Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Methods Article - JoVE Produced Video
Manuscript Number:	JoVE62702R1
Full Title:	Humanized mediator release assay as read-out for allergen potency
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Additional Information:	
Question	**Response**
Please specify the section of the submitted manuscript.	Immunology and Infection
Please indicate whether this article will be Standard Access or Open Access.	Open Access ($3900)
Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations.	Salzburg, Austria
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TITLE:

Humanized Mediator Release Assay as a Read-Out for Allergen Potency

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KEYWORDS:

allergy, RBL assay, allergenicity, allergen, mediator-release, histamine, IgE, basophils, AIT

SUMMARY:

Here we present the mediator release assay, using a rat basophilic leukemia cell line transfected with the human IgE receptor, to simulate the degranulation of effector cells typically observed in type 1 allergic reactions. This method investigates the biological activity of allergens in a highly sensitive, reproducible, and tailorable manner.

ABSTRACT:

Mediator release assays analyze *in vitro* immunoglobulin E (IgE)-mediated degranulation and secretion of mediators by effector cells, such as mast cells and basophils, upon stimulation with serial dilutions of putative allergens. Therefore, these assays represent an essential tool that mimics the *in vivo* degranulation process, which occurs upon allergen exposure in sensitized patients or in skin prick tests. Additionally, these assays are usually employed to investigate the allergenic potential of proteins and the reactivity of patients' sera's reactivity. Herein, we describe a simple 2-day protocol using an immortalized rat basophil leukemia cell line transfected and humanized with the human high-affinity IgE plasma-membrane receptor (FcεRI). This variant of the mediator release assay is a robust, sensitive, and reproducible *in vitro* cell-based system without the need to immobilize the antigen to solid matrices. The protocol consists of the following steps: (1) complement inactivation of human sera, (2) harvesting, seeding, and passive sensitization of the cells, (3) stimulation with antigen to cause mediator release, and (4) measuring of β-hexosaminidase activity as a surrogate for the released inflammatory mediators, such as histamine. The assay represents a useful tool to assess the capacity of the allergen-IgE cross-linking to trigger cell degranulation and can be implemented to standardize allergen extracts, to compare patients' reactivity to minor or major allergens and to allergenic extracts (pollen, cat dander, etc.), to investigate the potency of allergen homologs, isoforms, and fold-variants (e.g., hypoallergenicity), as well as the effects of ligands on the allergenic activity. A more recent application includes the use of the assay to monitor the treatment efficacy in the course of allergen immunotherapy.

INTRODUCTION:

Type I hypersensitivity reactions, characterized by Immunoglobulin E (IgE) production specific for a respective antigen, affect nearly a third of the world's population. These reactions are

associated with several allergic manifestations such as asthma and rhinoconjunctivitis and can even lead to systemic life-threatening reactions[1]. Unlike *in vivo* tests, immunochemical approaches, such as the enzyme-linked immunosorbent assay (ELISA), are solely suitable for investigating the target binding of antibodies but do not address the functional aspect of proteins that can cause immediate hypersensitivity reactions. The immobilization of the allergens on solid supports (e.g., ELISA plates) could cause changes in their structural integrity and the destruction of allergy relevant epitopes[2]. Even skin prick tests (SPT), the most common tool to confirm sensitization against certain allergens, have their limits concerning the detection of symptomatic IgE-mediated food allergy or allergen availability[3,4]. In order to find an ethical, highly specific, sensitive, and cost-effective method for testing the biological potency of allergens to cause a type I hypersensitivity reaction, the so-called mediator release assays have been established.

The principle of these assays relies on events following the sensitization phase and the accompanying ability of IgE to bind to the α-chain of the high-affinity receptors expressed on the surface of effector cells, such as mast cells and basophils. IgE is mainly produced by plasma cells in the mucosal-associated lymphoid tissue. Although it is the least abundant immunoglobulin (around 0.05% in non-atopic individuals) in the blood, it possesses an extraordinarily high biological activity being the main cause for allergic symptoms. The half-life of IgE can increase from 2–3 days to several weeks and even months when bound to its receptors on effector cells. Subsequent binding of an antigen to the variable region of two receptor-bound IgE molecules leads to their cross-linking followed by the induction of a downstream signaling cascade in the effector cell leading to degranulation and the release of several pro-inflammatory mediators causing vasodilation, such as histamine, serine proteases (e.g., tryptase), and prostaglandins[5–7]. The secretion of cytokines such as interleukin 4 (IL-4) and IL-13 are responsible for the maintenance of the inflammatory T helper 2 (Th2) response and the class-switching of B cells to IgE-producing plasma cells[5,8,9]. On the other hand, released thromboxane causes bronchoconstriction, and leukotrienes stimulate smooth muscle contraction as well as vascular leakage, and play a crucial role in airway inflammation leading to asthma or allergic rhinitis[10,11].

Research tools for analyzing most of the aforementioned mediators have been established, although with some major disadvantages. Tryptase assays are suitable clinical approaches for the measurement of systemic anaphylaxis through mast cell activation but their sensitivity and specificity in allergy diagnoses is too inaccurate compared to gold standard methods such as SPT. On the other hand, cysteinyl leukotriene assays are not capable of diagnosing allergies to β-lactams or nonsteroidal anti-inflammatory drugs[12]. Protocols for the measurement of histamine as a major mediator released in allergic reactions were already established in the 1960s. Once released in the peripheral blood, histamine is immediately degraded by histamine methyltransferases resulting in a plasma half-life of only a few minutes, making its analysis quite challenging[13]. Aside from its instability, the monitoring of histamine was shown to have a low specificity and sensitivity for drug allergies as well as commercial food proteins and wasp venoms[12].

In vitro models with effector cell lines have been introduced as an alternative to the labor-intensive procedures of isolation and cultivation of basophils from allergic patients to perform release assays. Therefore, the rat basophilic leukemia- (RBL-) based assay using the RBL-2H3 cell line has been established[3]. Since this cell line is not capable of binding human IgE, it was

first transfected with the α-, β-, and γ-chain of the human IgE plasma-membrane receptor (FcεRI). Several clones have been generated and tested for expression levels and homogeneity of the human α-chain, of which the clone RBL-30/25 emerged as the most promising candidate for *in vitro* testing. The signaling cascade induced upon receptor activation of the transfected clone was tested via calcium mobilization assays. As an indicator for degranulation and surrogate for histamine release, the lysosomal enzyme β-hexosaminidase was measured, which has the significant advantage of higher stability[14]. The mediator release using RBL-30/25 cells reaches up to 100% and is, therefore, used to test sera derived from allergic patients. The assay was tested for the mediator release after challenging sensitized cells with commercial allergen extracts. This led to the finding that there is a tremendous variation in the composition (of up to 60-fold regarding the total protein content) of allergen extracts derived from different manufacturers and used for diagnostic (e.g., SPT) or therapeutic approaches[3,15,16].

