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Title: Cell-free *In Vitro* mRNA Translation in *Drosophila* Lysates

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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: 16
Number of Shots: 53 (5 SC)

Interviews

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

INTRODUCTION:

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

- 1.1. **Patricio Reza:** Our research employs a *Drosophila* cell-free translation system to study protein synthesis and translational regulation across various stages of insect development.

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. **Mariana Valdés-Barreto:** This protocol accurately recapitulates *in vivo* translation events specific to insect development, unlike the lysates from distant metazoans.

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. **Mariana Valdés-Barreto:** This protocol allows researchers to study mRNA translation and translational control mechanisms using *Drosophila* embryo lysates.

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. **Patricio Reza:** The critical challenges are maintaining mRNA integrity and minimizing lysate variability through consistent embryo aging and homogenization.

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

~~How can future studies build on this work? Describe the questions or research directions that can be investigated next.~~

1.5. **Patricio Reza:** Future studies can extend this protocol to other insect species for evolutionary comparisons and to investigate novel translational dynamics during embryogenesis.

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.

Videographer's Note: I forgot to note the headshots:

5177 - Greco Hernández

5211 - Noemi Baranda

5259 - Patricio Reza

5266 - Mariana Valdez

5281 - Dora Vélez

Protocol

Videographer: Please always record an audio slate while filming each shot mentioned in the shotlist.

2. Template Plasmid Construction and *In vitro* mRNA Transcription

Demonstrator: Patricio Reza

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

- 2.1. To begin, open the **Benchling** cloning software [1]. Select a suitable plasmid to insert the construct, containing a 5 prime untranslated region, a Firefly luciferase, or FLuc (*F-luck*) reporter cistron, a 3 prime untranslated region, and a polyadenylate, or polyA (*poly-A*) tail downstream of a T7 (*T-seven*) promoter [2-TXT]. Use the unique restriction site of KpnI (*K-P-N-one*) immediately downstream of the polyA tail to linearize each plasmid construct before transcription [3]. Verify the clean, well-resolved peaks of the constructs obtained by sequencing, before use as templates for *in vitro* transcription [4].
 - 2.1.1. WIDE: Talent opening the plasmid construction software on the computer.
Videographer: Please obtain CU shot as well for this shot.
 - 2.1.2. SCREEN: 70844_screenshot_1.mp4. 00:04–00:06; 00:07–00:10; 00:11–00:14.
TXT: Plasmid: pTZ57R/T
 - 2.1.3. SCREEN: 70844_screenshot_1.mp4. 00:34–00:38
 - 2.1.4. SCREEN: 70844_screenshot_4.mp4. 00:11–00:17
- 2.2. Resolve the linearized DNA by 1 percent agarose gel electrophoresis at 100 volts for 1 hour [1].
 - 2.2.1. Talent loading the linearized DNA samples onto a 1 percent agarose gel.
- 2.3. Gel-extract the linearized DNA [1] ~~Check DNA integrity and proper linearization by agarose gel electrophoresis [2].~~ Place it in a microtube and add agarose digestion buffer, or ADB (*A-D-B*) for DNA purification [2]. Incubate the tube in a thermoshaker [3], then transfer the contents to a column and centrifuge to recover the linearized DNA [4]. Use an *in vitro* transcription kit to transcribe the linearized DNA according to the manufacturer's instructions [5]. Load the thermocycler and select the appropriate program [6]. **Note:** For added shots, the descriptions were provided by the author and the shot numbers were provided by the videographer. Corresponding narrations were updated in accordance with the shot description. The numbers and narration are adjusted to accommodate the added shots.
 - 2.3.1. Talent excising and recovering the DNA band from the gel.

Added shot: 2.3.1.1. Excised DNA band put in microtube and talent adding ADB for DNA purification.

Added shot: 2.3.1.2. Talent putting tubes with excised DNA gel in a thermoshaker.

