NCL Method ITA-38

Analysis of Nanoparticle Effects on IgE-Dependent Mast Cell Degranulation

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This protocol assumes an intermediate level of scientific competency with regard to techniques, instrumentation, and safety procedures. Rudimentary assay details have been omitted for the sake of brevity.

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1. Introduction

Mast cells are myeloid-derived immune cells normally present in connective tissues, especially under the skin, around nerves, in lungs and intestines, and near blood and lymph vessels. In health, mast cells play an important role in the host's protection from certain bacteria (e.g., *Staphylococcus aureus*, *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, *Listeria monocytogenes*, and *Mycoplasma pneumoniae*) and parasites [1]. Through Fcε receptors expressed on the cell surface, mast cells bind antigen-specific IgE and can respond to antigens through a process known as degranulation. During degranulation, activated mast cells release secondary messengers like histamine, heparin, cytokines, growth factors, tryptase, and β-hexosaminidase which activate the immune system to eliminate the antigen-bearing pathogen [2]. When released in excessive amounts and/or when the negative regulation of mast cells is altered, the same secondary messengers underline pathological reactions such as those experienced by individuals with allergies and asthma [3]. Mast cells can have a pro-tumorigenic or anti-tumorigenic role in cancer, depending on the type of cancer, the degree of tumor progression, and the location of the mast cells [4].

Engineered nanomaterials present in the environment have been shown to exaggerate allergies and asthma [5]. Moreover, IgEs specific to nanotechnology's most popular component—polyethylene glycol (PEG)—have been reported in people at a frequency exceeding that theoretically expected and in association with a true allergy to drug products, including but not limited to nanotechnology formulations [6, 7].

Certain disorders, such as mastocytosis—a condition characterized by an abnormally high number of mast cells—increase the risk of developing anaphylaxis [8, 9]. Abnormal activation of mast cells is associated with symptoms including flushing, itching, hives, abdominal pain, diarrhea, nausea, vomiting, anemia, bleeding disorders, bone and muscle pain, and mood changes [10]. Patients with mastocytosis are at greater risk of developing anaphylaxis from exposure to drug products, including but not limited to PEGylated lipid nanoparticles used for mRNA delivery [11, 12].

Collectively, the current knowledge around mast cells' role in health and pathophysiological processes and the potential contribution of nanomaterials to triggering or exaggerating mast cell-mediated pathologies warrant additional studies. This document

describes a protocol for in vitro assessment of IgE-mediated mast cell degranulation and the ability of nanomaterials to alter this normal process of mast cell biology.

2. Principle

This protocol is used to detect and quantify IgE-dependent release of β-hexosaminidase from the human mast cell line LAD2. First, these cells are sensitized with biotinylated IgEs. After sensitization, the cells are exposed to streptavidin. This exposure mimics allergen-mediated crosslinking of the IgE receptor (FcεR), causing the LAD2 cells to degranulate, thus releasing mediators such as β-hexosaminidase. Treatment with nanoparticles before IgE sensitization allows for determination of the nanoparticles' potential effects on IgE-dependent degranulation. The effect of nanoparticles on mast cell degranulation is important for understanding if and how these nanomaterials contribute to mast cell-mediated inflammation and hypersensitivity reactions.

3. Reagents, Materials, and Equipment

Note: The NCL does not endorse any of the suppliers listed below; these reagents were used in the development of the protocol, and their inclusion is for informational purposes only. Equivalent supplies from alternate vendors can be substituted. Please note that suppliers may undergo a name change due to a variety of factors. Brands and part numbers typically remain consistent but may also change over time.

3.1 Reagents for LAD2 Cell Line Maintenance

3.1.1 LAD2 cells (refer to Section 4 for additional information)

3.1.2 StemPro®-34 medium + supplied nutrient supplement (Gibco, 10640-019)

3.1.3 Penicillin/streptomycin (P/S) solution (GE Life Sciences, Hyclone, SV30010)

3.1.4 L-glutamine (GE Life Sciences, Hyclone, SH30034.01)

3.1.5 Recombinant human stem cell factor protein (rhSCF) (Gibco, PHC2111)

3.1.6 HyPure™ cell culture grade water (Cytiva, SH30529.02)

3.1.7 Phosphate buffered saline (PBS) (GE Life Sciences, SH30256.01)

3.1.8 0.4% Trypan blue (Gibco, 15250-061)

3.1.9 CyroScarless DMSO-free (BioVerde, CPL-A1)

3.2 Reagents for Degranulation Assay

3.2.1 Human IgE biotin monoclonal antibody (Abbiotech, 250206)

3.2.2 Streptavidin (SAV) (Invitrogen, 434301)

3.2.3 Tyrode's solution with 10 mM HEPES and 0.25% BSA (Boston BioProduct, Inc, BSS-370)

3.2.4 Citric acid ($C_6H_8O_7$) (Sigma-Aldrich, 251275)

3.2.5 Sodium phosphate dibasic heptahydrate ($Na_2HPO_4 \cdot 7H_2O$) (Sigma-Aldrich, 431478)

3.2.6 Glycine ($C_2H_5NO_2$) (Sigma-Aldrich, 50046)

3.2.7 1N Hydrochloric acid (HCl) and 1N sodium hydroxide (NaOH)

3.2.8 Sodium hydroxide pellets (Sigma-Aldrich, S5881)

3.2.9 p-Nitrophenyl-N-acetyl-B-D-glucosaminide (PNAG) (EMD Millipore, 487052)

3.2.10 Triton X-100 solution (Sigma-Aldrich, 93443)

3.2.11 HyPure™ cell culture grade water (Cytiva, SH30529.02)

3.2.12 4-nitrophenol (pNP) (Millipore-Sigma, N7660-100ML)

3.2.13 β-hexosaminidase B (β-Hex) (R&D Systems, 8907-GH-020)

3.3 Equipment and Materials

3.3.1 Sterile 100 mL bottle

3.3.2 Pipettes, 0.05–25 mL

3.3.3 Nalgene filtration product; 0.2 µm aPES (Thermo Fisher Scientific, 295-3345)

3.3.4 T25, T75 flasks

3.3.5 Polypropylene tubes, 15 and 50 mL

3.3.6 Microcentrifuge tubes

3.3.7 Hemocytometer slide and coverslip

3.3.8 Weigh paper

3.3.9 Scoop

3.3.10 Graduated cylinders (25–100 mL)

3.3.11 Beakers (100 mL)

3.3.12 Magnetic stir rods

3.3.13 96-well round-bottom plates

3.3.14 96-well flat-bottom plates

3.3.15 12- or 6-well flat-bottom plates

3.3.16 Reservoirs

3.3.17 Water purification system

3.3.18 Water bath

3.3.19 Sonication bath

3.3.20 Vortex

3.3.21 Centrifuge

3.3.22 Balance

3.3.23 Stir plate

3.3.24 pH meter

3.3.25 Refrigerator, 2-8˚C

3.3.26 Freezers, -20˚C and -80˚C

3.3.27 Liquid nitrogen freezer, -150˚C

3.3.28 Light microscope

3.3.29 Cell culture incubator with 5% CO_2 and 95% humidity

3.3.30 Cell culture incubator with 0% CO_2 and 95% humidity

3.3.31 Spectrophotometer capable of measuring OD at 405 nm and 620 nm (e.g., SpectraMax M5)

3.3.32 Biohazard safety cabinet approved for level II handling of biological material

4. LAD2 Cell Line Maintenance

The LAD2 cells used to develop this protocol were obtained from Dr. Metcalfe's lab at the NIAID, NIH, under a Materials Transfer Agreement restricting the transfer of these cells to other labs. However, this cell line is now commercially available through several sources, including but not limited to:

a. https://www.abmgood.com/Human-Mast-Cell-Line-LAD2-t8157.html

b. https://www.biocat.com/products/T8157-GVO-ABM

c. https://www.paludanlab.dk/shop/0171-t8157-human-mast-cell-line-lad2-size-1x106-cells-1-0-ml-8109#attribute_values=

4.1 Supplemented StemPro®-34 Media

100 mL StemPro®-34 Media

2.6 mL thawed nutrient supplement

1 mL of 200 mM L-glutamine

1 mL of 100X Pen/Strep

Filter solution through 0.2 µm aPES filter

Important: Prepare in a sterile bottle. The supplemented StemPro®-34 media prepared in this step lasts 30 days when stored at 2–8˚C in the dark. Make 100 mL at a time; 2.6 mL nutrient supplement should be added per 100 mL of media. Thaw nutrient supplement overnight at 4˚C. Once the nutrient supplement is thawed, prepare single-use aliquots to avoid repeated freeze/thaw cycles.