Herein, we provide a detailed description of the RBL protocol to perform the mediator release assay using serum from allergic donors. During passive sensitization, IgE in the serum is captured by the high affinity FcεR1 receptor expressed on the surface of the basophilic cells. Upon antigen-stimulation, bound IgEs specific for the antigen are cross-linked, triggering cell degranulation and the release of the mediator β-hexosaminidase. The activity of β-hexosaminidase is subsequently measured using a suitable substrate. For the assay, huRBL-2H3 cells were used, and termed huRBL in the following protocol. The protocol describes a standard antigen dilution series with 8 steps diluted 1:10 ranging from 1 µg/mL to 0.1 pg/mL of allergen.

PROTOCOL:

Ethical approval to use sera derived from birch pollen allergic patients was obtained from the Dutch ethical committee (approval number: NL65758.018.18).

1. Safety procedures

1.1. Work under sterile conditions using a biological safety class 2 workbench during the first day of the experiment (Biosafety Level 2). Follow the safety guidelines of the institution for the usage of human serum.

2. Complement inactivation of human sera

2.1. Harvest a dense culture of P3X63Ag8.653 cells (termed Ag8 cells henceforth), from the cell culture flask and transfer them into a centrifugation tube.

2.1.1. Use the following culture medium for these cells: Modified Eagles´s Minimum Essential Medium with reduced serum concentration, 1% Penicillin-Streptomycin (100 units Pen., 0.1 mg/mL Strep.), 5% heat-inactivated fetal calf/bovine serum (FCSi).

2.2. Centrifuge Ag8 cells for 5 min at 250 x *g* at room temperature.

2.3. Re-suspend the cell pellet to a final concentration of approximately 1 x 10^6 cells/mL in huRBL medium (Minimum Essential Medium Eagle with Alpha Modification, 4 mM L-Glutamine, 5% FCSi, 1% G418 (100% stock: 10 g/125 mL dH_2O).

NOTE: Maintain Ag8 cells by passaging for future use as well.

2.4. Dilute human sera 1:10 in Ag8 cell suspension. Final serum dilution in the assay will be 1:20.

NOTE: For sera with low specific IgE a 1:5 (1:10 final dilution) can be used.

2.5. Incubate for 1 h at 37 °C and 5%–7% CO_2.

3. Harvesting and seeding of huRBL cells

3.1. Aspirate the medium from a T-75 cell culture flask carefully without touching the huRBL cells (huRBL cells are adherent). Ensure that the cells are around 50%–90% confluent.

NOTE: Depending on the cell confluence, the cell content of a dense T-75 cell culture flask is usually enough for one to two 96-well plates.

3.2. Wash the cells twice by adding 10 mL of Dulbecco's phosphate-buffered saline (DPBS). Add DPBS to the opposite side of the flask and not directly onto the cells.

3.3. Aspirate DPBS and add 5 mL of pre-warmed 1x trypsin-EDTA (0.05%/0.02% EDTA diluted in DPBS) for cell detachment.

3.4. Incubate the flask for 5 min at 37 °C.

3.5. Detach cells by carefully tapping the flask.

3.6. Transfer the cell suspension into a 15 mL centrifugation tube and fill up with huRBL medium or DPBS to dilute the trypsin-EDTA.

3.7. Centrifuge the cells at 250 x *g* for 5 min at room temperature.

3.8. Aspirate the supernatant and resuspend the pellet in 5 mL of huRBL medium for cell counting.

3.9. Count the cells and dilute them in huRBL medium to obtain a final concentration of 2 x 10^6 cells/mL.

3.10. Use a sterile 96-well plate and add 50 µL of huRBL cell suspension per well, which is equivalent to 1 x 10^5 cells/well.

4. Passive sensitization of huRBL cells

4.1. Centrifuge the pre-incubated Ag8/serum suspension for 5 min at 250 x *g*.

4.2. Transfer 50 µL of the centrifuged Ag8/serum suspension to each well containing huRBL cells without disturbing the Ag8 cell pellet.

4.2.1. Include the no antigen control, which are sensitized, but unstimulated cells (do not add antigen), serving as an indication for the bottom signal plateau/background. Background and maximum lysis control wells do not need to be sensitized with serum. Add 50 µL of huRBL medium to control wells instead.

4.3. Cover the plate with the lid and incubate overnight at 37 °C and 5%–7% CO_2.

5. Antigen-stimulated degranulation and mediator release

5.1. Prepare the antigen dilution in 1x Tyrode's buffer (9.5 g/L Tyrode's salts, 0.1% bovine serum albumin (BSA), 0.5 g/L Sodium hydrogen carbonate ($NaHCO_3$) in dH_2O) in advance. A final amount of 100 µL per well is needed.

NOTE: Not every allergen, either purified from natural sources or recombinantly produced, might be stable in 1x Tyrode's buffer. Therefore, perform stability tests in 1x Tyrode's buffer prior to the assay procedure. Alternatively, dilute 1x Tyrode's buffer in deuterium oxide (D_2O) to increase the signal-to-noise ratio of the assay.

5.2. Make 8 dilutions of the antigen of interest of a 1:10 dilution series in reaction tubes starting with either 10 or 1 µg/mL of protein.

NOTE: Always test the dilution series beforehand. Alternatively, adapt the 1:10 dilution series (e.g., 1:5, 1:20, or 1:30) in order to cover the full release curve. In addition, the starting concentration can vary depending on the experimental setup.

5.3. To wash huRBL cells plated on the 96-well plate, remove the sera-containing cell medium first by carefully aspirating, inverting, and tapping the plate on absorbent paper.

5.4. Wash cells with 200 µL of 1x Tyrodes's buffer per well. Treat all wells similarly.

NOTE: Add the washing solution slowly to the cells in order to not disturb them.

5.5. Leave it for approximately 30 s and repeat the washing step three times in total.

5.6. After adding the washing solution for the final time, leave the solution in the wells until ready to continue with adding the antigen dilution.

NOTE: Avoid exposing cells to air for too long.

5.7. Transfer 100 µL of antigen solution to each well containing the pre-sensitized huRBL cells.

NOTE: If analyzing several different parameters, transfer the individual samples of the dilution series into an additional non-binding 96-well plate (use the same layout as on the huRBL plate) and transfer them afterward with a multichannel pipette directly on the huRBL cell plate. This way, exposing the cells to air for too long can be avoided, which might result in poor assay performance (lower/no signal).

5.8. Cover control wells (maximum lysis and non-sensitized background cells) with 100 µL of 1x Tyrode's buffer. Do not stimulate these control wells with the antigen.

5.8.1. Additionally, add 100 µL of 1x Tyrode's buffer to the sensitized no-antigen wells of the dilution series, which is needed to take antigen-independent spontaneous release of sensitized cells into account during data analysis.

5.9. Incubate huRBL cells for 1 h at 37 °C and 5%–7% CO_2.

6. Fluorescence measurement of β-hexosaminidase activity

6.1. Treat the wells of the maximum lysis control with 10 µL of 10% Triton X-100 per well and mix properly in order to lyse the cells completely and obtain the 100% release of β-hexosaminidase.

