

**PREPARING TEST SAMPLES
USING ATS ANTIBODY SECONDARY CONJUGATES**

On Day Two of your Cytotoxicity Assay, before adding anything to the plates, determine the amount of antibody and Secondary Conjugate needed for the experiment.

TO DETERMINE THE AMOUNT OF ANTIBODY NEEDED:

- Make a stock tube that contains 1 μM of antibody in a volume of 150 μl .

Example for an antibody that has a concentration of 1.55 mg/ml:

(The standard molecular weight for a typical IgG is 1.6×10^5 mg/mmol. Consult the data sheet that came with your antibody to determine the molecular weight of your antibody.)

$$150 \mu\text{l} (1 \mu\text{M}) = \frac{1.55 \text{ mg/ml} \cdot (x)}{1.6 \times 10^5 \text{ mg/mmol}}$$

$$150 \mu\text{l} (\mu\text{M}) = 9.7 \times 10^{-6} \text{ M} \cdot (x)$$

$$150 \mu\text{l} (\mu\text{M}) = 9.7 \mu\text{M} \cdot (x)$$

$$(x) = \frac{150 \mu\text{l}(\mu\text{M})}{9.7 \mu\text{M}}$$

$$(x) = 15.46 \mu\text{l} \quad (\text{The volume of antibody to be used in this experiment.})$$

- Since the total volume in the Stock Tube will be 150 μl , subtract the calculated volume from 150 μl . Bring the volume to 150 μl with a Secondary Conjugate solution (see below).

Example: $150 \mu\text{l} - 15.46 \mu\text{l} = 136.54 \mu\text{l}$ of Secondary Conjugate solution

TO PREPARE YOUR SECONDARY CONJUGATE SOLUTION:

- 100 ng of Secondary Conjugate will be added to each well. Since each well will receive 10 μl of solution (Antibody + Secondary Conjugate + Media), the stock tube must be set up to have a concentration of 100 ng/10 μl . To make the solution test samples and have enough material for controls, make at least 1.5 ml of Secondary Conjugate solution.

Example: $\frac{100 \text{ ng}}{10 \mu\text{l}} = \frac{X}{1500 \mu\text{l}}$ $X = 15,000 \text{ ng}$
 $X = 15 \mu\text{g}$ of Secondary Conjugate is needed

- Calculate the volume of Secondary Conjugate needed in μl .

Example for a Secondary Conjugate that has a concentration of 3.42 mg/ml = 3.42 $\mu\text{g}/\mu\text{l}$:

$$\frac{3.42 \mu\text{g}}{1 \mu\text{l}} = \frac{15 \mu\text{g}}{X} \quad \quad 3.42 \mu\text{g} \cdot X = 15 \mu\text{g}$$

$$X = 4.4 \mu\text{l} \text{ of Secondary Conjugate}$$

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- In a test tube bring the calculated volume of Secondary Conjugate to 1.5 ml with medium.
- Vortex well for 5 seconds.

TO PREPARE THE DILUTIONS OF YOUR TEST SAMPLES:

- Prepare 8 micro-centrifuge tubes. Label them 1 - 8.
- Tube 1 will be the stock tube.
- In tubes 2-8 add 135 μ l of the Secondary Conjugate solution.
- In Tube 1 add the calculated volume of 1 μ M antibody then add the calculated volume of Secondary Conjugate solution.
- Mix well by vortexing for 5 seconds.
- Let the stock tube sit and incubate in the hood at room temperature for 30 minutes.
- After 30 minutes has passed, prepare the rest of the antibody dilutions.
- Pipette 15 μ l from Tube 1 (stock tube), and transfer it to Tube 2.
- Vortex Tube 2 for 5 seconds.
- Pipette 15 μ l from Tube 2 and transfer it to tube 3.
- Vortex well for 5 seconds.
- Repeat for subsequent tubes until you get a total of 150 μ l in tube 8.

After finishing with the dilutions, each micro-centrifuge tube has Secondary Conjugate at a concentration of 100 ng/10 μ l and Tube 1 contains the primary antibody at a concentration of 1 μ M. Each tube from 1 down to 8 has a 1:10 dilution of the previous tube's antibody molar concentration.

ADDING YOUR TEST SAMPLES TO YOUR PLATE:

- Take the 96-well plate from the incubator into the hood. Add 10 μ l of medium (room temperature) to the wells in the control columns (B2-G2 and B11-G11).
- Begin adding the serial dilutions to the plate starting with tube 8 (the tube with the lowest concentration of antibody).
- Transfer 10 μ l to each well in the column B10-G10. Add the material to the side of the well. Do not touch the bottom of the well, so the cells on the bottom are not disturbed. Do not pipette up and down.
- Repeat for Tube 7 into wells (B9-G9) working backward to Tube 1 (B3-G3).
- Label the lid and the base with a number to avoid switching plate lids.
- Once finished, put the plate back into the incubator.
- Incubate for 72 hours.

See "Cytotoxicity Assay for Targeted Toxins *in vitro*" for more information.