

Table of Contents

Solutions

Complete Cell Culture Medium	
Tall-104	3
Jurkat T	4
Raji	4
K-562	4
Cell Culture Supplements	
L-Glutamine	4
Pen-Strep	4
Bulk Interleukin-2	5
Heat-inactivated Fetal Bovine Serum	5
Ringer's Solutions	
Stock Solutions	6
2 mM Ca ²⁺ Normal Ringers	6
Zero Ca ²⁺ Ringers	7
Zero Ca ²⁺ Ringers + EGTA	7
Squeeze & Spray Bottles	
100% Bleach	8
10% Bleach	8
100% Ethanol	8
1% Alconox	8
70% Ethanol	8
DNA Preparation Components	
Pre-NZY	9
NZY	9
LB	9
Antibiotics	
Kanamycin	9
Ampicillin	9
Reagents for LAMP assay	
PMA	10
Thapsigargin	10
FACS Buffer	10
16% PFA→4%	10

Tasks

Incubator Care	
Water at Bottom	11
Water Jacket	11
CO2 Tank	11
Dishwashing	12
Autoclaving	13-15
Biohazardous Waste	16
Liquid Nitrogen Tank	17
LB Agar Plates	17

Protocols

LAMP Assay	18
Transforming DNA into Competent Cells	19
E.Coli Cultures	
Starter Culture	20
Maxiprep Overnight	20
Miniprep Overnight	21
Making Glycerol Stock	21
DNA Quantification	22
Freezing Cell Lines	23
Thawing Cell Lines	23
1% Agarose Gel	24
Conjugating CD107a (LAMP) to Alexa-647	25-26
Harvesting Mouse Splenocytes	27
Where to get things	
Stockroom	28
Cell Culture Facility	28
Biotech Center	28
Fisher	29
Airgas	29

Cell Culture Medium

Tall-104 Complete Medium:

400 mL total volume

40 mL heat-inactivated Characterized FBS (10%)

4 mL Pen-Strep (1%)

8 mL L-Glutamine (2%)

348 mL Iscove's medium

80 uL IL-2 (8X10⁴ IU) added after sterile-filtered (200 IU/mL)

See Bulk Interleukin-2 section for more information

1. Set water bath to 25°C.
2. From the -20°C freezer take out New Characterized Fetal Bovine Serum (FBS), L-Glutamine (LG) & Pen-Strep (PS).
3. Place frozen components into rack or floaty without submerging caps.
4. When all components are thawed, obtain 500 mL filter system from Rm. 324 and Iscove's medium base from the cold room.
5. Take all components to the hood. Pipette Iscove's medium base to upper reservoir. To it add FBS, LG & PS. Use individually wrapped pipettes for Iscove's & FBS and micropipettor for the LG & PS.
6. Cover upper reservoir and bring to benchtop, attach to vacuum line (clear tube with orange cap) and filter sterilize by turning on the vacuum.
7. During filtration go to the -20°C freezer back in lab and obtain required amount of Interleukin-2 (IL-2) [200 IU/mL so 80 uL for a 400 mL batch of medium].
8. Return to hood, unscrew filter reservoir and without unnecessary agitation, add IL-2 to filtered medium then screw on provided cap.
9. Label: Complete Tall Medium for Cell Culture ONLY, "today's date" & your initials (e.g. 8/25/2010 & MYDR) & store in 4°C fridge.

Cryoprotectant Medium

1 mL for each cryovial tube to freeze

50% HI Characterized FBS

40% Complete Tall Medium

10% sterile DMSO (using glass pipette to take out of bottle)

1. Since FBS is expensive, if you are planning to if you are using a 40 mL aliquot, thaw it, transfer to smaller aliquots & re-freeze. Don't throw away!
2. Combine components in falcon tube and bring to 25°C in waterbath.

Complete Cell Culture Medium

Jurkat T-cells & Raji Medium:

200 mL total volume
20 mL FBS (10%)
2 mL Pen-Strep (1%)
2 mL L-Glutamine (1%)
176 mL RPMI medium

K-562 Medium:

200 mL total volume
20 mL FBS (10%)
2 mL Pen-Strep (1%)
2 mL L-Glutamine (1%)
176 mL Iscove's medium

- 1) Combine thawed components in upper reservoir of filter system.
- 2) Filter, label & store in 4°C fridge.

*** For other cell lines, please see the datasheets in ATCC or @
www.atcc.org.***

LG (L-Glutamine)

- 1) The LG comes at 100 mL frozen from Gemini via Fisher in -80°C. Thaw the bottle in 25°C water bath. Spray exterior with 70% ethanol then dry.
- 2) In the hood, make 1 mL aliquots and label with LG. Freeze in rack then transfer to jar labeled L-Glutamine in -20°C Freezer

PS (Pen-Strep)

- 1) The PS comes at 100 mL frozen from Gemini via Fisher in -80°C. Thaw the bottle in 25°C water bath. Spray exterior with 70% ethanol then dry.
- 2) In the hood, make 1 mL aliquots and label with PS. Freeze in rack then transfer to jar labeled Pen-Strep in -20°C Freezer.

Bulk Interleukin-2 (IL-2)

Please NOTE: IL-2 is very sensitive and CANNOT be vortexed!

The goal is to agitate the IL-2 as little as possible.

Mix only by gently pipetting up & down.

- 1) We receive our bulk IL-2 from NCI-Frederick. It is stored in a metal tube on the third shelf of the -20°C freezer. It comes in the form of lyophilized powder and contains 5 million IU. Tall medium contains 200 IU/mL.
- 2) Reconstitute lyophilized powder in 1 mL 0.9% NaCl in filter-sterilized H_2O .
- 3) Divide into (5) 200 uL aliquots, each containing 1 million IU. Label 4 of the tubes as such: “ 1×10^6 U IL-2 in 200 uL 0.9% NaCl” & date. These can be stored in the IL-2 box on the perimeter.
- 4) Take the 5th aliquot and add 800 uL Iscove’s medium. Gently mix by pipetting up and down. Follow this step when more IL-2 is needed for remaining 4 aliquots.
- 5) Make 40 uL aliquots into 0.7 mL microcentrifuge tubes. Each will contain 4×10^4 IU so label as such.
- 6) Place into IL-2 box in -20°C freezer.
- 7) Use 20 uL of IL-2 for 100 mL Complete Tall Medium, i.e. 40 uL for 200 mL & 80 uL for 400 mL.

