

12 Pipette the appropriate amounts of IM and the final solutions from protocols section "Growing Agro to the appropriate OD_{660}" and "Harvesting Sp zoospores for transformation" into the appropriate microcentrifuge tube in the order they appear in Figure 3.

Note

This will prevent cross contamination between strains.

Pipetting time can be sped up by using an M4 repeat pipettor (Eppendorf #4982000322) to dispense 50 µL volumes at a time, ensuring the proper amount is dispensed and that the liquid does not splash due to the force of dispensing. This is best done by dispensing along the tube walls just above the water line.

13 Gently pipette to mix the contents of the microfuge tube.

14 Transfer all 200 µL of an *Agro-Sp* co-culture to one of the premade depressions on a room temperature IM plate.

Note

All 4 co-culture tubes for one plasmid can be placed onto one IM plate. One tube per quadrant. Start on the left side and then go to the right side to lower the risk of contamination (or the opposite for left handed people).

15 GENTLY slide the IM plates to a place where they will not be disturbed.

Note

Avoid picking up the plates, as this may result in the pools of co-culture to combine, disturbing the ratios established and potentially lowering transformation efficiency or success.

16 Leave the plates to dry at Room temperature for 12-24 hours.

17 Seal plates with parafilm, invert, and grow in a closed chamber (such as a plastic storage container) at Room temperature for 4 days.

Note

Evidence of growth appears as an opaque, roughly circular area on the agar. If not all of the quadrants have obvious growth, that is okay. As long as there is at least one quadrant that shows growth, *Protocol 6: Selecting for Spizellomyces punctatus transformants* can be done, though success at gaining selectable transformants may be lower.