

CatchPoint™ Cyclic-GMP Fluorescent Assay Kit

Product # R8065

Quantity: 768, 60 µL reactions

Introduction

About the cGMP Assay

The CatchPoint™ cyclic-GMP Fluorescent Assay Kit measures levels of 3', 5'-cyclic guanosine monophosphate (cGMP) or guanylate cyclase activity via a competitive immunoassay for cGMP. The assay requires only a single washing step, and readings can be taken in as little as 10 minutes or as long as 24 hours following substrate addition, since no termination step is needed. The cGMP Fluorescent Assay Kit is suitable for use in cell-based assays.

Principle of the Assay

The cGMP in the sample or standard competes with horseradish peroxidase (HRP)-labeled cGMP conjugate for binding sites on the anti-cGMP antibodies (Fig. 1). In the absence of cGMP, most of the HRP-cGMP conjugate is bound to the antibody. Increasing concentrations of cGMP competitively decrease the amount of bound conjugate, thus decreasing measured HRP activity.

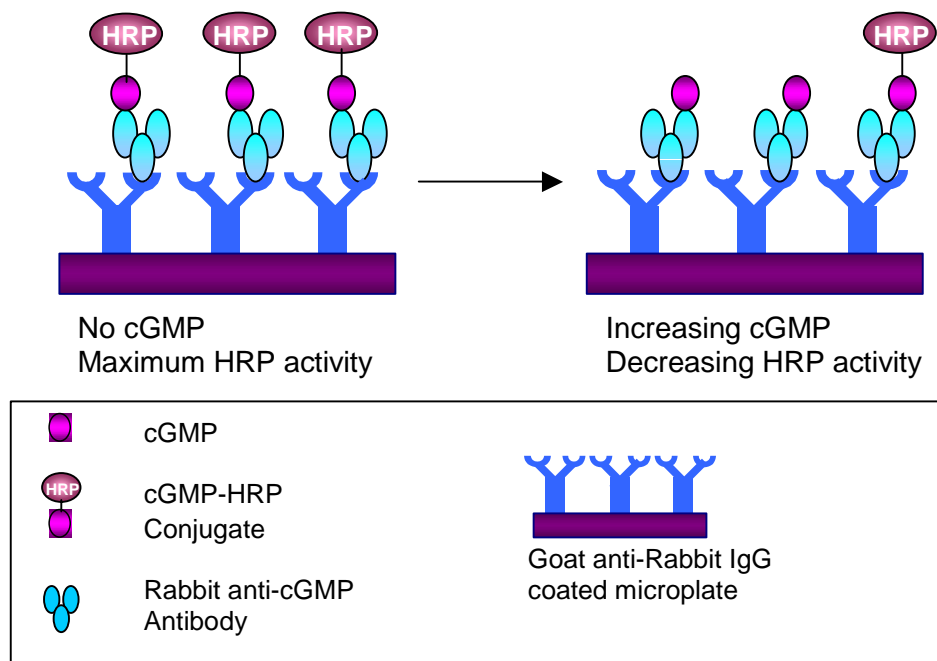


Figure 1. Principle of the CatchPoint cGMP assay system

Applications

The kit is designed for use in applications where cGMP is generated in biochemical assay systems, such as assays of guanylate cyclase activity. In addition, this kit can be used for cell-based assays to measure intracellular cGMP levels following stimulation with peptide hormones or stimulants such as nitric oxide (NO), sodium nitrate, and nitroprusside to soluble or particulate guanylate cyclases.

Materials and Equipment

Kit Components The following table lists the kit components.

