FINAL SCRIPT: APPROVED FOR FILMING

Submission ID #: 68874
Scriptwriter Name: Sulakshana Karkala
Project Page Link: https://review.jove.com/account/file-uploader?src=21006618

Title: On-site DNA Detection of Trypanosomatid Parasites and *Nosema ceranae* Through Alkaline Lysis Coupled to RPA/CRISPR/Cas12a System

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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? **No**

3. **Filming location:** Will the filming need to take place in multiple locations? **Optionally but not as a requirement**

   50 kms. The laboratory is located in Granada city. The apiaries are located in the town of Lanjarón.

Current Protocol Length
Number of Steps: 17
Number of Shots: 37

Introduction

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

1.1. **Luis Miguel de Pablos:** Our main interests are focused on trying to understand the life cycles, mechanisms of transmission and improving diagnostics of the main pathogens of bees with special interest in trypanosomatid parasites.

1.1.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:4.1*

What are the most recent developments in your field of research?

1.2. **Luis Miguel de Pablos:** The advent of new Isothermal nucleic acid amplification techniques, which enable exponential amplification of nucleic acids at constant temperatures, are allowing the detection of pathogens neither using complex infrastructures nor laboratory equipments.

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

What technologies are currently used to advance research in your field?

1.3. **Jennifer Solano:** In my field of molecular target identification and selection for pathogen detection and diagnosis, we use isothermal RPA combined with CRISPR-Cas12a for rapid, sensitive, and portable molecular diagnostics.

1.3.1. INTERVIEW: Named Talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B.roll:2.7*

What are the current experimental challenges?

1.4. **Jennifer Solano:** Current challenges include optimizing assay sensitivity and specificity, minimizing false positives, simplifying sample preparation, and developing robust, field-deployable diagnostics adaptable to diverse pathogens and conditions.

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

What research gap are you addressing with your protocol?

1.5. **Jessica Carreira de Paula:** We have addressed the need for a fast, simple and field-adaptable method to detect honey bee pathogens, overcoming limitations of traditional molecular diagnostics.

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.

Testimonial Questions (OPTIONAL):

Videographer:

- ***Please ensure that all testimonial shots are captured in a wide-angle format, while also maintaining sufficient headspace, given that the final videos will be rendered in a 1:1 aspect ratio.***
- ***Also, kindly note that testimonial statements will be presented live by the authors, offering their spontaneous perspectives.***

How do you think publishing with JoVE will enhance the visibility and impact of your research?

1.6. **Luis Miguel de Pablos**: (authors will present their testimonial statements live)

Can you share a specific success story or benefit you've experienced—or expect to experience—after using or publishing with JoVE? (This could include increased collaborations, citations, funding opportunities, streamlined lab procedures, reduced training time, cost savings in the lab, or improved lab productivity.)

1.7. **Jessica Carreira de Paula**: (authors will present their testimonial statements live)

Ethics Title Card

All procedures were conducted in accordance with institutional and national guidelines for working with invertebrates and using the lowest number of bees needed for proof of concept

2. Extraction and Isothermal Amplification of Pathogen DNA from Honeybee Tissue Using HotSHOT Lysis

Demonstrator: Jessica Carreira de Paula

2.1. To begin, obtain an anesthetized honeybee **[1].** With a sterile scalpel, dissect the abdomen of the bee **[2].** Transfer the dissected tissue into a 1.5-milliliter microcentrifuge tube **[3]**.

2.1.1. WIDE: Talent holding an anesthetized honeybee.

2.1.2. Talent dissecting the abdomen of the bee using a sterile scalpel on a clean workbench.

2.1.3. Talent transferring the dissected tissue into a labeled 1.5 milliliter microcentrifuge tube.

2.2. Add 400 microliters of HotSHOT *(Hot-shot)* buffer to the tube **[1]**. Then use a disposable pestle to macerate the tissue thoroughly **[2]**. Vortex the tube to further homogenize the tissue **[3]**.

