

# ZAP Antibody Internalization Kit

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Screening large numbers of antibodies can be prohibitively expensive in both cost and time. The ability to perform a diagnostic screen that is amenable to high-throughput methods, prior to direct conjugation of those antibodies, is a great cost-benefit in the development of an effective targeted conjugate. Targeted conjugates are widely used to escort payloads to specific cell populations *in vitro* and *in vivo* for both basic research and pharmaceutical development. The development of an effective and specific targeted conjugate is a long and costly process. A molecule that targets the marker of choice (a Targeting Agent) must be identified, produced, and specificity must be characterized. Desirable traits of a Targeting Agent include high specificity and rapid internalization. The Targeting Agent can be an antibody, peptide, protein, or any other molecule that recognizes a cell-surface marker. Antibodies often make the best targeting agent, and the choice of the correct antibody is crucial to the specificity and performance of payload delivery.

The ZAP products produced by **Advanced Targeting Systems, Inc.** allow a large number of targeting agents to be screened quickly and cost-efficiently for specificity, functional binding, internalization, and EC<sub>50</sub> determination. The wide selection of these secondary conjugates identified by the ZAP moniker provide ideal tools for screening potential targeting agents. They are constructed using either species-specific polyclonal anti-IgG antibodies, or streptavidin (for use with biotinylated targeting agents), that are chemically attached to Saporin, the most potent of the plant ribosome-inactivating proteins. The mechanism of action of ZAP-based conjugates is detailed in Figure 1.

Use of a secondary conjugate eliminates the time-consuming and expensive step of conjugating each targeting-agent-candidate to the payload: the ZAP product can simply be added to cells, in culture conditions, together with the targeting candidate. Once the materials have been administered, the targeting molecule directs the ZAP to the cells of interest, the complex is bound by the targeted cells, internalized, and the Saporin protein is released within the cytosol to inactivate the ribosomes. Cells not expressing the target do not bind or internalize the ZAP-targeting agent complex, and are not affected. Saporin has no binding chain, and no means of getting into cells on its own (free Saporin is cleared via the kidney).

The ZAP Targeting Agent Screening Kit contains all of the materials needed to screen **your** targeting agent. Included, in addition to the selected ZAP products, are controls and developing reagents for the assay. All the user provides are the materials specific to their experiment (the targeting agent, cells expressing the target, and culture reagents). Recommended protocols for use are detailed in this booklet, and are specific to the particular kit chosen (Whole-ZAP, Fab-ZAP, or Streptavidin-ZAP). Examples of predicted assay results are also included for comparison, and a successful assay provides an EC<sub>50</sub> useful in determining if the candidate-targeting agent should be pursued at the next level.

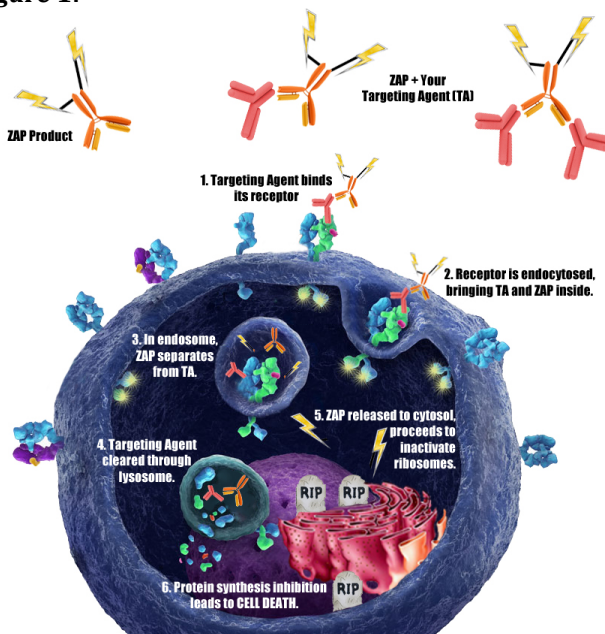
## Kit Components

- Instruction booklet
- Flash drive with video tutorial
- Saporin, 50 micrograms
- Control conjugate (kit specific)
  - Fab IgG-SAP, 10 micrograms
  - Whole-ZAP, 25 micrograms
- Secondary conjugate (kit specific)
  - Fab-ZAP, 10 micrograms
  - Whole-ZAP, 25 micrograms
- Developing reagents
  - XTT, 5.5 milliliters
  - PMS, 100 microliters

### Provided by user:

- 96-well tissue culture treated, flat bottom plates with lids
- Cells expressing marker of interest
- Culture media for the cells
- Microcentrifuge tubes (1.5 ml size)

Figure 1:



\*View PDF for enlarged figure

## Background and General Information

### Cell Culture

- Cells should be chosen that have significant levels of expression of the desired marker (either native, or transfected). Cell sorting using a flow cytometer can enrich a population of cells for the marker of interest.
- Cells are typically plated in the late afternoon (~16 hours prior to administration of the products). This is considered Day 1 of the assay.
- The number of cells per well are determined by the proliferation rate of the cells being used. Typical ranges include 1000-5000 cells per well of a 96-well plate, though as few as 100 cells and as many as 10,000 per well can be used in particular cases.
- The goal is to have the untreated cells approach 70-80% confluence on the morning of day five of the assay.

