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Luciferase Activity Assay for Neurospora crassa

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Circadian Clock Control ...

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Abstract

Here, we describe an assay for screening for luciferase activity and performing a circadian luciferase rhythm assay in the filamentous fungus *Neurospora crassa*.

Troubleshooting

Luciferase Activity assay for *Neurospora crassa*

A. Screening for Luciferase Activity-Positive Transformants

Grow strains on solid Vogel's media with 2% glucose (V2G) with appropriate antibiotic. These include strains to be tested and positive and negative controls for luciferase activity. When enough conidia have formed, prepare the following:

1. UV-treat 96-well EnVision Opti-Plate for 15 mins. If reusing a plate, treat the plate first with 95% ethanol, dry and UV treat.
2. Prepare luciferase assay media enough for the number of plates needed.Using the multichannel pipettor, transfer media into plates.
   1 well = 150 uL media 1 plate = 96 wells = 14.4 mL

	8 mL 1x Vogel's	20 uL 10 mM luciferin
	15 mL 1x Vogel's	37.5 uL 10 mM luciferin
	30 mL 1x Vogel's	75 uL 10 mM luciferin

1. Using a sterile stick, inoculate conidia from each strain into each well. Do not forget to run positive and negative controls for luciferase activity.
2. Cover plate with Breathe-Easy clear cover.
3. Place the plate inside the EnVision and run the assay to check/measure for luciferase activity. Alternatively, leave the plate for 5 hrs maximum at 30^{o}C, the repeat the measurement.

B. 7-Day Rhythm Check

Preparing the plates

Grow strains on slants to generate sufficient conidia (typically 7 days at 30^{o}C). Suspend conidia in ~1000 uL sterile H_2O when ready to inoculate on 96-well plate.

1. UV-treat 96-well EnVision Opti-Plate for 15 mins. If reusing a plate, treat the plate first with 95% ethanol, dry and UV treat. Ideally, you want fresh plates for 7-day runs.
2. Prepare the luciferase assay media enough for the number of plates needed in an Erlenmeyer flask. Leave the stirrer during autoclaving.
   1 well = 150 uL media 1 plate = 96 wells = 14.4 mL

	Component	50mL	100mL	150mL	200mL	Final Concentration
	50x Vogels	1mL	2mL	3mL	4mL	1x
	0.1 mg/ml Biotin	25µL	50µL	75µL	100µL	0.05mg/µL
	Glucose	0.015g	0.03g	0.045g	0.06g	0.03%
	Arginine	0.025g	0.05g	0.075g	0.1g	0.05%
	Quinic Acid	0.96g	1.92g	2.88g	3.84g	1.9%
	pH to 6.0					
	BactoAgar	0.9g	1.8g	2.7g	3.6g	1.5%
	Autoclave					

	10mM Luciferin	125µL	250µL	375µL	500µL	25µM

1. In the hood: Pour some media into a reusable/autoclvable reservoir and, using a multichannel pipettor, plate 150µL into each well. (You can do this using a single pipette, but it's easier and more consistent with the multichannel pipettor.)

a.Keep the flask of media in 65ºC between pours. Use a new reservoir every time you refill.

b.To avoid pipetting bubbles, I usually "prime" the tips with the media a few times (pipette up and down). If you do pipette bubbles, simply pop them with a clean tip after you use up your current reservoir of media and before you fill a new reservoir.

c.The media will start to solidify in ~10-15min, so pipette quickly.

2. Once all of your plates are filled with media, cover them with plastic wrap and foil. Dry plates overnight in the hood with the lights off or foil cover on. Drying for longer than 24hrs is not recommended.

a.Note: The foil over your plates should protect it from any light, including UV. But ideally, you should try to avoid any light, if possible.

Counting cells for inoculation

It's very important to have a controlled cell count between strains because different concentrations of cells from the same strain produce different phases.

1. Turn on the spectrophotometer to let it warm up.
2. Make a conidial suspension in ~1.5mLs of sterile dH_2O. (You could also make your suspension in larger amounts of water; I just use the Eppendorf tubes for everything.)
3. Let your suspension settle for 1-3min, to let most of the hyphae/agar/clumps sink to the bottom. (This is especially useful for strains with clumping conidia like Δ*csp-1*.)
4. Pipette 1mL of your settled suspension into a spectrophotometer cuvette. Avoid the settled "pellet" at the bottom.
5. Set wavelength to 420nm on the spectrophotometer. Blank with 1mL of water (in a different cuvette). Measure sample.

a.OD_{420} = 1 = 5×10^6conidia/mL

b. Transfer sample from cuvette to a clean Eppendorf tube. Dilute sample to **1×10^5conidia/mL**. (An example for dilution is below.)

c.More conidia (1×10^6) results in rhythms that don't persist the entire assay length (~1 week) and less conidia (1×10^4) results in more variation between technical replicates.

Example: OD_{420} = 0.765

Calculation Cell count = $0.765 * 5\times10^6$ =3.825×10^6 conidia/mL

For 500µL of 1×10^5: (500 * 1×10^5 conidia/mL)/ 3.825×10^6 conidia/mL = 13.07 µL of conidial suspension + 486.93µL dH_2O

Inoculating Plate

1. Generally, the plate setup is one strain per row. This way, there are 12 technical replicates per strain. (So, the max number of strains on one plate is 8.)

a.If you have less than 8 strains on a plate, spread out the strains, to avoid crosstalk. (Crosstalk between wells isn't too much of a problem; I have, however, seen background levels show a rhythm.)

2. Vortex your suspension dilution thoroughly. Pipette 5µL into each well.

a.I will usually cover all other rows on the plate with foil, just to avoid contamination between strains.

3. Once the plate is filled, cover the plate with an BreatheEasy membrane. Evenly apply pressure with the roller. Keep top layer of plastic on.

a.If you have multiple plates, be sure to mark them somehow!

4. Incubate plate in 30ºC for ~24hrs. Don't put your plates on top of a light source, since this will cause condensation on the top film. Condensation will ruin your rhythmic data. If you notice condensation building up on your plates after a few hours, simply move them to be directly *under* a light source. They should dry up.

Using EnVison

1. After incubation, remove the top layer of plastic off the film. Double check that the Percival holding the EnVison is at DD, 25ºC.
2. Cut the film around the edges of the plate, so there is no overhang of film. Sometimes the film over the edge causes the plates to stick together in the stacker.
3. Stack the plates, with the first plate you want read on the very bottom (and in ascending order from there). Place them in the stacker in the envision. Tape the Percival door shut, to remind everyone to not open!
4. Start-up EnVison software and use the "Start Wizard" to start a protocol.

a.We use the protocol with 99 assay repeats and 90min intervals between counts.