SOP Title: Preparation of Bacterial Glycerol Stock for Plasmid Purification		
Document ID: 20002	Version	1.0
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Effective Date: 10 Dec 21		

Written by:		
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1. PURPOSE

1.1 The purpose of this procedure is to prepare the bacterial glycerol stock for at -65 to -90°C storage.

2. SCOPE

2.1 This procedure applies to the Vaccine, Immunity and Cancer Directorate Laboratories.

3. REFERENCES

3.1 Addgene plasmid long-term storage instruction

3.2 10009: General Record Review

3.3 15000: Waste Disposal at the Advanced Technology Research Facility

3.4 20004: Plasmid Purification Using a QIAGEN Kit

3.5 20010: Plasmid Purification Using a Zymo Research Kit

3.6 26000: Biosafety Cabinet (BSC) Use and Maintenance

3.7 26005: Use and Maintenance of a 2-8°C Refrigerator

3.8 26007: Use and Maintenance of the Fisher Scientific Isotemp GDP10 Water Bath

3.9 26008: Use and Maintenance of Incubator Shakers

3.10 26009: Use and Maintenance of Pipettes

3.11 26014: Use and Maintenance of Laboratory Convection Incubators

3.12 26016: Operation, Use and Maintenance of the Water Purification Systems

3.13 26030: Use and Maintenance of a -80°C Freezer

4. RESPONSIBILITIES

4.1 The Research Associate, hereafter referred as analyst, is responsible for reviewing and following this procedure, and documenting assay information.

4.2 The Scientific Manager or designee is responsible for training personnel in this procedure and reviewing associated documentation.

4.3 The Quality Assurance Specialist is responsible for quality oversight and approval of this procedure.

5. DEFINITIONS

5.1 Amp – Ampicillin

5.2 Blas – Blasticidin

5.3 Kan – Kanamycin

5.4 LB – Luria broth

5.5 SOP – Standard Operating Procedure

5.6 TB – Terrific Broth

5.7 TBD - To be determined

5.8 TOC – Total Oxidizable Carbon

5.9 Type I Water - Ultrapure/Reagent Grade/critical applications (Resistivity >18 MΩ-cm and TOC < 50 ppb)

5.10 Zeo - Zeocin

6. REAGENTS, MATERIALS AND EQUIPMENT

6.1 Reagents

6.1.1 Addgene Plasmid Bacterial Stab (transformed bacteria in stab culture format stable at 2-8°C for at least 2 weeks)

6.1.2 Ampicillin Solution, 100 mg/mL (Teknova, Cat # A9626 or equivalent

6.1.3 Clorox Bleach, Concentrated (FNLCR Warehouse, Cat #68100251 or equivalent)

6.1.4 Fast-Media® Amp Media (Invivogen, Cat # fas-am-b or equivalent)

6.1.5 Fast-Media® Kan Media (Invivogen, Cat # fas-kn-b or equivalent)

6.1.6 Fast-Media® Blas TB (Invivogen, Cat # fas-bl-l or equivalent)

6.1.7 Fast-Media® Zeo TB (Invivogen, Cat # fas-zn-l or equivalent)

6.1.8 Fast-Media® Amp Agar (Invivogen, Cat # fas-am-s or equivalent)

6.1.9 Fast-Media® Kan Agar (Invivogen, Cat # fas-kn-s or equivalent)

6.1.10 Fast-Media® Blas Agar (Invivogen, Cat # fas-bl-s or equivalent)

6.1.11 Fast-Media® Zeo Agar (Invivogen, Cat # fas-zn-s or equivalent)

6.1.12 Kanamycin Solution, 100 mg/mL (Teknova, Cat # K2135 or equivalent)

6.1.13 LB Broth, 1 L Bottle (Teknova, Cat # L8000-12 or equivalent)

6.1.14 Plasmids in DH5a Bacteria, Laboratory Transformed

6.1.15 5x Terrific Broth with Kanamycin (Teknova, Cat # T8211-12 or equivalent)

6.1.16 10x Terrific Broth (Teknova, Cat # T7009 or equivalent)

