**Submission ID #: 61275**
**Scriptwriter Name: Bridget Colvin**
**Project Page Link:** https://www.jove.com/account/file-uploader?src=18688923

# Title: Phage Therapy Application to Counteract *Pseudomonas aeruginosa* Infection in Cystic Fibrosis Zebrafish Embryos

**Authors and Affiliations: Marco Cafora[1,2], Francesca Forti[3], Federica Briani[3], Daniela Ghisotti[3], and Anna Pistocchi[2]**

[1]Dipartimento di Scienze Cliniche e Comunità, Università degli Studi di Milano, Via Santa Barbara
[2]Dipartimento di Biotecnologie Mediche e Medicina Traslazionale, Università degli Studi di Milano, LITA, via Fratelli Cervi
[3]Dipartimento di Bioscienze, Università degli Studi di Milano, Via Celoria

**Corresponding Author:**
Anna Pistocchi
anna.pistocchi@unimi.it

**Co-authors:**
marco.cafora@unimi.it
francesca.forti@unimi.it
federica.briani@unimi.it
daniela.ghisotti@unimi.it

 

# Author Questionnaire

**1. Microscopy**: Does your protocol involve video microscopy, such as filming a complex dissection or microinjection technique? **Y**

If **Yes**, can you record movies/images using your own microscope camera?

**Y**

**2. Software:** Does the part of your protocol being filmed demonstrate software usage? **N**

**3. Filming location:** Will the filming need to take place in multiple locations (greater than walking distance)? **Y, 10 minutes driving**

# Introduction

**1. Introductory Interview Statements**

**REQUIRED:**

1.1. **Marco Cafora**: This protocol facilitates the preparation and administration of a virulent phage cocktail against *Pseudomonas aeruginosa* in zebrafish embryos **[1]**.

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

**REQUIRED:**

1.2. **Francesca Forti**: The main advantage of this technique is that it allows an in vivo assessment of the phage efficacy in counteracting the bacterial infection **[1]**.

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

**OPTIONAL:**

1.3. **Anna Pistocchi**: The phage cocktail is efficient in both wild-type and cystic fibrosis zebrafish models, opening the possibility of applying phage therapy to bacterial infections in cystic fibrosis patients **[1]**.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can skip for time*

**OPTIONAL:**

1.4. **Marco Cafora**: Phage therapy can also be applied to counteract specific bacterial infections in other model systems **[1]**.

1.4.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can skip for time*

# Protocol

## 2. Phage Stock and Phage Cocktail Preparation

2.1. To prepare a phage stock for the experiment, add 1.25 x $10^7$ phages to an $OD_{600}$ (O-D-six hundred) of 0.05 *P. aeruginosa* culture to a multiplicity of infection of 1 x $10^{-3}$ **[1-TXT]** and incubate the culture for 3-4 hours with shaking until the OD600 drops to 0.1-0.3 **[2]**.

2.1.1. WIDE: Talent adding phage to culture, with phage container visible in frame **TEXT: $OD_{600}$: optical density at 600 nm**

2.1.2. Talent placing culture onto shaker

2.2. At the end of the incubation, incubate the lysate with 1 microgram/milliliter of DNase and RNase for 30 minutes at 37 degrees Celsius **[1]** and pellet the cells by centrifugation **[2-TXT]**.

2.2.1. Talent adding DNase and RNase to culture, with DNase and RNase containers visible frame

2.2.2. Talent placing tube(s) into centrifuge **TEXT: 30 min, 5000 x g, 4 °C**

2.3. At the end of the centrifugation, filter the supernatant through a 0.8-micrometer-pore diameter **[1]** and add 58 grams/liter of sodium chloride and 105 grams/liter of PEG6000 (peg-six thousand) **[2-TXT]**.

2.3.1. Talent filtering supernatant

2.3.2. Talent adding NaCl and/or PEG6000 to tube, with both containers visible in frame **TEXT: PEG: polyethylene glycol**

2.4. Incubate the solution at 4 degrees Celsius overnight **[1]**. The next morning, precipitate the phage by centrifugation **[2-TXT]**.

2.4.1. Talent placing tube at 4 °C

2.4.2. Talent placing tube(s) into centrifuge **TEXT: 30 min, 20,000 x g, 4 °C**

2.5. At the end of the centrifugation, remove the supernatant **[1]** and carefully dissolve the phage pellet in 15 milliliters of tris-sodium chloride buffer **[2-TXT]**.

