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Title: Production and Testing of Antimicrobial Peptides and Their Mimics

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**
If **Yes**, how far apart are the locations?
- 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. **Yes**

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**

Best 5 answers are chosen

INTRODUCTION:

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

- 1.1. **Nathan Carey:** This protocol describes a method for producing and evaluating the antimicrobial activity and cytotoxicity of the cationic peptide Mel4 and peptide mimic RK758.
 - 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. **Nathan Carey:** Fragmented, non-standardized Anti-microbial peptide studies limit reproducibility and translation; this protocol integrates synthesis, testing, and mechanistic analysis into a unified workflow.
 - 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. **Nathan Carey:** This protocol enables understanding of different production methods, cost-effectiveness, and evaluation of peptides and mimics for drug development.
 - 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. **Nathan Carey:** Follow-up analyses after this procedure can include mechanistic studies, stability testing, formulation optimization, resistance development, and advanced efficacy evaluation in relevant models.

- 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. **Nathan Carey**: Future studies can optimize scalable production of peptides and mimics, improve cost-effectiveness, and evaluate in vivo efficacy, safety, and clinical translation.

- 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 capture all testimonial shots in a wide-angle format with sufficient headspace, as the final videos will be rendered in a 1:1 aspect ratio. Testimonial statements will be presented live by the authors, sharing their spontaneous perspectives.

- Testimonial statements will **not appear in the video** but may be featured in the journal's promotional materials.
- **Provide the full name and position** (e.g., Director of [Institute Name], Senior Researcher [University Name], etc.) of the author delivering the testimonial.
- Please **answer the testimonial question live during the shoot**, speaking naturally and in your own words in **complete sentences**.

What problem in your field of research does your JoVE article address?

1.6. **Naresh Kumar, Professor, School of Chemistry, University of New South Wales**

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

What value do you expect this video article to have for other researchers working in your field?

1.7. **Naresh Kumar, Professor, School of Chemistry, University of New South Wales:**
(authors will present their testimonial statements live)

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

Protocol

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

2. Preparation of RK758 and AMP Mel4 Peptide Solutions and Bacterial Inoculum

Demonstrator: George Enniful

2.1. To begin, obtain the anthranilamide peptide mimic RK758 and the highly cationic antimicrobial peptide Mel4 [1]. Dissolve the peptides in sterile water at a high concentration of 20 millimolar [2] and filter-sterilize the peptide stocks using a 0.22 micrometer syringe filter [3].

2.1.1. WIDE: Talent placing the vials with peptides on the work bench. **TXT: Antimicrobial Peptide (AMP) Mel4: KNKRKRRRRRRGGRR**

2.1.2. Talent adding sterile water to the peptide vials and mixing them. **Authors: Let us skip media preparation steps as those are standard procedures**

2.1.3. Talent adding the dissolved peptide solution into a syringe filter.

2.2. Streak the bacterial isolate to be tested onto Trypticase Soy agar to obtain pure and well-isolated colonies [1-TXT]. Incubate the plate aerobically at around 36 degrees Celsius plus for 18 to 24 hours [2].

2.2.1. Talent streaking bacterial isolate onto a Trypticase Soy agar plate in a hood. **TXT: *K. pneumoniae* JIE 2709 and *E. coli* NCTC 13846 were used in this study**

2.2.2. Talent placing the agar plate into an incubator.

2.3. Next day, select 3 to 5 morphologically similar and well-isolated colonies using a sterile inoculating loop [1]. Transfer the colonies into a sterile tube containing 5 to 10 milliliters of cation-adjusted Mueller Hinton Broth [2]. Gently rub the loop against the inner wall of the tilted tube to obtain a homogeneous suspension [3].

2.3.1. Talent picking isolated colonies from the agar plate using a sterile inoculating loop.

2.3.2. Talent transferring colonies into a sterile tube containing broth. **Authors: I am retaining MHB broth here as the same is used in the subsequent steps**

2.3.3. Talent rubbing the loop against the inner wall of a tilted tube to mix the suspension.

2.4. Incubate the broth culture at around 36 degrees Celsius while shaking at 180 revolutions per minute for 18 to 24 hours [1].

2.4.1. Talent placing the tube into a shaking incubator.

2.5. After incubation, centrifuge the overnight culture at 3000 g for 10 minutes at 25 degrees Celsius [1]. Discard the supernatant and wash the bacterial pellet three times with sterile PBS [1].