4.2 Recombinant Human Stem Cell Factor (rhSCF)

a. Centrifuge the vial briefly before opening.
b. Reconstitute the protein in 1 mL sterile deionized cell culture-grade water to a final concentration of 100 µg/mL.
c. Prepare single-use (5–10 and 20–25 µL) aliquots of the 100 µg/mL stock and store at -20˚C.

4.3 Complete StemPro®-34 Media

Add 1 µL 100 µg/mL rhSCF per 1 mL of supplemented StemPro®-34 Media from Step 4.1 to a final rhSCF concentration of 100 ng/mL.

Important: Complete media should be prepared fresh each time it is used in this assay; add rhSCF to the supplemented media immediately before using it with cells. Do not store.

Note: Some studies report an rhSCF concentration of 200 ng/mL [13]; however, NCL did not observe any issues with cell growth with an rhSCF concentration of 100 ng/mL.

4.4 Thawing of LAD2 Cells

Important: LAD2 cells should be frozen in CryoScarless DMSO-free preservation solution at $5x10^6$ cells in 1 mL.

a. Prepare 15 mL complete StemPro®-34 medium as described in Step 4.3.
b. Warm the complete StemPro®-34 medium in a 37˚C water bath.
c. Obtain a vial of LAD2 cells for thawing.
   Note: ~$5x10^6$ cells should be cultured in 10–15 mL in a T25 or T75 flask so that the cell concentration is approximately 0.3–$0.5x10^6$ cells/mL.
d. Immediately after thawing cells (in a 37˚C water bath), set aside ~50 µL LAD2 cell suspension to check viability.
   Note: Make sure the cells have been gently de-clumped or the counting procedure will be inaccurate.

4.5 Counting the LAD2 Cells

a. Dilute 0.4% trypan blue 1:2 in PBS to a final concentration of 0.2% (1 part trypan blue:1 part PBS).
b. Mix 0.2% trypan blue with a low dilution of cells (Dil 5–10 of cells depending on the concentration of cells).
c. Count numbers of live and dead cells (blue) using a hemacytometer.
   Note: Viability should be >80%. If viability is <80% or there are issues with counting, continue cultures and check viability often.
d. Calculate viability:

$$\%\, Viability = \frac{Total\ cell\ count - dead\ cell\ (blue) count}{Total\ cell\ count}\ x\ 100\%$$

e. Calculate cell concentration:

$$Total\ live\ cells = \frac{Cell\ count\ in\ all\ 4\ quadrants}{4}\ x\ dilution\ factor\ x\ 10^4 mL\ of\ cell\ solution$$

4.6 Transfer the Thawed LAD2 Cells

a. Add 9–14 mL of complete StemPro®-34 medium to a culture flask.
b. Transfer the 1 mL of $5x10^6$ thawed LAD2 cells in CryoScarless DMSO-free preservation solution to the flask.

4.7 Removal of Cell Debris

Note: Cell debris should be removed 1–4 days after thawing LAD2 cells.

a. Prepare 15 mL complete StemPro®-34 medium and warm in a 37˚C water bath.
b. Obtain the culture flask with cells and use cell suspension to rinse the bottom of the flask, transferring the contents to a 15 mL conical tube.
c. Centrifuge sample for 5 min at 200x*g*.
d. Aspirate the supernatant.
e. Add 2 mL of warmed 15 mL complete StemPro®-34 medium to the conical tube.
f. Pipette cell suspension up and down to thoroughly resuspend the sample.
g. Transfer 50 µL of cell suspension to a 0.6 mL Eppendorf tube for counting.
h. Count the LAD2 cells according to Step 4.5.
i. Adjust the volume of LAD2 cells (in a 15 mL conical tube) to an appropriate volume using a warmed complete StemPro®-34 medium such that cell concentration remains 0.3–0.5x10^6 cells/mL.
j. Transfer the LAD2 cell suspension to a T25 or T75 flask and place in a 37˚C incubator with 5% CO_2 and 95% humidity.

4.8 Weekly Maintenance of LAD2 Cells

Important:

- Perform hemidepletions (splits) weekly, where half the media is replaced with fresh media.
- Cells grow slowly initially but double within 10–14 days. **After the first couple of passages, cells double every 4–6 days and must be split accordingly and cultured in T75 flasks.**
- Keep cell count under 0.8x10^6 cells/mL. Ideally, cells should be kept between 0.25–0.5x10^6 cells/mL.
- It is best to thaw and start new stocks of LAD2 cells every 6 months.

4.8.1 Count cells to determine the hemidepletion method.

Note: To get an accurate count, one may need to concentrate (10X) a small volume of cells.

a. Obtain a 100 µL sample from the flask and transfer it to an appropriately labeled Eppendorf tube.
b. Place the T75 flask with cells back in the incubator.
c. Centrifuge the 100 µL cell sample for 5 min at 200x*g*.
d. Aspirate the supernatant.
e. Add 10 µL warmed complete StemPro®-34 medium to tube and resuspend cell sample.
f. Pipette cell suspension up and down to thoroughly resuspend.
g. Count the LAD2 cells using a 5-fold dilution of cells in 0.2% trypan blue using a hemocytometer.

4.8.2 If the count is between 0.50–0.8x10^6 cells, split cells into two flasks, replenishing with half fresh complete StemPro®-34 medium. If the count is below 0.5x10^6 cells, centrifuge cells at 200x*g* for 5 minutes, remove half the media, replenish with half fresh complete StemPro®-34 medium, then add cells to a T75 flask.

4.9 Freezing the LAD2 Cells

Note: The cell suspensions should be frozen down at 5x10^6 cells/mL.

a. Transfer cell suspension to be frozen to a conical tube and centrifuge cell sample for 10 min at 450x*g*.
b. Aspirate supernatant, leaving about 100 µL of the supernatant.
c. Resuspend the cells in cryopreserve (CryoScarless DMSO-free) at 5x10^6 cells/mL.
d. Transfer 1 mL of LAD2 cells to a cryo vial.
e. Transfer vials to a Nalgene freezing container and place them at -80˚C.
f. Transfer vials to liquid nitrogen the following day for longer storage.

5. Preparation of Solutions and Reagents

5.1 Human IgE Biotin Monoclonal Antibody

Human IgE biotin monoclonal antibody comes in solution at 1 mg/mL (0.1 mL total). Make aliquots of antibody to avoid multiple freeze-thaw cycles. 1 µL of 1

mg/mL will be needed to prepare the working concentration (1 µg/mL) of antibody that will be added to the samples. Store at -20˚C.

5.2 Streptavidin (SAV)

SAV is obtained as a powder (1 mg per vial). The SAV should be reconstituted with 1 mL cell culture-grade water to a final stock concentration of 1 mg/mL. Store at 4˚C.

