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

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Author Questionnaire

1. **Microscopy**: Does your protocol require the use of a dissecting or stereomicroscope for performing a complex dissection, microinjection technique, or something similar? **No**

2. **Software:** Does the part of your protocol being filmed include step-by-step descriptions of software usage? **No**

3. **Interview statements:** Considering the COVID-19-imposed mask-wearing and social distancing recommendations, which interview statement filming option is the most appropriate for your group? **Please select one**.

   ☒ Interviewees wear masks until the videographer steps away (≥6 ft/2 m) and begins filming, then the interviewee removes the mask for line delivery only. When the take is captured, the interviewee puts the mask back on. Statements can be filmed outside if weather permits.

4. **Filming location:** Will the filming need to take place in multiple locations? **No**

Current Protocol Length

Number of Steps: 16
Number of Shots: 39

Introduction

1. Introductory Interview Statements

REQUIRED:

1.1. **Lorenz Aglas:** The assay is an important research tool to simulate the degranulation of basophils induced by the cross-linking of allergens and patient´s IgE. Thus, type 1 allergic reactions can be mimicked.

1.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

1.2. **Litty Johnson:** The assay is robust, reproducible, and tailorable. Moreover, it is highly sensitive, as it can be performed with small amounts of recombinant or purified allergens or even with complex allergen extracts.

1.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

OPTIONAL:

1.3. **Mario Wenger:** The method is used to diagnose allergies as it analyzes the reactivity of patient´s IgE to allergens. Also, this method is suitable to analyze cross-reactivity and to monitor AIT-treatment-efficacy.

1.3.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.

Ethics Title Card

1.4. Procedures involving human subjects have been approved by the Dutch ethical committee at Amsterdam University Medical Centers, Amsterdam, The Netherlands.

Protocol

2. Complement Inactivation of Human Sera

2.1. Begin by harvesting Ag8 *(A-G-8)* cells from the cell culture flask **[1-TXT]** and transfer the cells into a centrifugation tube **[2]**. Pellet down the cells by centrifugation for 5 minutes at 250 x *g* at room temperature **[3]**.

2.1.1. WIDE: Talent harvesting the cells. **TEXT: P3X63Ag8.653 cells**

2.1.2. Talent transferring the cells in the centrifuge tube.

2.1.3. Talent placing the tube in the centrifuge. *Videographer: Obtain multiple usable takes because this will be reused in 4.1.1*

Author's NOTE: Shots 2.1.1 and 2.1.2 were combined in one shot and labelled as 2.1.1 + 2.1.2.

2.2. Aspirate the supernatant **[1]** and resuspend the cell pellet to a final concentration of approximately 1 x 10^6 cells per milliliter in huRBL *(humanized R-B-L cells)* medium **[1-TXT]**. Dilute human sera 1 to 10 in Ag8 cell suspension for the final serum dilution of 1 to 20 in the assay **[2]** and incubate for 1 hour at 37 degrees Celsius and 5 to 7% carbon dioxide **[3]**.

2.2.1. Talent resuspending the pellet. **TEXT: See the text for huRBL medium preparation details.**

2.2.2. Talent diluting the human sera.

2.2.3. Talent placing the tube in the incubator. *Videographer: Obtain multiple usable takes because this will be reused in 3.3.3 and 4.2.3*

3. Humanized-RBL Cell Culture

3.1. When the huRBL cells reach 50 to 90% confluency **[1]**, aspirate the medium from a T-75 cell culture flask carefully without touching the adhered huRBL cells **[2]**. Wash the cells twice by adding 10 milliliters of DPBS *(D-P-B-S)* to the opposite side of the flask and not directly onto the cells **[3]**. *Videographer: This step is important!*

3.1.1. LAB MEDIA: Figure 1B

3.1.2. Talent aspirating the medium in the flask.

3.1.3. Talent adding DPBS in the flask.

3.2. Aspirate DPBS **[1]**, add 5 milliliters of pre-warmed 1 times trypsin-EDTA *(E-D-T-A)* for cell detachment **[2]** and incubate for 5 minutes at 37 degrees Celsius **[3]**. Gently tap the flask to detach the cells **[4]**. *Videographer: This step is important!*

3.2.1. Talent aspirating DPBS.

3.2.2. Talent adding trypsin-EDTA solution in the flask.

3.2.3. Talent placing the flask in the incubator.

3.2.4. Talent tapping the flask on the bench.

3.3. Transfer the cell suspension into a 15-milliliter centrifugation tube **[1]** and fill the tube with huRBL medium or DPBS to dilute the trypsin-EDTA **[2]**. Centrifuge the cells at 250 x *g* for 5 minutes at room temperature **[3]**.

