

Chapter 12

Use of Humanized RS-ATL8 Reporter System for Detection of Allergen-Specific IgE Sensitization in Human Food Allergy

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Abstract

Allergen-specific Immunoglobulin E (IgE) determination lies at the heart of diagnosis of sensitization to food and other allergens. In the past few years, reporter systems capable of detecting the presence of allergen-specific IgE have been developed by several labs. These rely on humanized rat basophil leukemia cell lines stably transfected with reporter genes such as firefly luciferase. In this chapter, we describe protocols for the use of the RS-ATL8 cell line (IgE cross-linking-induced luciferase expression; EXiLE) in 96-well and 384-well formats. We also describe optional treatment steps for enveloped virus and complement inactivation.

Key words Reporter system, Luciferase, Humanized RBL, IgE, Allergy, RS-ATL8, EXiLE

1 Introduction

Immunoglobulin E (IgE) is the antibody responsible for Type I allergic reactions via activation of the tetrameric high-affinity IgE receptor FcεRI, present on basophils and mast cells. Several cellular diagnostic techniques are used for the detection of specific IgE (sensitization) to suspected allergens. The most common diagnosis is the skin prick test (SPT), in which allergens are injected intradermally, and the subsequent wheal-and-flare reaction induced by the activation of resident mast cells measured against histamine and negative controls. While this method is simple, relatively safe, and cheap, as it requires no expensive instrumentation or reagents for detection, it is not amenable to the testing of large numbers of allergens. More importantly in the context of food allergy, skin prick testing, which is widely accepted in testing of environmental allergens such as tree or grass pollens, is more controversial for food allergens due to a high number of false positive reactions. Such false positive SPT tests may also occur in individuals who are tolerant

and thus cannot always be taken as an indication of clinical sensitivity to the offending allergen-containing food [1]. Furthermore, SPT does not reflect the more complex process of allergen degradation during transit through the gastrointestinal tract. The ability of proteins to resist conditions found in the GI tract (e.g., to withstand the actions of low pH, pepsin, bile acids, and trypsin) is of relevance to the ability of allergens to induce sensitization and, more importantly in the context of allergy testing, to trigger cell activation via the FcεRI receptor. Finally, false negative SPT test results can be obtained due to the intake of antihistamines by the patient. Patients to be tested should avoid taking antiallergic drugs before the test. For example, in Japan, 3–7 day-cessation is encouraged for histamine-release test of patients' basophils. However, such cessation of the drug may affect the patients' QOL.

A second cellular test method is based on measuring the activation of basophilic granulocytes in whole blood (Basophil Activation Test, BAT). BATs are more elaborate and expensive than SPT, as they require flow cytometry equipment; fluorescent antibodies for labeling of basophil surface activation markers, as well as trained personnel. About 10–20% percentage of individuals ("nonresponders") will not respond to IgE-dependent stimulation due to spleen tyrosine kinase (a.k.a. syk) deficiency [2], thus appear as negative, but this does not imply that these individuals are not atopic, as the nonresponder phenotype is not limited to nonatopics [3]. Due (at least in part) to the lack of standardization across laboratories, BATs are not widely used in allergy clinics.

An alternative technique, which is also currently not widely used due to a set of perceived limitations (discussed in detail in a recent review [4]), is the use of humanized rat basophilic leukemia (RBL) reporters. This system uses the RBL cell line, which as a minimum requirement needs to be stably transfected with the alpha chain of human FcεRI [5], for use with human blood samples. The requirement for the alpha chain is based on the fact that human IgE does not bind to the rat IgE receptor with high affinity [6]. Assessment of cell activation by allergens was in the past performed by measuring beta-hexosaminidase activity using fluorescent substrates, a method that is increasingly displaced by the use of more sensitive luciferase- [7, 8] or fluorescence-based [9] humanized reporter cell lines. Importantly, this system is not affected by the use of most antiallergic drugs. This is illustrated in Fig. 1, in which the luciferase-based RS-ATL8 reporter system (EXiLE), which is described in this chapter, is used in the presence of seven different drugs used for the treatment of allergic diseases.

As shown in Fig. 1, with the exception of Cyclosporin A, none of the tested drugs interferes with the use of the RS-ATL8 reporter cell line in concentrations achieved in vivo. The $C_{\max}$ value given for each drug represents the maximum (peak) achievable concentration in a specified compartment after one single administration.