5.3 Tyrode's Solution (with 10 mM HEPES and 0.25% BSA)

This solution is commercially available. It is kept frozen at -20˚C. Thaw the solution in a 37˚C water bath. After thawing, the solution should be aliquoted in 50 mL conical tubes to prevent multiple freeze-thaw cycles.

5.4 Buffer

The buffer contains 40 mM citric acid and 20 mM sodium phosphate dibasic heptahydrate in dH_2O at a final pH of 4.5. The prepared buffer can be used for at least 7 days. If making 50 mL of buffer, add 384.2 mg citric acid and 268.1 mg sodium phosphate dibasic heptahydrate to 40 mL dH_2O. Adjust pH to 4.5 using 1N NaOH/1N HCl and adjust final volume to 50 mL using dH_2O. Filter solution using 0.2 µm aPES filter. Store at 4˚C.

5.5 0.4 M Glycine Buffer

0.4 M Glycine buffer is prepared in dH_2O at a final pH of 10.7. Prepared glycine buffer can be used for at least 7 days. If making 50 mL glycine buffer, add 1.5 g glycine to 40 mL dH_2O. pH of 0.4 M glycine buffer is resistant to change, so NaOH can be used. Adjust pH using 4–5 NaOH pellets and then fine-tune pH to 10.7 using 1N NaOH/1N HCl and adjust final volume to 50 mL using dH_2O. Filter solution using 0.2 µm aPES filter. Store at 4˚C.

5.6 Treatment Controls

If there is sufficient space, the following treatment controls should be included on the plates: Baseline negative control (media) is a cell sample that receives appropriate media instead of nanoparticle treatment. Negative control (1X PBS) is a cell sample that receives 1X PBS instead of nanoparticle treatment. Vehicle control is a cell sample that receives the buffer used to store/reconstitute the nanoparticle (at the same concentration/dilution) instead of nanoparticle treatment.

6. Preparation of Nanoparticles

This assay requires 0.3–0.5 mL nanoparticle at 10X the highest final tested concentration. The nanoparticle concentration is selected based on the plasma concentration of the nanoparticle at the intended therapeutic dose. For the purpose of this protocol, this concentration is called "theoretical plasma concentration." Considerations for estimating theoretical plasma concentration were reviewed elsewhere and are summarized in Box 1 below.

Box 1. Example Calculation to Determine Nanoparticle Concentration for In Vitro Tests

In this example, we assume a mouse dose of 123 mg/kg. Therefore, the scaled equivalent human dose would be:

$$Human\ dose = \frac{mouse\ dose}{12.3} = \frac{123\ mg/kg}{12.3} = 10\ mg/kg$$

Blood volume constitutes approximately 8% of the body weight. Therefore, an average human of 70 kg body weight has approximately 5.6 L of blood. Assuming all the nanoparticle injected goes into the systemic circulation, this provides a rough approximation of the potential maximum nanoparticle concentration in blood, which is used as the in vitro test concentration.

$$in\ vitro\ concentration_{human\ matrix} = \frac{human\ dose}{human\ blood\ volume} = \frac{70\ kg * 10\ mg/kg}{5.6\ L} = 0.125\ mg/mL$$

The assay can evaluate 4 test samples—either 4 different nanoparticles at one concentration or 1 nanoparticle at four concentrations: 10X (or, when feasible, 100X, 30X, or 5X) of the theoretical plasma concentration, theoretical plasma concentration, and two 1:5

serial dilutions of the theoretical plasma concentration. When the intended therapeutic concentration is unknown, the highest final concentration is 1 mg/mL or the highest reasonably achievable concentration.

For example, if the final theoretical plasma concentration to be tested is 0.2 mg/mL, 20 mg/mL stock will be prepared and diluted 10-fold (2 mg/mL), followed by two 1:5 serial dilutions (0.4 and 0.08 mg/mL). When 10 µL of each of these samples are combined in a culture plate well with 90 µL of cells, the final concentrations of nanoparticles are 0.008, 0.04, 0.2, and 2 mg/mL. Each nanoparticle concentration is plated 6 times for the Control Plate plus another 4–6 times for the cell-free samples on the Control Plate (*see* Plate Maps 3 and 4 in the Appendix for an example). For the Experimental Plate, each nanoparticle concentration is plated 12 times (*see* Plate Map 5 in the Appendix for an example).

7. Preparation of Standards

Important: These standards must be prepared fresh each time the protocol calls for quantifying β-hexosaminidase release (i.e., in Steps 9.4.2 and 9.4.3).

7.1. 4-Nitrophenol (pNP) Working Stock

Prepare 4-nitrophenol (pNP) working stock by diluting the 10,000 µM commercial stock 5-fold in Tyrode's buffer to a final pNP concentration of 2000 µM. Use this working stock to prepare pNP standards following the table below.

Note: The standard solution is yellow and clear. Prepare fresh. Vortex each standard well before proceeding to the next step. Discard after use.

Sample	*Final pNP Conc. µM*	*Preparation Procedure*
P-Standard 1	1500	Combine 300 µL of the working stock with 100 µL of Tyrode's buffer
P-Standard 2	1000	Combine 250 µL of P-Standard 1 with 125 µL of Tyrode's buffer
P-Standard 3	500	Combine 125 µL of P-Standard 2 with 125 µL of Tyrode's buffer
P-Standard 4	250	Combine 125 µL of P-Standard 3 with 125 µL of Tyrode's buffer
P-Standard 5	125	Combine 125 µL of P-Standard 4 with 125 µL of Tyrode's buffer
P-Standard 6	62.5	Combine 125 µL of P-Standard 5 with 125 µL of Tyrode's buffer
P-Standard 7	31.3	Combine 125 µL of P-Standard 6 with 125 µL of Tyrode's buffer
Blank	0	Use 150 µL of Tyrode's buffer

7.2. β-Hexosaminidase B (β-Hex) Working Stock

Prepare the β-hexosaminidase B (β-Hex) working stock solution by diluting the commercial stock with a concentration of 0.377 mg/mL 4000 times in Tyrode's solution to a final concentration of 94.24 ng/mL. Follow the table below to prepare β-Hex standards.

Note: Vortex each standard well before proceeding to the next step. Prepare fresh and discard leftovers after use.

Sample	*Final β-Hex Conc. ng/mL*	*Preparation Procedure*
B-Standard 1	47.1	Combine 125 µL of working stock with 125 µL of Tyrode's buffer
B-Standard 2	23.6	Combine 125 µL of B-Standard 1 with 125 µL of Tyrode's buffer
B-Standard 3	11.8	Combine 125 µL of B-Standard 2 with 125 µL of Tyrode's buffer
B-Standard 4	5.89	Combine 125 µL of B-Standard 3 with 125 µL of Tyrode's buffer
B-Standard 5	2.95	Combine 125 µL of B-Standard 4 with 125 µL of Tyrode's buffer
B-Standard 6	1.47	Combine 125 µL of B-Standard 5 with 125 µL of Tyrode's buffer
B-Standard 7	0.736	Combine 125 µL of B-Standard 6 with 125 µL of Tyrode's buffer
B-Standard 8	0.368	Combine 125 µL of B-Standard 7 with 125 µL of Tyrode's buffer
Blank	0	Use 150 µL of Tyrode's buffer

8. IgE-Dependent Mast Cell Degranulation in the Absence of Nanoparticles

Important:

- This procedure is used for assay performance qualification or validation to assess a new batch of mast cells or any change in reagents over time. It is recommended that this assay be performed first to qualify the test system before assessing nanoparticles.
- This protocol is based on Measuring Mast Cell Mediator Release Basic Protocol 1 from Kuehn et al [12].
- The assay should include the following controls: Plate Blanks (No cells, +IgE +SAV); Negative Control (LAD2, -IgE -SAV); IgE Control (LAD2, +IgE -SAV); SAV control (LAD2, -IgE +SAV); Positive Control (LAD2, +IgE + SAV). The assay should be run in triplicate. See example Plate Map 1 in the Appendix.

