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Targeting Trends

Reporting the latest news in Molecular Surgery

Drug-free selection of stable transfectants using targeted toxin technology and a vector expressing cell-surface carbohydrate-digesting enzyme

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Isolation of stable transfectants is one of the important steps for exploring biological functions of gene of interest. Most studies have employed drug resistance genes, such as the neomycin resistance gene (*neo*), to eliminate unwanted, untransfected cells after transfection. In such cases, the drug resistance genes are integrated into host chromosomes upon transfection so that they synthesize proteins capable of degrading drugs present in medium. However, this method often causes unwanted byproducts that are occasionally toxic to cells if they are continuously cultivated in the drug-containing medium. Therefore, drug-free selection of stable transfectants is necessary. Previously, we have assessed this problem and have provided a way to obtain transfectants efficiently in the absence of drug selection. Our system is based on co-transfection with a vector carrying a gene of interest (GOI) and a vector (pCAG/EndoGalC) carrying a gene encoding *Clostridium perfringens*-derived endo-ß-galactosidase C (EndoGalC), which cleaves a specific cell-surface carbohydrate, called the α-Gal epitope, expressed in most *(continued on page 6)*

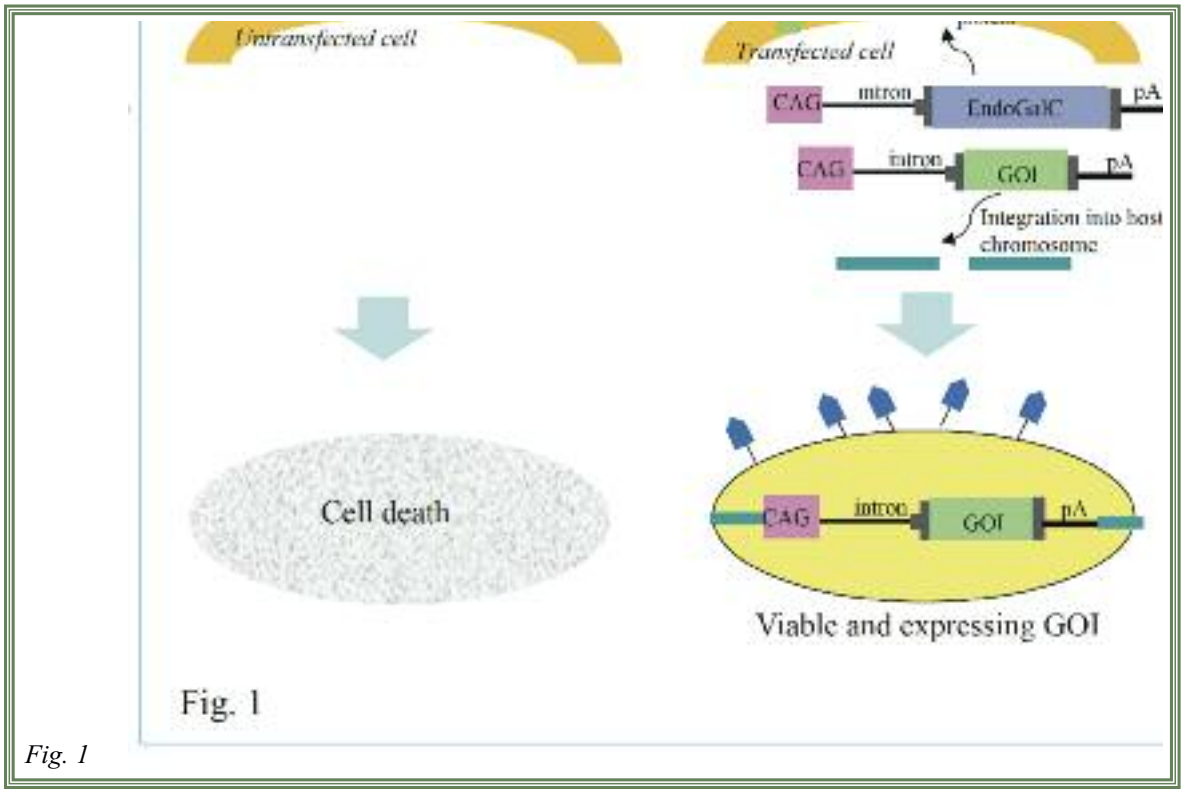

Fig. 1

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Denise Higgins, Editor

ADVANCED TARGETING SYSTEMS

Beta-Testing Program: Great Price / Great Opportunity

ATS is pleased to announce Beta-release of a wide array of targeted toxins for use in eliminating specific cell types. This Beta-Testing Program will make new conjugates available to our customers sooner.

Each of the Beta products has:

Saporin activity confirmed,
Peptide sequences published/confirmed, and/or
Antibody binding specificity published/confirmed.

Check out these Beta Products - Available Now!

Nociceptin-SAP

Eliminates nociceptin-receptor expressing cells.

This targeted toxin recognizes cells that express the nociceptin receptor. Nociceptin-SAP is a bonded toxin between nociceptin and the secondary conjugate Streptavidin-ZAP (IT-27) containing the ribosome-inactivating protein, saporin. Nociceptin (Orphanin FQ) is a 17-amino acid peptide widely distributed within the central and peripheral nervous system functioning as an endogenous agonist of the Nociceptin receptor (NOP) formerly known as the opioid receptor-like 1 receptor (ORL1). Nociceptin has been confirmed to play a role in a variety of physiological functions involving not only the CNS and PNS but non-neuronal systems as well. These functions include pain, gastrointestinal motility, locomotion, learning and memory, neurotransmitter and hormone release, renal function, neuronal differentiation, sexual and reproductive behavior and anxiety.