Added shot: 2.3.1.3. Talent removing the tube with the melted gel, transferring it to a column, and centrifuging it to recover linearized DNA.

2.3.2. ~~SCREEN: Show analysis of DNA integrity and linearization of the recovered DNA by agarose gel electrophoresis results.~~

2.3.3. Talent preparing the in vitro transcription reaction.

Added shot: Talent putting individual 0.2 milliliter polymerase chain reaction, or PCR tubes with transcription mix in the thermocycler and selecting the *in vitro* transcription program. (Note: This shot addition was provided by the author but not mentioned in the videographer's note.)

2.4. Treat all transcription reactions with RNase (*R-N-ace*)-free DNase (*D-N-ace*) to remove the DNA template after transcription [1]. Purify the transcript using lithium chloride precipitation, following the manufacturer's instructions [2]. Quantify the purified transcript by spectroscopy [3]. Assess messenger RNA, or mRNA (*M-R-N-A*) integrity by 1 percent agarose gel electrophoresis at 100 volts for 1 hour [4]. Aliquot the mRNA and store it at minus 80 degrees Celsius until use [5].

2.4.1. Talent adding RNase-free DNase enzyme to the transcription reactions.

2.4.2. Talent performing RNA purification by LiCl precipitation method.

2.4.3. SCREEN: 70844_screenshot_2.mp4. 00:32–00:36. Video Editor: Please emphasize the text “ng/μL: 743.168”

2.4.4. Talent loading RNA samples on agarose gel for evaluating messenger RNA integrity. Author's Note: Use shot 2.4.4.1 for this shot.

2.4.5. Talent placing the mRNA aliquots into -80 °C storage.

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

3. Large-scale *Drosophila* Culture and Maintenance

Demonstrator: Mariana Valdés-Barreto

3.1. Set up a cylindrical acrylic cage, approximately 1 meter long and 50 centimeters in diameter, with an approximately 30-centimeter diameter opening in the upper part, for the reference *Drosophila melanogaster* wild-type strain *Oregon-R* [1]. Place a mesh to cover the upper opening and seal it with tape on all four sides [2].

3.1.1. LAB MEDIA: IMG_0232.MOV. 00:00 – 00:10

- 3.1.2. LAB MEDIA: IMG_0232.MOV. 00:11 – 00:20 and LAB MEDIA: IMG_0234.MOV. 00:25 – 00:32; 00:38 – 00:42
- 3.2. Place wet paper on top of the mesh to provide water for the flies, replacing it every day [1]. Keep the front part of the cage open and covered with fabric for access [2].
- 3.2.1. LAB MEDIA: IMG_0235.MOV. 00:00 – 00:19
- 3.2.2. LAB MEDIA: IMG_0235.MOV. 00:31 – 00:43
- 3.3. From the amplified fly stock, place approximately 1,000 young flies inside the cage maintained at 22 to 25 degrees Celsius [1]. To prepare the feed, top the agar-fruit juice plates with a spoonful of rehydrated baker's yeast [2], then add 200 microliters of vinegar uniformly throughout the plate [3]. Carefully insert the feed plate through the front opening of the cage and seal the fabric to contain the flies [4].
- 3.3.1. LAB MEDIA: IMG_0236.MOV. 00:01 – 00:03; 00:13 – 00:27 and LAB MEDIA: IMG_0238.MOV. 00:21 – 00:25
- 3.3.2. LAB MEDIA: IMG_0239.MOV. 00:06 – 00:12
- 3.3.3. LAB MEDIA: IMG_0239.MOV. 01:00 – 01:20
- 3.3.4. LAB MEDIA: IMG_0239.MOV. 01:40 – 01:49; 01:55 – 02:02
- 3.4. To collect the embryos, remove the plates from the cage [1]. ~~Use a stack of sieves of varying sizes to collect embryos between days 3 and 10 for lysate preparation [2].~~
- 3.4.1. Talent removing the plates from the cage.
- 3.4.2. ~~Talent collecting embryos with stacked sieves.~~ **Author's Note:** Shot 3.4.2 is the same as 4.1.4, so it can be removed from here.

