ELISPOT PROTOCOL

Need to keep the plate sterile. Perform the steps (D0 and D1) under a laminar flow hood.

D0:

- Use Millipore ELISPOT plates (cat#MAIPSWU10). Cut lanes as per need
- To activate the PVDF membrane, add 50 ul of 70% EtOH for less than 2 min
- Wash the plate 6X with sterile H_2O
- Coat (with- LVS3 2ul/ml; Goat-anti-Mouse IgM-unlabeled 10ul/ml; WT or Rag1 μB LPS 25ul/ml PBS) 50 ul, O/N at 4 °C

D1:

- Wash the plate 4X with sterile PBST
- Block with RPMI (1X) + 1X ELISA/ELISPOT buffer at RT for 2 hrs
- Wash with sterile PBS (2X)
- Add 50 ul RPMI+P/S in the wells so that the membrane does not go dry
- Add cells ($5x10^5$ and/or $5x10^4$) in 150 ul RPMI+P/S+ amphotericin B
- Add 25 ml of RPMI+P/S+ amphotericin B to the bottom of the plate
- Without shaking, place it in the incubator for 3 days

D4:

- Perform the steps under non-sterile conditions
- Wash the plate (3X) with PBS, 3X with water (pressure wash), 3X with PBST
- Add 100 ul G-anti-mouse-HRP (1:5000) diluted in 1X ELISA/ELISPOT buffer
- Incubate the plate at RT for 2 hrs
- Wash 5X PBST
- Add 50ul 1X **ELISPOT** TMB-substrate
- Wash with H2O to stop the development of color
- Wash also the bottom of plate, air dry and keep at 4 °C

Take pictures and count the spots

CFSE-labeling for *in vivo* Monitoring of Adoptively Transferred Cells

1. Prepare lymphocyte suspension in 5 ml RPMI with 10% FCS
2. Count cells
3. Prepare 5 μM CFSE
    a. Add 10 μl of 10 mM stock CFSE into 20 ml HBSS (serum free)
    b. Equilibrate at RT in the dark for 10 min
4. Spin cell suspension 5 min at 1400 rpm; remove media
5. Resuspend cells at $5x10^6$/ml in 5 μM CFSE (when resuspending break up pellet with 200 ul HBSS [without CFSE] then add total amount of CFSE directly). The addition process is very important if you wish to track dividing cells because you need a very uniformly stained population.
6. Incubate at RT in the dark for 8min (exactly; do not go over with this step)
7. Immediately add 20 ml (or equivalent staining volume) of HBSS/20% FCS and spin cells for 5min at 1400 rpm 4°C
8. Remove media and add 20 ml of HBSS (serum free); spin cells for 5 min at 1400 rpm
9. Repeat wash
10. Count cells with trypan blue to verify viablility
11. Resuspend CFSE labeled cells at $2.5x10^7$/ml (or at appropriate concentration for your adoptive transfer experiments) in HBSS (serum free)
12. Pass cells through 40 μm filter to remove any cell aggregates
13. Keep cells in foil on ice until adoptive transfer of 200 μl IV with 28G½ insulin syringe
    a. Adoptive Transfer: perform remainder in animal facility procedure room
    b. Heat beaker of water on hot plate to warm (you should be able to touch without pain for a short period)
    c. Put mouse into illuminated mouse holder for IV injection
    d. Soak gauze pads in water and then wrap the tail for 10-20s with gauze
    e. Remove the gauze and proceed immediately with injection

*Be sure to remove some cells prior to and after labeling to verify CFSE incorporation by flow.

*The presence of FCS in the staining buffer inhibits incorporation of CFSE but adding HBSS with serum immediately after is needed to maintain the viability of the cells after staining as CFSE is highly toxic to cells. After serum rescue it is essential to remove all serum prior to transfer.

