**ApoBb.1 Nanoluc Assay (2020-11-15\_ MRH)**

**Materials:**

* unskirted PCR plate
* 1x Tricaine
* Microseal B clear plastic cover for unskirted PCR plate
* Black Perkin Elmer plate

**Solutions:**

* **Homogenization Buffer:** 
  + Is made at x2 because we use 50.0uL of this and add 50.0uL of EM with the larvae, diluting it to x1.
  + can be stored in -20C for up to 1 week, freeze thaw twice.
  + Use a 15mL conical tube or a 5mL epi tube for mixing
  + Mix the following for one 96-well plate:

|  |  |
| --- | --- |
|  | X2 |
| 0.5M EGTA | 400.0uL |
| Sucrose | 1.0g |
| DI H20 | 4.1mL |
| Total | 5.0mL |
| Dissolve one complete mini EDTA-free protease inhibitor tables (lives in deli case) | 1 tablet |

* Or make a larger volume in a 50mL conical tube using the EDTA-free protease inhibitor tablet, NOT the mini tablet

|  |  |
| --- | --- |
|  | X2 |
| 0.5M EGTA | 2.0mL |
| Sucrose | 5g |
| DI H20 | 20.5mL |
| Total | 25.0mL |
| Dissolve one complete EDTA-free protease inhibitor tables (lives in deli case) | 1 tablet |

* **Nanoluc Reaction Buffer:**
  + make fresh each time, do not reuse left over
  + NanoGlo Buffer & Substrate live in Freezer #3

|  |  |  |
| --- | --- | --- |
|  | 1x (1 well) | 1x for 96-well plate |
| 1x PBS | 30.0uL | 3.3 mL |
| NanoGlo Buffer | 10.0uL | 1.1 mL |
| Substrate | 0.2uL | 22.0 uL |
| Total | 40.2uL | 4.422 mL |

* **DNA Lysis Buffer:**
* Can be stored at room temperature for long time

|  |  |
| --- | --- |
| 100mM NaOH | 0.2g NaOH dissolved into 50mL Milli-Q H2O |
| 100mM Tris | 0.788g Tris dissolved into 50mL Milli-Q H2O |

* **Native Gel:**
* Mix the following in a 50mL conical tube to make 4 gels

|  |  |  |  |  |
| --- | --- | --- | --- | --- |
|  | **4x gels** | **3x gels** | **2x gels** | **1x gel** |
| 5x TBE | 6.4mL | 4.8 mL | 3.2 mL | 1.6 mL |
| Milli-q H20 | 22.9mL | 17.1 mL | 11.4 mL | 5.7 mL |
| 40% Polyacrylamide:Bis-Acrylamide 19:1 (lives in deli case) | 2.4mL | 1.8 mL | 1.2 mL | 0.6 mL |
| \*\*de-gas\*\* |  |  |  |  |
| 10% APS in Milli-q H20 | 250.0 uL | 187.5 uL | 125.0 uL | 62.5 uL |
| TEMED (lives in flammable cabinet) | 20.0 uL | 15.0 uL | 10.0 uL | 5.0 uL |

* de-gas this mixture for 30min
* Prepare 10% APS by added 0.05g APS into 500uL milli-q water, and add desired amount
* Add desired amount of TEMED (lives in flammable cabinet)
* Mix and cast gels
* Add combs last
* Let sit overnight in walk-in
* Gels are good for about one week, kept at 4C and moist
* **Gel Imaging Solution:**
* 1mL 1x TBE + 2.0uL substrate (yellow cap or purple cap)/gel
* **5x TBE**

|  |  |  |
| --- | --- | --- |
| Tris Base | 54g | 5.4 g |
| Boric Acid | 27.5g | 2.75 g |
| 0.5M EDTA pH8.0 | 20mL | 2.0 mL |
| Total | Up to 1L with Milli-Q H20 | Up to 100mL with Milli-q H20 |

* Filter after mixing
* Store at room temperature
* **0.5M EGTA pH 8.0**
* store at room temperature

|  |  |
| --- | --- |
| EGTA disodium salt dihydrate | 18.61g |
| Milli-q H20 | 80.0mL |
| Total | 100mL (after adjusting pH) |