4. Translation Lysate Preparation from 0–12 h-old Drosophila Embryos

Demonstrator: Mariana Valdés-Barreto

- 4.1. Prepare embryo lysis buffer [1] and store 1 milliliter aliquots [2] at minus 80 degrees Celsius [3]. Use a sieve to collect the embryos [4], then wash them extensively with tap water, followed by embryo buffer containing 0.9 percent sodium chloride and 0.03 percent Triton X-100 [5].
- 4.1.1. Talent preparing embryo lysis buffer
- 4.1.2. LAB MEDIA: Table 2. *Video Editor: Please use the composition specified for “2) Embryo lysis buffer (for 7 mL)” here.*
- 4.1.3. Talent placing the 1 mL aliquots of the buffer into -80 °C storage.
- 3.4.2. Talent collecting embryos with a sieve. **Note:** Shot 4.1.4 was filmed as 3.4.2, and the shots 3.4.2 and 4.1.5 are combined.

- 4.1.5. Talent washing embryos with tap water followed by embryo buffer.
- 4.2. Dechorionate the embryos for 5 minutes in a solution containing 50 percent embryo buffer and 50 percent household bleach [1]. Wash the embryos extensively with tap water [2], then dry them with paper [3] and weigh them [4].
 - 4.2.1. Talent immersing embryos in the dechoronation solution. **TXT: Household bleach: NaOCl 4–6% and NaOH 0.02–0.1%**
 - 4.2.2. Talent washing the embryos thoroughly. **Videographer's Note: 4.2.2 - is slated as: "4.2.2 & 4.2.3" but it is only 4.2.2**
 - 4.2.3. Talent drying the embryos with paper.
 - 4.2.4. Talent weighing the embryos.
- 4.3. Transfer the packed embryos to a glass tissue grinder homogenizer on ice [1]. Add 1 milliliter of embryo lysis buffer per 1 gram of embryos [2]. Homogenize the embryos on ice with 20 strokes [3]. Transfer the homogenate to 1.5 milliliter microcentrifuge tubes [4].
 - 4.3.1. Talent transferring embryos into the homogenizer. **Author's Note: Shots 4.3.1, 4.3.2 and 4.3.3 were combined.**
 - 4.3.2. Talent adding embryo lysis buffer.
 - 4.3.3. Talent homogenizing the embryos on ice.
 - 4.3.4. Talent transferring homogenate into microcentrifuge tubes.
- 4.4. Centrifuge the homogenate at 15,900 *g* for 20 minutes at 4 degrees Celsius [1]. Recover the interphase in 50 microliter aliquots on ice [2]. Store the aliquots at minus 80 degrees Celsius immediately [3].
 - 4.4.1. Talent placing the tubes into the centrifuge.
 - 4.4.2. Talent collecting the interphase into aliquots.
 - 4.4.3. Talent placing the aliquots into -80°C storage.

5. *In Vitro* Translation and Quantitation of Synthesized Luciferase Protein

Demonstrator: Patricio Reza

Videographer: Please record the computer screen for the shots labeled as **SCREEN** as back-up

- ~~5.1. Set up translation reactions in individual 0.2 milliliter polymerase chain reaction, or PCR (P-C-R) tubes on a thermocycler [1]. Prior to the *in vitro* translation reaction, denature the mRNA by heating to 65 degrees Celsius for 2 minutes [2] and quickly chill on ice for 2 minutes [3].~~ **Note: This step was rearranged and merged with step 5.2**

