Using and Maintaining Listeriaphage

FILE NAME: Listeria Phage Protocol_07292016

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SECTION 1 INTRODUCTION

1.1 Purpose

This document establishes guidelines for the purification, quantification, and maintenance of Listeria phage stocks.

1.2 Scope

This SOP applied to the food safety and MQIP labs.

1.3 Caution

Phage stocks are at a greater risk of contamination than bacterial stocks. Even one contaminant phage particle can outcompete and overwhelm a phage stock during subsequent amplifications (e.g., if the contaminant phage replicates faster and/or has a larger burst size). Make sure that pipet tips are sterile and ALWAYS use aseptic technique when working with phages. Pipettor barrels should be wiped off with ethanol before use, and make sure that pipette barrels never enter a tube containing phage (only the sterile tip should enter a tube with phage).

1.4 Definitions

Bacteriophage (phage): a virus that infects bacteria and can either instantly kill a bacterial cell or integrate its DNA into the host bacterial chromosome.
***Listeria* phage:** A phage with specificity to bacteria of the genus *Listeria*, mostly serotype specific, but some have a broad host range. *Listeria*-infecting phage may also be used.
Plaque: Observed clearing on an agar plate, which is due to lysis of the bacterial lawn by phage
PFU: Plaque forming unit, the number of viable phages present in a volume capable of forming plaques.
Confluent Lysis: Plate where plaques are overlapping to a point that the entire plate is essentially covered with phages

Host Strains for *Listeria* phages:

Listeria strain	FSL designation	Lineage	Serotype
FSL J1-175	FSL J1-0175	I	1/2b
FSL J1-208	FSL J1-0208	III	4a
MACK	FSL F6-0367	II	1/2a
F2365	FSL C6-0003	I	4b

1.5 Safety

L. monocytogenes is a BSL-2 pathogen. Appropriate protective measures need to be taken when working with *L. monocytogenes*. All waste from experiments involving *Listeria monocytogenes* needs to be treated as BSL-2 waste.

SECTION 2 MATERIALS

<u>Media and stock solutions:</u> Autoclave for 30 min after mixing media/solutions.
All media prepared can be stored for 6 months in appropriate conditions

LB MOPS (Lysogeny broth buffered with 3-(N-morpholino)propanesulfonic acid)
10g tryptone
5g yeast extract
5g NaCl
10.5g MOPS-free acid (50mM final concentration)
1.7g NaOH
1L ultrapure H20
store at room temperature

LB MOPS + Glucose + Salts plating agar
10g tryptone
5g yeast extract
5g NaCl
1L ultrapure H20
10.5g MOPS-free acid (50mM final concentration)
1.7g NaOH
1g glucose
2.03g $MgCl_2 \cdot 6H_2O$ (10mM $MgCl_2$ final concentration)
1.47g $CaCl_2 \cdot 2H_2O$ (10mM $CaCl_2$ final concentration)
15g Agar (1.5% agar)
1L ultrapure H20
After plates are poured store in cold room

LB MOPS + Glucose + Salts top overlay agar
10g tryptone
5g yeast extract
5g NaCl
1L ultrapure H20
10.5g MOPS-free acid (50mM final concentration)
1.7g NaOH
1g glucose
2.03g $MgCl_2 \cdot 6H_2O$ (10mM $MgCl_2$ final concentration)
1.47g $CaCl_2 \cdot 2H_2O$ (10mM $CaCl_2$ final concentration)
7g Agar (0.7% agar)
1L ultrapure H20
Heat and mix until agar is fully dissolved
Make 100 – 200 ml aliquots prior to autoclaving
Store at room temperature

1M Tris-Cl pH 7.5
12.1g Tris base
80mL H20
add 6N HCl until a pH of 7.5 is reached
adjust total volume to 100 ml with ultrapure H2O
Store at room temperature

Gelatin solution (2% w/v)
2g gelatin
100ml H2O
Heat and mix until gelatin is dissolved
Store at room temperature