Heat-Inactivated Fetal Bovine Serum

- 1) Obtain 500 mL bottle of *Characterized* FBS from -80°C freezer and place in 25°C water bath to thaw.
- 2) Once thawed, remove bottle and set water bath to 50°C . Once it comes to temperature, return bottle of FBS to water bath and set timer for 1 hour.
- 3) During that hour, gently swirl bottle every ten minutes.
- 4) Once the hour is over, remove bottle, dry with paper towels and spray down with 70% ethanol. Bring to cell culture hood.
- 5) Get a bag of sterile 50 mL falcon tubes, label with “40 mL HI FBC for Talls”, today’s date and place 13 of them in Styrofoam rack.
- 6) Using individually-wrapped 50 mL sterile serological pipette tips, pipette 40 mL into each falcon tube.
- 7) Ensure the caps are secure and place rack in -20°C freezer.

Ringer's Solutions

Stock solutions found on door of 4°C Fridge

Stock Solution	FW	Amount to add
450 mM KCl	74.56 g/mol	3.36 g to 100 mL dH ₂ O
0.1 M MgCl ₂	203.31 g/mol	2.03 g to 100 mL dH ₂ O
0.5 M CaCl ₂	110.99 g/mol	5.55 g to 100 mL dH ₂ O

-All stock solutions are made up in ultrapure water in the carboy from the Lynes' Lab in beakers on stirplate then filter sterilized in 250 mL filter system.

500 mL of 2 mM Ca²⁺ Normal Ringers

Final Concentration of Component in Solution	Amount to add of Stock Solutions or to weigh out
4.5 mM KCl	5 mL of 450 mM KCl stock
1 mM MgCl ₂	5 mL of 0.1 M MgCl ₂ stock
2 mM CaCl ₂	2 mL of 0.5 M CaCl ₂ stock
10 mM Glucose	0.9g of D-Glucose weighed out
155 mM NaCl	4.53 g of NaCl weighed out
5 mM HEPES	0.6 g of HEPES weighed out

- 1) In 600 mL beaker with stirbar, combine all components and add ~ 450 mL Lynes' Lab water. Place on stir plate and mix for about 5 minutes.
- 2) Check pH, usually ~5.1. Adjust pH to 7.35-7.45 using 10 N NaOH by adding drop by drop with P200 micropipettor set to 100 uL. (usually 8-12 drops).
- 3) Transfer contents of beaker to 500 mL volumetric flask and then add more water to bring total volume to 500 mL.
- 4) Place in upper reservoir of 500 mL filter system, attach to vacuum to filter sterilize. Cap, label with "2 mM Ca²⁺ Normal Ringer's", the pH, date, initials & store in 4°C fridge.

500 mL of Zero Ca²⁺ Ringers

Final Concentration of Component in Solution	Amount to add of Stock Solutions or to weigh out
4.5 mM KCl	5 mL of 450 mM KCl stock
3 mM MgCl ₂	1.5 mL of 1 M MgCl ₂ stock
10 mM Glucose	2.5 mL of 2 M Glucose stock added in hood
145 mM NaCl	4.53 g of NaCl weighed out
5 mM HEPES	0.6 g of HEPES weighed out

-Follow instructions for Normal Ringer's.

500 mL of Zero Ca²⁺ Ringers + EGTA

Final Concentration of Component in Solution	Amount to add of Stock Solutions or to weigh out
4.5 mM KCl	5 mL of 450 mM KCl stock
3 mM MgCl ₂	1.5 mL of 1 M MgCl ₂ stock
10 mM Glucose	2.5 mL of 2 M Glucose stock added in hood
155 mM NaCl	4.53 g of NaCl weighed out
5 mM HEPES	0.6 g of HEPES weighed out
1 mM EGTA	2.5 mL of 200 mM stock added after filtered

-Follow instructions for Normal Ringer's.

Squeeze & Spray Bottle Solutions

100% Ethanol 5 gallon drum*:

- To make ethanol more manageable, transfer 1 gallon from 5 gallon drum to the 100% ethanol jug stored under hood in flammable cabinet using funnel found on drying rack above sink

100% Bleach:

- Fill squeeze bottle to line with 100% bleach* from under sink

10% Bleach:

- In graduated cylinder, measure 50 mL 100% bleach* from under sink
- Fill squeeze bottle to line with dH₂O

1% Alconox:

- Measure 5 g Alconox powder* from 4 lb carton from under sink
- Place 5 g of Alconox powder in 500 mL 1% Alconox squeeze bottle
- Add 500 mL of dH₂O and place on stir plate.
- The bottle should have a stirbar in it so turn stir plate on until mixed.

100% Ethanol:

- Fill squeeze bottle to line with 100% ethanol jug from flammable cabinet under the hood.

70% Ethanol in SPRAY Bottle:

- Using funnel, fill 750 mL spray bottle with gallon jug of 100% ethanol to the line, fill to top with dH₂O

*Alconox Detergent Powder 4 lb. carton, Bleach 1 gallon jug and 100% ethanol 5 gallon drum come from stockroom in TLS Rm. #175

Pre-NZY

In a 250 mL bottle combine:

- 2.2 g NZY broth powder (Fisher BP2465-500)
- 100 mL dH₂O

- 1) Place in autoclave tray with 1/2" water, place cap on top of jar (do not screw it on), mark with autoclave tape, run Liquid 20 cycle, let cool
- 2) Color tape label with pre-NZY, initials & date; store in 4°C fridge

NZY

In 15 mL falcon tube combine:

- 9.775 mL pre-NZY
- 125 uL 1M MgCl₂
- 100 uL 2M Glucose (sterile so open in hood)

Label with NZY, initials & date; store in 4°C fridge

LB broth

In a 1 L bottle combine:

- 10 g LB powder (Fisher #BP1427-500)
- 500 mL dH₂O

- 1) Place in autoclave tray with 1/2" water, place cap on top of jar (do not screw it on), mark with autoclave tape, run Liquid 20 cycle, let cool.
- 2) Color tape label with LB, initials & date; store in 4°C fridge or coldroom.

