Journal of Visualized Experiments

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--Manuscript Draft--

Article Type:	Invited Methods Collection - Author Produced Video
Manuscript Number:	JoVE62448R3
Full Title:	Screening peptides that activate MRGPRX2 using engineered HEK cells.
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TITLE:

Screening Peptides that Activate MRGPRX2 using Engineered HEK Cells

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KEYWORDS:

Mast cells, MRGPRX2, Fura-2, Calcium assay, HEK cells, Plate reader.

SUMMARY:

Techniques for generating a library of short peptides that can activate mast cells via the MRGPRX2 receptor are described. Associated techniques are easy, inexpensive, and can be extended to other cell receptors.

ABSTRACT:

Identifying ligands specific to therapeutically significant cell receptors is crucial for many applications, including the design and development of new therapeutics. Mas related G-protein receptor-X2 (MRGPRX2) is an important receptor that regulates mast cell activation and, thus, directs the general immune response. Numerous ligands for MRGPRX2 have been identified and include endogenous peptides like PAMPs, defensins, LL-37 and other protein fragments (i.e., degraded albumin). Further identification of MRGPRX2 specific ligands requires the screening of a large number of peptides (i.e., peptide library); however, mast cells are difficult and expensive to maintain *in vitro* and, therefore, not economical to use for screening large numbers of molecules. The present paper demonstrates a method to design, develop, and screen a library of small peptide molecules using MRGPRX2 expressing HEK cells. This cell line is relatively easy and inexpensive to maintain and can be used for *in vitro* high-throughput analysis. A calcium sensitive Fura-2 fluorescent dye to mark intracellular calcium flux upon activation was used to monitor the activation. The ratio of fluorescence intensity of Fura-2 at 510 nm against excitation wavelengths of 340 and 380 nm was used to calculate calcium concentration. The peptide library used to verify this system was based on the endogenous proadrenomedullin N-terminal 12 (PAMP-12) secretagogue, which is known to bind MRGPRX2 with high specificity and affinity. Subsequent

peptides were generated through amino acid truncation and alanine scanning techniques applied to PAMP-12. The method described here is simple and inexpensive yet robust for screening a large library of compounds to identify binding domains and other important parameters that play an important role in receptor activation.

INTRODUCTION:

Mast cells are an integral part of the immune system and play a crucial role in both innate and adaptive immune responses. Mast cells are primarily activated either by the binding of an antigen to the immunoglobulin E (IgE) - FcεRI receptor complex, or by the recently discovered mas related G-protein receptor-X2 (MRGPRX2)[1]. MRGPRX2 activation has been linked to several immune and inflammatory diseases, and hence, it is important to understand the binding mechanism of the receptor to its ligands[2]. To do so, a library of small peptide molecules was developed and screened against MRGPRX2 receptors that were overexpressed in HEK cells. In the study, the peptide library was constructed using the simple and versatile techniques of alanine scanning and amino acid truncation. Alanine scanning involves replacing specific amino acids with an alanine residue. Alanine being small and neutral, strips the peptide of the specific properties conferred by the replaced residue and consecutively highlights the significance of the respective physiochemical properties of the amino acid in receptor interactions. On the contrary, in amino acid truncation, peptide sequences are designed such that it lacks one or more amino acid residues from the N-terminal, C terminal, or both. This set of peptides was used to identify the amino acid sequences crucial to MRGPRX2 binding.

Experience with human mast cells lines (LAD-2) has shown that these cells are difficult to culture and maintain *in vitro*: a doubling time of two weeks, expensive medium supplements, and direct attention required during passaging[3]. These attributes make the cells unsuitable for large scale screening of potential ligands. Herein, stably transfected HEK cells expressing MRGPRX2 receptor (HEK-X2) were used to screen the peptide library[1]. HEK-293 cells are widely used and studied for the heterologous expression of surface receptors due to their high transfection efficiency, faster doubling rate, and the need for non-expensive medium supplements to be cultured in laboratory[4]. The protocol to transfect HEK-293 cell line has been demonstrated and is well established[5]. HEK-293 cells stably expressing MRGPRX2 receptor (passage 13-19) were activated with the peptides generated through N-truncation, C-truncation, N+C-truncation, and alanine scanning[1]. Wild type HEK cells (HEK-WT) (passage 16-21) were used as control. Intracellular calcium release upon activation was monitored to study the MRGPRX2 based activation.

Cell activation by MRGPRX2 is followed by a cytosolic calcium mobilization. This regulated intracellular calcium release in mast cells is regulated by the store operated calcium entry (SOCE), coordinated by the stromal interaction molecule 1 (STIM1); and is central to the immune response cascade[6,7]. Various methods have been used to detect intracellular calcium concentration, including patch-clamps and fluorescent dyes[8]. Of all the techniques available, fluorometric calcium dyes in conjugation with various detection techniques are being widely used[9]. Two types of fluorometric dyes that have gained interests are namely, single wavelength dyes like Fluo-4, and dual wavelength ratiometric dyes like Indo -1 and Fura-2. The advantage that dual wavelength ratiometric dyes bring over single wavelength dyes is that the ratiometric

dyes correct for experimental errors like dye loading, photo bleaching, and focusing[10, 11].

Fura-2 acetoxymethyl ester (Fura-2 AM) is a cell permeating, green-fluorescent dye whose excitation shifts to a lower wavelength upon calcium-binding. Experimentally, Fura-2 is excited at 340 and 380 nm, while the emission is recorded at 510 nm. Upon calcium binding, the fluorescent intensity at 340 nm increases while that of 380 nm decreases, as shown in **Figure 1**. Data is represented as a ratio of fluorescence intensity after excitation at 340 nm (F340) to that of intensity after excitation at 380 nm (F380) i.e., F340/F380. The F340/F380 ratio is proportional to intracellular calcium, the value of which can be calculated by the Grynkiewicz equation[12]. Since the fluorescence signal is obtained from the excitation of the dye at two wavelengths (340 nm and 380 nm), the ratio of the fluorescence signals corrects for experimental factors like dye loading, dye leakage, photobleaching, and cell densities.

PROTOCOL:

1 Design and development of peptide library

1.1 To identify the ligands of the mast cell MRGPRX2 receptor based on a known ligand i.e., PAMP-12[13], follow the steps below.

1.1.1 Generate N-truncated peptide library by truncating the N-terminal amino acid residues of the ligand, in succession, by solid-phase peptide synthesis (SPPS).

1.1.2 Generate C-truncated peptide library by truncating the C-terminal amino acid residues of the known ligand, in succession, by SPPS.

1.1.3 Based on the results of 1.1.1 and 1.1.2, generate an N+C-truncated peptide library using SPPS by truncating the desired residues from the N and C-terminal, respectively.

1.1.4 Use solid-phase peptide synthesis to synthesize the peptides[13].

1.1.5 Modify the N-terminal to acetyl (Ac) group and C-terminal to amide group.

1.1.6 Characterize the peptide for its purity using high-pressure liquid chromatography (HPLC) and for the mass using a mass spectrophotometer.

1.2 To study the significance of specific amino acids within the parent PAMP-12 molecule, follow the steps below.

1.2.1 Generate an alanine scanning peptide library by replacing the respective amino acid residues in the peptide molecule with alanine, one at a time using SPPS. Modify the N-terminal to acetyl (Ac) group and C-terminal to amide group.

1.2.2 Characterize the peptide for its purity using high-pressure liquid chromatography (HPLC) and for the mass using a mass spectrophotometer.

NOTE: Ensure that the synthesized peptides are of high purity. Characterize the peptides using mass spectroscopy and HPLC.

2 *In vitro* cell culture

2.1 Culture HEK-X2 and HEK-WT cells by following the steps below.

2.1.1 Prepare culture medium by supplementing high glucose DMEM with 10% fetal bovine serum (FBS), 2 mM L-Glutamine, 100 U/mL of penicillin and 100 µg/mL of streptomycin.

2.1.2 Passage the cells in tissue culture (TC) treated T-75 culture flasks and grow in an incubator at 37 °C containing 5% CO_2, till they are 75-80% confluent.

2.1.3 Once 75% confluent, wash the cells and add 2-3 mL of trypsin for 2 – 3 min. Incubate in a 37 °C, 5% CO_2 incubator to detach the cells.

2.1.4 Once the cells have detached, collect the cells in trypsin. Add 6-9 mL of fresh medium.

2.1.5 Centrifuge the cells at 1620 x *g* for 3-5 min.

2.1.6 After centrifugation, discard the supernatant to collect the pellet. Resuspend the cells in a fresh culture medium. Dilute the cells as per the desired concentration.

NOTE: HEK cells are fast-growing cells and hence optimize the cell medium supplements. HEK cells are adherent cells; passage them in TC-treated culture flasks to support adhesion.

2.2 Prepare a 96 well assay plate for the experiment.

2.2.1 Add 200 µL of the cell suspension in each well, with a concentration of 200,000 cells/mL, to seed 40,000 cells/well.

