Standard Operating Procedures

Huang Lab, May 2013

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Safety Training Agreement

Huang Research Lab

The following online training courses are required prior to working in the laboratory.

For students and postdocs working on chemical synthesis only, the following trainings are required:

- Hazardous Waste Initial Training
- Chemical Hygiene & Laboratory Safety
- Security Awareness
- Compressed Gas Cylinder Safety

These training courses are available at:
http://www.oeos.msu.edu/mastercourse/search_results_compact.htm?-DB=training-mastercourse&-Lay=Training%20Course%20Form&-format=search_results_compact.htm&-sortorder=ascending&-sortfield=Course%20Name&-max=All&-Find

In addition, site-specific training is required for general safety considerations. This will cover:

____Eyewash location and use

____Safety shower location

____Spill kit locations

____Addition of contact information to list and location of list

____Hood use (sashes, emergency purge)

____Fire extinguisher locations

____Fire emergency alarms

____Lab security (doors should be locked, suspicious people around the lab, etc.)

____Location of hazardous waste and biological waste disposal

____Use of appropriate safety equipment (lab coat, goggles, shoes, shields, gloves, etc.)

____Location of chemical hygiene plan and MSDS forms

____Read and sign SOP for Disposal of Reactive and Pyrophoric Chemicals

• MSDS forms are available on the EHS website:

http://www.aware.msu.edu/MSDS/search.htm?-DB=MSDS&-Lay=Form&-format=search.htm&-view

• The chemical hygiene plan is available at:

http://www.ehs.msu.edu/chemical/programs_guidelines/chem_hygiene/chem_hygiene_plan/chp_full.pdf

Printed copies are available on request.

For Students and postdocs working on biological projects, the following trainings are required:

- Biosafety principles
- Autoclave safety
- Bloodborne pathogen initial
- Hazardous waste initial training
- Chemical hygiene & laboratory safety
- Safety awareness

These training courses are available at:

http://www.oeos.msu.edu/mastercourse/search_results_compact.htm?-DB=training-mastercourse&-Lay=Training%20Course%20Form&-format=search_results_compact.htm&-sortorder=ascending&-sortfield=Course%20Name&-max=All&-Find

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____Use of appropriate safety equipment (lab coat, goggles, shoes, shields, gloves, etc.)

____Location of chemical hygiene plan and MSDS forms

Signature of Person Giving Site-Specific Training_____

Printed Name of Person Giving Training_____

Date of Site-Specific Training _____

I have completed and understand all of the above site-specific and online training on the date provided.

Signature of Person Receiving
Training_____

Printed Name of Person Receiving
Training_____

Date_____

Standard Operating Procedure for:

Disposal of Reactive and Pyrophoric Chemicals

Some compounds that we use in our lab are extremely sensitive and reactive to moisture and oxygen, which are primarily stored in the dessicator for this very reason. On occasion one may need to dispose of one of these types of compounds. If one needs to neutralize some highly reactive compound, such as Na metal, NaH, n-butyl lithium, etc., it should be done with care and following protocol.

Ideally, the compound will be loaded in a flask filled with nitrogen for neutralization. If the compound is dry, e.g. Na or NaH, a high boiling, aprotic solvent such as **toluene** should be added to dilute the killing agent. In addition, if the material to be killed is quite concentrated, it may be a good idea to dilute before beginning the quench. Ideally, the solution will be magnetically or mechanically stirred during the neutralization process.

Any neutralization should be done under a flow of nitrogen in a fume hood to prevent any side reaction with oxygen or moisture in the air. Neutralization can be performed using isopropyl alcohol. In cases where a very large amount of compound is being disposed of, the flask should be cooled with an ice water bath from the beginning. If the reaction becomes warm at any time, the flask should be placed in an ice water bath.

At all times, chemical resistant gloves, goggles or safety glasses, and a fire-resistant lab coat will be worn.

An example of a setup is shown below. Obviously, the size of the container will depend on the amount of material to be neutralized. A securely clamped round bottom flask could also be used. Be certain that your flask is large enough to accommodate what you are disposing of and the reagent you will be adding. The sum total of solution that you expect to have in the end will preferably be only about half your container size.

The nitrogen flow should be on a bubbler. Many of the common reagents disposed of such as Na, NaH, butyllithium, methyllithium, etc., generate a gaseous by-product. In addition, the quenching process is inherently exothermic. If the flask is not vented through a bubbler pressure can build during the additions.

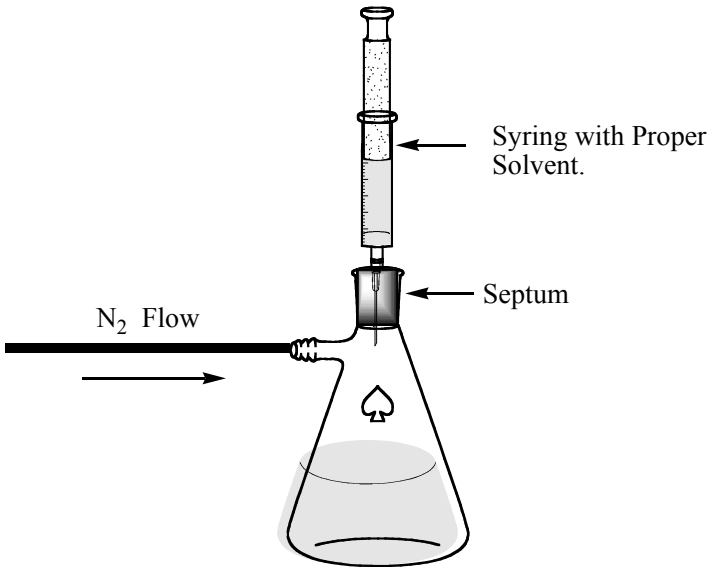
All possible fuel sources in the hood (every solvent) should be under a nitrogen atmosphere. The killing agent, e.g., isopropanol, is best kept in a syringe for easy addition to the compound being disposed of. In addition, the syringe will keep the vapors of the killing agent contained. There should be no waste containers, solvent bottles, reagent bottles, etc in the hood where compounds are being disposed of. The bottle for the killing agent should never be in the hood. The syringe can be refilled in a different hood.

Caps, septa, etc. from the compound being neutralized may have pyrophoric material on them. They should be placed in a metal container and handled cautiously until they are known to be safe for disposal.

The proper way of neutralizing a compound is to start with small portions of isopropanol. After a portion is added, wait for reaction to cease if there is a clear indication of reaction. This is easily done when volatile components are generated like when quenching $LiBu^n$. In other cases, it may be advantageous to alter the set up with a thermometer so that a rise in temperature can be observed. This is readily done by putting a hole in a septum with a pair of scissors to insert the thermometer; the syringe may then be put in the same septum.