Kanamycin (Kan^r)

- 1) The kanamycin powder is in the antibiotic box on the top shelf of the 4°C fridge. The MW is 582.58 and stock is 10 mg/ mL in dH₂O.
- 2) Kan^r aliquots are stored in the kanamycin box in -20°C Freezer.

Ampicillin (Amp^r)

- 3) The ampicillin powder is in the antibiotic box on the top shelf of the 4°C fridge. The MW is 371.39 and stock is 50 mg/ mL in dH₂O.
- 4) Amp^r stock is stored in a 15 ml falcon tube wrapped in tinfoil in 4°C fridge.

PMA (Phorbol Myristate Acetate)

The M.W. of PMA is 616.8 and we receive 1 mg in lyophilized powder form. Each small tube used for LAMP assays, in PMA jar contains 5 uL of 50 uM PMA in DMSO.

- 1) Resuspend 1 mg PMA in 1621 uL of DMSO to create the 1 mM stock. Make 100 uL aliquots and label. Store in the white container PMA comes in.
- 2) Dilute the 100 uL of 1 mM PMA stock by adding 1900 uL of DMSO. This will be 50 uM. Make 250 uL aliquots and label.
- 3) Make the 5 uL aliquots of 50 uM PMA to be used for LAMP assays from one of the 250 uL aliquots.

TG (Thapsigargin)

The M.W. of TG is 650.8 and we receive 1 mg in lyophilized powder form. Each small tube for LAMP assay in TG jar contains 5 uL of 1 mM TG in DMSO.

- 1) Resuspend 1 mg TG in 1537 uL of DMSO to create the 1 mM stock. Make 250 uL aliquots and label. Store in the white container TG comes in.
- 2) Make the 5 uL aliquots of 1 mM TG to be used for LAMP assays from one of the 250 uL aliquots.

400 mL FACS Buffer

- 0.1% NaN₃ (400 mg)
 - 5% (20 mL) FBS thawed in 25°C water bath
 - PBS from FCCM
- 1) Zero the balance with 15 mL falcon tube. Measure out 400 mg of NaN₃ and place in tube. Add 10 mL of PBS and vortex until dissolved.
 - 2) Obtain 500 mL filter system and place 370 mL in the upper reservoir.
 - 3) Add 20 mL thawed FBS and the 10 mL of NaN₃ in PBS.
 - 4) Attach to vacuum to filter. Cap, label & store in 4°C fridge.

16% PFA → 4% PFA

- 1) The 16% PFA (Electron Microscopy Sciences #15710) comes in ampules.
- 2) Transfer contents of ampule to the glass jar labeled 16% PFA in the fume hood in Rm. 325. Break the top of the ampule by wrapping top with kimwipes and snapping off away from you. Hold the ampule and glass jar mouth to mouth and gently tap to transfer.
- 3) To make 4% PFA, dilute with PBS 1:4 in a 50 mL falcon tube. (i.e. 5 mL 16% PFA + 15 mL PBS). Label, date and store in 4°C fridge.

Cell Culture Incubator Care

Water

- 1) The water level in the incubator should be checked weekly.
- 2) If the water level is less than 1/3 in the stainless pan or less than 300 mL in the 600 mL beakers make sure to add water from the 5L dH₂O carboy. The blue color is due to cupric sulfate.

Water jacket

- 1) If the “low water” light comes on, get another person to assist you.
- 2) In drawer under microscope, get out clear tubing attached to glass pipette. Attach the tubing to the faucet spout of the little sink located behind the microscope. Do not turn water on yet.
- 3) On incubator, remove orange port, insert glass pipette and hold in place.
- 4) Have the person assisting you gradually turn on the water, making sure it is entering the port and not leaking out.
- 5) Continue filling 1-2 minutes after the “low water” light goes off.
- 6) Turn off water, re-insert parafilm-covered orange port and return tubing with glass pipette to drawer.

CO₂ Tank (Airgas # CDBD-200) *see Where to get things: Airgas

- 1) If the CO₂ level is below 400 psi (right gauge), you need to change the tank.
- 2) If the incubator is gassing, wait a few minutes. Do not open door until after the tank is changed.
- 3) Fire code requires all CO₂ tanks to be strapped to table, so unstrap the two tanks and maneuver them to switch their position. Unscrew cap from new tank, remove airgas label and screw cap onto used tank.
- 4) Turn off the used tank by turning knob clockwise. Using wrench, loosen & remove regulator and transfer to new tank. Turn new tank on ensuring there is about 900 psi (right gauge) and inlet pressure is 20 psi (left gauge).
- 5) Order a new CO₂ tank from Airgas.

New system of CO₂ tank (automatic tank switch)

If tank is switched automatically, alarm will go off on the incubator, press any key to silence the alarm for 15min.

Check new tank air pressure, the right gauge should read ~750 psi. If the air pressure is off, it means the new tank is either not open or without air. Twist the top valve on the tank to make sure it's open. If the pressure is still off, manually switch to a new tank.

To switch a tank manually, remove metal cover from the new tank, remove the blue seal. Untwist the regulator from the old tank using wrench, and attach it to the new tank where the blue seal was. Turn on the top valve and make sure the pressure is about 750 psi.

Cancel the incubator alarm following manual. (long press "setup" key twice)

Dishwashing

Glassware & Other

All glassware used in the lab should be washed with Alconox detergent using the bottle brush, rinsed with dI water at least 3 times, allowed to dry on dryer rack then autoclaved as directed in “Autoclave Use”

Endofree Centrifuge tubes

are an exception to most rules and their cleaning should be as follows:

1. After maxiprep is complete, rinse tube with 100% ethanol, remove any markings, fill with 100% ethanol and place in rack with cap up
2. Leave tube overnight, the next day, pour out $\sim 1/2$ the volume, replace cap and shake vigorously to loosen and release any residual DNA
3. Rinse and fill with 100% ethanol and place in rack with cap up
4. Repeat Step 2
5. Look to see if there is any DNA, threadlike precipitate, if there is not, pour out remaining ethanol and transfer tube to metal drying rack upside down without cap on kimwipes
6. Once completely dry, replace cap and return to endofree tube box

Autoclave Use

Types of Cycles:

- Dry- use when there are absolutely NO liquids
- Liquid- use when there is any sort of liquid, waste or solution
 - Things containing liquid should be placed in an autoclave tray with at least 1/2" of water in the bottom to prevent glass from shattering

Using the autoclave:

- See below to prepare things for autoclaving
- Determine type of cycle (dry or liquid)
- Open autoclave door by pressing foot pedal once
- Place autoclave trays and or biohazard buckets in autoclave
- Close autoclave door by pressing foot pedal once again
- On touch screen press
 1. "Cycle Select"
 2. "20 LIQUID" for liquid **OR** "20 DRY" for dry
 3. "Start Cycle"
- Complete usage chart by filling in Lab name/your name/date/time
- Make note of cycle completion time by cycle countdown clock
- Buzzer will sound when cycle is complete, return to autoclave with oven mitts from cabinet above biohazardous packaging materials
- Open autoclave door by pressing foot pedal once

be careful of steam
- Remove items carefully and bring to lab, place on cleared counter avoiding things that may melt (such as styrofoam ice buckets)

Autoclave Use (cont.)