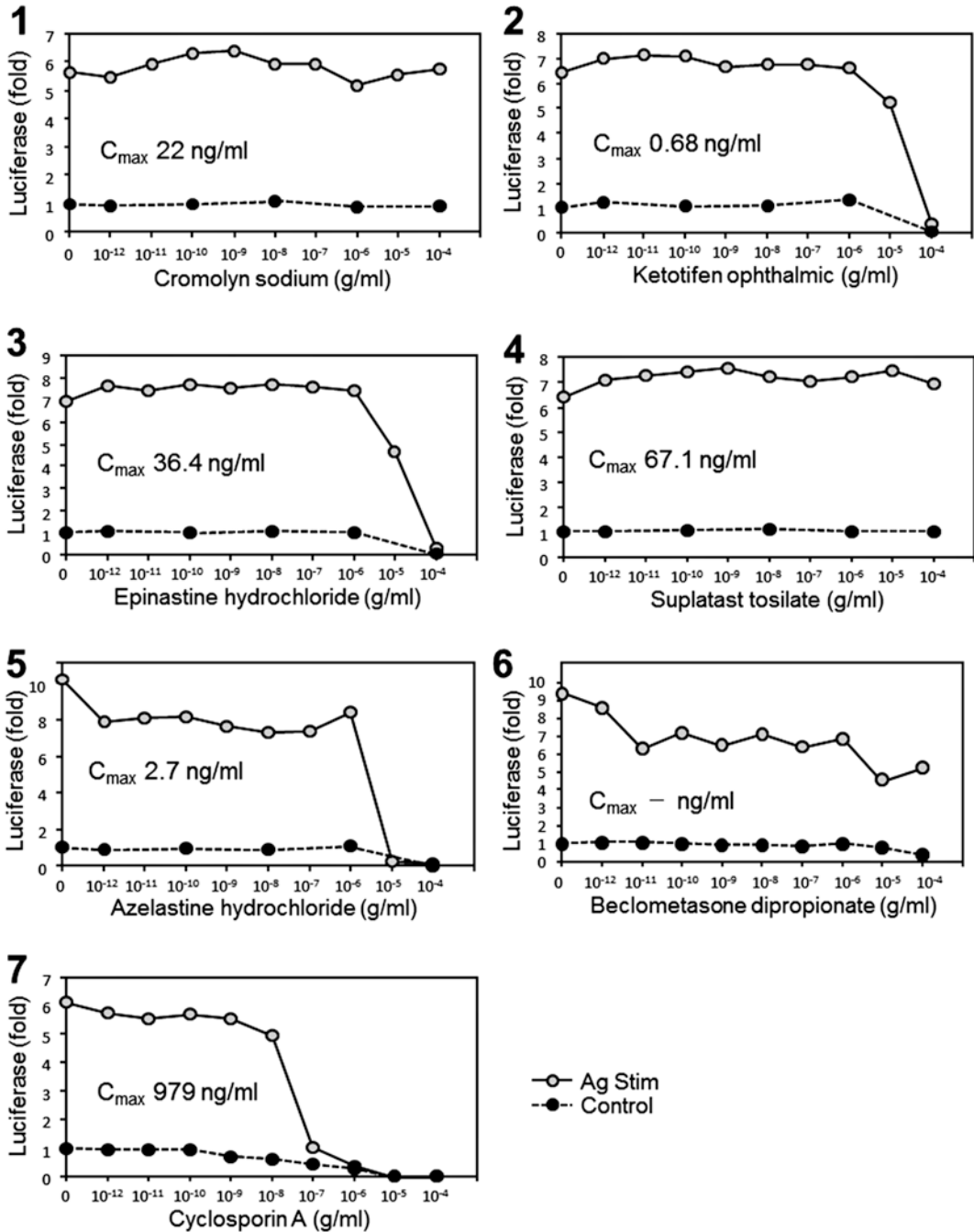


Fig. 1 Performance of the RS-ATL8 reporter system (EXiLE) in the presence of seven different drugs used for treatment of allergic symptoms. Shown is the n-fold increase in luciferase activity over unstimulated cells, using reporter cells sensitized with egg white allergic patient's serum (diluted 1:100) 3 h after stimulation with egg white extract (100 ng/mL), in the presence of different concentrations of the named drugs. (1) Cromolyn sodium; (2) Ketotifen ophthalmic; (3) Epinastine hydrochloride; (4) Suplatast tosilate; (5) Azelastine hydrochloride; (6) Beclometasone dipropionate; (7) Cyclosporin A. C_{max} values obtained from the package inserts of each drug

The sharp decrease seen for Ketotifen (2), Epinastine hydrochloride (3), and Azelastine hydrochloride (5) are due to direct cytotoxic effects on the cells at the highest concentrations (all well above the $C_{\max}$, i.e., not normally achieved in vivo). Beclometasone dipropionate (6) does not have a $C_{\max}$ value, as it is used externally. The only drug that can exert an inhibitory effect at physiologically relevant concentrations is Cyclosporin A. This can be explained by its effects on calcineurin, which is an essential molecule in the signaling pathway used by the RS-ATL8 reporter system, as explained in the following text.

Due to their high sensitivity and their robustness, these newer reporter systems are amenable to high-throughput screening in different formats, either in 96-/384-well format or in the case of the NFAT-DsRed reporter, also in allergen array-based assays [9]. The best available reporter cell line is currently the RS-ATL8 reporter cell line [10], which reports IgE-dependent cytosolic NFAT dephosphorylation by Ca^{2+} - and calmodulin-dependent calcineurin activation and subsequent NFAT translocation to the nucleus, where it binds to a synthetic NF-AT promoter, inducing luciferase expression.

