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Title: Amikacin Protection Assay for Quantification and Visualization of *Escherichia coli* Cell Invasion

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**
- 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: 54

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 are slightly edited in accordance with journal guidelines.

INTRODUCTION:

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

- 1.1. **Sydney Field:** We focus on characterizing how Adherent-Invasive *E. coli* invade and survive within intestinal epithelial cells and identifying bacterial factors that drive this process.

- 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. **Sydney Field:** Gentamicin resistant strains limit traditional invasion assays. This amikacin-based protocol enables accurate quantification of intracellular bacteria.

- 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. **Sydney Field:** This protocol enables accurate quantification of intracellular bacteria and assessment of bacterial invasion in epithelial cells.

- 1.3.1. 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?**~~

- 1.4. **Sydney Field:** Additional methods such as immunofluorescence microscopy can be used to further characterize bacterial invasion and validate intracellular localization.

- 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. **Sydney Field:** Future studies can identify novel virulence factors, define intracellular survival mechanisms, and determine host pathways targeted during Adherent-Invasive *E. coli* infection.

- 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

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

2. Preparation of Adherent-Invasive *E. coli* (AIEC) and Infection Media

Demonstrator: Sydney Field

- 2.1. To begin, gather all reagents and media required for the invasion assay [1].
 - 2.1.1. WIDE: Talent standing at the bench and arranging media, reagent tubes/bottles.
- 2.2. Now, retrieve the frozen glycerol stock of adherent-invasive *Escherichia coli* or AIEC strain from the minus 80 degrees Celsius freezer [1]. Using a sterile wooden stick, gently scrape bacteria from the stock [2] and streak it onto a Luria-Bertani or LB agar plate to obtain single colonies [2]. Incubate the plate at 37 degrees Celsius overnight [3].
 - 2.2.1. Talent removing the glycerol stock from the freezer and placing on a table.
 - 2.2.2. Talent scraping bacteria from a frozen glycerol stock using a sterile wooden stick.
 - 2.2.3. Talent streaking bacteria onto a LB agar plate for single colonies.
 - 2.2.4. Talent placing the agar plate into an incubator.
- 2.3. The next day, using a sterile wooden stick, pick a single colony from the agar plate [1]. Inoculate the colony into 3 milliliters of LB broth [2] and incubate the culture at 37 degrees Celsius at 200 revolutions per minute overnight [3].
 - 2.3.1. Talent picking a single bacterial colony from the agar plate using a sterile wooden stick.
 - 2.3.2. Talent inoculating the colony into a tube containing 3 milliliters of LB broth.
 - 2.3.3. Talent placing the culture tube into a shaking incubator.
- 2.4. Then, perform a 1 to 10 dilution of the overnight culture in 1 milliliter of LB broth [1]. Measure the optical density at 600 nanometers of the diluted culture using a spectrophotometer [2].
 - 2.4.1. Talent adding overnight bacterial culture into LB for dilution.
 - 2.4.2. Talent placing the sample into a spectrophotometer to measure optical density

at 600 nanometers.

2.5. Transfer 100 microliters of the overnight culture into a 1.5-milliliter microcentrifuge tube [1] and centrifuge at 6000 *g* for 10 minutes at 4 degrees Celsius [2].

2.5.1. Talent pipetting 100 microliters of bacterial culture into a 1.5 milliliter microcentrifuge tube.

2.5.2. Talent placing the tube into a centrifuge.

2.6. Aspirate the supernatant carefully without disturbing the cell pellet [1] and resuspend the pellet in 1 milliliter of serum and antibiotic-antimycotic-free media [2].

2.6.1. Talent aspirating supernatant from the microcentrifuge tube while leaving the pellet undisturbed.

2.6.2. Talent resuspending the bacterial pellet in media by pipetting.

2.7. Calculate the required volume of bacterial suspension to achieve a multiplicity of infection or MOI of 10 using the provided formula [1-TXT].

2.7.1. Talent making calculations and writing in a note book.

TXT:

$$\text{Volume of bacterial suspension } (\mu\text{L}) = \frac{10 \text{ MOI} \times (1 \times 10^5 \text{ host cells}) \times 1000}{(\text{OD}_{600} \text{ measured} \times 10 \text{ dilution factor}) \times (2.4 \times 10^9)}$$

2.8. To prepare the infection media, transfer the calculated volume of bacterial suspension into a sterile 1.5-milliliter microcentrifuge tube [1] and make up the volume to 1 milliliter with serum and antibiotic-antimycotic-free media [2]. Mix gently by pipetting up and down several times [3].