6.2. Add 50 µL of substrate solution into a new non-binding 96-well plate. Substrate solution for one 96-well plate: 5 mL of 0.1 M citric assay buffer, pH 4.5; and 80 µL of 10 mM 4-methylumbelliferyl N-acetyl-β-D-glucosaminide.

6.3. Transfer 50 µL of cell supernatant of all wells to the new plate containing the substrate solution.

NOTE: Pipette the supernatant carefully off the huRBL plate in order to not disrupt the huRBL cells.

6.4. Incubate the plate with substrate solution and cell supernatant for 1 h at 37 °C to allow conversion of the fluorogenic substrate.

NOTE: Keep the huRBL plate for cell viability assay.

6.5. Add 100 µL of stopping solution (15 g/L glycine, 11.7 g/L NaCl dissolved in dH_2O, pH 10.7) per well.

6.6. Measure the fluorescence at 360 nm excitation and 465 nm emission using a plate reader.

7. Data analysis

7.1. For basic calculations of percentage release, use any spreadsheet software.

7.2. For the background subtraction/baseline removal, subtract the average of the background wells from all other wells.

7.3. Calculate the mean of maximum lysis wells and express data of the dilution series in percentage. This way one can express data as a percentage of cell release normalized to the maximum enzyme release caused by cell lysis.

7.4. Complete dose-response mediator release curves are represented best as XY graphs with the antigen concentration on a log on the X-axis and percentage of mediator release on the Y-axis.

7.5. Add the values of the no-antigen control as a dashed line to indicate the background or the bottom plateau.

NOTE: Several similarly treated sera can be compared using this normalization strategy. For direct comparison, it is further recommended to calculate the half maximum release, which is the antigen concentration (in ng/mL) necessary for half maximal release defined as the average of the maximal and minimal values per curve. The antigen concentration to stimulate half maximal release is calculated by interpolation of the half maximal release value into a logarithmic regression line.

REPRESENTATIVE RESULTS:

The mediator release assay, based on huRBL cells (**Figure 1A and B**), results in a bell-shaped dose-response curve (**Figure 1C**). For simplified data representation, the antigen concentration necessary for the half maximum mediator release can be calculated using linear regression (**Figure 1D**). A cell viability assay is performed to exclude cytotoxic effects derived from either the sensitizing serum or the antigen used for stimulation (**Figure 1E**). The assay can be used to test the reactivity of different sera to a certain antigen. In our case, 4 out of 5 sera, derived from birch pollen allergic patients, responded to Bet v 1 stimulation. Serum #1 showed the highest mediator release (**Figure 2**). Serum #5 did not respond to Bet v 1 stimulation and, thus, might react to other birch pollen allergens (e.g., Bet v 2, profilin). These data indicate that Bet v 1 is a potent allergen responsible for IgE-mediated allergic symptoms. By using the huRBL assay, the cross-reactivity of IgE to homologous allergens can be assessed (**Figure 3**). Here, both birch pollen allergic patients respond well to Bet v 1, whereas only patient #2 responded also to Cor a 1, the Bet v 1-homologous food allergen found in hazelnuts. Based on these data, patient #2 most likely has higher Cor a 1-cross-reactive IgE levels than patient #1, resulting in oral allergy symptoms upon hazelnut consumption. Even the assessment of the hypoallergenic nature of mutant variants of allergens (decreased potency) can be analyzed and compared to their wild-type counterpart (**Figure 4**). In the provided example, the release curve of the fold variant shifted towards a higher antigen concentration compared to the wild-type allergen, resulting in a significantly higher concentration of antigen necessary to provoke half maximal release (**Figure 4B**). These data imply that the generated mutant/fold variant in less allergenic compared to the wild-type protein. This reduced potency to trigger IgE-mediated degranulation highlights the hypoallergenic character of the fold variant. Based on this assay, the fold variant is an interesting candidate for allergen-specific immunotherapy since it might cause reduced IgE-associated side effects during the treatment.

FIGURE LEGENDS:

Figure 1: Humanized RBL cells and a representative bell-shaped curve of IgE-allergen cross-linking-induced β-hexosaminidase release. RBL cells are adherent to the culture flasks, which gives them a rod-like shape as they are trying to attach themselves (**A**). An ideal level of confluence for cells to be harvested is no more than 90% (**B**). Cells are shown under magnification of 40x and 10x, respectively. Cells that were sensitized with human serum of a birch pollen allergic individual reacting upon challenge with recombinant Bet v 1 (rBet v 1), the major birch pollen allergen (**C**). As surrogate for mediator release, the β-hexosaminidase

activity is measured in cell supernatants. The bell-shaped curve results from a monovalent occupation of antigen epitopes on IgE due to the excess of allergen, which competitively inhibits the allergen-IgE cross-linking at high antigen concentrations. Another explanation for the low release at high allergen concentrations is the inhibition of intracellular pathways in presence of excess antigen. For determination of the allergen concentration necessary to obtain half maximal release, a logarithmic regression line based on the experimental values representing the linear part of the slope of the mediator release curve was used (**D**). The red dotted line represents the logarithmic regression line used for calculation. The formula of the regression line is shown in red. The half maximal release is defined as: half maximal release = (minimum release value + maximum release value)/2. In the example, the calculated half maximal release was 20.6%. The representative human serum used in this experiment was diluted 1:20 for incubation with huRBL cells, and the antigen concentration used for stimulation ranged from 100 µg/mL to 0.004 pg/mL of Bet v 1. A cell viability assay, in this case a MTT assay, was performed with the remaining cells after antigen stimulation to assess the influence of the sensitizing serum as well as of the antigen dilution on cell viability and cell count (**E**). Untreated background cells and lysed cells (maximum lysis) are shown as dotted line.

Figure 2: Representative curves of β-hexosaminidase percent release of five different human sera. The same antigen concentration range of rBet v 1 was incubated with huRBL cells that were sensitized with sera of different birch pollen sensitized individuals. There is a clear difference of percent release between the different patients corresponding to the severity of their symptoms. Notice that patient #5 is non-reactive to the major birch pollen allergen Bet v 1. All five human sera used to obtain these mediator release curves were diluted equally 1:20 for incubation with huRBL cells.

Figure 3: Cross-reactivity of IgE derived from sera of birch pollen sensitized patients with the Bet v 1 homologous hazelnut allergen Cor a 1. Two representative sera of patients sensitized to birch pollen strongly react to Bet v 1 as well as to a lesser degree to the homologous allergen Cor a 1. Patient 2 is shows a significant reaction to Cor a 1, and thus will likely exhibit oral allergy symptoms upon hazelnut consumption, compared to patient 1 where the mediator release is almost negligible. The dotted line represents the no-antigen control, which are cells sensitized with the human sera but not stimulated with an allergen, and, thus, serves as indication for the bottom signal plateau/background.