Octreotide-SAP

Eliminates cells that express somatostatin receptors.

Octreotide-SAP is a bonded toxin between octreotide peptide and the ribosome-inactivating protein, saporin. Octreotide is a somatostatin analog and binds to somatostatin receptors on cell surfaces, predominantly somatostatin receptor subtypes 2 & 5. It is an octapeptide that mimics natural somatostatin pharmacologically, though it is a more potent inhibitor of growth hormone, glucagon, and insulin secretion than the natural hormone and has a much longer half-life. Octreotide affects neurotransmission and cell proliferation via interaction with G protein-coupled somatostatin receptors and inhibition of the release of numerous secondary hormones. It is indicated for symptomatic treatment of carcinoid syndrome and acromegaly. It is also finding increased use in treatment of polycystic diseases of the liver and kidney. Octreotide-SAP eliminates cells that express somatostatin receptors.

Azido-ZAP

Combines with alkyne-containing molecule in click chemistry reaction to eliminate molecules containing a free alkyne group.

Click chemistry can be used when methods such as direct labeling or the use of antibodies are not applicable nor efficient. The click chemistry label is small enough that tagged molecules (e.g., nucleotides, sugars, and amino acids) are acceptable substrates for the enzymes that assemble these building blocks into biopolymers. The small size of click detection molecules allows them to easily penetrate complex samples, including intact, supercoiled DNA, with only mild permeabilization required.

Beta Products have not been characterized or reported in scientific literature. This provides researchers with special Beta-pricing and the opportunity to be the first to publish using the material. The researcher who first publishes data will receive a $500 credit for use on ATS products.

Targeting Teaser

Last quarter's puzzle was posted incorrectly online. We are sorry for any inconvenience. It has been corrected and can be solved online. Win a jigsaw puzzle!

Solve the Teaser at www.ATSbio.com/news/15q2_teaser.html

Targeting Topics: Recent Scientific References

Reviewed by Matthew Kohls

Cortically projecting basal forebrain parvalbumin neurons regulate cortical gamma band oscillations.

Kim T, Thankachan S, McKenna JT, McNally JM, Yang C, Choi JH, Chen L, Kocsis B, Deisseroth K, Strecker RE, Basheer R, Brown RE, McCarley RW.