2.2.2 Grow the cells for 24 h in a 37 °C, 5% CO_2 incubator.

NOTE: Optimize the cell density per well based on the plate size and the cell strain type. Conduct the experiment in triplicates in a black TC-treated 96 well plate with flat transparent bottom.

3 Fura-2 AM calcium assay

3.1 Prepare dye by following the steps below.

3.1.1 Use Fura-2 AM dye for the experiment.

3.1.2 Prepare HEPES-Tyrode's buffer (HTB) buffer containing 25 mM HEPES buffer, 120 mM NaCl, 5 mM KCl, 1 mg/mL glucose, 1 mg/mL bovine serum albumin (BSA) and freshly added 1.8 mM $CaCl_2$ in autoclave sterilized water.

3.1.3 Add 50 µL of DMSO in 50 µg Fura-2 AM vial to prepare 1 mM stock solution of Fura-2 AM dye. Add 1 µL of 1 mM Fura-2 AM dye per mL of fresh medium to prepare the dye loading medium having 1 µM dye concentration.

3.1.4 Remove the 96 well plate from the incubator and discard the medium. Replace the medium with a fresh dye loading medium. Add 200 µL of dye loading medium in each well. Incubate the cells for 30–40 min in a 37 °C, 5% CO_2 incubator.

3.1.5 After 40 min of incubation, remove the medium. Wash the cells with the HTB buffer. Add 100 µL of HTB buffer for fluorescence reading. Take the plate for fluorescence reading.

NOTE: Optimize the concentration of dye in the dye loading medium. Dye leakage and photobleaching are possible concerns associated with the dye. Add $CaCl_2$ fresh into the HTB buffer to avoid precipitation.

4 Cell activation and fluorescent reading

NOTE: Fluorescence plate reader with an automated pipetting system allows for the automatic transfer of compounds from a compound source to the assay plate without taking the plate out of the plate reader.

4.1 While the cells are being incubated, set the **Plate Reader**.

4.1.1 Set the **Temperature** to 37 °C.

4.1.2 In **Settings,** select **Flex**.

4.1.3 Set the **Read Mode** to **Fluorescence** and **Bottom read**.

4.1.4 In **Wavelengths,** set the number of **Wavelengths** to 2. Set the **Excitation** to 340 nm and 380 nm. Set the **Emission** to 510 nm.

4.1.5 Leave the **Sensitivity** to **Default**.

4.1.6 In **Timing**, set the **Interval** to 3.9 s. Set **Run Time** to 94 s to get 25 reads.

4.1.7 Next, select the **Assay Plate Type**.

4.1.8 Next, select the **Wells to Read**.

4.1.9 In **Compound Transfer**, set **Transfers** to 1 and **Initial Volume** to 100 µL. Set the **Pipette Height** to 100 µL, **Volume** to 50 µL, and **Time Point** to 36 s, to add the compound at the 10th reading.

4.1.10 Next, select the **Compound Source** plate type.

4.1.11 Leave **Triturate** to **Not Used**.

4.1.12 Select the tips in the **Pipette Tips Layout**.

4.1.13 For **Compound and Tip Columns**, ensure that the compounds to be transferred are in column 1 of the compound plate. Set the **Tip Column** to 1 and **Compound Column** to 1.

4.1.14 Leave the **Autocalibrate** as **ON**.

4.1.15 Click **OK**.

4.2 When the temperature has reached to 37 °C, load the plates into the **Plate Reader**.

4.2.1 Press the **Reading Chamber** to put the **Assay Plate** into the fluorescence **Plate Reader**.

4.2.2 Press the **Source** to put the **Compound Plate**. Prepare the **Compound Plate** by adding 200 µL of respective peptides, ionomycin and ethylene glycol-bis(β-aminoethyl ether)-N,N,N′,N′-tetraacetic acid (EGTA)-Triton X-100 solutions.

4.2.3 Press the **Tip Rack** to put the **Tip Box**. Use a black tip to avoid tip autofluorescence.

4.2.4 Once the plates are loaded, review the settings of the software and press **Read**.

5 Data analysis

5.1. Determine the calcium concentration from the fluorescence ratio by the Grynkiewicz equation -

$$[Ca^{2+}] = K_d \frac{F_{380min}}{F_{380max}} \frac{(R - R_{min})}{(R_{max} - R)}$$

where, K_d is the dissociation constant of Fura-2 AM, R is the emission ratio after excitation at 340 nm and 380 nm (F340/F380) for respective peptides, R_{max} is the maximum fluorescence ratio observed by the addition of 50 µL of 30 µM ionomycin, R_{min} is the minimum fluorescence observed by the addition of 50 µL of 100 mM EGTA/2.5% Triton X-100, and $F380_{min}$ and $F380_{max}$ are the absolute fluorescence intensity of Fura-2 AM in calcium free and bound state, respectively.

NOTE: The machine dispenses the liquid with some force. Do not set the peptide dispensing height too close to the bottom of the plate; it may detach the cells. Use black pipette tips to avoid autofluorescence. Read the maximum fluorescence and the minimum fluorescence for each plate for each experiment.

REPRESENTATIVE RESULTS:

Table 1 contains the peptide sequences generated through terminal amino acid truncation and alanine scanning. As shown in **Table 1**, peptide sequence RKKWNKWALSR lacks N-terminal phenylalanine (F) with respect to its parent PAMP-12 and hence is a representative peptide in N-truncated library. Similarly, in FRKKWNKWALS, PAMP-12 C-terminal serine has been removed, representing a C-truncated peptide library derived from PAMP-12. In N+C-truncated peptide library, amino acid from both N and C-terminal are removed. Truncation of 4 amino acid from N-terminal and 1 residue from C-terminal of PAMP-12 results in WNKWALS. Peptide library derived from alanine scanning has one amino acid replaced with alanine, as with ARKKWNKWALSR, where N-terminal phenylalanine is replaced with alanine. Fura-2 AM dye was used to study the activation potential of peptides against MRGPRX2 transfected HEK cells[1]. The data was recorded on a fluorescence plate reader. If the peptide ligand activated the cell, the fluorescence pumped at 340 nm (F340) increases, while the same decreases for 380 nm wavelength (F380) (**Figure 1a**). For a blank (or for that matter non activating peptide or HEK-WT control) however, the relative increase and decrease would be respectively low, as shown in the **Figure 2a**. Calcium concentration is, however, represented by F340/F380 ratio as shown in **Figure 1b,2b**. The ratio F340/F380 can be further substituted in the Grynkiewicz equation to get the calcium concentration using in situ calibrations (**Figure 3**). The peptides listed in **Table 1** were characterized by mass spectroscopy (**Figure 4a**) and HPLC (**Figure 4b**) and found to be of high purity.

FIGURE AND TABLE LEGENDS:

Table 1: Representative peptide sequences generated after N, C and N+C – truncation, and alanine scanning. The N-terminal of the peptides were acetyl modified while the C-Terminal contained an amide group.

Figure 1: Representative data for an activating peptide. (**a**) The fluorescence signals for an activating peptide. Represented data corresponds to PAMP-12 (FRKKWNKWALSR). Peptide was added after generating a baseline for 10 reading cycles (36 s), as shown by the arrow. (**b**) The ratio of the fluorescence emission after excitation at 340 nm (F340) to that of fluorescence emission after excitation at 380 nm (F380) (F340/F380).

Figure 2: Representative data for the blank. (**a**) The fluorescence signals for the Blank. HTB was added after generating a baseline for 10 reading cycles (36 s), as shown by the arrow. (**b**) The ratio of the fluorescence emission after excitation at 340 nm (F340) to that of fluorescence emission after excitation at 380 nm (F380) (F340/F380).

Figure 3: Representative data for the standards for dye calibration. (**a**) Ionomycin was added at 10^{th} readings, as shown by the arrow, to get maximum fluorescence in Ca^{+2} bound state. EGTA-

Triton X-100 was added after 20 readings, as shown by the arrow, to get a minimum signal. (**b**) The ratio of the fluorescence emission after excitation at 340 nm (F340) to that of fluorescence emission after excitation at 380 nm (F380) (F340/F380). These values are further put in the Grynkiewicz equation to get the intracellular calcium concentration.

Figure 4: Characterization of a representative peptide to confirm the sequence and purity. (**a**) The theoretical mass of the representative peptide sequence WNKWAL was 857.90 Da, which was shown by the m/z ratio in mass spectroscopy. (**b**) Peptide's purity of 99% as confirmed by HPLC. This peptide belongs to N+C-truncated peptide library.