8.1 LAD2 Cell Preparation (Day 1)

8.1.1 Count LAD2 cells according to the procedure described in Step 4.5.

8.1.2 Centrifuge cell sample for 5 min at 450x*g* at room temperature.

8.1.3 Resuspend the cell sample in the supplemented StemPro®-34 medium from Step 4.1.

Note: Cells should be resuspended to a concentration of 0.278×10^6 cells/mL since 90 µL of cells are added to each well, and the cells are needed at a final concentration of 2.5×10^4 cells per well in 100 µL final volume.

8.2 Plate Preparation and Biotin-IgE Sensitization (Day 1)

8.2.1 Obtain and label a 96-well round-bottom plate. (Referred to as Treatment Plate; *see* Plate Map 1 in the Appendix for an example.)

8.2.2 Add 90 µL of thawed Tyrode's solution to Plate Blank wells.

8.2.3 Add 90 µL of cells to appropriate wells (all wells except Plate Blanks).

8.2.4 Obtain an aliquot of 1 mg/mL biotinylated IgE and make a 1 µg/mL working stock of biotinylated IgE (1 mL total) in supplemented StemPro®-34 medium from Step 4.1.

8.2.5 Add 10 µL of 1 µg/mL working stock of biotinylated IgE to each appropriate well (Plate Blanks, IgE Controls, Positive Controls).

Note: The final concentration of IgE is 100 ng/mL in 100 µL.

8.2.6 Add 10 µL of supplemented StemPro®-34 medium from Step 4.1 to all wells not receiving biotinylated IgE to make the final volume 100 µL (Negative Controls, SAV Controls).

8.2.7 Place the Treatment Plate in a 37˚C incubator with 5% CO_2 and 95% humidity overnight.

8.3 Addition of SAV (Day 2)

8.3.1 After IgE incubation, centrifuge the Treatment Plate at 450x*g* for 10 min at room temperature.

8.3.2 Aspirate the supernatants, making sure to leave the pellets untouched.

8.3.3 Wash the wells three times with Tyrode's solution to remove excess IgE.

a. Add 100 µL of Tyrode's solution to each well.
b. Centrifuge plate at 450x*g* for 10 min at room temperature.
c. Aspirate the supernatants.

8.3.4 Resuspend cells in 90 µL of Tyrode's solution.

8.3.5 Incubate cells in a 37˚C incubator with 5% CO_2 and 95% humidity for 5–10 min to equilibrate the cells with activation temperature.

8.3.6 Prepare 1 mL of 1 µg/mL working stock of SAV in Tyrode's solution from the 1 mg/mL SAV stock from Step 5.2.

8.3.7 Add 10 µL of 1 µg/mL SAV to each appropriate well (Plate Blanks, SAV Controls, Positive Controls). The total volume per well is 100 µL, and the final SAV concentration is 100 ng/mL.

8.3.8 Add 10 µL of Tyrode's solution to each well that did not receive SAV (Negative Controls, IgE Controls). The total volume per well is 100 µL.

8.3.9 Incubate the Treatment Plate in a 37˚C incubator with 95% humidity without CO_2 (0% CO_2) for 30 min.

8.4 PNAG Preparation and Treatment (Day 2)

8.4.1 During SAV incubation, prepare p-nitrophenyl N-acetyl-β-D-glucosaminide (PNAG) solution by dissolving 70 mg of PNAG in 20 mL of the buffer prepared in Step 5.4; sonicate in a sonication water bath until PNAG is completely solubilized. *Note:* 10 mL PNAG in buffer from Step 5.4 is needed per one 96-well plate.

8.4.2 Obtain two flat-bottom 96-well plates; label one "Supernatant" for measurement of secreted β-hexosaminidase activity and one "Lysate" to calculate the total amount (supernatants and lysates) to determine the percentage release (see Plate Map 1 in the Appendix for an example).

8.4.3 Aliquot 100 µL of PNAG solution into each well of the Supernatant and Lysate Plates.

8.4.4 After incubation (Step 8.3.9), centrifuge the Treatment Plate 450x*g* at 4˚C for 10 min to stop the reaction.

8.4.5 Carefully transfer 50 µL of the supernatant from each well of the Treatment Plate to the corresponding well of the Supernatant Plate from Step 8.4.3.

Note: It is important to ensure sure the supernatant does not have any leftover cells; collect supernatant carefully and avoid disturbing the pellet.

8.4.6 Incubate the Supernatant Plate in a 37˚C incubator with 95% humidity without CO_2 (0% CO_2) for 90 min.

8.4.7 Prepare 20 mL of 0.1% Triton X-100 solution by diluting 10% Triton X-100 in water.

8.4.8 Add 150 µL of 0.1% Triton X-100 solution to each well of the Treatment Plate to make the lysates. Resuspend carefully and transfer 50 µL lysates to corresponding wells of the Lysate Plate from Step 8.4.3.

8.4.9 Incubate the Lysate Plate in a 37˚C incubator with 95% humidity without CO_2 (0% CO_2) for 90 min.

8.4.10 After incubation, add 50 µL of 0.4 M glycine buffer into each well of both plates (Supernatant and Lysate Plates).

Note: The appearance and intensity of the yellow color indicates the extent of β-hexosaminidase activity.

8.5 Data Acquisition (Day 2)

Notes:

- The plate reader's maximum absorbance is 4. If values exceed 4, samples must be re-run at lower concentrations (i.e., at higher dilution). Lower concentrations could be achieved by using a lower cell concentration on Day 1 or by decreasing the volume of supernatant/lysate, increasing the volume of PNAG in the respective plate on Day 2, and adjusting the calculations accordingly.
- The steps below are for the Spectramax M5 reader (Molecular Devices) used at the NCL. If using a different reader, follow the instructions from the manufacturer of that reader.

8.5.1 Set up the instrument beforehand to have the correct acquisition settings.

8.5.2 Set up the instrument to read at both 405 nm and 620 nm.

8.5.3 Open the software.

Note: For the Spectramax M5 reader, the software used at the NCL is SoftProMax 7.0.3.

8.5.4 Label plate maps accordingly.

8.5.5 Apply the reduction equation: 405 nm read – 620 nm read.

8.5.6 On the instrument: Press the drawer, insert the Supernatant Plate without a lid, and then press the drawer again to close the instrument.

8.5.7 Click Read.

8.5.8 Repeat 8.5.6 and 8.5.7 for the Lysate Plate.

8.5.9 Save the file and export it to Excel, then save the Excel file as a .xlsx file.

8.5.10 Perform calculations as described in Step 10.1.

9. Nanoparticle Effects on IgE-dependent Mast Cell Degranulation

Notes:

- This procedure is used to assess nanoparticle effects on IgE-dependent degranulation of mast cells, to understand how nanoparticles may affect the normal function of these cells.
- Since some nanoparticles may affect the integrity of the cellular membrane, thereby causing spontaneous leakage of mast cell granules, this assay uses a Control Plate. The purpose of the Control Plate is to detect such IgE-independent/nanoparticle-triggered release of granules.
- The Experimental Plate used in this protocol tests the effects of nanoparticles on IgE-dependent degranulation.
- There is no clinically relevant value to compare the results to. This assay detects degranulation and compares it to the negative control. Statistical analysis is used to compare between the samples.
- If no nanoparticle-mediated degranulation is detected on the Control Plate, the data on the Experimental Plate can be used without adjustment. However, when nanoparticle-mediated degranulation is detected, the Experimental Plate data must be adjusted to consider this fact.