6.2 Materials

6.2.1 Aluminum Foil

6.2.2 Cryovial (Fisher Scientific, Cat # 12-565-163N or equivalent)

6.2.3 Erlenmeyer Flask with Baffles (Thomas Scientific, Cat # 1234D77 or equivalent)

6.2.4 250 mL Filter Bottle, 0.2 µM PES Membrane - Nalgene (Thomas Scientific, Cat # 1234K60 or equivalent)

6.2.5 Glycerol (Sigma-Aldrich, Cat #G7893-500mL)

6.2.6 Ice Pan (Thomas Scientific, Cat # 1200R42 or equivalent)

6.2.7 Petri Dish (FNLCR Warehouse, Cat # 66401175)

6.2.8 Pipette Tips

6.2.9 Inoculation Loops (Thomas Scientific, Cat # 1230Z29 or equivalent)

6.2.10 25 mL Serological Pipets (FNLCR Warehouse, Cat # 66401361 or equivalent)

6.2.11 Type I water (Life Technologies Cat # 15230204 or equivalent)

6.3 Equipment

6.3.1 Class II Biosafety Cabinet (BSC)

6.3.2 Convection Oven

6.3.3 -65 to -90°C Freezer

6.3.4 Microwave

6.3.5 Water Purification System

6.3.6 Orbital Shaker

6.3.7 Pipettes

6.3.8 2-8°C Refrigerator

6.3.9 Timer

6.3.10 Sterilized Graduated Cylinder with aluminum foil cover

6.3.11 Sterilized Beaker with aluminum foil cover

6.3.12 Water Bath

6.3.13 Silicone Hand Protector (Thomas Scientific Cat # 1150H33 or equivalent)

6.3.14 Heat Resistant Gloves (Thomas Scientific Cat # 1176R24 or equivalent)

7. HEALTH AND SAFETY CONSIDERATIONS

7.1 Proper safety precautions must be taken while working in a laboratory setting. This includes, but is not limited to, proper protective equipment such as lab coats, safety glasses, closed-toe shoes, and non-latex gloves.

7.2 Refer to the respective Safety Data Sheet when working with any chemicals.

7.3 Refer to “15000: Waste Disposal at the Advanced Technology Research Facility” regarding waste disposal processes at the Advanced Technology Research Facility.

8. PROCEDURE PRINCIPLES

8.1 All process relevant information is recorded on “20002-01: Preparation of Bacterial Glycerol Stock for Plasmid Purification Form.”

8.2 When available, all steps are performed in the BSC "26000: Biosafety Cabinet (BSC) Use and Maintenance."

8.3 To obtain sterile Type I water using the Water Purification System, sterile filter Type I water (see "26016: Operation, Use and Maintenance of Water Purification Systems") using a 0.2 µm PES filter. Reagent expires 2 months from date of preparation and is stored at room temperature.

8.4 Sterile filtered Type I water from the Water Purification Systems or purchased Distilled water from vendor may be used interchangeably as sterile Type I water throughout the procedure.

9. PROCEDURE

9.1 Preparing Agar Plate

Note: Procedure in section 9 is only performed when using Fast-Media® agar powder.

9.1.1 Pour the Fast-Media® Amp/Kan/Blas/Zeo Agar powder into a clean, autoclaved 1L borosilicated glass flask or borosilicate glass bottle.

Note: Make sure to use the appropriate antibiotic additive for bacteria being used; see Attachment 1 for reference.

9.1.2 Add 200 mL of sterile Type I water

9.1.3 Mix thoroughly by swirling the glass bottle or flask

9.1.4 Remove foil and place flask in the microwave and heat on MEDIUM power setting (about 450W) until bubbles start to appear (about 3 minutes)

9.1.5 Repeat steps 9.1.3 and 9.1.4 until medium is completely dissolved. Do not overboil. Use heat resistant gloves or silicone hand protectors to hold flask.

9.1.6 Replace the autoclaved foil cover on the flask and allow the medium to cool to about 50-55°C before use. At this temperature, the flask will be cool enough to touch and the agar is still liquid.

