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Scriptwriter Name: Poornima G

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Title: Rapid Production of Recombinant Human SLFN14 Ribonuclease and Stoichiometric Analysis by Mass Photometry

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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? **No**

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

- 3. Filming location:** Will the filming need to take place in multiple locations? **No**

- 4. Testimonials (optional):** Would you be open to filming two short testimonial statements **live during your JoVE shoot**? These will **not appear in your JoVE video** but may be used in JoVE's promotional materials. **No**

Current Protocol Length

Number of Steps: 25

Number of Shots: 51 (16 SC)

Introduction

Videographer: Obtain headshots for all authors available at the filming location.

Authors: Please note that **the questions will not appear on screen**. Please answer in **stand-alone full sentence with sufficient context**.

Answers will be slightly edited in accordance with the journal guidelines

INTRODUCTION:

~~REQUIRED:~~ Summarize your research focus and the main questions it aims to answer.

- 1.1. **Monica Pillon:** Our research program aims to characterize the function and regulation of understudied ribonucleases in eukaryotic gene control.
 - 1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

~~Describe the research gap or limitation in existing methods and how this protocol addresses them.~~

- 1.2. **Monica Pillon:** Recombinant production of eukaryotic ribonucleases is often challenging due to their complexity and cytotoxicity; this rapid protocol uses a mammalian cell line to overcome these limitations.
 - 1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera

CONCLUSION:

~~What does this protocol allow researchers to study or measure?~~

1.3. Cole Masuga: This protocol offers a rapid strategy to produce recombinant eukaryotic ribonucleases without the need for stabilizing mutations.

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

~~Describe the most important consideration or challenge when performing this protocol.~~

- 1.4. **Cole Masuga:** Additional considerations may be required for high-affinity RNA-binding proteins that have a propensity to co-purify with cellular nucleic acids.

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

~~Are there any additional methods or analyses that can be performed following this procedure?~~

- 1.5. **Cole Masuga:** The recombinant protein isolated from this protocol can be used for qualitative biochemical analyses and structural studies, including single particle cryoEM.

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

Videographer: Obtain headshots for all authors available at the filming location.

Protocol

2. Harvesting Mammalian Cells Expressing SLFN14^{Myc-Flag}

Demonstrator: Cole Masuga

- 2.1. To begin, seed mammalian HEK293 suspension cells and incubate them at 37 degrees Celsius [1-TXT]. Using trypan blue stain and a cell counter, verify that the cell count is between 1.2 and 1.8 million cells per milliliter [2] and that the viability is greater than 90 percent [3].
 - 2.1.1. WIDE: Talent placing the cell culture into the incubator set to 37 degrees Celsius.
TXT: 125 rpm; 8% CO₂; 75 - 80% Humidity; 24 - 32 h
 - 2.1.2. Talent mixing the cells with trypan blue in a tube.
 - 2.1.3. Talent loading cells into a cell counter.
- 2.2. In a conical tube, add 320 micrograms of purified plasmid DNA encoding the recombinant SLFN14-Myc-Flag (*S-L-F-N-14-mik-flag*) gene to 8 milliliters of serum-free transfection medium [1] and incubate for 5 minutes at 20 to 25 degrees Celsius [2].
 - 2.2.1. Talent pipetting purified plasmid deoxyribonucleic acid into a conical tube containing serum-free transfection medium.
 - 2.2.2. Talent placing the sample aside for incubation.
- 2.3. At the same time, add 960 micrograms of 0.22 micrometer filter-sterilized PEI to 8 milliliters of serum-free transfection medium [1-TXT].
 - 2.3.1. Talent pipetting filter-sterilized PEI into another tube with serum-free transfection medium. **TXT: PEI: Polyethylenimine; Incubate for 5 min (20 - 25 °C)**
- 2.4. Now, slowly add the PEI mixture dropwise to the recombinant plasmid DNA solution and incubate the combined solution for 15 minutes at 20 to 25 degrees Celsius [1]. Next, slowly add 16 milliliters of the DNA-PEI mixture dropwise to the prepared cells [2].
 - 2.4.1. Talent adding the PEI mixture dropwise into the deoxyribonucleic acid solution and, then setting the tube aside to incubate.
 - 2.4.2. Talent adding the DNA/PEI mixture dropwise into the cell culture flask using a pipette.

