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Title: Using the E1A Minigene Tool to Study mRNA Splicing Changes

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **Yes,** to show the cell confluence (step 2.2.1 script draft) using a conventional inverted light microscope and the transfection efficiency using a fluorescence microscope (step 3.1 script draft).

   If Yes, can you record movies/images using your own microscope camera?

   No

   If your protocol involves microscopy but you are not able to record movies/images with your microscope camera, JoVE will need to use our scope kit (through a camera port or one of the oculars). Please list the make and model of your microscope.

   Leica DMi1 and Leica DMIL LED FLUO.

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **Yes, all done**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees self-record interview statements. JoVE can provide support for this option.

4. **Filming location:** Will the filming need to take place in multiple locations? **Yes, walking distance**

   If **Yes**, how far apart are the locations? We will need to use the fume hood (Steps 4.3-5.4) in another location, but the laboratory is at a side building and we can go on foot.

Current Protocol Length

Number of Steps: 26
Number of Shots: 46

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Lívia AR Moura**: The overall goal of this protocol is to provide detailed guidance to use the minigene E1A tool for evaluating global mRNA splicing changes.

NOTE: Uploaded as: 1.1_(1).mp4 to AWS project page

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Dr. Fernanda Luisa Basei**: This is a rapid, cheap, and simple tool for evaluating the role of a protein with uncharacterized function in alternative splicing regulation without the necessity of special reagents or laboratory conditions.

NOTE: Uploaded as: 1.2_20210307_203627.mp4

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Protocol

2. Cell transfection

2.1. Begin by culturing HEK293 cells *(read as HEK-2-9-3)* with stable expression of the gene of interest **[1]**. Split the cells using 0.25% trypsin-EDTA **[2]**, then plate 3 x 10^5 cells per well in 6-well plates **[3]** and incubate them for 24 hours at 37 degrees Celsius in 5% carbon dioxide **[4]**.

2.1.1. WIDE: Establishing shot of talent taking cells out of the incubator.

2.1.2. Talent adding trypsin-EDTA to the cells.

2.1.3. Talent adding cells to a well.

2.1.4. Talent putting the plate with cells in the incubator.

2.2. After 24 hours, check the confluence **[1]** and transfect HEK293 stable cells only when 70 to 80% confluent **[2]**. Carefully remove the cell culture medium with a pipette **[3]**, then add 2 milliliters of complete DMEM *(D-M-E-M)* medium without antibiotics and put the plate back in the incubator **[4]**. *Videographer: This step is important!*

2.2.1. Talent using the microscope to check cells. *Videographer: Obtain multiple usable takes, this will be reused in 3.1.1.*

2.2.2. LAB MEDIA: 70 – 80% confluent cells. NOTE: There are two options at file uploader: 2.2.1_cell_confluent or 2.2.1_cell_confluent2 on AWS project page

2.2.3. Talent removing the medium.

2.2.4. Talent adding DMEM medium without antibiotics to the cells.

2.3. Prepare a tube with the transfection buffer **[1-TXT]**, then add 2 micrograms of pMTE1A DNA **[2]**, vortex, and add 2 microliters of transfection reagent **[3]**. Vortex again and incubate for 10 minutes at room temperature **[4]**. *Videographer: This step is important!*

2.3.1. Talent adding transfection buffer to a tube. **TEXT: 200 microliters/well**

2.3.2. Talent adding the DNA to the buffer.

2.3.3. Talent adding transfection reagent to the tube.

2.3.4. Talent vortexing the tube.

2.4. Remove plates from the incubator **[1]** and carefully add the transfection mixture dropwise **[2]**.

2.4.1. Talent taking cells out of the incubator.

2.4.2. Talent adding transfection mixture to the cells.

2.5. Six hours after the transfection, add tetracycline to HEK293 stable cells for Nek4 expression induction **[1-TXT]**.

2.5.1. Talent adding tetracycline to HEK293 stable cells. **TEXT: 0.5 µg/mL**

3. Cell treatment and collection

3.1. Twenty-four hours after the transfection, verify cell morphology **[1]** and transfection efficiency using a fluorescent microscope **[2]**.

3.1.1. *Use 2.2.1.*

3.1.2. LAB MEDIA: Cells 24-hours after transfection. NOTE: There are two microscope images at file uploader: 3.1.1_pEGFP_transfection and Nek4pEGFP_transfection

3.2. Use a pipette to remove the cell culture medium **[1]**, then add cell culture medium with the chemotherapeutics. Incubate the cells for another 24 hours **[2]**.

3.2.1. Talent removing medium from cells.

3.2.2. Talent adding medium with chemotherapeutics to the cells.

3.3. To collect RNA, discard the cell culture medium **[1]** and add 0.5 to 1 milliliter of RNA extraction reagent directly to the well. If wells are very confluent, use 1 milliliter of RNA extraction reagent to improve RNA quality **[2]**.