2.8.1. Talent pipetting the calculated volume of bacterial suspension into a microcentrifuge tube.

2.8.2. Talent adding media to the tube.

2.8.3. Talent mixing the infection media by pipetting up and down.

3. Plating Input AIEC Inoculum for Pre-Infection Quantification

- 3.1. Prepare a 96-well plate to determine the colony forming units per milliliter of the diluted overnight bacterial culture [1] by performing 10-fold serial dilutions in sterile 1x PBS [2].
 - 3.1.1. Talent placing a 96 well plate, diluted bacterial overnight culture and PBS containing tube on the bench.
 - 3.1.2. Talent adding PBS to the wells of the plate.
- 3.2. Transfer 200 microliters of the diluted overnight culture into the first well of the plate [1].
 - 3.2.1. Talent pipetting culture into the first well of a 96 well plate.
- 3.3. Pipette 20 microliters of the culture into the next row containing 180 microliters of 1x PBS [1]. Mix by pipetting up and down 10 times [2] and repeat the dilution process until reaching 10 to the power of minus 6 using a fresh pipette tip each time [3].
 - 3.3.1. Talent transferring culture into the next well containing phosphate buffered saline.
 - 3.3.2. Talent mixing the dilution by pipetting up and down.
 - 3.3.3. Talent continuing serial dilutions to the next wells and across the plate with fresh tips.
- 3.4. Now, plate 10 microliters of spot dilutions in triplicate from the 10 to the power of minus 2 to 10 to the power of minus 6 dilutions onto LB agar plates [1]. Allow the spots to absorb into the agar near a flame [2].
 - 3.4.1. Talent spotting diluted samples onto agar plates.
 - 3.4.2. Shot of placing the agar near a flame for quick absorption.
- 3.5. Incubate the plates overnight at 27 to 30 degrees Celsius to prevent colony overgrowth and obtain clear individual colonies [1].
 - 3.5.1. Talent placing agar plates into an incubator.

4. Infection of Caco-2 Epithelial Cell Monolayers with AIEC

4.1. Now, remove the healthy, confluent Caco-2 epithelial cell monolayers cultured in a collagen-coated 24-well culture plate from the incubator [1] and aspirate the complete media from each well [2].

4.1.1. Talent removing a 24 well plate from the incubator and placing in a hood.

4.1.2. Talent aspirating media from the wells of the plate.

4.2. Pipette 1 milliliter of serum and antibiotic antimycotic-free media into each well [1]. Aspirate the media to remove traces of media [2] and repeat the wash one more time [3] for a total of two washes [4].

4.2.1. Talent adding media to each well of the plate.

4.2.2. Talent aspirating media from the wells.

4.2.3. Talent adding the media to each well of the plate again.

Added shot 4.2.3A. Talent aspirating media from the wells again.

4.3. Now, add 1 milliliter of the previously prepared infection media containing AIEC into each well of the 24 well plate containing the Caco-2 cells [1].

4.3.1. Talent adding infection media to each well of the plate.

4.4. Centrifuge the 24 well plate at 500 g for 15 minutes at room temperature to synchronize bacterial contact with the epithelial monolayer [1] and incubate the plate at 37 degrees Celsius with 5 percent carbon dioxide for 3 hours to allow bacterial invasion to occur [2].

4.4.1. Talent placing the 24 well plate into a centrifuge.

4.4.2. Talent placing the plate into an incubator.

5. Amikacin Protection Treatment and Recovery of Intracellular Bacteria

5.1. Prepare antibiotic-containing media by adding 240 micrograms per milliliter of amikacin to pre-warmed serum and antibiotic antimycotic-free media [1].

5.1.1. Talent adding amikacin to media and mixing under a laminar hood.

5.2. After the infection period, aspirate the media from the Caco-2 cell monolayers [1] and wash each well with 1 milliliter of serum and antibiotic antimycotic-free media [2].

Aspirate the media [3] and repeat the wash once [4] more for a total of two washes [5].