Figure 4: Comparison of percent release between rBet v 1 wild type and a hypoallergenic fold-variant. The same serum of a birch pollen-sensitized individual was incubated with rBet v 1 wild type (wt) and a hypoallergenic fold-variant of the major birch pollen allergen (**A**). Even though mediator release is seen in both antigens, there is a clear shift toward higher antigen concentrations when comparing the fold-variant to wild type rBet v 1 for the same percent release. A standard way of comparing the difference in percent release of different antigens is calculating the concentration of antigen needed to attain half maximum release (**B**). This is usually performed in biological replicates (testing of the same antigen range for each allergen in different human sera). Usually, in order to draw any significant conclusions, the mediator release is performed with sera from at least 8 to 10 different patients. Here the results of four different sera are plotted as an example. A paired *t*-test was used for statistical analysis. *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

Table 1: Troubleshooting.

DISCUSSION:

The herein described huRBL cell-based mediator release assay is a robust method that can easily be performed and implemented in any laboratory. The only requirement is that cells need to be cultivated under sterile conditions. The assay is used to assess the likelihood of an allergen or allergenic source to evoke patients' IgE-crosslinking and basophil degranulation[17]. The assay can easily be adapted to any allergen or allergenic source as long as the patient´s serum with a high level of specific IgE, recognizing the allergen of interest, is available. It is recommended to perform a cell viability assay in addition to the mediator release assay in order to account for any potential cytotoxic effects that might result in poor assay performance. This might be due to incomplete complement-inactivation of the sera or other serum-derived cytotoxic effects. Even the antigen itself, for instance, because of proteolytic/enzymatic activity, can harm the huRBL cells. We are usually using a cell viability assay with MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) to evaluate potential cytotoxic effects. The assay can easily be performed with the huRBL cells left after the cell supernatant was collected and transferred (see step 6.3. of the protocol). Compared to other immunochemical methods, such as ELISAs and western blotting, for determining the allergenic potential of either individual allergens or complex extracts based on allergen-IgE binding, this assay can detect not only the binding of IgE to an allergen but can also measure the functionality of both, human IgE and the allergen, to provoke IgE-mediated basophil degranulation[18]. Thus, it can aid in studying the severity of allergic symptoms *ex vivo* using patients' sera. The assay is reported to be more consistent and efficient than the classical passive cutaneous anaphylaxis tests since the assay utilizes the RBL-2H3 cells, which are relatively easy to handle and produce less variability in results compared to primary cells, such as mast cells or human basophils[19,20]. In addition to this, the assay provides a good representation of the biological activity of allergens and can accurately estimate the total allergen content in a given complex sample[3]. For troubleshooting certain steps in the protocol please refer to **Table 1**.

Regarding the applicability of this version of the mediator release assay, it has mostly been used for research purposes but also for the standardization of allergenic extracts based on their biological activity. This includes the analysis of different batches of SPT solutions, provocation test solution, as well as extracts used for allergen-specific immunotherapy; as shown for pollen, cat dander, house dust mite, and peanut extracts, as well as bee venom[3,17,21]. The technique can be applied especially in diagnosing food allergies, as it can detect even minimal amounts of allergenic constituents in complex food products such as peanuts, milk, wheat, and eggs[22]. In this respect, is has also been reported as a valuable tool for the assessment of allergenicity of animal food allergens, such as tropomyosins, and can aid in distinguishing potent allergens from non-allergens[23]. As a research tool, the assay is used to study the impact of food processing as well as to evaluate the influence of ligand binding to allergens and its effect on allergenicity[24,25]. For instance, the binding of Bet v 1 to ligands was shown not to affect the allergen-IgE cross-linking, although it caused an increase in its thermal and proteolytic stability[25]. The assay can be used to compare patient's reactivity to minor and major allergens, as well as to investigate the cross-reactivity of allergen homologues and isoforms, as shown in our example using Bet v 1 and the homologous food allergen Cor a 1 (**Figure 3**). Regarding allergen isoforms, the mediator release assay was used to identify the major allergen Amb a 1.01 as the most potent IgE-reactive isoform in ragweed

pollen (*Ambrosia artemisiifolia*). In comparison, the other two identified isoforms in ragweed pollen extracts, Amb a 1.02 and Amb a 1.03, showed reduced reactivity to patients' IgE[26].

In recent years, the assay has been applied to study potential anti-allergic compounds and novel hypoallergenic variants of allergens, aiding in the identification of suitable candidates for allergen-specific immunotherapy[27,28]. Another novel approach is to use the assay to monitor treatment efficacy in the course of allergen-specific immunotherapy. In this regard, our research group developed a huRBL assay inhibition system, which correlated well with the reduction of the patient's symptom score during allergen-specific immunotherapy[29]. The assay has also been proposed to study the immunosuppressive effects of TGFβ1 on allergen-induced IgE-mediated degranulation[30].

The limitations of the assay are that even though the huRBL cells possess some features of mast cells or basophils, they do not completely mimic the natural function of these effector cells. For example, mast cells widely express the pattern recognition receptor Toll-like receptor 4 (TLR4), necessary for pathogen recognition, whereas it is completely deficient in the RBL-2H3 cells[31]. Due to this difference in functionality, the assay does not fully mimic the real-life situation, which needs to be kept in mind when interpreting the data. In addition, since the huRBL cells are cancerous basophilic cells, changes in culture conditions and prolonged culturing can lead to phenotypic differences leading to altered results among different laboratories[20]. Another aspect is the choice of allergen concentration that has to be taken into account when adapting this method since high allergen concentrations might result in non-IgE mediated degranulation due to the presence of high amounts of proteases or endotoxins[18]. Other limitations are the dependency on human sera with relatively high specific IgE levels (RAST class 5–6), and the need for cell culture systems, which remains an obstacle that needs to be overcome in order to implement the technique in the daily clinical routine.

Apart from these limitations, the huRBL assay represents a valuable research tool for the diagnosis and treatment of allergic diseases and can be used in a wide range of applications.

ACKNOWLEDGMENTS:

The authors would like to thank Prof. Dr. Stefan Vieths of the Department of Molecular Allergology, Paul-Ehrlich-Institut, Langen, Germany, for providing the humanized/FcεRI-transfected RBL cells and for giving his consent to write this research methodology paper. We want to thank Prof. Dr. Fatima Ferreira for providing excellent feedback. We further would like to thank Prof. Dr. Ronald van Ree and Dr. Jaap Akkerdaas of the Department of Experimental Immunology, Amsterdam University Medical Centers, location AMC, Amsterdam, The Netherlands, for giving their consent to publish representative data provided in this methods paper, which were generated in the course of the project BM4SIT – innovations for allergy (www.BM4SIT.eu). The work of the authors has been supported by the Austrian Science Fund (Project P32189), by the University of Salzburg priority program Allergy-Cancer-BioNano Research Centre, by the doctoral program Immunity in Cancer and Allergy—ICA funded by the Austrian Science Fund (FWF W01213), and by the BM4SIT project (grant number 601763) from the European Union's Seventh Framework Program FP7.

DISCLOSURES:

The authors have nothing to disclose.