DISCUSSION:

Calcium signaling is central to mast cell degranulation and has been widely used in the study of receptor-ligand interactions, ligand identification, and drug discovery[14]. MRGPRX2 is a recently discovered mast cell receptor that has been found to play a key role in many inflammatory diseases like itch, asthma, and atopic dermatitis, among others[2]. Furthermore, several approved drugs have been shown to elicit an inflammatory response through the MRGPRX2 receptor[15]. It is, therefore, imperative to study the ligand-receptor interaction, identify new ligands, and understand the activation mechanism. This study shows the use of common peptide techniques in designing a peptide library (**Table 1**) and in studying the MRGPRX2 based mast cell activation. The underlying calcium mobilization was employed as the indicator of mast cell activation.

Fura-2 is a calcium sensitive dye used to measure intracellular calcium concentration. The acetoxymethyl ester variant (Fura-2 AM) increases the permeability of the cell membrane yielding an easy method for quantifying the cytoplasmic calcium releases. The dye has frequently been used with various characterization techniques like flow cytometer, microscopy, and fluorimeters[9,16,17]. Though widely used, there are challenges associated with the dye, which need to be addressed before these can be efficiently employed. Intracellular calcium binding relies on the de-esterification of the dye, which is largely dependent on the dye loading conditions, cell system, and cell culture types. Partial de-esterification results in inadequate fluorescence signals. Furthermore, incomplete de-esterification also results in the localization of dye into the cell organelles resulting in inaccurate data. Leakage of de-esterified dye from the cell is another issue faced with Fura-2[10,11].

Apart from the dye loading, the use of the detection technique also plays an important role. The inability of flow cytometers to operate with UV lasers makes them unfavorable for the dye[9]. Similarly, fluorescent imaging techniques require advanced training in microscope operation. The transient nature of calcium release upon ligand activation requires a rapid response in the change in wavelengths and thus a faster shutter speed of the microscope. In addition, the use of specialized cell imaging chambers, use of coverslips and constant image focusing makes it a cumbersome technique for large-scale screening[16].

A significant amount of time was invested in the method optimization. Cuvette based fluorometer, wherein cells after detaching from the culture flask, were incubated with the dye-loaded medium in the dark and then were washed and resuspended in the HTB buffer for the

study. Cells suspended in HTB buffer were taken in a cuvette and were read using a photomultiplier based fluorometer. The detection systems could only hold few (2-4) cuvettes at a time and thus was not suitable for the large-scale screening of peptides. Further, HEK cells being adherent cells, the cell suspension reading showed great variations within individual repeats within an experiment. Consequently, fluorescent imaging technique was used, which again proved to be tedious and slow. The requirement for an advanced microscope with fast wavelength changing capability, microscope-specific cell chambers, and excellent imaging skills impeded the study. Additionally, *in situ* peptide simulation was time extensive and resulted in the loss of important data. Inefficient cell processing technique resulted in cell flotations during readings which made it difficult to focus and gave inconsistent results.

A fluorescence plate reader system with an automated pipetting system that can dispense compounds during experiments was used for the study. The fact that many samples are read in quick succession in a 96 well plate is an added benefit of this technique. The results showed that the readings were more consistent when cells were attached as compared to suspension. Cell counts of 40,000 cells/well in a TC-treated 96 well plate grown for a day gave the best results. A dye incubation time of 30–40 min at 37 °C incubator was optimum. However, when experiments were done with experimental repeats, variations in the corresponding wells of different columns were observed. To overcome this, a positive (PAMP-12) and a negative control (Blank) for each column of the plate was used. This gave more consistent and reproducible data. The method described here is an easy and versatile method, which can be efficiently employed for large-scale calcium-based screening. However, there are several factors which determines the quality of data and thus the method needs to be optimized for a given cell-instrument system.

ACKNOWLEDGMENTS:

SR and LDU would like to acknowledge Alberta Innovates Strategic Research Project, NRC, and NSERC-Discovery grant for this project.

DISCLOSURES:

Authors declare no competing interests.

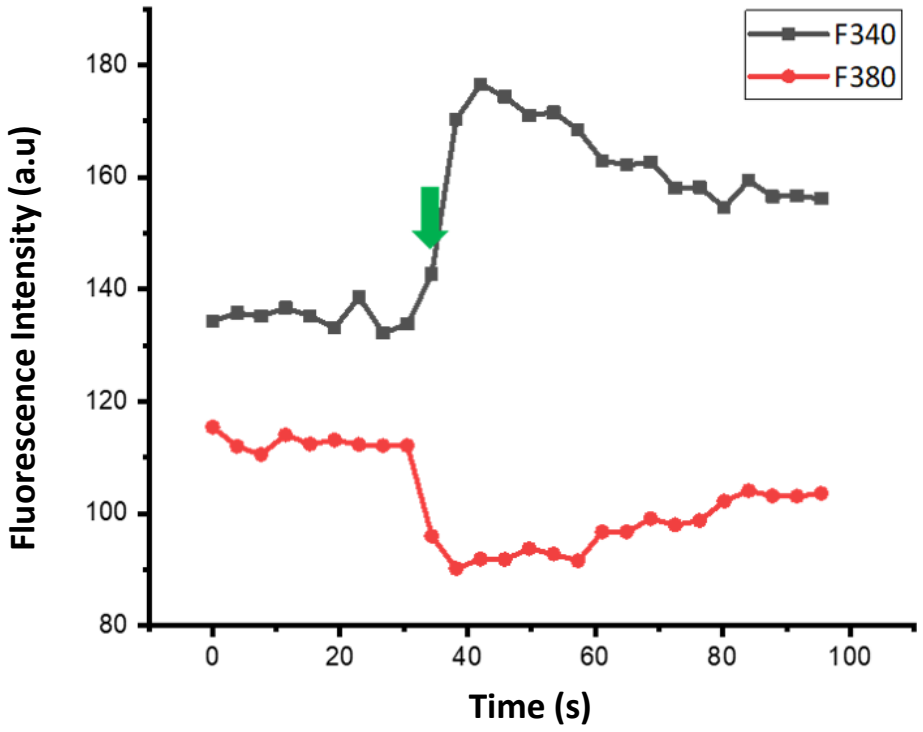
REFERENCES:

1. McNeil, B. D. et al. Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions. *Nature*. **519** (7542), 237–241 (2015).
2. Subramanian, H., Gupta, K., Ali, H. Roles of Mas-related G protein–coupled receptor X2 on mast cell–mediated host defense, pseudoallergic drug reactions, and chronic inflammatory diseases. *Journal of Allergy and Clinical Immunology*. **138** (3), 700–710 (2016).
3. Rådinger, M., Jensen, B. M., Kuehn, H. S., Kirshenbaum, A., Gilfillan, A. M. Generation, isolation, and maintenance of human mast cells and mast cell lines derived from peripheral blood or cord blood. *Current protocols in immunology*. **Chapter 7**, Unit-7.37 (2010).
4. Thomas, P., Smart, T. G. HEK293 cell line: A vehicle for the expression of recombinant proteins. *Journal of Pharmacological and Toxicological Methods*. **51** (3), 187–200 (2005).
5. Subedi, G. P., Johnson, R. W., Moniz, H. A., Moremen, K. W., Barb, A. High yield expression of recombinant human proteins with the transient transfection of HEK293 cells in suspension.

