We thank both reviewers for their thoughtful and constructive criticism. We believe that, after making the reviewers’ suggested changes, we are re-submitting an improved manuscript.

**Editorial comments:**  
  
\* Protocol text:  
Please see the highlighted portion of your protocol. We would like to clarify the parts of the protocol you would like to include in the video. The highlighted parts should include complete steps and important transitions between actions.   
\*For example, the very first highlighted text reads: "remove spent media from flask with sterile pipette." What media is being removed? What else is in that flask?   
Also, the highlighted portion can only be 3 pages or less. Remember that the non-highlighted protocol steps will remain in the manuscript and therefore will still be available to the reader.

In an effort to clarify the parts of the protocol that we would like to include in the video, the highlighted sections have been revised.

Although it has technically not been yellow-highlighted to indicate that we would like it to be part of the video section, we suggest that the video show the colored 96-well plate setup scheme.

Additionally, to avoid possible confusion regarding the word “spent,” where the phrase “spent media” once appeared in the manuscript, it has been replaced to “all media,” or “media” (the term “spent” has been deleted altogether).

**Reviewer #1:**  
*Manuscript Summary:*   
The authors of this method paper provide a detailed and thorough protocol for a high throughput assay for the measurement of RBL-2H3 mast cell degranulation in the context of assessing chemical inhibition by Triclosan. Their representative results demonstrate that Triclosan dissolved in aqueous buffer is capable of inhibiting antigen-mediated degranulation in a dose dependent manner.

Overall the writing quality was good and there is certainly enough detail to carry out the experiment. The introductory material provides sufficient detail to properly understand the system and scientifically relevant considerations for the experimental design.

Thank you.   
  
*Major Concerns:*  
I have no major concerns over the material covered in this methods paper. The authors provide sufficient information for successful execution of the protocol.  
  
*Minor Concerns:*  
(1) The authors show data indicating that calcium ionophore-triggered degranulation is also inhibited. However, the degranulation values displayed in figure 3 are somewhat concerning. The spontaneous degranulation is quite high (50% of the degranulation in those cells stimulated with ionophore but without Triclosan). This is likely, in part, a result of the fact that the absolute value of degranulation of cells treated with ionophore alone is only 6.9%. So while they see a significant reduction of ionophore-triggered degranulation in the presence of Triclosan, the actual reduction is perhaps not very biologically relevant, because the dynamic range of their data is only 3-4%. The doses used for both antigen and ionophore are quite low, which translate to degranulation percentages are also quite low, so their results are not unreasonable, it is just more difficult to make conclusions based on such small relative differences. That being said, the authors correctly point out in their discussion that balancing antigen and toxicant dose is an important factor.

We thank the reviewer for a close reading of the text and its figures, and for emphasizing that a greater dynamic range of the data would be desirable. In the manuscript we submitted, a concentration of 160 nM A23187 calcium Ionophore was used to elicit a degranulation response from RBL-2H3 cells. Thus, we have addressed the reviewer’s comments by repeating the ionophore experiment with a higher concentration of ionophore, 180 nM. This level of ionophore elicited an absolute value of degranulation of ~25% (much higher than the 6.9% in the first manuscript draft), whereas the absolute level of the “spontaneous” degranulation in this same experiment was approximately 4%. Please note that the overall data trends and the statistical significance are very similar to those reported in the first draft of the manuscript.

We are therefore submitting a revised Figure 3 that reflects these changes. The representative results and legend for Figure 3 have been changed to represent these revisions (changes are highlighted in green):

change in the “Results” section:

“RBL mast cells were incubated for 1 hour in “TCS-buffer” or “control buffer,” containing a calcium ionophore dose of 180 nM. In the absence of TCS, this concentration of A23187 elicited an average absolute degranulation response of 25.1% ± 4.7% (mean ± standard deviation). Inhibition of degranulation was found with as little as 1 µM TCS (0.63 ± 0.11 [mean ± SD]). As TCS concentration increases, so does the severity of the inhibition: at 5 µM, 0.21-fold ± 0.04-fold of the 0 µM TCS control levels; at 10 µM, 0.09 ± 0.05; at 15 µM, 0.077 ± 0.006; and at 20 µM, 0.09 ± 0.02 (means ± SD). In fact, from 5 µM and higher concentrations of TCS, levels of A23187-induced degranulation were found to be near the level of spontaneous control (where no A23187 is present at all).”

change in the figure legend:

“**Figure 3**: A representative degranulation response of RBL mast cells stimulated with 180 nM A23187 calcium Ionophore in the presence of TCS (0-20 μM).”