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Figure

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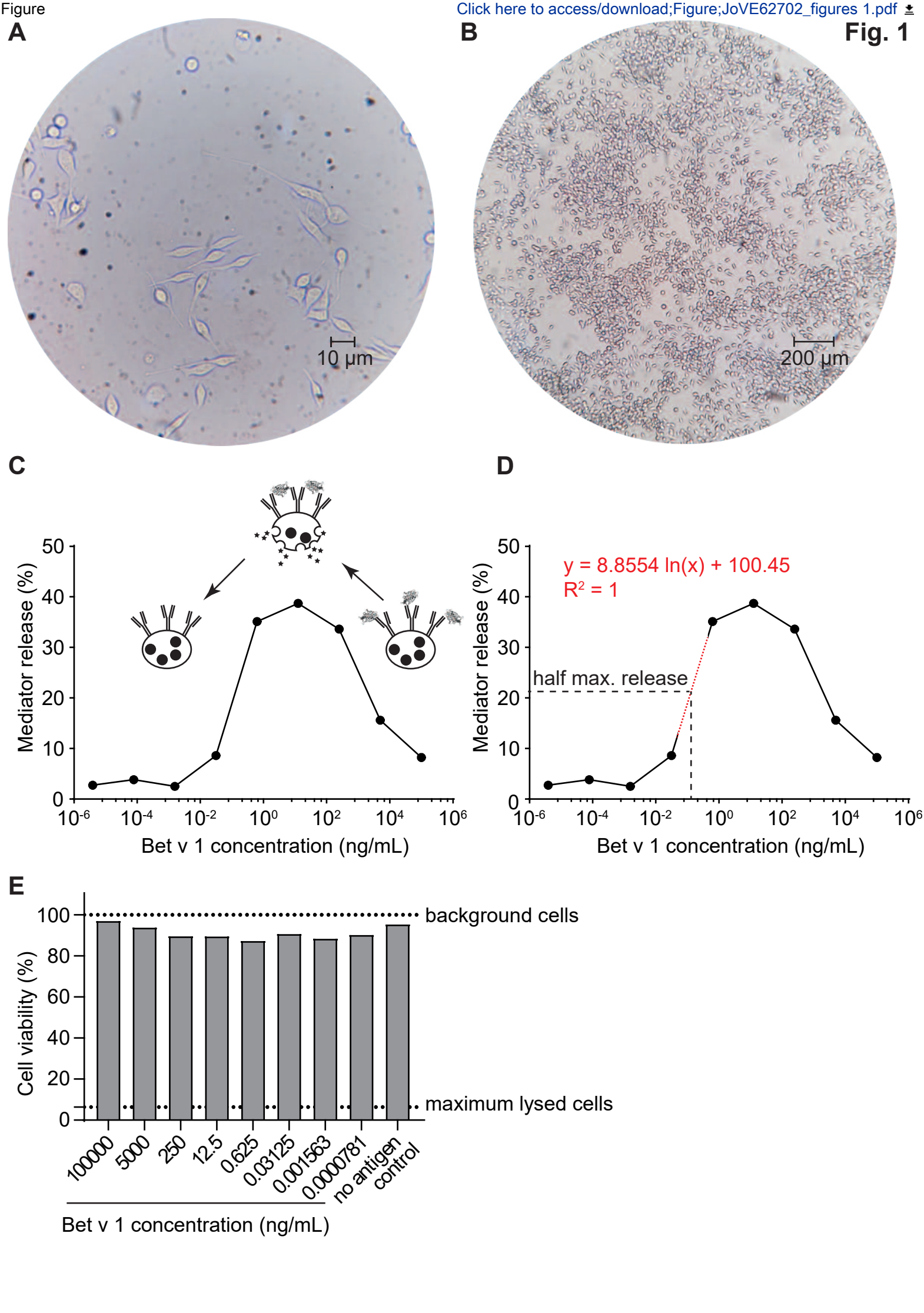
Fig. 1



Figure

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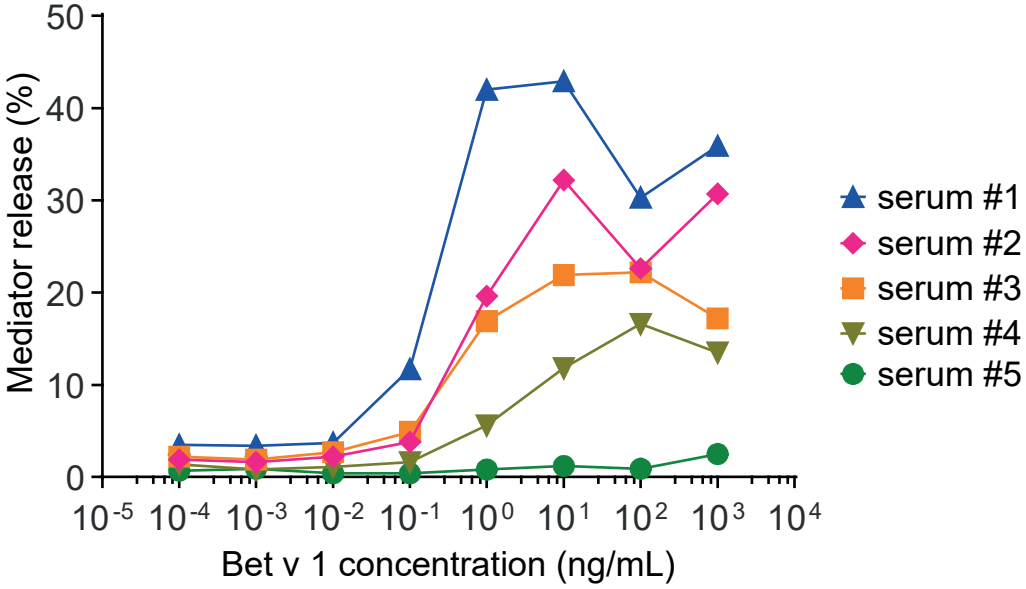
Fig. 2