9.1 LAD2 Cell Preparation (Day 0)

9.1.1 Count LAD2 cells according to the procedure described in Step 4.5.

9.1.2 Split cell sample into two samples, one to be used in the Control Plate and the other to be used in the Experimental Plate.

9.1.3 Centrifuge cell samples for 5 min at 450x*g* at room temperature.

9.1.4 Resuspend the cell samples in Tyrode's solution (for the Control Plate) or complete StemPro®-34 medium (for the Experimental Plate).

Note: Cells should be resuspended at a concentration of 0.278×10^6 cells/mL (NPs or controls will be added at an MRD10, so the final concentration will be 0.25×10^6 cells/mL, which will equate to 25,000 cells in 100 µL).

9.2 Plate Preparation (Day 0)—Experimental Cell Treatment Plate

Note: Cells are prepared in 6-well plates but can also be prepared in 12-well plates. If 12-well plates are preferred, the volumes described below should be adjusted accordingly.

9.2.1 Obtain and label a 6-well plate(s) as the Experimental Cell Treatment Plate.

Note: One well is needed for β-hexosaminidase controls (Negative Controls, SAV Controls, IgE Controls, Positive Controls) and two wells for each NP treatment, PBS control, and vehicle control. If nanoparticles are resuspended in PBS, then vehicle control can be skipped. One can also use a 12-well plate(s); however, the volume per well and the number of wells per treatment may need to be adjusted to provide sufficient number of cells. If testing more than 1 NP or multiple concentrations of a single NP adjust plate number accordingly (*see* Plate Map 2 as an example.)

9.2.2 Add 0.278×10^6 cells/mL (in complete StemPro®-34 medium) to each well. The final concentration of these cells will be 0.25×10^6 cells/mL after appropriate control solutions and nanoparticles have been added at MRD10.

Note: The total volume of cells/cell count will exceed the number of cells required for the 25,000 cells/well needed for Day 1. Six wells on a 96-well plate will be

needed per β-hexosaminidase control (Negative Controls, SAV Controls, IgE Controls, Positive Controls); twelve wells on a 96-well plate will be needed per nanoparticle treatment; and 8–12 wells will be needed per PBS control/vehicle control depending on well availability (*see* Plate Map 5 in the Appendix as an example). Vehicle control can be skipped if nanoparticles are resuspended in PBS. Therefore, double the number of cells needed.

For example, one nanoparticle concentration will require 12 wells, each with 25,000 cells/well = $0.3x10^6$ cells, so in the 12-well plate, $0.3x10^6$ cells would be treated in duplicate with one nanoparticle concentration per well. If using a 6-well plate, then $0.6x10^6$ cells total would be treated with one nanoparticle concentration per well. This will allow for a maximum of 50% cell death while still having enough cells for 25,000 cells/well in the 96-well plate on Day 1.

9.2.3 Add appropriate control media/solution/nanoparticle treatment at MRD10.
Notes: Add complete StemPro®-34 medium to the wells that contain cells for the β-hexosaminidase controls (Negative Control, SAV Controls, IgE Controls, Positive Controls). For the nanoparticle concentrations, an example would be to add 2.16 mL of 0.278 $x10^6$ cells/mL + 240 µL of the 10X nanoparticle concentration in a 6-well plate, or if using a 12-well plate, prepare two duplicate wells for each nanoparticle treatment where each well contains 1.08 mL of $0.278x10^6$ cells/mL+ 120 µL of the 10X nanoparticle concentration ($0.3x10^6$ cells/well).

9.2.4 Place Experimental Treatment Plate in a 37˚C incubator with 5% CO_2 and 95% humidity overnight, up to 24 hours.

9.3 Plate Preparation (Day 0)—Control Plate

Note: The Control Plate does not include an IgE overnight incubation or the 30 min SAV incubation. The 30-minute nanoparticle incubation replaces the SAV step.

9.3.1 Obtain and label a 96-well round-bottom plate. This plate is referred to as "Control Treatment Plate" (*see* Plate Map 3 in the Appendix for an example).

9.3.2 Add 90 µL of cells resuspended in Tyrode's solution at $0.278x10^6$ cells/mL to appropriate wells (wells with NP samples and Vehicle Control).

9.3.3 Add 10 µL nanoparticles to each appropriate well (sample and cell-free control wells).

Note: When 10 µL of nanoparticles are added to 90 µL of cells, the final volume in the well will be 100 µL, and the dilution of the nanoparticle sample will be 10-fold.

9.3.4 Add 10 µL of vehicle to appropriate wells. The final volume in each well will be 100 µL.

9.3.5 Add 100 µL Tyrode's solution to Plate Blank wells. The final volume per well will also be 100 µL.

9.3.6 Add 90 µL Tyrode's solution to cell-free wells. The final volume per well will be 100 µL.

9.3.7 No wells for standards are needed at this point. Standard wells are only needed in the Control Supernatant Plate when determining the β-hexosaminidase activity.

9.3.8 Place Control Treatment Plate in a 37˚C incubator with 5% CO_2 and 95% humidity for 30 min.

9.4 Control Plate: PNAG Preparation, Standard Preparation, Treatment, and Plate Read (Day 0)

9.4.1 During nanoparticle incubation, prepare p-nitrophenyl N-acetyl-β-D-glucosaminide (PNAG) solution by dissolving 70 g PNAG in 20 mL of buffer from Step 5.4 and sonicating the solution in a sonication water bath until PNAG is solubilized.

9.4.2 Prepare 4-nitrophenol (pNP) standards as described in Step 7.1.

9.4.3 Prepare the β-hexosaminidase B (β-Hex) standards as described in Step 7.2.

9.4.4 Obtain two flat-bottom 96-well plates. Label one "Control Supernatant" for measurement of secreted β-hexosaminidase activity in supernatants (*see* Plate Map 4 in the Appendix for an example) and the second plate "Control Lysate" to calculate the total amount (supernatants and lysates) to determine the percentage release (*see* Plate Map 3 in the Appendix for an example). The Control Supernatant Plate will contain the pNP and β-Hex standard curves.

9.4.5 Aliquot 100 µL Tyrode's solution into 4-nitrophenol standard wells on the Control Supernatant Plate.

9.4.6 Aliquot 100 µL of PNAG solution into the β-hexosaminidase standard wells on the Control Supernatant Plate.

9.4.7 Aliquot 100 µL of PNAG solution into the Plate Blank, cell-free wells (i.e., wells on the plate that do not have cells), vehicle control wells, and test-sample wells on the Control Supernatant Plate.

9.4.8 Aliquot 100 µL of PNAG solution into all wells of the Control Lysate Plate.

9.4.9 After the incubation from Step 9.3.8, centrifuge the Control Treatment Plate 450x*g*, at 4˚C for 10 min to stop the reaction.

9.4.10 Carefully transfer 50 µL of the supernatant from each well of the Control Treatment Plate to the corresponding well of the Control Supernatant Plate.
Note: It is important to ensure the supernatant does not have any leftover cells; collect supernatant carefully and avoid disturbing the pellet.

9.4.11 Add 50 µL of appropriate 4-nitrophenol standard to each appropriate 4-nitrophenol standard well of the Control Supernatant Plate.

9.4.12 Add 50 µL of appropriate β-hexosaminidase standard to each appropriate β-Hex standard well of the Control Supernatant Plate.

9.4.13 Incubate the Control Supernatant Plate in a 37˚C incubator with 95% humidity without CO_2 (0% CO_2) for 90 min.

9.4.14 Prepare 20 mL of 0.1% Triton X-100 solution by combining 200 µL of 10% Triton X-100 with 19.8 mL of water.

9.4.15 Add 150 µL of 0.1% Triton X-100 solution into the 50 µL of supernatants/cells remaining in the Control Treatment Plate. Resuspend carefully and transfer 50 µL of the generated lysates into the corresponding wells on the Control Lysate Plate.