2.5. Incubate the cells at 37 degrees Celsius and 125 revolutions per minute in an atmosphere of 8 percent carbon dioxide for 48 hours [1-TXT]. Then, decant 40 milliliters of transfected cells into eight conical tubes [2]. Harvest the transfected cells by centrifuging at 400 g for 5 minutes at 20 to 25 degrees Celsius [3].

2.5.1. Talent placing the transfected cell flask back into the incubator. **TXT: 75 – 80% humidity**

2.5.2. Talent pouring equal volumes of transfected cells from the flask into eight conical tubes arranged on the bench.

2.5.3. Talent placing the conical tubes into a centrifuge and closing the lid to begin centrifugation.

2.6. Resuspend each transfected cell pellet with 225 microliters of chilled Resuspension Buffer for a total of 900 microliters across four pellets [1]. Transfer the combined resuspended cells to a 2-milliliter microcentrifuge tube that has been pre-chilled on ice [2].

2.6.1. Talent pipetting chilled Resuspension Buffer into a tube with pellet.

2.6.2. Talent transferring the resuspended cell solution into a microcentrifuge tube resting on an ice-filled rack.

2.7. Incubate the microcentrifuge tube horizontally on a nutator for 30 minutes at 4 degrees Celsius [1]. Then, clarify the cell lysate by centrifugation at 21,300 g for 20 minutes at 4 degrees Celsius [2].

2.7.1. Talent placing the tube horizontally on a nutator and switching on the device to begin incubation.

2.7.2. Talent placing the microcentrifuge tube into the centrifuge and closing the lid to start the spin.

3. Protein Affinity Capture for Recombinant SLFN14^{Myc-Flag} Purification

3.1. Combine the clarified lysate with the equilibrated anti-Flag resin in a microcentrifuge tube [1] and incubate the mixture horizontally on a nutator for 60 minutes at 4 degrees Celsius [2].

3.1.1. Talent pipetting the clarified lysate into the tube containing anti-Flag resin.

- 3.1.2. Talent placing the microcentrifuge tube on its side on a nutator and starting the incubation. **NOTE: Use 2.7.1. The shot 3.1.2 was not filmed as I filmed numerous takes for 2.7.1**
- 3.2. Centrifuge the lysate-resin mixture at 8,000 *g* for 2 minutes at 4 degrees Celsius to pellet the resin [1]. Discard approximately 700 microliters of supernatant carefully without disturbing the resin pellet [2].
- 3.2.1. Talent placing the tube in a centrifuge and closing the lid to begin the spin.
- 3.2.2. Talent slowly aspirating the supernatant while visually monitoring the resin pellet to avoid disruption.
- 3.3. Now, resuspend the remaining resin-supernatant mixture and transfer it to an empty 800-microliter capacity spin column inserted into a 2-milliliter microcentrifuge tube [1]. Centrifuge the spin column at 1,000 *g* for 2 minutes at 4 degrees Celsius [2].
- 3.3.1. Talent pipetting up and down to resuspend the resin and transferring the entire contents into the spin column seated in a collection tube.
- 3.3.2. Talent placing the spin column into a centrifuge.
- 3.4. After discarding the flow-through, rinse the microcentrifuge tube used previously with 500 microliters of High Salt Wash Buffer and transfer the rinse to the spin column [1]. Centrifuge the spin column at 1,000 *g* for 2 minutes at 4 degrees Celsius and discard the flow-through [2].
- 3.4.1. Talent rinsing the tube with High Salt Wash Buffer, then pipetting the solution into the spin column.
- 3.4.2. Talent removing the tube from the centrifuge and discarding the flow-through.
- 3.5. Now, add 500 microliters of High Salt Wash Buffer to the spin column [1] and centrifuge at 1,000 *g* for 2 minutes at 4 degrees Celsius [2-TXT].
- 3.5.1. Talent adding High Salt Wash Buffer to the column.
- 3.5.2. Talent placing the column in centrifuge. **TXT: Repeat the wash step 3x more**
- 3.6. After 4 washes in total, add 55 microliters of 3-x-Flag Elution Buffer directly over the

resin [1]. Incubate the spin column vertically on a nutator for 60 minutes at 4 degrees Celsius [2].

