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Title: Establishment and Quantification of *De Novo* Lytic Infection by Cell-Free Kaposi's Sarcoma-Associated Herpesvirus

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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? **Yes, all done**

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**

   They are on the same floor, about 50 meters apart.

Current Protocol Length
Number of Steps: 18
Number of Shots: 35

Introduction

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

1.1. **Janvhi Machhar:** Tractable and reproducible systems to model KSHV primary lytic infection are limited. We established a reliable and robust *in vitro* platform for modeling KSHV de *novo* lytic infection.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 1*

What are the current experimental challenges?

1.2. **Janvhi Machhar:** It's challenging to model KSHV lytic infection because of its strong latency bias, and the few primary cells that support it are delicate and have a limited lifespan, which severely limits reproducibility and scalability.

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

What significant findings have you established in your field?

1.3. **Janvhi Machhar:** We developed a tractable and reproducible model in HCT116 cells that captures efficient KSHV lytic replication during primary infection, offering a unique platform to study early events in herpesvirus replication.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B roll: Figure 2*

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

Protocol

NOTE: LAB MEDIA/SCOPE timestamps for protocol were added at the postshoot stage. Please contact the postshoot note integrator (Sulakshana Karkala) for queries regarding lab media

2. Production of Cell-Free BAC16-Derived KSHV

Demonstrator: Qing Zhu

2.1. To begin, seed five T75 *(T-Seventy)* flasks with Dox-inducible iSLK *(I-S-L-K)* cells containing wild-type KSHV *(K-S-H-V)* BAC16 *(Bac-Sixteen)* at approximately 80 percent confluency **[1]**. Incubate the flasks at 37 degrees Celsius in a humidified incubator with 5 percent carbon dioxide for 24 hours **[2]**.

2.1.1. WIDE: Talent seeding T75 flasks with Dox-inducible iSLK cells using a pipette.

2.1.2. Talent placing the flasks in a 37 degrees Celsius incubator set at 5 percent carbon dioxide.

2.2. Add 1 microgram per milliliter of doxycycline and 1 millimolar sodium butyrate to each flask to induce lytic reactivation **[1-TXT]**.

2.2.1. Talent pipetting doxycycline and sodium butyrate into each flask. **TXT: Incubate for 76 h and 96 h**

2.3. Using phase-contrast microscopy, monitor the cells daily **[1]**. Collect the supernatant once more than 90 percent of cells show rounding, detachment from the flask surface, or visible lysis **[4]**.

2.3.1. LAB MEDIA: 2.3.1-shot-of-the-cell.tif.

2.3.2. Talent collecting the supernatant from the flasks after confirming cell rounding and lysis.

2.4. Then, centrifuge the collected supernatants at 1,500 *g* for 10 minutes at 4 degrees Celsius to remove cellular debris **[1]**. Filter the resulting supernatant through a 0.45 micrometer polyethersulfone membrane filter without disturbing the pellet **[2]**.

2.4.1. Talent placing the supernatant tubes into a centrifuge set at 1,500 g and 4 degrees Celsius.

2.4.2. Talent filtering the supernatant through a 0.45 micrometer polyethersulfone

membrane filter.

2.5. Transfer the filtered supernatants to ultracentrifuge tubes **[1]**. Centrifuge the tubes at 25,000 *g* for 3 hours at 4 degrees Celsius using an SW28 *(S-W-Twenty-Eight)* rotor to pellet the viral particles **[2]**.

2.5.1. Talent transferring filtered supernatant into ultracentrifuge tubes.

2.5.2. Talent loading the tubes into an ultracentrifuge with an SW28 rotor and setting it to 25,000 g at 4 degrees Celsius.
Videographer's Note: Settings on the centrifuge were incorrect for this shot. Please blur or crop

2.6. Carefully discard the supernatant into a container with 10 percent bleach **[1]**. Resuspend the viral pellet in 1 milliliter of serum-free DMEM *(D-M-E-M)* **[2]**.

2.6.1. Talent pouring off the supernatant into a bleach-containing waste container.

2.6.2. Talent resuspending the viral pellet in 1 milliliter of serum-free DMEM using a pipette.

3. De Novo Infection of HCT116 Cells

3.1. Seed 1 × 10^5 HCT116 *(H-C-T-One-One-Six)* cells per well in a 12-well tissue culture plate **[1]**. Incubate the plate at 37 degrees Celsius in a humidified incubator with 5 percent carbon dioxide for 24 hours **[2]**.

3.1.1. Talent seeding 1 × 10^5 HCT116 cells in each well of a 12-well plate using a pipette.

3.1.2. Talent placing the culture plate inside a 37 degrees Celsius, 5 percent carbon dioxide incubator.

3.2. Dilute the concentrated virus stock in 250 microliters of pre-warmed, serum-free DMEM to achieve a multiplicity of infection of 10 **[1]**.

3.2.1. Talent pipetting virus stock into a microcentrifuge tube containing 250 microliters of pre-warmed, serum-free DMEM.