Proc Natl Acad Sci U S A Epub2015.

Measurements of cortical EEG capture gamma band oscillations (GBO). Abnormalities in these GBO have been found in some neuropsychiatric disorders such as Alzheimer's disease and schizophrenia. The authors analyzed GBO neuronal groups by administering 650-ng bilateral icv injections of mu p75-SAP (Cat. #IT-16) to mice to determine the role of basal forebrain cholinergic neurons in the generation of GBO. The results indicate GABAergic basal forebrain neurons containing parvalbumin were important for GBO integrity, but cholinergic neurons in the basal forebrain were not involved.

alphaCGRP is essential for algesic exocytotic mobilization of TRPV1 channels in peptidergic nociceptors.

Devesa I, Ferrandiz-Huertas C, Mathivanan S, Wolf C, Lujan R, Changeux JP, Ferrer-Montiel A.

Proc Natl Acad Sci U S A 111(51):18345-18350, 2014.

The sensitization of transient receptor potential vanilloid 1 (TRPV1) can lead to the development and maintenance of chronic pathological pain conditions. In this work the authors determined that TRPV1 receptors use membrane insertion mechanisms in order to potentiate neuronal excitability. In order to specifically link this activity to peptidergic neurons the authors treated rat primary dorsal root ganglion cultures with 10 mM rIB4-SAP (Cat. #IT-10) to deplete the non-peptidergic neurons.

Monoclonal Antibodies Targeting LecLex-Related Glycans with Potent Anti-Tumor Activity.

Jia CX, Vankemmelbeke M, McIntosh RS, Clarke PA, Moss R, Parsons T, Spendlove I, Zaitoun AM, Madhusudan S, Durrant LG.

Clin Cancer Res 2015.

In this work the authors characterized two monoclonal antibodies that target glycans containing Lewis carbohydrate antigens. One of the methods used was to combine varying concentrations of the antibodies with 50 ng mouse Fab-ZAP (Cat. #IT-48) and apply the conjugates to cells for 72 hours. The antibodies were demonstrated to have efficient internalization, supported by potent *in vivo* anti-tumor activity.

Light-controlled endosomal escape of the novel CD133-targeting immunotoxin AC133-saporin by photochemical internalization - A minimally invasive cancer stem cell-targeting strategy.

Bostad M, Olsen CE, Peng Q, Berg K, Hogset A, Selbo PK.

J Control Release 206(28):37–48, 2015.

Previously the authors demonstrated the use of photochemical internalization of a custom conjugate consisting of a CD133 antibody coupled to saporin (ATS Custom conjugation). Several cancer cell lines were plated, and incubated in the presence of a photosensitizer with either CD133-SAP at 8.6 pM or Saporin (Cat. #PR-01) at 24 pM. The different concentrations equalized the number of saporin molecules in each sample. A light source was used to initiate the internalization of the molecules. The results indicate that this is a viable strategy for the targeted treatment of cancer stem cells.

High-content analysis of antibody phage-display library selection outputs identifies tumor selective macropinocytosis-dependent rapidly internalizing antibodies.

Ha KD, Bidlingmaier SM, Zhang Y, Su Y, Liu B.

Mol Cell Proteomics 13(12):3320-3331, 2014.

Macropinocytosis, the internalization of large endocytic vesicles called macropinosomes, is upregulated in Ras-transformed cancers. To date, large-scale antibody generation strategies have not incorporated a selection method for antibodies. In this work the authors demonstrate screening and validation of the antibodies that utilize the macropinosome pathway. One method used was to biotinylate the antibodies and combine them with Streptavidin-ZAP (Cat. #IT-27) at a 1:1 ratio. The conjugate was applied to cells in a concentration curve starting at 200 nM in order to demonstrate internalization and cell killing.

T-box transcription regulator Tbr2 is essential for the formation and maintenance of Opn4/melanopsin-expressing intrinsically photosensitive retinal ganglion cells.