Non-sterile lab supplies:

- Plastics

Micropipettor tips

- Blue, yellow and clear tips should be placed in respective tip boxes while wearing gloves and taped shut with a small piece of autoclave tape (white lined marking tape that will turn black once sterile)
- Place in white autoclave tray (boxes may be stacked)

Microcentrifuge tubes

- 1.5 and 0.7 mL tubes placed in clear Nalgene jars
DO NOT SCREW LID ON!
- Apply small piece of autoclave tape to lid of jar and place jar into corner of autoclave tray, rest the lid on lip of jar leaving at least 1.5" opening (jars are NOT to be stacked)

if you don't leave an opening, the lid will be sealed onto the jar and ruined beyond use

Centrifuge bottles

- 500 mL flat-bottomed & 200 mL conical-bottomed bottles should be clean and dry and *gaskets removed caps*
- Put a small piece of autoclave tape on cap, place tubes and caps into red autoclave bag (not screwed together) and put into autoclave tray

50 mL endofree centrifuge bottles should NOT be autoclaved

5L Carboy with spigot

- Usually contains only water so should be rinsed and dry
- Turn spigot to horizontal position, place but DO NOT SCREW cap on top, apply large piece of autoclave tap to cap and place in autoclave tray
- If autoclaving water in carboy, add ~1/2" water to tray

Autoclave Use (cont.)

Racks

- Cone racks, tube holder, microcentrifuge tube racks do not need to be autoclaved constantly but at least once every 3 months
- Prior to autoclaving, wash and dry racks, wrap in tinfoil and place in autoclave tray

GLASSWARE

Without lids: beakers, Erlenmeyer flasks, volumetric flasks, filter flasks, graduated cylinders, etc.

- Prior to autoclaving, all glassware should be clean & dry
- Cover openings with tinfoil, apply a small piece of autoclave tape to tinfoil and place into autoclave tray

With lids: screw-cap bottles

- Prior to autoclaving, all glassware should be clean & dry
- Place but DO NOT SCREW caps onto bottles, apply small piece of autoclave tap to caps and place into autoclave tray

METALS

- Shelves from incubators, metal water trays from incubators
- Spatulas, once washed & dried, wrap in tinfoil for autoclaving

Biohazardous Waste: anything containing cell culture material

- 7.5 gal infectious waste containers aka Red pipette waste buckets
 - With a gloved hand push anything sticking out as far into bucket as it will go
 - Place directly on rack in autoclave, run a liquid cycle
- Orange autoclave bags filled with tips, plates, bleached flasks, etc. should be tied and placed in large autoclave tray then placed on rack in autoclave, run a liquid cycle

****Please note: any biohazardous waste will smell very bad after autoclaving, do not be alarmed this is normal****

Things that should not be autoclaved:

- Centrifuge tubes for EndoFree DNA preps (see dishwashing for their specific care)
- Anything rubber including gaskets for centrifuge bottles

Biohazardous Waste

Biohazardous waste is anything that contains/ed cell culture material including: tissue culture flasks, serological pipettes, micropipettor tips, microcentrifuge tubes, falcon tubes, well plates, etc.

Benchtop waste should be put into small waste buckets

Small waste buckets should be regularly emptied into large orange autoclave bag (Fisher 01-814C)

Once full, tie orange autoclave bags shut, place in large autoclave tray (this is secondary containment), run a liquid cycle

- 7.5 gal infectious waste containers aka Red pipette waste buckets
 - With a gloved hand push anything sticking out as far into bucket
 - Place directly on rack in autoclave, run a liquid cycle
- Orange autoclave bags filled with tips, plates, bleached flasks, etc. should be tied and placed in large autoclave tray then placed on rack in autoclave, run a liquid cycle

****Please note: any biohazardous waste will smell very bad after autoclaving, do not be alarmed this is normal****

Once biohazardous wastes are autoclaved and cooled to room temperature they must be packaged to be picked up by EH&S

- Large cardboard boxes are provided in flattened state. To create box, take larger portion and press two opposing corners toward each other, thus opening the square. Flip box upside down so the four flaps are on top. Fold two opposing flaps to middle and then the opposite two (DO NOT overlay or the pickup guy will be annoyed) Secure with three strips of clear packing tape in left drawer by door. Now construct the lid by folding the edges to create a lip, no tape required. Once the box is constructed, place one biohazard bag as a box liner with opening up. Place two autoclaved items (biohazard buckets or autoclaved bags into liner. Tie and tape shut. Place pre-printed sticker identifying BSP 325 to tied liner the close with lid. Use two strips of packing tape to secure lid to box and check off "Treated" box and apply another sticker in top right rectangular box. Fill in BSP 325 in designated area.
- Go to www.ehs.uconn.edu → Biological Health & Safety → Forms → Biological Waste Pickup or Supply Delivery. (See sample)
- Fill out form with your name, BSP Rm. 325 & 486-8907; number of large boxes to be picked up; number of large boxes to be delivered as are being picked up as well as (4) 7.5 gallon A sharps containers.
- Then submit. Pickups are Tuesdays and Thursdays. Leave all waste material in lab and do NOT place in hallway.

Liquid Nitrogen Tank

To measure level of liquid nitrogen in tank, take yard stick and place in center of tank, remove and look to see to what level the stick is frosted, the range is 1" to 15". If it is at or below 5", fill the tank.