*The concentration of CFSE may need to be adjusted depending on its application. You may wish to drop the staining concentration to 2.5 μM if you are having difficulty with toxicity (2.5 μM is probably the first one you should try). If you wish to track cells in vivo for extended times you may need to increase the CFSE. However, this may also lead to increased toxicity and subsequent adjustments such as decreased incubation time or increased cell concentration (10^7/ml). The recommended range is 1 μM – 10 μM.

2 Kits available

- Vybrant CFDA SE Cell Tracer Kit (Molecular Probes, Cat # V-12883)
    - 10 vials lyophilized CFSE at 500ug/vial
- CellTrace CFSE Cell Proliferation Kit (Molecular Probes, Cat # C34554)
    - 10 vials lyophilized CFSE at 50ug/vial

I recommend the smaller kit as CFSE is not very stable and toxicity rapidly increases during storage. Do not use CFSE after 6 months at -20°C.

Mouse IgM (total) Detection (ELISA)

- Coat plate with 50µl of Goat anti-mouse IgM unlabeled pAb in Bicarbonate Buffer (50mM PH9.6) overnight at 4 °C. The stock Ab (Southern Biotech 1020-01) concentration is 1µg/µl; use 2 µg/ml (2 µl/ml) to coat i.e. dilution of 1:500
- Wash plate with PBS-T (tween 0.05%) 3X
- Block with PBS-1%BSA for 1 hour at 37 °C
- Wash three times
- Add mice BALF (dilution 1:10and 1:30) 50µl for 1.45-2 hours at 37 °C
  Dilution buffer: PBS-T + BSA 0.5%

- Wash three times (long wash with 5 min interval)
- Incubate with Goat anti- mouse IgM-HRP (Southern Biotech 1140-05; 1:5,000) in PBS-T + BSA 0.5% 1 hour at RT
- Wash three times (long wash with 5 min interval)
- Add substrate 50µl, wait till it turns blue
- Stop the reaction with 25µl 0.18M H_2SO_4
- Read at 450nm

For Total-IgA- use Coat plate with 50µl of Goat anti-mouse IgM unlabeled Ab in Bicarbonate Buffer (50mM PH9.6) overnight at 4 °C. The stock Ab (Southern Biotech 1040-01) concentration is 1µg/µl; use 2 µg/ml to coat i.e. dilution of 1:500

For anti-Ft ELISAs, coat with Ft lysate (1:100 in PBS, O/N, 4 °C)

Bt Lysate	1:20 in PBS, O/N 4 °C
Sa Δ Lgt lysate	1:20 in PBS, O/N 4 °C
Sa lysate	1:20 in PBS, O/N 4 °C
E coli BL21 lysate	1:20 in PBS, O/N 4 °C
LPMX lysate	1:20 in PBS, O/N 4 °C
CR lysate (no NP-40)	1:20 in PBS, O/N 4 °C
Ft lysate (no NP-40)	1:100 in PBS, O/N 4 °C
poop lysate (WT-6/3/150-add 1% NP-40)	1:100 in PBS, O/N 4 °C
WT-poop lysate with NP-40 (8/25/15)	1 ul/ml in PBS, O/N 4 °C
Rag1 poop lysate with NP-40 (8/25/15)	1 ul/ml in PBS, O/N 4 °C
BEI LPS	1 ul/ml in PBS, O/N 4 °C