SM Buffer with Gelatin (1L)
5.8g NaCl (100mM final concentration)
2g MgSO4•7H2O (8mM final concentration)
50mL 1M Tris-Cl 7.5pH (50mM final concentration) (if making 1M Tris-Cl 7.5pH at the same time you can use it here before autoclaving)
5mL Gelatin (2% w/v) (if making gelatin at the same time you can use it here before autoclaving)
adjust total volume to 1L with ultrapure H2O
Make 100 – 200 ml aliquots prior to autoclaving
Store at room temperature

Glassware and equipment:

4mL amber glass vials with rubber-lined cap (Wheaton #224982)
Used for long-term storage of phage stocks. Clean and autoclave vials prior to use.

16mm No-Wire Racks (Scienceware Cat. No. 185140016)
Used to hold the amber vials.

16x125 glass tubes with red caps
Should be autoclaved prior to use. Are in general lab stock, but prepare your own if using copious amounts.

Waterbath
Set to 50ºC to keep overlay agar molten.

Colony counter

5mL Strippettes and pipet-aid
p1000, p200 or p100, and p20 pipetors and Tips
Cell scrapers

SECTION 3 PROCEDURES

3.1 Protocol for enumerating phage stocks by spot assay

(for determining the approximate concentration of phage stocks).
**Preheat water bath to 50℃ to allow top agar to equilibrate.
**Spot assays should always be done in duplicate (i.e. technical replicate).

Overnight Cultures (Day 0):

1. Prepare overnight cultures of host bacteria. Inoculate 5 mL BHI broth with a single colony of the host bacteria. Streak plate should be no more than two weeks old.

Preparation of host overlay (Day 1):

1. Place a bottle of LB MOPS + Glucose + Salts Top Agar 0.7% in a container with water (should be at the same level as the agar) in the microwave. Melt agar completely and place bottle in pre-set water bath (50ºC) to equilibrate for at least 20 min.

2. Pipette 300 µL of a 1/10 dilution (30 µL of O/N culture in BHI, 270 µL of LB MOPS) of the host strain O/N culture into a 16 mm culture tube. The dilution of the host strain O/N (in BHI media) should be diluted into LB MOPS media. The dilution should be made no more than 1 hour before plating.

3. Using a 5 mL serological pipet, pipet 3.5 mL molten top agar into the tube (with 300 µL of host dilution) and briefly pulse vortex on a low setting to mix.

4. Pour mixture over LB MOPS plate and immediately (and carefully) swirl plate to evenly distribute the molten agar before it solidifies.

5. Let plate sit to solidify (try not to move the plate before the top layer solidifies because doing so may result in a bad lawn; solidification takes ~10-15 min).

Spotting phage dilutions (Day 1):

1. Transfer 10 µL of phage stock into 990 µL of SM buffer with gelatin to get a 10^{-2} dilution of the phage stock (sing this volume allows you to conserve stock volume).

2. Transfer 100 µL of the 10^{-2} phage dilution into 900 µL of SM buffer (with gelatin) to get a 10^{-3} dilution of the phage stock.

3. Continue to prepare the 1/10 phage stock dilutions, as in step 2, until you reach the dilution point where there would be an expected 1–10 PFU per spot (base this off the last known titer of the phage stock; e.g., the stock titer should never increase over time). For example, if at the last known titering event the concentration was 1×10^{7} PFU/mL, you should spot 5 µL of a 1×10^{-4} dilution to get about 5 plaques per spot.

4. Starting with lowest dilution pipet 5µL onto solidified plate with top layer. Spot each dilution in triplicate.

5. Spot all the dilution down to 10^{-2}.

6. Allow spots to dry by leaving the plate uncovered under a flame or in the biohood (should take ~10 min, can also leave them covered to dry outside of flame length but drying of the spots will take longer). **Do not move the plates excessively before the spots are dried as the spots may run together.