Figure

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Fig. 3

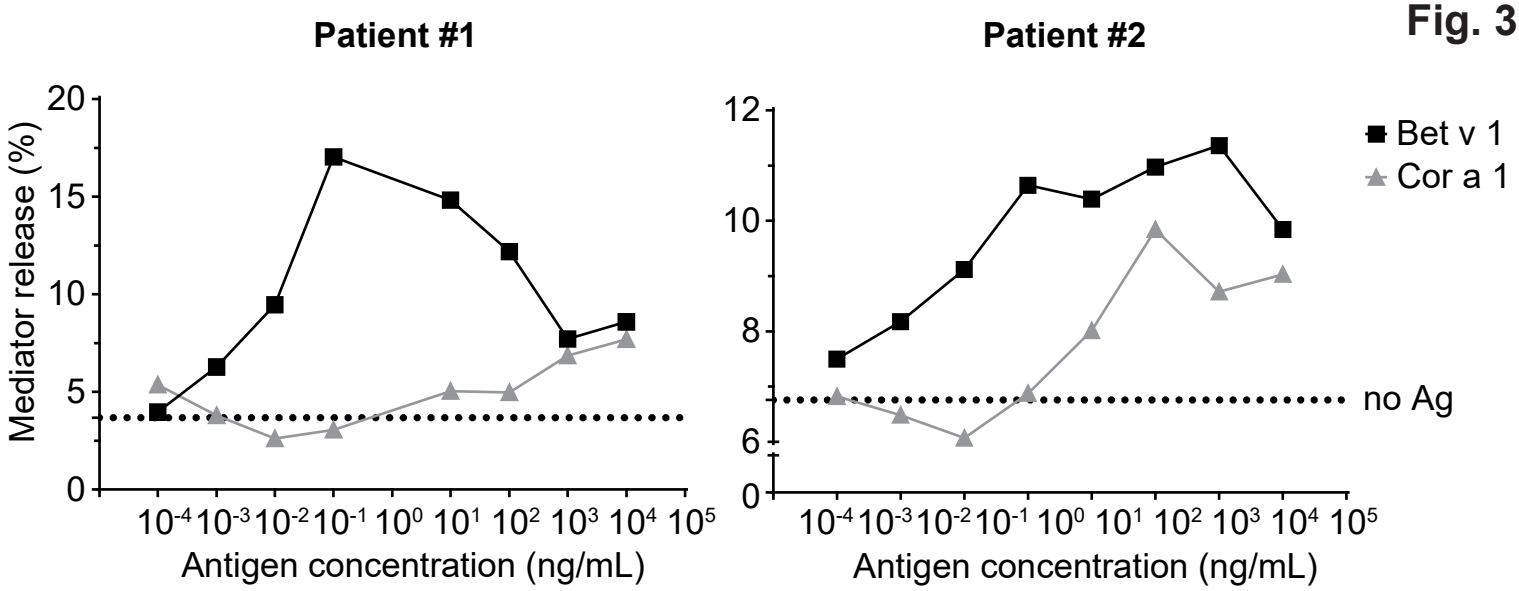

Fig. 4

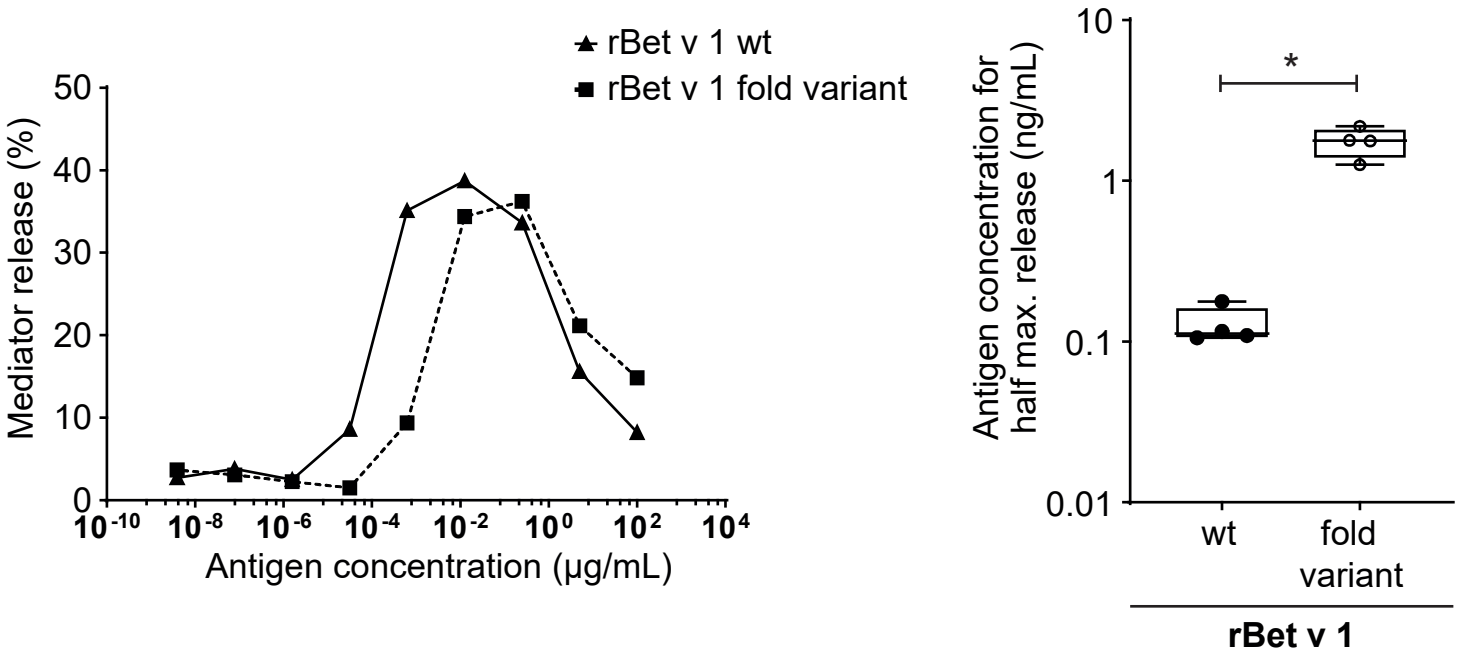

Figure 1

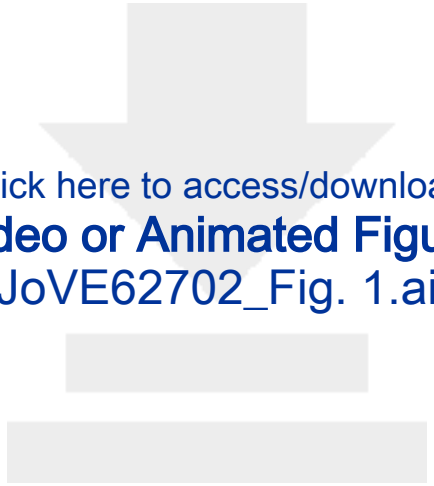
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Figure 2

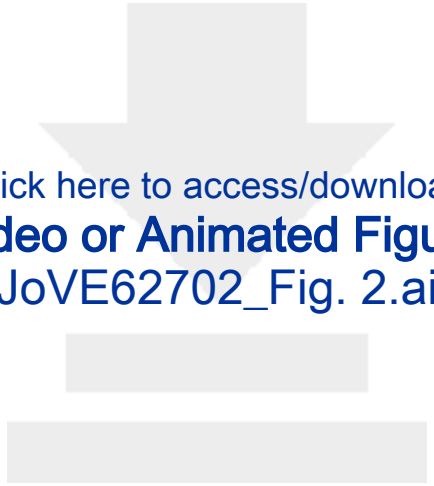
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Figure 3

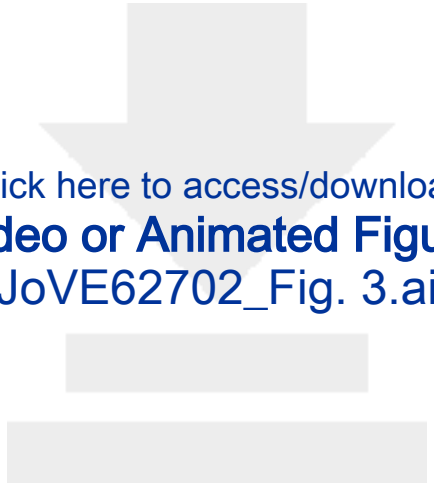
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Figure 4