- 5.1.1. ~~Talent arranging reaction tubes on the thermocycler.~~
- 5.1.2. ~~Talent placing messenger RNA into a heating device.~~
- 5.1.3. ~~Talent transferring messenger RNA onto ice.~~
- 5.2. To set up triplicate reactions in individual 0.2 milliliter polymerase chain reaction, or PCR (*P-C-R*) tubes, prepare a master mix scaled to the required number of reactions prior to adding the required volume in each PCR tube [1]. Then, add 5 microliters of embryo lysate and 30 nanogram of mRNA [2], to a final volume of 17 microliters [3]. ~~Prepare a master mix scaled to the required number of reactions [3]~~ Place the reaction mixture tubes in a thermocycler to denature the mRNA at 65 degrees Celsius for 2 minutes, then chill at 4 degrees for 2 minutes prior to the *in vitro* translation [4].
 - 5.2.3. Talent preparing the scaled master mix. **Note: Shots 5.2.1 and 5.2.3 were combined.**
 - 5.2.1. Talent adding 5 μ L of embryo lysate and 30 ng mRNA.
 - 5.2.2. LAB MEDIA: Table 2: *Video Editor: Please use the composition specified for “4) Reaction cocktail for one reaction (17 μ L)” here.*
 - 5.2.3. ~~Talent preparing the scaled master mix.~~ **Note: This shot was moved before and combined with 5.2.1**
 - 5.2.4. Talent placing the reactions into the thermocycler. **Videographer’s Note: Shot 5.3.1 was combined with 5.2.4**
- 5.3. Next, program the thermocycler with an initial step of 5 minutes at 4 degrees Celsius, followed by 60 minutes at 25 degrees Celsius, and a final 10 minutes at 4 degrees Celsius [1]. To stop the reaction, add 20 microliters of cold 1X passive lysis buffer and mix thoroughly [2]. Place the reactions at room temperature [3] and proceed directly to luciferase quantitation [4].
 - 5.3.1. SCREEN: Display the thermocycler program settings and confirm the temperature profile. **Videographer’s Note: Shot 5.3.1 was not shot as a close-up and is combined with 5.2.4**
 - 5.3.2. Talent adding passive lysis buffer to the reactions. **Videographer’s Note: Shots 5.3.2, 5.3.3, and 5.3.4 were combined. Shot 5.3.4 is when the talent places the samples in the pink rack.**
 - 5.3.3. Talent placing the reactions at room temperature. **(Note: This condition was changed to room temperature by the authors during filming. The narration is updated accordingly.)**
 - 5.3.4. Talent ~~storing samples or~~ preparing them for analysis.
- 5.4. ~~To quantify the synthesized FLuc activity, bring the reaction mixture and the luciferase substrate to room temperature [1].~~ Add 50 microliters of luciferase substrate to 10 microliters of translation reaction [2]. Record relative light units, or RLU (*R-L-U*) using

an automatic or manual luminometer, programmed to collect 3 readings at 3-second intervals [3].

5.4.1. ~~Talent equilibrating reaction samples to room temperature.~~ **Note: This shot was deleted by the authors.**

5.4.2. Talent adding luciferase substrate to the reaction mixture.

5.4.3. Talent loading samples into the luminometer. *Videographer: Please capture the luminometer screen settings configured for 3 measurements collected at 3-second intervals.*

Videographer: Please record the computer screen for the shots labeled as SCREEN as back-up