3.3.1. Talent discarding medium.

3.3.2. Talent adding RNA extraction reagent to a well.

3.4. Homogenize the cells with the pipette **[1]** and transfer them to a 1.5-milliliter centrifuge tube. proceed immediately to the RNA extraction or store the samples at -80 degrees Celsius **[2]**. *Videographer: This step is important!*

3.4.1. Talent homogenizing the cells by pipetting. NOTE: There is an additional shot showing a close up of the well

3.4.2. Talent transferring the sample to a centrifuge tube.

4. RNA extraction and cDNA synthesis

4.1. Thaw the samples in a fume hood and incubate for 5 minutes at room temperature. Add 0.1 to 0.2 milliliters of chloroform **[1]** and agitate vigorously, then incubate for 3 minutes at room temperature **[2]**.

4.1.1. Talent adding chloroform to a sample.

4.1.2. Talent agitating the sample.

4.2. Centrifuge for 15 minutes at 12,000 x *g* and 4 degrees Celsius **[1]**, then collect the upper aqueous phase and transfer it to a new 1.5-milliliter centrifuge tube **[2]**. Collect around 60% of the total volume, but do not collect the DNA or the organic phase **[3]**.

4.2.1. Talent putting the samples in the centrifuge and closing the lid.

4.2.2. Talent collecting the upper phase.

4.2.3. Talent adding upper phase to the microcentrifuge tube.

4.3. Add 0.25 to 0.5 milliliters of isopropanol **[1]** and agitate the sample by inversion 4 times **[2]**. Incubate for 10 minutes at room temperature, then centrifuge at 12,000 x *g* for 10 minutes at 4 degrees Celsius **[3]** and discard the supernatant **[4]**.

4.3.1. Talent adding isopropanol to the sample.

4.3.2. Talent inverting the sample.

4.3.3. Talent placing the sample in the centrifuge and closing the lid.

4.3.4. Talent removing supernatant.

4.4. Wash the RNA pellet twice with ethanol **[1]** and centrifuge at 7,500 x *g* for 5 minutes **[2]**, then discard the ethanol. Remove excess ethanol by inverting the tube on a paper towel **[3]**, then leave the tube open inside a fume hood to partially dry the pellet for 5 to 10 minutes **[4-TXT]**.

4.4.1. Talent washing the RNA pellet with ethanol.

4.4.2. Talent placing the tube in the centrifuge.

4.4.3. Talent discarding the ethanol and inverting the tube on a paper towel.

4.4.4. Open tube inside the fume hood. **TEXT: Dry the pellet for 30 min**

4.5. Resuspend the RNA pellet in 15 microliters of DEPC-treated water **[1]**. Quantify total RNA and verify RNA quality by measuring absorbance at 230, 260, and 280 nanometers **[2]**. To verify total RNA quality, run a 1% agarose gel pre-treated with 1.2% of a 2.5% sodium hypochlorite solution for 30 minutes **[3]**. *Videographer: This step is important!*

4.5.1. Talent resuspending the RNA pellet.

4.5.2. Talent measuring absorbance.

4.5.3. Image of gel.

4.6. Perform cDNA synthesis using 1 to 2 micrograms of total RNA. Combine RNA, 1 microliter of oligo-dT, 1 microliter of dNTP, and nuclease free water to 12 microliters **[1]**. Incubate the reaction in the thermocycler for 5 minutes at 65 degrees Celsius **[2]**.

4.6.1. Talent combining the reagents in a tube.

4.6.2. Talent placing the tube in the thermocycler and programming it.

4.7. Remove samples from the thermocycler and add 4 microliters of reverse transcriptase buffer, 2 microliters of DTT, and 1 microliter of ribonuclease inhibitor **[1]**. Incubate at 37 degrees Celsius for 2 minutes **[2]**.

4.7.1. Talent adding reagents to the tube.

4.7.2. Talent placing the tube back in the thermocycler or incubator.

4.8. Add 1 microliter of thermo-stable reverse transcriptase and incubate at 37 degrees Celsius for 50 minutes. Inactivate the enzyme at 70 degrees Celsius for 15 minutes **[1]**.

4.8.1. Talent adding reverse transcriptase to the tube.

~~4.8.2. Talent programming the thermocycler for the incubation and heat deactivation.~~

5. pMTE1A minigene PCR and gel analysis

5.1. Perform PCR as described in the text manuscript **[1]**, then load 20 to 25 microliters of the PCR product on a 3% agarose gel containing nucleic acid stain and run at 100 Volts for approximately 1 hour **[2]**.

5.1.1. Talent programming the thermocycler.

5.1.2. Talent loading the product onto a gel.

5.2. Photograph the gel, avoiding any band saturation, and quantify the bands using an image processing software **[1]**. The bands at 631, 493, and 156 base pairs correspond to the 13S, 12S and 9S isoforms, respectively **[2]**. *Videographer: This step is important!*

5.2.1. Talent imaging the gel.

5.2.2. Gel image.

5.3. From the software's **File** menu, open the image file and convert it to greyscale, adjust brightness and contrast, then remove outlier noise if necessary **[1]**.