9.4.16 Incubate the Control Lysate Plate in a 37˚C incubator with 95% humidity without CO_2 (0% CO_2) for 90 min.

9.4.17 After incubations (Steps 9.4.13 and 9.4.16), add 50 µL of 0.4 M glycine buffer into each well of both the Control Supernatant and Control Lysate Plates.
Note: The appearance and intensity of the yellow color indicates the extent of β-hexosaminidase activity.

9.4.18 Read Control Supernatant and Control Lysate Plates on a plate reader according to Step 8.5 if using a SpectraMax M5 reader or following the manufacturer's instructions for your reader.

9.5 Experimental Plate: IgE Sensitization (Day 1)

9.5.1 Obtain the 6-well or 12-well plate(s) with samples from incubation in Step 9.2.4 (Experimental Cell Treatment Plate).

9.5.2 Transfer samples to 15 mL conical tubes. Centrifuge samples for 10 min at 450x*g*. Aspirate supernatants.

9.5.3 Wash the samples 2X with warmed supplemented StemPro®-34 medium.

a. Add 2 mL of media to each tube prepared in step 9.5.2.
b. Centrifuge sample for 10 min at 450x*g* at room temperature.
c. Aspirate the supernatants.

9.5.4 Resuspend each cell pellet in 1 mL supplemented StemPro®-34 medium.

9.5.5 Count the cells according to Step 4.5.

9.5.6 Adjust the cell concentrations to 0.278×10^6 cells/mL with supplemented StemPro®-34 medium.

Note: These cells will be added to a round bottom 96-well plate in step 9.5.8.

9.5.7 Obtain a 96-well round bottom plate and add 90 µL of Tyrode's solution to appropriate wells (Plate Blank).

9.5.8 Add 90 µL of cells from step 9.5.6 to the appropriate well (*see* Plate Map 5 in the Appendix for an example.).

9.5.9 Obtain a 1 mg/mL aliquot of biotinylated IgE and make a 1 µg/mL working stock of biotinylated IgE (1 mL total) in supplemented StemPro®-34 medium.

9.5.10 Add 10 µL of 1 µg/mL working stock of biotinylated IgE to each appropriate well of Plate Blanks, IgE Controls, Positive Controls, Nanoparticle Samples/Controls with IgE and SAV.

Note: The final concentration of IgE is 100 ng/mL in 100 µL.

9.5.11 Add 10 µL of supplemented StemPro®-34 medium to all wells not receiving biotinylated IgE to make the final volume 100 µL. These wells include the

Negative Controls, SAV Controls, and Nanoparticle Samples/Controls without IgE or SAV.

9.5.12 Place the Experimental Treatment Plate in an incubator in a 37˚C incubator with 5% CO_2 and 95% humidity overnight.

9.6 Experimental Plate: Addition of SAV (Day 2)

9.6.1 After IgE incubation, centrifuge the Experimental Treatment Plate with cells at 450x*g* at room temperature for 10 minutes.

9.6.2 Aspirate the supernatants, making sure to leave the pellets untouched.

9.6.3 Wash the wells three times with Tyrode's solution to remove excess IgE.

a. Add 100 µL of Tyrode's solution to each well.

b. Centrifuge plate for 10 min at room temperature at 450x*g*.

a. Aspirate the supernatants.

9.6.4 Resuspend cells in 90 µL of Tyrode's solution. The final volume per well will be 100 µL in total.

9.6.5 Incubate cells in a 37˚C incubator with 5% CO_2 and 95% humidity for 5–10 min to equilibrate the cells with activation temperature.

9.6.6 Prepare 1 mL of 1 µg/mL working stock of SAV in Tyrode's solution from 1 mg/mL SAV stock.

9.6.7 Add 10 µL of 1 µg/mL SAV to each appropriate well (Plate Blanks, SAV Controls, Positive Controls, and Nanoparticle-treated Samples and Controls with IgE and SAV). Total volume will be 100 µL with a final 100 ng/mL SAV concentration.

9.6.8 Add 10 µL of Tyrode's solution to each well that did not receive SAV (Negative Controls, IgE Controls, and Nanoparticle Samples and Controls without IgE or SAV). Total volume will be 100 µL.

9.6.9 Incubate the Experimental Treatment Plate in a 37˚C incubator with 95% humidity without CO_2 (0% CO_2) for 30 min.

9.7 Experimental Plate: PNAG Preparation, Treatment, and Plate Read (Day 2)

9.7.1 During SAV incubation, prepare p-nitrophenyl N-acetyl-β-D-glucosaminide (PNAG) solution in citrate buffer by dissolving 70 mg of PNAG in 20 mL of the buffer from Step 5.4 and sonicating the solution in a sonication water bath at room temperature until PNAG is solubilized.

Note: You will need 10 mL of PNAG solution per one 96-well plate.

9.7.2 Obtain two flat-bottom 96-well plates and label one "Experimental Supernatant" to measure secreted β-hexosaminidase activity in supernatants and one "Experimental Lysate" to calculate the total amount (supernatants and lysates) to determine the percentage release (*see* Plate Map 5 in the Appendix for an example.)

9.7.3 Aliquot 100 µL of PNAG solution into each well of the Experimental Supernatant and Experimental Lysate plates.

9.7.4 After the incubation from Step 9.6.9, centrifuge the Experimental Treatment Plate 450x*g*, at 4˚C for 10 min to stop the reaction.

9.7.5 Carefully transfer 50 µL of the supernatant of each well of the Experimental Treatment Plate to the corresponding well of the Experimental Supernatant Plate.

Note: It is important to ensure sure the supernatant does not have any leftover cells; collect supernatant carefully and avoid disturbing the pellet.

9.7.6 Incubate the Experimental Supernatant Plate in a 37˚C incubator with 95% humidity without CO_2 (0% CO_2) for 90 min.

9.7.7 Make 20 mL of 0.1% Triton X-100 solution by combining 200 µL of the 10% Triton X-100 stock with 19.8 mL of water.

9.7.8 Add 150 µL of 0.1% Triton X-100 solution into the 50 µL of supernatants/cells remaining in the original Experimental Treatment Plate. Resuspend carefully and transfer 50 µL of generated lysates into the corresponding well on the Experimental Lysate Plate.

9.7.9 Incubate the Experimental Lysate Plate in a 37˚C incubator with 95% humidity without CO_2 (0% CO_2) for 90 min.

9.7.10 At the end of the incubation from Steps 9.7.6 and 9.7.9, add 50 µL of 0.4 M glycine buffer to each well on the Experimental Supernatant and Experimental Lysate Plates.

Note: The appearance and intensity of the yellow color indicates the extent of β-hexosaminidase activity.

9.7.11 Read the Experimental Supernatant and Experimental Lysate Plates on a plate reader according to Step 8.5 if using a Spectramax M5 reader or the manufacturer's instructions for your plate reader.

10. Calculations

10.1 Calculate percent degranulation according to the following formula:

$$\%\ Release = \frac{2\ x\ (Supernatant - Plate\ Blank)}{(Supernatant - Plate\ Blank) + 4\ x\ (Lysates - Plate\ Blank)}\ x\ 100\%$$

Note: Additional information about this formula is available in reference [12].

10.2 Calculate the percent coefficient of variation (%CV) for each control and study sample using the following formula:

$$\%\ CV = \frac{Standard\ Deviation}{Mean\ \%\ Release}\ x\ 100\%$$

11. Acceptance Criteria

11.1 The %CV for each positive control, negative control, and test sample should be within 30%.

11.2 Positive and negative control should have an acceptable %CV for the run to be accepted.

11.3 Within an acceptable run, if %CV for a study sample does not meet the acceptance criterion in step 11.1, the sample should be re-analyzed.