(2) I found some sections of the protocol a little hard to follow. For example, while I understood the concept behind the 96 well plate scheme, it was a difficult easily determine what was what on the layout provided. A table of what samples to include might be clearer than the list of given in step 1.1. 

For the sake of clarity, we have added tables in addition to the 96-well plate layouts. The following tables were added to section 1.1 and section 5.1, respectively, of the manuscript.

Antigen Table

|  |  |
| --- | --- |
| **Treatment** | **Triplicates** |
| Stimulated, 0 µM TCS | A7, B7, C7, F4, G4, H4 |
| Stimulated, 0.001 µM TCS | F6, G6, H6 |
| Stimulated, 0.1 µM TCS | A4, B4, C4 |
| Stimulated, 1 µM TCS | A6, B6, C6 |
| Stimulated, 5 µM TCS | F5, G5, H5 |
| Stimulated, 10 µM TCS | A3, B3, C3 |
| Stimulated, 15 µM TCS | A5, B5, C5 |
| Stimulated, 20 µM TCS | F7, G7, H7 |
| Stimulated, plus highest [TCS] | F3, G3, H3 |
| Spontaneous, no TCS  (includes mocks) | A10, A11, A12, B10, B11, B12  A1, A2, A8, B1, B2, B8, C1, C2, C8, F1, F2, F8, G1, G2, G8, H1, H2, H8 |
| TX-100, no TCS | D10, D11, D12, E10, E11, E12 |
| No cells background, plus highest [TCS] | G10, G11, G12, H10, H11, H12 |

Ionophore Table

|  |  |
| --- | --- |
| **Treatment** | **Triplicates** |
| Stimulated, 0 µM TCS | A7, B7, C7, F4, G4, H4 |
| Stimulated, 0.001 µM TCS | F6, G6, H6 |
| Stimulated, 0.01 µM TCS | F3, G3, H3 |
| Stimulated, 0.1 µM TCS | A4, B4, C4 |
| Stimulated, 1 µM TCS | A6, B6, C6 |
| Stimulated, 5 µM [TCS | F5, G5, H5 |
| Stimulated, 10 µM TCS | A3, B3, C3 |
| Stimulated, 15 µM TCS | A5, B5, C5 |
| Stimulated, 20 µM TCS | F7, G7, H7 |
| Spontaneous, with DMSO, no TCS  (includes mocks) | A10, A11, A12, B10, B11, B12  A1, A2, A8, B1, B2, B8, C1, C2, C8, F1, F2, F8, G1, G2, G8, H1, H2, H8 |
| TX-100, with DMSO, no TCS | D10, D11, D12, E10, E11, E12 |
| No cells background, with DMSO,  plus highest [TCS] | G10, G11, G12, H10, H11, H12 |

(4) The addition of a trouble shooting section (either in the discussion or in an accompanying table), should readers have experimental difficulties, would be a useful.

Although it was not specifically labeled as a troubleshooting section in the text, we had detailed possible confounding effects in paragraphs 5 and 6 of the Discussion section. To make it clearer that we are addressing troubleshooting in these paragraphs, we have added the following paragraph to the “Discussion” section, as well as a table:

In the event that troubleshooting is required, several potential aspects of this protocol should be reviewed. For example, it may be that (1) spontaneous release levels are too high (greater than ~7% of lysis values); (2) a dose-response with either stimulant and/or test chemical is not observed; or (3) the TCS concentration in solution is too low (lower than 20 μM). In the first case, a high spontaneous level could be an indication of the cells’ being in culture too long or being contaminated with mycoplasma; therefore, try these experiments with RBL-2H3 cells that have been in culture between 2-20 weeks, and regularly test for mycoplasma. If a stimulant dose response is not observed, the dissolved stimulant concentration may be too low, and stocks should be re-made. As an example, calcium ionophore typically is provided as a thin film, to be reconstituted with DMSO, requiring careful attention and much vortexing. Additionally, a new ionophore stock with a different lot number could have a different potency simply due to lot-to-lot variation; therefore, a degranulation dose response is recommended with each newly purchased ionophore stock. It is also worth noting that an apparent lack of effect with a given test chemical could be an indication that this chemical may require a longer incubation period in order to cause an effect. If you are not achieving a high TCS yield in solution, check that the temperature has remained constant (50°C ± 5°C) while the granules are dissolving into buffer. The thermometer should never touch the bottom of the flask, a position that would result in an overestimate of the temperature of the solution. Also, make sure there is constant vigorous stirring and that the 90 minute countdown is not started until the temperature has first reached 50°C.