2.2.1. Talent pipetting 400 microliters of HotSHOT buffer into the microcentrifuge tube.

2.2.2. Talent macerating the tissue inside the tube using a disposable pestle.

2.2.3. Talent vortexing the tube for homogenization.

2.3. Next, incubate the tube in a heating block set to 95 degrees Celsius for 10 minutes to lyse the cells **[1]**. After incubation, transfer the tube immediately onto ice to cool **[2]**.

2.3.1. Talent placing the tube in a heating block at 95 degrees Celsius.

2.3.2. Talent transferring the tube onto an ice rack immediately after incubation.

2.4. Neutralize the reaction with 400 microliters of 40 millimolar Tris-HCl resulting in a final concentration of 20 millimolar Tris-HCl in the solution **[1]**.

2.4.1. Talent pipetting 400 microliters of 40 millimolar Tris-HCl into the tube.

2.5. For isothermal amplification by RPA *(R-P-A)*, first prepare the master mix according to the number of reactions required **[1-TXT]**. Use Diethyl Pyrocarbonate -treated water instead of amplification-ready cell lysis for the negative control, and pathogen DNA for the positive control **[2]**.

2.5.1. Talent pipetting reagents into a master mix tube at a clean workstation. **TXT:**

RPA: Recombinase Polymerase Amplification
AND
LAB MEDIA: Table 1
Video Editor: Please play both shots side by side

2.5.2. Talent preparing separate tubes labelled for negative and positive controls.

2.6. Transfer 46.5 microliters of the master mix into 0.2-milliliter PCR tubes **[1]**.

2.6.1. Talent pipetting 46.5 microliters of the master mix into multiple 0.2 milliliter PCR tubes.

2.7. Add magnesium acetate and template DNA to the caps of each PCR tube according to specified volumes **[1]**. Briefly centrifuge the tubes to mix and initiate the RPA reaction **[2]**. Then incubate the tubes in a thermocycler at 39 degrees Celsius for 40 minutes to carry out the RPA amplification reaction **[3]**. Alternatively, incubate the samples using DNA-free Eppendorf tubes in a thermal block **[4]**.

2.7.1. Talent pipetting magnesium acetate and DNA into the caps of PCR tubes.
AND
LAB MEDIA: Table 2
Video Editor: Please play both shots side by side

2.7.2. Talent placing tubes into a centrifuge and starting a brief spin.

2.7.3. Talent placing PCR tubes into a thermocycler and starting incubation.

Added shot: talent inserting PCR tubes into a thermal block.

3. Rapid Molecular Detection of Pathogen DNA in Honey Bees

Demonstrator: Jennifer Solano Parada

3.1. First, prepare a 100 micromolar crRNA *(Crisper-R-N-A)* stock solution **[1].** Reconstitute 10 nanomoles of lyophilized crRNA in 100 microliters of Diethyl Pyrocarbonate-treated water **[2-TXT].**

3.1.1. Shot of vial containing crRNA.

3.1.2. Talent pipetting 100 microliters of DEPC-treated water into a vial containing 10 nanomoles of lyophilized crRNA. **TXT: Store at - 20 °C**

3.2. To prepare a 1-micromolar working solution, mix 1 microliter of the 100 micromolar stock with 99 microliters of Diethyl Pyrocarbonate -treated water in a 0.2-milliliter tube **[1]**.

3.2.1. Talent preparing a 1:100 dilution by mixing 1 microliter of crRNA stock with 99 microliters of DEPC-treated water in a 0.2 milliliter tube.

3.3. To prepare the Cas12a *(Kahs-Twelve-A)* enzyme solution, dilute the 100 micromolar solution to a 1 micromolar working solution by mixing 1 microliter of the enzyme with 99 microliters of the diluent provided in the kit **[1]**.

3.3.1. Talent pipetting 1 microliter of Cas12a enzyme and 99 microliters of diluent into a microcentrifuge tube for dilution.

