ADVANCED TARGETING SYSTEMS

ZAP Biotin-Z Internalization Kit

Screening large numbers of molecules 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 molecules, 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.

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_{50} 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 the targeting candidate and collectively applied to plated cells. 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 Biotin-Z Internalization Kit contains all of the materials needed to screen **your** targeting agent. Included in the kit are Streptavidin-ZAP, 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_{50} useful in determining if the candidate-targeting agent should be pursued at the next level.

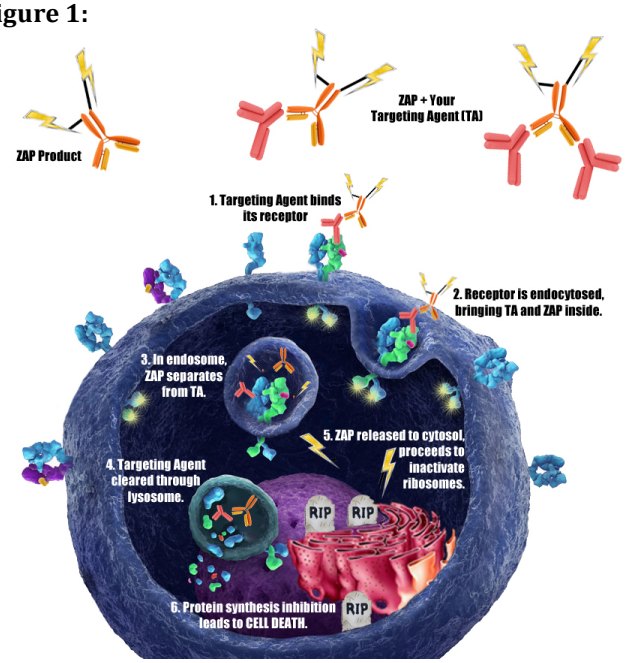
Kit Components

- Instruction booklet
- Flash drive with video tutorial
- Saporin, 50 micrograms
- Secondary conjugate (kit specific)
  - Streptavidin-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 (≥1 μM) 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 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

- **Streptavidin-ZAP** is a product uniquely designed for use with biotin-labeled molecules. The extremely strong interaction between streptavidin and biotin allows this product to also be used for initial *in vivo* studies. The streptavidin used in the product is in the tetramer form, so is capable of binding up to four biotins per molecule of Streptavidin-ZAP. It is recommended that for this particular ZAP product one use it in an equimolar ratio with the targeting agent.

Control-SAP (not 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.
- An alternative control would be a non-biotinylated version of the targeting agent.
- 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 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

		µl/well		Total µl 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 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.
- 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 µl of cell culture media to tubes 2-7. To the first tube, add 105 µl of culture media (150 µl – 45 µl).

Desired Conc. (µM)	*	Desired volume (µl)	=	Saporin Conc. (mg/ml) / Saporin Molec. Weight (Da)	*	Volume of Saporin Needed (µl)
10	*	150	=	1.0 / 3.0E+04	*	X
		1500	=	3.3E-05 M	*	X
		1500	=	X		

33 µM		
45 µl	=	X

- Add 45 µl of Saporin 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.

Streptavidin-ZAP

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 + Streptavidin-ZAP are used in an equimolar ratio and 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.

Targeting Agent

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 / µM	=	X		
		µl	=	X		

- Also calculate the volume of Streptavidin-ZAP you will need for an equimolar solution of Targeting Agent to ZAP in the table below.