3.3. Aspirate the culture medium from each well **[1]** and add 250 microliters of the diluted virus directly onto the cells **[2]**.

3.3.1. Talent aspirating the culture medium from each well using a vacuum aspirator.

3.3.2. Talent pipetting 250 microliters of the virus dilution into each well.

3.4. Centrifuge the plate at 1,500 *g* for 1 hour at 30 degrees Celsius to synchronize infection

[1]. Immediately transfer the plate to a 37 degrees Celsius incubator and continue incubation for 1 hour **[2]**.

3.4.1. Talent placing the plate into a centrifuge and setting it to 1,500 g and 30 degrees Celsius for 1 hour.

3.4.2. Talent moving the plate from the centrifuge into a 37 degrees Celsius incubator.

3.5. Aspirate the viral inoculum from each well **[1]**. Wash the cells three times with pre-warmed PBS to remove residual virus **[2]**. Add 1 milliliter of complete DMEM to each well **[3]**.

3.5.1. Talent aspirating the inoculum from the wells.

3.5.2. Talent washing each well three times with pre-warmed phosphate-buffered saline using a pipette.

3.5.3. Talent adding 1 milliliter of complete DMEM to each well. **TXT: Monitor GFP expression at 36 h, 60 h, and 96 h post-infection**

3.6. Then, collect 1 milliliter of culture supernatant from each well **[2]**.

3.6.1. Talent pipetting 1 milliliter of culture supernatant from each well into labeled collection tubes.

4. Infectious Units Assay (IU assay)

4.1. Seed 1 × 10^4 SLK cells per well in a 96-well plate 24 hours before infection **[1-TXT]**.

4.1.1. WIDE: Talent seeding SLK cells into a 96-well plate using a multichannel pipette. **TXT: Seed cells to reach ~60–70% confluence on infection day**

4.2. Label nine 1.5-milliliter microcentrifuge tubes for a serial two-fold dilution series **[1]**.

4.2.1. Talent labeling each microcentrifuge tube with dilution ratios using a permanent marker.

4.3. Add 200 microliters of virus supernatant to Tube 1 **[1]**. Add 100 microliters of pre-warmed, serum-free DMEM to each of Tubes 2 through 9 **[2]**. Begin the serial dilution by transferring 100 microliters from Tube 1 to Tube 2 and mixing thoroughly by pipetting **[3-TXT]**.

4.3.1. Talent pipetting virus supernatant into Tube 1.

4.3.2. Talent adding DMEM into Tubes 2 through 9.

4.3.3. Talent transferring 100 microliters from Tube 1 to Tube 2 and mixing. **TXT: Repeat the process sequentially through tube 9**

Videographer's Note: Take 2 is better shot for 4.3.3

4.4. Now, aspirate the culture medium from SLK cells **[1]**. Add 100 microliters of each virus dilution to the designated wells, using 27 wells in total **[2]**.

4.4.1. Talent aspirating media from the 96-well plate using a multichannel aspirator.

4.4.2. Talent dispensing 100 microliters of each dilution into designated triplicate wells.

4.5. Centrifuge the 96-well plate at 1,500 *g* for 1 hour at 30 degrees Celsius to promote viral adsorption **[1]**. Transfer the plate to a 37 degrees Celsius incubator with 5 percent carbon dioxide and incubate for an additional hour **[2]**.

4.5.1. Talent placing the 96-well plate in a centrifuge set at 1,500 g and 30 degrees Celsius.

4.5.2. Talent transferring the plate from the centrifuge to a 37 degrees Celsius carbon dioxide incubator.

4.6. Remove the inoculum from each well **[1]**. Then, gently add 100 microliters of complete DMEM to each well **[2]**. Incubate the plate for 24 hours at 37 degrees Celsius **[3]**. Then, analyse the GFP positive cells **[4].**

4.6.1. Talent aspirating the inoculum from all wells in the 96-well plate.

4.6.2. Talent adding 100 microliters of complete DMEM to each well using a multichannel pipette.

4.6.3. Talent placing the plate back in the 37 degrees Celsius incubator.

4.6.4. LAB MEDIA: 4.6.4-shot-of-the-GFP-pisitive-cells.tif

Results

5. Results

5.1. A two-fold serial dilution of BAC16-derived virus stock showed a progressive decrease in the number of GFP–positive SLK cells, confirming a measurable and dilution-sensitive infectious titer **[1].** The calculated infectious titer of the BAC16-derived virus stock was approximately 1.3 million infectious units per milliliter **[2]**.

5.1.1. LAB MEDIA: Figure 2A. *Video editor: Highlight the downward trend in the bar graph, moving left to right across the dilution factors*.

5.1.2. LAB MEDIA: Figure 2B.

5.2. GFP expression in HCT116 cells increased over time following de novo infection, with the most intense signal observed at 60 hours post-infection **[1],** indicating robust viral replication at that timepoint **[2].**

5.2.1. LAB MEDIA: Figure 2C. Video editor: *Highlight the three green micrographs under the 36 h, 60 h, and 96 h timepoints, emphasizing the brightest image at 60 h*.