Once the compound seems to be neutralized with isopropanol, this is followed by adding ethanol, methanol, and lastly water using the same protocol. It is a good idea to swirl the solution around, especially when dealing with solids like Na or K metal to make sure no oxides are covering an unreacted metal. After adding water and it doesn't react, you can dispose of the solution in a waste container.

If there are any questions concerning the procedure, they should be asked to the research director or to senior research personnel prior to starting the neutralization. If you are disposing of potentially hazardous compounds for the first time, notify the research director so that hands on training can be done.



Example set up: The flask should be stirred magnetically, tightly clamped in a hood, and the nitrogen flow should be on a bubbler. See the text for the full description.

I have read and understand the material in this standard operating procedure for the neutralization of reactive compounds.

Signature _____

Printed Name_____

Date_____

Standard Operating Procedure for Synthesis of *p*-Toluenesulfenyl Chloride

Sulfuryl chloride (4.8 mL, 60 mmol) is added, at 0ºC over 10 minutes, to a solution of 4-methylbenzenethiol (6.20 g, 50 mmol) in anhydrous hexanes (25 mL), and the mixture is stirred at the same temperature for 1 h. The reaction vessel should be kept under a constant flow of nitrogen. It is important a gas outlet (nitrogen bubbler) is connected as large amount of gas is generated during the reaction.

The mixture is warmed to room temperature over one hour, and stirred at room temperature for one more hour. After removal of hexanes and excess sulfuryl chloride under vacuum, *p*-TolSCl (5.6 g, d=1.1, 70%) is obtained as a red liquid by vacuum distillation (50 ºC, 1 mmHg) using a short-path distillation apparatus.[1] ^{1}H-NMR (400 MHz, $CDCl_3$) δ 7.65-7.60 (m, 2H), 7.25-7.20 (m, 2H), 2.40 (s, 3H).

[1] Compound **1** (*p*-TolSCl) is moisture sensitive and should be kept in a dessicator at -20 ^{o}C, in small round bottoms flasks capped with rubber septa. The compound is corrosive to rubber. Therefore, the inside of the rubber septum should be wrapped with TEFLON tape. *p*-TolSCl is stable for more than six months when properly stored. When frozen as a solid at -20 ^{o}C, *p*-TolSCl is orange. It should be discarded when the solid turns green.

Standard Operating Procedure for Preactivation Based Glycosylation Reaction

A solution of a glycosyl donor (0.050 mmol) and freshly activated molecular sieve MS 4 Å (150 mg) in CH_2Cl_2 (2 mL) was stirred at room temperature for 30 minutes, cooled to −60°C, and AgOTf (39 mg, 0.15 mmol) dissolved in Et_2O (1 mL) was added, **without the solution touching the wall of the flask**. After 5 minutes, orange colored *p*-TolSCl (7.9 μL, 0.060 mmol) was added through a microsyringe. Since the reaction temperature was lower than the freezing point of *p*-TolSCl, **the reagent was added directly into the reaction mixture to prevent it from freezing on the flask wall**. The characteristic yellow color of *p*-TolSCl in the reaction solution dissipated within a few seconds, indicating depletion of *p*-TolSCl.[2] When the donor was completely consumed (TLC, ~ 5 minutes at −60°C), a solution of acceptor (0.050 mmol) in CH_2Cl_2 (0.2 mL) was added slowly and dropwise along the flask wall via a syringe. The reaction mixture was stirred and allowed to warm to −10°C in 2 hours. CH_2Cl_2 (20 mL) was added, and the mixture was filtered (Celite pad). The Celite was washed with CH_2Cl_2 **until no organic compounds were present in the filtrate (TLC)**. The combined CH_2Cl_2 solutions were washed successively with a saturated aqueous solution of $NaHCO_3$ (2 x 20 mL) and water (2 x 10 mL). The organic layer was dried (Na_2SO_4), concentrated, and chromatography gave the desired, amorphous oligosaccharide.

[2] The temperature at which preactivation occurs is not crucial as long as it does not exceed -50 ^oC.

Catalytic Hydrogenolysis

General Procedure:

Reaction substrates were added in a round-bottom flask followed by addition of Pd/C (6 mol%). The flask was flushed with N_2 and the solvent (THF or MeOH) was then added into the flask with a syringe. The flask was then capped with a H_2 balloon (with a three-way flask adaptor), degased quickly and refilled with H_2 three times. The mixture was allowed to react under the atmosphere of H_2 for a time. Upon completion, Pd/C was removed by filtration through celite and the solution was concentrated for further purification or reaction.

Cautions:

1. Solvents must be added under the atmosphere of N_2 because it may catch fire when mixed with Pd/C!

Standard Operating Procedure: SpeedVac

Read the instructions before you start!!

Note: The speedvac is only for aqueous samples. DO NOT use ANY organic solvents!!

To Start:

1. Sign the logbook. Write down your name and start time.
2. Check the cold trap to see if there is any residual liquid left. If yes, empty and clean the trap.
3. Turn on the refrigerating unit. This needs to be done at least **One Hour** before the vaccum is turned on.

To Use:

1. Make sure the refrigerating unit has been on for at least one hour.
2. Load your samples into the rotor chamber. Make sure the rotor is properly balanced and it is securely tightened by turning the knob in the middle.
3. Turn on the rotor. If you want to heat your sample to speed up the evaporation, turn on the heater.
4. Wait 20 seconds till the rotor has reached full speed to prevent sample loss when pump is turned on.
5. Turn on the pump by toggling the 'Power' switch to ON.
6. Turn the 3 way valve (yellow mark on the valve pointing to the yellow mark on the tube) to connect the pump with the rotor chamber.
7. Wait 1-2 minutes until the pump becomes very quiet (the needle on the vaccum gauge should be pointing around 2T).
   Note: A noisy pump means there is a leak in the system and thus, your samples will NOT dry. Fix the leak!

To Stop:

1. Toggle the pump 'Power' switch to OFF.
2. Turn the 3 way valve (yellow mark pointing down towards the table) to release the vacuum to the pump and then turn the valve with the yellow mark pointing towards the ceiling to release vacuum in the rotor chamber.
3. Turn off the rotor and turn off the heating if you have turned the heating on.
4. Open door and retrieve the samples.
5. Turn off the refrigerating unit.
6. Clean the trap!!
7. Sign the logbook and write down the end time.

