

## Introduction:

Toxin neutralization assays assess the ability of antibodies within sample sera to protect cells in culture (usually macrophages, such as J7747 cell line, or other B cell lines) from toxin. The cells are exposed to sample sera, usually from an immunized animal, which is diluted down the plate. Toxin is then added, and the cells incubated. Any live cells can be visualized using reagents such as diphenyltetrasolium bromide (MTT) which will be metabolized by live cells to give a vivid color (for MTT, the resulting color is purple formazan crystals). The further toward death the cells are, the less color they will develop. Completely dead cells will give no color. In the presence of any antibody towards the toxin, the antibody should bind the toxin will therefore help reduce the effect of the toxin on the cells, and should prevent them from dying off. The plates the cells have grown on can then be analyzed on a plate reader to assess the absorbance from each well and therefore the concentration at which the toxin can kill half the cells.

## 1. Preparation of reagents

### Assay media

Use an appropriate culture media depending on the cell line used. The following is a general example:

DMEM	450 ml
10% FBS	50 ml
2 mM glutamine	5 ml
100 U penicillin / 0.1 mg/ml streptomycin	5 ml

Use aseptic technique working in a Class II safety hood.

### Assay media 5% serum

2.5 ml heat inactivated fetal bovine serum

Make up to 50 ml with assay media

Discard any unused reagent at the end of the assay.

### MTT stock

1g fresh MTT (use fume hood).

Make up to stock concentration of 4.5 mg/ml with PBS (if exactly 1mg = 222 mls PBS)

This can be stored at 4°C for 1 month

### Solubilising buffer

5 g SDS

100 ml sterile water. (may need mixing on a stirrer plate to dissolve)

900 ml isopropanol (90%)

2.5 ml concentrated hydrochloric acid.

## 2. Preparation of samples and controls

1. Prepare sera/samples by diluting in assay media 5% FBS as required into bijoux or eppendorfs. 75 µl of sample per lane or as required. Samples may require dilution as required. Samples can be assayed in duplicate or triplicate (usually done in triplicate (use total 500µl))
2. Prepare control as required, e.g. control sera if available. Diluted in assay media

## 3. Preparation of dilution plate

1. Using solvent proof marker pen, label dilution plates with plate number, sample numbers and positive and negative control wells.
2. Add 75 µl assay media 5% FBS to all wells except the top row A (see fig 1)

- Add an additional 75  $\mu$ l (total 150  $\mu$ l) assay media 5% FBS to wells H7 to H12 (last 6 wells of bottom row). These will be the negative control wells (see fig 1)
- This is an example of a plate to run samples in triplicate:

**Figure 1: Layout of 96 well plate**

	1	2	3	4	5	6	7	8	9	10	11	12
A	none	none	none	none	none	none	none	none	none	none	none	none
B	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l
C	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l
D	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l
E	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l
F	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l
G	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l	75 $\mu$ l
H	75 $\mu$ l Pos	75 $\mu$ l Pos	75 $\mu$ l Pos	75 $\mu$ l Pos	75 $\mu$ l Pos	75 $\mu$ l Pos	150 $\mu$ l Neg	150 $\mu$ l Neg	150 $\mu$ l Neg	150 $\mu$ l Neg	150 $\mu$ l Neg	150 $\mu$ l Neg

Pos = wells for positive control  
Neg = wells for negative control

- Add 150  $\mu$ l of sample to the top row A

**Figure 2:**

	1	2	3	4	5	6	7	8	9	10	11	12
A	150 $\mu$ l S1	150 $\mu$ l S1	150 $\mu$ l S1	150 $\mu$ l S2	150 $\mu$ l S2	150 $\mu$ l S2	150 $\mu$ l S3	150 $\mu$ l S3	150 $\mu$ l S3	150 $\mu$ l cont	150 $\mu$ l cont	150 $\mu$ l cont

Samples usually put on in triplicate as figure 2 where

S1 = sample 1

S2 = sample 2

S3 = sample 3

Cont = control eg control sera

- Dilute the samples down the plate 1:2, or as required, DOWN TO ROW G ONLY to leave negative controls in row H..