Mao CA, Li H, Zhang Z, Kiyama T, Panda S, Hattar S, Ribelayga CP, Mills SL, Wang SW.

J Neurosci 34(39):13083-13095, 2014.

Opsin 4/melanopsin-expressing intrinsically photosensitive retinal ganglion cells (ipRGCs) are responsible for controlling non-image-forming visual functions in the retina. The findings show that opsin 4 is only expressed in Tbr2-positive ipRGCs, no ipRGCs are found if Tbr2 is deleted before RGC specialization, and most ipRGCs are eliminated when Tbr2 is deleted from established ipRGCs. An antibody against melanopsin (Cat. #AB-N39) was used at a 1:1000 dilution for immunohistochemical analyses.

TrkA in vivo function is negatively regulated by ubiquitination.

Kiris E, Wang T, Yanpallewar S, Dorsey SG, Becker J, Bavari S, Palko ME, Coppola V, Tessarollo L.

J Neurosci 34(11):4090-4098, 2014.

The high affinity nerve growth factor receptor, trkA, plays an intrinsic role in the regulation of various aspects of the mammalian nervous system. The post-translational attachment of ubiquitin to trkA plays a role in the final disposition and function of many proteins; in this work the authors investigate the result of trkA ubiquitination. By removing a 3 amino acid sequence from the receptor the ubiquitination of TrkA was reduced which resulted in an increase in TrkA protein levels and activity. In mice containing this mutation, the rise in TrkA activity was accompanied by enhanced thermal sensitivity and inflammatory pain. Anti-trkA (Cat. #AB-N03) was used at a concentration of 1:500 in immunohistochemistry.

(continued on page 4)

Targeting Topics: Recent Scientific References

(continued from page 3)

Characteristic patterns of dendritic remodeling in early-stage glaucoma: evidence from genetically identified retinal ganglion cell types.

El-Danaf RN, Huberman AD.

J Neurosci 35(6):2329-2343, 2015.

The loss of retinal ganglion cells (RGC) is the second-most common cause of blindness worldwide. Using several mouse transgenic cell lines, the authors investigated the changes that occur on the establishment of elevated ocular pressure. Anti-melanopsin (Cat. #AB-N39) at 1:1000 was used to illuminate the morphology of the M1 intrinsically photosensitive RGC.

Individual Differences in Acute Pain-induced Endogenous Analgesia Predict Time to Resolution of Postoperative Pain in the Rat.

Peters CM, Hayashida KI, Suto T, Houle TT, Aschenbrenner CA, Martin TJ, Eisenach JC.

Anesthesiology 2015.

The authors investigated the relationship between preoperative Conditioned Pain Modulation (CPM) and the time course of recovery from surgery. CPM was evaluated using forepaw capsaicin injections into rats. During the study, lesioned rats received 5-µg intrathecal injections of anti-DBH-SAP (Cat. #IT-03), followed 14 days later by a partial L5 spinal nerve ligation surgery. Mouse-IgG-SAP (Cat. #IT-18) was used as a control. CPM was partially blocked in the lesioned animals, suggesting descending noradrenergic signaling is important in the time course of recovery from surgery.

New mouse retinal stroke model reveals direction-selective circuit damage linked to permanent optokinetic response loss.

Joly S, Guzik-Kornacka A, Schwab ME, Pernet V.

Invest Ophthalmol Vis Sci 55(7):4476-4489, 2014.

The authors used a mouse model of 'retinal stroke' to better delineate the optokinetic response deficits at the cellular level. Damage was found in the processes of starburst amacrine cells (SACs), and to a lesser extent, the dendrites. Anti-melanopsin (Cat. #AB-N38) at 1:2500 was used for immunohistochemistry.

Neutral aminoaciduria in cystathionine beta-synthase-deficient mice; an animal model of homocystinuria.

Akahoshi N, Kamata S, Kubota M, Hishiki T, Nagahata Y, Matsuura T, Yamazaki C, Yoshida Y, Yamada H, Ishizaki Y, Suematsu M, Kasahara T, Ishii I.

