00:00– Welcome to the instructional video of the protocol for *Agrobacterium*-mediated transformation of the chytrid fungus *Spizellomyces punctatus*. This process involves transforming *Agrobacterium*, inducing its virulence genes, and co-culturing with *Spizellomyces* cells to facilitate plasmid DNA incorporation into the *Spizellomyces* genome.

00:25– This protocol involves many steps that require adequate planning. Here is an example of the timing of the entire process, all of which will be described in further detail later in this video.

00:35– Briefly*, Agrobacterium* electroporation should occur at least 4 days prior to your intended transformation day. The goal is to have colonies of your transformed *Agrobacterium* or lawns of individual colonies ready for transformation day. It is best to have active plates that have been growing at 28degC for at least 24 hours prior to transformation day. Ensure you keep 25% glycerol stocks of viable colonies at -80degC to avoid needing to redo electroporation should *Spizellomyces* transformation be unsuccessful.

1:07– Prior to transformation day, ensure all materials mentioned later in this video are prepared and properly sterilized.

1:14– At least 36 hours before your intended transformation time, plate your *Spizellomyces* cultures, and then transfer these cells to new plates around 18 hours before your intended transformation time.

1:25– About 12 hours before transformation, begin growing transformed *Agrobacterium* in liquid media.

1:33– You will need to return 12-24 hours after transformation day to seal and invert the co-culture plates.

1:40– 4 days after transformation day, the co-culture plates should be harvested and plated onto selection media.

1:47– 4-6 days after plating onto selection, if transformation was successful, individual *Spizellomyces* colonies will appear and can be picked for amplification and further downstream analysis.

<u>MATERIALS:</u>

2:02– To complete this protocol, you will need to prepare the following sterile materials:

- 2:06– Sterile 25mm syringe filters preloaded with Grade 1 Whatman paper, 20 mL syringes
- 2:14– 18G needles
- 2:19– Razor blades and long forceps
- 2:23– Sterile MilliQ water, or equivalent

- 2:27– Dilute salts solution, made from the given stock solutions
- 2:32– K1 agar plates, with and without selection antibiotics for your plasmid
- 2:38– LB broth and agar plates with and without selection antibiotics for your plasmid
- 2:46– A plate of wild-type Agrobacterium that has been growing at 28degC for at least 24 hours
- 2:52– Induction Media liquid and agar plates (“IM plates”)
- 3:01– 2.5x Minimal salts solution for the induction media
- 3:06– MES with Acetosyrigone for the induction media

IM DIVETS:

3:11– You will also need to create small depressions in IM plates to contain the mixture of chytrid and agrobacterium while it is absorbed by the IM plate.

3:20– To do this, first divide the bottom of an IM plate into four equal sections. In each quadrant, draw a circle to indicate where each depression will be. We will use a glass tube to make the depressions in the agar plates, this tube will be cleaned between plates in 70% ethanol.

3:48– Add enough ethanol to a 50 mL conical to cover only the first inch of the glass tube.

4:02– Next, carefully burn off the ethanol. Dip the tube back in the ethanol and repeat this process 3 times to ensure full sterilization and to warm the glass to better sculpt the agar.

4:16– Gently press the sterile end of the glass tube into the center of one of the circles drawn on the bottom of an IM plate. Move the tube in a circular motion, being careful not to rip the agar or make the circle larger than the quadrant. Aim for depressions of approximately 1 inch diameter. Do not worry if they are not perfectly circular. As long as they hold the liquid and you can move the plates without the liquid overflowing you should be ok. Practice this step until you don't scar the media surface. Repeat this for each quadrant. Prepare one full plate per plasmid to be transformed.

AGRO EPOR (at least 1 week prior to transformation):

5:15– The first step of the transformation protocol is electroporation of *Agrobacterium* with your plasmids of interest.

5:21– This process requires everything to be at 4 degrees Celsius, thus setting up an ice box with the given materials like the example here and ensuring the centrifuge you are using is pre-chilled prior to starting is important.

5:36– Here, we start with a lawn of *Agrobacterium* without any plasmid on a regular LB plate. To make this lawn, start with a fresh streak from frozen stock*. Agrobacterium* should be grown, sealed and inverted at 28degC for 2-3 days until colonies are visible. This plate can be stored at 4degC for about one month and used as a stock from which to streak more plates if necessary.

Pick a colony and resuspend in 1mL of water. Spread onto a non selective plate and incubate at 28C until a lawn is formed. This may take up to 48 hours.

6:24– First, harvest the lawn of *Agrobacterium* without any plasmid by taking 1 mL of cold, sterile water and pipetting it over the plate at least 3 times.

6:55– Ensure you are holding the plate at an angle to collect the bacteria at the bottom, gently scraping along the agar if needed.