\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_\_

**Procedure Steps:**

**\*Ice down sonication horn, step 2a-d, before arraying larvae in plate for sonication\***

1. **Array larvae into an unskirted PCR plate**
   1. If needed dechorionate the embryos with #5 forceps before plating
   2. Pipette 50.0uL of homogenization buffer/well in an unskirted PCR plate
   3. Anesthetize with 1x Tricaine (MESOB)
   4. Cut the tip off a p200 tip
   5. Set pipette to 50uL
   6. Pipette individual larvae in 50uL of EM into the unskirted PCR plate, make sure the larvae are at the bottom of the plate
   7. Cover plate with a Microseal B clear plastic cover
2. **Sonication**
   1. Sonicator is in room 218A
   2. Prepare sonicator by taking both drain tubes and clamping them up with the large white tube clamps
   3. Add 3-4 hands full of ice to the cup horn, then add DI water to the 17mm mark using the ruler that lives near the sonicator (sonicator works best when cold)
   4. While the sonicator is cooling plate out larvae for sonication
   5. Take covered plate to sonicator on ice
   6. Put plate into sonicator cup horn, close door, put on ear protection
   7. Turn on sonicator (blue/silver box) back right
   8. Question- Using micro tip? NO
   9. Select the second yellow icon
   10. Select modify program, the read out on the screen says “Amplitude 100\_Process Time 30sec\_Pulse-ON 2sec\_Pulse-OFF 1 sec
   11. Select Run
   12. Select Start
   13. After 4 pulses select pause and check to make sure the larvae are getting sonicated and rotate the plate ¼ turn when you put it back in for the remaining pulses, this step can be repeated as needed
   14. After sonication turn off machine, put plate on ice, unclamp drain tubes and put into drain bottle

**3. DNA Extraction**

1. In a new non-skirted PCR plate pipette in 10.0uL 50mM NaOH/well
2. Add 10.0uL homogenized samples/well
3. Heat at 98C for 15min
4. Cool to room temperature
5. Add 1/10th volume (2.0uL) 1M Tris pH 8.0
6. Use 2-5uL per PCR reaction

**4. Nanoluc Plate Reader**

1. Prepare nanoluc reaction buffer
2. Pipette 40.0uL nanoluc reaction buffer into the wells of a black perkin elmer plate
3. Add 40.0uL of homogenized samples
4. Take plate to room 378
5. Open Softmax Pro Software (icon is a magnifying glass)
6. Use buttons on machine to set temp to 25C
7. Use purple metal ring when putting plate into machine
8. Select protocol-> Basic -> endpoint
9. Double click on the grey box on the right side of screen
10. Select Lumin. Reading 500ms
11. UNCLICK Read from bottom box
12. Select OK, select Read
13. After reading highlite the whole plate -> edit -> copy plate data -> paste into excel sheet and save on USB drive
14. Close software

**5. Analyze plate reader data**

1. open excel sheet with data
2. highlight data, click Conditional Formatting and select green to red
3. 1.0E+05 or lower means little to no lipoprotein; 2-4E+06 is medium; 4-7E+06 is high but keep in mind too high of a number can mean a dead fish.
4. Take the average, stdev, and cov
5. You will want to run three samples closest to the average on the native gel.

**6. Make 3.0% Native Gels (32mL makes 4 gels)**

1. Assemble gel casting set-up
2. Jay has glass with RainX on his bench, non-rainx glass is next to sink
3. Use 1.0mm glass, clean with soapy water and rinse well with tap water
4. Mix the following in a 50mL falcon tube to make 4 gels

X4 gels

5x TBE 6.4mL

MilliQ H20 22.9mL

Polyacrylamide:Bis-Acrylamide (19:1) 2.4mL

1. De-gas pouring mixture into E. flask, connect the grey connection to tubing, apply green stopper, turn on vacuum for 30min
2. While degasing prepare 10% APS by weighing 0.05g APS into 500.0uL milli-q water
3. After degas pour back into falcon tube, rinse e flask with tap water
4. Add 250.0uL 10% APS and 20.0uL TEMED, mix briefly and cast gels
5. About 4mL per gel, add comb last, let polymerize overnight in the walk-in