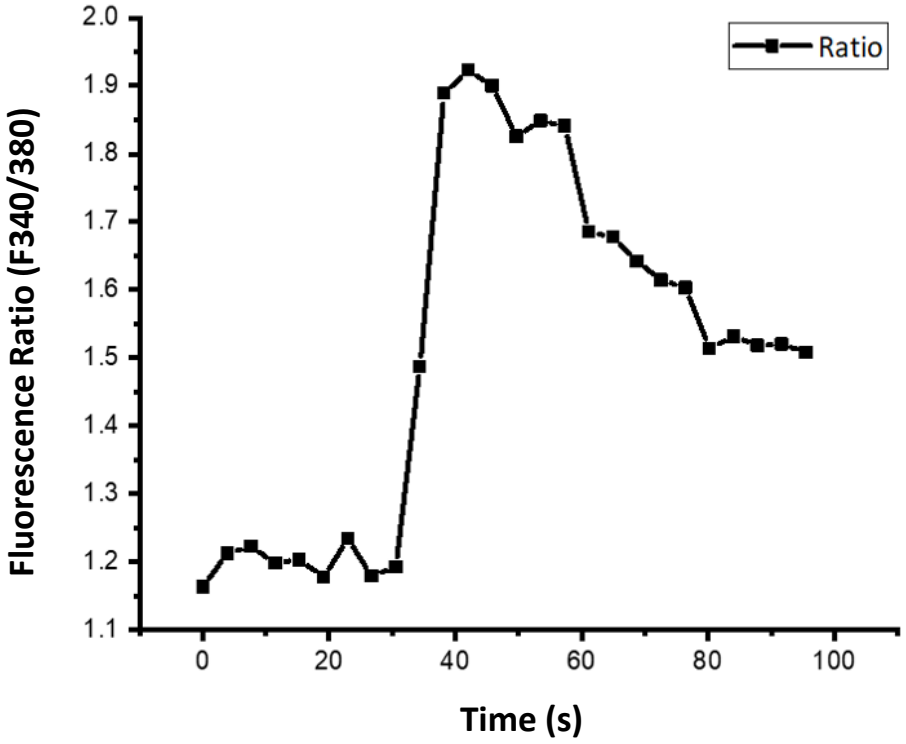
Journal of Visualized Experiments. (106), e53568 (2015).
6. Occhiuto, C. J. et al. Store-operated calcium entry via STIM1 contributes to MRGPRX2 induced mast cell functions. *Frontiers in Immunology*. **10**, 3143 (2020).
7. Baba, Y. et al. Essential function for the calcium sensor STIM1 in mast cell activation and anaphylactic responses. *Nature Immunology*. **9** (1), 81–88 (2008).
8. Hoth, M., Penner, R. Depletion of intracellular calcium stores activates a calcium current in mast cells. *Nature*. **355** (6358), 353–356 (1992).
9. Assinger, A., Volf, I., Schmid, D. A novel, rapid method to quantify intraplatelet calcium dynamics by ratiometric flow cytometry. *PLoS One*. **10** (4), e0122527 (2015).
10. Roe, M. W., Lemasters, J. J., Herman, B. Assessment of Fura-2 for measurements of cytosolic free calcium. *Cell Calcium*. **11** (2–3), 63–73 (1990).
11. Malgaroli, A., Milani, D., Meldolesi, J., Pozzan, T. Fura-2 measurement of cytosolic free Ca2+ in monolayers and suspensions of various types of animal cells. *The Journal of Cell Biology*. **105** (5), 2145–2155 (1987).
12. Grynkiewicz, G., Poenie, M., Tsienb, R. Y. A New Generation of Ca2 + Indicators with Greatly Improved Fluorescence Properties. *Journal of Biological Chemistry.* **260** (6), 3440–3450 (1985).
13. Lu, L., Parmar, M. B., Kulka, M., Kwan, P., Unsworth, L. D. Self-assembling peptide nanoscaffold that activates human mast cells. *ACS Applied Materials and Interfaces*. **10** (7), 6107–6117 (2018).
14. Lansu, K. et al. In silico design of novel probes for the atypical opioid receptor MRGPRX2. *Nature Chemical Biology*. **13** (5), 529–536 (2017).
15. Navinés-Ferrer, A. et al. MRGPRX2-mediated mast cell response to drugs used in perioperative procedures and anaesthesia. *Scientific Reports*. **8** (1), 11628 (2018).
16. Johnson, M. Calcium imaging of store-operated calcium (Ca 2+) entry (SOCE) in HEK293 cells using Fura-2. *Calcium Signalling*. 163–172 (2019).
17. Tinning, P. W., Franssen, A. J. P. M., Hridi, S. U., Bushell, T. J., McConnell, G. A 340/380 nm light-emitting diode illuminator for Fura-2 AM ratiometric Ca2+ imaging of live cells with better than 5 nM precision. *Journal of Microscopy*. **269** (3), 212–220 (2018).