3.4. Now, prepare a 100 micromolar stock solution of the FAM *(Fam)* probe, by resuspending it in 1 microliter of Diethyl Pyrocarbonate -treated water per 1 nanomole of probe **[1-TXT]**. For the 10-micromolar working solution, mix 90 microliters of DEPC-treated water **[2]** with 10 microliters of the 100-micromolar stock **[3]**.

3.4.1. Talent pipetting DEPC-treated water into a vial containing lyophilized probe, matching the volume to the nanomole content. **TXT: Store at - 20 °C**

3.4.2. Talent pipetting 90 microliters of DEPC-treated water into a tube.

3.4.3. Talent pipetting 10 microliters of the 100 micromolar probe stock to the labelled tube.

3.5. Next, prepare the CRISPR reaction mix excluding the amplicons in a 1.5-milliliter microcentrifuge tube **[1]**. Vortex the suspension to mix well **[2]** then distribute it into PCR tubes **[3].**

3.5.1. Talent preparing a reaction mix in a 1.5 milliliter microcentrifuge tube without adding the amplicons.
AND
LAB MEDIA: Table 3
Video Editor: Please play both shots side by side

3.5.2. Talent vortexing the tube.

3.5.3. Talent pipetting the mixture into multiple PCR tubes.

3.6. Now pipette 4 microliters of RPA amplicon into each tube **[1]**. Place the tubes in a thermocycler set to 37 degrees Celsius **[2]** and incubate for 120 minutes with fluorescence recorded every minute **[3]**. Alternatively, incubate the samples using DNA-free Eppendorf tubes in a thermal block **[4]**.

3.6.1. Talent pipetting 4 microliters of RPA amplicon into labeled PCR tubes.

3.6.2. Talent inserting PCR tubes into a fluorescence-capable thermocycler.

3.6.3. Shot of the program parameters being input into the machine.
Videographer: please capture the instrument screen for this step

Added shot: Talent inserting PCR tubes into a thermo block.

3.7. At the end of the incubation, transfer the reactions into 0.2-milliliter tubes for visualization using a gel documentation system **[1]**.

3.7.1. Talent transferring samples to 0.2 milliliter tubes and placing them into a gel documentation system for imaging.
Videographer: This is an optional step. Please record if necessary

3.8. To detect biotin probe in a lateral flow test, prepare the flow test components in a 1.5-milliliter tube **[1]**. After vortexing the solution, distribute it into a PCR plate **[2].** Then add 4 microliters of amplicon to the respective wells **[3]**.

3.8.1. Talent preparing a mix for lateral flow detection in a 1.5 milliliter tube.
AND
LAB MEDIA: Table 4
Video Editor: Please play both shots side by side

3.8.2. Talent distributing the mix into a PCR tubes.

3.8.3. Talent adding 4 µL amplicon solution into the tubes.

3.9. Incubate the PCR tubes at 37 degrees Celsius for 120 minutes using a thermocycler or thermal block without fluorescence reading **[1]**. Then transfer the samples to 0.2-milliliter tubes **[2]**.

3.9.1. Talent placing the PCR plate into a thermal block and starting the incubation.

3.9.2. Shot of the samples being pipetted into 1.5 mL tubes.

3.10. Then add 50 microliters of running buffer and 10 microliters of the reaction mixture to each tube **[1]**. Insert immunostrips and incubate for 15 minutes at room temperature **[2]**. Read results after 10 minutes of immersion **[3]**.

3.10.1. Talent pipetting running buffer and reaction mix to each 0.2 milliliter tube.

3.10.2. Talent inserting immunostrips into the tubes.

3.10.3. Shot of the immunostrips after incubation.

Results

4. Results

4.1. Recombinase polymerase amplification detected *Lotmaria passim* in 16 out of 32 honeybee samples **[1]**, whereas quantitative polymerase chain reaction identified only 12 positives, demonstrating higher sensitivity of the RPA method **[2]**.