Note: May use water bath set at 50 ± 5°C per "26007: Use and Maintenance of the Fisher Scientific Isotemp GDP10 Water Bath"

9.1.7 Place 8 to 10 petri dishes on a flat surface and transfer approximately 20 mL of LB agar into each petri dish to completely cover the bottom of the dish.

9.1.8 Replace the lid of each petri dish and let the petri dish sit at room temperature until the agar has solidified.

9.1.9 Label the plates with date of preparation and antibiotic additive. See Attachment 2 for label example. Wrap petri dishes with parafilm.

9.1.10 Dishes should be stored UPSIDE DOWN at 2-8°C and given an expiration date of ONE MONTH.

9.2 Preparing Liquid Media

9.2.1 Preparing Liquid Media from Invivogen Fast-Media®.

9.2.1.1 Pour the Fast-Media® Amp/Kan/Blas/Zeo powder into a clean, autoclaved 1L borosilicated glass flask or borosilicate glass bottle.

Note: Make sure to use the appropriate antibiotic additive for bacteria being used; see Attachment 1 for reference.

9.2.1.2 Add 200 mL of sterile Type I water

9.2.1.3 Mix thoroughly by swirling the glass bottle or flask.

9.2.1.4 Remove foil and place flask in the microwave and heat on MEDIUM power setting (about 450W) until bubbles start to appear (about 3 minutes)

9.2.1.5 Repeat steps 9.2.1.3 and 9.2.1.4 until medium is completely dissolved. Do not overboil. Use heat resistant gloves or silicone hand protectors to hold flask.

9.2.1.6 Replace the autoclaved foil cover on the flask and allow the medium to cool to about room temperature up to 37°C before use. At this temperature, the flask will be cool enough to handle without heat resistant gloves.

Note: May use water bath set at 37 ± 2°C per “26007: Use and Maintenance of the Fisher Scientific Isotemp GDP10 Water Bath”

9.2.2 Preparing Liquid Media from sterile liquid source

9.2.2.1 Prepare 200 mL growth medium with antibiotic in a clean, autoclaved flask; see Attachment 1 for reference.

9.2.2.2 Dilute stock media as needed using sterile Type I water.

9.2.2.3 For example, Teknova LB media is used neat without addition of Type I water. Teknova 10X TB media is prepared by mixing 20 mL of 10X TB media with 180 mL sterile Type I water.

9.2.2.4 If Kanamycin is required, add to growth media at a final concentration of 50 µg/mL (1:2,000 dilution of 100 mg/mL stock concentration).

9.2.2.5 For example, 200 µL of 100 mg/mL Kanamycin solution into 400 mL growth media

9.2.2.6 If Ampicillin is required, add to growth media at a final concentration of 100 µg/mL (1:1,000 of 100 mg/mL stock concentration).

For example, 200 µL of 100 mg\mL Ampicillin solution in 200 mL growth media.

9.3 Preparation of 50% Glycerol

9.3.1 If using previously prepared “Autoclaved 50% Glycerol”, record lot number on 20002-01 form.

9.3.2 Mix equal amount of sterile Type I water and 100% Glycerol in an autoclavable container.

9.3.3 Autoclave 50% Glycerol for 15 minutes on liquid cycle. Follow liquid autoclave procedure per 15000.

9.3.4 Label the bottle as “Autoclaved 50% Gycerol” with Data Reference, preparation date, analyst initials and one year expiration date. Store at room temperature.

9.4 Streaking a Plate from a Plasmid Bacterial Stab Culture or a Liquid Culture

9.4.1 Pre-warm agar plate with appropriate antibiotic selection for at least 20 minutes at 37 ± 2°C in the Oven per “26014: Use and Maintenance of Laboratory Convection Incubators” or Orbital Shaker per “26008: Use and Maintenance of Incubator Shakers”.

9.4.2 Label the bottom of the plate with the lot number, plasmid name, antibiotic resistance, date, and analyst initials per Attachment 2.

