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Title: Assessing Cellular Target Engagement by SHP2 (PTPN11) Phosphatase Inhibitors

Authors and Affiliations:

Lester J. Lambert[1], Celeste Romero[1], Douglas J. Sheffler[1], Maria Celeridad[1], Nicholas D. P. Cosford[1], Lutz Tautz[1]

[1]Cancer Metabolism & Signaling Networks Program, NCI-Designated Cancer Center, Sanford Burnham Prebys Medical Discovery Institute, La Jolla, CA, USA.

Corresponding Authors:

Lutz Tautz (tautz@SBPdiscovery.org)

Email Addresses for All Authors:

llambert@SBPdiscovery.org
Celeste.Romero@UTSouthwestern.edu
dsheffler@SBPdiscovery.org
mceleridad@SBPdiscovery.org
ncosford@SBPdiscovery.org
tautz@SBPdiscovery.org

Author Questionnaire

1. **Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **Yes, short walk 300 yards**

Current Protocol Length

Number of Steps: 16
Number of Shots: 45

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Lutz Tautz:** The tyrosine phosphatase SHP2 is a key mediator of cell signaling, promoting cell survival and proliferation. My team is focused on finding small-molecule SHP2 inhibitors for the treatment of cancer.

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

1.2. **Lester J. Lambert:** Our technique provides a rapid and powerful method of establishing cell penetrance as well as target engagement of small-molecule SHP2 inhibitors in cells, assuring that discovery resources are efficiently directed.

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

OPTIONAL:

1.3. **Lutz Tautz:** SHP2 is an attractive target in cancer therapy, and several SHP2 inhibitors are in clinical trials. Our technique will facilitate the discovery of next generation inhibitors with improved efficacy profiles.

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

Introduction of Demonstrator on Camera

1.4. **Lutz Tautz:** Demonstrating the procedure will be Celeste Romero, a Research Assistant, and Douglas Sheffler, a Research Assistant Professor from our laboratory.

1.4.1. INTERVIEW: Author saying the above.

1.4.2. The named demonstrator(s) looks up from workbench or desk or microscope and acknowledges the camera.

Protocol

2. Transfection of HEK293T Cells

Pronounce HEK293T: "Heck" "two" "nine" "three" "T"

2.1. To begin, detach HEK293T cells from the plates using 3 milliliters of cell detachment reagent **[1]**. Dilute the cells with 12 milliliters of growth media **[2]** and collect them by centrifugation at 1,400 *x g* for 4 minutes **[3]**.

2.1.1. WIDE: Establishing shot of talent adding detachment reagent to the cells.

2.1.2. Talent adding growth media to the plate, with the media container in the shot.

2.1.3. Talent putting the cells in the centrifuge and closing the lid.

2.2. Resuspend the cell pellet in 10 milliliters of growth media **[1]** and measure the concentration and viability of the cells with trypan blue and a cell counter **[2]**. Plate 700,000 exponentially growing cells into each well of a 6-well culture plate **[3-TXT]** and incubate them for 24 hours at 37 degrees Celsius and 5% carbon dioxide **[4]**. *Videographer: This step is important!*

2.2.1. Talent resuspending the cells pellet.

2.2.2. ~~Talent counting cells.~~ *Videographer: Obtain multiple usable takes because this shot will be reused in 3.6.3.* NOTE: Use 3.6.3 A – D here

2.2.3. Talent seeding cells into wells. **TEXT: Reserve one well for each plasmid**

2.2.4. Talent putting the plate in the incubator and closing the door. *Videographer: Obtain multiple usable takes because this shot will be reused in 2.4.3.*

2.3. On the next day, dilute 2 micrograms of plasmid DNA into 200 microliters of transfection buffer **[1-TXT]**. Vortex the plasmid DNA for 10 seconds **[2]** and centrifuge it at 1,400 *x g* for 4 minutes **[3]**.