To fill tank:

1. Grab blue LN₂ gloves and check to see the orange Liquid Nitrogen cage key is attached to handle of LN₂ tank.
2. Carefully wheel entire LN₂ tank to center elevator and take to the basement.
3. Turn right and follow hallway to the Physics building. Left after the double doors, right down the hall to the loading dock room.
4. Use the key to open the cage on the left, wheel tank over to left fill-up tank.
5. Remove metal cover and Styrofoam insert from LN₂ tank.
6. Place the metal dispensing line into mouth of LN₂ tank and turn fill-up tank on by turning knob on top counter-clockwise a couple of times.
7. While you wait, fill out the form on the computer including: amount taken as 15 L, Adam Zweifach, your name, MCB-3125.
8. Once you see the liquid nitrogen boiling at the bottom of the mouth of the tank, turn off the fill-up tank by turning knob on top clockwise until it is completely off.
9. Replace Styrofoam insert & metal cover. *be careful of spillover*
10. Shut cage door and remember to check that it is locked.
11. Return to BSP building and take the center elevator up.

LB Agar Plates

In a 500 mL Erlenmeyer flask combine:

- 1.5 g Agar powder (Fisher #S70210)
 - 100 mL LB broth*
- 1) Place in autoclave tray with 1/2" water, cover with tinfoil, mark with autoclave tape, run 20 Liquid cycle, bring down to temperature in 50°C waterbath for 10 minutes.
 - 2) Label 7 petri dishes along edge with date, LB & antibiotic type (Kan^r or Amp^r); invert so lids lift straight off
 - 3) Once LB Agar is cooled to 50°C, add antibiotic (500 uL Kan or 100 uL Amp), swirl and pour evenly into dishes, tilt to spread.

Be careful not to contaminate plates by spitting, speaking or coughing

- 4) Let plates set for 1 hour (cover if Amp because light-sensitive)
- 5) Invert and place in plate box in 4°C coldroom (wrap in tinfoil if Amp)

LAMP Assay

- 1) Pre-warm 2 mM Ca²⁺ Normal Ringer's (NR) or 2% NR (BSA w/v) in 25°C water bath for 10 minutes.
- 2) Use ~250,000 cells per condition. Count cells and put enough volume for all conditions into 1.5ml or 15ml centrifuge tube(s). Spin the cell-containing centrifuge tube(s) for 30 seconds at 13,200 rpm (1.5ml tubes) or for 10min at 2250 rpm (15ml tubes). Aspirate supernatant without disturbing white cell pellet.
- 3) Add 1 mL NR to wash cells (pipet cells up and down with 1ml volume pipet for 5 times to mix, spin and aspirate supernatant). Prepare stimulating (STIM) or unstimulating (UNSTIM) solution.
- 4) STIM solution is comprised of 50 nM PMA & 1 uM TG in NR. This is made by adding 1 uL 50 uM PMA & 1 uL 1 mM TG to 48ul NR. Vortex the microcentrifuge tube to mix. Then add 5ul of STIM solution to 95ul of cell resuspension.

UNSTIM solution is comprised of 2ul DMSO in 48ul NR. Vortex to mix and add 5ul of UNSTIM solution to 95ul of cell resuspension.

- 5) Resuspend cells in 95ul NR per condition. Add 5ul of STIM/UNSTIM solution into respective condition. To each tube, add 1 uL of LAMP antibody (LAMP conjugated to Alexa-647 is located in the "LAMP" box in the 4°C fridge).
- 6) Pipette up and down with 100ul volume pipet and flick the tubes to mix. MIX WELL!

NOTE: DO NOT vortex!

- 7) Incubate for 50 minutes at room temperature (25°C) on the rotor in dark box.
- 8) At end of incubation period, use RED micropipets to add 100ul 2%PFA to each tube. Mix and transfer the content to microtiter tubes. Place micro tubes in flow tubes and take to flow cytometer for analysis.

Transforming XL1-Blue Competent cells with DNA of interest

Prior to starting, make sure there is NZY broth (1 mL per condition on the top shelf of the 4C fridge, if not prepare according to NZY recipe) and LB Agar plates of the target antibiotic resistance (either Kanamycin or Ampicillin)

- 1) Get bucket of ice filled from ice machine
- 2) From top section of -80°C freezer, take one tube of XL1-Blue cells; use **Subcloning** grade for DNA received and **Supercompetent** grade for ligation or PCR products.
- 3) Thaw on ice then transfer 50 uL of Subcloning grade or 100 uL of Supercompetent grade to microcentrifuge tube for each transformation reaction. Keep on ice.
- 4) Add 0.5 uL of experimental DNA (at least 50 ng) to the competent cells. Return competent cell stock to -80°C freezer.
- 5) Incubate on ice for 30 minutes, flicking every 5 minutes.
- 6) Preheat water bath to 42°C and pre-warm NZY broth.
- 7) After 30 minute incubation, heat pulse competent cells + DNA by submerging microcentrifuge above contents for exactly 45 seconds.
- 8) Return tube to ice for 2 minutes.
- 9) Add 1 mL of pre-warmed NZY to each tube.
- 10) Using shaker in the warm room (Rm. 335), incubate tubes for 1 hour shaking at 250 rpm. At this time, also obtain LB agar plate(s) with correct resistance from the cold room (Rm. 330) and place on shelf in warm room for duration of incubation.
- 11) Retrieve both tube(s) and plate(s) from warm room.
- 12) Using P200 micropipettor, place 100 uL of transformation mixture to the center of the agar plate. Gently pour 8-15 glass beads and spread mixture by swirling plate with cover on, horizontally. After about 30 seconds or when fully spread, discard glass beads into waste bucket.
- 13) Invert plate so lid is on the bottom (and condensation will not pool on the agar) and return to warm room shelf for overnight incubation.
- 14) After overnight incubation, look to confirm there are colonies. If there are none, leave in warm room and check back in a couple of hours. If there are colonies, take from warm room, wrap with parafilm and store inverted (lid down) in cold room.

E.Coli Cultures

Starter culture from plate colonies

- 1) For each colony you plan to pick, bring 1 mL of LB to 37°C in water bath in 15 mL falcon tube.
- 2) After warmed, add appropriate amount of antibiotic, either 1 uL/mL of ampicillin or 5 uL/mL of kanamycin. Vortex to mix.
- 3) Put 1 mL of LB + antibiotic mix into 5 mL capped culture tubes for each colony you plan to pick and label with DNA & number.
- 4) Using a sterilized yellow micropipettor tip, gently scrape a single, isolated colony from plate. Carefully drop into 1 mL LB + antibiotic in culture tube. Replace cap.
- 5) Incubate for 5-8 hours in warm room on shaker @ 250-300 rpm.
- 6) After incubation period, prepare for maxiprep overnight culture.