9.4.3 Using a sterile pipette tip, touch the bacteria growing within the punctured area of the stab culture. See Figure 1 below. Or using a sterile loop or pipette tip, touch the bacterial liquid culture.

Figure 1: Bacterial Stab

Figure 2: Plate Streak

9.4.4 Run the tip or loop lightly over a section of the plate to spread the bacteria over approximately one-fourth of the surface area of the plate, as shown in Figure 2, to streak the first quadrant.

9.4.5 Using a new sterile pipette tip or sterile edge of a loop, pass through the first quadrant and dilute the bacteria over the second quadrant of the plate, and continue diluting bacteria over the third and fourth quadrant of the plate to achieve single colonies.

9.4.6 Incubate the plate overnight (12-18 hours) at 37 ± 2°C, at the designated growth temperature using the Convection Incubator per 26014 or Orbital Shaker per 26008.

Note: Some plasmids require a different temperature or growth condition. Refer to the Scientific Manager and manufacturer plasmid information page for specific growth conditions.

Note: DO NOT use a CO_2 culture incubator.

9.4.7 After the incubation check for single colonies. A single colony should appear as a separate white dot growing on the solid medium, see Figure 2.

9.4.8 Once single colonies are obtained, proceed to Section 9.5, Inoculating an overnight liquid culture.

9.5 Inoculating an Overnight Liquid Culture from a Single Colony

9.5.1 Obtain appropriate liquid media for the bacteria desired (Section 9.2)

9.5.2 Label a sterile tube or flask with Plasmid Name, Lot number, Bacteria Type, Total volume in tube, date, and analyst Initials per Attachment 2.

9.5.3 Add 10 mL liquid media to the labeled sterile tube or flask

9.5.4 Using a sterile pipette tip or loop, select a single colony from the streaked agar plate (Section 9.4)

9.5.5 Drop the tip or loop into the liquid media then swirl.

9.5.6 Loosely cover the culture with sterile aluminum foil or a cap that is not air-tight.

9.5.7 Incubate bacterial culture at 37 ± 2 °C at 250 ± 10 RPM for 12-18 hours in the Orbital Shaker per 26008.

9.5.8 Note: Some plasmids may specify a different temperature, follow instructions provided with each plasmid.

9.6 Creating Glycerol Stock of the Plasmid

9.6.1 Label cryovials with the following information per Attachment 2 with plasmid name, lot number, preparation date, analyst initials, volume, and liquid media composition.

9.6.2 Add equal volumes of 50% glycerol and overnight liquid culture to a sterile tube or bottle and mix gently.

9.6.3 Aliquot 1 mL of bacterial glycerol stock to each labeled cryovial.

9.6.4 Place cryovial into a box labelled per Attachment 2 and freeze at -65 to -90°C.

Note: Vials are single use.

Table 1: Approximate Time Bacterial Cultures Remain Stable

Condition	Temperature (°C)	Time (approximate)
Agar plates	4 (2-8)	4 weeks
Stab cultures	4 (2-8)	2 weeks
Glycerol Stock	-65 to -90	1-10 years