Standard Operating Procedures: Lypholizer

Important: Organic solvents or acidic solutions are NOT allowed on this machine!!

Use rotavapor first if your samples contain organic solvents or acids.

1. Sign your name in the logbook.
2. Remove all the water left in the drying chamber using paper towel. If there is a lot of water left in the drying chamber, drain it from the draining hose, which is located on the left side of the cooling unit.
3. Check to make sure all valves are in the OFF position.
4. Turn on the power switch on the right side of the cooling unit.
5. Press the panel switch labeled REFRIGERATION AUTO. (When the temperature of the cooling unit drops below – 40 °C, the vacuum pump will start automatically.)
6. Freeze your sample using dry ice-isopropanol until it becomes a solid and wipe your sample to remove all isopropanol.
7. Place your sample in a lypholizer chamber and connect the chamber to the sample valve on the drying chamber.
8. Turn the plastic valve knob to the VACUUM position to open the valve. (The bevel on the knob should be positioned toward the sample port to apply vacuum to the sample)
9. When you finish drying the sample, press the Vacuum switch on the control panel to turn the pump off.
10. Release vacuum to your sample by turning the plastic valve knob 180° to the OPEN position. Take your sample off the valve.
11. Release system vacuum by turning the plastic valve to VACUUM position.
12. After the system vacuum is released, turn the valve to OFF position.
13. Press the Refrigeration switch next to the illuminated LED to turn off the refrigeration system.
14. Turn of the main power switch on the right side of the cooling unit.

Standard Operation Procedures: HPLC

1. Choose Columns. You can get column information by searching the serial number on the column.
2. Prepare HPLC solvent (Only HPLC grade solvent can be used. If it is not HPLC grade, the solvent needs to be filtered first through 0.22um filter.)
3. All samples need to be filtered by 0.22um filter before injection.
4. Open the Pump, Degaser, Detector, Collector (System Control), and then open the computer and start the EZstart on desktop (the system controller unit must be turned on before EZstart program). Several seconds later, there is an alarm that means the computer has connected to the machine.
5. If there is no solvent inside the lines between Degaser and Pump, do not connect column, do the following steps:

1) Turn the button to Open on the Pump A and B;

2) Connect a big syringe to the needle at the end of the line below the button, and pull the plunger until the lines between the Degaser and Pump are full of solvent;
3) Turn the button to CLOSE;
4) Press the "PUMP" on the Pump A and B. The pumps start to work. If the flow rate is ok and no bubble inside all the lines, stop the pumps by pressing "PUMP" again.

6. Connect the column (Note the flow direction).
7. Equilibrate column until the baseline is stable:
8. Set up the method and inject sample to trigger the method:

1) Note the max volume indicated on sample loop;
2) Turn the button to "Load", inject your sample, turn back to "Inject", and then take the syringe out.

9. When you finish your method, wash the column and then fill the column with desired solvent;
10. Take the column off and close everything.

Tips:

1. If you do not have enough time to stay in front of HPLC, check "shut down" in the window popped up when you run the method. This means the pumps will stop pumping when

the method is finished. Otherwise, the pumps are still working even your method is finished. If you do not have enough solvent and you are not here, your column will be dry.

2. When you set up the method, make sure you set enough time for the Detector.

3. The first equilibrate time is very long (2 ~ 4 h, depend on your column) if you add TFA in your solvent. Do not worry about this. The following equilibrate time between different methods usually is very short (20-30 min).

4. Do not use 100% water to wash C18 column. Do not keep column for long time with TFA, because TFA is corrosive, which will damage the column.

Troubleshooting

Problem

1) **The message "waiting for trigger" does not show up before loading sample, or bug report window pops up.**
   Solution: Close the program and system controller unit. Turn on the system controller unit first, then, open the program. Wait till here the beep signal. Then, start setting the method again.
2) **Sample has been loaded, but the running acquisition has not been started yet. (Usually causes the first problem).**
   Solution: Hold the injection button in the "load" position, so that you will not lose the sample. Start solving the first problem. When the message "waiting for trigger" comes back, put the button down to "inject".
3) **The warning signal with "MAX Pressure" indicated at the pump monitor.**
   Solution: Stop both pumps. Check the flow rate in the first tab in method window. Change the flow rate to proper rate for the column you are using.

MTS Cell Viability Assay for drug and drug-loaded nanoparticles

Required Materials

Microplate Reader
Multichannel pipettes
96-well plates
MTS reagent: CellTiter 96® AQ_{ueous} One Solution (Promega, Cat. G3582)
(Note: the MTS reagent is extremely light-sensitive, so care should be taken to keep it from direct, prolonged light)

Prcedures

1. Cells (5000 cells/well, 200 μL) were cultured in serum-containing media in a 96-well plate and incubated at 37 °C for 24 h. For best results, cells in the log phase of growth should be employed and final cell number should not exceed 10^6 cells/cm^2.To minimize experimental errors, a minimum of 4–8 replicates of experimental and no-cell control samples should be used.
2. The media was removed and the cells were washed with PBS (2 times).
3. Various amounts of drug and drug-loaded NPs were added, and the plate was incubated at 37 °C for 24 h.
4. The test reagent was removed and the cells were washed with PBS (2 times).
5. Serum-containing media (150 μL/well) was added and the plate was incubated at 37 °C for 72 h.
6. MTS reagent (20 μL/well) was added and the plate was incubated at 37 °C for 3 h in the dark. To add MTS, first transfer enough reagent to a tray reservoir, and then add MTS reagent to each well using a multi-well pipette.
7. A brown color appeared in the wells containing live cells. The optical absorbance was measured on a microplate reader at 490 nm.

Resuscitation of Frozen Cell Lines

Procedures

1. Read the cell line data sheet to establish specific requirements for your cell line.
2. Prepare the flasks - label with cell line name, passage number and date.
3. Collect a vial of cells from liquid nitrogen storage wearing appropriate personal protective equipment.
4. Quickly transfer the vial to a 37°C waterbath until only one or two small ice crystals. It is important to thaw rapidly to minimise any damage to the cell membranes. Note: Do not totally immerse the vial as this may increase the risk of contamination.
5. Wipe the vial with a tissue soaked in 70% alcohol prior to opening it in a microbiological safety cabinet.
6. Pipette the whole content of the vial into a sterile tube (e.g. 15 ml capacity), and then slowly add 10ml complete medium and gently agitate the cell suspension.
7. To remove cryoprotectant, pellet the cells by centrifugation at 1500rpm for 5 minutes, aspirate the supernatant and re-suspend cell in fresh medium.
8. Seed the cells in a 10cm culture dish (total volume of complete medium is 12ml).
9. Examine cells microscopically (phase contrast) every day and subculture as necessary.