7. Flip plates upside down and incubate 18-24 hours at the incubation temperature indicated in the phage database (30ºC is the most common temp used for *Listeria* phages).

Enumeration of plaques (Day 2):

1. Remove plates from the incubator.

2. Count each spot in the dilution with a countable number of spots after putting the plate on the Colony Counter. Enter the number of plaques in each spot into the database in the spot titering section of the titer table.

3. Enter the dilution spotted in the appropriate column and enter the volume spotted in the appropriate column.

4. Enter any notes regarding the purity of the stock or any abnormalities in the note section.

5. If the total number of calculated PFU's in the stock is below 1×10^6 (e.g. 10mL of 1×10^5 PFU/mL stock, 1mL of 1×10^6 PFU/mL stock, or 0.1 mL 1×10^7 PFU/mL stock) make a note that the stock needs to be amplified (to do this, follow the protocol for amplifying phage stocks found in section 3.5).

3.2 Protocol for full plate titration of phage stock

(Should be used for more precise measurement of stock concentration)
**Preheat water bath to 50℃ to allow top agar to equilibrate.
**Everything done here is done on duplicate plates

Overnight cultures (Day 0)

1. Prepare overnight cultures of host bacteria. Inoculate 5 mL BHI broth with a single colony of the host bacteria. Streak plate should be no more than two weeks old.

Phage and host overlap (Day 1)

1. Place a bottle of LB MOPS + Glucose + Salts Top Agar 0.7% in a container with water (should be at the same level as the agar) in the microwave. Melt agar

completely and place bottle in pre-set water bath (50ºC) to equilibrate for at least 20 min.

2. Pipette 300 µL of a 1/10 dilution of the host strain O/N culture into a 16 mm culture tube. The dilution of the host strain O/N (in BHI media) should be diluted into LB MOPS media. The dilution should be made no more than 40 min before plating.

3. Transfer 100 µL of phage stock into 900 µL of SM buffer with gelatin to get a 10^{-1} dilution of the phage stock.

4. Continue to dilute the phage stock in 1/10 increments, as in step 3, until you reach the dilution point where there would be an expected 30–300 PFU per plate (base this off the last known titer of the phage stock; e.g., the stock titer should never increase over time). For example, if at the last known titering event the concentration was 1×10^{7} PFU/mL, you should plate 100 µL of a 1×10^{-4} dilution to get about 100 plaques per plate.

5. Pipet 100µL of the phage dilution into tube (with 300 µL host dilution).

6. Using a 5 mL serological pipet, pipet 3.5 mL molten top agar into the tube (with 300 µL of host dilution) and briefly pulse vortex on a low setting to mix.

7. Pour mixture over LB MOPS plate and immediately (and carefully) swirl plate to evenly distribute the molten agar before it solidifies.

8. Let plate sit to solidify (try not to move the plate before the top layer solidifies because doing so may result in a bad lawn; solidification takes ~10-15 min).

9. Flip plates upside down and incubate overnight at the incubation temperature indicated in the phage database (30ºC is the most common temp used for *Listeria* phages).

Enumeration of plaques (Day 2):

1. Remove plates from incubator.

2. Count each plate with a countable number of plaques by putting the plate on the Colony Counter. Enter the number of plaques in each spot into the database in the plate titering section of the titer table.

3. After entering the information for each individual plate enter the dilution and plating factor used in the appropriate column.

4. Enter any notes regarding the purity of the stock or any abnormalities in the note section.

5. If the total number of calculated PFU's in the stock is below 1×10^6 (e.g. 10mL of 1×10^5 PFU/mL stock, 1mL of 1×10^6 PFU/mL stock, or 0.1 mL 1×10^7 PFU/mL stock) make a note that the stock needs to be amplified (to do this, follow the protocol for amplifying phage stocks found in section 3.5).