10. ATTACHMENTS

10.1 Attachment 1: List of Plasmids and Characteristics

10.2 Attachment 2: Petri Dish, Flask, Cryovial, and Box Labels

10.3 Attachment 3: 20002-01: Preparation of Bacterial Glycerol Stock for Plasmid Purification Form.

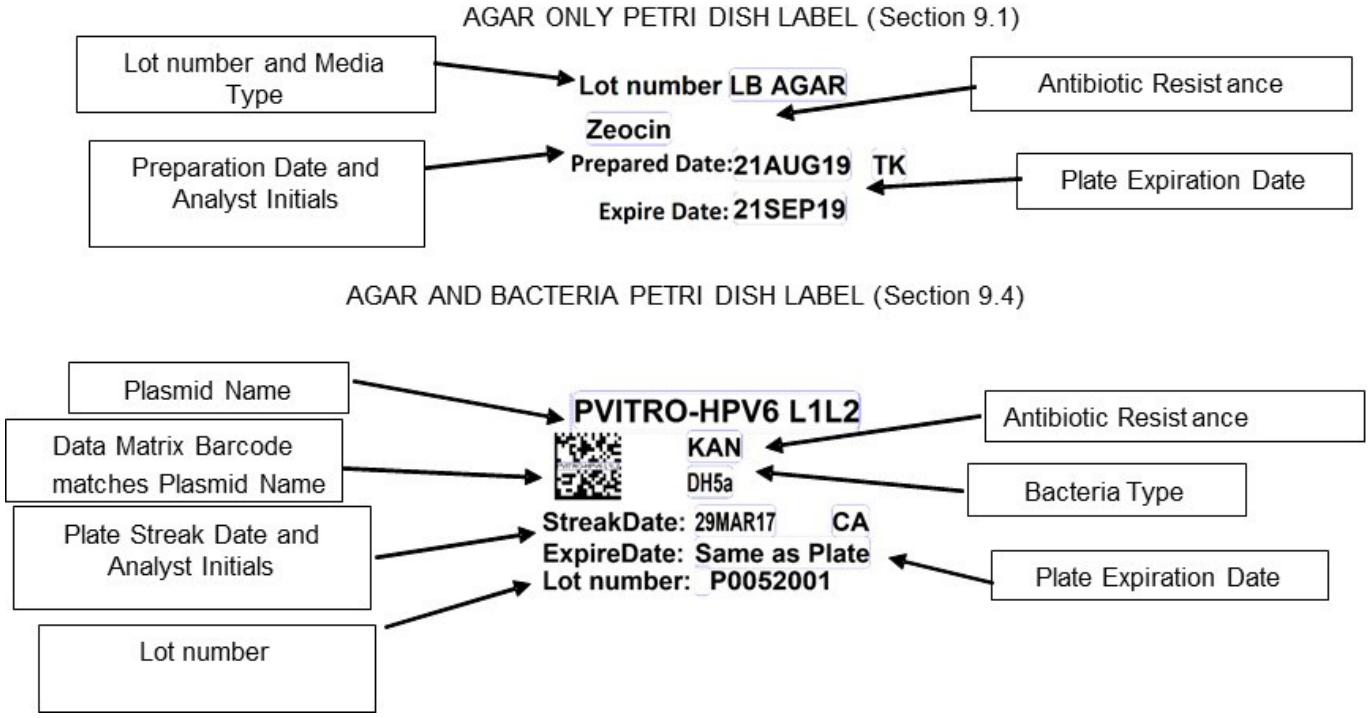
11. REVISION HISTORY

Version	Change	Reason
1.0	New SOP numbering from HSL_LAB_002	Reflect current practices

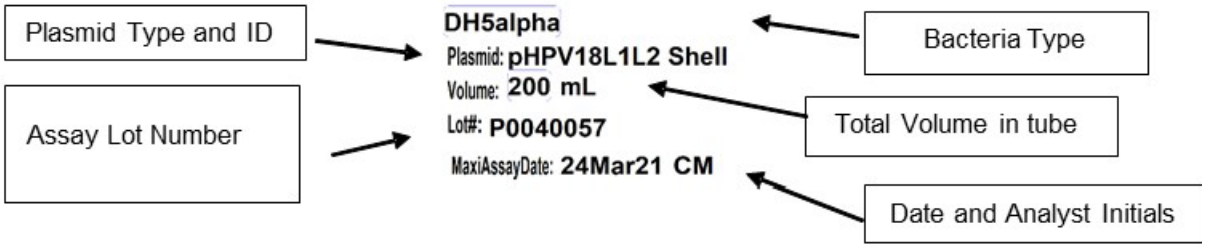
Attachment 1: List of Plasmids and Characteristics