Am J Physiol Renal Physiol 306(12):F1462-76, 2014.

The authors utilized a mouse model for homocystinuria in order to examine renal amino acid reabsorbtion. Some of the immunohistochemistry experiments used anti-Met (Cat. #AB-T036). It was found that loss of cystathionine β-synthase causes hyperexcretion of both glucogenic and ketogenic neutral amino acids, as well as histidine.

TRPV1 expression level in isolectin B4-positive neurons contributes to mouse strain difference in cutaneous thermal nociceptive sensitivity.

Ono K, Ye Y, Viet CT, Dang D, Schmidt BL.

J Neurophysiol jn.00973.2014, 2015.

In order to determine whether IB4-positive trigeminal sensory neurons affect pain sensitivity, the authors administered 2 µg of rIB4-SAP (Cat. #IT-10) to the right infraorbital foramen. Saporin (Cat. #PR-01) was used as a control.

Macrophages are needed in the progression of tuberculosis into lung cancer.

Li J, Pan Y, Zhang B, Chen Q.

Tumour Biol 2015.

Approximately 30% of lung carcinomas also have tuberculosis lesions. The authors investigated the potential link between inflammatory processes and cancer in the lung. Mice with established tuberculosis infections received weekly 20 µg tail vein injections of Mac-1-SAP (Cat. #IT-06) in order to eliminate macrophages. Six months later the mice receiving Mac-1-SAP had a significantly lower incidence of lung carcinoma than control animals.

Dual targeting NG2 and GD3A using Mab-Zap immunotoxin results in reduced glioma cell viability in vitro.

Higgins SC, Fillmore HL, Ashkan K, Butt AM, Pilkington GJ.

Anticancer Res 35(1):77-84, 2015.

Human glioma-derived cell lines were sequentially incubated with anti-NG2 and anti-GD3A coupled to Mab-ZAP (Cat. #IT-04) at 1 µg/ml and 5 µg/ml for 72 hours each. The combination therapy was significantly more effective than single therapy in eliminating the glioma cells.

Activation of the mouse primary visual cortex by medial prefrontal subregion stimulation is not mediated by cholinergic basalo-cortical projections.

Nguyen HN, Huppe-Gourgues F, Vaucher E.

Front Syst Neurosci 9:1, 2015.

Mice received 1 µg icv injections of mu p75-SAP (Cat. #IT-16) to eliminate NGFr-positive cells. The results indicate a link between the prelimbic and infralimbic cortices and the primary visual cortex.

Preliminary results from a phase I study of substance P-saporin in terminal cancer patients with intractable pain.

Frankel AE, Nymeyer H, Lappi DA, Higgins D, Ahn C, Noe C.

J Clin Oncol 32 (suppl 31):191, 2014.

Existing pain therapies are insufficient to control cancer pain in 10-15% of patients. Substance P (SP) and its receptor, neurokinin-1 (NK-1r) have been determined to play a major role in spinal transmission of chronic pain. Animal studies have demonstrated that disruption of the NK-1r pathway alleviates chronic pain caused by a variety of stimuli. The authors are conducting a Phase I clinical trial in humans (NCT02036281) assessing the ability of SP-SAP to treat intractable chronic pain due to cancer. Patients have received intrathecal injections of 1, 2, or 4 µg of SP-SAP with no evidence of toxicity or neurological or cardiac abnormalities. Doses will escalate up to 90 µg.

Targeting Talk: Product Q&A

Q: I ordered a targeted toxin. Will it come in powder form? How do I re-dissolve it?