We have used RS-ATL8 cells in both 96- and 384-well formats. Using the latter format has the advantage of further reducing the amount of serum needed for assessment of allergen-specific IgE, which can be of particular advantage when dealing with small pediatric blood samples. However, due to the well-known bell-shaped dose dependency of FcεRI-mediated activation, some caution needs to be taken when adapting protocols from 96- to 384-well format. This is because transition affects the surface and volume to a different extent, and the plate geometries are different, as demonstrated in the following text.

As shown in Fig. 2a for a typical 96-well plate, D1 and D2 are not equal; hence to determine its volume, a **Conical Frustum Shape** needs to be used for general calculations. Accordingly, the top radius is 3.5 mm, while the bottom radius is 3.25 mm and the height is 11.2 mm. The total volume can be calculated as follows:

Volume of a conical frustum: $V = (1/3)\pi h(R_1^2 + R_2^2 + (R_1R_2))$
 $= 401 \text{ mm}^3 = 0.401 \text{ cm}^3$, where R_1 is $D_1/2$ and $R_2 = D_2/2$ and $h = H_3$, whereas the base surface can be calculated as

Base surface area of a conical frustum S (a circle) $= \pi \times R_2^2 = 38.5 \text{ mm}^2 = 0.38 \text{ cm}^2$, where $R_2 = D_2/2$. This is the total surface on which the reporter cell lines can grow as a cell monolayer in a 96-well plate in each well.

In contrast, the shape of a 384-well plate is an (inverted) **truncated square pyramid** with the square base surface area (Fig. 2b). Accordingly, the volume is determined as

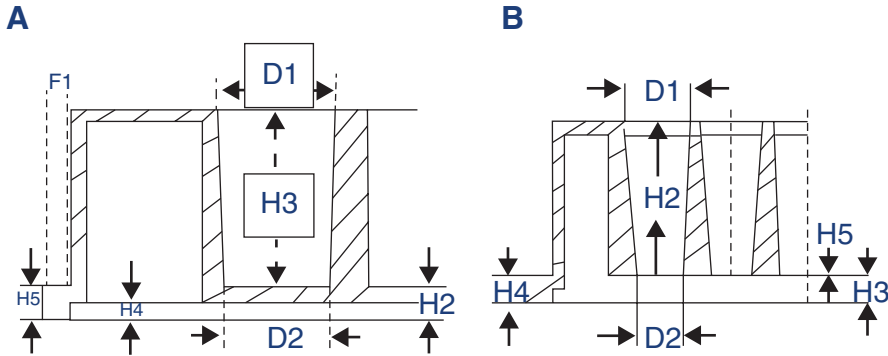


Fig. 2 (a) Dimensions of a typical 96-well plate. Total height of plate 14.5 ± 0.2 mm, $H2$: Vertical distance from inside well bottom to resting plane 3.2 mm, $H3$: Inside depth of well 11.2 mm, $H4$: Vertical distance from external well bottom to resting plane 2.1, $H5$: Height of flange 2.85 ± 0.15 , $D1$: Well diameter, top 7.05 ± 0.2 mm, $D2$: Well diameter, bottom 6.5 mm. (b) Typical 384-well plate dimensions. $H1$: Plate total height 14.4 mm, $H2$: Vertical distance from inside well bottom to resting plane 2.7 mm, $H3$: Inside depth of well 11.6 mm, $H4$: Height of flange 2.5 mm, $H5$: Bottom thickness 0.25 mm, $D1$: Well diameter, top 3.7 mm, $D2$ Well diameter, bottom 2.5 mm

Volume of a truncated square pyramid $V = 1/3 (a^2 + ab + b^2)h$, where $a = R_2 = D_2/2$, $b = R_1 = D_1/2$, and $h = H_2$. Accordingly, the volume is determined as $V = 1/3(R_2^2 + R_1R_2 + R_2^2)h$, giving 26.27 mm^3 or 0.026 cm^3 , while the base surface area (square) is $S = R_2^2$, where $R_2 = D_2/\sqrt{2} = 3.13 \text{ mm}^2 = 0.031 \text{ cm}^2$.

So while the volume is reduced by a factor of 15.4 when transitioning from 96- to 384-well format, the surface is only decreased by 12.6-fold. Therefore, optimal conditions established for 96-well plates should take into account this difference when adapted to 384-well plates, for example, when deciding which concentration of optimal anti-IgE should be used as positive control in the assay. Without taking this into account, it is possible to reduce the efficiency of stimulation by entering the supraoptimal stimulation range. This is illustrated by a typical experiment shown in Fig. 3, in which RS-ATL8 cells sensitized overnight with the serum of a donor allergic to milk are stimulated with a wide range of allergen concentrations (1 pg/mL to 0.1 mg/mL). As can be seen, supra-optimal stimulations (leading to decreased measured reporter gene activation) are obtained with allergen concentrations higher than 100 ng/mL. The optimum stimulation conditions are therefore obtained in a relatively narrow range, although activation can also be measured outside this window. As could be expected and also shown in Fig. 3, the overall intensity of luciferase-induced chemiluminescence obtained is higher in 96-well plates, due to the higher cell numbers in each well. Also, the signal-to-noise ratio is slightly better in 96-well plates, but this may be offset by the advantage of using smaller volumes of serum and reagents when using 384-well plates.