COMPANY	*PLASMID*	*BACTERIA STRAIN*	*ANTIBIOTIC*	*ANTIBIOTIC CONC.*	*GROWTH MEDIA*
ADDGENE	PVITRO-HPV6 L1L2	E.COLI_DH5A	KANAMYCIN	50 µg/mL	LB or TB
ADDGENE	PVITRO-HPV52 L1L2	E.COLI_DH5A	KANAMYCIN	50 µg/mL	LB or TB
ADDGENE	PVITRO-HPV31 L1L2	E.COLI_DH5A	KANAMYCIN	50 µg/mL	LB or TB
ADDGENE	PVITRO-HPV18 L1L2	E.COLI_DH5A	KANAMYCIN	50 µg/mL	LB or TB
ADDGENE	PVITRO-HPV11 L1L2	E.COLI_DH5A	KANAMYCIN	50 µg/mL	LB or TB
ADDGENE	PVITRO-HPV33 L1L2	E.COLI_DH5A	KANAMYCIN	50 µg/mL	LB or TB
ADDGENE	P11L2W	E.COLI_DH5A	KANAMYCIN	50 µg/mL	LB or TB
ADDGENE	P58SHELL	E.COLI_DH5A	AMPICILLIN	100 µg/mL	LB or TB
ADDGENE	P18SHELL	E.COLI_DH5A	AMPICILLIN	100 µg/mL	LB or TB
ADDGENE	P31SHELL	E.COLI_DH5A	AMPICILLIN	100 µg/mL	LB or TB
ADDGENE	P52SHELL	E.COLI_DH5A	AMPICILLIN	100 µg/mL	LB or TB
ADDGENE	P11L1W	E.COLI_DH5A	KANAMYCIN	50 µg/mL	LB or TB
ADDGENE	P45SHELL	E.COLI_DH5A	AMPICILLIN	100 µg/mL	LB or TB
ADDGENE	P16SHELL	E.COLI_DH5A	AMPICILLIN	100 µg/mL	LB or TB
ADDGENE	P6SHELLR	E.COLI_DH5A	AMPICILLIN	100 µg/mL	LB or TB
SCHILLER	PFWB (EGFP)	E.COLI_DH5A	ZEOCIN	25 µg/mL	LB or TB
SCHILLER	PYSEAP	E.COLI_DH5A	BLASTICIDIN	75 µg/mL	LB or TB
SCHILLER	PHPV16L1H	E.COLI_DH5A	AMPICILLIN	100 µg/mL	LB or TB

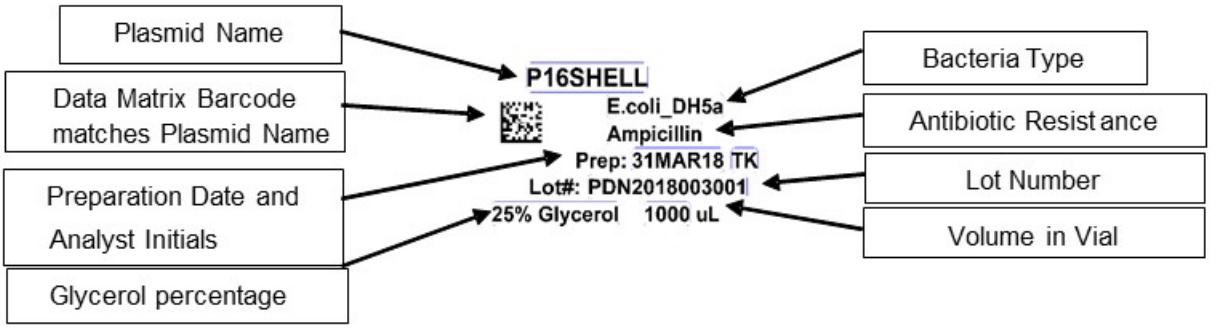
Attachment 2: Petri Dish, Flask, Cryovial, and Box Labels



Flask Label (Section 9.5)



Cryovial Label (Section 9.6)



BOX LABEL FOR BACTERIAL GLYCEROL STOCK

Attachment 3: 20002-01: Preparation of Bacterial Glycerol Stock for Plasmid Purification Form.