5.3.1. SCREEN: 62181_screenshot_1.mp4. 0:00 – 0:19.

5.4. Draw a rectangle around the first lane with the **Rectangle Selection** tool and select it through the **Analyze**, **Gels**, and **Select First Lane** command or by using the keyboard shortcut **[1]**.

5.4.1. SCREEN: 62181_screenshot_1.mp4. 0:20 – 0:27.

5.5. Use the mouse to click and hold in the middle of the rectangle on the first lane and drag it over to the next lane. Go to **Analyze**, **Gels**, and **Select Next Lane**. Repeat this step for all remaining lanes **[1]**.

5.5.1. SCREEN: 62181_screenshot_1.mp4. 0:28 – 0:44. *Video Editor: Feel free to speed up the clip after lane 2 is done.*

5.6. After all the lanes are highlighted and numbered, go to **Analyze**, **Gels**, and **Plot Lanes** to draw a profile plot of each lane **[1]**.

5.6.1. SCREEN: 62181_screenshot_1.mp4. 0:45 – 0:50.

5.7. Use the **Straight-line selection** tool to draw a line across the base of each peak corresponding to each band, leaving out the background noise **[1]**.

5.7.1. SCREEN: 62181_screenshot_1.mp4. 0:51 – 1:26. *Video Editor: Speed this up.*

5.8. After all the peaks from every lane have been closed off, select the **Wand** tool and click inside each peak. Measurements should pop up in the **Results** window for each highlighted peak. Consider only the 13S, 12S and 9S peaks corresponding to 631, 493, and 156 base pairs bands **[1]**.

5.8.1. SCREEN: 62181_screenshot_1.mp4. 1:27 – 1:53. *Video Editor: Speed this up if needed.*

5.9. Copy intensity data for a spreadsheet editor and Sum the intensity from all 3 bands for each sample, then calculate the percentage for each isoform relative to the total. Plot the percentages of each isoform or the differences in the percentage relative to untreated samples **[1]**.

5.9.1. SCREEN: 62181_screenshot_1.mp4. 1:53 – 2:18.

Results

6. Results: Effects of Cisplatin and Paclitaxel on minigene E1A splicing pattern

6.1. A plasmid containing the minigene from E1A was used to observe the effect on alternative splicing **[1]** by evaluating the proportion of mRNA from each isoform produced **[2]**.

6.1.1. LAB MEDIA: Figure 1 A and B.

6.1.2. LAB MEDIA: Figure 1 B.

6.2. Basal expression of E1A isoforms variants depended on the cell line and time of E1A expression **[1]**. The HEK293-stable cell line or HEK293 recombinase containing site showed a higher expression of 13S **[2]** in comparison to HeLa cells that showed similar levels of 13S and 12S isoforms after 48 hours of E1A expression **[3]**.

6.2.1. LAB MEDIA: Figure 1 C.

6.2.2. LAB MEDIA: Figure 1 C. *Video Editor: Emphasize the 13S bands for the HEK293 cell lines.*

6.2.3. LAB MEDIA: Figure 1 D. *Video Editor: Emphasize the 12S and 13S bars for HeLa-PLKO.*

6.3. Cells exposed to cisplatin showed a shift in 5´SS splicing selection favoring 12S expression **[1]**. This effect was observed in HEK293 stably expressing the Flag empty vector as well as isoform 1 of Nek4 *(pronounce 'neck-four')* **[2]**.

6.3.1. LAB MEDIA: Figure 2 A. *Video Editor: Emphasize the + lanes.*

6.3.2. LAB MEDIA: Figure 2 B - D.

6.4. Changes in alternative splicing after paclitaxel treatment were very discrete, but the directions of the changes were opposite in Flag and Nek4 expressing cells **[1]**.

6.4.1. LAB MEDIA: Figure 3 E.

Conclusion

7. Conclusion Interview Statements

7.1. **Dr. Fernanda Luisa Basei**: When performing this protocol, it is important to use untransfected and non-reverse transcriptase controls to avoid misinterpretation with endogenous E1A expression, mainly in case of using HEK293 cells.

NOTE: Uploaded as: 7.1_20210307_204110.mp4 on AWS project page

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 4.6.1.*

7.2. **Lívia AR Moura**: After obtaining positive results, one can evaluate the alternative splicing of specific genes related to the chemotropic response can be evaluated.

NOTE: Uploaded as: 7.2 (1).mp4

7.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

7.3. **Dr. Fernanda Luisa Basei**: When some effect is observed, this simple protocol can direct studies to more consistent pathways where the protein plays a role regulating alternative splicing in chemotherapy response.

NOTE: Uploaded as: 7.3_20210307_204629.mp4

7.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.