5.2.1. Talent aspirating media from the wells after infection.

5.2.2. Talent adding fresh media to each well.

5.2.3. Talent removing the fresh media from each well again.

Added shot 5.2.3A. Talent adding fresh media to each well again.

Added shot: Reuse 5.2.3

5.3. Then, pipette 1 milliliter of 240 micrograms per milliliter amikacin-containing media into each well [1]. In parallel, treat another set of cells with 100 micrograms per milliliter gentamicin for comparison [2] and incubate the cells at 37 degrees Celsius with 5 percent carbon dioxide for 1 hour to eliminate extracellular bacteria [2].

5.3.1. Talent adding amikacin antibiotic containing media to each well.

5.3.2. Talent adding gentamicin to another plate with cells.

5.3.3. Talent placing the plates into an incubator.

5.4. After incubation, aspirate the media from each well [1]. Add 1 milliliter of PBS to wash away adherent bacteria [2]. Aspirate the PBS [3] and repeat the wash one more time [4] for a total of two washes [5].

5.4.1. Talent aspirating media from the plate.

5.4.2. Talent adding phosphate buffered saline to each well.

5.4.3. Talent aspirating PBS from the well.

Added shot 5.4.3A. Talent adding fresh PBS to each well.

Added shot: Reuse 5.4.3

5.5. Now, add 200 microliters of 1 percent Triton-X-100 diluted in PBS into each well [1] and incubate on an orbital shaker at 90 revolutions per minute for 10 minutes to lyse epithelial cells [2].

5.5.1. Talent adding Triton X 100 solution to each well.

5.5.2. Talent placing the plate onto an orbital shaker.

5.6. Pipette up and down approximately 30 times per well to detach the cells from the plate [1]. Next, perform 10-fold serial dilutions of the cell lysates in a 96-well plate using PBS [2].

5.6.1. Talent pipetting up and down in the wells to detach cells.

5.6.2. Talent preparing serial dilutions of cell lysates in a 96 well plate by aspirating and adding lysates from previous wells to next wells.

5.7. Plate 10 microliters of spot dilutions in triplicate from the undiluted to 10 to the power of minus 3 dilutions onto LB agar plates [1]. After the spots dry, incubate the plates overnight at 27 to 30 degrees Celsius to obtain clear individual colonies [3].

5.7.1. Talent spotting cell lysate dilutions onto agar plates.

5.7.2. Talent placing plates for incubation after spot absorption.

5.8. Following incubation, remove the plates and count well-isolated colonies from the appropriate spot dilutions containing more than 7 colonies per spot across the triplicates [1]. Finally, calculate the inoculum and retrieved colony forming units per milliliter, and percent invasion using the equations [3 and 4].

5.8.1. Talent removing the culture plates from the incubator.

~~5.8.2. Talent opening the plate and showing/pointing to the clear colonies in certain spots.~~

~~5.8.3. Talent looking at the plate and writing the number in a notebook.~~

5.8.4. TEXT on 5.8.3's BACKGROUND

$$CFU_{\text{Inoculum/Well}} = (\text{Average number of colonies})(\text{Dilution factor})(\text{Volume from infection media in } \mu\text{L}) \times 100$$

$$CFU_{\text{Retrieved}} = (\text{Average number of colonies})(\text{Dilution factor}) \times 100$$

$$\text{Invasion (\%)} = \frac{CFU_{\text{Retrieved}}}{CFU_{\text{Inoculum}}} \times 100$$

Results

6. Results

6.1. Treatment with either gentamicin or amikacin following infection of Caco-2 cell monolayers with invasive AIEC strain NRG857c (*N-R-G-8-5-7-C*) or the non-invasive *Escherichia coli* DH5 alpha control strain resulted in similar invasion profiles [1]. These findings indicated that amikacin was as effective as gentamicin in eliminating extracellular bacteria and accurately reflected intracellular bacterial burden [2].

6.1.1. LAB MEDIA: Figure 1. *Video editor: highlight the two bars labeled “NRG857C” and then “DH5α”*

6.1.2. LAB MEDIA: Figure 1. *Video editor: Show the right panel titled “Amikacin”*

6.2. Treatment of gentamicin-resistant clinical *Escherichia coli* isolates obtained from patients with inflammatory bowel disease with gentamicin resulted in highly variable bacterial recovery values, ranging from around 21 to 2788% [1]. In contrast, amikacin treatment dramatically reduced bacterial recovery across all isolates, demonstrating its effectiveness and enabling more reliable invasion measurements [2].