Frederick National Laboratory for Cancer Research sponsored by the National Cancer Institute		Vaccine, Immunity and Cancer Directorate Standard Operating Procedure Form	
Form Title: Preparation of Bacterial Glycerol Stock for Plasmid Purification Form			
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Bacterial Glycerol Stock Description _____ **Lot #**_____

Equipment

Equipment Name	Equipment ID	Calibration Due Date
BSC	☐ HSL_009 ☐ Other	
-80 ±10°C Freezer	☐ HSL_022 ☐ HSL_105 ☐ Other	
2-8°C Refrigerator	☐ HSL_029 ☐ HSL_043 ☐ Other	☐ N/A
☐ N/A Water Bath	☐ HSL_010 ☐ Other	
☐ N/A Orbital Shaker	☐ HSL_011 ☐ HSL_050 ☐ Other	
☐ N/A Convection Oven	☐ HSL_025 ☐ Other	
☐ N/A Microwave	☐ HSL_053 ☐ Other	☐ N/A
Pipette _____µL	PIP_	
☐ N/A Pipette_____µL	PIP_	
☐ N/A Milli-Q		

Reagents

Reagent Name	Lot Number	Expiration Date
☐ N/A Fast-Media Amp Media		
☐ N/A Fast-Media Kan Media		
☐ N/A Fast-Media Blas TB		
☐ N/A Fast-Media Zeo TB		
☐ N/A Fast-Media Amp Agar		
☐ N/A Fast-Media Kan Agar		
☐ N/A Fast-Media Blas Agar		
☐ N/A Fast-Media Zeo Agar		
☐ N/A LB		
☐ N/A TB		
☐ N/A Kanamycin		
☐ N/A Ampicillin		
☐ N/A Zeocin		

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Reagent Name	Lot Number	Expiration Date
☐ N/A Autoclaved 50% Glycerol		
☐ N/A 100% Glycerol		
☐ N/A Type I Water		

Agar Plates:

Volume of Type I Water (mL)	Fast-Media, Agar Used (Amp/Kan/Blas/Zeo)	# Fast-Media Pouches Added	# Plates Prepared
Performed by/date:			

Streaking and Inoculation of Agar Plates:

☐ Plate Pre-Warm ☐ N/A			
Agar Plate (Amp/Kan/Blas/Zeo)	Bacterial Culture Description		Lot Number
Agar Plate Inoculation			
Incubation Temperature (°C):		Incubation Start Time/Date:	
Performed by/Date:			
Incubation End Time/ Date:		Total Hours Incubated:	
Performed by/Date:			
Expiration Date of Agar Plate (4 weeks):			
Storage Location of Agar Plate:			
Reviewed by/Date:			

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Liquid Media Preparation for Bacteria Culture:			
Fast-Media Pouches ☐ N/A Section, LB or TB used			
Type I Water Sterile Filtered	Volume of Water (mL)	# of Fast-Media Pouches Added	Total # of Flasks Prepared
☐ Yes ☐ N/A			
LB or TB ☐ N/A Section, Fast-Media Pouches used			
Volume of LB or TB (mL)	Volume of Type I water Sterile Filtered (mL) ☐ N/A	Volume of Prepared Antibiotic Added (µL)	Total # of Flasks prepared

Overnight Liquid Culture Inoculation			
Incubation Temperature (°C):		Incubation Start Time/Date:	
Performed by/Date:			
Incubation End Time/ Date:		Total Hours Incubated:	
Performed by/Date:			
Reviewed by/Date:			

50% Glycerol Preparation: ☐ N/A

Volume of Type I Water (mL)	Volume of 100% Glycerol (mL)	Date Autoclaved
Performed by/date:		

Bacterial Glycerol Stock Preparation:

Volume of Bacterial Culture (mL)	Volume of 50% Glycerol (mL)	# of Vials per Bacterial Plasmid

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Final Glycerol Stock Storage (Check box as performed)	
☐ Freeze at -80 ±10°C ☐ Update freezer inventory	
Performed by/Date:	
Expiration Date of Glycerol Stock (1-10years):	
Storage Location of Bacterial Glycerol Stock:	

Comments:
☐ N/A

Performed by/date:	
Reviewed by/date:	

Lot Number: _____

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