2.5.1. Talent placing the culture tube into a centrifuge.

2.5.2. Talent discarding the supernatant and adding PBS to the cell pellet.

2.6. Now, dispense 2 milliliters of PBS into a sterile container [1]. Add 50 to 100 microliters of the washed bacterial suspension and vortex for 5 to 10 seconds [2].

2.6.1. Talent dispensing PBS into a sterile container.

2.6.2. Talent adding washed bacterial suspension into the container and vortexing the mixture.

2.7. Add 200 microliters of this bacterial suspension into a 96-well plate [1]. Measure the optical density at 600 nanometers using a spectrophotometer to obtain a value of 0.1 corresponding to approximately $1 \text{ to } 2 \times 10^8$ colony forming units per milliliter [2].

2.7.1. Talent pipetting bacterial suspension into a 96-well plate. **TXT: Blank: 200 μ L 1x PBS**

2.7.2. Talent placing the plate into a compatible spectrophotometer and recording the optical density reading in a notebook.

2.8. Dilute the adjusted bacterial suspension 1 to 100 in the cation-adjusted Mueller Hinton Broth to obtain a final inoculum of about 10^6 colony forming units per milliliter [1].

2.8.1. Talent diluting the bacterial suspension in cation-adjusted Mueller Hinton Broth.

3. Minimum Inhibitory Concentration (MIC) Assay Plate Setup and Analysis

Demonstrator: Jiawei Shen

3.1. Label a sterile flat-bottomed 96-well plate with the bacterial strain, antimicrobial peptide name, date, and scientist initials [1]. Mark the locations for test, blank, and

control wells on the plate lid [2].

3.1.1. Talent labeling a sterile 96-well plate in the laboratory workspace.

3.1.2. Talent marking well positions on the plate lid with a marker.

3.2. Dispense 200 microliters of antimicrobial peptide solution at the highest concentration into the first column of the plate [1] and then add 100 microliters of cation-adjusted Mueller Hinton Broth to the remaining wells from column 2 to column 12 [2].

3.2.1. Talent pipetting antimicrobial peptide solution into the first column of a 96-well plate.

3.2.2. Talent dispensing broth into wells across columns 2 to 12 using a multichannel pipette.

3.3. Using a multichannel pipette, perform twofold serial dilutions of the antimicrobial peptide from column 1 through column 10 [1].

3.3.1. Talent performing serial dilution across the 96-well plate using a multichannel pipette.

3.4. Next, add 100 microliters of the prepared bacterial inoculum at 10^6 colony forming units per milliliter to each well except the blank wells [1]. Dispense 100 microliters of cation-adjusted Mueller Hinton Broth to the blank wells to reach a final volume of 200 microliters [2-TXT].

3.4.1. Talent adding bacterial inoculum to wells of the 96-well plate.

3.4.2. Talent adding broth to designated blank wells. **TXT: Prepare triplicate wells for each concentration; Repeat the assay for RK758 peptide and comparator antibiotic colistin**

3.5. Cover the plate with its lid and seal with cling wrap to prevent evaporation [1]. Incubate the plate at 36 degrees Celsius for 18 to 24 hours [2].

3.5.1. Talent placing the lid and sealing the plate with cling wrap.

3.5.2. Talent placing the sealed plate into an incubator.

3.6. After incubation, measure the optical density at 600 nanometers of each well using a microplate reader [1]. Calculate the percentage reduction in growth relative to the untreated growth control and identify the minimum inhibitory [2-TXT].

- 3.6.1. Talent placing the 96-well plate into a microplate reader.
- 3.6.2. Talent looking at the computer screen and noting in a book. **TXT: Additionally, determine Minimum Bactericidal Concentration (MBC)**

4. Seeding L929 Mouse Fibroblast Cells and Incubation with Antimicrobial Peptides (AMPs)

Demonstrator: Elias Shiferaw Mekonen

4.1. Seed L929 mouse fibroblast cells in a 75 square centimeter flask containing DMEM supplemented with 10 percent FBS and 1 percent antibiotics including streptomycin sulfate and penicillin G [1-TXT]. Incubate the cells at 37 degrees Celsius with 5 percent carbon dioxide until reaching 75 to 80% confluency [2]. Once confluent, wash the cells with PBS [3], then add 0.25% trypsin-EDTA to detach the cells [4]. Mix 50 microliters of cell suspension with 50 microliters of 0.4 percent Trypan Blue [5] and load 10 microliters of the mixture into a hemocytometer [6].