2.5.1. Supernatant being removed

2.5.2. Talent adding TN buffer to tube, with TN buffer container visible in frame **TEXT: See text for all buffer and solution preparation details**

2.6. To purify the phages by cesium chloride density gradient, first stratify 2 milliliters of four different cesium chloride solutions in polyallomer ultracentrifuge tubes for an SW41 (S-W-forty-one) rotor **[1-TXT]** and add 3.5 milliliters of the phage suspension to each tube **[2]**.

2.6.1. Talent adding CsCl to tube(s) *Videographer: Important step* **TEXT: See text for CsCl solution preparation details**
2.6.2. Talent adding phage to tube(s) *Videographer: Important step*

2.7. **Marco Cafora**: Cesium chloride is toxic. Always adopt proper safety procedures when handling and discarding this compound **[1]**.

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

2.8. When all of the tubes have been loaded, place the tubes into the rotor, taking care that the tubes are balanced **[1-TXT]**, and centrifuge the samples for 2 hours at 100,000 x g and 4 degrees Celsius **[2]**.

2.8.1. Talent placing tubes into rotor **TEXT: Weight difference between facing tubes ≤ 0.01g**
2.8.2. Talent inputting centrifuge settings/starting centrifuge

2.9. At the end of the centrifugation, use a syringe with 19-gauge needle to aspirate the white layer between the d=1.5 and the d=1.4 density regions **[1]** and transfer the suspensions to into new SW60-size polyallomer tubes **[2]**.

2.9.1. Shot of layers, then white layer being aspirated *Videographer: Important/difficult step*
2.9.2. Talent adding suspension to tube *Videographer: Important/difficult step*

2.10. Centrifuge the suspensions for at least 16 hours at 150,0000 *x g* and 4 degrees Celsius **[1]** and transfer the visible bands into dialysis tubes with a 6000-Dalton cut off **[2]**.

2.10.1. Talent placing tubes into rotor
2.10.2. Shot of layers, then white layer being aspirated

2.11. Then dialyze the collected samples two times against 500 milliliters of water for 20 minutes per dialysis **[1]** and overnight against 500 milliliters of tris-sodium chloride buffer **[2]**.

2.11.1. Talent starting dialysis
2.11.2. TN buffer being added to system 2.11.1. and 2.11.2. merged

2.12. The next morning, filter the resulting phage stock with a 0.22-micrometer-pore filter **[1]** and store the stock at 4 degrees Celsius **[2]**.

2.12.1. Talent filtering stock
2.12.2. Talent placing stock at 4 °C

~~2.13. For phage cocktail preparation, mix equal volumes and concentrations of four different phage preparations with an overall titer of 5 x $10^8$ plaque forming units/milliliter **[1]**.~~

~~2.13.1. Talent adding preparation(s) to tube, with phage stock containers visible in frame~~

## 3. Zebrafish Embryo Cystic Fibrosis Transmembrane Conductance Regulator (*cftr*) Morpholino Microinjection

3.1. One the day of the microinjection, dilute two 1-micromolar morpholino stock solutions in sterile water to a final concentration of 0.25-picomolar/embryo to obtain a 5-microliter morpholino injection solution **[1-TXT]** and add 0.5 microliters of phenol red to each solution **[2]**.

3.1.1. WIDE: Talent adding water to tube(s), with morpholino containers visible in frame **TEXT: *i.e.*, ATG-MO and splice-MO morpholinos**
3.1.2. Talent adding phenol red to tube(s)

3.2. Next, use a 20-microliter micropipette with a fine gel loading tip to load a microinjection needle with the entire volume of morpholino mix solution **[1]** and secure the needle to a micromanipulator connected to a stand under a stereomicroscope **[2]**.

3.2.1. Talent loading needle with solution
3.2.2. Talent securing needle to stand, with microscope visible in frame

3.3. Then, with one- or two-cell zebrafish embryos arranged on a glass slide placed within a 96-millimeter-diameter Petri dish **[1]**, penetrate the chorion and the yolk with the microinjection needle tip **[2]** …

~~3.3.1. LAB MEDIA: To be provided by Authors: Embryos being arranged~~
3.3.2. SCOPE: Shot of arranged embryos, then chorion and yolk being penetrated 3.3.2. and 3.4.1. merged

3.4. … and inject 2 nanoliters of the morpholino mixture into the embryo **[1]**.

3.4.1. SCOPE: Embryo being injected

## 4. Zebrafish Embryo *P. aeruginosa* (PAO1) Bacteria and Phage Cocktail Microinjection

4.1. At 26 hours post-fertilization, load a microinjection needle with approximately 5 microliters of *P. aeruginosa* inoculum **[1-TXT]** and insert the needle dorsally to the starting point of the duct of Cuvier at which the duct starts spreading over the yolk sac **[2]**.

