ADVANCED TARGETING SYSTEMS
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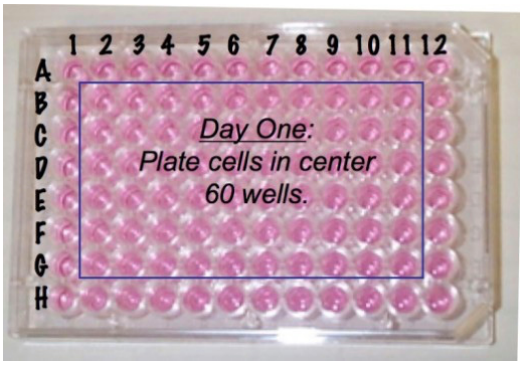
Cytotoxicity Assay Protocol for ZAP Antibody Internalization Kits

This is an example protocol. Please follow good laboratory technique and safety guidelines.
Working dilutions must be determined for each lot.
Please contact us if you have questions. www.ATSbio.com

DAY 1

Cell Culture



Plating (example) - Day 1:

- Plate cells at 2500 cells/well for a typical 3-plate cytotoxicity assay.
- Cells should be lifted from the flask and counted.
- Only the center 60 wells have cells added to them. Add 100 µl of culture media to the outer ring of wells to avoid evaporation of the sample wells during the course of the assay.

For the present example, the calculation for the number of cells needed is as follows:

Number of wells		Cells/well		Total cells needed
200	x	2500	=	500,000

Number of wells		µl/well		Total µl needed
200	x	90	=	18,000

- The cells will need to be resuspended very thoroughly, and create a single cell suspension as best as possible via a combination of pipetting up and down plus vortexing.
- Add 100 microliters of media ONLY to each of the outer ring of cells. Using a multi-channel pipettor quickens the process.
- Pour the cell suspension into a reservoir that is easy to pipet from. Add 90 microliters of the cell suspension to each of the center 60 wells on each plate.
- Label each plate in some fashion with the cell type, # of cells/well, and date. Place the plates into the proper culture environment overnight.

Use the online calculators at ATSbio.com

DAY 2

ZAP Media

Preparing ZAP spiked media needed (example) – Day 2:

- These ZAP Antibody Internalization kits are used at a constant concentration within the assay. Doing so ensures that the effects witnessed in the assay are attributable to the targeting agent alone.
- The recommended effective concentration is 4.5 nanomolar in the wells. The recommended volume is 1500 µl. In order to best titrate the targeting agent, it is suggested that the media used for the titration be spiked with the ZAP product at 45 nM such that a 1:10 dilution during addition to the wells results in a proper final concentration.
- View the formula below to determine the volume of ZAP needed. The concentration and molecular weight for the ZAP product can be found on the tube label and data sheet. These numbers may vary with each lot number and product.

Desired concentration (45 nM)	x	Desired volume (1500 µl)	=	ZAP conc (mg/ml) / ZAP mol wt (kDa)	x	Volume of ZAP needed (µl)

Prepare ZAP spiked media (example) – Day 2:

- For a final volume of 1500 µl, add the calculated volume of ZAP product to the calculated volume of culture media and vortex. Use the formula below to determine the volume of culture media needed.

Desired volume (1500 µl)	-	Calculated Volume of ZAP (µl)	=	Volume of culture media needed (µl)

- Label tube as “ZAP Media” so as not to mix it up with the regular culture media.

DAY 2 (continued)

Targeting Agent

Determine Targeting Agent needed to react with ZAP product (example) – Day 2:

- View the formula below to determine the volume of Targeting Agent needed. Input your known concentration and molecular weight for the agent.

Desired concentration (µM)	x	Desired volume (µl)	=	Targ. Agent conc (mg/ml) / Targ. Agent mol wt (kDa)	x	Volume of Targeting Agent needed (µl)

Prepare Targeting Agent-ZAP Conjugate samples (example) – Day 2:

- Samples should be added to the plates in the morning (~16 hours after the cells are plated).
- Targeting Agent dilutions will be prepared at 8 concentrations, diluting from 10 nM to 1 fM, in 1:10 dilution increments. These are final concentrations in the well, but will be added to the well in a 10 µl volume, therefore the beginning concentration will be 10-fold higher, 100 nM.
- In a microcentrifuge rack, line up 8 tubes, labeling them 1-8. Add 135 µl of ZAP Media to tubes 2-7.
- To the first tube, add the calculated amounts of Targeting Agent and ZAP media for a final volume of 150 µl. Use the formula below to determine the volume of culture media needed.