As a general example guide, if samples are used in triplicate, the plate format will then be:

**Figure 3:**

	1	2	3	4	5	6	7	8	9	10	11	12
A	S1	S1	S1	S2	S2	S2	S3	S3	S3	Cont	Cont	Cont
B	S1d1	S1d1	S1d1	S2d1	S2d1	S2d1	S3d1	S3d1	S3d1	Cd1	Cd1	Cd1
C	S1d2	S1d2	S1d2	S2d2	S2d2	S2d2	S3d2	S3d2	S3d2	Cd2	Cd2	Cd2
D	S1d3	S1d3	S1d3	S2d3	S2d3	S2d3	S3d3	S3d3	S3d3	Cd3	Cd3	Cd3
E	S1d4	S1d4	S1d4	S2d4	S2d4	S2d4	S3d4	S3d4	S3d4	Cd4	Cd4	Cd4
F	S1d5	S1d5	S1d5	S2d5	S2d5	S2d5	S3d5	S3d5	S3d5	Cd5	Cd5	Cd5
G	S1d6	S1d6	S1d6	S2d6	S2d6	S2d6	S3d6	S3d6	S3d6	Cd6	Cd6	Cd6
H	pos	pos	pos	pos	pos	pos	neg	neg	neg	neg	neg	neg

S1, S2 etc. = sample 1, sample 2 etc.  
D1, D2 etc = dilution 1, dilution 2 etc  
Cont / C = control reference serum (positive control)  
Pos = toxin positive control (toxins added to wells)  
Neg = toxin negative control (culture medium only)

#### 4. Addition of toxin to dilution plates

*This dilution plate is set up for dilution of toxin only, and not the plate that already contains the cells.*

1. Prepare toxin by dilution in assay. Prepare the correct concentration (this will require optimization) for addition of 75  $\mu$ l to pipette 75  $\mu$ l of toxin mix to each well of the dilution plates.
2. Add to columns 1-6 first and down the plate from A to H (including positive control wells in row H which should contain toxin). Then columns 7-12 down the plate from A to G (missing out the negative control wells in row H which should not contain toxin).
3. Place in CO<sub>2</sub> 37°C incubator for 30 minutes (this time will require optimization).

#### 5. Transfer onto cells

1. Take the cell plates (seeded the previous day) into the safety cabinet. Carefully take off the cell culture media from the wells. Proceed immediately to step 2 to prevent any damage to the cells from drying.
2. Carefully transfer 100  $\mu$ l from each well of the dilution plates into each well of the corresponding 96 well culture plate (cells seeded previous day). Transfer from one column of wells at a time and change pipette tips between each sample.
3. Place cell plates back into 37°C CO<sub>2</sub> incubator for 3 hours.

#### 6. Addition of MTT

1. Pipette 10 ml of stock MTT into 50 ml centrifuge tube. Dilute 1:3 to correct concentration (1.5  $\mu$ g/ml), by adding 20 ml warm assay media 5% FBS and mix well.
2. Pipette 50  $\mu$ l of diluted MTT into each well of assay cell plates (this may require optimization) Add to one column of wells at a time across the plate.
3. Place cell plates back into incubator for 1 hour (this time may require optimisation)

#### 7. Addition of Solubilizing buffer

1. Take cell plates out of the incubator and carefully remove the media from all the wells. Then proceed immediately to step 2 to prevent any damage to the cells from drying
2. Add 100  $\mu$ l of solubilizing buffer to each well. This can be added to one column of wells at a time across the plate.
3. Tape plates together and place on shaker (on a gentle speed) for 30 minutes to dissolve the MTT. This may require some optimisation. The plates are then ready to read.

#### 8. Reading plates

1. Inspect plate to ensure dissolution of the MTT crystals and read at 570 nm using a plate reader.

#### 9. Calculations

Programs are available to analyze the plate reader data. For example, these programs can be used to generate a four parameter logistic curve fit. This should then give an ED<sub>50</sub>% result. This is the dilution at which there is 50% neutralization of toxin (ED<sub>50</sub>) calculated as the dilution at which half the cells in the well are dead, half way down the calculated curve. This should be at approximately the 4<sup>th</sup> 2-fold dilution of test sample.