7:37– Pipette the harvested *Agro* into an empty, pre-chilled 1.5mL microcentrifuge tube, and place it back on ice.

7:53– Spin down the harvested *Agro* for five minutes at 4000 rcf using a rotor that has been pre-chilled to 4degC.

8:04– After this, you should have a pellet of bacteria. Discard the supernatant and gently resuspend the pellet with 1 mL of cold, sterile water. Do not vortex the cells.

9:08– Spin down the bacteria as before, then repeat this washing a total of 3 times before moving on to the next step.

9:29– Once you've washed the *Agro* 3x with water, resuspend the pellet with 300uL of sterile, cold 10% glycerol. You will need roughly 50 uL of bacteria per plasmid to be transformed, so be sure to harvest enough *Agro* to account for your needs, keeping in mind that one plate is enough for 5-10 electroporations. Place the resuspended *Agro* back on ice.

9:57– Next, aliquot 50 uL of *Agro* into new, pre-chilled 0.5mL microcentrifuge tubes. One per plasmid to be transformed.

10:59– Add 1uL of sterile water to one aliquot of *Agro* as a control, and then add 1uL of purified plasmid to its own aliquot of bacteria. Ensure proper mixing by gently tapping the tube. Aim for at least 100 ng/uL of DNA from a standard miniprep.

11:57– Next, transfer each *Agro* and plasmid mixture to its own pre-chilled electroporation cuvette. Here, we use 2mm cuvettes. Place the cuvettes back on ice.

12:40– Then, for each plasmid and control, prepare the recovery media by adding 150uL of SOC medium to fresh culture tubes. Do this with enough time in advance so that the SOC reaches room temperature by the time you go to electroporate, an estimated 30-40min..

13:29– Now it's time for electroporation. To make this process as efficient as possible, you will need your culture tubes with SOC medium, your ice box with the electroporation cuvettes, a flame source, a p200 micropipette, and kim wipes by your electroporator. Please use an exponential decay electroporator.

13:52– The settings for the electroporator are as follows: 2400 Volts, 25 microFarad, and 200 ohms.

14:04– Make sure to dry off your cuvette so there is no water present. Failure to do so may lead to current arcing and failed electroporation. Place the cuvette into the port and turn on your flame prior to delivering the charge. Deliver the charge and meanwhile remove some SOC medium from the appropriate test tube. The best way to do this is to set your p200 to 150 uL, but not take up the full 150,

14:41– Then gently dispense the SOC medium into the cuvette from the port and take up all the remaining liquid from the cuvette. Pipette this back into the test tube you originally took from. Do this for each plasmid to be transformed.

15:02– Place the tubes to shake at 225 rpm at 28 deg C. for 4 hours. During this time, pre-warm the correct amount LB plates with or without selection antibiotic to 28 deg C.

15:21– Once the 4 hours is done, remove the tubes and plates from the incubator.

15:35– Place between 4 and 6 sterile glass beads onto each LB plate.

15:49– Then, pipette 10uL of the recovered Agro onto the appropriate plate. To help distribute this small volume, add 40uL of water into the plate and then add 10uL of the culture to the water puddle. The transformation efficiency of Agro is high, do not plate more than 10uL or you risk overgrowth and lack of pickable colonies.

16:17– Gently shake the plate to spread the bacteria with the beads. Once all liquid is absorbed, seal, invert, and place the plates back into the incubator at 28 deg C. 4 days later, colonies should appear on the plates.

2 DAYS B4 TRANSFORMATION:

16:32– Two days before you plan on transforming *Spizellomyces*, you need to prepare the cultures so you have an adequate pool of zoospores for the protocol.

16:41– To do this, around 40 hours before your planned transformation, split your *Spizellomyces* cultures onto K1 plates without any antibiotics of any kind by flooding active culture plates with 1-2mL of DS and filtering zoospores using a 25mm syringe filter preloaded with Grade 1 Whattman paper. You can combine all the harvested zoospores into a conical if need be. A good rule of thumb is that one plate is enough to seed 2-3 new plates.

17:14– Then, inoculate one new plate per plasmid to be transformed with 1mL of zoospores and spread the cells along the agar by gently shaking. Again, it is important to not grow Spizellomyces on any antibiotics in the few days prior to transformation day, as this may result in lowered transformation efficiency.

DAY B4 TRANSFORMATION:

17:48– Several steps must be taken late on the eve of the transformation day

17:54– First, prepare the cultures of transformed *Agro* for use on transformation day. Here, we have the results of *Agro* transformation after 4 days of growth. Pick several colonies into their own test tube containing 5mL of LB media with appropriate selection antibiotics. This should not be done from frozen 25% glycerol stocks of previously picked colonies. If you must take from a frozen stock, streak the cells onto appropriate LB plates and let them grow for 2-4 days before transferring cells to liquid media.