Representative data for an activating peptide

a Fluorescence signals for an activating peptide

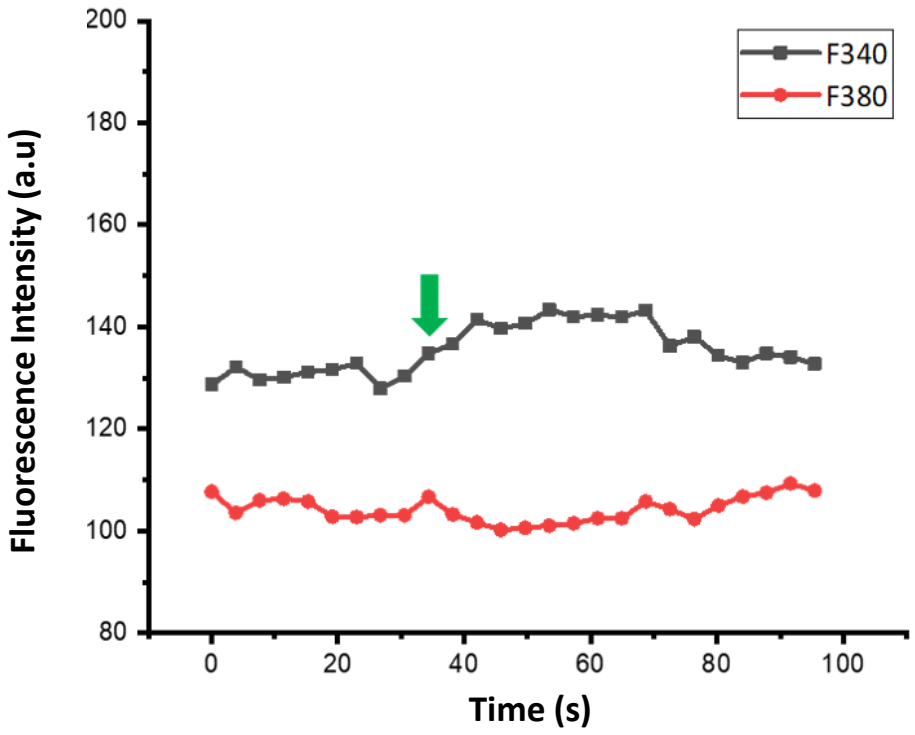


b Fluorescence ratio (F340/380) for an activating peptide

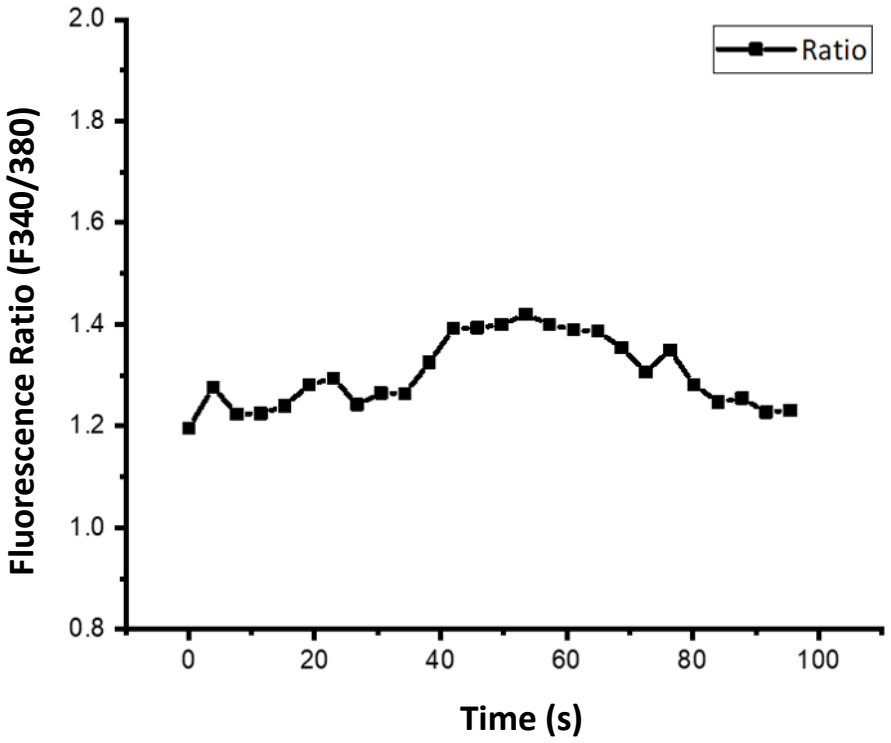


Representative data for the blank

a Fluorescence signals for the blank

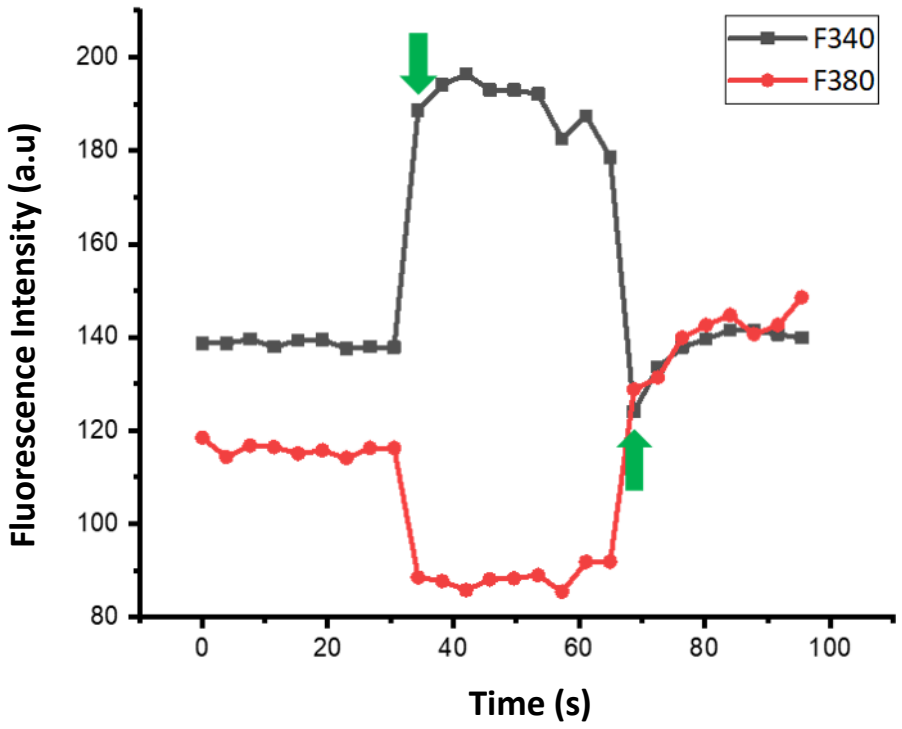


b Fluorescence ratio (F340/380) for the blank

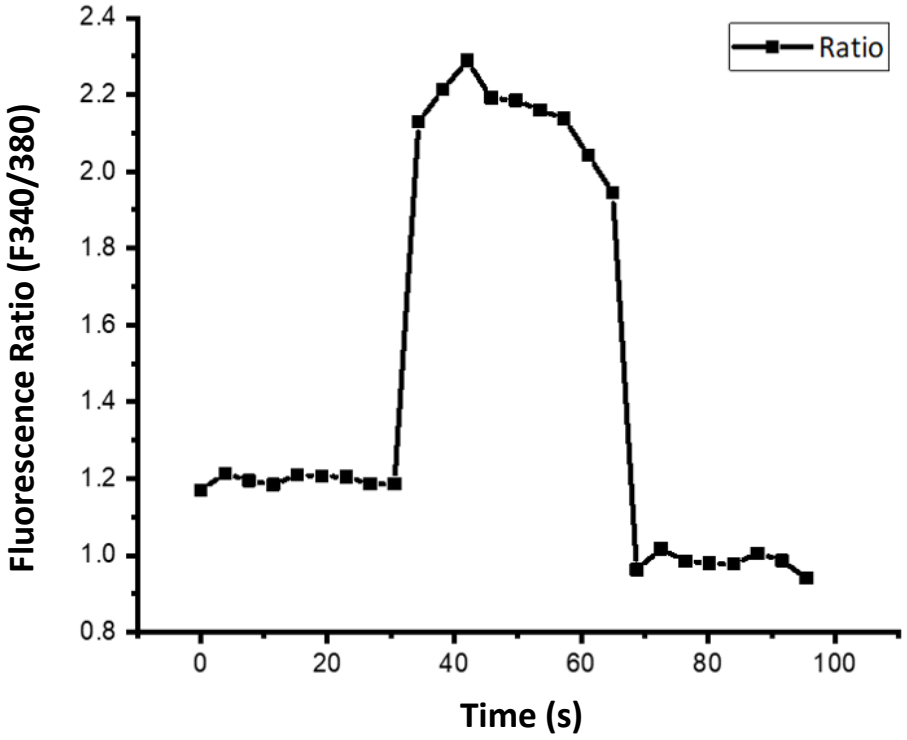


Representative data for the standards for dye calibration

a Fluorescence signals for the standards

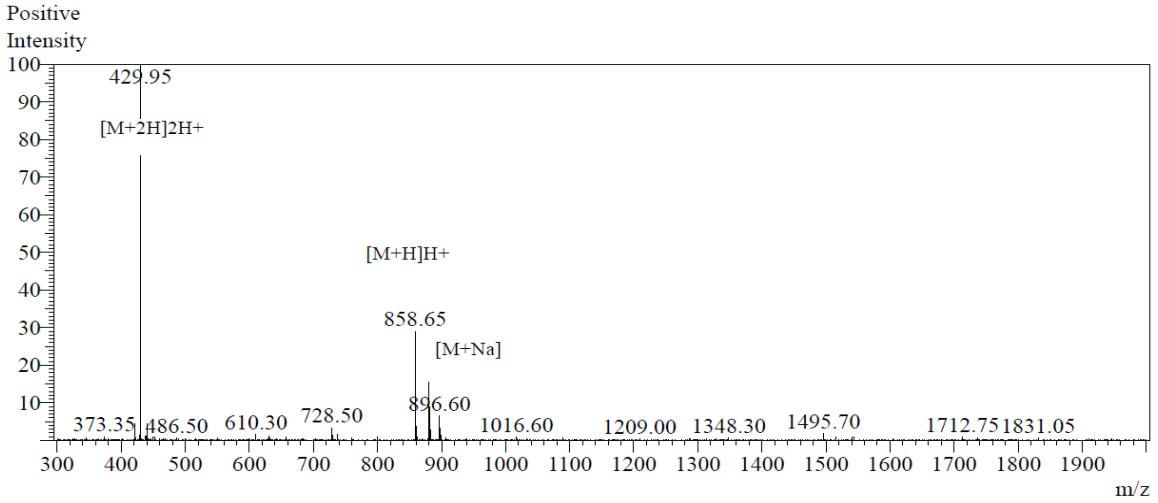


b Fluorescence ratio (F340/380) for the standards

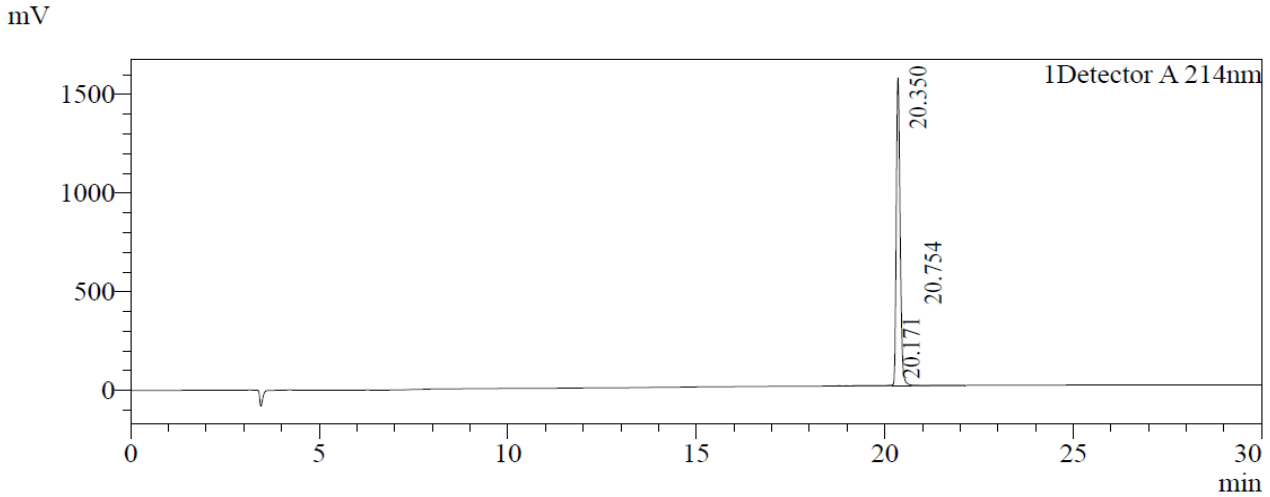


Characterization of a Representative Peptide to Confirm the Sequence and Purity

a Mass spectroscopy of a representative peptide with mass 858 Da



b HPLC chromatogram of a representative peptide with 99% purity



Peptide Technique	**Representative Peptide**
Parent peptide	Ac-FRKKWNKWALSR-Amide
N-Truncation	Ac-RKKWNKWALSR-Amide
C-Truncation	Ac-FRKKWNKWALS-Amide
N+C-Truncation	Ac-WNKWALS-Amide
Alanine Scanning	Ac-ARKKWNKWALSR-Amide

Changes to be made in the manuscript

Changes to be made by the Author(s):

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

Response: Thanks for the suggestion, the manuscript has been thoroughly proofread.

2. Please provide an email address for each author.

Response: Thanks for the comment, the email addresses of each author have been added.

3. For in-text citations, use "…established1,2." instead of "..established.1,2"

Response: Thanks for the comment, mentioned style has been incorporated.

4. Maintain a 0-inch left indent throughout the text and indicate new paragraphs using single-line spacing.

Response: Thanks for the comment, mentioned style has been incorporated.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. e.g., lines 112, 114, 116, etc.

Response: Thanks for the comment; method to synthesize peptides has been mentioned.

6. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

Response: Thanks for the suggestion, suggested changes have been made.

7. Use "mL, µL" instead of "ml, uL/ul". Use "h, min, s" instead of "hours/hrs/hr; minutes/mins; seconds/secs/sec" – follow for the figure labelling as well. Express centrifugation speed as "x g" instead of "rpm". Add a single space between the quantity and its unit e.g., "37 oC" instead of "37oC", etc.