3.6.1. Talent pipetting the elution buffer carefully onto the resin bed in the spin column.

3.6.2. Talent placing the spin column upright on a nutator and beginning the incubation.

3.7. Centrifuge the column as demonstrated earlier to collect the eluted recombinant SLFN14Myc-Flag protein [1].

3.7.1. Talent placing the column in a centrifuge and starting the spin.

3.8. Add 100 microliters of the combined anti-Flag resin eluate to the center of the spin desalting column bed without disrupting the resin [1]. Elute the recombinant SLFN14Myc-Flag protein by centrifuging the spin desalting column at 700 g for 2 minutes at 4 degrees Celsius [2].

3.8.1. Talent carefully pipetting 100 microliters of eluate onto the center of the column bed without touching or disturbing the resin.

3.8.2. Talent placing the spin desalting column into a centrifuge and starting the spin.

3.9. Measure the absorbance at 260 and 280 nanometers from 2 microliters of recovered recombinant protein using a microvolume spectrophotometer [1].

3.9.1. Talent loading 2 microliters of sample onto the spectrophotometer pedestal and starting the absorbance reading.

4. Analyzing Broad Range Protein Standards

4.1. Add 10 microliters of 0.22 micrometer filtered Low or High Salt Desalting Buffer into the empty well [1] and close the lid [2].

4.1.1. Talent pipetting Desalting Buffer into the sample well. **NOTE: 4.1.1 and 4.1.2 were merged into one shot**

4.1.2. Talent gently closing the lid over the sample well.

4.2. Under **Find focus**, click **droplet dilution** in the Acquire-MP software to determine the correct focus of the sample well [1].

4.2.1. SCREEN: 4.2.1.mp4 00:00-00:20.

4.3. Then, add 10 microliters of the 5x calibrant working stock to the buffer droplet and gently pipette up and down to mix the solution [1] and close the lid over the sample [2].

4.3.1. Talent pipetting 5x calibrant into the droplet and mixing gently with a pipette.

4.3.2. Talent lowering the lid over the sample well after mixing.

4.4. Examine the instrument settings to confirm the values for motion, signal, saturation, and sharpness are stable and optimal [1]. Click **record** in the Acquire-MP software to record a 60-second video [2]. Save the result file with a descriptive title and timestamp in the **.mpr (dot-M-P-R)** format [2].

4.4.1. SCREEN: 4.4.1-4.4.3.mp4 00:16-00:22.

4.4.2. SCREEN: 4.4.1-4.4.3.mp4 00:23-00:30.

4.4.3. SCREEN: 4.4.1-4.4.3.mp4 01:22-01:24 and 01:48-01:52.

5. Creating a Calibration Curve and Calculating the Molecular Mass and Stoichiometry of Recombinant Protein

5.1. Under **Measurements**, click the **+ open (plus open)** button and navigate to the calibrant result file and double click on the listed protein standards file to open the results [1].

5.1.1. SCREEN: 5.1.1-5.1.2.mp4 00:01-00:14.

5.2. Under **Plot configuration**, adjust the contrast boundaries to 0.0 and minus 0.03, respectively. Manually define a Gaussian curve for the first four protein standard peaks by dragging the cursor across each individual peak [1].

5.2.1. SCREEN: 5.2.1-5.2.2.mp4

5.3. Then, click **Create** and select **Mass calibration** from the menu [1]. Under the **Calibrant** header, assign each fitted calibrant peak to its known molecular mass of 86, 172, 258, and 344 kilodaltons [1].