12. References

1. Trivedi, N.H., Guentzel, M.N., Rodriguez, A.R., Yu, J.J., Forsthuber, T.G., and Arulanandam, B.P. Mast cells: multitalented facilitators of protection against bacterial pathogens. *Expert Rev Clin Immunol*. **2013**, 9(2), 129-138. DOI: 10.1586/eci.12.95. PMID: 23390944.
2. National Cancer Institute. Available from: https://www.cancer.gov/publications/dictionaries/cancer-terms/def/mast-cell.
3. Penn, R.B. Mast cells in asthma: Here I am, stuck in the middle with you. *Eur Respir J*. **2020**, 56(1). DOI: 10.1183/13993003.01337-2020. PMID: 32616549.
4. Derakhshani, A., Vahidian, F., Alihasanzadeh, M., Mokhtarzadeh, A., Lotfi Nezhad, P., and Baradaran, B. Mast cells: A double-edged sword in cancer. *Immunol Lett*. **2019**, 209, 28-35. DOI: 10.1016/j.imlet.2019.03.011. PMID: 30905824.
5. Dobrovolskaia, M.A., Shurin, M.R., Kagan, V.E., and Shvedova, A.A. Ins and Outs in Environmental and Occupational Safety Studies of Asthma and Engineered Nanomaterials. *ACS Nano*. **2017**, 11(8), 7565-7571. DOI: 10.1021/acsnano.7b04916. PMID: 28737932.
6. Stone, C.A., Jr., Liu, Y., Relling, M.V., Krantz, M.S., Pratt, A.L., Abreo, A., Hemler, J.A., and Phillips, E.J. Immediate Hypersensitivity to Polyethylene Glycols and Polysorbates: More Common Than We Have Recognized. *J Allergy Clin Immunol Pract*. **2019**, 7(5), 1533-1540 e1538. DOI: 10.1016/j.jaip.2018.12.003. PMID: 30557713.
7. Szebeni, J., Storm, G., Ljubimova, J.Y., Castells, M., Phillips, E.J., Turjeman, K., Barenholz, Y., Crommelin, D.J.A., and Dobrovolskaia, M.A. Applying lessons learned from nanomedicines to understand rare hypersensitivity reactions to mRNA-based SARS-CoV-2 vaccines. *Nat Nanotechnol*. **2022**, 17(4), 337-346. DOI: 10.1038/s41565-022-01071-x. PMID: 35393599.
8. The Mayo Clinic. Available from: https://www.mayoclinic.org/diseases-conditions/systemic-mastocytosis/symptoms-causes/syc-20352859.
9. Li, J.Y., Ryder, C.B., Zhang, H., Cockey, S.G., Hyjek, E., Moscinski, L.C., Sagatys, E., and Song, J. Review and Updates on Systemic Mastocytosis and Related Entities. *Cancers (Basel)*. **2023**, 15(23). DOI: 10.3390/cancers15235626. PMID: 38067330.
10. Rama, T.A. and Castells, M. Triggers of Anaphylaxis in Mastocytosis Patients: Evidence of the Current Drug-Avoidance Recommendation. *Current Treatment Options in Allergy*. **2023**, 10(4), 442-457. DOI: 10.1007/s40521-023-00349-2. PMID: WOS:001068389300001.
11. Kozma, G.T., Meszaros, T., Berenyi, P., Facsko, R., Patko, Z., Olah, C.Z., Nagy, A., Fulop, T.G., Glatter, K.A., Radovits, T., Merkely, B., and Szebeni, J. Role of anti-polyethylene glycol (PEG) antibodies in the allergic reactions to PEG-containing Covid-19 vaccines: Evidence for immunogenicity of PEG. *Vaccine*. **2023**, 41(31), 4561-4570. DOI: 10.1016/j.vaccine.2023.06.009. PMID: 37330369.
12. Kuehn, H.S., Radinger, M., and Gilfillan, A.M. Measuring mast cell mediator release. *Curr Protoc Immunol*. **2010**, Chapter 7, Unit7 38. DOI: 10.1002/0471142735.im0738s91. PMID: 21053305.
13. Radinger, M., Jensen, B.M., Kuehn, H.S., Kirshenbaum, A., and Gilfillan, A.M. Generation, isolation, and maintenance of human mast cells and mast cell lines

derived from peripheral blood or cord blood. *Curr Protoc Immunol*. **2010**, Chapter 7, Unit 7 37. DOI: 10.1002/0471142735.im0737s90. PMID: 20814942.

13. Abbreviations

B-Hex	β-hexosaminidase
BSA	bovine serum albumin
DMSO	dimethyl sulfoxide
NC	negative control
NIAID	National Institutes of Allergy and Infectious Diseases
NIH	National Institutes of Health
PBS	phosphate buffered saline
PC	positive control
PEG	poly(ethylene glycol)
PES	polyethersulfone
PNAG	p-nitrophenyl-N-acetyl-B-D-glucosaminide
pNP	4-nitrophenol
P/S	penicillin/streptomycin
rhSCF	recombinant human stem cell factor
SAV	Streptavidin

14. Appendix

Plate Map 1. Example plate map for set up of **Treatment Plate**. Plate maps for Treatment, Supernatant, and Lysate plates are the same. This plate is used in Step 8. The Treatment Plates uses a round-bottom plate. The Supernatant and Lysate Plates use flat-bottom plates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells
B	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Plate Blank (+IgE +SAV) No Cells	Empty	Empty	Empty	Empty	Empty	Empty
C	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well
D	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well
E	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	NC (-IgE -SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well	SAV Control (-IgE +SAV) 25K LAD2/well
F	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well
G	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well
H	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	IgE Ctrl (+IgE -SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well	PC (+IgE +SAV) 25K LAD2/well

Plate Map 2. Example **Experimental Cell Treatment** Plate. This plate is used in Step 9.2. This plate can be comprised of either 6-well or 12-well plate(s), depending on the researcher's preference. The cells from these plates will later be transferred to corresponding wells on example Plate Map 5, Experimental Treatment Plate for treatment with SAV and IgE.

6-well Plates

	1	2	3
A	β-Hex Controls (NC, SAV Ctrl, IgE Ctrl, PC)	PBS Ctrl	Vehicle Ctrl
B	β-Hex Controls (NC, SAV Ctrl, IgE Ctrl, PC)	PBS Ctrl	Vehicle Ctrl

	1	2	3
A	TS1	TS2	TS3
B	TS1	TS2	TS3

	1	2	3
A	TS4	Empty	Empty
B	TS4	Empty	Empty

12-well Plate

	1	2	3	4
A	β-Hex Controls (NC, SAV Ctrl, IgE Ctrl, PC)	β-Hex Controls (NC, SAV Ctrl, IgE Ctrl, PC)	TS1	TS1
B	PBS Ctrl	PBS Ctrl	TS2	TS2
C	Vehicle Ctrl	Vehicle Ctrl	TS3	TS3

	1	2	3	4
A	TS4	TS4	Empty	Empty
B	Empty	Empty	Empty	Empty
C	Empty	Empty	Empty	Empty