Response: Thanks for the suggestion, suggested changes have been made.

8. Line 139: Please specify the concentration.

Response: Thanks for the comment, mentioned concentration has been added.

9. 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. E.g. Flexstation3, etc.

Response: Thanks for the comment, the manuscript has been reviewed to remove mentioned commercial names.

10. 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.”

Response: Thanks for the comment, the protocol has been changed to ‘imperative tense’ where lacking.

11. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and a maximum of 4 sentences per step. Also, consider combining the “notes” for the specific steps into one. E.g. Combine lines 208-213 as a single note, etc.

Response: Thanks for the comment, Protocols have either been shortened, combined, or merged.

12. Please expand the representative results section, in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. Data from both successful and sub-optimal experiments can be included.

Response: Thanks for the comment, the representative section has been expanded.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Do not abbreviate journal names. Please include volume and issue numbers for all references.

Response: Thanks for the comment, changes have been made.

14. Please sort the Materials Table alphabetically by the name of the material.

Response: Thanks for the suggestion, suggested changes have been made.

Reviewer #2:

Manuscript Summary:
In this manuscript, the authors indicate a method to design, develop, and screen a library of peptides which can differentially activate mast cells through MRGPRX2. The authors state that the library was generated by amino acid truncation and alanine scanning using PAMP12 as the parent peptide. MRGPRX2 expressing HEK cells were treated with peptides and MRGPRX2 activation assessed via calcium flux measured with a calcium sensitive Fura-2 fluorescent dye. The authors assert that based on this method, they were able to identify the MRGPRX2 binding domain of PAMP-12 and were also able to highlight important physiochemical properties of ligands that facilitates the receptor-ligand interaction and receptor activation.

Major Concerns:
1. The abstract and introduction have a lot of focus on the generation of a peptide library and how it was used to "identify MRGPRX2 binding domain of PAMP-12 and were also able to highlight the important physiochemical properties of ligands that facilitates the receptor-ligand interaction, and receptor activation". These statements are misleading as no data whatsoever is included to support these statements. These statements should be removed, or supportive data should be included (or referenced if demonstrated elsewhere).

Response: Thanks for the comment. Respective corrections have been made.

2. Although this manuscript is focused on the method, the manuscript would benefit from expanding the results section. Very few data in general were included in this manuscript; essentially, the purity of one peptide as well as calcium flux from only one positive control and one negative control peptide was shown. The figures don't support some of the main statements in the abstract/introduction. Also, table 1 doesn't provide much value on its own as it's only a repetition of what is verbally said in the text. A table with all peptides analyzed would have been more useful.

Response: Thanks for the comment. However, and as stated, the purpose of the manuscript was not to demonstrate the scientific advancement that was achieved using the herein discussed method, but to describe a technique, with all its benefits and challenges, which will help the research community in their scientific pursuit. Having mentioned that, a comprehensive description of the Representative Results has been added and peptide sequences have been mentioned.

3. Since HEK cells express other GPCRs that could cause calcium flux if activated by the peptides, the parental HEK cells should be tested side by side the HEK-X2 cells as a control. Although mentioned on line 85 that wild type HEK was used as a control, no results are shown, and this important topic is not really discussed.

Response: Thanks for the comment. As mentioned in the manuscript, the HEK-WT were used as controls. The results of these studies are in the process of submission. However, as far as HEK-WT results are concerned, the representative results would be no different from the non-activating peptide as

mentioned in Figure 3 of the manuscript. We have added this in the Representative Results section, and Figure 3 description has been rephrased to highlight the suggestion.

4. The discussion could be more focused, and the point of several statements could be made clearer and more succinct.
a) The discussion regarding the dye (lines 263-280) is unfocused leaving the reader unclear as to the take home message and relation to the method used in some cases.

Response: Thanks for the comment. Mentioned lines have been rephrased to make it more comprehensive.

b) Paragraph starting on line 282: Too much attention to personal past failures rather than on how the field has evolved.

Response: Thanks for the comment, and yes, that is correct. However, I would not call them as “personal failures”. Optimizing a technique takes both time and effort. One has to go through a lot of ‘betters’ to get to the ‘best’; and that is what we have highlighted. This being an article describing a method, we feel it is ideal to highlight the challenges that was faced as well; so that a novice researcher would know what not to do when using this method in their scientific quest.

c) Some of the optimization statements could have been included in the manuscript as figures to strengthen the manuscript, especially for a methods journal with a focus on reproducibility. Otherwise the statement "data not shown" should be used.

Response: Thanks for the comment, comprehensive notes have been added after each Protocol which are crucial in optimizing the method.

Minor Concerns:
1. Line 60: FcER1 binds to IgE which binds to the antigen. Antigen doesn't bind directly to FcER1.

Response: Thanks for the comment, this has been corrected.

2. Line 102: Figure 1 does not depict the statement on this line.

Response: Thanks for the comment, this has been corrected.

3. Line 109: Section 1 of protocol is missing a lot of detail; for instance, exactly what method was used to truncate the peptides? How were the amino acids replaced with alanine? How many peptides in total were generated in the library? Either extra detail or a reference to the method used would be helpful.

Response: Thanks for the comment, this has been corrected.

4. Line 125: Information on how the HEK cells were transfected with MRGPRX2 would be useful, as well as confirmation data that the cells express MRGPRX2.

Response: Thanks for the comment, respective reference has been added in the Representative Result section.

5. It's unclear from the protocol exactly when and where is the ionomycin and the EGTA/TX-100 added to the cells.

Response: Thanks for the comment, this has been corrected both in figures and their description.

6. Line 150: Fura-2 should be Fura-2 AM.

Response: Thanks for the comment, this has been corrected.

7. Line 198: "100mM, 2.5% EGTA - TritonX-100" is a confusing way of representing the concentration of both agents. Suggest: 100mM EGTA/2.5% TritonX-100.

Response: Thanks for the comment, this has been corrected.

8. Lines 216/217: indicate which data is in Figure 1a and which data is in Figure 1b. e.g. "characterized by mass spectroscopy (Figure 1a) and HPLC (Figure 1b)".

Response: Thanks for the comment, this has been corrected.

9. Line 220: Same comment as above; indicate which data is in Figure 2a and which data is in Figure 2b.

Response: Thanks for the comment, this has been corrected.

10. Where any replicates performed? This is not discussed.

Response: Thanks for the comment, this has been corrected in the Protocol section.

11. Lines 255-257: This sentence should be re-written as it's very awkward sounding.

Response: Thanks for the comment, the lines have been proofread.

12. Table of materials had some formatting issues/missing fields. Also, the catalog number was not indicated for several of the reagents.

Response: Thanks for the comment, the table has been formatted and catalog numbers have been added.

Reviewer #3:

1. The time point of Figure 2-4 were indistinct, especially in Figure 4. When did you add Ionomycin and Triton-100 X? Also, Trition-100 X is usually applied as a lysis cell membrane. How do you detect intracellular calcium levels even after cell fragmentation? In general, this is when the extracellular ions are already free to come in.

Response: Thanks for the comment. The addition time points have been mentioned in Protocols and shown in figure as well.

You are correct, Triton-100 X is indeed used as a cell permeating agent. In the experiment, Triton-X is used with EGTA. EGTA is a Calcium chelating agent which will bind to calcium ion resulting in the minimum fluorescence. It is hence used as a control in the experiment.

2. Please describe in detail how you truncate and construct peptides?

Response: Thanks for the comment, this has been added.

3. Endogenous proadrenomedullin N-terminal 12 was used as the only parent ligand for MRGPRX2. More examples using other parent ligand, such as substance P, LL-37, should be shown as you suggesting that the approach can be used as universal method to explore target residues.

Response: Thanks for the comment, the complete article containing all results from the study is under submission.

4. Data for confirmation of high-expressed MRGPRX2 HEK293 cells should be presented. For example, western blot.

Response: Thanks for the comment, a reference for this has been added.

Changes to be made in the video

Changes to be made by the Author(s) regarding the video:

1. Please increase the homogeneity between the video and the written manuscript. Ideally, all figures in the video would appear in the written manuscript and vice versa. The video and the written manuscript should be reflections of each other.

Response: Thanks for the comment. Introduction, conclusion and narration has been reshot to include texts form the manuscript.

2. Furthermore, please revise the narration to be more homogenous with the written manuscript. Ideally, the narration is a word for word reading of the written protocol and is in imperative tense as in the written manuscript.

Response: Thanks for the comment, changes have been made.

3. Please ensure that the narration should line up with what is being shown in the video.

Response: Thanks for the comment, changes have been made.

4. Please ensure that the protocol section headings are the same in the text and the video.

Response: Thanks for the comment, changes have been made.

5. 1.24: Please remove Materials and methods and change the section heading to In vitro cell culture instead without any numbers preferably.

Response: Thanks for the comment, changes have been made.

6. Instead of having a narration please include an ethics card to show the protocol follows the institutional guidelines.