Desired volume (150 µl)	-	Calculated Volume of Targeting Agent (µl)	=	Volume of ZAP Media needed (µl)

- Perform serial dilutions by pipetting 15 µl from tube 1 into tube 2. Pipet up and down several times to mix. Vortex the tube briefly. Repeat by pipetting 15 µl from tube 2 into tube 3. Continue to repeat the steps through tube 8.

DAY 2 (continued)

Control-SAP

Determine Control-SAP needed (example) – Day 2:

- View the formula below to determine the volume of Control-SAP needed. The concentration and molecular weight can be found on the tube label and data sheet. These numbers may vary with each lot number and product.

Desired concentration (µM)	x	Desired volume (µl)	=	Control-SAP conc (mg/ml) / Control-SAP mol wt (kDa)	x	Volume of Control-SAP needed (µl)

Prepare Control samples (example) – Day 2:

- Samples should be added to the plates in the morning (~16 hours after the cells are plated).
- Control-SAP dilutions will be prepared at 8 concentrations, diluting from 10 nM to 1 fM, in 1:10 dilution increments. These are final concentrations in the well, but will be added to the well in a 10 µl volume, therefore the beginning concentration will be 10-fold higher, 100 nM.
- In a microcentrifuge rack, line up 8 tubes, labeling them 1-8.
- To the first tube, add the calculated amounts of Control-SAP and regular culture media for a final volume of 150 µl. Use the formula below to determine the volume of culture media needed.

Desired volume (150 µl)	-	Calculated Volume of Control-SAP (µl)	=	Volume of culture media needed (µl)

- Add 135 µl of regular culture media to tubes 2-7.
- Perform serial dilutions by pipetting 15 µl from tube 1 into tube 2. Pipet up and down several times to mix. Vortex the tube briefly. Repeat by pipetting 15 µl from tube 2 into tube 3. Continue to repeat the steps through tube 8.

DAY 2 (continued)

Saporin

USE THE CALCULATORS AT ATSBIO.COM

Determine Saporin needed (example) – Day 2:

- View the formula below to determine the volume of Saporin needed. The concentration and molecular weight can be found on the tube label and data sheet. These numbers may vary with each lot number and product.

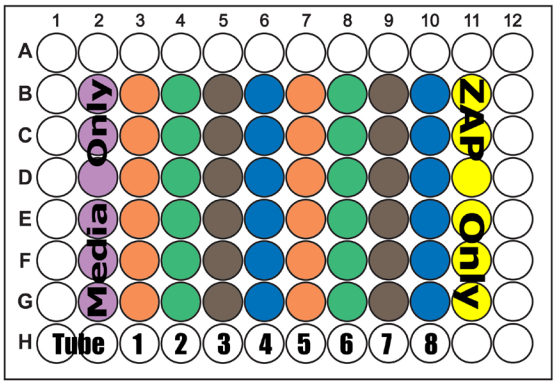
Desired concentration (µM)	x	Desired volume (µl)	=	Saporin conc (mg/ml) / Saporin mol wt (kDa)	x	Volume of Saporin needed (µl)

Prepare Saporin samples (example) – Day 2:

- Samples should be added to the plates in the morning (~16 hours after the cells are plated).
- Saporin dilutions will be prepared at 8 concentrations, diluted from 1 µM to 1 pM, in 1:10 dilution increments. These are final concentrations in the well, but will be added to the well in a 10 µl volume, therefore the beginning concentration will be 10-fold higher, 10 µM.
- In a microcentrifuge rack, line up 8 tubes, labeling them 1-8. Add 135 µl of cell culture media to tubes 2-7. To the first tube, add 105 µl of culture media (150 µl - 45 µl).
- Add the calculated volume of Saporin (45 µl) to tube #1, bringing the final volume to 150 µl.
- Perform serial dilutions by pipetting 15 µl from tube 1 into tube 2. Pipet up and down several times to mix. Vortex the tube briefly. Repeat by pipetting 15 µl from tube 2 into tube 3. Continue to repeat the steps through tube 8.