2.3.1. Talent diluting plasmid DNA into transfection buffer, with the buffer container in the shot. **TEXT: pICP-ePL-N-SHP2-WT or pICP-ePL-N-SHP2-E76K**

2.3.2. Talent vortexing the DNA.

2.3.3. Talent putting the DNA in the centrifuge and closing the lid.

2.4. Add 4 microliters of transfection reagent to the diluted DNA, vortex it for 10 seconds and repeat the centrifugation **[1]**. Incubate the DNA at 23 degrees Celsius for 10 minutes, then add the transfection mix to the attached HEK293T cells in the 6-well plate **[2]** and return the cells to the incubator for another 24 hours **[3]**. *Videographer: This step is important!*

2.4.1. Talent adding transfection reagent to the DNA and vortexing it.

2.4.2. Talent adding the transfection mix to the cells.

2.4.3. *Use 2.2.4.*

3. Incubation of Cells with SHP2 Inhibitors

NOTE to VO: (in case shp2 comes up in narration) Pronounce: SHP2 → "Ship" "two"

3.1. To prepare assay plates, dilute inhibitor solutions in DMSO for a stock concentration of 10 millimolar and dispense them into a 384-well low dead volume source plate for immediate use **[1]**. *Videographer: This step is important!*

3.1.1. Talent dispensing inhibitor solutions into a 384 well low dead volume source plate.

Authors Note: On the day of shooting there was a little confusion on our part about shots under 3.2. The shots were obtained by the videographer, but we are not sure he had the right number for these shots on his whiteboard cue card.

3.2. Spot the desired volume of inhibitors or vehicle using a liquid handler **[1]** into 384 well real-time PCR plates at a target final volume of less than 0.5% DMSO **[2]**. Seal the plates using a plate sealer with inert gas purging **[3]**.

3.2.1. Talent feeding assay and source plates into the liquid handler.

3.2.2. Talent taking the spotted assay plate from the liquid handler.

3.2.3. Talent sealing the plate.

3.3. To prepare the transfected cells, preincubate growth media and cell detachment reagent in a 37-degree Celsius water bath **[1]**. Remove the cells from the incubator **[2]** and gently aspirate the media from the wells **[3]**. *Videographer: This step is difficult and important!*

3.3.1. Talent putting the media and reagent in the water bath.

3.3.2. Talent taking the cells out of the incubator.

3.3.3. Talent aspirating media from the wells.

3.4. Add 0.3 milliliters of the cell detachment reagent to each well **[1]** and gently rock the plate back and forth to thoroughly cover the surface of the plate bottom **[2]**. Incubate the plate at 23 degrees Celsius for 2 minutes **[3]**.

3.4.1. Talent adding detachment reagent to the plate.

3.4.2. Talent rocking the plate.

3.4.3. Talent leaving the plate to incubate.

3.5. Add 1 milliliter of growth media to each well and gently pipette the cells in the well **[1]**, then transfer them to a 15 milliliter Falcon centrifuge tube **[2]**. Centrifuge the cells at 1,400 *x g* for 4 minutes to collect the cells **[3]**. *Videographer: This step is difficult and important!*

3.5.1. Talent adding media and pipetting the cells in a well.

3.5.2. Talent transferring the cells to the centrifuge tube.

3.5.3. Talent putting the tube in the centrifuge and closing the lid.

3.6. Gently aspirate the media **[1]**, then resuspend the cell pellet in 2 milliliters of growth media **[2]**. Ensure that the cell viability is greater than 90% using trypan blue and a cell counter **[3]**.

3.6.1. Talent aspirating the media.

3.6.2. Talent resuspending the cells.

3.6.3. *Use 2.2.2.*

3.7. Dilute cells to a concentration of 125 cells per microliter, keeping the cells in suspension for no more than 2 hours for optimal viability **[1]**.

3.7.1. Talent diluting the cells.

3.8. For incubation with SHP2 inhibitors, dispense the cells into a sterile single channel solution trough **[1]**. Centrifuge the previously prepared 384-well real-time PCR plate at 2,500 *x g* for 5 minutes **[2]**, then remove the seal and use a 125-microliter multichannel pipette to add 5 microliters of the diluted cells to the desired wells **[3]**.

3.8.1. Talent dispensing the cells in the trough.

3.8.2. Talent putting the PCR plate in the centrifuge and closing the lid. *Videographer: Obtain multiple usable takes because this shot will be reused in 4.2.2.*

3.8.3. Talent adding cells to the plate.

3.9. Centrifuge the plate at 42 *x g* for 30 seconds without a lid **[1]**, then attach a lid **[2]** and incubate the plate for 1 hour at 37 degrees Celsius and 5% carbon dioxide **[3]**.