Response: Thanks for the comment, changes have been made.

7. Please do not use personal pronouns in the protocol section of the video.

Response: Thanks for the comment, changes have been made.

8. 4:30: We cannot have commercial terms in our manuscript text or video. Please use a generic term and cover the commercial term in the video. The goal of this policy is to focus on science rather than to present a technique as an advertisement for a specific item.

Response: Thanks for the comment, changes have been made.

9. 4:37 onwards: Please zoom the shots to remove the SoftMax Pro from the video.

Response: Thanks for the comment, changes have been made.

10. 7:54: Please show changing of the tips when the buffer is being added to the plate.

Response: Thanks for the comment, changes have been made.

11. 9:11: Please change the section title to Representative Results instead.

Response: Thanks for the comment, changes have been made.

12. Figure 1: Please use an arrow to show the time point when the peptide was added.

Response: Thanks for the comment, changes have been made.

13. Please remove the figure legends from the figure.

Response: Thanks for the comment, changes have been made.

14. What activating and non-activating ligands are used in your case?

Response: Thanks for the comment, peptide has been mentioned.

15. • this video was submitted at 60 frames per second. please note that it will be converted to 30 frames per second before being published.

Response: Thanks for the comment, video has been edited to 30 frames.

16. • A "jump cut" is a type of edit where the camera does not move but the action changes instantly. This type of edit is distracting and jarring for the viewers, and we suggest using a quick dissolve (or fade) to move quickly between similar shots. Some other instances of where using dissolves instead of a jump cut are listed below: 1:47 2:25 2:45 3:40 3:43 3:57 4:18 4:24 4:27 7:48 8:13

Response: Thanks for the comment, 'Fade In' and 'Fade out' have been incorporated.

17. • 2:50-3:14,4:02-4:27, 5:35-5:43, 8:00-8:12, 8:25-8:34 - There are long silences that do not serve the video. Consider editing down the video to only the essential parts of the protocol, and having these parts on-screen during the narration. We recommend no period of video longer than 3 seconds without corresponding audio explaining what is happening on screen

Response: Thanks for the comment, mentioned sections have been edited.

18. • 4:27 - there is no action in this shot (such as opening the incubator and placing the cells within), and the footage is shaky. It may look more professional to simply use a still here.

Response: Thanks for the comment, it has been replaced with an image.

19. • 10:23 - please hold on this last slide a moment more to give viewers time to read more of it.

Response: Thanks for the comment, it has been held longer.

JoVE62448 Reviewers Comments

Editorial and production comments:

Changes to be made by the Author(s):

1. Please employ professional copyediting services. There continues to be awkward phrases throughout the manuscript.

Response: We thank the reviewer for their comment.

Action: The manuscript has been thoroughly edited.

2. Last line of the introduction: Please revise for clarity.

Response: We thank the reviewer for their comment.

Action: We changed this sentence from – “Data being ratio of two fluorescence intensities, effect of experimental factors like dye loading, photobleaching, dye leakage, cell density are corrected”

to – “Since the fluorescence signal is obtained from the excitation of the dye at two wavelengths (340 nm and 380 nm), the ratio of the fluorescence signals corrects for experimental factors like dye loading, dye leakage, photobleaching and cell densities”.

3. Additional details are required:
2.1.3: Is fresh media the culture media from step 2.1.1.? How long is the trypsin reaction and at what conditions?

Response: We thank the reviewer for their comment. Yes, it is washed with fresh media.

Action: This information, and trypsin reaction conditions have been added to the manuscript (page 3, lines 150-153).

2.1.4: Please be more specific with the centrifugation speed. What happens after centrifugation?

Response: We thank the reviewer for their comment.

Action: The centrifugation speed was 1620 g, this information along with centrifugation details have been added to the manuscript (page 4, line 155).

3.1.5: What wavelengths? 340/380 nm?

Response: Sorry, we could not find ‘section 3.1.5’ in the manuscript. However, the manuscript has been proofread to incorporate the wavelengths wherever required.

Reviewers' comments:
Reviewer #2:

Minor Concerns:
Comment #1: There's still significant proofreading required throughout manuscript.

Response: We thank the reviewer for their comment.

Action: The manuscript has been thoroughly edited.

Comment #2: Line 49; antigen binds to the IgE/FcεR1 complex, not the FcεR1 receptor directly.

Response: Thanks for the correction, this is correct.

Action: This fact has been added to the manuscript (page 1, lines 62-63).

Comment #3: Section 1.1: There's still some detail missing in the methods of section 1.1. Suggest adding additional detail or add references in this section of the methods (similarly to sections 2-5) to fill in the gaps for the methods for truncation of amino acids by SPPS, alanine scanning, mass spectroscopy and HPLC.

Response: We thank the reviewer for the suggestions.

Action: Characterization details have been incorporated in the manuscript (page 3, section 1.1 and 1.2, lines 117–140).

Comment #4: Line 126: WT should be written out in full, then abbreviated.

Response: Thank you for pointing this out.

Action: We have used the first occurrence of WT in the text and have added the full name (page 2, line 87).

Comment #5: Line 157: Write out in full what HTB is, then use abbreviation.

Response: Thank you for pointing this out.

Action: We have used the first occurrence of HTB in the text and have added the full name (page 4 line 168).

Comment #6: Line 199: Ideally write the volume and concentration as: "add 50 µl of 30µM ionomycin". This comment applies throughout the methods section for consistency.

Response: Thank you for pointing this out.

Action: We have used the format of volume then concentration throughout.

Comment #7: Fig 4a is not indicated in the results section.

Response: Thanks for the comment.

Action: We have reaffirmed that 'Figure 4a and 4b' was mentioned in the beginning of the sentence for Figure 4 in the results section.

Comment #8: Line 259: Should be "… fluorescence and ratio data, respectively, for dye calibration".

Response: Thanks for the comment.

Action: This format has been adopted.

Reviewer #4:

Major Concerns:
None

Minor Concerns:
the need for a fluorescence plate reader with automated pipetting system before the plate read may not be available to many labs - this may introduce more noise to the time-sensitive Ca2+ readout. Have the authors characterized the variability in the assay when conducted without access to automated pipetting?

Response: Thank you for highlighting the very pressing 'time-sensitivity' in Ca^{+2} measurement from HEK cells.

Lines 308-320 of the revised manuscript, started with a fluorometer. It was labour intensive in terms of number of peptides that can be run within a given period of time. We have studied approx. 40 peptides (in review manuscript), multiplying this with number of replicates and repeats it took a lot of time, and we observed significant variations in the results. We then moved to a plate reader with manual addition. Though, it did help us in running multiple peptides, pulling the plates out to add the peptides and then again to add the standards, again interfered with the results. Finally, we used the plate reader with automated pipetting system, with one control per plate which did give better results, but we got the best when we used both a positive and negative controls for each column of the well (lines 329–330).

Action: None taken.

Reviewer #5:

Major Concerns:

1. The key feature of the described procedure is the use of engineered HEK cells expressing the MRGPX2 receptor. Therefore, the authors should provide further specific details how these cells

were produced. How did they clone the X2 receptor, what plasmid was used, how constructed, what promotor, method of transfection, stable versus transient transfection?, if stable transfected what selection method was used (which antibiotic), were X2-expressing cells further selected for level of X2 expression, if transient transfected what was the variation between different experiments, how was expression determined (flow cytometry?). All these details are essential to describe here and make this procedure unique and useful for other researchers, otherwise this is just a description of "another" procedure to measure calcium fluxes in cells.

Response: Thank you for the comment. The cells used in the study were HEK-293 stably expressing MRGPRX2 receptor. Detailed procedure for transfection and quality control has been described [B. D. McNeil et al, 2015].

Reference

McNeil, Benjamin D., et al. "Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions." Nature 519.7542 (2015): 237-241.

Action: The reference has been added in the text (page 1, line 81; page 2, line 86).

Minor Concerns:

2. Line 49: mast cells are activated by antigen through crosslinking of IgE bound to the FcεRI Receptor

Response: Thanks for the comment, we agree with the reviewer.

Action: Action: This fact has been added to the manuscript (page 1, lines 62-63).

3. Line 134: fresh medium, aspirate medium, and add 2-3 ml trypsin or is medium not aspirated?

Response: Thanks for the comment.

Action: The statement has been clarified to read: "Once 75% confluent, wash the cells gently with 2-3 mL fresh culture medium, remove the medium and add 2-3 mL of trypsin for 2 – 3 minutes in 37 ^{o}C, 5% CO_2 incubator to detach the cells. Gently tap the flask to detach the cells", (page 3, lines 150-153).

4. Line 138: 1620-4500 x g is quite wide range. Is this correct?

Response: Thanks for pointing this out, we agree that it is a wide range.

Action: It has been corrected to 1620 g (page 4, line 155).

5. Line 138: Resuspend the cells in culture medium as per desired concentration. Add culture medium

Response: Thanks for the comment.