Streptavidin-ZAP

Desired Conc. (µM)	*	Desired volume (µl)	=	Strep-ZAP Conc. (mg/ml) / Strep-ZAP Molec. Weight (Da)	*	Volume of ZAP Needed (µl)
0.1	*	150	=	Tube Label / Data Sheet	*	X

15	=	M	*	X

15 / µM	=	X

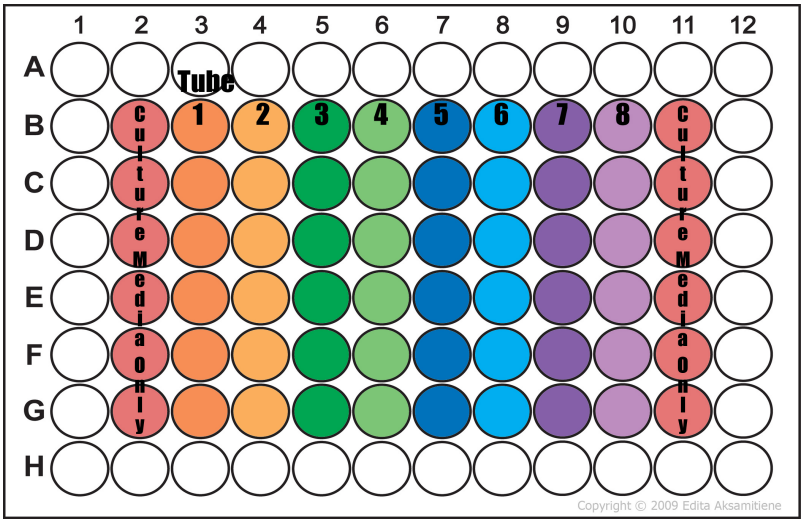
µl	=	X

- In a microcentrifuge rack, line up 8 tubes, labeling them 1-8. Add 135 µl of regular culture media to tubes 2-7.
- To Tube 1, add the calculated volume of Streptavidin-ZAP plus the calculated volume of Targeting Agent. Pipet up and down to mix and incubate at room temperature for 15 minutes.
- Add culture media to Tube 1 to bring final volume up 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 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 µl 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 µl per well.



- Incubate all plates under normal culture conditions for 72 hours.

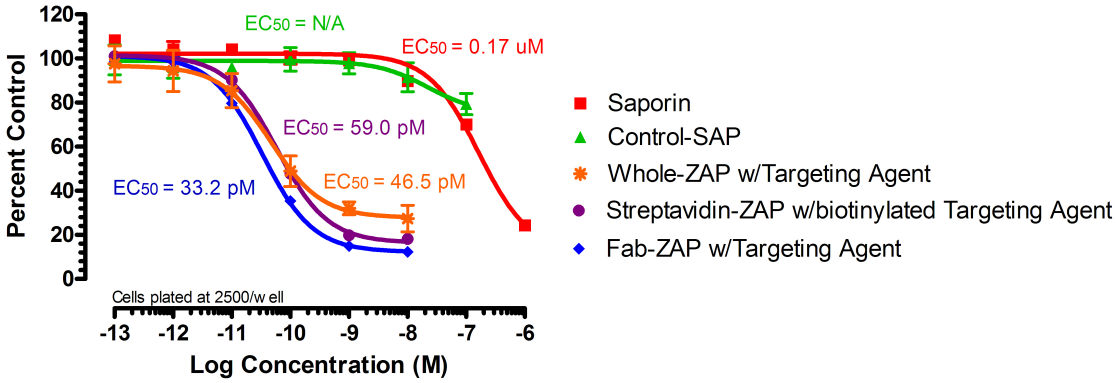
RESULTS

Developing the Assay (example) – Day 5:

- Warm 5.5 milliliters of PBS in a 15 ml conical tube to 37ºC.
- 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 µl of the XTT/PMS solution to each of the interior 60 wells and the A1 blank well or each plate.
- Incubate plates at 37ºC. 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 development stain is a total protein stain for what remains in the well upon Day 5. Staining of the protein is appropriate for this assay because Saporin's toxicity comes from protein-synthesis inhibition.
- Protein content (darkness of stain in well) in the untreated wells is considered to be 100% of control.



Materials & Safety

- Need to include here any relevant safety information regarding the developing reagents

- 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.

References

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

Other ZAP Kits

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