4.1.1. WIDE: Talent loading needle with inoculum, with inoculum container visible in frame *Videographer: Important/difficult step* **TEXT: See text for PAO1 inoculum preparation details**
4.1.2. SCOPE: Needle being inserted

4.2. Inject 1-3 nanoliters of the PAO1 (P-A-O-one) inoculum into the embryo, making sure that the volume expands directly within the duct and enters into the circulation **[1]**.

4.2.1. SCOPE: PAO1 inoculum being injected/duct expanding 4.1.2. and 4.2.1 merged

4.3. After the injection, transfer the embryo into one of two new Petri dishes containing fresh E3 medium supplemented with phenyl thiourea **[1]** for a 30-minute or 3-hour incubation at 28 degrees Celsius **[2]**.

4.3.1. Talent adding embryo to dish
4.3.2. Talent placing dish into incubator

4.4. Next, load a microinjection needle with approximately 5 microliters of the phage cocktail **[1]** and fix the needle to the microinjector **[2]**.

4.4.1. Talent loading needle with cocktail, with cocktail container visible in frame
4.4.2. Talent fixing needle to microinjector

4.5. Then inject 1-3 nanoliters of phage cocktail into the duct of Cuvier of each embryo previously injected with bacteria **[1]** and place the embryos into one of two new Petri dishes containing fresh E3 medium supplemented with phenyl thiourea at 28 degrees Celsius **[2]**.

4.5.1. SCOPE: Embryo being injected
4.5.2. Talent placing dish(es) into incubator

## 5. Time-Lapse Imaging Preparation

5.1. At four hours post infection, use a plastic pipette to transfer an anesthetized embryo into a glass bottom dish **[1]** and fill the dish with warm low-melting-point agarose solution **[2]**.

5.1.1. WIDE: Talent placing embryo into dish
5.1.2. Talent adding agarose to dish

5.2. When the agarose is cold, gently filling the dish with E3 medium supplemented with anesthetic solution **[1]**.

5.2.1. Talent adding medium to dish, with medium container visible in frame

5.3. Place the dish under the stereomicroscope **[1]** and use a pipette tip to position the embryo in the desired orientation **[2]**.

5.3.1. Talent placing dish under microscope
5.3.2. SCOPE: Embryo being oriented

5.4. Then place the dish under a fluorescent stereomicroscope with a fluorescent filter for GFP (G-F-P)-positive bacteria for up to 18 hours post infection **[1-TXT]** and image the embryo for the progression of GFP expression at 9, 14, and 18 hours post infection **[2]**.

5.4.1. Talent placing dish under microscope **TEXT: GFP: green fluorescent protein**
5.4.2. LAB MEDIA: Figure 4

## 6. Bacterial Burden and PAO1 Infection Embryonic Lethality Evaluation

6.1. To determine the bacterial burden, at 8 hours post infection, transfer 15 anesthetized, microinjected embryos into a 1.5-milliliter centrifuge tube **[1-TXT]** and replace the anesthetic solution with 300 microliters of 1% Triton X-100 in PBS **[2]**.

6.1.1. WIDE: Talent adding embryo(s) to tube **TEXT: Anesthesia: 1% Tricaine**
6.1.2. Talent adding Triton X-100 to tube, with Triton X-100 container visible in frame

6.2. Pass the needles through the sterile, 27-gauge needle of an insulin syringe at least 15 times to homogenize the embryos **[1]** and prepare serial dilutions of the homogenate by transferring 100 microliters of the resulting into 900 microliters of sterile PBS per dilution **[2]**.

6.2.1. Embryos being aspirated and released
6.2.2. Talent adding homogenate to tube, with PBS and homogenate containers visible in frame

6.3. To select for the naturally ampicillin-resistant *P. aeruginosa* strain, plate 100 microliters of the dilutions onto LB agar supplemented with ampicillin **[1]** and incubate the overnight at 37 degrees Celsius **[2]**.

6.3.1. Talent adding dilution to plate
6.3.2. Talent placing plate into incubator

6.4. The next day, count the number of colonies **[1]** to allow calculation of the total number of colony forming and the average number of colony forming units/infected embryo **[2]**.

6.4.1. Shot of plate with colonies *Video Editor: please add numbers to show counting OR no animation*
6.4.2. LAB MEDIA: Figure 2

6.5. To evaluate the lethality of the *P. aeruginosa* infection, score the injected embryos at 20 hours post infection under a stereomicroscope **[1]** by counting the number of dead, white, opaque embryos **[2]**.