Subculture of Adherent Cell Lines

Procedures

1. View cultures using an inverted microscope to assess the degree of confluency and confirm the absence of bacterial and fungal contaminants.
2. Remove spent medium.
3. Wash the cell monolayer with PBS without Ca^{2+}/Mg^{2+} using a volume equivalent to half the volume of culture medium. Repeat this wash step if the cells are known to adhere strongly.
4. Pipette trypsin/EDTA onto the washed cell monolayer (2ml for 10cm-culture dish), rotate flask to cover the monolayer with trypsin, and decant the excess trypsin.
5. Return the dish to the incubator and leave for 2-10 minutes.
6. Examine the cells using an inverted microscope to ensure that all the cells are detached. The side of the dish may be gently tapped to release any remaining attached cells.
7. Resuspend the cells in a small volume of fresh serum-containing medium to inactivate the trypsin. Remove 100-200µl and perform a cell count.
8. Transfer the required number of cells to a new labeled dish containing pre-warmed.
9. Incubate the cells in a CO2 incubator.
10. Repeat this process as demanded by the growth characteristics of the cell line.

Subculture of Suspension Cell Lines

Procedures

1. View cultures using an inverted phase contrast microscope. Cells growing in exponential growth phase should be bright, round and refractile.
2. Take a small sample (100-200µl) of the cells from the cell suspension and count the cells.
3. Calculate cells/ ml and re-seed the desired number of cells into freshly prepared culture dish containing appropriate volume of complete medium. (Note: do not centrifuge to subculture unless the pH of the medium is acidic (medium turns yellow) which indicates the cells have overgrown and may not recover. If this is so, centrifuge at 1500rpm for 5 minutes, reseed the cells.)
4. Repeat this every 2-3 days.

Cryopreservation of Cell Lines

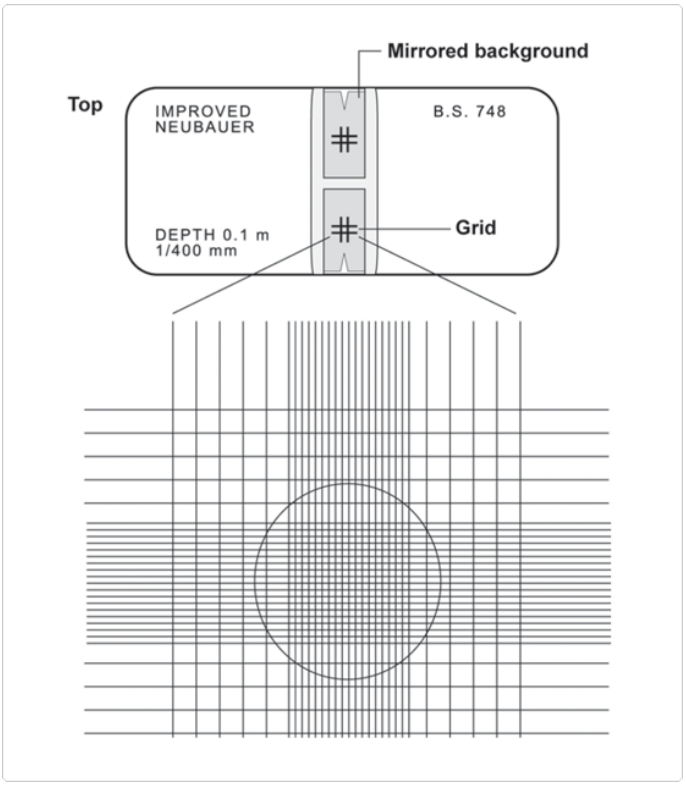
Procedures

1. View cultures using an inverted microscope to assess the degree of cell density and confirm the absence of bacterial and fungal contaminants. Harvest cells in the log phase of growth. For adherent cell lines harvest cells as close to 80 - 90% confluency as possible.
2. Bring adherent cells into suspension using trypsin/ EDTA as described previously and re-suspend in a volume of fresh medium at least equivalent to the volume of trypsin. Suspension cell lines can be used directly.
3. Remove a small aliquot of cells (100-200µl) and perform a cell count . Ideally, the cell viability should be in excess of 90% in order to achieve a good recovery after freezing.
4. Centrifuge the remaining culture at 1500rpm for 5 minutes.
5. Re-suspend cells at a concentration of 2-4x106 cells per ml in freeze medium (90%FBS and 10%DMSO).
6. Pipette 1ml aliquots of cells into cyroprotective vials that have been labeled with the cell line name, passage number, cell concentration and date.
7. Place the vials at -80^{o}C at least overnight.
8. Transfer the frozen vials to a liquid nitrogen storage vessel and record the location.

Counting Cells in a Haemocytometer

Procedures

1. Clean the chamber and cover slip with alcohol. Dry and fix the coverslip in position.
2. Harvest the cells, and under sterile conditions remove 100-200µl of cell suspension.
3. Add 10 µL of the cells to both sides of the haemocytometer. Do not overfill.
4. Place the haemocytometer in the inverted microscope under a 10X objective. Use phase contrast to distinguish the cells.
5. Count the cells in the large, central gridded square (1 mm^2). The gridded square is circled in the graphic below. Multiply by 10^4 to estimate the number of cells per mL. Prepare duplicate samples and average the count. Ideally >100 cells should be counted in order to increase the accuracy of the cell count.



Flow cytometry protocol

Solutions and Reagents

Phosphate Buffered Saline (1X), Formaldehyde (methanol free), FACS buffer (PBS, pH 7.4, 0.1% NaN_3, 1% FBS), Permeabilization buffer (0.1 % Saponin in PBS), Primary antibody Fluorescent labeled secondary antibody, Trypsin and cell growth medium

For adherent cells

- culture cells till they are over 80% confluent
- after culture, remove growth medium and wash twice with PBS (2mL each).
- Add 2 mL trypsin and keep at $37^{O}C$ to detach the cells
- add cell growth medium to inactivate trypsin and transfer to a centrifuge tube
- Centrifuge at 1600 rpm for 5 mins. Resuspend the pellet in 10 ml growth medium and count the cells
- Add $3.0X10^5$ cells to each FACS tube, centrifuge to remove supernatant

For non adherent cells

- culture cells till they are over 80% confluent
- after culture, collect the cells in a centrifuge tube.
- Centrifuge at 1600 rpm for 5 min. Decant the supernatant and resuspend the pellet in 10 ml growth medium and count the cells
- Add $3.0X10^5$ cells to each FACS tube, centrifuge to remove supernatant

Intracellular Staining

1. Fix cells in 4% formaldehyde in PBS (200 ul) for 30 mins on ice. Vortex prior to adding the formaldehyde and 15mins into the fixation vortex again and let it fix for additional 15mins
2. After 30mins of fixation add 1 ml of permeabilization buffer, vortex, add permeabilization buffer to full and incubate on ice for 30mins.
3. Centrifuge to remove supernatant and wash 2-3 times with permeabilization buffer, vortexing after every centrifugation.
4. Add primary antibody (1:200) or appropriate dilution in permeabilization buffer to each tube, vortex and incubate at room temperature for 30mins, vortexing again after the end of the first 15mins
5. Wash twice with permeabilization buffer, add 50 ul of secondary antibody (1:100) or appropriate dilution in permeabilization buffer, vortex, incubate for a total of 30 mins, vortexing again at the end of the first 15mins.