3.9.1. ~~Talent putting the plate without a lid in the centrifuge.~~ NOTE: Use 3.8.2.

3.9.2. Talent attaching a lid seal.

3.9.3. Talent putting the plate in the incubator and closing the door.

4. Isothermal or Thermal Profile Gradient Heat Pulse and Lysis Detection

4.1. Program the thermocycler according to manuscript directions for either the thermal profile gradient or isothermal experiment **[1]**. Start the thermocycler heat pulse

program **[2]** and place the assay plate on the thermoblock **[3]**. When the program is finished, remove the assay plate **[4]**. *Videographer: This step is important!*

4.1.1. Shot of gradient on screen of thermocycler.

4.1.2. Talent starting thermocycler heat pulse program.

4.1.3. Talent placing assay plate on thermoblock.

4.1.4. Talent removing assay plate from thermoblock.

4.2. Supplement each well of the assay plate with 5 microliters of lysis detection master mix **[1]**, centrifuge the plate at 42 *x g* for 30 seconds **[2]**, and store it at 23 degrees Celsius in darkness for 30 to 60 minutes **[3]**.

4.2.1. Talent adding lysis detection mix to the plate.

4.2.2. *Use 3.8.2.*

4.2.3. Talent putting the plate in a dark place.

4.3. Measure chemiluminescence using a microplate reader **[1]** with the optimized integration time **[2]**.

4.3.1. Talent programming the plate reader.

4.3.2. Chemiluminescent signal developing on screen.

Results

5. Results: Cellular Target Engagement of Allosteric SHP2 Inhibitors with SHP2-WT and SHP2-E76K Proteins

NOTE to VO: Pronounce SHP099: “Ship” “Oh” “9” 9”

NOTE to VO: Pronounce RMC-4550: “R” “M” “C” “4” “5” “5” “zero”

5.1. The thermal gradient experiment for Wild Type SHP2 resulted in a sigmoidal cellular thermal profile with a narrow melting transition that is typical for a folded protein **[1]**. Incubation of Wild Type SHP2 with the allosteric inhibitor SHP099 stabilized the thermal profile to a significant and measurable degree **[2]**.

5.1.1. LAB MEDIA: Figure 4 A.

5.1.2. LAB MEDIA: Figure 4 A. *Video Editor: Emphasize the blue line.*

5.2. The stabilization of SHP2 also tracked with the potency of SHP099-like allosteric compounds **[1]**. At 10 micromolar, the more potent RMC-4550 produced a greater degree of stabilization than SHP099 for Wild Type SHP2 **[2]**.

5.2.1. LAB MEDIA: Figure 4 A and B.

5.2.2. LAB MEDIA: Figure 4 A and B. *Video Editor: Emphasize the blue lines in both plots.*

5.3. Due to their unique mechanism, SHP099-like allosteric inhibitors are less effective against many SHP2 oncogenic mutants, including SHP2-E76K. Consistent with these known properties, only marginal thermal stabilization of SHP2-E76K by SHP099 is observed. **[1]**.

5.3.1. LAB MEDIA: Figure 4 C.

5.4. The expression level of the ePL-tagged protein can dramatically influence the signal intensity. Thermal profiles for transient and stably integrated Wild Type cells under identical assay conditions are shown **[1]**. Normalization of the disparate curves affords comparable thermal profiles, revealing the wide range of the EFC detection system **[2]**.

5.4.1. LAB MEDIA: Figure 4 D.

5.4.2. LAB MEDIA: Figure 4 E.

5.5. Taking advantage of the thermocycler ability to produce thermal gradients on the "short" axis of a 384-well plate, a series of isothermal titrations can be performed on a single plate **[1]**. At an optimal temperature, an isothermal titration using a full 10-point dose-response yielded a useful EC-50 for RMC-4550 **[2]**.

5.5.1. LAB MEDIA: Figure 5 A.

5.5.2. LAB MEDIA: Figure 5 B.

Conclusion

6. Conclusion Interview Statements

6.1. **Lester J. Lambert:** The principles of this assay could be used to monitor the degradation of proteins in cells induced by PROTAC compounds.

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

6.2. **Lutz Tautz:** We are using this cellular thermal shift technique to discover inhibitors of mutant SHP2 in leukemias. Drugs specific against frequent SHP2 oncogenic mutants will have a significant impact for the treatment of these cancers.

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