6.5.1. Talent at microscope, counting embryos
6.5.2. SCOPE: Shot of embryos

6.6. Then calculate the half-maximal lethal concentration 50 dose of *P. aeruginosa* that resulted in the death of 50% of the injected embryos at 20 hours post infection **[1]**.

6.6.1. LAB MEDIA: Figure 3A

# Protocol Script Questions

**A.** Which steps from the protocol are the most important for viewers to see?
2.6., 2.9., 4.1.

**B.** What is the single most difficult aspect of this procedure and what do you do to ensure success?
2.9. For phage stock the whole preparation is critical and susceptible to variability correlated to the lytic efficiency of phages.
4.1., 4.5. The insertion of the needle into the duct of cuvier might require expertise in zebrafish embryos manipulation and knowledge of their anatomy.

# Results

**7. Results: Representative Phage Therapy Effects on *Pseudomonas aeruginosa* Infection in Cystic Fibrosis (CF) Zebrafish Embryos**

7.1. To validate the cystic fibrosis phenotype, the impaired position of internal organs **[1]**, such as the heart **[2]**, liver **[3]**, and pancreas can be evaluated **[4]**.

7.1.1. LAB MEDIA: Figure 1
7.1.2. LAB MEDIA: Figure 1 *Video Editor: please emphasize dark tissue in images*
7.1.3. LAB MEDIA: Figure 1 *Video Editor: please emphasize dark tissue indicated by liv text and arrow*
7.1.4. LAB MEDIA: Figure 1 *Video Editor: please emphasize dark tissue indicated by p text*

7.2. The bacterial burden is reduced by phage therapy in cystic fibrosis embryos infected with *P. aeruginosa* **[1]**.

7.2.1. LAB MEDIA: Figure 2 *Video Editor: please emphasize grey data bar*

7.3. In 48-hour post fertilization cystic fibrosis embryos infected with GFP-positive *P. aeruginosa* bacteria **[1]**, a 30 colony forming units per embryo dose demonstrates a 50% lethality at 20 hours post infection **[2]**.

7.3.1. LAB MEDIA: Figure 3A
7.3.2. LAB MEDIA: Figure 3A *Video Editor: please emphasize data point along line at 50% lethality and 30 cfu/embryo*

7.4. Phage therapy is equally effective at 20 hours post infection **[1]** when delivered 30 minutes or 7 hours after bacterial injection **[2]**.

7.4.1. LAB MEDIA: Figure 3B
7.4.2. LAB MEDIA: Figure 3B *Video Editor: please emphasize grey data bar*

7.5. Here a cystic fibrosis- and GFP-positive-*P. aeruginosa*-injected embryo at 4, 9, 14, and 18 hours post infection is shown **[1]**.

7.5.1. LAB MEDIA: Figure 4 *Video Editor: please sequentially emphasize embyros in top part of images from left to right*

7.6. In contrast, this cystic fibrosis plus *P. aeruginosa* plus phage-injected embryo demonstrates a reduced fluorescence due to the phage action against the bacteria **[1]**.

7.6.1. LAB MEDIA: Figure 4 left image *Video Editor: please sequentially emphasize embryos in bottom part of images from left to right*

7.7. The inflammatory response generated by *P. aeruginosa* infection in cystic fibrosis embryos **[1]** is significantly increased, as revealed by the expression of the pro-inflammatory cytokines TNF (T-N-F)-alpha and interleukin-1-beta following *P. aeruginosa* injection **[2-TXT]** compared to control injected zebrafish **[3]**.

7.7.1. LAB MEDIA: Figure 5

7.7.2. LAB MEDIA: Figure 5 *Video Editor: please emphasize CF+PAO1 data bars* **TEXT: TNF: tumor necrosis factor**

7.7.3. LAB MEDIA: Figure 5 *Video Editor: please emphasize CF data bars*

7.8. The expression of both inflammatory cytokines is reduced in phage cocktail-co-injected animals, however **[1]**.

7.8.1. LAB MEDIA: Figure 5 *Video Editor: please emphasize CF+PAO1+phage data bars*

# Conclusion

**8. Conclusion Interview Statements**

8.1. **Anna Pistocchi**: The phages must be carefully purified to remove any contaminating endotoxin that may be retained during the preparation **[1]**.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera (2.6.-2.11.)

8.2. **Anna Pistocchi**: The phage preparations can be analysed by electron microscopy to assess the virion morphology **[1]**.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can skip for time*

8.3. **Anna Pistocchi**: It would be interesting to test whether the infections in human patients by bacteria other than *P. aeruginosa* can be cured with phage therapy in the Cystic Fibrosis zebrafish model **[1]**.

8.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera *Videographer: Can skip for time*