A: Our Saporin conjugate products are all provided in sterile PBS solution within a concentration range of 0.5 - 3 mg/ml. Saporin is an extremely safe 'toxin' to handle in standard laboratory environments when in solution for several reasons. Solutions in general are easier to corral and keep contained than powders and consequently are less likely to accidentally end up on an individual's skin, tongue, or in one's eyes. As a lyophilized product, Saporin would also be present at an extremely high concentration such that there is cause for concern should it contact the body of the user in any way. Lastly, our Saporin conjugates have historically required dilution prior to use for both *in vitro* and *in vivo* procedures. As such, it is much easier to ensure the amount of material you, as a customer, are receiving and the subsequent dilution is accurately adjusted to your desired concentration when providing these products already in solution. If upon receiving a Saporin conjugate you believe the product to be lyophilized or in a powder form, please contact us immediately, prior to opening the vial.

Q: I'm interested in your anti-DBH-saporin toxin for lesioning central catecholaminergic neurons. I see from the product description that the antibody used is a mouse monoclonal -- designed to specifically target rat DBH. My interest is to produce targeted lesions in mouse transgenic. Will this product still work specifically? Thanks.

A: Unfortunately, we do not have really good data to support the use of our Anti-DBH-SAP (Cat. #IT-03) in mice. There is significant homology between mouse and rat DBH, however the actual antigen for both the mouse monoclonal we use in the immunotoxin and an alternate unpurified rabbit polyclonal, is native bovine DBH enzyme. For further background information there are two references where our product was used in mice. The reference summaries from previous issues of *Targeting Trends* are listed below.

An early sympathetic nervous system influence exacerbates collagen-induced arthritis via CD4+ / CD25+ cells.[1] The sympathetic nervous system can play conflicting roles in collagen-induced arthritis (CIA). CD4+CD25+ T cells can play an immunoregulatory effect in this system depending on the expression of the FoxP3 transcription factor. Mice received 5-µg intraperitoneal injections of anti-DBH-SAP to induce an early sympathectomy. The results indicate that the sympathetic nervous system increases disease severity in CIA by stimulating some of the proinflammatory aspects of CD4+CD25+ T cells.

An opposing time-dependent immune-modulating effect of the sympathetic nervous system conferred by altering the cytokine profile in the local lymph nodes and spleen of mice with type II collagen-induced arthritis.[2] In this work the authors examined the role of the sympathetic nervous system (SNS) in late stages of chronic arthritis. 5 µg intraperitoneal injections of anti-DBH-SAP in mice were used to confirm that previous 6-OHDA injections caused a sympathectomy. The results demonstrate that the SNS supports inflammation during the asymptomatic phase of arthritis, but inhibits inflammation during the chronic symptomatic phase.

1. Harle P, Pongratz G, Albrecht J, Tarner IH, Straub RH *Arthritis Rheum* 58(8):2347-2355, 2008.
2. Harle P, Mobius D, Carr DJ, Scholmerich J, Straub RH *Arthritis Rheum* 52(4):1305-1313, 2005.

Q: Recently we used your flow cytometry services (Cytometry Research, ATS subsidiary). Based on post flow data analysis needs, I am providing the assay group list below (withheld for confidentiality).

A Yes, all these antibodies meet the requirement of being excited by our 488nm laser, however the specific combinations you list use fluorescent probes that have emission wavelengths that are too similar and would actually be detected on the same fluorescent channel. In essence, you would be unable to differentiate which target was being detected.

Here are suggestions for common combinations of commercially available fluorescent probes if you are interested in analyzing three colors simultaneously. All these probes can be excited at 488nm and will work on our equipment, so just make sure you don't have more than one probe from each channel.

FL1 Channel	FL2 Channel	FL-3 Channel
Alexa Fluor 488	Phycoerythrin (PE)	PE/Cy5
DyLight 488	Cy3	PE/Cy5.5
FITC		PerCP
GFP		PerCP/Cy5.5
		PE/Cy7

Drug-free selection of stable transfectants using rIB4-SAP

(continued from page 1)

mammalian cells, except in humans and Old World monkeys.