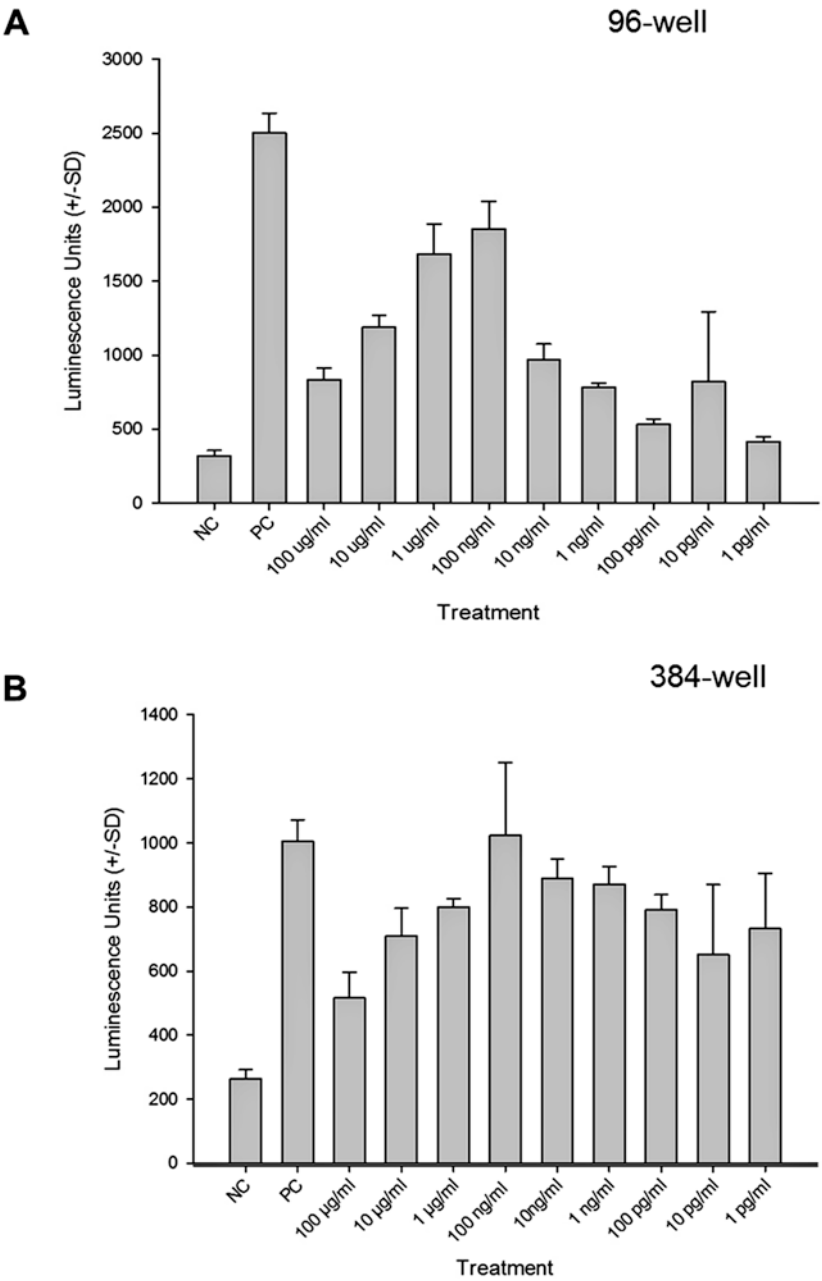


Fig. 3 RS-ATL8 cells were sensitized overnight with 1:50 diluted serum from a donor allergic to cow's milk for 16 h in a 96-well (a) or 384-well (b) plate. After one wash, cells were stimulated with a wide range of concentrations of fractionated allergen and activation measured as luminescence 4 h after stimulation. Data are shown as mean $\pm$ s.d. for triplicate determination

As pointed out by Dibbern and coauthors, who studied sera from peanut-sensitive patients [11], some sera can have toxic effects on RBL cells leading to apparently increased spontaneous (unstimulated) levels of beta-hexosaminidase. The cytotoxicity could be reduced by preabsorbing the sera on the parental RBL-2H3 cell line, which does not express the human FcεRI alpha chain and therefore does not reduce the availability of human IgE in the sample. An example of such treatment is shown in Fig. 4.