6.2.1. LAB MEDIA: Figure 2. *Video editor: Show the left panel titled “Gentamicin” and highlight the bars across the strains axis*

6.2.2. LAB MEDIA: Figure 2. *Video editor: Show the right panel titled “Amikacin” and highlight the bars across the strains axis*

1. Adherent-invasive

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

IPA: /əd'hɪrənt ɪn'veɪsɪv/

Phonetic Spelling: uh·dheer·uhnt in·vay·siv

2. Escherichia

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

IPA: /ˌɛ-shə-'ri-kē-ə/

Phonetic Spelling: esh·uh·ree·kee·uh

3. Luria-Bertani

Pronunciation link: <https://www.howtopronounce.com/luria-bertani>

IPA: /'lʊriə bər'tɑːni/

Phonetic Spelling: lur·ee·uh ber·taa·nee

4. Inoculate

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

- IPA: /ɪˈnɑːkjəˌleɪt/
Phonetic Spelling: ih·naa·kyuh·layt
5. Optical
Pronunciation link: <https://www.merriam-webster.com/dictionary/optical>
IPA: /ˈɑːptɪkəl/
Phonetic Spelling: aap·tuh·kuhl
 6. Spectrophotometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
IPA: /ˌspektrəfoʊˈtɑːmətər/
Phonetic Spelling: spek·truh·foh·taa·muh·ter
 7. Microcentrifuge
Pronunciation link: <https://www.howtopronounce.com/microcentrifuge>
IPA: /ˌmaɪkroʊˈsentrɪfjuːdʒ/
Phonetic Spelling: my·kroh·sen·truh·fyooj
 8. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentrəˌfjuːdʒ/
Phonetic Spelling: sen·truh·fyooj
 9. Aspirate
Pronunciation link: <https://www.merriam-webster.com/dictionary/aspirate>
IPA: /ˈæspəˌreɪt/
Phonetic Spelling: as·pɪuh·rayt
 10. Resuspend
Pronunciation link: <https://www.howtopronounce.com/resuspend>
IPA: /ˌriːsəˈspend/
Phonetic Spelling: ree·suh·spend
 11. Multiplicity
Pronunciation link: <https://www.merriam-webster.com/dictionary/multiplicity>
IPA: /ˌmʌltəˈplɪsəti/
Phonetic Spelling: mul·tuh·pliss·uh·tee
 12. Infection
Pronunciation link: <https://www.merriam-webster.com/dictionary/infection>
IPA: /ɪnˈfekʃən/
Phonetic Spelling: in·fek·shuhn
 13. Inoculum
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculum>
IPA: /ɪˈnɑːkjələm/
Phonetic Spelling: ih·naa·kyuh·luhm
 14. Quantification
Pronunciation link: <https://www.merriam-webster.com/dictionary/quantification>
IPA: /ˌkwɑːntəfəˈkeɪʃən/
Phonetic Spelling: kwaan·tuh·fuh·kay·shuhn
 15. Colony-forming
Pronunciation link: <https://www.merriam-webster.com/dictionary/colony>

