NO Assay Procedure

Seeding primary cells

1. Collect spleens from birds in incomplete RPMI
2. Press spleen through a cell strainer (40u) using a syringe plunger and 4ml of RPMI
    a. Be careful to leave cells on ice at all times when possible
    b. Be sure to keep the tissue wet at all times to reduce cell death
3. Collect filtered contents in a 15ml tube and fill to 5ml mark
4. In another 15ml tube, place 5ml of histopaque (1.077 g/ml)
5. Overlay the cell suspension onto histopaque without mixing
6. Spin cells at 1,200 xg for 10 minutes at 10C with no brake
7. Collect cell layer
8. Wash with 5ml complete RPMI by spinning at 300 x g for 10 minutes
9. Discard Supernatant and resuspend in 5ml complete RPMI

Preparation for counting

1. Discard supernatant from T75 Flask
2. Add 5mL PBS, rock, let sit 1 minute to wash, then remove supernatant
3. Add 3mL of Trypsin, rock, incubate 5 minutes
4. Add 5mL of RPMI to neutralize Trypsin
5. Collect supernatant in 15mL tube
6. Centrifuge 15mL tube at 2,000 RPMI for 5 minutes
7. Discard Supernatant
8. Resuspend and wash in 10ml RPMI (2,000 RPMI for 5 minutes)
9. Discard Supernatant
10. Resuspend in 5mL Complete RPMI (amount based on pellet size)
11. Ready for counting

Plating

1. After counting, spin down cells and resuspend in enough Complete RPMI to obtain a concentration of 1 x 10^6 cells/well. Typically, you plate cells in 200ul, so your intended concentration is 1 x 10^6 cells/200ul.
2. Place 200ul of cells+media into each well (96 well, flat bottom, cell culture plate)

Incubation and challenge

1. Incubate cell culture plates 24 hours to allow cells to adhere to the bottom of the plate
2. Challenge with LPS (use literature to find an appropriate concentration) and any other pathogens of interest (in separate wells). Be sure to have negative controls
3. Read plates at 24 and 48 hour timepoints.
    a. Note: you cannot read the same well multiple times, so you will have a 24 hour plate and a 48 hour plate.

b. If you have enough cells you can do additional time point. Remember, 48 hours is the most critical timepoint, so if not enough cells, do the 48 hour timepoint only

Standard curve preparation

1. Create a 1M sodium nitrate standard curve

2. - Add 3.45 g of sodium nitrite to 50 ml of distilled water (1 M stock)
3. - Diluted 10X in 3 series the 1M 1 ml in 9 ml of distilled water to get a 1mM or 1000 µM stock
4. - Prepare the standards in duplicates as indicated below

	Medium (µl)	Stock (µl)	Final concentration (µM)
1000	500	500	500
500	500	500	250
250	500	500	125
125	500	500	62.5
62.5	500	500	31.25
31.25	500	500	15.625
15.625	500	500	7.8125
0	500		0

NO Procedure

1. Spin down plate at 650 xg for 4 minutes
2. Take 100ul of supernatant from each well and place into a normal (for flow cytometry) 96 well plate
3. (Do this step in the dark) Add 100ul RICCA solution to each well. Incubate at room temperature for 5 minutes
4. Read on plate reader at 540nm

Plate Setup example

	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	Rep 1 Pen 1	Rep 1 Pen 2	Rep 1 Pen 3	Rep 1 Pen 4	Rep 1 Pen 5	Rep 1 Pen 6	Rep 1 Pen 7	Rep 1 Pen 8	Rep 1 Pen 9	Rep 1 Pen 10	Rep 1 Pen 11	Rep 1 Pen 12
B	Rep 2 Pen 1	Rep 2 Pen 2	Rep 2 Pen 3	Rep 2 Pen 4	Rep 2 Pen 5	Rep 2 Pen 6	Rep 2 Pen 7	Rep 2 Pen 8	Rep 2 Pen 9	Rep 2 Pen 10	Rep 2 Pen 11	Rep 2 Pen 12
C	Rep 3 Pen 1	Rep 3 Pen 2	Rep 3 Pen 3	Rep 3 Pen 4	Rep 3 Pen 5	Rep 3 Pen 6	Rep 3 Pen 7	Rep 3 Pen 8	Rep 3 Pen 9	Rep 3 Pen 10	Rep 3 Pen 11	Rep 3 Pen 12
D	Rep 1 Pen 13	Rep 1 Pen 14	Rep 1 Pen 15	Rep 1 Pen 16	Rep 1 Pen 17	Rep 1 Pen 18	Rep 1 Pen 19	Rep 1 Pen 20	Rep 1 Pen 21	Rep 1 Pen 22	Rep 1 Pen 23	Rep 1 Pen 24
E	Rep 2 Pen 13	Rep 2 Pen 14	Rep 2 Pen 15	Rep 2 Pen 16	Rep 2 Pen 17	Rep 2 Pen 18	Rep 2 Pen 19	Rep 2 Pen 20	Rep 2 Pen 21	Rep 2 Pen 22	Rep 2 Pen 23	Rep 2 Pen 24
F	Rep 3 Pen 13	Rep 3 Pen 14	Rep 3 Pen 15	Rep 3 Pen 16	Rep 3 Pen 17	Rep 3 Pen 18	Rep 3 Pen 19	Rep 3 Pen 20	Rep 3 Pen 21	Rep 3 Pen 22	Rep 3 Pen 23	Rep 3 Pen 24
G	STD 1	STD 2	STD 3	STD 4	STD 5	STD 5	STD 6	STD 7				
H	STD 1	STD 2	STD 3	STD 4	STD 5	STD 5	STD 6	STD 7				