Figure 4 shows how pretreatment of cytotoxic sera can increase the stimulation levels obtained after allergen stimulation, as this allows the use of higher serum concentrations, achieving more efficient sensitization of the FcεRI receptor (and possibly also higher receptor surface expression) during overnight incubation. One important difference between the traditional use of beta-hexosaminidase for the assessment of activation compared with a reporter gene product such as luciferase is that cytotoxicity of sera can increase the background by damaging cells and releasing their granular content (including the measured beta-hexosaminidase), whereas it is much less likely to induce luciferase expression that is under the control of a synthetic NFAT promoter in the RS-ATL8

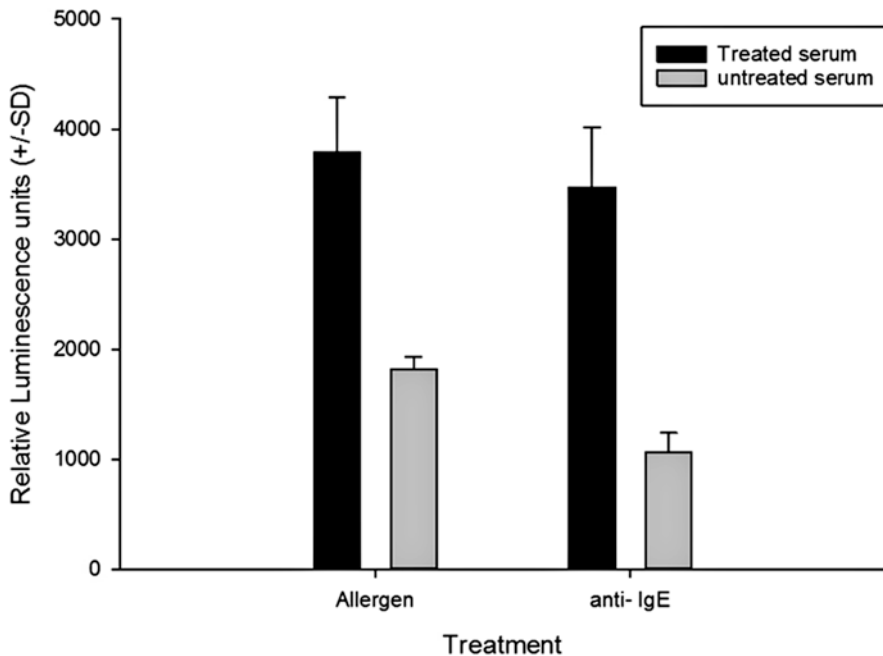


Fig. 4 Serum from a donor allergic to tufted grass was diluted 1:20 in cell culture medium and incubated for 1 h in a 96-well plate that had been coated with RBL-2H3 cells grown to full confluence. After 1 h, the supernatant was transferred to human FcεRI-expressing RS-ATL8 cells for 16 h for sensitization. After a wash, cells were stimulated with 10 ng/mL of *Holcus lanatus* pollen extract as the allergen. Luminescence was measured 4 h after stimulation. Shown is one experiment as mean ± s.d. for triplicate determination

cells. Thus, reporter cell lines, such as RS-ATL8 cells, are less prone to serum cytotoxicity-dependent artefacts than the traditional beta-hexosaminidase measurements with nonreporter, humanized RBL cell lines. However, also in the case of RS-ATL8, cytotoxicity will reduce signal strength as less viable cells will be available to produce luciferase upon stimulation.

Finally, in some cases, it may be necessary or desirable to treat sera in order to reduce the biohazard levels by neutralizing enveloped viruses such as HIV or Hepatitis B. This can be achieved by pretreating sera with a mixture of 0.3% (v/v) TNBP (tri-*N*-butylphosphate) and 1% (v/v) Tween-80 (polyoxyethyleneorbital) [12]. However, we have previously shown that after this treatment, serum dilutions of less than 1:100 cannot be used for RS-ATL8 sensitization, as the detergents damage the cells [4]. Detergents can be removed after serum treatment using SDR HyperD® detergent removal resin as described in Subheading 3. An example of detergent removal is shown in Fig. 5.

Without detergent removal, any serum dilution lower than 1:50 would have completely destroyed the cells during overnight incubation, and 1:100 dilutions would have given reduced levels of activation compared with the nondetergent treated control (not shown).