Results

6. Results

- 6.1. Analysis of efficiency variability among six independent lysate preparations using 0–12-hour-old embryos showed similar translational efficiency, although some variability was present [1]. Lysate number 6 was significantly different from all other lysates [2].
 - 6.1.1. LAB MEDIA: Figure 5. *Video editor: Show the 6 bars labeled Lysate #1 through Lysate #6*
 - 6.1.2. LAB MEDIA: Figure 5. *Video editor: Highlight the bar labelled Lysate #6*
- 6.2. Comparison of translation efficiency among different age embryos showed that lysates from 0–2-hour-old embryos produced the lowest firefly luciferase activity [1], whereas lysates from 0–18-hour-old embryos produced the highest activity [2].
 - 6.2.1. LAB MEDIA: Figure 6. *Video editor: Highlight the bars labelled 0–2 hours.*
 - 6.2.2. LAB MEDIA: Figure 6. *Video editor: Highlight the bars labelled 0–18 hours.*
- 6.3. Comparison of the translation efficiency of 30-percent or 70-percent lysate volumes of *Drosophila* 0–18-hour-old embryo with commercial rabbit reticulocyte and wheat germ lysates showed that rabbit reticulocyte lysates were the most efficient translation system [1], and *Drosophila* embryo lysates were the least efficient [2].
 - 6.3.1. LAB MEDIA: Figure 7. *Video editor: Please highlight the 30% and 70% bars on X-axis label "Rabbit"*
 - 6.3.2. LAB MEDIA: Figure 7. *Video editor: Please highlight the 30% and 70% bars on X-axis label "Fly"*

Pronunciation Guide:

- Template
Pronunciation link: <https://www.merriam-webster.com/dictionary/template>
IPA: /'tem-plət/
Phonetic Spelling: tem·plit
- Plasmid
Pronunciation link: <https://www.merriam-webster.com/dictionary/plasmid>
IPA: /'plaz-məd/
Phonetic Spelling: plaz·mid
- Benchling