5.3.1. SCREEN: 5.3.1-5.4.1.mp4 00:01-00:08.

5.3.2. SCREEN: 5.3.1-5.4.1.mp4 00:09-00:19.

- 5.4. Verify the calibration curve shows an R-squared value close to 1 and a maximum mass error below 2 percent [1]. Under **Measurements** in the DiscoverMP software, click the **+ open** button to select the result file for SLFN14Myc–Flag protein [1].
 - 5.4.1. SCREEN: 5.3.1-5.4.1.mp4 00:20-00:24.
 - 5.4.2. SCREEN: 5.4.2-5.5.2.mp4 00:00-00:04.
- 5.5. Then, under **Calibrations**, double click on the calibration curve file to apply the calibration [1]. Manually define a Gaussian curve for each observed SLFN14Myc–Flag species by dragging the cursor across each peak to obtain a calculated molecular mass value [2].
 - 5.5.1. SCREEN: 5.4.2-5.5.2.mp4 00:05-00:12.
 - 5.5.2. SCREEN: 5.4.2-5.5.2.mp4 00:14-00:23.
- 5.6. Finally, calculate the average molecular mass for each oligomeric species from three independent measurements, along with the standard deviation [1]. Determine the stoichiometry of recombinant SLFN14Myc–Flag protein by evaluating the average calculated molecular mass relative to integer multiples of the theoretical protomer mass [2].
 - 5.6.1. SCREEN: 5.6.1-5.6.2.mp4 00:00-00:05.
 - 5.6.2. SCREEN: 5.6.1-5.6.2.mp4 00:06-00:10.

Results

6. Results

6.1. SLFN14Myc–Flag protein was purified from HEK293 cells using anti-Flag affinity chromatography followed by size exclusion chromatography [1]. A representative SDS-PAGE gel showed successful purification of the protein, with a strong band appearing in the polished elution lane [2].

6.1.1. LAB MEDIA: Figure 2B

6.1.2. LAB MEDIA: Figure 2B. *Video editor: Highlight the dark band in lane "P" pointed by the black arrow.*

6.2. Mass photometry of protein standards showed distinct peaks corresponding to 86, 172, 258, and 344 kilodaltons [1], and produced a calibration curve with a perfect R^2 value of 1 and maximum mass error of 0.3% [2].

6.2.1. LAB MEDIA: Figure 3A. *Video editor: Show the four peaks labeled S86, S172, S258, and S344 on the mass distribution plot.*

6.2.2. LAB MEDIA: Figure 3B. *Video editor: Zoom in on the linear plot and box showing $R^2 = 1.00$ and MME = 0.3%.*

6.3. Under physiological salt conditions, SLFN14Myc–Flag protein formed two species with molecular masses of about 242 kilodaltons and 472 kilodaltons, both higher than the theoretical dimer and tetramer masses [1].

6.3.1. LAB MEDIA: Figure 3C. *Video editor: Highlight the labeled peaks for the SLFN14Myc–Flag dimer at 241 kilodaltons and tetramer at 474 kilodaltons.*

6.4. An elevated 260 by 280 nanometer absorbance ratio of 1.6 ± 0.0047 indicated the presence of nucleic acids in the SLFN14Myc–Flag sample [1], which was confirmed by RNA-specific dye quantification detecting approximately 3.5 micrograms of RNA [2].

6.4.1. LAB MEDIA: Figure 4A. *Video editor: Show the absorbance value "A260/A280 = 1.6" next to the cartoon of SLFN14 with RNA bound.*

6.4.2. LAB MEDIA: Figure 4A. *Video editor: Highlight the red label "RNA ~3.5 μ g" near the eluted sample graphic.*

6.5. In high salt conditions, SLFN14Myc–Flag protein formed two species with molecular

masses of around 111 and 219 kilodaltons, which matched theoretical monomer and dimer values, suggesting RNA release [1].

6.5.1. LAB MEDIA: Figure 4B. *Video editor: Highlight the peak labeled "SLFN14 monomer (111 kDa)" and the peak labeled "SLFN14 dimer (218 kDa)".*