4.1.1. LAB MEDIA: Figure 5A. *Video editor: Please highlight RPA bar*

4.1.2. LAB MEDIA: Figure 5A. *Video editor: Please highlight PCR bar*

4.2. The detection limit of quantitative polymerase chain reaction was approximately 9.6 parasites, as shown by the amplification curve with the lowest visible signal **[1]**.

4.2.1. LAB MEDIA: Figure 5B. *Video editor: Highlight the rightmost curve labeled "9.67 parasites"*

4.3. The detection limit of recombinase polymerase amplification matched that of qPCR **[1]**, detecting as low as 6 picograms corresponding to 96 parasites **[2]**.

4.3.1. LAB MEDIA: Figure 5C. *Video editor: Highlight the curve labeled "96.7 parasites"*

4.3.2. LAB MEDIA: Figure 5D. *Video editor: Highlight the Biotin strips from "10^7" to "10^2".*

Pronunciation Guide:

1. Trypanosomatidae

- **Pronunciation link:** https://www.merriam-webster.com/medical/Trypanosomatidae Merriam-Webster
- **IPA (AmE): /ˌtraɪ.pə.noʊˌsoʊ.məˈtaɪ.di/**
- **Phonetic spelling: *TRY-puh-no-soh-muh-TY-dee***

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2. Trypanosomatid

- **Pronunciation link:** https://www.howtopronounce.com/trypanosomatid How To Pronounce
- **IPA (AmE): /ˌtraɪ.pə.noʊ.soʊˈmæt.ɪd/**
- **Phonetic spelling: *TRY-puh-no-soh-MAT-id***

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3. CRISPR

- **Pronunciation link:** https://dictionary.cambridge.org/us/pronunciation/english/crispr Cambridge Dictionary
- **IPA (AmE): /ˈkrɪspɚ/**
- **Phonetic spelling: *KRIS-per***

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4. Cas12a (Cas Twelve-A)

- **This doesn't always appear in standard dictionaries, but generally is pronounced as:**
  IPA (AmE): /kæs ˈtwelv ə/
  Phonetic spelling: *CAS-TWELV-uh*
  (i.e., "Cas" as in "cass", "12" pronounced "twelve", then "-a" as "uh")

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5. Recombinase Polymerase Amplification (RPA)

- **While full technical terms may not all have dictionary entries, each component can be broken down:**
  - **Recombinase – /ˌriː.kəmˈbɪn.eɪs/ – *REE-kuhm-BIN-ace***
  - **Polymerase – /pəˈlɪm.əˌreɪs/ – *puh-LIM-uh-rays***
  - **Amplification – /æm.plə.fəˈkeɪ.ʃən/ – *am-pluh-fuh-KAY-shun***

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6. Alkaline

- **Pronunciation link: (if needed—for example Merriam-Webster)**
  (Not a specialized term, but often mis-pronounced)
  IPA (AmE): /ˈæl.kə.laɪn/
  Phonetic spelling: *AL-kuh-lyn*

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7. HotSHOT (Hot-Shot) Lysis

- **"HotSHOT" is a trademarked or colloquial term in molecular biology; pronounced like *hot short* or *hot-shot*.**

- **IPA (AmE): /hɑːtʃʌt/ (Hot + SHOT)**
- **Phonetic spelling: *HOT-shot***

- **Lysis**
  - **Pronunciation link: https://dictionary.cambridge.org/pronunciation/english/lysis Cambridge Dictionary**
  - **IPA (AmE): /ˈlaɪ.sɪs/**
  - **Phonetic spelling: *LYE-sis***

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8. Alkaline Lysis

- **IPA (AmE): /ˈæl.kə.laɪn ˈlaɪ.sɪs/**
- **Phonetic spelling: *AL-kuh-lyn LYE-sis***

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9. Lotmaria passim (pathogen name)

- **Lotmaria – /lɒtˈmeɪ.ri.ə/ – *lot-MAY-ree-uh* (approximate, as scientific names can vary but usually follow Latin rules)**
- **passim – /ˈpæs.ɪm/ – *PASS-im***