   NOTE every decantation step to remove supernatant leaves behind ~150 µL of buffer this should be put into consideration when making antibody dilutions.

6. Wash twice with permeabilization buffer
7. Add FACS (buffer to full and let it sit at room temperature for 10 mins
8. Centrifuge, add 150 ul FACS buffer, vortex and ready for FACS

Surface staining

1. No fixation and permeabilization of the cells. After counting and removal of supernatant, repeat steps 3-8 in intracellular staining substituting permeabilization buffer with FACS buffer.

Western blot

A. The following buffers are required:

Buffer recipes:

1. Resolving gel:
   1.5 M Tris-HCl pH=8.8 (4°C)
   27.2 g Tris base
   80 mL MilliQ H_2O
   Dissolve, adjust pH to 8.8 with 6N HCl
   Bring volume to 100 mL
2. Stacking gel:
   0.5 M Tris-HCl pH=6.8 (4°C)
   6.1 g Tris base
   80 mL MilliQ H_2O
   Dissolve, adjust pH=6.8 with 6N HCl
   Bring volume to 100 mL
3. Running buffer: Dilute to 1X before use, 2 L per run
   Running buffer (10X)
   30.3 g Tris base
   144.0 g Glycine
   10.0 g SDS
   Bring volume to 1 L with MilliQ H_2O
   pH~8.3
4. Transfer buffer: Dilute to 1X before use, 2 L per run
   Towbin 5% buffer (4°C) (10X)
   30.3 g Tris base
   144.0 g Glycine
   Bring volume to 1000 mL with MilliQ H_2O
   Before use dilute to 1X as follows: 7 parts MilliQ H_2O), 2 parts HPLC grade methanol and 1 part 10X
5. Wash buffer
   TBS buffer (10X) RT
   61 g Tris base
   90 g NaCl
   1 L MilliQ H_2O
   Adjust pH with HCl to 7.6
6. Wash buffer working solution
   TBST (with 0.1% tween-20)
   100 mL TBS (10X)
   900 mL MilliQ H_2O
   1 mL Tween-20
7. Blocking buffer/antibody diluents
   10% Non-fat dairy milk-TBST (4°C)
   10 g Non-fat dairy milk

100 mL TBST buffer

8. 6x SDS PAGE sample loading buffer (-20°C)

7 mL 4X tris-HCl/SDS pH 6.8
3 mL Glycerol
1 g SDS
1.2 mg Bromophenol blue
Dilute to 10 mL with MilliQ H_2O

B. Preparation of cell culture extracts:

Materials: RIPA buffer (Sigma), Protease inhibitor cocktail (Sigma), PMSF (Tepe lab), Cold cell culture grade PBS, Cell scrapers for adherent cells (Biochem Store), ep-tubes, ice

Procedures:

1. Pre-cool desired number of ep-tubes on ice for 1 h before the preparation of cell extracts
2. Prepare extract buffer as follows:
    a. For 10 mL culture plate: 500 µL RIPA buffer (Sigma) + 20 µL Protease inhibitor cocktail + 2.5 µL Phenylmethyl sulfonyl fluoride (PMSF, phosphatase inhibitor). Cool on ice. Always prepare in excess

3. For adherent Cells:
    a. Remove the supernatant media using an aspirator
    b. Wash the cells with ice cold cell culture grade PBS twice 2 x 2 mL
    c. Remove the residual PBS by tipping the flask
    d. Make sure all the PBS has been removed, residual PBS leads to dilution of the proteins
    e. Add cold lysis buffer
    f. Rock the plate under cold condition till cell debris starts to float around (Approx. 10-15 min)
    g. Scrape the plate surface using cell scraper (3010-Biochem store)
    h. Tip the plate to collect all the liquid in one area
    i. Pipet the contents of the plate in precooled ep-tubes
    j. Sonicate in ice containing sonicator for 10 sec.
    k. Centrifuge the samples at high for 5 min at 4°C.
    l. Transfer the supernatant to a new pre-cooled ep-tube, label and store at -80°C, discard the pellet

4. For non-adherent cells:
    a. Centrifuge the cells in a 10 mL tube at 4°C
    b. Remove the supernatant and resuspend the cells in 1 mL ice cold PBS buffer
    c. Transfer to the ep-tube and centrifuge again for 5 min
    d. Wash once more with 1 mL PBS and centrifuge
    e. Remove all the PBS

f. Add 500 µL of the lysis buffer and place the tube on the rocker under cold condition till cell debris starts to
g. float around (Approx. 10-15 min)
h. Sonicate in ice containing sonicator for 10 sec.
i. Centrifuge the samples at high for 5 min at 4°C.
j. Transfer the supernatant to a new pre-cooled ep-tube, label and store at -80°C

5. Store the remaining lysis buffer in an ep-tube together with the lysates at -80°C

C. Determination of protein concentration by Bradford Assay.

Material required: BSA, MilliQ water, Bradford reagent,

Procedures:

1. Prepare BSA stock solution (1 µg/2µl).
2. Prepare standard curve as given below in 96 well plate in
3. Pipette 1.5 and 3 µL of sample into empty wells of the plate, prepare in duplicates, dilute with appropriate amounts of MilliQ water to a volume of 50 µL.
4. Add Bradford reagent and let them sit at RT for 15 minutes
5. Turn on the plate reader instrument
6. Read the absorbance at 595 ηM
7. Plot the standard curve in excel and extrapolate the concentration of samples. Find the mean of the two concentrations from the two volumes of sample used.