- 4.1.1. Talent adding cells into the 75 sq.cm flask containing media. **TXT: DMEM: Dulbecco's Minimum Essential Medium; Seed 10^5 cells/mL**
- 4.1.2. Talent placing the flask into a carbon dioxide incubator.
- 4.1.3. Talent adding phosphate-buffered saline in a flask to wash the cells and **LAB MEDIA** as an inset.
- 4.1.4. Talent adding trypsin-ethylenediaminetetraacetic acid to the flask.
- 4.1.5. Talent mixing cell suspension with Trypan Blue dye.
- 4.1.6. Talent loading the stained cell mixture into a hemocytometer.

4.2. Count viable unstained cells and dead blue cells in the four corner squares of the hemocytometer [1] and calculate the cell viability using the formula [2].

- 4.2.1. Talent placing the hemocytometer under a microscope.
- 4.2.2. TEXT ON 3.6.2's BACKGROUND:

$$\text{Viability (\%)} = (\text{Live} / [\text{Live} + \text{Dead}]) \times 100$$

4.3. Next, adjust the final cell concentration to 5×10^4 cells per milliliter using DMEM [1]. Seed 100 microliters of the cell suspension into each well of a sterile 96-well plate [2] and incubate for 24 hours at 37 degrees Celsius with 5 percent carbon dioxide to allow cell adherence [3].

- 4.3.1. Talent diluting the cell suspension to the required concentration.
- 4.3.2. Talent pipetting cell suspension into wells of a 96-well plate.

4.3.3. Talent placing the plate into a carbon dioxide incubator.

4.4. Next, prepare working solutions of Mel4 and RK758 in DMEM without FBS and antibiotics using the previously prepared stock solution [1-TXT].

4.4.1. Talent adding Mel4 and RK758 to a tube beside which several other tubes are placed or adding to a fresh 96-well plate labelled 'Peptide dilutions'. **TXT: Concentrations: 1,000, 500, 250, 125, 62.5, 31.25, 15.6, 7.8, and 3.9 μ M**

4.5. Now, remove the culture medium from each well [1] and add 100 microliters of the corresponding working solution to each well [2-TXT].

4.5.1. Talent aspirating medium from wells of the 96-well plate.

4.5.2. Talent adding test solutions into the wells. **TXT: Negative control: DMEM; Positive control: DMSO; Blank: PBS**

5. MTT Assay to Determine Cytotoxicity of Mel4 or RK758

5.1. After 24 hours of Mel4 or RK758 exposure, aspirate the test media carefully without disturbing the L929 mouse fibroblast cells [1].

5.1.1. Talent aspirating media from wells of a 96-well plate without disturbing attached cells.

5.2. Add 100 microliters of the 0.5 milligrams per milliliter MTT working solution prepared in DMEM to each well [1]. Cover the plate with aluminum foil [2] and incubate for 2 hours at 37 degrees Celsius until purple formazan crystals form [3].

5.2.1. Talent adding MTT solution into each well of the plate.

5.2.2. Talent covering the plate with aluminum foil.

5.2.3. Talent placing the plate into an incubator.

5.3. After incubation, examine the cells for violet precipitation under the microscope [1]. Remove the MTT solution carefully from each well [2] and add 100 microliters of dimethyl sulfoxide to each well to dissolve the formazan crystals [3].

5.3.1. Talent seeing 96 well plates for violet precipitation under microscope.

5.3.2. Talent aspirating MTT solution from the wells.

5.3.3. Talent adding dimethyl sulfoxide into each well.

5.4. Place the plate on a shaker for 5 minutes to ensure complete dissolution [1] and measure the absorbance at 570 nanometers using a microplate reader [2].

5.4.1. Talent placing the plate on a shaker.

5.4.2. Talent inserting the plate into a microplate reader and initiating measurement.

5.5. After subtracting the blank absorbance values, calculate cell viability using the equation and then calculate IC50 or CC50 values [1].