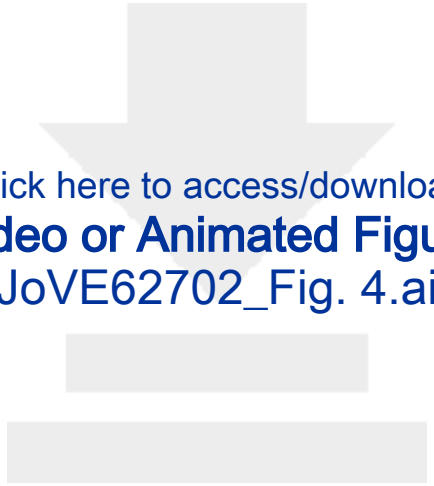
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Table

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Potential questions and troubleshooting
Assay-to-assay variability due to altered cell responsiveness
Sera contains low levels of specific IgE
Not enough cells to perform the assay
Cytotoxic effects of sera, i. e. due to incomplete complement-inactivation
Low signal
Allergen is not stable in Tyrode's buffer (e. g. precipitation)
Problems finding the right starting concentration for the respective allergen
Poor assay performance indicated by low/no signal
How do I know if the bottom signal plateau is reached?
Do I need a positive control in addition to the maximum lysis wells?
How many wells do I need?
How many sera should I test? And do I need replicates?

Solution
Ensure that the cell passage cycle number does not exceed 20 to 30 passages. Make frozen stocks at early pa
Rather rely on biological replicates (use of different sera) than technical ones.
A lower final serum dilution can be used (i. e. 1:10) instead of 1:20. Conversely, sera containing high levels of
Make sure the confluency in a T-75 flask is around 50-90%. Passage more flasks.
Perform a cell viability assay in addition to the mediator release assay. Increase Ag8 concentration to avoid in
Improve signal-to-noise ratio of the assay by diluting the 1x Tyrode´s buffer in deuterium oxide (D_2O) instead
Make stability tests in 1x Tyrode's buffer prior to the assay procedure. Substitution of Tyrode's buffer is not r
Adaption of dilution series to cover the full release curve (more dilution steps, 1:20 dilution instead of 1:10).
Avoid cytotoxic effects from either sera or antigen stimulation (e.g. enzymatic allergens). Wash and soak the
Add "no antigen" controls to your plate. These are sensitized cell, which were only stimulated with 1x Tyrode
As additional positive control a serum and antigen combination known to cause degranulation can be used o
That depends on your titration series, the number of antigens and sera you want to analyze. Plan the layout
Although the assay is quite robust, there is some assay-to-assay variability due to altered cell responsiveness.

ssages for future experiments.
specific IgE can be further diluted (1:30 or 1
ıcomplete complement-inactivation.
of dH_2O, or by using a sera with higher leve
ecommended.
cells carefully. Avoid exposure to air for too

´s buffer but without an allergen.
r an anti-FcεR1 antibody.
for the 96-well plates according to how many sera/antigens you are going to test. Do not forget to adc
. Therefore, it is recommended to rather rely on biological replicates (using different sera) than on tecl

d the "no antigen controls", the background cells (non-sensitized, non-stimulated) as well as the maxi
hnical replicates. A minimum of eight different sera is sufficient to analyze allergens. However, as sho

num lysis wells.
ʍn in Fig. 4B, significant results can already be obtained using less sera.

Table 1: Table of materials

Product name
4-Methylumbelliferyl N-acetyl-β-D-glucosaminide
96-well plate for huRBL cells (Nunc MicroWell 96-Well, Nunclon Delta-treated, flat-bottom microplate)
96-well plate for substrate solution and cell supernatant (Greiner Bio-One non-treated 96-well microplates)
Bovine serum albumin (BSA)
Citric acid
Dulbecco's phosphate-buffered saline (DPBS without calcium and magnesium)
G418
Glycine
Heat-inactivated fetal calf/bovine serum (FCSi)
L-Glutamine (200 mM)
Minimum Essential Medium Eagle with Alpha Modification, with ribonucleosides, deoxyribonucleosides and sodiu
Opti-MEM reduced serum medium, GlutaMAX supplement
Penicillin-Streptomycin (10K units Pen. 10 mg/mL Strep.)
Sodium chloride (NaCl)
Sodium hydrogen carbonate (NaHCO3)
Triton X-100
Trypsin-EDTA
Tyrode’s salt

Company	Catalog number
Sigma	M2133
ThermoFisher Scientific	167008
Fisher Scientific	655101
Sigma	10735078001
Applichem	131018
Sigma	D8537
Sigma	A1720
Applichem	A3707
Sigma	F0804
Sigma	G7513
Sigma	M8042
Gibco/ThermoFisher Scientific	51985034
Sigma	P4333
Applichem	A2942
Applichem	131638
Sigma	X100
Sigma	59418C
Sigma	T2145

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Reply: Manuscript was proofread precisely.

2. Please provide an institutional email address for each author.
Reply: Institutional email addresses of the authors have been added in line 6-7.

3. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
Reply: Commercial language, including trademark symbols or registered symbols as well as company names, has been removed from the manuscript.

4. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee (Data in Figure 3 shows the use of human sample)
Reply: Ethical statement for the usage of human blood was added. See lines 106 to 108 of the revised manuscript.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
Reply: The protocol was adjusted to the imperative tense and safety procedures were added (e.g. lines 149, 172 and 192 of the revised manuscript). For safety procedures see lines 110 to 129.

6. Line 98-114: The Protocol should contain only action items that direct the reader to do something. Please move the discussion about the protocol to the Discussion.
Reply: The part was partially moved into the discussion section (lines 314 to 321) as well as to the "troubleshooting" table suggested by reviewer #2 (see troubleshooting table).

7. For SI units, use standard abbreviations when the unit is preceded by a numeral throughout the protocol. Abbreviate liters to L to avoid confusion. Examples: 10 mL, 8 µL, 7 cm2
Reply: All SI unit abbreviations have been changed accordingly.

8. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

Reply: Essential steps for the video where highlighted in yellow.

9. Please do not use the &-sign or the word “and” when listing authors in the references. Authors should be listed as last name author 1, initials author 1, last name author 2, initials author 2, etc. End the list of authors with a period. Title case and italicize journal titles and book titles. Do not use any abbreviations. Article titles should start with a capital letter and end with a period and should appear exactly as they were published in the original work, without any abbreviations or truncations.
Reply: The endnote style was changed according to the editor´s comments.

10. Please submit each figure individually as a vector image file to ensure high resolution throughout production: (.psd, ai, .eps.).
Reply: Figure were uploaded as .ai files.