Volume of BSA std (uL)	DI water (ul)	Bradford reagent (uL)	Total volume (uL)	Final BSA conc. (ug/ml)	Abs
0	50	200	250	0	
1	49	200	250	2	
2	48	200	250	4	
3	47	200	250	6	
4	46	200	250	8	
5	45	200	250	10	
6	44	200	250	12	
7	43	200	250	14	

D. Gel preparation

Procedures:

Materials: 1.5 M tris pH 8.8, 10% w/v SDS, 30% acrylamide-Bis, 10% Ammonium persulfate (APS), TEMED, 0.5 M tris pH 6.8

1. Place the glass plates (big one in the back and small one in the front) on the gel caster. Pour some water to inspect for leakage. While waiting start preparing the resolving gel. Resolving Gel: Bottom part of the gel, can be of different composition. 10 mL can be used to cast two gels. Make it in either 15 mL or 50 mL centrifuge tube.

3. If no leakage, pour off the water from the caster plates and dry with filter paper.
4. Pour the resolving gel in the cast using a Pasteur pipet. Pour till the bottom line of the green horizontal bar. Pipet a small layer of butanol on top of the gel to keep it from drying/dehyadrating. Keep the remaining gel prep in the tube to test gelling time.
5. Allow the gel to solidify.
6. Prepare the stacking gel, once the gel gets almost solidified.
7. Stacking gel: Top part of the gel where the loading wells are located. This gel has the same composition irrespective of the resolving gel. 5 mL is enough to cast two gels. Make it in either 15 mL or 50 mL centrifuge tube.
8. Pour off the butanol and dry the gel with filter paper.
9. Pour the stacking gel all the way till the edge of the short plate.
10. Place the comb and let it set. (For storage, place the glass plates in a plastic container and submerge it in running buffer, keep the plastic container in a ziplock to prevent evaporation. Store at 4°C.)

Resolving gel recipe

No	`Material	18%	12%	10%
1	1.5M Tris pH 8.8	2.5 mL		
2	30% Bis acrylamide	6 mL	4 mL	3.3 mL
3	10 % SDS	100 µL	100 µL	100 µL
4	10% APS	50 µL	50 µL	50 µL
5	MilliQ water	To 10 mL	To 10 mL	To 10 mL
6	Degas for 10 mins			
7	TEMED	15 µL	15 µL	15 µL

Stacking gel recipe

No	`Material	18%
1	0.5M Tris pH 6.8	1.25 mL
2	30% Bis acrylamide	6 50 µL
3	10 % SDS	50 µL
4	10% APS	25 µL
5	MilliQ water	To 5 mL
6	Degas for 10 mins	
7	TEMED	10 µL

E. Gel electrophoresis, electrophoretic protein transfer and protein visualization

Procedures:

1. After the gel has solidified, remove the combs carefully.
2. Transfer the gel cast to clamping frame with the taller glass facing front. Add running buffer in between the frame till it flows over into the outside chamber. Add the buffer till the appropriate markings.
3. Let the assembly sit for a little bit to monitor any leaks from the clamping frame.
4. Rinse the wells with running buffer using a syringe.
5. Load protein samples prepared based on the Bradford assay results. Add 8 µL of ladder (Bio-Rad, dual colored) in one of the wells for reference; usually the first wel.
6. Run the gel at 200 V for 1 h. or till the blue loading dye has ran off.
7. After the gel has completed the run, blots are then transferred to either a PVDF or nitrocellulose membrane.
8. Cut the appropriate membrane with same dimensions as that of the resolving gel; usually 3.25 by 2.25 inches for min gels. (Handle with gloves at all times, the residual proteins from fingers can generate artifacts.) Remove the protecting blue paper, cut the right hand upper corner to indicate front of the membrane
9. Wet the membrane in HPLC grade methanol for 30 sec. followed by incubation in the transfer buffer (Towbin).
10. Carefully pry apart the gel cast plates and cut the stacking gel part off and add the resolving part of gel to the previously incubating PVDF membrane submerged in Towbin buffer for 15 min.
11. Fill transfer tank with Towbin buffer halfway to the mark. Add stir bar and the ice pack.
12. Add some Towbin buffer to the set-up dish. Soak the sponges in the front compartment and place the cassette with its black side down in the rare compartment.
13. Place the soaked sponge over the black part followed by the filter paper. The paper should become wet immediately. Roll it to ensure it lays flat without any wrinkles and air bubbles.

14. Carefully place the equilibrated gel on the filter paper. Roll out any air bubbles with the roller.
15. Place the membrane on top of the gel
16. Again roll out the air bubbles. Place another piece of the filter paper on top of the gel. Complete the assembly by placing the sponge on top.
17. Close the cassette and place it in the tank with red side in front.
18. Use the residual Towbin buffer from the tray as well as from the equilibrating case to fill up the rest of the tank. Fill the tank up to the fill mark.
19. Run at 60 V for 1.5 h.
20. Upon completion, remove the cassette from the tank, open it up and look at the membrane. The gel colors (molecular marker) should have transferred on the membrane.
21. Treat with 5 ml of blocking agent (10% milk in TBST) for more than 1 h at RT with the front of the membrane facing up. (The right upper corner should be previously cut)
22. Upon completion, decant the blocking solution and wash with TBST 3 times for 1 min each.
23. Treat the membrane with 5-10 mL of 1° antibody (1:1000 diluted, or as given by the manufacturer in 1% milk TBST) at RT for not more than 2 hrs or 4°C overnight if desired.
24. Store the antibody back in the centrifuge tube for further use at -20°C. Antibody can be recycled 3 times before discarding.
25. Wash the membrane with TBST 3 times for 15 min. each.
26. Treat with HRP-conjugated secondary antibody diluted as per manufacturer instructions in 1% milk TBST for 1 h at RT.
27. Upon completion, recycle the antibody. This antibody can also be used 3 times.
28. Wash the membrane with TBST 3 times for 15 min each.
29. Decant the TBST and spray the membrane with developing solution.
30. Pick up the membrane using tweezers and drain the excess developing solution off between two Watman paper
31. Place the membrane in seran wrap, secure it in the developing cassette with tapes, make sure the face is up and take for developing
32. For developing take the developing cassette, a timer together with autoradiography film.
33. In the dark room, open the cassette, keep the blot ready and set the timer. Turn off the fluorescent light. Make sure the red light is on. Turn off the fluorescent light. Remove the autoradiography film from the box in dark, and place it in the cassette. Fold the upper right corner to ensure marking for front of the film.
34. Close the cassette and keep the blot down for desired time like 30 sec, 1 min or 2 min. Do not open the cassette till the timer goes off and then remove the film . The machine will develop the film and releases it after its done. Protein of interest is marked by dark bands on the developed film.

