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Title: Postmortem Diagnosis of Rabies in Animals by the Updated, Multiplexed LN34 Real-Time Reverse Transcription-Polymerase Chain Reaction Assay

Authors and Affiliations:

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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? **No**

Current Protocol Length
Number of Steps: 22
Number of Shots: 55

Introduction

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

1.1. **Crystal Gigante:** Rabies is 100% fatal, requiring rapid, accurate diagnosis for life-saving treatment. Reliable laboratory testing is crucial for rabies control. The CDC developed a step-by-step protocol for rabies testing by quantitative reverse transcriptase PCR, or RT-PCR, using the validated pan-lyssavirus LN34 assay, enabling laboratories to improve diagnostic accuracy and support rabies prevention efforts **[1].**

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

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

1.2. **Crystal Gigante:** For decades, rabies testing relied on the Direct Fluorescence Antibody Test (DFA/FAT). However, many international laboratories now also recommend RT-PCR for rabies diagnosis due to its superior accuracy and sensitivity **[1]**.

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

What are the current experimental challenges?

1.3. **Crystal Gigante:** The DFA test for rabies requires specialized microscopes, cold-chain storage, and trained staff, but PCR and RT-PCR are now more widely used for pathogen testing. Many labs already have PCR and RT-PCR equipment and expertise, and its reagents are readily available from multiple vendors, unlike DFA reagents, which often face shortages **[1]**.

1.3.1. 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?

1.4. **Crystal Gigante:** Cost is a major barrier to rabies testing in regions of the world with the most human rabies cases. CDC has developed a multiplexed LN34 assay that improves quality controls and decreases the cost per sample tested **[1]**.

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

What advantage does your protocol offer compared to other techniques?

1.5. **Crystal Gigante:** RT-PCR is increasingly being used for rabies diagnostics because it is widely used for testing other pathogens, and the results are easy to interpret. But, using a validated test is extremely important. Our protocol provides step-by-step instructions, including details about safety for those performing the test, best practices, and recommended quality controls **[1]**.

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

General NOTE: Please cut any footage showing someone's hand outside of the biological safety cabinet while working under the biological safety cabinet.

2. Sheep Brain Tissue Collection and Homogenization

Demonstrator: Lillian Orciari

NOTE: In this section, please cut any footage showing moving samples with a scalpel. Tissue should be transferred using plastic forceps.

2.1. To begin, wear personal protective equipment, including safety glasses, a closed front gown, and two pairs of gloves **[1]**. Clean and disinfect the work surface with quaternary ammonium compound or QAC *(Q-A-C)* disinfectant for 2 minutes **[2]**. Lay out a plastic-lined absorbent pad on the work surface **[3]**.

2.1.1. WIDE: Establishing shot of talent with personal protective equipment on and walking into the room.

2.1.2. Talent spraying QAC disinfectant onto the work surface.

2.1.3. Talent placing a plastic-lined absorbent pad on the work surface.

2.2. Using a clean single-use scalpel, collect tissue representing a full cross-section of the brain stem and cerebellum *(/ˌsɛr.əˈbɛl.əm/)* **[1-TXT]**.

2.2.1. Talent using a scalpel to collect brain stem and cerebellum tissue. **TXT: Postmortem sheep brain purchased from a local butcher is used** NOTE: After the video, add a still shot showing the scalpel cutting cross-section of the brainstem.

2.3. For homogenization and RNA *(R-N-A)* extraction, finely mince the required tissues using a single-use scalpel **[1]**. Smear the minced tissue with a swab **[2]** and transfer the swab to a tube prefilled with homogenization buffer and beads **[3-TXT]**.

2.3.1. Talent mincing tissue using a scalpel.

2.3.2. Talent smearing the tissue with a swab.

2.3.3. Talent transferring the swab with tissue to the homogenization buffer tube. **TXT: Place remaining tissues in a conical tube for long-term storage**

2.4. Remove and discard the outer glove using QAC disinfectant diluted 1:256 *(one to two*

fifty-six), clean and disinfect the workstation, equipment, and outside of the sample tubes **[1]**. NOTE: VO is modified to compensate for the removed shot.