Maxiprep overnight culture from Starter Culture

- 1) Bring 100 mL of LB in 500 mL Erlenmeyer flask to 37°C in water bath. Take appropriate amount of kanamycin out of -20°C freezer if using kanamycin. (5uL/mL = 500 uL for 100 mL LB).
- 2) Once warmed, add appropriate amount of antibiotic to LB. 100 uL of ampicillin or 500 uL of kanamycin thus creating overnight broth.
- 3) Obtain starter cultures from warm room. Look to see cloudiness of starter culture. If visibly cloudy, add 100 uL of starter culture to overnight broth. If not visibly cloudy, add 200 uL of starter culture to overnight broth.
- 4) Incubate overnight (12-16 h) in warm room (37°C) on shaker @ 300 rpm.

Prior to starting Endofree Maxiprep (EF Maxiprep)

Look in DNA lineage database to see if there is a glycerol stock of the DNA you are preparing. If there is, continue to EF Maxiprep. If not, on morning after maxiprep overnight culture incubation, save 1 mL of culture in microcentrifuge tube, label and place in 4°C fridge.

E.Coli Cultures (cont.)

Miniprep overnight culture from plate colonies

- 1) For each miniprep you plan to do, bring 5 mL of LB to 37°C in water bath in 15 or 50 mL falcon tube.
- 2) After warmed, add appropriate amount of antibiotic, either 5 uL/mL of ampicillin or 25 uL/mL of kanamycin. Vortex to mix.
- 3) Put 5 mL of LB + antibiotic mix into 16 mL capped culture tubes for each colony you plan to pick and label with DNA & number.
- 4) Using a sterilized yellow micropipettor tip, gently scrape a single, isolated colony from plate.
- 5) Carefully drop into 5 mL LB + antibiotic in culture tube. Replace cap.
- 6) Incubate for overnight (12-16 hours) in warm room on shaker @ 300 rpm.

Making Glycerol Stocks

- 1) In falcon tube, mix 30% glycerol in LB solution. I.E. 3 mL glycerol + 7 mL LB. Vortex to mix thoroughly. Be careful to draw glycerol up slowly because the viscosity makes it difficult to pipette.
- 2) Add 750 uL of maxiprep overnight culture OR miniprep overnight culture to 750 uL of 30% glycerol in LB solution. Place in cryovial, label & place in glycerol stock box in -80°C freezer. Remember: add to DNA lineage database.

Glycerol is Fisher BP229-1

DNA Quantification & Protein Ratio

- 1) Uncover and turn on spectrophotometer (BioMate 3 in Rm. 324) by flipping switch on back left of machine.
- 2) Prepare your samples by diluting 2 uL of DNA sample in 140 uL of dH₂O. Remember to label and also to have a tube of dH₂O for blanking. You will also need a piece of paper (your lab notebook), a pen & a calculator.
- 3) Once BioMate3 is calibrated (automatic), arrow down to select “DNA/PROT (260/280” and hit enter.
- 4) Look to make sure you selected the correct test, then hit “Run Test”.
- 5) Obtain the Quartz Cuvette from its box in the drawer under the BioMate3. ***Be Careful not to drop or scratch the cuvette, they are very expensive***
- 6) Using gel loading pipette tip & the P200 micropipettor, place 70 uL of dH₂O into the slotted sample chamber of Quartz cuvette without bubbles.
- 7) Open lid of BioMate3 and place cuvette into cuvette holder with the “6Q” facing you.
- 8) Close lid and hit “Measure Blank”. Once it finishes, hit “Measure Sample” to confirm the blank reads as 0.000 ± 0.002.
- 9) Remove dH₂O from sample chamber of cuvette with gel-loading tip.
- 10) Place 70 uL of 1:70 diluted DNA sample into cuvette and return to cuvette holder inside BioMate3. Close lid and hit “Measure Sample”.
- 11) There will be several readings on the screen once the scan completes.
- 12) Take note of the “Ratio” result. A good range is 1.800-2.000.
- 13) The value under the “Conc ug/mL” result needs to be multiplied by 70 (dilution factor) and divided by 1000 to convert to ug/uL.
- 14) The concentration value should be added to EF maxiprep tube of DNA.
- 15) Once finished with samples, hit “Esc” twice.
- 16) Remove cuvette and rinse with dH₂O 3-5 times. Return to box in drawer.
- 17) Turn off BioMate3 and cover.

Freezing Cell Lines

- 1) Prepare appropriate cryoprotectant for the cells you are going to freeze.
- 2) Count cells and determine amount needed for each vial you plan to freeze. 15 million per vial for Tall-104 cells and 5 million for other cell lines.
- 3) For each vial, spin down cell culture amount in 15 mL falcon tube. 10 minutes at 1100 rpm will suffice.
- 4) Label the cryovial(s) with name of cell line, date of freeze, generation number if known and lot number if known.
- 5) Take the spun down cells, cryovial(s) in cryovial rack and cryoprotectant medium to the hood.
- 6) Pour supernatant of spun down cells into new 50 mL falcon tube.
- 7) Gently and as sterile as possible, resuspend cell pellet in 1 mL of cryoprotectant medium. Transfer to labeled cryovial in cryovial rack.
- 8) Place entire cryovial rack in -80C freezer for 24 hours.
- 9) In the meantime, figure out where in the LN2 freezer the cryovials are going to be placed. The LN2 freezer inventory is under Lab Documents.
- 10) Once you put the cryovials in the LN2 freezer, fill out all columns of the inventory including the In/Out Log.