18:29– Place the test tubes to shake at 225 rpm at 28 deg C for 12 hours or overnight.

18:40– Meanwhile, split your *Spizellomyces* cultures again the same way as you did yesterday to ensure a more synchronized population of zoospores. Do this around 16 hours prior to your planned transformation. This will help increase chances of getting transformants.

TRANSFORMATION DAY:

18:59– By this point, you should have prepared all the needed materials to complete the Agrobacterium-mediated transformation of *Spizellomyces* and it is now transformation day!

19:17– First, aliquot 4mL of sterile Induction Media into one culture tube for each plasmid to be transformed.

19:29– Then, remove the 12 hour or overnight cultures of *Agro* from the incubator.

19:35– Using Induction Media, dilute the Agro culture to 0.15 OD660nm. Under our laboratory conditions, this works out to be 500uL of overnight culture resuspended into 4mL of IM.

19:50– To do this, we aliquot 500uL of culture into 1.5 mL microcentrifuge tubes and complete the following steps.

20:06– Pellet the bacteria by spinning at 4500 rcf for 5 minutes.

20:18– Take the tubes out and ensure the presence of a pellet.

20:27– Remove the supernatant.

20:41– From the appropriate pre-filled culture tube, pipette 500uL of Induction Media and use this to resuspend the pellet of *Agro*. Return the resuspended pellet to the tube where the induction media came from. In our experience, this gives us an OD660nm of 0.15, but this should be empirically determined for each lab.

21:02– Place the culture tubes to shake at 225rpm at 28 deg C until the OD660nm reaches about 0.6. Under our laboratory conditions, this will take about 4 hours, but this should be empirically determined for each lab.

21:17– MEANWHILE: set up four 1.5 mL centrifuge tubes per plasmid to be transformed. These tubes will have varying ratios of *Spizellomyces* and *Agro* culture to increase the chances of recovering transformants. Pre-fill the tubes with the given amounts of Induction Media to reduce pipetting time later in the procedure.

21:45– ONE HOUR B4 AGRO IS READY: Flood your plates of *Spizellomyces* with 700 uL of sterile dilute salts solution to begin collecting zoospores. Additionally, remove the proper amount of IM plates with divots from the fridge so they can reach room temperature.

22:15– TWENTY MIN B4 AGRO IS READY, harvest the *Spizellomyces* zoospores.

22:21– To do this, take 1mL of DS or IM solution and pipette along the agar at an angle several times. Take the resuspended spores from the first plate and use this liquid to wash a second plate. Continue this process until you have pooled all zoospores into a tube of the appropriate size for the volume you need.

23:02– Here, we use a 50mL conical. To further remove clumps and older sporangia that can clog a syringe filter, you can pass this suspension of cells through a 40um mesh filter.

23:23– Next, use an 18 gauge needle and an appropriately sized syringe to take up all the unfiltered spores from the pool of spores you just collected. Using the needle can damage spores, but you can alternatively load the syringe directly using a p1000 set to 1000uL to reduce drag on the spores.

24:03– Remove the 18 gauge needle, if you used one, and use a sterile 25mm syringe filter pre-loaded with whatman grade 1 filter paper to filter the zoospores into a new tube.

24:29– Ensure the filter system is locked into place before pushing liquid through it.

24:39– FILTER BY PRESSING SLOWLY. You will need about 300uL of zoospores per plasmid to be transformed. If you have more volume than you need, consider concentrating the zoospores by centrifuging at 2500rcf for 5 minutes and resuspending the pellet in induction media.

24:59– CO-CULTURING SP AND AGRO: Now, you should have all the materials necessary to co-culture *Spizellomyces* and *Agro*.

24:06– First, dispense the proper amount of filtered *Spizellomyces* zoospores into the microcentrifuge tubes you prepared earlier. Here, we use an M4 repeat pipettor, set to dispense 50uL with each expulsion. Dispense 2-3 times back into the stock of cells before pipetting into the microcentrifuge tubes to ensure accuracy of the volume delivered. This method makes it easier and faster to pipette the 50 and 100uL of cells into the appropriate tubes.

25:36– Next, remove the *Agro* in IM from the incubator, gently mix using a pipette and ensure that the OD660nm is ~0.6.

25:47– Then, dispense the proper volume of the *Agro* culture into the designated microcentrifuge tubes for the plasmid of interest. To speed up the process, you can have two p200 pipettes with one set to 50uL and another set to 100uL.

26:35– Finally, use one IM plate with premade divots for all four co-culture tubes for one plasmid of interest. Mix gently by pipetting up and down a couple of times, then pipette 200uL of each

co-culture microcentrifuge tube onto the middle of the divot starting with the left side and going across to avoid cross-contamination.