Transient expression of EndoGalC should lead to resistance in some transfected cells to isolectin BS-I-B_4 conjugated to saporin (rIB4-SAP; #IT-10; Advanced Targeting Systems Inc.), which causes cell death of α-Gal epitope-expressing untransfected cells, mainly because rIB4-SAP, internalized via specific binding to the cell-surface α-Gal epitope, inhibits protein synthesis.[1,2] During the period (~3 days after transfection) of transient expression of exogenous DNA (pCAG/EndoGalC), expression of α-Gal epitope on the cell surface is lost, allowing the cell to survive rIB4-SAP treatment (as schematically depicted in Fig. 1). Concomitantly, the co-introduced plasmid carrying the GOI has a chance to be integrated into host chromosomes. Untransfected cells continue to express the α-Gal epitope, which is specifically recognized by rIB4-SAP, and are eliminated after about 10 days of cultivation (Fig. 1). Thus, the surviving cell population is expected to express the GOI and the α-Gal epitope, since pCAG/EndoGalC introduced into a cell is lost during the 10-day cultivation after rIB4-SAP treatment. This concept has been previously explored by us,[3] in which we demonstrated that the GOI could be effectively integrated into host chromosomes via the *piggyBac* system after rIB4-SAP treatment.

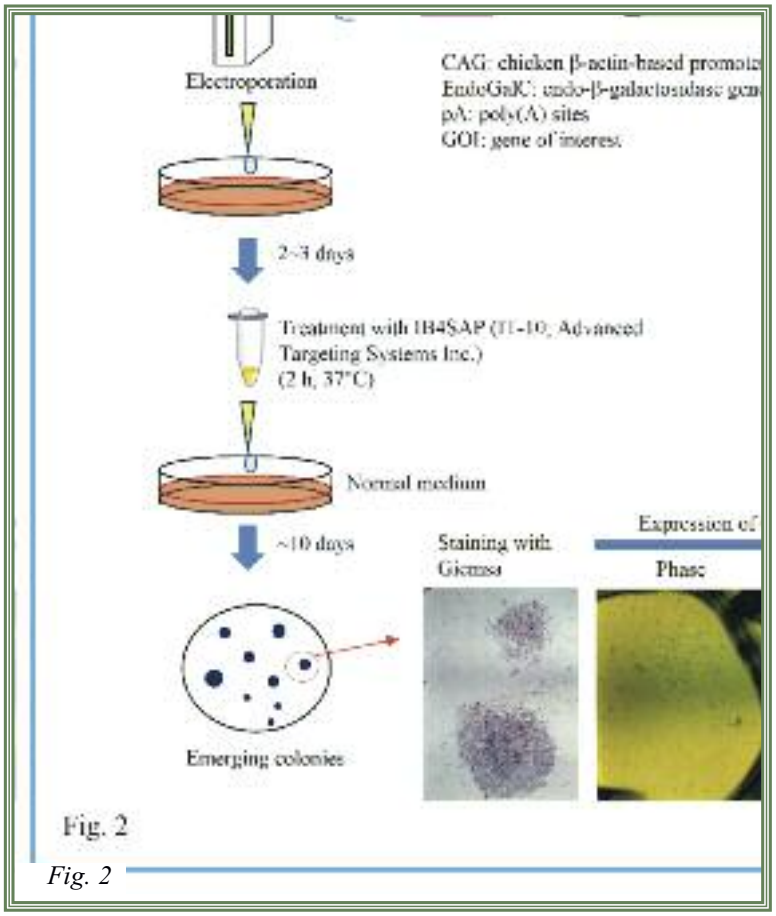

Fig. 2

Fig. 2

This drug-free acquisition of stably transfected cells is a very simple and convenient system. We prepared only two vectors, an EndoGalC-expression vector (in circular form) and a vector (in linearized form) carrying a GOI (Fig. 2). These vectors were mixed with trypsinized porcine fetal fibroblasts and were then delivered to the cells via electroporation. Immediately after electroporation, all transfected cells were seeded onto a dish containing normal medium and cultured for 2–3 days. The cells were then trypsinized and treated with 80 μg/mL rIB4-SAP in a 0.5-mL microfuge tube for 2 h at 37°C. Then, these cells were seeded in normal medium and cultured for ~10 days until colonies developed. The colonies were stained with Giemsa and were seen to express the GOI (tdTomato), as shown in the images at the bottom of Fig. 2. This system does not require selective drugs such as G418. Therefore, it does not require a pilot study to test the effectiveness of the drugs using untransfected cells. Furthermore, it will be useful for gene delivery to cells that are resistant to several selective drugs.