Thawing Cell Lines

- 1) Prepare appropriate complete cell culture medium. Put 9 mL of complete cell culture into (2) 15 mL falcon tubes. Warm to 37°C in waterbath.
- 2) In the LN2 freezer inventory find the cell line of interest and make a note of canister/cane location. Fill out In/Out Log and adjust Qty column.
- 3) Using blue cryogloves and tweezers take cryovial vial out of LN2 freezer and bring directly to 37°C water bath. Submerge above level of cell freeze but make sure the water does not go above the seal line.
- 4) Once the cell freeze is almost completely thawed, remove from water bath and spray entire exterior of cryovial with 70% ethanol. Dry then bring to hood with one of the medium falcon tubes.
- 5) Carefully (sterile) transfer contents of cryovial to medium in falcon tube.
- 6) Spin at 1100 rpm for 10 minutes to remove DMSO from cryoprotectant.
- 7) Back in hood, pour off supernatant, gently resuspend in 6 mL prewarmed medium. Transfer to small culture flask, label and place in incubator.

1% Agarose gel (small)

In a 500 mL Gel-Designated Erlenmeyer flask combine:

- 0.5 g Agarose powder (Fisher BP164-100)
- 50 mL 1X TAE solution (Fisher BP1335: 10X TAE diluted in dH₂O)

- 1) Place weigh boat on top, microwave for 1 minute
- 2) While gel in microwave, tape the open edges of the gel casting form and place in tray
- 3) Using some sort of insulation (pot holder, hot hands, paper towel) carefully remove flask from microwave, cool gel down by running under cold water and swirling, once the temperature is such that you can touch the bottom of the flask to your forearm return to benchtop
- 4) Add 2 uL ethidium bromide (Fisher BP1302-10) and swirl gently
- 5) Pour into taped gel form with as few bubbles as possible, place well comb into grooves & move any bubbles to the side with micropipettor tip
- 6) Allow gel to set 20 minutes, with even pressure pull well comb straight to remove from comb, peel off tape completely and place into grooves of min sub cell (Biorad)
- 7) The top of the gel (part with the wells) should be closest to the black
- 8) Make sure liquid (1X TAE solution) covers gel completely
- 9) Load wells with sample
- 10) Place lid on top of corresponding colors (red to red & black to black)

Conjugating CD107a (LAMP) to Alexa-647

Things you need:

BD Bioscience 555798 (Purified CD107a [LAMP-1] Antibody) (stored at 4°C) Millipore 42423 (Microcon Ultracel YM-50 Centrifugal Column) (stored at RT in Ab Conj drawer) 13 x 100 mm glass tube (stored at RT in Ab Conj drawer)

Molecular Probes A-20186 (Alexa Fluor 647 Monoclonal Antibody Labeling Kit)

Component A: Alexa Fluor 647 reactive dye (stored at -20°C)

Component B: Sodium Bicarbonate OR 1M in dH₂O pH 8.3 – 8.5 (stored at 4°C)

make sure to check pH prior to starting

Component C: Purification Resin (should be molasses consistency)

30 kDa size exclusion resin (stored at 4°C)

will need to be taken out of fridge 1 hr prior to use

Component D: Spin column (stored at RT in Ab Conj drawer)

Component E: 2 mL collection tube (stored at RT in Ab Conj drawer)

Protocol:

1. Add 110 uL of dH₂O to the microcentrifuge tube that comes with the Microcon Ultracel YM-50 column. Mark level of dH₂O and aspirate. Place YM-50 column into microcentrifuge tube and apply 220 uL of BD purified CD107a antibody to it. (200 uL will be from 1 vial of antibody and the remaining 20 uL comes from the supplementary stock of CD107a antibody.)
2. Spin at 3000 rpm in bench-top microcentrifuge at 1 minute intervals for 4 minutes and then 30 second intervals until volume in bottom tube reaches 110 uL. (Confirm volume with micropipette.) [It should take about 5' 45"]
3. Place liquid from top of column into new microcentrifuge tube and measure volume, which should be about 91 uL. Check the pH using pH paper if you have not yet done so. The pH should be between 8.3-8.5. Add 1/10 volume of 1 M Sodium Bicarbonate buffer (Component B) to the concentrated antibody liquid from step 2.
4. Take out the bottle of purification resin (Component C) from the door of the 4°C fridge.
5. Turn off the lights as the dye is extremely light-sensitive. Obtain 1 vial of reactive dye (Component A) from -20°C freezer. Transfer 100 uL of the protein solution to the vial of reactive dye (Component A). Peel off label and gently pipette up and down to fully dissolve dye. Solution will be bright blue.
Violent agitation can result in protein denaturation so GENTLY mix
6. Incubate for 1 hour at room temperature away from light (i.e. in a drawer). Every 10 minutes, gently pipette solution to increase labeling efficiency.
7. When 15 minutes of incubation time remains, prepare spin column. Place spin column in 13 x 100 mm glass tube. Stir purification resin (Component C) with sterile metal spatula and using 1 mL micropipettor add 1 mL of the suspension to column and allow it to settle. Continue to add more suspension until total volume is about 1.5 mL.
8. Allow column buffer to drain from column by gravity after initial drops are drawn out by mild suction. Centrifuge in Centra CL3R for 3 minutes at 1100 x g (2550 rpm). (Resin at top will appear "crunchy" and additional buffer should have come through.)

Conjugating CD107a (LAMP) to Alexa-647 (cont.)

9. Load 100 uL of reaction solution dropwise onto the center of the column. Allow solution to absorb into gel bed. Place spin column in empty collection tube and centrifuge for 5 minutes at 1100 x g (2550 rpm).
10. The collection tube will contain 120-150 uL of blue solution; top of the column will be blue. Discard spin column and cap collection vial. REMEMBER to protect from light!
11. To determine the concentration of protein and degree of labeling, prepare a 1:5 dilution in 8 uL PBS (Total volume = 10 uL), to measure the absorbance at 280 nm and 650 nm. This is done using the NanoDrop of the Jockusch lab located in Rm 320 of the Pharmacy building. The lab phone number is 486-6215 and the keypad code is 3256.
12. The NanoDrop is linked to the computer and it's program is ND-1000. Select the UV-Vis button and remove folded kimwipe from under the NanoDrop lever. Apply 2 uL of PBS to the sample stage and carefully close the lever. Click yes to measure initial blank. Once it is completed you will be able to adjust the two wavelength parameters: λ_1 to 250 nm & λ_2 to 650 nm. Click the Blank button so it is read at the two new wavelengths.
13. Place the folded kimwipe on the sample stage & gently close the lever to remove PBS.
14. Remove kimwipe, apply 2 uL of 1:5 dilution sample to sample stage, close lever and click "Measure". Take down readings.
15. Repeat step 13 twice to get multiple readings to average. Fold new kimwipe and place under lever. Exit ND-1000 program and return to Zweifach lab.
16. Take the average of the measurements at each wavelength and then multiply by 10 because the 2 following equations are for 1 cm pathlength but the UV-Vis program has a 1 mm pathlength. The dilution factor is 5.