Pronunciation link: No confirmed link found

IPA: No verified American IPA found

Phonetic Spelling: bench·ling

- Untranslated

Pronunciation link: <https://www.merriam-webster.com/dictionary/untranslated>

IPA: /,ən-tran(t)s-'lā-təd/

Phonetic Spelling: un·trans·lay·tid

- Luciferase

Pronunciation link: <https://www.merriam-webster.com/dictionary/luciferase>

IPA: /,lū-sə-fə-'rās/

Phonetic Spelling: loo·suh·fuh·rays

- Cistron

Pronunciation link: <https://www.merriam-webster.com/dictionary/cistron>

IPA: /'si-,strän/

Phonetic Spelling: see·stron

- Polyadenylate

Pronunciation link: <https://www.merriam-webster.com/dictionary/polyadenylate>

IPA: /,pä-lē-ə-'de-nə-,lāt/

Phonetic Spelling: pol·ee·uh·den·uh·layt

- Promoter

Pronunciation link: <https://www.merriam-webster.com/dictionary/promoter>

IPA: /prə'mō-tər/

Phonetic Spelling: pruh·moh·ter

- Restriction

Pronunciation link: <https://www.merriam-webster.com/dictionary/restriction>

IPA: /ri-'strik-shən/

Phonetic Spelling: ree·strik·shun

- KpnI

Pronunciation link: No confirmed link found

IPA: No verified American IPA found

Phonetic Spelling: kay·pee·en·one

- Linearize

Pronunciation link: <https://www.merriam-webster.com/dictionary/linearize>

IPA: /'li-nē-ə-,rīz/

Phonetic Spelling: lin·ee·uh·ryze

- Sequencing

Pronunciation link: <https://www.merriam-webster.com/dictionary/sequencing>

IPA: /'sē-kwən-sɪŋ/

Phonetic Spelling: see·kwun·sing

- Electrophoresis

Pronunciation link: <https://www.merriam-webster.com/dictionary/electrophoresis>

IPA: /i-ˌlek-trō-fə-'rē-səs/

Phonetic Spelling: ih·lek·troh·fuh·ree·suhs

- Agarose

Pronunciation link: <https://www.merriam-webster.com/dictionary/agarose>

IPA: /'a-gə-,rōs/

Phonetic Spelling: ag·uh·rohs

- Microtube

Pronunciation link: <https://www.merriam-webster.com/dictionary/microtube>

IPA: /'mī-krō-,tüb/

Phonetic Spelling: my·kroh·toob

- Thermoshaker

Pronunciation link: No confirmed link found

IPA: No verified American IPA found

Phonetic Spelling: ther·moh·shay·ker

- Centrifuge

Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>

IPA: /'sen-trə-,fyüj/

Phonetic Spelling: sen·truh·fyooj

- Thermocycler

Pronunciation link: No confirmed link found

IPA: No verified American IPA found

Phonetic Spelling: ther·moh·sy·kler

- RNase

Pronunciation link: <https://www.merriam-webster.com/dictionary/RNase>

IPA: /'är-,en-,ās/

Phonetic Spelling: ar·en·ays

- DNase

Pronunciation link: <https://www.merriam-webster.com/dictionary/DNase>

IPA: /'dē-,en-,ās/

Phonetic Spelling: dee·en·ays

- Lithium

Pronunciation link: <https://www.merriam-webster.com/dictionary/lithium>

IPA: /'li-thē-əm/

Phonetic Spelling: lith·ee·um

- Chloride
Pronunciation link: <https://www.merriam-webster.com/dictionary/chloride>
IPA: /'klôr-īd/
Phonetic Spelling: klor·ide
- Precipitation
Pronunciation link: <https://www.merriam-webster.com/dictionary/precipitation>
IPA: /pri-ˌsi-pə-'tā-shən/
Phonetic Spelling: pree·sip·uh·tay·shun
- Spectroscopy
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectroscopy>
IPA: /spek-'tră-skə-pē/
Phonetic Spelling: spek·tros·kuh·pee
- Aliquot
Pronunciation link: <https://www.merriam-webster.com/dictionary/aliquot>
IPA: /'a-lə-'kwät/
Phonetic Spelling: al·uh·kwot
- Drosophila
Pronunciation link: <https://www.merriam-webster.com/dictionary/Drosophila>
IPA: /drə-'sä-fə-lə/
Phonetic Spelling: dru·sof·uh·luh
- Melanogaster
Pronunciation link: <https://www.merriam-webster.com/dictionary/melanogaster>
IPA: /mə-'la-nō-'ga-stər/
Phonetic Spelling: muh·lan·oh·gas·ter
- Rehydrated
Pronunciation link: <https://www.merriam-webster.com/dictionary/rehydrate>
IPA: /ˌrē-hī-'drāt/ (rehydrate)
Phonetic Spelling: ree·hy·dray·tid
- Embryo
Pronunciation link: <https://www.merriam-webster.com/dictionary/embryo>
IPA: /'em-brē-ō/
Phonetic Spelling: em·bree·oh
- Lysate

Pronunciation link: <https://www.merriam-webster.com/dictionary/lysate>

IPA: /'lī-, sāṭ/

Phonetic Spelling: ly·sayt

- Dechorionate

Pronunciation link: No confirmed link found

IPA: No verified American IPA found

Phonetic Spelling: dee·kor·ee·uh·nayt

- Homogenizer

Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenizer>

IPA: /hə-'mä-jə-, nī-zər/

Phonetic Spelling: huh·moj·uh·ny·zer

- Homogenate

Pronunciation link: <https://www.merriam-webster.com/dictionary/homogenate>

IPA: /hə-'mä-jə-, nāt/

Phonetic Spelling: huh·moj·uh·nayt

- Interphase

Pronunciation link: <https://www.merriam-webster.com/dictionary/interphase>

IPA: /'in-tər-, fāz/

Phonetic Spelling: in·ter·fayz

- Denature

Pronunciation link: <https://www.merriam-webster.com/dictionary/denature>

IPA: /dē-'nā-chər/

Phonetic Spelling: dee·nay·cher

- Luciferin

Pronunciation link: <https://www.merriam-webster.com/dictionary/luciferin>

IPA: /lü-'si-fə-rən/

Phonetic Spelling: loo·sif·uh·rin

- Luminometer

Pronunciation link: <https://www.merriam-webster.com/dictionary/luminometer>

IPA: /,lü-mə-'nä-mə-tər/

Phonetic Spelling: loo·muh·nom·uh·ter