**7. Running Native Gel**

1. Run in cold room, have 1x TBE prechilled in cold room
2. Put gels into gel case with small glass facing you, if only running one gel use gel dam for other side of gel case
3. Remove combs
4. Put into gel box and add fresh 1x TBE to center of gel case to the top
5. Use a p1000 and ‘wash out’ each well by pipetting some fresh 1x TBE just added into each well
6. Run empty gels at 50V for 30min to acclimate the gels, in the mean time prepare the samples for running
7. Prepare samples for gel: 12.0uL nanoluc positive sample + 3.0uL loading dye, ladder is 12.0uL Dil-LDL + 3.0uL loading dye
8. Gel is read left to right with small glass facing you
9. Load 12.5uL of each sample/well
10. Should always have two things plugged into the power box, if needed plug in the agarose gel box
11. Set to 50volts and run 30min (0.01A and 1W is good)
12. Check to see if more 1x TBE needs to be added after 30min
13. Set to 125 volts and run 2 hours (0.03A and 4W)

**8. Native Gel Imaging**

1. Prepare imaging solution with 1mL 1x TBE + 2.0uL substrate (yellow cap is preferred but purple cap is ok)
2. Gather a tray with imaging solution, green wedge, P1000 & P2 and tips, plastic sheets, thumb drive, and paper towels
3. Get gel(s) and go to licor FC imager
4. Login and select appropriate work station
5. Select Odyssey-FC
6. Along the top of the screen UNCLICK 700 & 800
7. Along the top CHECK 600 & Chemi, then slide bar to 0.5 for 600 and 2 for chemi
8. Use green wedge to break the seal and remove the small piece of glass
9. Onto a black tray put a paper towl and the large glass with gel and add 1mL TBE/substrate to middle of gel slowly
10. Use plastic sheet and apply from bottom to top, lifting a few times to mix the TBE/substrate onto the gel, leave plastic cover on for remaining imaging steps
11. Carefully remove the paper towel
12. Let sit at room temperature for 3-4min to equilibrate
13. Open FC tray with giant eject button and put black tray into machine
14. Click ‘acquire’
15. In the display tab select the curves tab to adjust the gamma to a straight line
16. Both the 600 and chemi boxes have to be set white to black gradients
17. Export zip files to thumb drive

**9. Page Gel Quantification Steps, to be used with the associated excel file**

1. Unzip the raw data, note that the name of the zipped file does not show with the unzipped files and will need to be transferred
2. Open ImageJ
3. Plugins -> Bio-Formats -> Bio-Formats Importer -> and navigate to the 600nm file (the gel image with the ladder)  and open
4. Adjust the display so the outline of the bottom glass plate is visible. Image -> Adjust -> Brightness/contrast -> slide the Maximum bar to the left until the bottom glass is visible.
5. Rotate the image so it is square. Image -> Transform -> Rotate -> check the Preview to get crosshairs on the image -> type a number in the Angle box and hit enter to get gel square. Once image is square, record the Angle value in the “Gel image rotation (degree)” box under the calculations tab in the excel file.
6. Enable the “Vertical profile” option by selecting Edit -> Options -> Plots, in the popup window select “Vertical profile” and OK
7. Draw a rectangle (region of interest/ROI) around the ladder.
8. Then select Edit -> Selection -> Specify and make the “Width” 50 and the “Height” 500. (keep this box open to record the Y-axis value)
9. Using only the ARROW KEYS move the box to where the top of it is in the middle of the loading well for the ladder and then arrow up 20 times
10. Record the Y-axis value in the excel file
11. Select Analyze -> Plot Profile -> List -> Edit -> Select All -> Edit -> Copy and then paste into the first two rows under the “Paste ladder + raw data” tab
12. This process is now repeated for all the lanes in the chemi.tif file using the width of 50, height of 500 and the recorded y-axis value

**How to make a merged sudo-colored gel:**

1. Using imageJ open Plugins -> Bio-Formats -> Bio-Formats Importer -> and navigate to the 600nm file (the gel image with the ladder)  and open
2. Adjust the display so the outline of the bottom glass plate is visible. Image -> Adjust -> Brightness/contrast -> slide the Maximum bar to the left until the bottom glass is visible.
3. Repeat steps 1 and 2 for Chemi.tif
4. Set the color you want for each gel. For the ladder I like to set it to yellow and for the gel we use cyan hot. Image -> Lookup Tables -> select desired color.
5. To merge select Image -> Color -> Merge Channel and assign yellow to 600.tig and cyan to chemi.tif, and check the box for  “Keep source images” then “OK”
6. Save the image however you want