**Table for Troubleshooting.**

|  |  |  |
| --- | --- | --- |
| **Problem** | **Potential Reason** | **Solution** |
| TCS stock is determined to be < 20 μM | Non-uniform heating of the solution | Ensure that the thermometer is positioned so that it is suspended in the solution and is not touching the bottom of the flask. |
|  | Stirring is not vigorous enough | Increase magnetic stirring on stir-plate to achieve a level of stirring that is vigorous without causing the solution to jump out of the flask. Ensure that an appropriately sized magnetic stir-bar is used. |
|  | Problems with spectrophotometer | Allow for proper warm up of UV lamp (typically 10 minutes), or replace bulb if necessary. |
| Spontaneous degranulation levels are too high (>~7%) | Cells have acquired abnormal genetic mutations due to too much time in culture | Perform experiments with a new cell thaw. |
|  | Cells are dying because of mechanical shearing | When adding buffer or treatment to plates that have adherent cells, be careful not to force cells to detach, by adding these volumes carefully to the sides of the microwells. Practice using the combitip. |
| IgE/DNP-BSA does not cause release of beta-hexosaminidase over spontaneous release levels | IgE is older than 30 days or has been subjected to freeze/ thaw | Use a new, properly stored aliquot of IgE. |
|  | DNP-BSA has not been properly mixed | Be sure to carefully add the small volume of DNP-BSA to the conical tube and to vortex thoroughly. |
| A23187 ionophore does not cause release of beta-hexosaminidase over spontaneous release levels | A23187 stock has not been properly reconstituted | Product arrives as a "thin film," and must be reconstituted with care and much vortexing. Transfer reconstituted stock to a new 1.5-mL tube for storage. |
|  | A23187 stock has not been properly stored | Stocks are light sensitive. Once reconstituted, parafilm the top, and store wrapped in foil at -20°C. If there is a question about the storage of a stock, discard and begin tests with a new stock. |
| 180 nM of A23187 ionophore does not elicit the same level of relative degranulation response | Lot-to-lot variation of A23187 ionophore | Perform a dose response experiment for each new lot of ionophore. It is also recommended that stocks from the same lot be tested, due to potential variability in the reconstitution process. |

(5) The third paragraph on pg. 4 the following phrase is used: "also very much recent attention being paid", this phrasing is awkward, and I would suggest rewording.

The sentence was reworded: “Beyond its potential as a therapeutic aid in allergic skin disease (or, conversely, as an immunotoxicant), TCS may also be an endocrine disruptor.”

*Additional Comments to Authors:*  
Overall I found this manuscript well thought out and executed.

Thank you.

**Reviewer #2:**  
*Manuscript Summary:*   
Weatherly et al. reported to use a microplate assay as a high-throughput to assess the effect of Triclosan on RBL-2H3 mast cell degranulation. In addition, the authors reported a method to resolve triclosan into aqueous, which is different than using the traditional organic solvent. This article provided a detailed description of a method for detecting mast cell degranulation. The rationale and background for the method is adequately explained.

*Major Concerns:*  
Traditional method (beta-hexosaminidase) for examining mast cell degranulation should be used as positive control for evaluating the microplate assay. 

In this manuscript we describe using β-hexosaminidase as a measure for mast cell degranulation. We believe the reviewer is referring to β-hexosaminidase assays that, historically, were performed with samples measured one-at-a-time in a fluorimeter, rather than, as we describe here, using a 96-well plate, a microplate reader, and batteries of toxicant tests. As stated in the manuscript, our methods are an extension of Naal et al. (2004), who previously rigorously demonstrated concordance between the “old” β-hexosaminidase assay and this assay[1](#_ENREF_1). Additionally, prior to new toxicant-test assays, we always perform antigen and/or ionophore-dose responses (in the absence of test chemical) to ensure that the cells are responding normally, compared with the previous literature. To make this clearer in the text, we have added lines to the first paragraph of our “Discussion” section:

Prior to the Naal *et al.*[1](#_ENREF_1) assay, mast cell degranulation had been routinely assessed via β-hexosaminidase[2-4](#_ENREF_2" \o "Soto, 1988 #1670), but these early methods utilized fluorimeters in which one sample was read at a time. Importantly, Naal *et al*. established direct concordance between this more high-throughput method utilizing a microplate reader and the earlier method in which samples were read one-at-a-time in a fluorimeter. In sum, Naal *et al.*[1](#_ENREF_1) greatly improved the speed, power, simplicity, and reliability of the assay by adapting it to a high-throughput microplate platform, as well as by incorporating several changes to the workflow. Here, we have further adapted this assay for a study of various test chemicals, in particular, here, the ubiquitous drug TCS.

*Minor Concerns:*  
Other factors, such as Stem cell factor should be used as a positive control to evaluate the impact of Triclosan on mast cell degranulation.

The reviewer has made an interesting point–that there are multiple ways to stimulate degranulation of RBL-2H3 mast cells. There exist several methods of RBL-2H3 stimulation in addition to the corsslinking of IgE-bound receptors (multivalent antigen) and ionophore induced Ca2+ influx. One of these methods is stimulation via exposure to compound 48/80 along with quercetin[5](#_ENREF_5" \o "Senyshyn, 1998 #1843). Another, as suggested by this reviewer, is stimulation via stem cell factor. Yet another is crosslinking IgE-bound receptors with an anti-IgE IgG antibody. In fact, we have previously found that Triclosan’s (TCS’s) inhibition of IgE-mediated degranulation is not dependent upon the type of crosslinker used (whether antigen or anti-IgE IgG antibody)[6](#_ENREF_6), and this paper is cited in our *JoVE* manuscript. In light of this reviewer’s insightful comment, we have added the following statement to our “Discussion” section:

In addition to the multivalent antigen DNP-BSA and the calcium ionophore A23187, there exist numerous other methods of RBL-2H3 stimulation. One of these methods is stimulation via exposure to compound 48/80 along with quercetin5. Another is crosslinking of IgE-bound receptors with an anti-IgE IgG, as we previously tested along with TCS exposure6. Many other stimulation methods exist, and each of these methods addresses a different mechanistic aspect of mast cell degranulation. This plate reader assay can be adapted for use with many of these alternative stimulators, further expanding its utility.

Other edits:

-Due to her assistance with producing a revised Figure 3 and her anticipated role in the filming aspects of this manuscript, we are including Juyoung Shim as an additional author of the manuscript.

-At this time, we also have a publication number assigned, and this number now appears in the “Acknowledgements” section: “This is Maine Agricultural & Forest Experiment Station publication number 3311.”

1 Naal, R., Tabb, J., Holowka, D. & Baird, B. In situ measurement of degranulation as a biosensor based on RBL-2H3 mast cells. *Biosensors & Bioelectronics* **20**, 791-796, doi:10.1016/j.bios.2004.03.017 (2004).

2 Soto, E. O. & Pecht, I. A monoclonal-antibody that inhibits secretion from rat basophilic leukemia-cells and binds to a novel membrane component. *Journal of Immunology* **141**, 4324-4332 (1988).

3 Pierini, L., Harris, N. T., Holowka, D. & Baird, B. Evidence supporting a role for microfilaments in regulating the coupling between poorly dissociable IgE-Fc epsilon RI aggregates and downstream signaling pathways. *Biochemistry* **36**, 7447-7456, doi:10.1021/bi9629642 (1997).

4 Aketani, S., Teshima, R., Umezawa, Y. & Sawada, J. Correlation between cytosolic calcium concentration and degranulation in RBL-2H3 cells in the presence of various concentrations of antigen-specific IgEs. *Immunology Letters* **75**, 185-189, doi:10.1016/s0165-2478(00)00311-4 (2001).

5 Senyshyn, J., Baumgartner, R. A. & Beaven, M. A. Quercetin sensitizes RBL-2H3 cells to polybasic mast cell secretagogues through increased expression of Gi GTP-binding proteins linked to a phospholipase C signaling pathway. *Journal of Immunology* **160**, 5136-5144 (1998).

6 Palmer, R. K. *et al.* Antibacterial agent triclosan suppresses RBL-2H3 mast cell function. *Toxicology and Applied Pharmacology* **258**, 99-108, doi:10.1016/j.taap.2011.10.012 (2012).