2.4.1. ~~Talent removing and discarding the outer glove~~. NOTE: Shot removed.

2.4.2. Talent spraying and wiping down the workstation, equipment, and sample tubes.

2.5. Homogenize samples with a mini bead beater for 60 seconds **[1]** and visually inspect the tubes **[2-TXT]**. Allow the samples to sit for 5 minutes at room temperature **[3]**.

2.5.1. Talent placing sample tubes in the bead beater and starting the device.

2.5.2. Talent inspecting sample tubes for large tissue pieces. **TXT: Repeat homogenization if large tissue pieces remain**

2.5.3. Shot of labeled samples containing tubes placed on a working platform. NOTE: After the video, add a still shot showing two tubes, homogenized tissue (left) and non-homogenized (right) with TXT underneath as "Homogenized tissue" and "Non-homogenized tissue"

2.6. Finally, clean and disinfect the workstation, equipment, and outside of sample tubes with QAC disinfectant before proceeding to RNA extraction **[1-TXT]**.

2.6.1. Talent disinfecting the workstation and tubes. **TXT: Alternatively, store samples at -16 °C for long-term**

3. RNA Extraction from Sheep Brain Tissue

Demonstrator: Rene Edgar Condori

3.1. To begin, perform surface decontamination of the biological safety cabinet using QAC *(Q-A-C)* diluted to 1:256 *(one to two fifty-six)* **[1]**. Perform additional cleaning to remove dust or other environmental contaminants **[2]**. NOTE: VO is swapped for the swapped shots.

3.1.2. Talent spraying QAC disinfectant and wiping the BSC work surface. NOTE: Shots 3.1.1 and 3.1.2 are swapped.

3.1.1. WIDE: Talent cleaning the BSC work surface with a cloth to remove dust.

3.2. Lay out a plastic-lined absorbent work pad on the working platform **[1]**. Then, arrange the reagents and the samples in the biosafety cabinet **[2]**.

3.2.1. Talent placing a plastic-lined absorbent pad inside the BSC.

3.2.2. Talent arranging reagents and samples in the BSC.

3.3. Lay out all collection tubes in a clean rack for microcentrifuge tubes **[1]**. Prefill one 1.5 milliliter microcentrifuge tube with 300 microliters of 100% ethanol for each brain sample **[2-TXT]**.

3.3.1. Talent laying out microcentrifuge tubes in a rack.

3.3.2. Talent prefilling 1.5 milliliter tubes with 300 microliters of ethanol. **TXT: For small tissue or bat samples, use 600 µL of ethanol**

3.4. Centrifuge the homogenized sheep brain samples at 10,000 to 16,000 *g* for 2 minutes in a tabletop microcentrifuge **[1]**. Transfer the clear pink supernatant into a new sterile tube containing 100% ethanol **[2]**.

3.4.1. Talent loading sample tubes into a tabletop microcentrifuge.

3.4.2. Talent transferring the clear pink supernatant to a new tube with ethanol.

3.5. Transfer 600 microliters of the ethanol-supernatant mixture to a spin column in a collection tube **[1]**. Centrifuge until the liquid passes through the column **[2]**. Discard the flow-through **[3]** and transfer each column to a new collection tube **[4]**.

3.5.1. Talent transferring the ethanol-supernatant mixture to a spin column.

3.5.2. Talent placing the tube in a centrifuge.

3.5.3. Talent discarding the flow-through.

3.5.4. Talent placing the spin column in a new collection tube.

3.6. Add 400 microliters of RNA *(R-N-A)* prewash buffer to each column **[1]**. Centrifuge the mixture at 10,000 to 16,000 *g* for 30 seconds **[2]**. Discard the flow-through and return each column to the same collection tube **[3]**.