3.3.1. Talent adding cell suspension in 15 mL tube.

3.3.2. Talent adding huRBL medium or DPBS in the tube.

3.3.3. *Use 2.2.3*

3.4. Aspirate the supernatant **[1]** and resuspend the pellet in 5 milliliters of huRBL medium for cell counting **[2]**. After counting the cells, dilute the cells in huRBL medium to obtain a final concentration of 2 x 10^6 cells per milliliter **[3]**.

3.4.1. Talent aspirating the medium.

3.4.2. Talent resuspending the pellet.

3.4.3. Talent diluting the cells with huRBL medium.

3.5. Add 50 microliters of huRBL cell suspension per well equivalent to 1 x 10^5 cells per well in a sterile 96-well plate **[1]**.

3.5.1. Talent adding huRBL cell suspension in the 96-well plate.

4. Passive Sensitization of Humanized-RBL Cells

4.1. Centrifuge the pre-incubated Ag8-serum suspension **[1-TXT]** and transfer 50 microliters of the centrifuged Ag8-serum suspension to each well containing huRBL cells without disturbing the Ag8 cell pellet **[2]**.

4.1.1. *Use 2.1.3* **TEXT: Centrifugation: 5 min, 250 x *g*, RT**

4.1.2. Talent transferring centrifuged Ag8-serum suspension in the wells.

4.2. Use sensitized-unstimulated cells without antigen as no antigen control for indication of the bottom signal plateau or background and do not sensitize the background and maximum lysis control wells **[1]**. Cover the plate with the lid **[2]** and incubate overnight at 37 degrees Celsius and 5 to 7% carbon dioxide **[3]**.

4.2.1. Shot of plate with the wells labeled as no antigen control, background control, and maximum lysis control.

4.2.2. Talent placing the lid on the plate.

4.2.3. *Use 2.2.3*

5. Antigen-stimulated Degranulation and Mediator Release

5.1. Aspirate the sera-containing cell medium **[1]**, invert and tap the plate on the absorbent paper to empty the plate for washing huRBL cells **[2]**. Wash the cells 3 times by adding 200 microliters of Tyrodes's buffer per well and incubate for approximately 30 seconds per wash for the first two washes **[3]**. *Videographer: This step is important!*

5.1.1. Talent aspirating the medium in the wells.

5.1.2. Talent inverting and tapping the tube over an absorbent paper.

5.1.3. Talent adding Tyrodes's buffer in the wells.

5.2. After adding Tyrodes's buffer for the third time **[1]**, aspirate the buffer and leave the solution in the wells until ready to add the antigen dilution **[2]**. *Videographer: This step is important!*

5.2.1. Talent adding Tyrodes's buffer in the wells.

5.2.2. Talent aspirating the buffer.

5.3. Transfer 100 microliters of antigen solution to each well containing the pre-sensitized huRBL cells but do not stimulate the maximum lysis and non-sensitized background cells with antigen **[1]**. *Videographer: This step is important!*

5.3.1. Talent adding antigen solution in the wells.

5.4. Add 100 microliters of Tyrode's buffer in the maximum lysis control wells, non-sensitized background control wells, and sensitized no-antigen wells **[1]**. Incubate the cells for 1 hour at 37 °C and 5 to 7% carbon dioxide **[2]**.