11. Figure 1: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate Figure Legend (Figure 1A,B). Please revise the X-axis units to “ng/mL” instead of “ng/ml” (Figure 1C,D) and include a Y-axis title in Figure 1D.
Reply: Figure 1 was changed accordingly and the scale bar was added.

12. Figure 2/3: Please revise the X- axis units to “ng/mL” instead of “ng/ml
Reply: Figure 2 and 3 were changed accordingly.

13. Figure 4: Please revise the X- axis units to “µg/mL” instead of “µg /ml (Figure 4A) and the Y axis units to “ng/mL” instead of “ng/ml” (Figure 4B). Please include the details of the statistical analysis performed in the figure legend.
Reply: Figure 4 was changed accordingly and statistical test was included in the respective figure legend.

14. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.
Reply: Trademark and registered symbols have been removed from the Table of Equipment and Materials.

Reviewers' comments:
Reviewer #1:

Major Concerns:

1. Line 50 - "although is the least abundant immunoglobulin" should be "although it is the least abundant immunoglobulin" or "although the least abundant immunoglobulin"
Reply: According to the comment, the proposed change has been made. See line 55 of the revised manuscript.

2. Line 176 - "Soak the cells shorty" should be changed to "Soak the cells shortly" or "Soak the cells briefly"
Reply: The spelling error has been corrected. See line 197 of the revised manuscript.

3. Line 185 - What poor assay performance can be expected? High background or low signal?
Reply: Additional information about the assay performance has been included. See line 206 of the revised manuscript.

4. Line 154-155 Shouldn't serum be added to control wells? It is possible something in the serum could alter the amount of B-Hexosamindase/amount of background release.
Reply: The authors would like to thank reviewer #1 for this highly valuable comment. In fact, in addition to the background cells (no sera and no antigen), we always include "no antigen" controls (sensitized cells which were only stimulated with the tyrodes buffer and no antigen. The release of these cells serves as good indicator for the baseline level/indicator for spontaneous release caused by sensitization of the cells (see figure 3). Indeed, cytotoxic effects derived from sera i.e. incomplete complement-inactivation would result in poor assay performance (no signal due to cytotoxicity). See lines 172-174 of the revised manuscript.

5. Better to measure % release for each well (by lysing all cells, transferring 50uL of lysate to another plate and adding substrate), rather than comparing to samples that were not exposed to serum. This better accounts for changes in total available B-Hexosaminidase due to slower cell growth, cell detachment, etc. caused by serum/antigen exposure, random chance or technical errors. Ultimately this corrects for the fact that different wells may have different amount of cells/different amount of B-Hexosaminidase, especially important after overnight incubation.
Reply: We agree with Reviewer #1 that additional controls are necessary in order to take factors such as slower cell growth, cell detachment, and other technical errors into account. However, lysing all cells with Triton X-100 would only result in maximal release, and the degranulation effect would not be interpretable anymore. Although, it would make sense to add this control for each stimulation of sensitized cells and then to normalize each well to the respective max. lysis control. The assay depends on rather high amounts of patient sera, which are usually limited. Therefore, although important, the implementation of such control step might be difficult. For now, variations in cell count, viability as well as detachment are considered using the MTT assay (cell viability assay). We have pointed out in the text the importance of the "no antigen control" and the MTT assay, see lines 172-174 of the revised manuscript as well as the "troubleshooting" table.

6. As the authors mention in the discussion, RBL cells can show variability in the amount of degranulation they exhibit. For this reason a positive control to show that HuRBL can degranulate should be suggested to remove the possibility of false negatives. Ideally, a control serum/antigen combo previously known to cause significant degranulation or patient serum and anti-IgE.
Reply: A positive control to distinguish between false-positive and true-positive results is indeed relevant. We have added information about using a positive control in the new table of revised manuscript, see "troubleshooting" table.

7. Were LDH/viability assays done for figures 2/3/4? Could the authors provide one to give an example of how cell viability might affect apparent mediator release.
Reply: We have included representative data of the cell viability assay in figure 1. In general, cytotoxic effects in this assay result in reduced (no/hardly any signal) or very noisy signal (not representative for a dose-response curve). We have incorporated this information in the text of the revised manuscript, see lines 314 to 321, Figure legend 1, and the "troubleshooting" table.

8. Could the authors expand on how to calculate the half maximal antigen concentration? Is it simply finding the half maximal release and finding the part of the curve that intersects with this value? If so, how to linear curves and logarithmic regression lines play a role as described in Figure 1.
Reply: The half maximal antigen concentration is determined via interpolation of the half maximal release value into a logarithmic regression line. The regression line is necessary to provide a fit model for all experimental values on the linear part of the curve. However, other regression models/curve fitting models would work as well (e.g. 4P curve fitting), although such analysis models are not recommended mediator release data, since the release curves are usually not comparable with ideal sigmoidal curves (with a stable bottom and top plateau) as the release tends to decrease again at higher concentrations. We have included more information of how to determine the half maximal antigen concentration, see lines 246 to 249 of the revised manuscript.

Minor Concerns:

1. The authors recommend keeping the huRBL plate for cell viability testing via MTT assay (step 6.4), however the viability data are not provided. It would be helpful to include the corresponding RBL-2H3 cell viability data for any of the mediator release data sets presented in the manuscript to illustrate the reliability of the assay.
Reply: We have added representative cell viability data. See reply to major concern 7.

2. It would be helpful if the authors could provide some insight into the maximum passage cycle number up to which the humanized RBL-2H3 cells can produce reliable data in this assay setup.
Reply: Additional information about the maximum passage cycle number has been provided. See "troubleshooting" table of the revised manuscript.

Reviewer #2:
Minor Concerns:

Line 35 - immunoglobulins are by definition antibodies, therefore IgE antibodies is rather redundant. Please correct along the manuscript.
Reply: We have changed the manuscript accordingly. See lines 53, 59 and 264 of the revised manuscript.

Lines 35-37 - this is not the correct citation for this information. Please revise.
Reply: The sentence has been revised and the correct citation for this information has been added. See lines 39 to 42 of the revised manuscript.

Lines 107-110. The sentence is difficult to follow. Please revise.
Reply: The sentence has been moved to the discussion section as suggested by the Editor and revised in order to be more understandable. See lines 314 to 321 of the revised manuscript.

Discussion - This humanized mediator release assay can be also used for allergenicity assessment of novel animal foods, as well as to discriminate real allergenic proteins from "false" allergens. For more information please consult Klueber et al. 2020 https://doi.org/10.1111/cea.13503 or similar.
Reply: We would like to thank reviewer #2 for this highly relevant comment. We have added in the discussion section of the revised manuscript that the huRBL assay is a valuable tool for the assessment of allergenicity of animal food allergens and to discriminate real allergens causing IgE-mediated symptoms from "false" allergens. See line 338 of the revised manuscript.

Table - a table summarizing potential troubleshooting of the application of this assay would also be of interest for researchers, who want to implement this assay. Please consider adding a table with some potential examples.
Reply: A summarizing table with the potential troubleshooting of the application has been added. See additional table.