3.3 Storage of phage stock in the library

1. Wash 4 mL amber vials 9x in normal water 3x in distilled water 1x in ultra-pure water.

2. Put amber vials in rack and put the lids on before autoclaving but make sure they are still loose (standard autoclave practice).

3. Label dry autoclaved vials as follows:

   side label:

Phage name	Stock ID – (A or B)
netid	date

   top label:

Phage name
Stock ID – (A or B)

4. Put half of the stock into the "A" vial and the other half into the "B" vial.

5. Store phage stocks at 4℃ and keep the "A" and "B" stocks in separate areas in case of emergency.

3.4 Purification of phage stocks

(For the purification of a contaminated stock).
**Preheat water bath to 50℃ to allow top agar to equilibrate.
**Everything done here is done on duplicate plates

Overnight cultures (Day 0)

1. Prepare overnight cultures of host bacteria. Inoculate 5 mL BHI broth with a single colony of the host bacteria. Streak plate should be no more than two weeks old.

Phage and host overlap (Day 1)

1. Place a bottle of LB MOPS + Glucose + Salts Top Agar 0.7% in a container with water (should be at the same level as the agar) in the microwave. Melt agar

completely and place bottle in pre-set water bath (50ºC) to equilibrate for at least 20 min.

2. Pipette 300 µL of a 1/10 dilution (30 µL of O/N culture in BHI, 270 µL of LB MOPS) of the host strain O/N culture into a 16 mm culture tube. The dilution of the host strain O/N (in BHI media) should be diluted into LB MOPS media. The dilution should be made no more than 1 hour before plating.

3. Transfer 100 µL of phage stock into 900 µL of SM buffer with gelatin to get a 10^{-1} dilution of the phage stock.

4. Continue to dilute the phage stock in 1/10 increments, as in step 3, until you reach the dilution point where there would be an expected 1–10 PFU of the phage you are trying to purify per plate (base this off the last known titer of the phage stock; e.g., the stock titer should never increase over time). For example, if at the last known titering event the concentration was 1×10^{7} PFU/mL, you should plate 100 µL of a 1×10^{-5} dilution to get about 10 plaques per plate.

5. Pipet 100µL of the phage dilution into tube (with 300 µL host dilution).

6. Using a 5 mL serological pipet, pipet 3.5 mL molten top agar into the tube (with 300 µL of host dilution) and briefly pulse vortex on a low setting to mix.

7. Pour mixture over LB MOPS plate and immediately (and carefully) swirl plate to evenly distribute the molten agar before it solidifies.

8. Let plate sit to solidify (try not to move the plate before the top layer solidifies because doing so may result in a bad lawn; solidification takes ~10-15 min).

9. Flip plates upside down and incubate overnight at the incubation temperature indicated in the phage database (30ºC is the most common temp used for *Listeria* phages).

Plaque picking (Day 2):

1. Remove plates from incubator.

2. From the plates with the lowest number of plaques present take a sterile blunt pipet tip and pick 1-4 plaques. Make sure that plaques picked are far from any possible contaminant plaques.

3. Transfer each individual plaque into 300-500µL sterile SM Buffer w/Gelatin in a 1.5mL Eppendorf Tube

4. Let the plaques sit for at least 12 hours at 4℃, but they can sit for up to a week.

5. Take 100µL of the SM buffer that has had the plaque in it and make a dilution series down to 10^{-7}.

6. Repeat the "purification of phage stocks" protocol to this point with all the dilutions made in step 5.

7. Repeat until on the higher dilutions there is no contaminant phage plaques and then repeat once more.

8. Take the purified phage and amplify according to the protocol to amplify phage stocks (section 3.5).

3.5 Amplification of phage stocks

(If amplifying a stock with a very low number (to the point where you cannot achieve confluent lysis) of phages present go through protocol to purify the stock section 3.4 before amplifying it.)

**Preheat water bath to 50℃ to allow top agar to equilibrate.

**Everything done here is done on duplicate plates

Overnight cultures (Day 0)

1. Prepare overnight cultures of host bacteria. Inoculate 5 mL BHI broth with a single colony of the host bacteria. Streak plate should be no more than two weeks old.

