



Chapter 13

NPY-mRFP Rat Basophilic Leukemia (RBL) Reporter: A Novel, Fast Reporter of Basophil/Mast Cell Degranulation

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Abstract

Humanized rat basophilic leukemia (RBL) reporter cell lines are increasingly used for the detection of allergen-specific IgE and other purposes, such as the detection of allergens and standardization of allergen preparations. Existing reporter systems have many strengths and advantages but can be expensive or require longer incubation times. The new NPY-mRFP reporter cell line addresses such problems, as it requires neither expensive substrates nor overnight incubation for detection of activation. The fusion of Neuropeptide Y (NPY) with monomeric Red Fluorescent Protein (mRFP) results in localization of the fluorescent protein in granules. As NPY-mRFP is preformed in granules, the reporter system activation can be assessed using fluorescence measurements after as soon as 45–60 min, as described in this chapter, without the need to add any substrates.

Key words Reporter system, NPY-mRFP, RBL, IgE, Degranulation, Allergy

1 Introduction

Humanized rat basophilic leukemia (RBL) reporter systems are increasingly replacing the older generation of humanized RBL cell lines, due to their ease of use and higher sensitivity [1, 2]. The two most currently used humanized RBL reporter systems are RS-ATL8 [3] and NFAT-DsRed [4]. Both systems report the translocation of NFAT from the cytosol to the nucleus, which is known to occur as a consequence of Ca^{2+} influx after IgE-/Fc ϵ RI-dependent activation [5]. Therefore, the main application of humanized RBL reporter systems or the older non-humanized, non-reporter systems, has been the detection of allergen-specific IgE, e.g., in food allergy [6–9], but also the evaluation of the potential allergenicity of parasite antigens [10], as well as the detection of allergens or standardization of allergen preparations [11–13].

As previously described [2], the humanized reporter systems have a series of advantages over the older RBL cells, which used measurement of beta-hexosaminidase as a readout for activation.

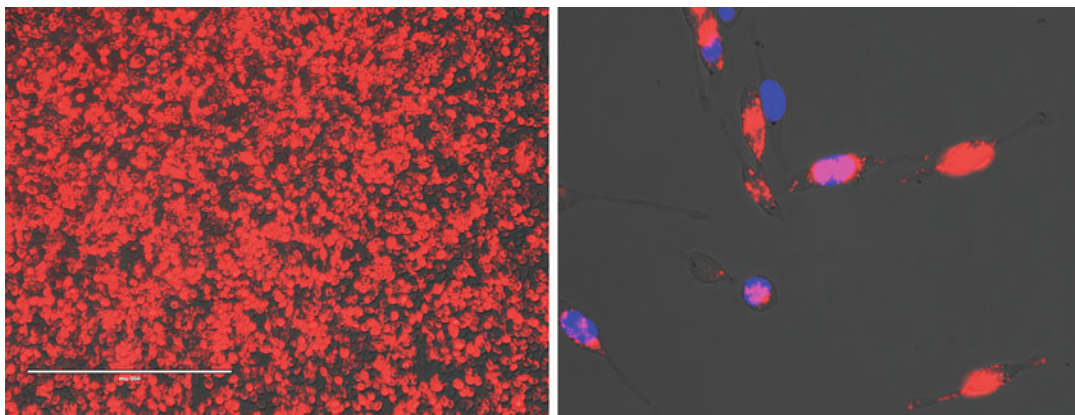


Fig. 1 Fluorescence microscopy images of unstimulated NPY-mRFP RBL cells at lower (100 $\times$) and higher (400 $\times$) magnification, showing strong red fluorescence localized to the granules. Nuclei were stained with 4',6-diamidino-2-phenylindole (DAPI) nuclear stain in the right image

However, both available reporter systems also have some disadvantages. The luciferase reporter RS-ATL8 is unsuitable for use in allergen arrays, as the substrate requires cell lysis and uses expensive luciferase substrates, while the NFAT-DsRed reporter needs an overnight incubation before the results can be evaluated (*see* Chapter 12).

To address this need, we created a new RBL reporter system, which is characterized by lower running costs and shorter times of detection. The system exploits the targeting of neuropeptide Y (NPY) to the RBL granules, which results in granular localization of fluorescent proteins fused with it [14] (*see* Fig. 1).

This is explained in more detail in our recent review [1]. There are several key advantages over the NFAT RBL reporters; the system does not require any costly reagents, as degranulation is assessed by measuring the red fluorescent protein contained in the granules; incubation times are short, as degranulation will be terminated within 45–60 min after the addition of the stimulus; and finally, the system reports the degranulation event directly, rather than parameters associated with RBL activation, such as NFAT nuclear translocation. This means that the newly developed NPY-mRFP RBL reporter cell line can also be used for mechanistic studies of degranulation or degranulation inhibitors.

2 Materials

All reagents and supplies used for cell culture should be sterile. Follow local waste disposal regulations.