ELISA Protocol

General protocol

1. A 96-well microtiter plate was first coated with a solution MUC1 peptide in $NaHCO_3/Na_2CO_3$ buffer[3] (10 μg mL^{-1}) and then incubated at 4 °C overnight [4]. (100ug/100ul aliquot, + 10ml buffer solution for 1 plate)
2. The plate was then washed four times with PBS/0.5% Tween-20 (PBST), followed by the addition of 1% (w/v) BSA in PBS to each well and incubation at room temperature for one hour.
3. The plate was washed again with PBST and mice sera were added in 0.1% BSA/PBS. The plate was incubated for two hours at 37 °C and washed.
4. A 1:2000 diluted horseradish peroxidase (HRP)-conjugated goat anti-mouse IgG, IgM, IgG1, IgG2b, IgG2c, or IgG3 (Jackson ImmunoResearch Laboratory) in 0.1% BSA/PBS was added to each well respectively. The plate was incubated for one hour at 37 °C.
5. The plate was washed and a solution of 3,3,5′,5′-tetramethylbenzidine (TMB) was added. Color was allowed to develop for 15 min and then a solution of 0.5 M H_2SO_4 was added to quench the reaction.
6. The optical density was then measured at 450 nm. The titer was determined by regression analysis, with log10 dilution plotted with optical density. The titer was calculated as the highest dilution that gave three times the absorbance of normal mouse sera diluted at 1:1600.

[3] $NaHCO_3/Na_2CO_3$ buffer (0.05 M, pH = 9.6): Na_2CO_3 (159 mg), $NaHCO_3$ (293 mg), NaN_3 (20 mg) {Sodium azide is an inhibitor of peroxidase}, H_2O 100 mL;

[4] Do not incubate at 37 °C to avoid elimination of GalNAc from peptide backbone in the basic condition;

Solution preparation

0.01M PBS for dilution (2 Liters) (can prepare 10 Liters, 8 for TPBS)

Dissolve 1 tablet PBS in 200 ml then filtered by 0.22 μm 45ml filter (get from Biochem stockroom)

Washing solution _0.5% v/v TPBS (8 Liters)

Mix 5ml tween in 995 ml 0.01M PBS, then filtered by 0.22 μm 45ml filter (can use the same one after filter PBS)

Blocking solution (1% BSA in PBS) 50ml

Dissolve 5ml of 10% BSA in 45ml 0.01M PBS

Sample diluting solution (0.1% BSA in PBS) 200ml

Dilute 1% BSA in PBS 10X. 20ml 1% BSA in PBS + 180 ml PBS.

TMB solution (for 1 plate), TMB: 3,3,5,5-Tetramethyl benzidine

Weight 5mg of TMB in 25 ml centrifuge tube with Aluminum foil cover to protect light. Add 2ml of DMSO to dissolve well.

Citric solution 18ml/plate

Na_2HPO_4 (MW 141.96) = 7.5 g

Critic acid monohydrate (MW 210.14) = 4.57 g

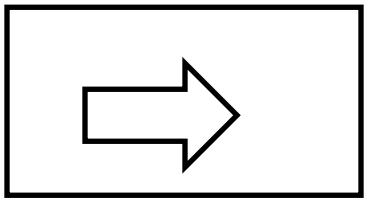
Q-H_2O = 1 L

0.5M H_2SO_4 in DI H_2O

5.43ml 98% conc. H_2SO_4 + DI H_2O up to 100ml

Procedures

0) Label plate, tube!
1) Four plates/day is maximum load.
2) **Coating**
    a. 8-10 mg/ml of antigen **in the antigen buffer**
    b. **100ul/well** (*use multichannel pipet*)
    c. cover with a plastic sheet
    d. Incubate the plate in fridge 4C overnight
3) **Washing**
    a. **200ul of PBST** each well, **4 times** from different side (lift-right, and then right-left.) (*Don't need to change pipet tips, 1 tray for 2 washing, use 1 tray for whole day*)
    b. Dry plate by hitting plate on the sheet.
4) **Blocking**
    a. **Prepare 1% BSA** (take **5ml 10%BSA +45ml PBS**)
    b. **Prepare 0.1% BSA** (take **5ml 1%BSA +45ml PBS_prepare2 tubes**)
    c. Use another tray, not washing tray.
    d. Add **200ul of 1% BSA** in PBS each well. (*don't need to cover*)
    e. Incubate the plate at **room temperature** (in rt incubator) for **1 hour**.
5) **Sample preparation**
    a. Use **0.1% BSA in all dilution**
    b. **See dilution diagram**
6) **Washing**
    a. After blocking incubation is done, wash 4 times with PBST
7) **Antibody titer incubation**
    a. Add prepared dilution **100ul each well**, 4 well each dilution (4 data)
    b. Add from edge to middle
    c. Direction



d. Cover with the plastic sheet

e. Incubate in **37C** incubator for **2 hours**

8) Washing

a. After incubation is done, wash 4 times with PBST

9) Secondary IgG or IgM incubation

a. For one plate, take **6ul of IgG** (orIgM) solution into 15 ml centrifuge tube.

b. Add **12 ml 0.1% BSA**

(If ¼ plate, use 1.5 ul in 3ml 0.1% BSA)

c. Add **100 ul each well** by **12 tip-**using multichannel pipet.

d. Cover with plastic sheet.

e. Incubate at **37C** (*in 37C incubator*) for **1 hour.**

10) Take substrates out from fridge

11) After **about 30min,** Turn on the plate reader instrument

12) Prepare the substrates

a. Cover 50ml centrifuge tube with Al foil.

b. For 1 plate, weight **5mg of TMB** into the centrifuge tube.

c. Add **2ml DMSO** to dissolve well

d. Add **18ml of citric buffer**

e. Add **20ul of H_2O_2**

f. Vortex

13) Washing

a. Washing with **200ul** of PBST 4 times

14) **Substrate incubation** (should be done in time manner)

a. Add the substrate in a tray.

b. Add the substrate solution **200ul/well** by using **8 tip** multichannel micropipette

c. Direction

d. Incubation for **15min** 

15) Quench the reaction

a. Add **50 ul of 0.5M H_2SO_4 each well** by using **8 tip** multichannel micropipette in the **same direction when adding the substrate**.
b. The solution color will turn from **blue to yellow**

16) Read plate

a. Set window **450 nm**, mix time = **60 second**, mix speed= **M**

Graph:

- Linear graph should have R^2 >0.95XX
- Use 0.3 to determine titer
- OD should be lower than 0.2
- Log 0.3 = -0.52288
- Plug -0.52288 in Y in eq to get titer.