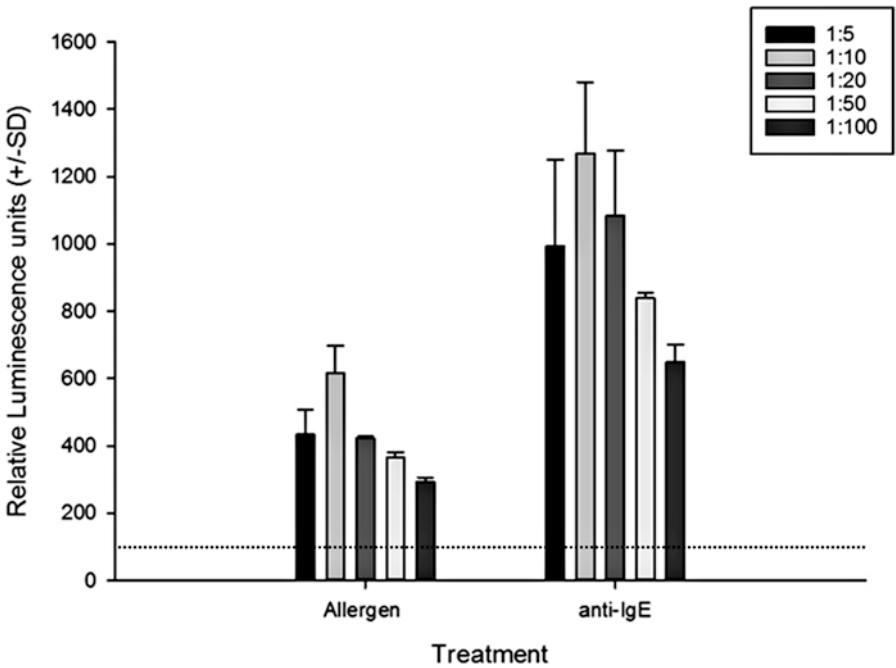


Fig. 5 Detergent removal effect. Serum from donor allergic to tufted grass was treated with detergents for 1 h and subsequently incubated using SDR HyperD® Detergent Removal resin for 30 min. RS-ATL8 cells were sensitized with different dilutions (1:5, 1:10, 1:20, 1:50, or 1:100) of the detergent- and resin-treated serum and stimulated with tufted grass pollen extract (allergen) or anti-IgE antibody (anti-IgE). Shown is one experiment as mean $\pm$ s.d. for triplicate determination. The *dotted line* indicates the negative control (=spontaneous activation) level

In this chapter, we describe the use of RS-ATL8 for the detection of allergen-specific IgE in human serum samples. We describe two slightly different protocols, one for 96-well and one for 384-well format, with optional treatment steps for the reduction of cytotoxicity by adsorption on parental RBL-2H3 cells and viral inactivation with detergents, with subsequent detergent removal via SDR HyperD® detergent removal resin.

2 Materials

2.1 Reagents

1. Humanized Rat basophilic leukemia cell line: RS-ATL8 can be obtained through an MTA from Ryosuke Nakamura (National Institute of Health Sciences, Tokyo, Japan) and RBL-2H3 cells, e.g., from the German Collection of Microorganisms and Cell Cultures, DSMZ, Germany (ACC312) or ATCC (ATCC® CRL-2256™).
2. Cell culture medium: Minimum Essential Medium (MEM), 10% fetal bovine serum (FBS), 2 mM L-glutamine (if not present in the MEM formulation). Also, it is recommended to add a standard antibiotic mixture such as 100 U/mL penicillin and 100 µg/mL streptomycin.
3. Washing buffer: Dulbecco's Phosphate Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+} .
4. Freezing medium: DMSO (dimethyl sulfoxide, cell culture grade) 10% v/v and complete cell culture medium.
5. Cell dissociation reagents: Trypsin-EDTA or cell scraper (not highly recommended) (*see Note 1*).
6. Geneticin G418 (eukaryotic antibiotic to kill cells which may have lost the HsFcεRI transfected gene) (*see Note 2*).
7. Hygromycin B (eukaryotic antibiotic to kill the cells that have lost the transfected NFAT luciferase reporter gene) (*see Note 2*).
8. Sensitizing agents: Sera from allergic donors.
9. Stimulating agents: Allergens and anti-human IgE (control).
10. Reagents for luciferase activity detection: ONE-Glo Luciferase Assay System (Promega).
11. Detergent treatment: Add 44.5 µL 20% (v/v) Tween 80 (polyoxyethylene (20) sorbitan monooleate) in DPBS and 2.7 µL TNBP (tri(*n*-butyl)phosphate) to 1 mL of serum.
12. Resin: SDR HyperD® Detergent Removal resin.
13. 70% v/v industrial methylated spirits (IMS) in deionized water and tissue for aseptic cleaning.
14. Polyclonal anti-human IgE antibody to be used as positive control. We use goat anti-human IgE polyclonal IgG from Vector Labs, UK.

2.2 Supplies

1. Cell culture well plate: 96- and 384-well clear and white plates.
2. Electronic pipette.
3. Variable volume pipettes and tips.
4. 75 cm² cell culture flasks.
5. Sterile plastic centrifuge tubes (25, 50 mL tubes).
6. Visual counting method: Hemocytometer slide (e.g., improved Neubauer chamber).

2.3 Equipment

1. Microbiological safety cabinet (MSC) class II.
2. Incubator: Adjusted to a constant temperature of 37 °C and 5% CO₂ to maintain the required physiological pH (7.2–7.4).
3. Routine cell culture visualization: Inverted phase contrast microscope.
4. Centrifuge to spin down cells (suited for 50 mL centrifuge and 1.5 mL Eppendorf tubes).
5. Luminescence microplate reader.