Treatment

Adding Samples to the Plates (example) – Day 2:



- This kit is designed to be used with up to three 96-well plates. One plate for Saporin, one plate for Control-SAP, and one plate for the Targeting Agent in conjunction with the ZAP product.
- It is recommended that the lid of the plate be labeled with the reagents to be added to the plate.
- All materials are added to the plates in 10 µl volumes.
- Samples are added to the plate in 6 replicates, one concentration per well-column.
- Add 10 µl of culture media to plate-column 2.
- Add 10 µl of ZAP media alone to plate-column 11 as an internal control.
- Incubate all plates under normal culture conditions for 72 hours.

DAY 5

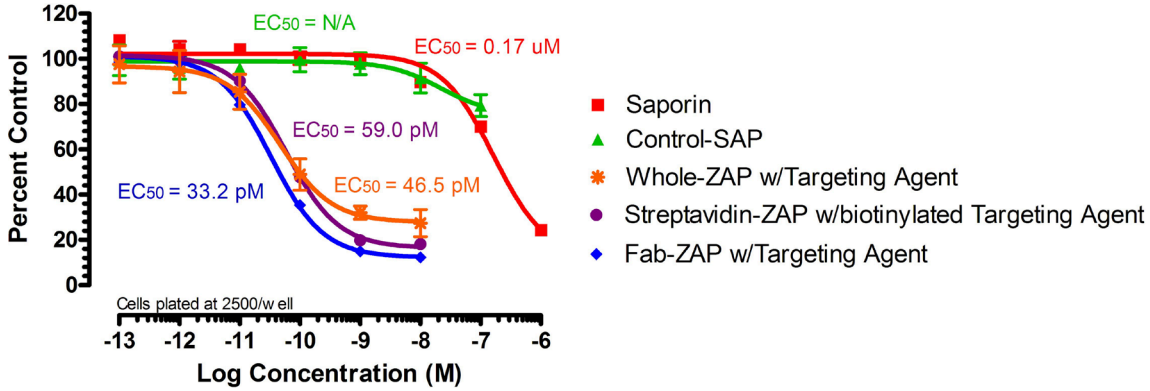
Results

Developing the Assay (example) – Day 5:

- Warm 5.5 ml of PBS in a 15-ml conical tube to 37°C.
- Thaw XTT vial to room temp and vortex thoroughly. Add entire contents of XTT tube to pre-warmed PBS and vortex again.
- Add 92 µl of PMS to the XTT/PBS tube and vortex thoroughly.
- Add 50 µl of the XTT/PMS solution to each of the interior 60 wells and the A1 blank well or each plate.
- Incubate plates at 37°C. Plates should be read approximately every 30 minutes to determine assay completion. (Typical total incubation time is 2 hours, but this will vary by cell type)
- To read plates, shake gently for 10 seconds in plate reader prior to reading. Read the plates at 450 nm. Optical density readings for the control wells should be >0.3 for best results.

Sample Data Presentation:

- Cytotoxicity data is typically analyzed by comparing well readings of the treated wells to those of the control wells, expressed as a percentage.
- The number of viable cells remaining on the day of development is measured via cell metabolism of a colorimetric molecule within the developing reagents.
- The darkness of color in the untreated wells is considered to be 100% of control.



Materials & Safety

Good laboratory technique must be employed for the safe handling of this product. This requires observation of the following practices:

- Wear appropriate laboratory attire, including lab coat, gloves and safety glasses.
- Do not pipet by mouth, inhale, ingest or allow product to come into contact with open wounds. Wash thoroughly any part of the body which comes into contact with the product.
- Avoid accidental autoinjection by exercising extreme care when handling in conjunction with any injection device.
- This product is intended for research use by qualified personnel only. It is not intended for use in humans or as a diagnostic agent. Advanced Targeting Systems is not liable for any damages resulting from the misuse or handling of this product.

See data sheets enclosed with kit for individual component safety and handling information.