27:00– Gently slide the plates to a safe location where they will not be disturbed. Leave the plates to dry for 12-24 hours before sealing with parafilm and inverting them. Incubate inverted in a sealed container at room temperature. Evidence of growth should appear after about 4 days from the beginning of co-culturing.

27:31– SELECTION OF TRANSFORMANTS: Once *Spizellomyces* and *Agro* have been co-cultured for 4 days, you can now begin the process of selecting transformants.

27:41– First, prepare one 50mL conical tube with 30mL of DS for each plasmid transformed.

27:56– Your co-culture plates should look something like this.

28:01– Rehydrate the quadrants of each co-culture plate by using 1mL of DS from the corresponding conicals you just prepared. Dispense the 1mL among the individual quadrants.

28:40– After a few minutes, it's time to scrape the plates to harvest the cells on them. Use one razor blade for each IM plate you have. Ensure your forceps are long enough to prevent injury.

28:54– First, flame both sides of a single edged razor blade to sterilize it. The metal will oxidize, turning an array of blues and purples, this is what you want. Then, tilt the IM plate and gently scrape its surface, collecting the cells in the DS pooled at the bottom. Rotate the plate as needed to scrape as much of the growth off as possible.

29:41– A 1000uL pipette tip can be used to scrape off any growth that remains on the razor blade. Sterilize the razor blade with the flame again before continuing. After you have thoroughly sterilized the blade, you should discard it into a sharps container.

30:01– Using the same pipette tip you used to remove excess growth from the razor blade, resuspend the cells into the appropriate conical you prepared earlier. Then, take up the remaining liquid from the scraped IM plate.

30:14– Dispense this CAREFULLY into the appropriate conical. There will be a density gradient between the scraped cells and the DS, you want to maintain this gradient at this point. From the top of the gradient, take up another 1mL of DS.

30:28– Wash the surface of the scraped IM plate with the DS several times, and then return as much of the liquid as you can to the conical it came from.

31:00– Your scraped IM plate should look roughly like this, where the opaque areas of growth are no longer visible.

31:09– Next, invert the conicals containing the harvested co-cultures several times and then vortex for about 1-2 seconds to ensure proper resuspension and to dislodge any *Agro* still stuck to *Spizellomyces*.

31:36– Now, centrifuge to pellet only the *Spizellomyces.* Spin for 10 minutes at 1000 rcf at room temperature.

31:52– Once done, you should have a pinkish pellet of chytrid cells. This pellet is fragile and should be handled with care.

32:10– Next, orient your tube so you gently pour off the supernatant with the high side of the pellet facing up. This orientation lessens the loss of chytrid cells while pouring.

32:25– Resuspend the pellet with 500 uL of fresh DS.

33:06– Prepare K1 selection plates with 4-5 sterile glass beads. Here we use Hygromycin B and also Carbenicilin and Tetracycline to kill any remaining *Agrobacterium*. Ensure proper control plates are being used in addition to this.

33:25– Take 200uL of the resuspended *Spizellomyces* and dispense it onto the K1 plate. Gently shake the plate to spread the cells.

34:09– Once the liquid has dried, remove the beads from the plate.

34:28– Invert, seal, and incubate the plates at room temperature. Here, we add a little water to a beaker in a chamber to ensure humidity so the plates do not dry out. This is recommended practice. After 4 to 6 days, colonies should begin to appear if transformation was successful.

<u>PICKING COLONIES:</u>

34:50– If colonies are present on your plates they should be a little rough, white, and opaque.

35:00– Start by aliquoting 50uL of DS for each colony you are going to pick.

35:46– Then, use a sterile 18G needle to pick a colony. You can use a new needle for each colony or you can sterilize your needle using the flame between each colony you pick to prevent cross contamination.

36:17– To begin picking a colony, gently lift it from the agar using the needle, being careful not to poke deeply into the agar.

36:37– Resuspend the colony in the 50uL of DS you prepared earlier. You may need to swirl the needle to dislodge the colony into the liquid. Sterilize your needle using the flame between each colony you pick to prevent cross contamination.

36:58– Then, you can plate up to 4 colonies from the same plasmid transformation on a single K1 plus selection plate. Simply divide the plate using a marker on the bottom. No need to make divets in these plates.

37:14– Using a p200 set to 50uL, swirl and pipette gently but with enough force to break up the pellet. Then, pipette the 50uL of resuspended cells onto the center of the appropriate section of the plate. After 2-3 days, rehydrate these sections with 100uL of DS each and plate each clone

on its own K1 selection plate. Repeat the culturing until there is enough *Spizellomyces* to cryopreserve. This can take up to a week.