Graph Pad Prism6

- File –> New project –> column
- Enter replicate 1st one → create
- Copy 5 (or 4) data in the graph (M1-5)
- Paste transport
- Y – add data title
- Data1 -> name of data
- Click graph → detail → titer style → ^mean

Mouse Spleen Cell Isolations

Culture Medium: RPMI 1640, 10% FBS, 10 mM HEPES, 50 μM Mercaptoethanol, 2 mM L-glutamine, 100 μg/mL penicillin G, and 100 U/mL streptomycin.

Wash Medium: everything same as culture medium, except 2% FBS was used (put in the ice);

ACK buffer (Invitrogen, A10492-01, 100 mL);

70-μm nylon mesh (*BD, 352350, Cell Strainer, white*);

Surgical scissors and forceps (sterile);

Dissecting board.

Procedures:

1) Anesthetize the mice by CO2, then sacrifice by cervical dislocation;

2) Place mouse on a dissecting board on their right sides and douse the chest and abdominal fur with 70% ethanol;

3) Using surgical scissors, create an incision on the left side of mouse about 2.5 cm in length between the last rib and the hip joint, cutting the skin but not the peritoneal wall;

4) With a fresh pair of sterilized surgical scissors, create an incision about 2.5cm long in the exposed peritoneal wall, in the same orientation as the skin incision. Grasp the spleen using sterilized forceps and pull it through the incision in the peritoneal wall. While holding the spleen with forceps, separate the spleen from connective tissue by using fine forceps or scissors;

5) Put the spleen in the petri dish. Using the topside of plunger of 5 ml syringe to grind the spleen gently;

6) Pipette and transfer the 10 ml washing medium from petri dish to 15 ml tube, add 2 ml washing medium to wash the petri dish, and transfer to the same tube;

7) Centrifuge (2000, 5 min, 4 °C);

8) Aspirate the supernatant and loosen the cell pellets by stroking the tubes against a rack or by flicking the ends, the add 3 ml red blood cell lysis buffer at room temperature for 5 min with gentle shaking; Then add 9 ml cold washing medium and centrifuge;

9) Aspirate the supernatant entirely, and then loosen the cell pellets as in step 8. Gently resuspend the pellet with 10 ml medium;

10) Filter the medium with 70 μm nylon mesh to another 50 ml tube;

11) Generally, you will have 8 ~ 10 million cells/ml. To count cells by microcopy, you need dilute 10 times (10 μL to 100 μL) and then dilute again by adding trypan blue (25 μL) to 25 μL cell suspension.

Mouse Bone Marrow Dendritic Cells Isolation

Reagents:

1 Culture Medium: RPMI 1640 with L-glutamine and phenol red, 50 μM 2-mercaptoethanol, antibiotic-antimycotic, 20 ng/ml murine GM-CSF (Peprotech).

<*Note 1*: Calculate how much culture medium you will use, and only make a little more for use. The effect of GM-CSF will gradually lose with time when kept at 4 °C.>

<*Note 1*: When you receive GM-CSF, please do aliquot with 0.1% BSA and keep at -20 °C or -80 °C. Dilute with culture medium and filter with 0.22 μm sterile filter before use.>

2 Culture Plate:

1) Growing DC: non-tissue treated 10 cm culture plates (BD: 351029)
2) Maturation Experiment: tissue treated 24 well culture plates.

Generation of BMDC:

Please visit:

http://www.jove.com/video/773/generation-bone-marrow-derived-murine-dendritic-cells-for-use-2

to see how to isolate bone marrow cells from mouse.

<*Note 3*: all centrifugations are done at room temperature>

Day 0: Plate 10 ml cells at a density of 2.5 x 10^5 cells/ml in each non-tissue-coated polystyrene petri plates;

Day 3: Change all supernatant with fresh medium;

<*Note 4*: when change medium, do it slowly, do not dislodge the cluster of dendritic cells; do not shake plates>

Day 6 and Day 8: Collect all supernatant and centrifuge at *room temperature*. At the same, add 5 ml fresh medium to the plate. The cell plates resuspended in 5 mL fresh medium, pipet gently, and then add back to plate.

Day 9: Non-adherent cells are collected by gently pipetting. Now the cells can be transferred to tissue culture plastic dish for maturation experiment.

Maturation Experiment:

Purified BMDC were further seeded at 1 x 10^6/ml/well in 24-well plates with GM-CSF-supplemented medium as usual; then LPS (sigma) at 1 μg/ml was added to the culture. Medium alone-treated DCs were used as controls. After 24 h, DCs were removed by vigorous pipetting and were resuspended in FACS buffer for analysis. Supernatants were harvested and frozen at -80 °C prior to analysis for cytokines/chemokines.

Elispot

Reagents:

1. Culture Medium: RPMI 1640, 10% FBS, 10 mM HEPES, 50 μM Mercaptoethanol, 2 mM L-glutamine, 100 μg/mL penicillin G, and 100 U/mL streptomycin.

2. 96 well Plate: Millipore (S2EM004M99)

3. Washing Buffer: PBS with 0.1% Tween-20 (PBST)

4. Dilution Buffer: 1% BSA-PBST

5. Blocking Buffer: 5% BSA

6. Secondary antibodies: Goat anti-mouse IgG (HRP).

<*Note 1:* we will change it to Goat anti-mouse IgG (biotin) + Neutravidin (HRP) to improve sensitivity later. >

6. Staining Kit: AEC kit from Sigma (AEC101)

Procedures:

1. Coat the 96-well plate with 100 μl antigen/well overnight at 4 °C, and cover the plate with parafilm.

2. Wash 3x with PBST;

3. Block with 300 μl/well 5% BSA-PBST at room temperature for 1h;

4. Wash 3x with PBST, 3x RPMI 1640, and add 200 μl/well culture medium 10 min before loading cells.

5. Plate 100 μl serial dilutions of spleen or bone marrow cells to each well, cover with lid and put in cell incubator overnight;

6. Wash 9x with PBST, 3x with dH_2O;

7. Add 100 μl/well goat anti-mouse IgG (HRP), with 1:1000 dilution in 1% BSA-PBST. Incubate at room temperature for 1h;

8. Wash 3x with PBST, 3x with dH_2O;

9. Use 8 mL miliQ water and add AEC Chromogen kit: 4 drops Acetate Buffer, 2 drops of AEC Chromogen, 2 drops of 3% hydrogen peroxide;

10. Develop until spots become visible;

11. Stop development with dH_2O wash 3x.

12. Dry overnight at room temperature, and then count with machine.