3 Method

3.1 Cell Culture

Preserving a sterile environment is the most important aspect of the maintenance of cell culture in a laboratory. Work should be carried out in a clean and sterile class II Microbiological safety cabinet, by routinely wiping the cabinet with IMS before and after completion of work and the equipment, if necessary.

1. Cell defrosting: Defrost the cells using either the water bath or your hand to warm the cells until they begin defrosting. Then transfer the 1 mL cells to a centrifuge tube and add 9 mL of cell culture medium and centrifuge for 5 min with $300 \times g$ to remove the supernatant that contains DMSO (*see Note 3*).
2. Remove the supernatant and resuspend the cell pellet in 10 mL prewarmed medium (*see Note 4*), then transfer to 25 cm² cell culture flask and place in the incubator.
3. Maintain transgene expression by including 1 mg/mL G418 and 600 µg/mL of Hygromycin in the medium every two weeks (*see Note 2*).
4. When cells are confluent, passaging (splitting) them requires the removal of the old medium and washing with 10 mL Ca²⁺/Mg²⁺-free DPBS (*see Note 5*), followed by addition of 2 mL of trypsin–EDTA and incubation in the incubator for 10 min (or until cells are detaching, which may happen earlier). After tapping the flask corners firmly but carefully with your knuckles, add 8 mL of warm medium. Then, transfer 9 mL to a centrifuge tube for further experiments (Subheading 3.3) and retain

1 mL of cells in the flask. Add 9 mL (*see Note 6*) of medium to the flask and keep in the incubator for further culture.

5. Cryopreservation: For long-term storage of cells, cryopreservation is needed. This requires harvesting of the cells, counting (*see Note 7*), resuspension in medium at a density of 5×10^6 cells/0.9 mL. After that, transfer 900 μ L to each labeled vial and add also 100 μ L of cell culture grade DMSO to obtain 10% v/v (*see Note 8*). Then, transfer the cryovials to Mr. Frosty (filled with isopropanol) freezing container (*see Note 8*). Store the container at -80°C overnight and transfer to liquid nitrogen vapor phase the next day and store until required.

3.2 Serum Treatment (Optional)

From **step 4** in Subheading 3.1, either proceed directly to cell sensitization as described in Subheading 3.3 or follow the optional procedures for complement inactivation and enveloped virus removal described in this section.

1. Heat the sera for 5 min at 56°C in a water bath.
or
2. Incubate the sera in the desired (e.g., 1:50) dilution with RBL-2H3 cells for 1 h (*see Note 9*).
3. If removal of enveloped viruses is required, add Tween 80 and TNBP to serum as described in **item 11** Subheading 2.1, and mix carefully using a mixer for 1 h. If detergent has been used, it needs to be removed before the sensitization step as it will damage the cells otherwise. Prepare 5 mg/mL of the detergent removal resin in DBPS then mix 1:1 (v/v) with the serum. Then, incubate in a mixer at room temperature for 30 min. To remove the resin and the detergent bound to it, centrifuge for 5 min at $1000 \times g$ and use the supernatant (*see Note 10*).

3.3 Cell Sensitization

To assess the presence of allergen-specific IgE in the tested serum, the reporter cells require to be sensitized overnight (or for a minimum of 8 h). This step will increase the levels of IgE receptor expression, thus sensitivity of the test will be increased. This is achieved by incubating the cells overnight with serum diluted 1:100 without pretreatment, or any other desired dilution (usually between 1:10 and 1:50) with pretreatment. Optimal dilution and the necessity to pretreat or not will depend on the cytotoxicity of the tested serum and the concentration of allergen-specific IgE, so will vary from sample to sample (*see Note 11*).

1. From Subheading 3.1, **step 4**, transfer the remaining cells to a centrifuge tube. Transfer 10 μ L to the hemocytometer slide for cell counting and centrifuge the remaining cells at $300 \times g$ for 5 min.

2. After calculating the number of cells in the tube, resuspend with the calculated amount of cell culture medium (*see Note 7*).
3. Mix the cells using the selected dilution of the serum (prepared in excess) and add 50 μL from each treatment in triplicate in a clear plate if using a 96-well plate, or 15.4 μL if using a 384-well plate using the optimum cell density (*see Note 7*).
4. Place the well plate in the cell culture incubator for 16–18 h.

3.4 Cell Stimulation

During the sensitization step, IgE will bind to the human Fc ϵ RI receptor and subsequent cross-linking by cognate allergen will reveal the presence of specific IgE in the serum during the cell stimulation step.

1. Prepare selected concentration of the allergen and control (anti-IgE) (*see Notes 12 and 13*).
2. Check the cell condition under a microscope (*see Note 14*).
3. Remove the old medium from the plate.
4. Add 15.4 or 50 μL of the allergen in the desired concentrations and the anti-IgE control to the cells in 384- and 96-well plates, respectively.

3.5 Luminescence Measurement

1. Add 15.4 or 50 μL of ONE-Glo Luciferase Assay System (Promega) to each well in a 384-well plate or in a 96-well plate, respectively (*see Note 15*).
2. Transfer 30.6 or 100 μL of the mixture from the clear plates to a 384- or 96-white well plate, respectively, and measure the luminescence response using the plate reader.