1. HEK293
Pronunciation link: <https://www.merriam-webster.com/dictionary/HEK>
IPA: /ˌeɪf.iː.ker tuː ˌnɑːm.tiː ˈθriː/
Phonetic Spelling: aych·ee·kay two·nine·tee three
2. mammalian
Pronunciation link: <https://www.merriam-webster.com/dictionary/mammalian>
IPA: /məˈmeɪliən/
Phonetic Spelling: muh·may·lee·uhn
3. trypan
Pronunciation link: <https://www.merriam-webster.com/dictionary/trypan>
IPA: /ˈtraɪ.pæn/
Phonetic Spelling: try·pan
4. plasmid
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /ˈplæz.mɪd/
Phonetic Spelling: ɹaz·mid
5. recombinant
Pronunciation link: <https://www.merriam-webster.com/dictionary/recombinant>
IPA: /rɪˈkɑːmbə.nənt/
Phonetic Spelling: ree·kom·buh·nuhnt
6. deoxyribonucleic
Pronunciation link: <https://www.merriam-webster.com/dictionary/deoxyribonucleic>
IPA: /diˌɔːksiˌraɪboʊnuːˈkliːk/
Phonetic Spelling: dee·ok·see·rye·boh·noo·klee·ik
7. transfection
Pronunciation link: <https://www.merriam-webster.com/dictionary/transfection>
IPA: /trænsˈfɛkʃən/
Phonetic Spelling: tranz·fek·shuhn
8. polyethylenimine
Pronunciation link: <https://www.merriam-webster.com/dictionary/polyethylenimine>
IPA: /ˌpɑːliˌeθəˈliːnɪmɪn/
Phonetic Spelling: pah·lee·eth·uh·leen·ih·meen
9. centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj

10. microcentrifuge
Pronunciation link: No confirmed link found
IPA: /,maɪkroʊ'sentrə,fjuːdʒ/
Phonetic Spelling: my·kroh·sen·truh·fyooj
11. lysate
Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>
IPA: /'laɪsɪt/
Phonetic Spelling: ly·sayt
12. chromatography
Pronunciation link: <https://www.merriam-webster.com/dictionary/chromatography>
IPA: /,kroʊmə'tɑːgrəfi/
Phonetic Spelling: kroh·muh·tog·ruh·fee
13. supernatant
Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
IPA: /,suːpər'neɪtənt/
Phonetic Spelling: soo·per·nay·tuhnt
14. eluate
Pronunciation link: <https://www.merriam-webster.com/dictionary/eluate>
IPA: /'iːluːeɪt/
Phonetic Spelling: ee·loo·ayt
15. spectrophotometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
IPA: /,spektrəʊfə'tɑːmɪtər/
Phonetic Spelling: spek·troh·fuh·tom·ih·ter
16. calibrant
Pronunciation link: <https://www.merriam-webster.com/dictionary/calibrant>
IPA: /'kælɪbrənt/
Phonetic Spelling: kal·uh·bruhnt
17. Gaussian
Pronunciation link: <https://www.merriam-webster.com/dictionary/Gaussian>
IPA: /'gaʊsiən/
Phonetic Spelling: gow·see·uhn
18. kilodalton
Pronunciation link: <https://www.merriam-webster.com/dictionary/kilodalton>
IPA: /,kɪlə'dɔːltən/
Phonetic Spelling: kil·uh·dawl·tuhn
19. stoichiometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/stoichiometry>
IPA: /,stɔɪki'ɑːmətri/
Phonetic Spelling: stoy·kee·om·uh·tree
20. oligomeric
Pronunciation link: <https://www.merriam-webster.com/dictionary/oligomeric>
IPA: /,ɑːlɪgə'merɪk/
Phonetic Spelling: ol·ih·guh·mer·ik

21. protomer
Pronunciation link: <https://www.merriam-webster.com/dictionary/protomer>
IPA: /'proutə,mər/
Phonetic Spelling: proh·tuh·mer
22. affinity
Pronunciation link: <https://www.merriam-webster.com/dictionary/affinity>
IPA: /ə'finəti/
Phonetic Spelling: uh·fin·uh·tee
23. photometry
Pronunciation link: <https://www.merriam-webster.com/dictionary/photometry>
IPA: /foʊ'tɑ:mətri/
Phonetic Spelling: foh·tom·uh·tree
24. physiological
Pronunciation link: <https://www.merriam-webster.com/dictionary/physiological>
IPA: /,fiziə'lɑ:dʒɪkəl/
Phonetic Spelling: fiz·ee·uh·loj·ih·kuhl
25. nucleic
Pronunciation link: <https://www.merriam-webster.com/dictionary/nucleic>
IPA: /nu:'kli:ɪk/
Phonetic Spelling: noo·klee·ik