2.1 Reagents

1. RBL medium: Minimum Essential Medium (MEM), 10% v/v heat-inactivated fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin, 2 mM L-glutamine (freshly added when making up medium; *see Note 1*). Store at 4 °C.
2. Phenol red-free medium. The same as above, but without any phenol red.
3. NPY-mRFP reporter cell lines (humanized or non-humanized), available from the authors through a Material Transfer Agreement (MTA). The reporter cells are available in two versions: NPY-mRFP RBL cells, which do not express the human high-affinity IgE receptor, and *hμ*NPY-mRFP RBL, which expresses the human FcεRI alpha chain, enabling them to bind human IgE.
4. G418 sulfate (Geneticin) for humanized and non-humanized NPY-mRFP RBL.
5. Zeocin (for humanized NPY-mRFP RBL only).
6. Dulbecco's phosphate-buffered saline (DPBS) without Ca²⁺ or Mg²⁺.
7. Trypsin-EDTA or cell scrapers.
8. Patient sera.
9. Allergens.
10. Anti-human IgE.
11. CELLBANKER cryopreservation medium.

2.2 Supplies

1. Graduated pipettes and pipette tips.
2. 25 cm²/75 cm² cell culture flasks.
3. 50 mL centrifuge tubes.
4. Clear 96-well plates.
5. Black 96-well plates (for fluorescence assay).

2.3 Equipment

1. Pipettors for graduated pipettes/tips.
2. Water bath.
3. Class II microbiological safety cabinet.
4. Cell culture incubator: humidified incubator set to 37 °C and 5% CO₂.
5. Heat block.
6. Hemocytometer (e.g., Neubauer modified).
7. Centrifuge capable of holding 50 mL tubes.
8. Plate reader for fluorescence.

3 Method

3.1 General Cell Culture

Work in a class II microbiological safety cabinet using sterile techniques.

1. If starting from frozen stocks, partially defrost a vial of cells by warming in hand, and then add 1 mL pre-warmed RBL medium to fully defrost.
2. Transfer cells to a 25 cm² flask containing 9 mL RBL medium, and place in a cell culture incubator overnight.
3. When the cells are confluent in the 25 cm² flask (this may take several days, depending on the initial viability of defrosted cells), transfer cells to a 75 cm² flask by detaching adherent cells using trypsin-EDTA (*see steps 2–11* of Subheading 3.3).
4. Maintain cells in 10 mL RBL medium supplemented with 1 mg/mL G418 sulfate and 200 µg/mL Zeocin (*see Note 2*) in a 75 cm² flask, in the cell culture incubator.
5. Passage cells when confluent using trypsin-EDTA (*see steps 2–10* of Subheading 3.3 below). Replacing the flask with 20% cells will result in confluence after 3 days. Replace with 10% cells for confluence (*see Note 3*) after 4 days (refresh the medium after 2 or 3 days). Top up the level of liquid in the flask to 10 mL.
6. Freeze stocks of cells in larger batches, i.e., from several flasks. Centrifuge cells at $300 \times g$ for 5 min, resuspend in CELL-BANKER (*see Note 4*) to obtain a cell concentration of 5×10^6 cells/mL. Aliquot 1 mL cells/cryovial, transferring cryovials to a Mr. Frosty™ (Nalgene) container filled with isopropanol, and place in -80°C for several hours or overnight. After freezing, transfer the cryovials to a liquid nitrogen Dewar flask for long-term storage. Defrost a vial to check the viability of the batch.

3.2 [Optional] Treatment of Serum Before Sensitization

1. If necessary, heat human serum in an Eppendorf tube at 56°C for 5 min (*see Note 5*) on a heat block and then place it on ice.

3.3 Sensitization

Cells are sensitized for 16–20 h by incubating them in RBL medium with the desired dilution of previously heat-inactivated (if necessary) serum (*see Note 6*).

1. Pre-warm RBL medium to 37°C in a water bath.
2. Remove the medium from 75 cm² flask of confluent cells.
3. To the flask, add 10 mL DPBS without Ca^{2+} or Mg^{2+} (*see Note 7*).
4. Tilt the flask to wash the cells with DPBS.

5. Discard the DPBS in the flask.
6. Repeat the washing step (4 and 5).
7. Add enough trypsin-EDTA to the flask so that the cells are fully covered with a thin layer of fluid (*see Note 8*).
8. Incubate the flask for 5–15 min at 37 °C in a cell culture incubator, until clumps of cells are visibly detached (*see Note 9*).
9. Add 8 mL pre-warmed RBL medium to the flask and resuspend the cells (*see Note 10*).
10. Transfer the cells to a 50 mL tube. Some cells can be left behind in the flask for passaging (*see Subheading 3.1*).
11. Count a sample of cells in a hemocytometer and calculate the total cell number.
12. Pellet the cells by centrifuging at $300 \times g$ for 5 min.
13. Resuspend the cells to a concentration of 1×10^6 cells/mL using RBL medium.
14. Sensitize cells using appropriate concentration of serum (*see Note 5*).
15. Pipette 150 μ L cells/well of a 96-well plate.
16. Incubate the plate overnight in the cell culture incubator.