IIIB4 LPS	1 ul/ml in PBS, O/N 4 °C
μB LPS	5 ul/ml in PBS, O/N 4 °C
CR LPS	5/10 ul/ml in PBS, O/N 4 °C
Bt LPS	5/10 ul/ml in PBS, O/N 4 °C
Sabortus LPS	5 ul/ml in PBS, O/N 4 °C
Sa R95 LPS	5 ul/ml in PBS, O/N 4 °C
LVS1 (lab-LPS)	1 ul/ml in PBS, O/N 4 °C
New LVS LPS	1 ul/ml in PBS, O/N 4 °C
Φ Ft LVSLPS	60 ul/ml in PBS, O/N 4 °C
LPS-Ag	8 ul/ml in PBS, O/N 4 °C
LPS-R1Ag	8 ul/ml in PBS, O/N 4 °C
μB lipoprotein	5 ul/ml in PBS, O/N 4 °C
WbtA LPS (lab made)	10 ul/ml in PBS, O/N 4 °C
WbtM LPS (lab made)	10 ul/ml in PBS, O/N 4 °C
Rag1 μB LPS (8/31/15)	5 ul/ml in PBS, O/N 4 °C
WT μB LPS (8/31/15)	5 ul/ml in PBS, O/N 4 °C
LVS3 (lab-LPS)	1 ul/ml in PBS, O/N 4 °C
LVS4 (lab-LPS)	1 ul/ml in PBS, O/N 4 °C
LVS5 clean (lab-LPS)	1 ul/ml in PBS, O/N 4 °C
F novicida (lab-LPS)	1 ul/ml in PBS, O/N 4 °C
IIIB4 LPS Detox	1 ul/ml in PBS, O/N 4 °C
Bt2 LPS	5ul/ml in PBS, O/N 4 °C
P aeruginosa LPS	1 ul/ml in PBS, O/N 4 °C
K pneumoniae LPS	1 ul/ml in PBS, O/N 4 °C
P gingivilis LPS	5ul/ml in PBS, O/N 4 °C
Re lab FLU PR8 (Heat @ 65 °C for 1 hr)-don't use	7.5 ul/620 ul in PBS, O/N 4 °C ; 12 wells
Joe's FLU PR8 (Heat @ 65 °C for 1 hr)	2.5 ul/1200 ul in PBS, O/N 4 °C
Joe's FLU PR8 (Heat @ 65 °C for 1 hr) ELISPOT	5 ul/820 ul in PBS, O/N 4 °C

	BMDC sup (peri cell sup)	BALF	Sera	SI/	Omentum
mIL-6	1:100; 1:100 with LPS; 1:10 LVS or control	1:6	1:2-6		1:2
mIL-1b	1:2	1:2	-------		
mTNFa	1:20 (1:10)	1:3	----		1:2
IL18	1:1-1:2	1:2	1:2		
MCP1	1:5-	1:2	1:1		
IFNg		1:2	1:3		
KC	1:3 (1:6)	1:6	1:6		1:3
mIgM	1:2.5	5X Bt (1:1 Ft)	1:500	1:3	
mIgA		5X Bt	1:100	1:3	
mIgG			1:1000, 1:2500		

BONE MARROW HARVEST AND GENERATION OF BMDM or BMDC

- Collect aseptically hind leg bones of mice. Keep in PBS + P/S (1.5X) on ice.
- Wear gloves.
- Clean hood, forceps and scissors with 70% EtOH.
- Sterilize paper towels with 100% EtOH, let them dry on the grill of the hood.
- Take out required amount of PBS and add 1.5X P/S.
- For BM removal, use 10 ml syringe with 25 gauge needle (blue color).
- Tear paper towel into small pieces for removing extra flesh attached to bones.
- After cleaning the bones, keep them in PBS + P/S (1.5X) on the upper lid of a bacterial plate.
- Cut femurs in the middle while cut both ends of tibia carefully without loosing much bone marrow.
- Plunge the bone marrow out from the bones into the bacterial plate using a stream of PBS. Wash around the bones to collect all the bone marrow cells.
- After collecting all the bone marrow, pass through the BD Falcon "cell strainer" to get a single cell suspension.
- Centrifuge @ 1500 rpm for 10 min (use program "cell").
- Flick to loosen the pellet and add 1 ml red blood lysis buffer and mix again with P1000.
- Let it stand for 5 min at RT.
- Add 9 ml PBS + P/S (1.5X); centrifuge for 10 min using program "cell".
- In the mean time, add 12 ml RPMI + P/S (1.5X) + 12µl GM-CSF (for BMDC) or 12 ul of M-CSF (for BMDM) to a bacterial plate.
- After centrifugation aspirate the PBS, and resuspend the pellet with 1 ml of media from the bacterial plate using a P1000.
- Add the cells to the plate; mix properly and incubate in 37 °C incubator for 7 days; on D4 change 7 ml old media and replace 7 ml fresh media with 7µl GM-CSF/MCSF. (To change media, take out 7 ml old media in a 15 ml tube, centrifuge @ 1500 rpm for 10 min, aspirate media, add new media to cell pellet, mix the pellet well and add back to the plate)