References

1. Akasaka E, Watanabe S, Himaki T, Ohtsuka M, Yoshida M, Miyoshi K, Sato M. (2010) Enrichment of xenograft-competent genetically modified pig cells using a targeted toxin, isolectin BS-I-B4 conjugate. *Xenotransplantation* 17(1):81-89.
2. Sato M, Akasaka E, Saitoh I, Ohtsuka M, Nakamura S, Sakurai T, Watanabe S. (2013) Targeted toxin-based selectable drug-free enrichment of Mammalian cells with high transgene expression. *Biology (Basel)* 2(1):341-355.
3. Sato M, Inada E, Saitoh I, Matsumoto Y, Ohtsuka M, Miura H, Nakamura S, Sakurai T, Watanabe S. (2015) A combination of targeted toxin technology and the piggyBac-mediated gene transfer system enables efficient isolation of stable transfectants in nonhuman mammalian cells. *Biotechnol J* 10(1):143-153.

Amer Assoc Immunologists
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Chicago, IL
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Upcoming Events

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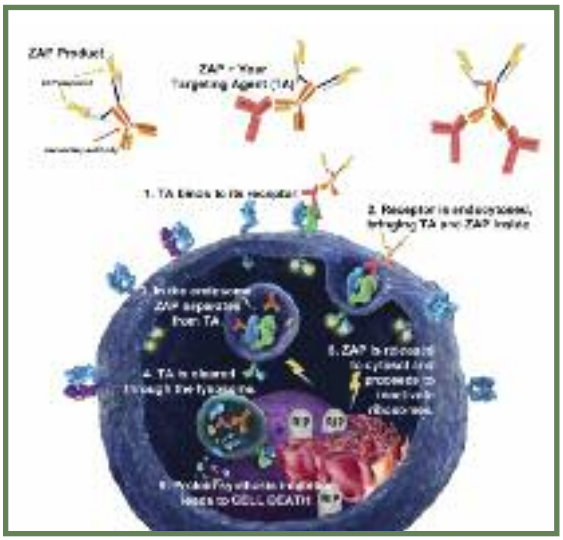
	Includes Biotin-Z Internalization Kit
	Zero Hands-On time for you

*Dependent upon peptide, but typically available for most proteins such as antibodies



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- Average chemical ratio of moles of biotin to moles of protein
- Average effective molecular weight

Kermit and friend after a busy morning of fun and games.

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Cells that do not have the receptor will not be affected.

The conjugate is internalized and SAPORIN breaks away from the antibody.

SAPORIN inactivates the ribosomes.

The result is **CELL DEATH**.

Targeting Teaser

Unscramble these five Jumbles **taken from last quarter's cover story**, one letter to each block, to solve the puzzle.

INSTOPPERY

EXCLOMP

STARVERE

COLOTOMONI

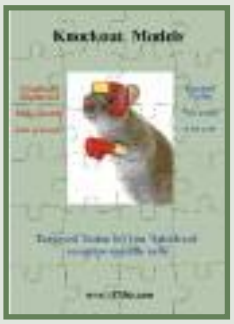
REIGNCOLICH

How using saporin conjugates affects the scientist's New Year.

Arrange the circled letters to form the answer, as suggested by the above clue.

ANSWER:
IT KEEPS RESEARCH RIGHT ON . . . ▢▢▢▢▢▢ !

WIN!



SOLVE the puzzle online with the correct solution by April 30, 2015.

WIN a ATS jigsaw puzzle!

www.atsbio.com/news/15q2_teaser.html