Phage and host overlap (Day 1)

1. Place a bottle of LB MOPS + Glucose + Salts Top Agar 0.7% in a container with water (should be at the same level as the agar) in the microwave. Melt agar completely and place bottle in pre-set water bath (50ºC) to equilibrate for at least 20 min.

2. Pipette 300 µL of a 1/10 dilution (30 µL of O/N culture in BHI, 270 µL of LB MOPS) of the host strain O/N culture into a 16 mm culture tube. The dilution of the host strain O/N (in BHI media) should be diluted into LB MOPS media. The dilution should be made no more than 1 hour before plating.

3. Transfer 100 µL of phage stock into 900 µL of SM buffer with gelatin to get a 10^{-1} dilution of the phage stock.

4. Continue to dilute the phage stock in 1/10 increments, as in step 3, until you reach the dilution point where there would be an expected 300–3000 PFU per plate (base this off the last known titer of the phage stock; e.g., the stock titer should never increase over time). For example, if at the last known titering event the concentration was 1×10^{7} PFU/mL, you should plate 100 µL of a 1×10^{-3} dilution to get about 1000 plaques per plate.

5. Pipet 100µL of the phage dilution into tube (with 300 µL host dilution) use the highest dilution and others until you plate the dilution you would expect confluent lysis (definition section 1.4), usually about 5000 plaques, on and plate one dilution lower in case lower than expected phage titer.

6. Using a 5 mL serological pipet, pipet 3.5 mL molten top agar into the tube (with 300 µL of host dilution) and briefly pulse vortex on a low setting to mix.

7. Pour mixture over LB MOPS plate and immediately (and carefully) swirl plate to evenly distribute the molten agar before it solidifies.

8. Let plate sit to solidify (try not to move the plate before the top layer solidifies because doing so may result in a bad lawn; solidification takes ~10-15 min).

9. Flip plates upside down and incubate overnight at the incubation temperature indicated in the phage database (30ºC is the most common temp used for *Listeria* phages).

Amplification of phage (Day 2):

1. Remove plates from incubator.

If there is any suspicion of contamination go through purification protocol (section 3.4)

2. Taking the plates with confluent lysis add 5mL of SM Buffer w/Gelatin and using a cell scraper "chop" up the top layer of agar. *Before doing this for the first time have someone who has done it before demonstrate or observe you chopping up the top layer*

3. Put the plates with the SM Buffer to shake at ~60rpm for 1-2 hours at room temperature.

4. Pipet as much of the SM buffer as you can into 15mL falcon tubes one falcon tube for each plate.

5. Pre-chill centrifuge to 4℃ and spin the falcon tubes at 18000G (3000RPM) for 15 min.

6. Pipet the liquid out of the falcon tube and syringe filter into a new tube. The top agar should be pelleted onto the bottom of the tube separate from the liquid, don't try to filter any of the top agar because it can and probably will clog the filter. You should mix the samples from the two different tubes making one stock.

7. After amplification perform a spot titer (section 3.1) to determine the approximate concentration of the new stock.

SECTION 4 DATABASE

4.1 Entering phage titer into the database

- Enter all relevant data into the phage database as soon as possible (consult the chart below if you need clarification regarding what information goes where)
- When entering spot titer data you enter the number of plaques counted for each spot separately before averaging al the spots together. Enter the dilution counted and the volume spotted to calculate the approximate concentration.
- When entering full plate titer data for a full plate titering event, enter the phage counts for each plate separately before averaging them together and then from that average calculate approximate concentration by multiplying the average number of plaques per plate times the dilution and plating factor.