5.5.1. TEXT ON 3.6.2'S BACKGROUND:

$$\text{Viability (\%)} = (A_{\text{Treated}} / A_{\text{NC (Cell)}}) \times 100$$

Results

6. Results

6.1. RK758 exhibited a minimum inhibitory concentration of 16 microgram per milliliter against both *Klebsiella pneumoniae* JIE 2709 and *Escherichia coli* NCTC 13846 [1].

6.1.1. LAB MEDIA: Table 3. *Video editor: Highlight the column RK758*

6.2. Mel4 showed minimum inhibitory concentrations of 62.5 microgram per milliliter for *Klebsiella pneumoniae* JIE 2709 [1] and 125 microgram per milliliter for *Escherichia coli* NCTC 13846 [2].

6.2.1. LAB MEDIA: Table 3. *Video editor: Highlight the value 62.5 microgram per milliliter for Mel4 under K. pneumoniae JIE 2709*

6.2.2. LAB MEDIA: Table 3. *Video editor: Highlight the value 125 microgram per milliliter for Mel4 under E. coli NCTC 13846*

6.3. Colistin demonstrated a minimum inhibitory concentration of 1 microgram per milliliter for *Klebsiella pneumoniae* JIE 2709 [1] and 8 microgram per milliliter for *Escherichia coli* NCTC 13846 [2].

6.3.1. LAB MEDIA: Table 3. *Video editor: Highlight the value 1 microgram per milliliter for colistin under K. pneumoniae JIE 2709*

6.3.2. LAB MEDIA: Table 3. *Video editor: Highlight the value 8 microgram per milliliter for colistin under E. coli NCTC 13846*

6.4. RK758 showed concentration-dependent inhibition of cell viability in L929 fibroblast cells with a CC50 value of 172.6 micromolar [1].

6.4.1. LAB MEDIA: Figure 6. *Video editor: Highlight the label "CC50: 172.6 micromolar" on the graph*

6.5. Mel4 showed slightly higher cytotoxicity than RK758 in L929 cells with a CC50 value of 147.5 micromolar [1].

6.5.1. LAB MEDIA: Figure 7. *Video editor: Highlight the label "CC50: 147.5 micromolar" on the graph*

1. Anthranilamide
Pronunciation link: <https://www.merriam-webster.com/dictionary/anthranilamide>
IPA: /, ænθrəˈnɪləˌmaɪd/
Phonetic Spelling: an·thruh·nil·uh·mide
2. Cationic
Pronunciation link: <https://www.merriam-webster.com/dictionary/cationic>
IPA: /, kætɪˈɑːnɪk/
Phonetic Spelling: kat·ee·on·ik
3. Antimicrobial
Pronunciation link: <https://www.merriam-webster.com/dictionary/antimicrobial>
IPA: /, æn.tɪ.maiˈkroʊ.bi.əl/
Phonetic Spelling: an·tee·my·kroh·bee·uhl
4. Millimolar
Pronunciation link: <https://www.merriam-webster.com/dictionary/millimolar>
IPA: /, mɪləˈmoʊlər/
Phonetic Spelling: mil·uh·moh·ler
5. Micrometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/micrometer>
IPA: /maɪˈkraːmətər/
Phonetic Spelling: my·krah·muh·ter
6. Trypticase
Pronunciation link: No confirmed link found
IPA: /ˈtrɪptɪˌkeɪs/
Phonetic Spelling: trip·tih·kays
7. Aerobically
Pronunciation link: <https://www.merriam-webster.com/dictionary/aerobically>
IPA: /erˈoʊbɪkli/
Phonetic Spelling: air·oh·bik·lee
8. Inoculating
Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculate>
IPA: /ɪˈnɑːkjəˌleɪt/
Phonetic Spelling: ih·nok·yuh·layt
9. Mueller Hinton
Pronunciation link: No confirmed link found
IPA: /ˈmjuːlər ˈhɪntən/
Phonetic Spelling: myoo·ler hin·tuhn
10. Homogeneous
Pronunciation link: <https://www.merriam-webster.com/dictionary/homogeneous>
IPA: /, hoʊməˈdʒiːniəs/
Phonetic Spelling: hoh·muh·jee·nee·uhs
11. Centrifuge
Pronunciation link: <https://www.merriam-webster.com/dictionary/centrifuge>
IPA: /ˈsentriˌfjuːdʒ/
Phonetic Spelling: sen·trih·fyooj