Plate Map 3. Example **Control Treatment Plate** and **Control Lysate Plate**. This plate is used in Step 9.3 and 9.4. The Treatment Plates uses a round-bottom plate. The Lysate Plate uses a flat-bottom plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Plate Blank	Cell-free PBS/Vehicle Ctrl	Cell-free PBS/Vehicle Ctrl	Cell-free PBS/Vehicle Ctrl
B	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Plate Blank	Cell-free PBS/Vehicle Ctrl	Cell-free PBS/Vehicle Ctrl	Cell-free PBS/Vehicle Ctrl
C	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Cell-free TS1	Cell-free TS1	Cell-free TS1
D	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Empty	Cell-free TS1	Cell-free TS1	Cell-free TS1
E	Cell-free TS2	Cell-free TS2	Cell-free TS2	Cell-free TS3	Cell-free TS3	Cell-free TS3	Cell-free TS4	Cell-free TS4	Cell-free TS4	Vehicle Ctrl	Vehicle Ctrl	Vehicle Ctrl
F	Cell-free TS2	Cell-free TS2	Cell-free TS2	Cell-free TS3	Cell-free TS3	Cell-free TS3	Cell-free TS4	Cell-free TS4	Cell-free TS4	Vehicle Ctrl	Vehicle Ctrl	Vehicle Ctrl
G	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
H	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4

Plate Map 4. Example **Control Supernatant Plate**. This plate is used in Step 9.4. The Supernatant Plate uses a flat-bottom plate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	4-nitrophenol 1500 µM	4-nitrophenol 1000 µM	4-nitrophenol 500 µM	4-nitrophenol 250 µM	4-nitrophenol 125 µM	4-nitrophenol 62.5 µM	4-nitrophenol 31.25 µM	4-nitrophenol 0 µM	Plate Blank	Cell-free PBS/Vehicle Ctrl	Cell-free PBS/Vehicle Ctrl	Cell-free PBS/Vehicle Ctrl
B	4-nitrophenol 1500 µM	4-nitrophenol 1000 µM	4-nitrophenol 500 µM	4-nitrophenol 250 µM	4-nitrophenol 125 µM	4-nitrophenol 62.5 µM	4-nitrophenol 31.25 µM	4-nitrophenol 0 µM	Plate Blank	Cell-free PBS/Vehicle Ctrl	Cell-free PBS/Vehicle Ctrl	Cell-free PBS/Vehicle Ctrl
C	β-Hex 47.1 ng/mL	β-Hex 23.6 ng/mL	β-Hex 11.8 ng/mL	β-Hex 5.9 ng/mL	β-Hex 2.95 ng/mL	β-Hex 1.475 ng/mL	β-Hex 0.738 ng/mL	β-Hex 0.369 ng/mL	β-Hex 0 ng/mL	Cell-free TS1	Cell-free TS1	Cell-free TS1
D	β-Hex 47.1 ng/mL	β-Hex 23.6 ng/mL	β-Hex 11.8 ng/mL	β-Hex 5.9 ng/mL	β-Hex 2.95 ng/mL	β-Hex 1.475 ng/mL	β-Hex 0.738 ng/mL	β-Hex 0.369 ng/mL	β-Hex 0 ng/mL	Cell-free TS1	Cell-free TS1	Cell-free TS1
E	Cell-free TS2	Cell-free TS2	Cell-free TS2	Cell-free TS3	Cell-free TS3	Cell-free TS3	Cell-free TS4	Cell-free TS4	Cell-free TS4	Vehicle Ctrl	Vehicle Ctrl	Vehicle Ctrl
F	Cell-free TS2	Cell-free TS2	Cell-free TS2	Cell-free TS3	Cell-free TS3	Cell-free TS3	Cell-free TS4	Cell-free TS4	Cell-free TS4	Vehicle Ctrl	Vehicle Ctrl	Vehicle Ctrl
G	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4
H	TS1	TS1	TS1	TS2	TS2	TS2	TS3	TS3	TS3	TS4	TS4	TS4

Plate Map 5. Example **Experimental Treatment Plate**. Plate maps for Experimental Treatment, Experimental Supernatant, and Experimental Lysate plates are the same. This plate is used in Steps 9.5–9.7. The Treatment Plates uses a round-bottom plate. The Supernatant and Lysate Plates use flat-bottom plates.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Plate Blank (+IgE +SAV) No cells	Plate Blank (+IgE +SAV) No cells	Plate Blank (+IgE +SAV) No cells	NC (-IgE -SAV)	NC (-IgE -SAV)	NC (-IgE -SAV)	SAV Control (-IgE + SAV)	SAV Control (-IgE + SAV)	SAV Control (-IgE + SAV)	IgE Control (+IgE -SAV)	IgE Control (+IgE -SAV)	IgE Control (+IgE -SAV)
B	Plate Blank (+IgE +SAV) No cells	Plate Blank (+IgE +SAV) No cells	Plate Blank (+IgE +SAV) No cells	NC (-IgE -SAV)	NC (-IgE -SAV)	NC (-IgE -SAV)	SAV Control (-IgE + SAV)	SAV Control (-IgE + SAV)	SAV Control (-IgE + SAV)	IgE Control (+IgE -SAV)	IgE Control (+IgE -SAV)	IgE Control (+IgE -SAV)
C	PC (+IgE +SAV)	PC (+IgE +SAV)	PC (+IgE +SAV)	PBS Ctr (-IgE -SAV)	PBS Ctr (-IgE -SAV)	PBS Ctrl (+IgE +SAV)	PBS Ctrl (+IgE +SAV)	Vehicle Ctrl (-IgE -SAV)	Vehicle Ctrl (-IgE -SAV)	Vehicle Ctrl (+IgE +SAV)	Vehicle Ctrl (+IgE +SAV)	Vehicle Ctrl (+IgE +SAV)
D	PC (+IgE +SAV)	PC (+IgE +SAV)	PC (+IgE +SAV)	PBS Ctr (-IgE -SAV)	PBS Ctr (-IgE -SAV)	PBS Ctrl (+IgE +SAV)	PBS Ctrl (+IgE +SAV)	Vehicle Ctrl (-IgE -SAV)	Vehicle Ctrl (-IgE -SAV)	Vehicle Ctrl (+IgE +SAV)	Vehicle Ctrl (+IgE +SAV)	Vehicle Ctrl (+IgE +SAV)
E	TS1 (-IgE -SAV)	TS1 (-IgE -SAV)	TS1 (-IgE -SAV)	TS2 (-IgE -SAV)	TS2 (-IgE -SAV)	TS2 (-IgE -SAV)	TS3 (-IgE -SAV)	TS3 (-IgE -SAV)	TS3 (-IgE -SAV)	TS4 (-IgE -SAV)	TS4 (-IgE -SAV)	TS4 (-IgE -SAV)
F	TS1 (-IgE -SAV)	TS1 (-IgE -SAV)	TS1 (-IgE -SAV)	TS2 (-IgE -SAV)	TS2 (-IgE -SAV)	TS2 (-IgE -SAV)	TS3 (-IgE -SAV)	TS3 (-IgE -SAV)	TS3 (-IgE -SAV)	TS4 (-IgE -SAV)	TS4 (-IgE -SAV)	TS4 (-IgE -SAV)
G	TS1 (+IgE +SAV)	TS1 (+IgE +SAV)	TS1 (+IgE +SAV)	TS2 (+IgE +SAV)	TS2 (+IgE +SAV)	TS2 (+IgE +SAV)	TS3 (+IgE +SAV)	TS3 (+IgE +SAV)	TS3 (+IgE +SAV)	TS4 (+IgE +SAV)	TS4 (+IgE +SAV)	TS4 (+IgE +SAV)
H	TS1 (+IgE +SAV)	TS1 (+IgE +SAV)	TS1 (+IgE +SAV)	TS2 (+IgE +SAV)	TS2 (+IgE +SAV)	TS2 (+IgE +SAV)	TS3 (+IgE +SAV)	TS3 (+IgE +SAV)	TS3 (+IgE +SAV)	TS4 (+IgE +SAV)	TS4 (+IgE +SAV)	TS4 (+IgE +SAV)