5.2.2. LAB MEDIA: Figure 2D. *Video editor: Highlight the corresponding three green-stained SLK cell images in the right column labeled 36 h, 60 h, and 96 h, emphasizing the most densely green image at 60 h.*

5.3. Flow cytometry analysis confirmed a peak in GFP–positive SLK cells at 60 hours post-infection, reaching 74.4% positivity **[1],** followed by a decrease to 62.7% at 96 hours **[2].**

5.3.1. LAB MEDIA: Figure 2E. *Video editor: Highlight the orange peak labeled "60 h" with the 74.4% annotation.*

5.3.2. LAB MEDIA: Figure 2E. *Video editor: Highlight the green peak labeled "96 h" with the 62.7% annotation.*

5.4. Quantitative PCR of viral DNA in supernatants showed a sharp increase in viral genome copies, peaking at 60 hours post-infection and declining thereafter **[1].**

5.4.1. LAB MEDIA: Figure 2F.

Pronunciation Guide:

1. **Kaposi's sarcoma-associated herpesvirus**
    - **Pronunciation link:** Cambridge Dictionary provides the pronunciation for *Kaposi's sarcoma*; the rest (herpesvirus) follows standard pronunciation patterns YouTube+11Cambridge Dictionary+11Wikipedia+11
    - **IPA (American):** /kəˌpoʊ.siːz sɑːrˈkoʊ.mə-əˈsoʊ.siˌeɪ.tɪd ˈhɜr.pizˌvɜr.əs/
    - **Phonetic Spelling:** kuh-POH-seez sar-KOH-muh-uh-soh-see-ay-tud HER-peez-vur-us
2. **KSHV (Kaposi's sarcoma-associated herpesvirus)**
    - **Pronunciation link:** Defined by its abbreviation; "K-S-H-V" pronounced as individual letters, matching the full term Cambridge Dictionary+1How To Pronounce+3Wikipedia+3Wikipedia+3
    - **IPA:** /keɪ-ɛs-eɪtʃ-viː/
    - **Phonetic Spelling:** kay-ess-aych-vee
3. **BAC16 (BAC Sixteen derived KSHV)**
    - **Pronunciation link:** Cambridge Dictionary for "BAC" covers the abbreviation Wikipedia+9Cambridge Dictionary+9YouTube+9How To PronouncePronounce Names+6DigiKey+6L-com+6Cambridge Dictionary
    - **IPA:** /biː.eɪ.siː ˈsɪkˌstiːn/
    - **Phonetic Spelling:** bee-ay-see SIX-teen
4. **Dox-inducible**
    - **Pronunciation link:** While not listed in dictionaries, derived from "doxycycline-inducible"—standard pronunciation rules apply
    - **IPA:** /dɒk.sɪnˈdju.sə.bəl/ (American: /dɑk.sɪnˈdju.sə.bəl/)
    - **Phonetic Spelling:** dok-sin-DYOO-suh-buhl
5. **Ultracentrifuge (Ultracentrifugation)**
    - **Pronunciation link:** The base word *ultracentrifuge* is pronounced per standard patterns
    - **IPA:** /ˌʌl.trəˌsɛn.trəˈfjuːdʒ/
    - **Phonetic Spelling:** uhl-truh-sen-truh-FYOODGE
6. **iSLK (cell line name)**
    - **Pronunciation link:** Treated as separate letters in common lab usage
    - **IPA:** /aɪ-ɛs-ɛl-keɪ/
    - **Phonetic Spelling:** eye-ess-el-kay
7. **HCT116 (cell line name)**
    - **Pronunciation link:** Standard scientific naming; pronounced as letters and numbers
    - **IPA:** /eɪtʃ-si-ti-wʌn-wʌn-sɪks/
    - **Phonetic Spelling:** H-C-T-one-one-six
8. **Multiplicity of Infection (MOI)**
    - **Pronunciation link:** Standard scientific phrase
    - **IPA:** /ˌmʌl.təˈplɪs.ɪ.ti əv ɪnˈfɛk.ʃən/
    - **Phonetic Spelling:** mul-tuh-PLIS-ih-tee of in-FEK-shun

9. **Phase-contrast microscopy**
    - **Pronunciation link:** Standard compound term in microscopy
    - **IPA:** /feɪz-ˈkɑn.træst maɪˈkrɑs.kə.pi/
    - **Phonetic Spelling:** faz-KON-trast my-KRAS-kuh-pee
10. **Serial two-fold dilution**
    - **Pronunciation link:** Common lab phrase
    - **IPA:** /ˈsɪə.ri.əl tuː-foʊld daɪˈluː.ʃən/
    - **Phonetic Spelling:** SEER-ee-uhl too-fold dy-LOO-shun