12. Supernatant
 Pronunciation link: <https://www.merriam-webster.com/dictionary/supernatant>
 IPA: /,su:pər'neɪtənt/
 Phonetic Spelling: soo·per·nay·tuhnt
13. Pellet
 Pronunciation link: <https://www.merriam-webster.com/dictionary/pellet>
 IPA: /'pelɪt/
 Phonetic Spelling: pel·it
14. Phosphate-buffered saline
 Pronunciation link: No confirmed link found
 IPA: /'fɑ:sfeɪt 'bʌfəd sə'li:n/
 Phonetic Spelling: fos·fate buf·erd suh·leen
15. Vortex
 Pronunciation link: <https://www.merriam-webster.com/dictionary/vortex>
 IPA: /'vɔ:r'teks/
 Phonetic Spelling: vor·teks
16. Spectrophotometer
 Pronunciation link: <https://www.merriam-webster.com/dictionary/spectrophotometer>
 IPA: /,spektrəfoʊ'tɑ:məʊtər/
 Phonetic Spelling: spek·truh·foh·tah·muh·ter
17. Optical density
 Pronunciation link: <https://www.merriam-webster.com/dictionary/optical>
 IPA: /'ɑ:ptɪkəl/
 Phonetic Spelling: op·tih·kuhl den·suh·tee
18. Inoculum
 Pronunciation link: <https://www.merriam-webster.com/dictionary/inoculum>
 IPA: /ɪ'nɑ:kjələm/
 Phonetic Spelling: ih·nok·yuh·luhm
19. Serial dilution
 Pronunciation link: <https://www.merriam-webster.com/dictionary/dilution>
 IPA: /dɑr'lu:fən/
 Phonetic Spelling: dye·loo·shuhn
20. Fibroblast
 Pronunciation link: <https://www.merriam-webster.com/dictionary/fibroblast>
 IPA: /'faɪbrə'blæst/
 Phonetic Spelling: fy·broh·blast
21. Confluency
 Pronunciation link: No confirmed link found
 IPA: /kən'flu:ənsi/
 Phonetic Spelling: kuhn·floo·uhn·see
22. Trypsin-EDTA
 Pronunciation link: No confirmed link found
 IPA: /'trɪpsɪn ,ɪ:di:ti:'eɪ/
 Phonetic Spelling: trip·sin ee·dee·tee·ay

23. Hemocytometer
Pronunciation link: <https://www.merriam-webster.com/dictionary/hemocytometer>
IPA: /ˌhiːmouˈsaɪˈtɑːmətər/
Phonetic Spelling: hee·moh·sy·tah·muh·ter
24. Cytotoxicity
Pronunciation link: <https://www.merriam-webster.com/dictionary/cytotoxic>
IPA: /ˌsaɪtəʊtɪkˈsɪsɪti/
Phonetic Spelling: sy·toh·tok·sis·ih·tee
25. Formazan
Pronunciation link: No confirmed link found
IPA: /ˈfɔːrməzæn/
Phonetic Spelling: for·muh·zan
26. Dimethyl sulfoxide
Pronunciation link: <https://www.merriam-webster.com/dictionary/dimethyl%20sulfoxide>
IPA: /daɪˈmɛθəl sʌlˈfɑːksaɪd/
Phonetic Spelling: dye·meth·uhl sul·fok·side
27. Absorbance
Pronunciation link: <https://www.merriam-webster.com/dictionary/absorbance>
IPA: /əbˈzɔːrbəns/
Phonetic Spelling: ub·zor·buhns
28. Klebsiella pneumoniae
Pronunciation link: <https://www.merriam-webster.com/dictionary/Klebsiella>
IPA: /ˌklebsiˈɛlə nuːˈmoʊni.iː/
Phonetic Spelling: kleb·see·el·uh noo·moh·nee·ee
29. Escherichia coli
Pronunciation link: <https://www.merriam-webster.com/dictionary/Escherichia%20coli>
IPA: /ˌɛʃəˈrɪkiə ˈkoʊlaɪ/
Phonetic Spelling: esh·uh·rik·ee·uh koh·ly
30. Colistin
Pronunciation link: <https://www.merriam-webster.com/dictionary/colistin>
IPA: /kəˈlɪstɪn/
Phonetic Spelling: kuh·lis·tin