### *Saporin as Positive & Negative control*

- Saporin is a 30 kDa protein that cannot enter cells on its own, it has no binding chain. As a ribosome inactivating protein (RIP) it is only active and effective inside the cytosol of a cell.
- Using Saporin in the ZAP cytotoxicity assay is an essential control. Very high levels of saporin ( $\geq 1 \mu\text{M}$ ) will cause non-specific death as a result of bulk-phase endocytosis by the treated cells. Using saporin in the assay starting at that concentration will ensure that the EC50 of non-specific killing is established.
- Testing multiple dilutions of Saporin not only establishes the EC50, but at lower concentrations of Saporin (typically  $< 50 \text{ nM}$ ) there is not discernable elimination of the tested cell population. This titration of Saporin provides the baseline cell death profile by which the experimental targeting agents will be compared.
- It is recommended that the dilution profile for Saporin start at a higher concentration than the test samples, but follow a similar dilution schedule (i.e. 1:3 or 1:10).

### *The ZAP Products*

- **Whole-ZAP products** are made by conjugating species-specific polyclonal anti-IgG to Saporin. The secondary antibody used in the product is bi-valent IgG against the whole IgG of the species chosen. As such, there will be some cross-reactivity to other immunoglobulin subtypes (IgA, IgM, IgE, etc.) due to light chain homology.
- **Fab-ZAP products** are made by using species-specific, polyclonal, mono-valent, anti-IgG attached to Saporin. The antigen is against the whole IgG of the species chosen. As such, there will be some cross-reactivity to other immunoglobulin subtypes (IgA, IgM, IgE, etc.) due to light chain homology. The use of Fab IgG prevents the theoretical possibility of false-positive killing that could result from the capping phenomenon suffered by bi-valent antibodies.
- **FabFc-ZAP products** also are manufactured using species-specific, polyclonal, mono-valent, anti-IgG attached to Saporin. The difference is that the FabFc antibodies are made against only the Fc portion of the species-specific IgG. This means that the ZAP conjugate will not bind other Ig subclasses and will also not bind surface Ig that is typically expressed on B-cells.

### *Control-SAP included*

- The best possible control for use in this assay is a molecule that mimics the Targeting Agent candidate in every way but binding capability. A typical example would be an isotype control for an antibody candidate.
- As a worthwhile alternative when those options are not available, the kit includes a species-specific non-binding control conjugate for use with the Whole, Fab, and FabFc-ZAP products. The antibodies used in these products come from normal serum of the species specified.

- Control conjugates should be used in the assay at the same concentration as the Targeting Agent candidate to demonstrate effects of a non-binding targeting agent at equivalent concentrations.

## Suggested Protocols:

### Plating (Example) – Day 1:

- Plate cells at 2500 cells/well for a typical 3-plate cytotoxicity assay:
  - Cells should be lifted from the flask (if need be) and counted.
  - Only the center 60 wells have cells added to them. Add 100  $\mu$ l 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

		$\mu$ l/well		Total $\mu$ l media
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.

## SAPORIN

### Preparing Saporin control dilutions (example) – Day 2:

- Controls and 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  $\mu$ M 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  $\mu\text{l}$  volume, therefore the beginning concentration will be 10-fold higher, 10  $\mu\text{M}$ .
- View calculations below, for Saporin at a concentration of 1.0 mg/ml, 45 microliters of material will be used.
- In a microcentrifuge rack, line up 8 tubes, labeling them 1-8. Add 135  $\mu\text{l}$  of cell culture media to tubes 2-7. To the first tube, add 105  $\mu\text{l}$  of culture media (150  $\mu\text{l}$  – 45  $\mu\text{l}$ ).

Desired Conc. ( $\mu\text{M}$ )	*	Desired volume ( $\mu\text{l}$ )	=	Saporin Conc. (mg/ml) Saporin Molec. Weight (Da)	*	Volume of Saporin Needed ( $\mu\text{l}$ )
10	*	150	=	$\frac{1.0}{3.0\text{E}+04}$	*	X
		1500	=	3.3E-05 M	*	X
		1500	=	X		
		33 $\mu\text{M}$	=	X		
		45 $\mu\text{l}$	=	X		

- Add 45  $\mu\text{l}$  of Saporin to tube #1, bringing the final volume to 150  $\mu\text{l}$ .
- Perform serial dilutions by pipetting 15  $\mu\text{l}$  from tube 1 into tube 2. Pipet up and down several times to mix. Vortex the tube briefly. Repeat by pipetting 15  $\mu\text{l}$  from tube 2 into tube 3. Continue to repeat the steps through tube 8.