4.2 Taking pictures and entering them into the database

- All titering events should have a picture associated with them
- Before taking pictures make sure that the pictures will be of as high quality as possible.
- Taking pictures on the lightbox is the best option but there is also a lab camera that can be used if it will provide pictures with as many/all plaques visible.
- For each set of duplicate plates only one plate needs to be photographed.
- To link pictures to the database take the picture needed from the unorganized pictures and rename it with the next 7 digit number in order and put the picture into the organized pictures folder (eg. 0000001.jpg).
- Insert a hyperlink to the location of the picture linking it into the database
- To insert a hyperlink in excel right click and select hyperlink then select the document tab and then select the image file needed to hyperlink it into the spreadsheet
- *IMPORTANT* Once a picture is linked to in the database it cannot be moved relative to the location of the database. Once the picture is named and in the organized pictures folder that is its permanent location.

Field name	Table	Description	Example
Phage	Phage table (main ID for phage table)	phage name	LP-009
Stock ID (unorganized)	Stock table	designation for specific stocks of phage strains	LP-009-A

Stock ID (organized)	Stock table	an organized stock designation system for phage strains	F-0001-A
Titration ID	Titration table	specific titering event designation	LP-009-A.1 (if unorganized) F-0001-A.1 (if organized)
Titration Date	Titration table	date titering information was recorded in the database (should be the day plates are counted)	11/30/2014
Isolation Host	Phage table	Bacterial strain phage was isolated from	MACK
Prefered Host	Phage table	Bacterial strain phage prefers and should be used for the specific phage	MACK
FSL Designation	Phage table	The FSL ID of the phage	FSL LP-0009
Master Stock ID	Phage table	Stock ID (organized) of the primary stock for this phage	F-0001-A (if organized) LP-0009-A (if unorganized)
Previous Master Stock ID(s)	Phage table	Stock ID of the previous primary stock for this phage	F-0001-A (if organized) LP-0009-A (if unorganized)
Preferred Incubation Temp (ºC)	Phage table	Temperature phage should be incubated	30
Stock created by	Stock table	who created the stock	tgd32
Stock created on	Stock table	date of stock creation	11/30/2014
Stock status (mL remaining)	Stock table	Volume remaining	4
Stock location	Stock table	Locations of the primary stock	Cold room, Incubator #54

Stock creation method	Stock table	how the stock was made either by plate lysate, in a liquid culture, or new stock made by organizing it	plate lysate, liquid culture, moving into new stock
Spot or Plate	Titration table	Which method was used for the titering event	spot
# plaques plate x spot x	Titration table	for spot titrations enter the number of plaques for each spot in an appropriate column, for plate titrations enter the number of plaques for each plate in appropriate column	20
Average plaques per spot combined	Titration table	average number of plaques for all spots in a spot titer	20
countable plaque dilution (10^-x)	Titration table	dilution the plaques were counted at	5
Volume per spot(µL)	Titration table	volume that was spotted in each spot	5
Spot concentration (approximate PFU/mL)	Titration table	Approximate concentration (PFU/mL of the phage stock based on the spot titration (formula=(Average plaques per spot combined/volume per spot)*(1000*dilution)	4.00E+08
Full plate plaque average	Titration table	average number of plaques of the two plates in a full plate titration	100
Full plate dilution and plating	Titration table	dilution of phage used for the titration with the plating factor accounted for	5

factor (10^-x)			
Full plate concentration	Titration table	Approximate concentration (PFU/mL of the phage stock based on the full plate titration (formula=full plate plaque average*10^(full plate dilution and plating factor)	1.00E+07
Host strain used (if different)	Titration table	Host strain used for titering event if different from the standard host strain for the phage	MACK
Image	Titration table		Link to image; images named as Titration ID; FSL_LP_009A1 (as of now the pictures have a separate number designation because there may be more than one picture of the same phage but for different titering events)
notes	notes	notes	there seemed to be a contaminant will perform full plate to follow up

4.3 Explanation of database columns

Phage table - phage specific information common across different stocks of the same phage
Stock table - Stock specific information that may be different for different stocks of the same phage
Titration table - Information about the specific titer event