3.6.1. Talent adding RNA prewash buffer to the spin columns.

3.6.2. Talent centrifuging the tubes.

3.6.3. Talent discarding the flow-through and placing the columns in the same tube.

3.7. Next, add 700 microliters of RNA wash buffer to each column **[1]**. Centrifuge at 10,000 to 16,000 *g* for 2 minutes **[2]** and carefully transfer the column into an RNase *(R-N-ace)*-free tube **[3]**. Discard the flow-through and the collection tube **[4]**. Then, remove and discard outer gloves **[5]**.

3.7.1. Talent adding RNA wash buffer to the spin columns.

3.7.2. Talent placing the tubes in a centrifuge.

3.7.3. Talent transferring spin columns to RNase-free tubes.

3.7.4. Talent discarding the collection tubes.

3.7.5. Talent removing and discarding the outer gloves.

3.8. Now, add 50 microliters of DNase *(D-N-ace)* and RNase-free water directly to the column matrix and incubate for 30 seconds **[1]**. Then, centrifuge at 10,000 to 16,000 *g* for 1 minute **[2]**. Carefully transfer RNA to a new screw-top flat-bottom accession-labeled microcentrifuge tube for RT-PCR *(R-T-P-C-R)* assay **[3-TXT]**.

3.8.1. Talent adding water to the spin columns to elute RNA.

3.8.2. Talent centrifuging the spin columns.

3.8.3. Talent transferring RNA to a labeled microcentrifuge tube. **TXT: Alternatively, store at -70 °C**

4. Pan-Lyssavirus LN34 Real-Time RT-PCR Assay

Demonstrators: Vaughn Wicker and Kimberly Wilkins

4.1. To begin, thaw one-step RT-PCR *(R-T-P-C-R)* buffer, no template control, nuclease-free water, primers, and probes on ice in the master mix preparation space. Place one-step RT-PCR enzyme on ice **[1].** NOTE: VO is modified for the removed shots**.**

4.1.1. WIDE: Establishing shot of talent placing the labeled container reagents on ice.

4.1.2. ~~Talent vortexing the tube containing primers, buffers, and probes~~. NOTE: Shot removed

4.1.3. ~~Talent placing enzyme tube and RNA sample tubes on ice~~. NOTE: Shot removed.

4.2. Label one microcentrifuge tube per assay for LN34 *(L-N-thirty-four)* and βA *(beta-Actin)* **[1]**. Calculate the volume of each reagent for the LN34 and βA mastermixes **[2]**. Designate wells for each sample in triplicate for the LN34 assay and singlicate for the βA assay using a 96-well plate map **[3]**.

4.2.1. Talent labeling tubes for LN34 and βA assays.

4.2.2. LAB MEDIA: Table 2

4.2.3. Talent marking the wells of a 96-well plate for the assay.

4.3. After vortexing and spinning, dispense 23 microliters of LN34 assay master mix into each LN34-assigned well **[1]**. Similarly, add 23 microliters of βA assay master mix into each βA-labeled well **[2]**.

4.3.1. Talent dispensing LN34 master mix from the labeled tube into labeled wells.

4.3.2. Talent adding βA master mix from the labeled tube into labeled wells

4.4. To set up negative template control reactions, add 2 microliters of PCR-grade water into each negative template control well **[1]**. Place the 96 well plate on ice **[2]**. Clean the workstation with 70% ethanol [**3]**.

4.4.1. Talent transferring PCR-grade water into NTC wells.

4.4.2. Talent placing the plate on ice.

4.4.3. Talent wiping the workstation with 70% ethanol.

4.5. Now, at the template addition workspace, thaw RNA samples and positive control single use aliquot of artificial RNA on ice **[1]**. After vortexing, briefly centrifuge tubes containing RNA samples **[2]**. Pipette 2 microliters of sample and positive control RNA into the corresponding well **[3]**.