BMDM/BMDC infection with bacteria

- Harvest medium containing suspension cells from the Petri dish and transfer to 15 ml tube. Wash plate with 2 ml of PBS to recover as many cells as possible-transfer to 15 ml tube. Wash again plate with 10 ml PBS-aspirate. Detach adherent cells with 2 ml of cell dissociation solution. Incubate 10-15 min 37C. Shake **vigorously** the plate to detach cells. Wash with P1000 but don't be too harsh-this may killed the cells. Transfer to another 15 ml tube-add PBS to balance with the other tube and spin 10 min 1500rpm.
- Aspirate media and resuspend cells in RPMI-FCS with appropriate growth factors. ***** DO NOT use pen/strep in the media*****
- Aliquot in 48 well plates (250 ul/well). One full 10 cm plate (two mice) will yield enough cells for at least two 48 well plate. $2x10^5$ cells/well
- **On the day of experiment**, thaw a frozen vial of bacteria, add 100 µl of warm LB media; incubate in the incubator at 37°C for 1 hr.
- Estimate the CFU/ml of each bacterial strain and prepare the necessary dilutions of the bacteria: Confirm titer by plating on LB agar.
    - Bp = MOI 10 (and 1)
    - CR, ETEC, St, Vibrio = MOI 10 (and 1); (infect for **1hr** after spinning)
    - Bt = MOI 10 and 100
    - LVS, SchuS4, Wbta = MOI (10 and 1); (infect for **4 hrs** after spinning)
- Infect the mouse BMDMs by adding 10 µl of bacterial suspension (adjust volume by executing preparative dilutions for different MOIs with RPMI)
- Spin the 48-well plate at 2000 RPM 10 minutes RT. This is the start up Time!
- Incubate 30 minutes at 37°C
- Wash in 500µl PBS and add 250µl **prewarmed** (at 37°C) fresh RPMI 10% FS with M-CSF/GM-CSF (20ng/mL), Kanamycin (400µg/ml final) and Gentamycin (400µg/ml final).

For each time point, (e.g.: after 1h, 2h, 4h):

- Transfer sup and cells to 1.5 ml tube, spin @5000 RPM for 10' at 4°C/RT, collect the supernatant with a p200 pipette and save them in a 96-well plate in the same order
- Add 250µl/well of Lysis buffer (1X) on the cell monolayer, incubate 10' at 37°C, collect cell lysates in 1.5 ml tubes and vortex, store at 4°C
- Plate on LB plates. 2 dilutions in H2O are typically used: 1:1 and 1:100. The lysates from uninfected wells will be used as total lysates in LDH release assay.

***5X sterile Lysis buffer. 2.5% Saponin + 5% BSA in PBS 1X. Filter with 0.45µm Millipore filter and store at 4°C. The day of the experiment, dilute the 5X Lysis buffer in H_20 and filter at 0.22µm.

**** B. cepacia grows slower than Bt or Bp. LVS, SchuS4 and Wbta need more time to grow.

Extraction, Purification and Characterization of Lipopolysaccharide from Escherichia coli and Salmonella typhi

LPS was extracted by hot phenol-water method as described previously with some modifications (14). In brief, bacterial suspensions (108 colony-forming units/mL) were centrifuged at 10,000×g for 5 min. The pellets were washed twice in PBS (pH = 7.2) (0.15 M) containing 0.15 mM CaCl2 and 0.5 mM MgCl2. Pellets were then resuspended in 10 ml PBS and sonicated for 10 min on ice.