5.4.1. Talent adding Tyrode's buffer in the wells.

5.4.2. Talent placing the plate in the incubator.

6. β-hexosaminidase Activity Measurement

6.1. Treat the maximum lysis control wells with 10 microliters of 10% Triton X-100 per well **[1]** and mix properly to lyse the cells completely for 100% release of beta-hexosaminidase **[2]**. Add 50 microliters of substrate solution into a new non-binding 96-well plate **[3-TXT]**. *Videographer: This step is important!*

6.1.1. Talent adding triton x-100 in the wells.

6.1.2. Talent mixing triton x-100 by pipetting in the wells.

6.1.3. Talent adding substrate solution in the wells. **TEXT: See the text for substrate solution preparation details.**

6.2. Transfer 50 microliters of supernatant from the wells of huRBL cells containing plate into the new plate containing the substrate solution **[1]** and incubate the plate for 1 hour at 37 degrees Celsius to allow conversion of the fluorogenic substrate **[2]**. *Videographer: This step is important!*

6.2.1. Talent transferring supernatant from the wells of huRBL cells containing plate to the wells of substrate solution containing plate.

6.2.2. Talent placing the plate in the incubator.

6.3. Add 100 µL of stopping solution per well and measure the fluorescence as described in the text manuscript **[1]**.

6.3.1. Talent adding stopping solution in the wells.

Results

7. Results: Effect of Allergen on β-hexosaminidase Release

7.1. The bell-shaped curve obtained in the beta-hexosaminidase activity assay **[1]**, indicating the monovalent occupation of antigen epitopes on IgE *(Immunoglobulin-E)* due to the excess of allergen, which inhibits the allergen-IgE cross-linking at high antigen concentrations **[2]**.

7.1.1. LAB MEDIA: Figure 1C

7.1.2. LAB MEDIA: Figure 1C *Video editor: Please emphasize the antigen-antibody reaction above the bell-shaped curve.*

7.2. The antigen concentration necessary for the half-maximum mediator release was calculated using the linear regression analysis **[1]**. The cell viability assay was performed to exclude the cytotoxic effects derived from either the sensitizing serum or the antigen used for stimulation **[2]**.

7.2.1. LAB MEDIA: Figure 1D *Video editor: Please emphasize the equation in red font color and red dotted line in the curve.*

7.2.2. LAB MEDIA: Figure 1E

7.3. Five different human serums were used for the beta-hexosaminidase activity assay **[1]**, and 4 out of 5 sera, derived from the birch pollen allergic patients, responded to Bet v-1 stimulation, demonstrating that Bet-v-1 is a potent allergen responsible for IgE-mediated allergic symptoms **[2]**.

7.3.1. LAB MEDIA: Figure 2

7.3.2. LAB MEDIA: Figure 2 *Video editor: Please emphasize curves of serum #1 to serum #4*

7.4. The cross-reactivity of IgE to homologous allergens was assessed **[1]**. The response to Bet v-1 was found in both patients **[2]**, but patient-2 also responded to Cor a-1, the Bet v-1-homologous food allergen indicating higher Cor-A-1-cross-reactive IgE levels in patient-2 **[3]**.

7.4.1. LAB MEDIA: Figure 3

7.4.2. LAB MEDIA: Figure 3 *Video editor: Please emphasize the black line with black square data points in both the graphs*

7.4.3. LAB MEDIA: Figure 3 *Video editor: Please emphasize the gray line with gray triangle data points in both the graphs.*

7.5. The hypoallergenic nature of mutant variants of the allergens was assessed and compared to their wild-type counterpart **[1]**.

7.5.1. LAB MEDIA: Figure 4

7.6. The release curve of the Bet v-1 fold variant shifted towards a higher antigen concentration compared to the wild-type allergen **[1],** resulting in a significantly higher concentration of antigen to provoke the half-maximal release, which makes the mutant less allergic and, therefore, a candidate for allergen-specific immunotherapy **[2]**.

7.6.1. LAB MEDIA: Figure 4A *Video editor: Please emphasize the black dotted line with square data points of "rBet v 1 fold variant"*

7.6.2. LAB MEDIA: Figure 4B *Video editor: Please emphasize the bar of fold variant.*

Conclusion

8. Conclusion Interview Statements

8.1. **Litty Johnson:** Do not forget to leave the washing solution on the cells until applying the antigen dilutions to avoid the cells from drying out, which would result in poor assay performance.

8.1.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera. *Suggested B-roll: 5.2.1*

8.2. **Lorenz Aglas:** The assay can be used for many different applications, such as the standardization of allergenic extracts based on their biological activity, including SPT solutions or extracts used for allergen-specific immunotherapy.

8.2.1. INTERVIEW: Named talent says the statement above in an interview-style shot, looking slightly off-camera.