Equation 1: Protein concentration (M) =
$$\frac{[A_{280} - (A_{650} \times 0.03)] \times \text{dilution factor}}{203,000}$$

Equation 2: Moles of dye per mole of protein =
$$\frac{[A_{650} \times \text{dilution factor}]}{239,000 \times M}$$

17. A good range for moles of dye per mole of protein is 3-7.
18. The MW of the antibody alone is 145 kDa and each mole of dye is 1.25 kDa so multiply the moles of dye per mole protein by 1.25 kDa. Add this to the 145 kDa, so if there were 3 moles of dye per mole of protein, it would be 148.75 kDa. 148,750 g in 1 L would be a 1 M solution, so a 1 mM solution would contain 148.75 g in 1 L, or 148.75 mg in 1 mL and a 1 uM solution would contain 148.75 ug in 1 mL or 14.875 ug in 100 uL.
19. Measure the volume of conjugated antibody actually produced and then cross-multiply to determine ug of conjugated antibody.

i.e. If 140 uL is the volume acquired then: 14.875 ug in 100 uL = X ug in 140 uL and X = 20.825 ug. This can then be used to dilute the solution to stock concentration of 0.045 ug/uL. Dilute a small amount at first to test on flow cytometer.

Harvesting Mouse Splenocytes

1. Obtain mouse from animal facility
2. Euthanize by CO₂ or cervical segregation
3. Spray down torso with 70% ethanol, absorb excess with Kimwipe
4. Midsagittal cut skin of abdomen, peel back, then muscle, peel back, continue cutting carefully layer by layer until spleen is exposed.
5. Remove spleen and place in 60 mm Petri dish containing 5 mL (5%) Complete Medium; if there are multiple spleens keep each in its own Petri dish on ice.
6. For each spleen place between two autoclaved frosted microscope slides (frosted parts facing spleen) and squish spleen until only the membrane (white part) remains.
7. Transfer the cell suspension from the Petri dish to a 50 mL falcon tube and spin 1100 rpm X 5 minutes at 4 C.
8. Pour off supernatant & resuspend pellet in residual supernatant by flicking
9. Add 1 mL sterilized (by filter) water (hypotonic will lyse RBCs)
10. After 10 seconds add 250 uL 5X NaCl in sterilized (by filter) water to return cell suspension to isotonic
11. Add (10%) Complete Medium until volume totals ~20 mL
12. Remove tissue by placing mesh basket atop 50 mL falcon tube and pouring cell suspension on. Allow flow by gravity.
13. Spin 1100 rpm X 5 minutes at 4 C. Pour off supernatant.
14. Resuspend pellet in 5 mL (10%) Complete Medium
15. Find cell density by Z2-Coulter instrument using a sample that has been diluted 400X in "isoton"
16. Target 10×10^6 cells/mL then if want to maintain a line dilute to 7.5×10^6 cells/mL; 2 mL per well of 24-well plate in (10%) Complete Medium

Complete Medium (sensitize with 2 ug/mL ConA)

(5% or 10%) Heat-inactivated FBS

50 uM beta-mercaptoethanol

1% non-essential amino acids

1 ug/mL Pen-Strep

DMEM Mediatech cellgro Dulbecco's Modification of Eagle's 1X
(Fisher MT-10-013-CV)

Where to get things:

Stockroom in TLS Rm. 175 **Ken Bernier 486-2296**

-bring FRS#

Micropipettor Tips (blue & yellow); please note tips for P2 are from Denville
Microcentrifuge Tubes (0.7 & 1.5 mL)

Colored labeling tape

Tissues

Paper towels

Sponges

100% Ethanol (5 gallon size)

Petri dishes of all sizes (for LB agar plates & imaging)

5 mL glass serological pipettes for DMSO

Orange autoclave bags for biohazardous waste

FCCM (Cell Culture Facility) 2nd floor in TLS

Dr. Terry Smith 486-2469

-Bottles are returned, washed and filled with DI H₂O, to TLS Rm. 286. The sheet must be completed with the quantity returned, "DAZ" PI's initials & your initials.

-New bottles are obtained by taking the blue refrigerator key from the cardboard box in the middle metal cabinet. Unlock the fridge, take out the desired products, lock the fridge, return key & fill out the sheet to the right of the fridge/freezer on the wall. Again, "DAZ" for PI's initials.

PBS, RPMI, Ultrapure Water

Biotech Center on Ground floor of BSP, left of Up 'n' Atom

486-5024

-Items are ordered by stopping in or calling with the company name, item's catalog number, item's description, your name and the lab # 486-8907.

-The Biotech Center will order the item and call the lab once it is received.

-When going to pick up an item, bring an FRS# (currently 299039) and check in with person at the desk in the Biotech Center.

Qiagen

Invitrogen

Bio-Rad

NE Biolabs

From Fisher online ordering

www.fishersci.com → User: azlab1 & PW: azfisher → My Hotlists → Personal: Select “Zweifach Lab” → Activate → scroll down to fill in quantities of items needed then press “add items to shopping cart” → Check out shopping cart → continue → Payment Method: Select “Invoice” → Enter PO Blanket Order # (currently D004335) → Continue → Submit Order → Print View → Print copy for pending folder → log off

Items are received on Tuesdays and Thursdays each week. Usually if you order before noon on Monday or Wednesday, you will receive it the following day.

1, 5, 10, 25 & 50 mL individually wrapped serological pipettes
25 & 175 mL culture flasks
250 & 500 mL filter systems
6 & 24-well plates
kimwipes

Airgas (Customer # XOI49)

800-750-4427 or www.airgas.com

Login → Username: azweifach1; Password: airgas1; Login → Mini Express Order: Part# “cdbl200”; Qty: “1”; “Submit Order” → Checkout → Select Payment Method: “On Account” → Enter Blanket PO# (currently D010307) → Ship to: Second option on pull down (Rm. 319) → Submit Order → Print copy and place in pending file → Log Out