### Whole-ZAP, Fab-ZAP, & FabFc-ZAP

#### Preparing ZAP spiked media (example; Whole, Fab, & FabFc ZAP products) – Day 2:

- These three ZAP product types 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. 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.

Desired Conc. (nM)	*	Desired volume ( $\mu\text{l}$ )	=	ZAP Conc. (mg/ml) ZAP Molec. Weight (Da)	*	Volume of ZAP Needed ( $\mu\text{l}$ )
45	*	1500	=	Tube Label Data Sheet	*	X

67500	=	M	*	X
67500	=	X		
nM				
μl	=	X		

- Add the calculated volume of ZAP product to 1.5 ml of culture media and vortex.
- Label tube as “ZAP media,” or something similar, so as not to mix it up with the regular culture media.

#### *Preparing Targeting Agent (test sample) dilutions (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.
- View calculations below for Your Targeting Agent. Input your known concentration and molecular weight for the agent.
- In a microcentrifuge rack, line up 8 tubes, labeling them 1-8. Add 135 μl of ZAP-spiked culture media to tubes 2-7. To the first tube, add 150 μl of ZAP-spiked culture media.

Desired Conc. (μM)	*	Desired volume (μl)	=	Sample Conc. (mg/ml) Sample Molec. Weight (Da)	*	Volume of ZAP Needed (μl)
0.1	*	150	=	mg/ml Da	*	X
15	=	M	*	X		
15	=	X				
μM						
μl	=	X				

- Remove the calculated volume of ZAP spiked media from tube 1 and discard. Add the calculated volume of Targeting Agent to tube 1, bringing the final volume to 150 μl.
- Immediately 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.

- Allow tubes to incubate at room temperature for 15 minutes.

## CONTROL-SAP

### Preparing 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  $\mu$ l volume, therefore the beginning concentration will be 10-fold higher, 100 nM.
- View calculations below, for Control sample using the concentration on the tube label and molecular weight given on the data sheet.
- In a microcentrifuge rack, line up 8 tubes, labeling them 1-8. Add 135  $\mu$ l of regular culture media to tubes 2-7. To the first tube, add 150  $\mu$ l of regular culture media.

Desired Conc. ( $\mu$ M)	*	Desired volume ( $\mu$ l)	=	Control Conc. (mg/ml) Control Molec. Weight (Da)	*	Volume of ZAP Needed ( $\mu$ l)
0.1	*	150	=	Tube Label Data Sheet	*	X
15	=			M	*	X
15	=			X		
$\mu$ M						
$\mu$ l	=			X		

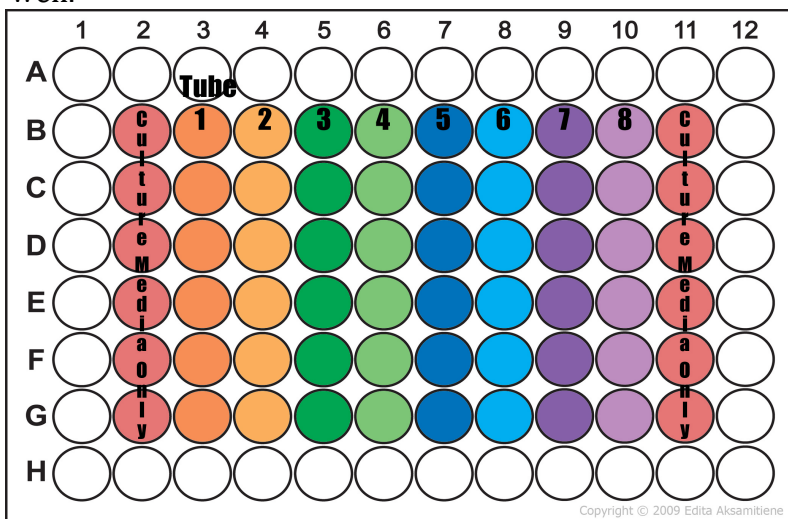
- Remove the calculated volume of culture media from tube #1 and discard. Add the calculated volume of Control sample to tube #1, bringing the final volume to 150  $\mu$ l.
- Perform serial dilutions by pipetting 15  $\mu$ l from tube 1 into tube 2. Pipet up and down several times to mix. Vortex the tube briefly. Repeat by pipetting 15  $\mu$ l 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 three 96-well plates. One plate for Saporin, one plate for Control-SAP, and one plate for the Targeting Agent sample in conjunction with the included 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  $\mu$ l volumes.
- Samples are added to the plate in 6 replicates, one concentration per well-column. Traditionally plate-column 2 and 11 have culture media ONLY added, 10  $\mu$ l per well.