Action: ‘Culture Medium’ has been added to the text (page 4, line 156).

6. Line 172: What is read here? Baseline fluorescence? In section 4 the actual reading starts.

Response: Thanks for pointing it out.

Action: It has been clarified in the manuscript to highlight that: “Take the plate for fluorescence reading” (page 4, line 181-182).

7. Line 187: Is the HTB buffer added in section 3 removed?

Response: Thanks for the comment. The peptides are added into the HTB buffer.

Action: This has been clarified in the manuscript (page 5, lines 204-206).

8. Authors use media (plural) throughout the ms while they mean medium (singular). Please check and correct.

Response: Thanks for pointing it out.

Action: It has been corrected wherever required.

9. How is a differential response evoked by X2 by different ligands calculated? Is the AUC calculated for each Ca2+ curve or peak height of the curve?

Response: Thank you for the comment. Qualitatively, an idea of relative calcium signal can be obtained through the fluorescence ratio for 340 to 380 nm (Figure 2 and 3). Furthermore, the fluorescence ratio of the peptides, along with those of the calibrations (Figure 4) can evaluated using the Grynkiewicz equation (protocol, section 5) to quantify the values.

Action: None taken.

Reviewer #6:

Major Concerns:

1. Authors should state the rationale of using PAMP-12 in the present study. Why not other well-known and studied peptide agonist were selected?

Response: Thank you for the comment.

PAMP-12 is a gold-standard peptide that is known to bind this receptor. Moreover, it is easy to synthesize using solid-state techniques as it relatively short [Ehsan Azimi et al, 2017].

Furthermore, our lab has had previous experiences in working with PAMP-12, which simplified our effort [Lei Lu et al, 2018].

References:

Azimi, Ehsan, et al. "Substance P activates Mas-related G protein–coupled receptors to induce itch." Journal of Allergy and Clinical Immunology 140.2 (2017): 447-453.

Lu, Lei, et al. "Self-assembling peptide nanoscaffold that activates human mast cells." ACS applied materials & interfaces 10.7 (2018): 6107-6117.

Action: None taken.

2.a The quality of figures (resolution) is poor, its very difficult to read.

Response: Thank you for the comment. The issue of quality of figures was brought to the Editors of the Journal while submitting the revised draft. We were told that, the issue lies in the PDF compilation and that it would not be carried forward.

Action: None taken.

2.b As authors mentioned somewhere, "the purpose of the manuscript was not to demonstrate the scientific advancement that was achieved using the herein discussed method, but to describe a technique, with all its benefits and challenges". It is very crucial to select the material in the experiments. What is rationale of using alanine analogs instead of some known ligands which do not binds with MRGPRX2?

Response: Alanine scanning helped us to systematically control the interaction between the peptide and the receptor of interest so that one could understand the related physicochemical attributes related to the activation of said receptor.

Selecting widely different molecules obfuscates the effects of individual amino acids or the changes within the region of individual amino acids on receptor activation.

Action: None taken.

3. As MRGPRX2 has shown a binding affinity with a lot of ligands, including few more ligands will improve the quality of results and manuscript overall.

Response: Although we understand the reviewer’s point of view, previous work has done this to some extent already [B. D. McNeil et al, 2015]. Moreover, our paper is not dedicated to understanding the results of ligand binding, rather to elucidate the techniques used to obtain reproducible and robust results. This technique can then be employed by a myriad of researchers to explore a multitude of research questions while ensuring their results are accurate.

Reference

McNeil, Benjamin D., et al. "Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions." Nature 519.7542 (2015): 237-241.

Action: None taken.

4. What is the accuracy and precision data of this novel method? Comparing this data with other/conventional methods will help to understand the accuracy and reliability of method.

Response: Thank you for the comment. We have compared released Ca^{+2}, as a measure of cell activation with other characteristics of mast cell degranulation, particularly, quantification of β-hexosaminidase. The results were very comparable. The study in its entirety has been submitted and is under revision. On the contrary, our initial trials with fluorometer and fluorescence gave varying results (page 8, lines 311-316).

Action: None taken.

5. The data from this method is also greatly depends on the transfection method, including the details of transfection methods will improve the quality. Details such as % of positive transfected cells, ideal passage no of cells etc. is advisable.

Response: Thank you for the comment. Cells used were HEK-293 stably expressing MRGPRX2 receptor. Detailed procedure has been described [B. D. McNeil et al, 2015]. HEK-X2 were used at passage number 13-19 while HEK-WT were used at passages 16-21.

Reference

McNeil, Benjamin D., et al. "Identification of a mast-cell-specific receptor crucial for pseudo-allergic drug reactions." Nature 519.7542 (2015): 237-241.

Action: The reference has been added in the text (page 1, line 81; page 2, line 86). Passage number has been mentioned in the discussion section (page 2, line 85 and 87).

6. How one can quantify the amount of calcium signal from this method? A procedure of quantifying calcium signal and use of relevant software is also needed.

Response: Thank you for the comment. There are two ways of looking into data – as shown in Figure 2b and 3b, a comparison of the ratios between compounds gives a qualitative result for the relative calcium signal. Furthermore, these ratios, along with those of standards/calibrations (Figure 4) are used with the Grynkiewicz equation (protocol, section 5) to quantify the results.

Action: None taken.

Changes to be made by the Author(s) regarding the video:

1. A ""hard cut"" is a type of edit where the camera does not move but the action changes instantly. This type of edit is distracting and jarring for the viewers, and we suggest using a quick dissolve (or fade) between clips to move quickly between similar shots. Dissolves don't have to be to a solid color--one shot can fade directly into another. Instances of where we reccomend using dissolves instead of a jump cut are listed below:
• 4:53
• 5:02
• 6:02
• 6:08
• 6:14
• 9:19
• 9:24
• 9:27
• 9:59"

Response: Thank you for pointing this out.

Action: Hard cuts have been dissolved throughout.

2. 3:28 - 5:25 - the video uses only one continuous shot here, showing the researcher adding the solution to a trough, adding the medium, and then using a multipipette to plate the mixture in a 96 well plate. The narration for this section includes more than this. For one, it seems to list 5 components of the final solution, yet only two additions to the trough are shown (3:37 and 4:06). Additionally, the narration mentions centrifugation and incubation, which are not shown. 5:02-5:12 is one of the few sections that seem to describe the plating that is being shown (i.e., where audio and video match).

Response: Thank you for the comment.

Action: This section has been reedited to incorporate the asked changes and to match the narration with the video.

3. 5:29 - consider keeping this on-screen longer to give viewers time to read it, or removing it entirely. The current brief flash is disorienting to viewers.

Response: Thank you for the comment.

Action: It has been replaced by a still.

4. 9:30 - consider using a black or white box here instead. the blue box draws attention to it and away from the action of interest in the shot.

Response: Thank you for the comment.

Action: It has been blackened.

Video:

1. Please change the title card format to Title, Authors and then Affiliation. Please see the text and do format.

Response: Thanks for the comment.

Action: The Title Card format in the video has been changed to match the text.

2. Please reduce introduction/interview section. No need to introduce yourself in the video as the name and affiliation is there in the text.

Response: Thanks for the comment.

Action: Speaker introduction from the Introduction Section has been trimmed.

3. 4:55 Please show that the plate is being covered with the plater cover, before taking it out of the hood.

Response: Thanks for the comment.

Action: Video has been reshot to include the plate covering. Similarly, video has also been reshot to show plate covering during dye loading, and while taking the plate for fluorescence reading.

4. Please do not show the Invitrogen tag for Fura 2 dye.

Response: Thanks for the comment.

Action: Invitrogen label on Fura-2 dye has been blackened. Centrifuge's company name has also been blackened.

5. 9:36 Hand is blocking the action.

Response: Thanks for the comment.

Action: Video has been reshot to show the action.

7. VIDEO COMMENTS:

• 5:09 - consider moving the audio from 5:09-5:13 up to 5:06 (when the Fura-2 is shown on-screen) and holding on that image for those few seconds, since the narration is talking directly about what is shown in the image. You can maintain the pacing by trimming out the first 3-4 seconds of the video footage starting at 5:09, since the researcher has not yet begun the most critical part of the described step.

Response: Thanks for the comment.

Action: Video showing preparation of Fura-2 stock solution has been added to match the narration.

8. AUDIO COMMENTS:
• Volume is GOOD from 3:14-3:58. All narration should be at this volume as per JoVE ASV guidelines. To this end, please
◘ slightly increase the volume of the narration 2:21-3:08, 5:30-5:36, 6:00-6:07, 6:09-7:19, 7:48-8:36, 10:18-10:40,
◘ slightly decrease the volume 3:59-4:03, 4:24-5:03
◘ decrease the volume 4:04-4:11, 4:19-4:23

Response: Thanks for the comment.

Action: Audio levels throughout has been adjusted to match the mentioned volume.