In order to eliminate contaminating protein and nucleic acids, treatment with proteinase K, DNase and RNase was performed prior to extraction step. For this purpose, proteinase K (100 µg/mL) (Roche, Mannheim, Germany) was added to the cell mixture and the tubes were kept at 65°C for an additional hour. Mixture was subsequently treated with RNase (40 µg/mL) (Roche, Mannheim, Germany) and DNase (20 µg/mL) (Roche, Mannheim, Germany) in the presence of 1 µL/mL 20% MgSO4 and 4 µL/mL chloroform and incubation was continued at 37°C overnight.At the next step, an equal volume of hot (65–70°C) 90% phenol was added to the mixtures followed by vigorous shaking at 65–70°C for 15 min. Suspensions were then cooled on ice, transferred to 1.5 mL polypropylene tubes and centrifuged at 8500×g for 15 min. Supernatants were transferred to 15 mL conical centrifuge tubes and phenol phases were re-extracted by 300 µL distilled water. Sodium acetate at 0.5 M final concentration and 10 volumes of 95% ethanol were added to the extracts and samples were stored at -20°C overnight in order to precipitate LPS.

Tubes were then centrifuged at 2000×g 4°C for 10 min and the pellets were resuspended in 1 ml distilled water. Extensive dialysis against double distilled water at 4°C was carried out at the next step until the residual phenol in the aqueous phases was totally eliminated. Final purified LPS product was lyophilized and stored at 4°C.

PMCID: PMC3558174

LPS-Lipoprotein clean up

At room temperature, 5 mg of smooth, Rc, and Re LPS were individually resuspended in 1 ml of endotoxin-free water containing 0.2% triethylamine (TEA). Each sample was split into two 500-µl aliquots, and one aliquot was stored at 4°C without further manipulation ("unextracted LPS").

Deoxycholate (DOC) was added to the remaining aliquot to a final concentration of 0.5%, followed by the addition of 500 µl of water-saturated phenol. The samples were vortexed intermittently for 5 min, and the phases were allowed to separate at room temperature for 5 min. Samples were placed on ice for 5 min, followed by centrifugation at 4°C for 2 min at 10,000 × *g*. The top aqueous layer was transferred to a new tube, and the phenol phase was subjected to re-extraction with 500 µl of 0.2% TEA/0.5% DOC. The aqueous phases were pooled and re-extracted with 1 ml of water-saturated phenol.

The pooled aqueous phases were adjusted to 75% ethanol and 30 mM sodium acetate and were allowed to precipitate at −20°C for 1 h. The precipitates were centrifuged at 4°C for 10 min at 10,000 × *g*, washed in 1 ml of cold 100% ethanol, and air-dried. The precipitates were resuspended in the original volume (500 µl) of 0.2% TEA.

Biotinylation of Pam3cys

1. Dissolved Pam3cys in water to make 1mg/ml (0.66mM). Vortex to resuspend. Dissolved Sulfo-NHS-LCLC-Biotin 3 mg in 45 ul water to make 100mM stock.
2. Prepared two mixtures of Pam3cys (0.66mM) with Biotin. Vortex
    A. 7X Biotin
    400ul of pam3cys (360ul of 1mg/ml pam3cys in water + 40 ul of 10XPBS). Added18.5 ul of 100mM biotin stock to it.

    B. 30X Biotin
    400ul of pam3cys (360ul of 1mg/ml pam3cys in water + 40 ul of 10XPBS). Added 80 ul of 100mM biotin stock to it.

3. Incubate 30 min. RT
4. Load in Dispodialyzer. Stir in a solution of PBS for 1hrs three times.
5. Keep the biotinylated pam3Cys at 4C. Had a cloudy appearance and seems to have lost its function on TLR2.