- If using Whole, Fab, or FabFc-ZAP spiked media, add 10  $\mu$ l of ZAP media alone to plate-column 11 as an internal control.
- Incubate all plates under normal culture conditions for 72 hours.

## RESULTS

### *Developing the Assay (example) – Day 5:*

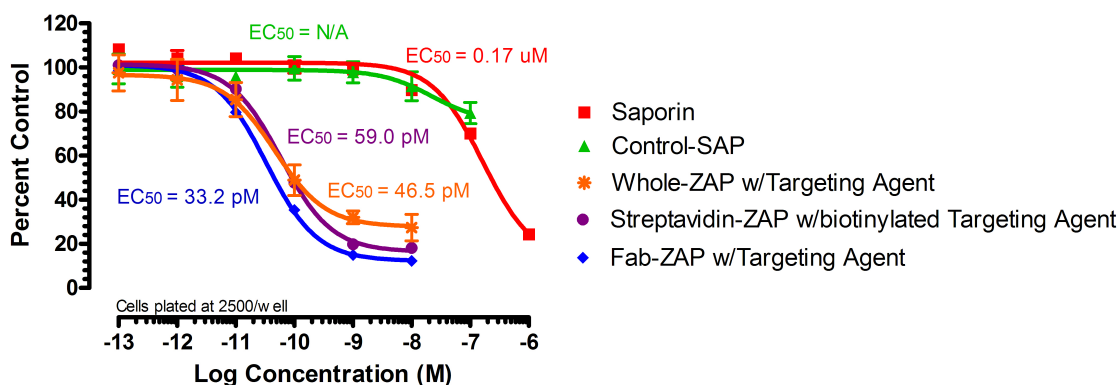
- Warm 5.5 milliliters of PBS in a 15 ml conical tube to 37°C.
- Thaw XTT vial to room temp and vortex thoroughly. Add entire contents of XTT tube to pre-warmed PBS and vortex again.
- Add 92 microliters of PMS to the XTT/PBS tube and vortex thoroughly.
- Add 50  $\mu$ l of the XTT/PMS solution to each 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

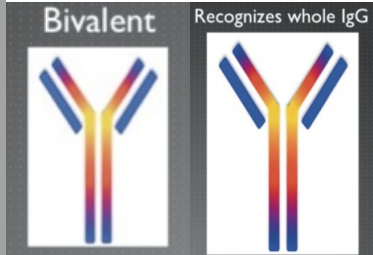
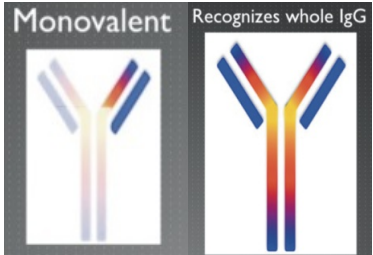
- Good laboratory technique must be employed for the safe handling of this product. This requires observation of the following practices:
  - Wear appropriate laboratory attire, including lab coat, gloves and safety glasses.
  - Do not pipet by mouth, inhale, ingest or allow product to come into contact 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.

## References

- Kohls MD, Lappi DA (2000) MabZAP: A tool for evaluating antibody efficacy for use in an immunotoxin. *BioTechniques* 28(1):162-165.



## Ordering

Your Ab	Available ZAP Products		
chicken IgY	<a href="#">Chick-ZAP</a> (IT-62)		
goat IgG	<a href="#">Goat-ZAP</a> (IT-36)		
guinea pig IgG	<a href="#">gPIG-ZAP</a> (IT-64)		
human IgG	<a href="#">Hum-ZAP</a> (IT-22)	<a href="#">Fab-ZAP human</a> (IT-51)	<a href="#">FabFc-ZAP human</a> (IT-65)
human IgM	<a href="#">Hug-M-ZAP</a> (IT-43)		
mouse IgG	<a href="#">Mab-ZAP</a> (IT-04)	<a href="#">Fab-ZAP mouse</a> (IT-48)	
mouse IgM	<a href="#">Anti-M-ZAP</a> (IT-30)		
rabbit IgG	<a href="#">Rab-ZAP</a> (IT-05)	<a href="#">Fab-ZAP rabbit</a> (IT-57)	
rat IgG	<a href="#">Rat-ZAP</a> (IT-26)	<a href="#">Fab-ZAP rat</a> (IT-55)	
	<b>All available individually (100 µg or 250 µg) or as a kit</b>		
	<p><i>made with bivalent antibodies that recognize the whole IgG</i></p> 	<p><i>made with monovalent (Fab) antibodies that recognize the whole IgG, without bivalent capping</i></p> 	<p><i>made with monovalent (Fab) antibodies that recognize Fc region only</i></p> 