3.6 Waste Disposal

Cell culture contaminated plastics, tissues, tips, and tubes must be autoclaved before disposal. Also, contaminated liquid can be treated with disinfectant overnight and then disposed of in accordance with legislation and/or local regulations for safe disposal of genetically modified organisms (GMOs).

4 Notes

1. The use of cell scraper is not recommended, because it can cause more cell death than Trypsin-EDTA. Also, the cells tend to clump and so, it is not easy to count them under the microscope.
2. G418 stock = 1 g/mL. Final = 0.5 mg/mL (1:2000 dilution), Hygromycin stock = 50 g/mL. Final = 200 $\mu\text{g/mL}$ (1:250 dilution). These antibiotics are relatively expensive but have to be used to prevent loss of transgene expression in the long term.

There is no need to include these antibiotics during washes or splitting of the cells, but they should be included at least twice a month. We have cultured the cells in the absence of antibiotics for 3 months without noticing any effects on their performance.

3. Freezing medium contains 10% DMSO, which is toxic to cells when they are defrosting. It is therefore important to rapidly reduce the DMSO concentration to 1% (where toxicity is less pronounced) by diluting the cells 1:10 in cell culture medium immediately after defrosting.
4. It is important to ensure that no air bubbles are generated during resuspension of the cells. It is best to flick the tube containing the cell pellet *before* adding the medium to loosen the pellet. When adding the medium, carefully pipette up and down a few times without sucking in or expelling all the liquid. This will avoid air bubbles that damage the cells.
5. The wash is intended to remove most of the culture medium for two reasons; the bovine serum in the medium contains serum trypsin inhibitor that would inactivate the trypsin used for detachment; furthermore, as cell attachment requires Ca^{2+} and Mg^{2+} , the use of $\text{Ca}^{2+}/\text{Mg}^{2+}$ -free DPBS followed by inclusion of EDTA (a divalent metal ion chelator) further supports cell detachment.
6. RS-ATL8 cells grow in a monolayer at the bottom of the flask. Perform 1/5 split for 2 days, 1/10 for 3 days, and 1/20 for 4 days for when you want the cells to be confluent again.
7. To calculate the needed amount of the medium to be resuspended,

$$\begin{aligned} \text{Number of the cells in the flask} &= \text{Number of cells counted} \\ &\times 10^4 \times \text{volume of cells in the flask (e.g., 10 mL)}. \end{aligned} \quad (1)$$

$$\begin{aligned} \text{Cell density needed (100,000 in 96-well or 50,000 in 384-well)} \\ \times 1000 \text{ (in 1 mL)} / 50 \text{ (in 96)} \text{ 15.4 (in 384)}. \end{aligned} \quad (2)$$

Divide Eq. 1 by Eq. 2 to obtain the volume needed.

8. DMSO is added to reduce the formation of ice crystals inside the cells, and Mr. Frosty (filled with isopropanol) freezing container allows controlled cooling down at a rate of approximately 1 °C/min from room temperature to -80 °C.
9. After incubating the RBL-2H3 with the serum in a well plate, to sensitize the RS-ATL8 simply transfer the supernatant to the wells containing the RS-ATL8. This can be on the same plate if numbers allow.

10. Use of a sterile syringe filter for resin/detergent removal is not recommended due to the small volume of the serum and the incurred volume loss during filtration.
11. Ideally the optimal serum dilution would be assessed in preliminary experiments; some sera can be used in 1:10 or 1:20 dilution without affecting cell viability. In practice, such experiments may not be possible due to lack of serum. In this case, we found it safest to use a 1:100 dilution without pretreating the serum.
12. Activation via the high-affinity IgE receptor follows a bell-shaped dose-dependent curve. Lower levels of activation will be obtained by either suboptimal or supraoptimal levels of allergen (or anti-IgE). The optimum will depend on issues such as the number and distance of IgE epitopes on the allergen, the binding affinity of the IgE antibodies, and other factors that are usually unknown. Ideally, the allergen concentration would be optimized using a fixed amount of serum during sensitization and challenging the cells with different dilutions of allergen (and anti-IgE antibody as a positive control). However, in most cases, either there will be not sufficient serum or reactivity will be unknown, so in practice, at least three different concentrations of allergen should be used (over at least three orders of magnitude). The assay is very sensitive, and we regularly see optimal allergen concentrations of 100 pg/mL or 1 ng/mL.
13. Optimum polyclonal anti-IgE concentration is 1 µg/mL when using goat anti-human IgE polyclonal IgG from Vector Labs. If using a different antibody, the optimal concentration needs to be determined experimentally.
14. Healthy cells have elongated shape and are attached to the bottom (flask or plate) or a more rounded shape if confluent. Dead cells have abnormal smaller shape and detach from the surface.
15. For a maximum response after adding the Luciferase reagent, the measurements should be carried out within 30 min.

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