4.5.1. Talent placing RNA samples and positive controls on ice. ~~**TXT: RNA samples extracted from sheep brains and previously extracted bat brain tissues were used**~~ NOTE: Text overlay removed per author's request**.**

4.5.2. Talent placing RNA sample tube in a centrifuge.

4.5.3. Talent adding RNA into designated wells.

4.6. After adding all samples, place the optical adhesive cover over the wells to seal them completely **[1]**. Centrifuge the plate at 500 *g* for 1 minute at room temperature **[2]**.

4.6.1. Talent sealing the 96-well plate with an optical adhesive cover.

4.6.2. Talent placing the sealed plate in a centrifuge.

4.7. Place the sealed plate into a real-time or quantitative PCR instrument calibrated for FAM *(fam as in 'FAM' ily)* and VIC/HEX *(Vic-Hex)* reporter dyes **[1]**. Set the instrument to the appropriate cycling parameters **[2-TXT]**.

4.7.1. Talent loading the sealed plate into the PCR machine.

4.7.2. Display of the PCR cycling parameters on the instrument. **TXT: Store RNA at ≤ -70 °C for long-term storage** *Video Editor: Split screen to show Table 3*

4.8. After the run is completed, set the threshold values to 0.2 for LN34 and 0.05 for beta actin. Check the control sample curves for any quality issues **[1]**.

4.8.1. Talent performing analyzation steps on computer and reviewing different views of data.

Results

5. Results

5.1. The LN34 assay showed successful amplification curves, with positive results crossing the threshold at distinct cycle threshold values when viewed on a logarithmic scale **[1]** and sigmoidal amplification curves on a linear scale for both LN34 and β *(beta)*-actin **[2]**. Negative results exhibited flat lines without amplification **[3]**.

5.1.1. LAB MEDIA: Figure 2A and 2C

5.1.2. LAB MEDIA: Figure 2B and 2D

5.1.3. LAB MEDIA: Figure 2E and 2F

5.2. Abnormal amplification curves were identified, with some displaying linear rather than sigmoidal increases in fluorescence, indicating atypical results **[1]**. The corresponding multicomponent plots showed irregular wavy fluorescence signals rather than expected smooth curves, highlighting the need to analyze amplification plots instead of relying solely on threshold cycle values **[2]**.

5.2.1. LAB MEDIA: Figure 3A

5.2.2. LAB MEDIA: Figure 3B

Pronunciation Guide:

Quaternary

- **Pronunciation link (for "quaternary"):** https://www.merriam-webster.com/dictionary/quaternary
- **IPA:** /ˈkwɑːrtəˌneri/
- **Phonetic Spelling:** KWAR-tuh-nair-ee

Scalpel

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/scalpel
- **IPA:** /ˈskæl.pəl/
- **Phonetic Spelling:** SKAL-puhl

Homogenization

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/homogenization
- **IPA:** /həˌmɑːdʒənəˈzeɪʃən/
- **Phonetic Spelling:** huh-MAH-juh-nuh-ZAY-shun

Microcentrifuge

- **Pronunciation link:** https://www.howtopronounce.com/microcentrifuge
- **IPA:** /ˌmaɪ.kroʊˈsɛn.trəˌfjuːdʒ/
- **Phonetic Spelling:** MY-kroh-SEN-truh-fyooj

Supernatant

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/supernatant
- **IPA:** /ˌsuː.pɚˈnæt.ənt/
- **Phonetic Spelling:** SOO-per-NA-tuhnt

Lyssavirus

- **Pronunciation link:** https://www.howtopronounce.com/lyssavirus
- **IPA:** /ˈlɪsəˌvaɪrəs/
- **Phonetic Spelling:** LIH-suh-vy-ruhs

Primers

- **Pronunciation link:** https://www.merriam-webster.com/dictionary/primer (biotech meaning)
- **IPA:** /ˈpraɪmɚ/
- **Phonetic Spelling:** PRY-mer