- IPA: /'kɑ:ləni 'fɔ:rmɪŋ/
Phonetic Spelling: kaa·luh·nee for·ming
16. Serial
Pronunciation link: <https://www.merriam-webster.com/dictionary/serial>
IPA: /'sɪriəl/
Phonetic Spelling: seer·ee·uhl
17. Phosphate-buffered
Pronunciation link: <https://www.merriam-webster.com/dictionary/phosphate>
IPA: /'fɑ:sfɛrt 'bʌfərd/
Phonetic Spelling: faas·fayt buf·erd
18. Confluent
Pronunciation link: <https://www.merriam-webster.com/dictionary/confluent>
IPA: /'kɑ:nfluənt/
Phonetic Spelling: kaan·floo·uhnt
19. Epithelial
Pronunciation link: <https://www.merriam-webster.com/dictionary/epithelial>
IPA: /,epə'ti:liəl/
Phonetic Spelling: eh·puh·thee·lee·uhl
20. Monolayers
Pronunciation link: <https://www.howtopronounce.com/monolayer>
IPA: /'mɒnoʊ,leɪərz/
Phonetic Spelling: mon·oh·lay·erz
21. Collagen-coated
Pronunciation link: <https://www.merriam-webster.com/dictionary/collagen>
IPA: /'kɑ:lədʒən 'koʊtɪd/
Phonetic Spelling: kaa·luh·juhn koh·tuhd
22. Synchronize
Pronunciation link: <https://www.merriam-webster.com/dictionary/synchronize>
IPA: /'sɪŋkrə,naɪz/
Phonetic Spelling: sing·kruh·nyz
23. Monolayer
Pronunciation link: <https://www.howtopronounce.com/monolayer>
IPA: /'mɒnoʊ,leɪər/
Phonetic Spelling: mon·oh·lay·er
24. Amikacin
Pronunciation link: <https://www.merriam-webster.com/medical/amikacin>
IPA: /,æmɪ'keɪsɪn/
Phonetic Spelling: am·ih·kay·sin
25. Gentamicin
Pronunciation link: <https://www.merriam-webster.com/medical/gentamicin>
IPA: /,dʒentə'maɪsɪn/
Phonetic Spelling: jen·tuh·my·sin
26. Extracellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/extracellular>

- IPA: /ˌɛkstrəsəˈljʊlər/
Phonetic Spelling: eks·truh·suh·lyuh·ler
27. Triton-X-100
Pronunciation link: <https://www.howtopronounce.com/triton>
IPA: /ˈtraɪtɒːn ɛks wʌn ˈhʌndrəd/
Phonetic Spelling: try·taan eks one hun·drid
28. Orbital
Pronunciation link: <https://www.merriam-webster.com/dictionary/orbital>
IPA: /ˈɔːrbɪtəl/
Phonetic Spelling: or·buh·tuhl
29. Lysates
Pronunciation link: <https://www.howtopronounce.com/lysate>
IPA: /ˈlaɪsɪts/
Phonetic Spelling: ly·sayts
30. Intracellular
Pronunciation link: <https://www.merriam-webster.com/dictionary/intracellular>
IPA: /ˌɪntrəsəˈljʊlər/
Phonetic Spelling: in·truh·suh·lyuh·ler
31. Inflammatory
Pronunciation link: <https://www.merriam-webster.com/dictionary/inflammatory>
IPA: /ɪnˈflæməˌtɔːri/
Phonetic Spelling: in·flam·uh·tor·ee
32. Escherichia coli
Pronunciation link: <https://www.merriam-webster.com/dictionary/E.%20coli>
IPA: /ˌɛ-shə-ˈri-kē-ə ˈkō-,lī/
Phonetic Spelling: esh·uh·ree·kee·uh koh·ly
33. Caco-2
Pronunciation link: <https://www.howtopronounce.com/caco-2>
IPA: /ˈkækoo tuː/
Phonetic Spelling: ka·koh two
34. Laminar
Pronunciation link: <https://www.merriam-webster.com/dictionary/laminar>
IPA: /ˈlæmənər/
Phonetic Spelling: lam·uh·ner
35. Antimycotic
Pronunciation link: <https://www.merriam-webster.com/medical/antimycotic>
IPA: /ˌænti maɪˈkɑːtɪk/
Phonetic Spelling: an·tee·my·kaa·tik
36. Microliters
Pronunciation link: <https://www.merriam-webster.com/dictionary/microliter>
IPA: /ˈmaɪkroʊ liːtərz/
Phonetic Spelling: my·kroh·lee·terz
37. Nanometers
Pronunciation link: <https://www.merriam-webster.com/dictionary/nanometer>

IPA: /'nænə,mi:tərz/

Phonetic Spelling: nan·uh·mee·terz

38. Revolutions

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

IPA: /,revə'lu:ʃənz/

Phonetic Spelling: rev·uh·loo·shunz

39. Incubate

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

IPA: /'ɪŋkjə,bert/

Phonetic Spelling: ing·kyuh·bayt

40. Incubation

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

IPA: /,ɪŋkjə'beɪʃən/

Phonetic Spelling: ing·kyuh·bay·shuhn