3.4 Stimulation

1. Check the plate under a light microscope to see if the cells are in a healthy state.
2. Remove the medium from the cells in the 96-well plate.
3. Carefully wash the cells once with phenol red-free cell culture medium (*see Note 11*).
4. Replace the medium with 50 μ L of phenol red-free cell culture medium.
5. Prepare appropriate dilutions of allergens or other stimuli in phenol red-free medium. Optimal concentrations may vary for each allergen and will need to be determined experimentally. Use an appropriate amount of anti-human IgE as a positive control (*see Note 12*).
6. Add 50 μ L stimuli to appropriate wells of the plate.
7. Incubate the plate in the cell culture incubator for 45–60 min.

3.5 Fluorescence Assay with NPY-mRFP RBL

1. Transfer supernatants to a black 96-well plate (*see Note 13*). The medium only should be used as a blank, in addition to the supernatant of unstimulated cells. This will give an idea of spontaneous degranulation.
2. Measure fluorescence at 530 nm (excitation) and 590 nm (emission).

4 Notes

1. Glutamine is considered a nonessential amino acid, and it may therefore appear counterintuitive to add this to the medium. However, glutamine can become “conditionally” essential under certain conditions, such as in the case of fast-growing cells. L-Glutamine is unstable at physiological pH and is rapidly degraded into NH_4^+ and pyroglutamate (a heterocyclic lactone form of glutamic acid). The degradation of glutamine depends on the type of medium, the pH, and the temperature. Increasing amounts of NH_4^+ can have toxic effects and will affect cell growth and behavior. For these reasons, L-glutamate is added freshly to the medium together with other components such as serum or antibiotics. It is then stored at 4 °C.
2. The NPY-mRFP is a stable transfectant. However, as for all stable transfectants, there is the risk that expression of the transgene is lost over time. This will depend on the transgene copy number, the chromosomal integration sites, and multiple other factors. Thus, keeping the antibiotics used for selection in the medium can help prevent this unwanted loss of expression. It is possible to omit the antibiotics for a short (days) time, e.g., to reduce the cost of the media, but this needs to be carefully weighed against the risks. In this case, G418 is added to prevent loss of the NPY-mRFP transgene, while Zeocin is added to prevent loss of the stably transfected human Fc ϵ RI α chain.
3. A 1/20 split (5% cells) is not recommended for routine passaging as this can cause “colonies” to form and unhealthy cells in the middle of the cell clusters.
4. While other commercial and self-made freezing media are possible, we have good experience with CELLBANKER. The manufacturers state that cells can be transferred to a –80 °C freezer directly after resuspending the pelleted cells in CELLBANKER, without the need for the controlled 1 °C/min cooling rate provided by the Mr. Frosty device, and that cells can also be stored at –80 °C instead of liquid nitrogen, but we have not tested this. If using DMSO-based freezing media, work quickly, since 10% v/v DMSO is toxic to cells at room temperature.
5. Human sera are frequently cytotoxic for RBL cells. This is in part due to the presence of activated complement, which is traditionally inactivated by heating at 56 °C for 30 min. This prolonged treatment however also induces irreversible conformational changes in IgE molecules, ablating its ability to bind to Fc ϵ RI. A shorter heat inactivation will be sufficient to inactivate complement while preserving the integrity of IgE. We have

had good results with this protocol, even with 20% serum concentration (1:5). Not all sera, however, require heat inactivation. In most cases, a 1:50 or 1:100 dilution will be sufficient to remove cytotoxicity without any heat treatment. Thus the heat treatment is described as optional.

6. The exact duration of the overnight sensitization time can vary, as long as it is performed for a minimum of 8–10 h. We routinely start the sensitization protocol at 3 p.m. and the stimulation at 9 a.m. the following day.
7. Once fully confluent, a 75 cm² flask contains enough cells to fill 2 96-well plates.
8. You can use cell scrapers to detach the cells instead of trypsin-EDTA. Simply refresh the RBL medium and scrape the cells. There is no need for a DPBS wash.
9. You can speed up the detachment process by repeatedly knocking the bottom of the flask with your knuckles, holding it tight with the other hand. Be careful, however, as the use of too much force can split the flask.
10. Make sure any visible clumps are broken up, by pipetting up and down. We routinely use a 5 mL serological pipette for this purpose, as these have a narrower tapered end. Make sure you do not create bubbles when pipetting up and down by always leaving some medium in the pipette when expelling the liquid and not taking up any air when aspirating it.
11. Phenol red has an absorption maximum at 550 nm, which is very close to the 530 nm excitation wavelength of mRFP, and would therefore interfere with the measurements.
12. The optimal amount of anti-IgE will depend on the type (species, clonality, affinity, etc.) used and needs to be determined empirically. Optimum concentrations can range between 100 ng/mL and 2 µg/mL which therefore need to be determined by testing the anti-IgE antibody over a wide range of concentrations (e.g., at least 5 orders of magnitude). This should result in a bell-shaped stimulation curve, with a clear sub-optimal and supraoptimal stimulation range.
13. In our lab, we have tested different types of multiwell plates. Black plates give the greatest sensitivity for fluorescence assays. However, their background fluorescence can vary greatly between manufacturers. Always give preference to black plates optimized for low autofluorescence.

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