Table 1: The CatchPoint cGMP Fluorescent Assay Kit (P/N R8065) contents

Reagent	Quantity	Description
384-well black plates (clear bottom)	2 plates	384-well plates coated with Goat anti-Rabbit IgG. Plates are pre-blocked and ready to use.
cGMP Assay Buffer	100 mL	cGMP Assay Buffer, pH 5.8
Rabbit anti-cGMP Antibody	2 vials	Antibody, lyophilized
cGMP Calibrator	2 vials	30 μ M cGMP Calibrator, lyophilized. On reconstitution with 5 mL assay buffer each vial contains 30,000 pmol cGMP / mL.
HRP-cGMP Conjugate	2 vials	HRP-cGMP Conjugate, lyophilized
10X Wash Concentrate	100 mL	10X Wash Concentrate, pH 7.4
Cell Lysis Buffer	50 mL	Cell Lysis Buffer, pH 7.3
Stoplight Red TM Substrate	450 μ L	One vial containing 100X Substrate
Substrate Buffer	100 mL	Substrate Buffer, pH 7.5

When the working concentrations of the above reagents are used as suggested, each kit provides sufficient reagents for two 384-well microplates with 60 μ L total assay volume.

Storage and Handling

All kit components are to be stored at 4°C.

IMPORTANT: Allow all the reagents to warm to room temperature prior to use.

Note: Small volumes of product will occasionally collect in the cap of the product vial during shipment. Gently tap the vial on a hard surface or briefly centrifuge the vial to collect any liquid trapped in the vial cap.

When stored properly, the kit components are stable for six months from the date of receipt.

The reconstituted working solutions of Rabbit anti-cAMP, cAMP Calibrator, and HRP-cAMP Conjugate are stable for 3 weeks at 4°C. To insure optimal performance of the reagents do not store below 4°C once reconstituted.

If the entire plate will not be used, protect any unused wells with a plate sealer and store at 4°C in the original foil wrapper protected from light.

IMPORTANT: The reconstituted substrate solution is sensitive to light. To insure optimal performance we recommend preparing a fresh stock solution (keep protected from light) and adding to the assay plate within 60 minutes. Following substrate addition readings can be taken at 10 minutes or as long as 24 hours.

Materials Required but not Provided

The following tables list the materials required but not supplied.

Table 2: Reagents and supplies

Reagent Item	Source
3% wt / vol hydrogen peroxide (H ₂ O ₂)	Major laboratory suppliers (MLS)
1.5 mL polypropylene capped centrifuge tubes	MLS
10 mL capped centrifuge tubes	MLS
Pipettors – adjustable volume	MLS
Multi-channel troughs	MLS

Table 3: Compatible Instruments available from Molecular Devices (MDC)

Equipment Item	Source
Analyst System One of the following: - Analyst™ AD - Analyst™ HT - Acquest™ - Screen Station™	MDC P/N 42-000-0096 MDC P/N 42-000-0100 MDC P/N 42-000-0102 MDC P/N 42-000-0151
Gemini XS™ System	MDC P/N 0200-3940
FlexStation™	MDC P/N 0200-4000
Embla 384™ plate washer	MDC P/N 0200-3906

CatchPoint cGMP Fluorescent Assay Kit Experimental Protocol

Preparing the Reagents

The following protocol provides sufficient reagents to run one 384-well plate, including 24 calibrators, 3 controls, and up to 357 samples.

Table 4: Preparation of reagents

Step	Action
1	Reconstitute one vial of 30 μ M cGMP Calibrator: Add 5 mL of cGMP Assay Buffer (pH 5.8) to the vial. Mix well to ensure dissolution of all contents. Store on ice or at 4°C.
2	Reconstitute one vial of HRP-cGMP Conjugate: Add 10 mL of cGMP Assay Buffer (pH 5.8) to one of the vials. Mix well to ensure dissolution of all contents. Store on ice or at 4°C.
3	Reconstitute one vial of Rabbit anti-cGMP Antibody: Add 10 mL of cGMP Assay Buffer (pH 5.8) to one of the vials. Mix well to ensure dissolution of all contents. Store on ice or at 4°C.
4	Dilute 10X Wash Concentrate 10-fold in deionized water, i.e. for one assay plate dilute 50 mL of 10X Wash Concentrate in 450 mL of deionized water.*
5	Prepare calibrators: The 30 μ M cGMP calibrator is diluted in cGMP Assay Buffer to prepare stock calibrators of 10000, 100, 33, 11, 3.7, 1.2, and 0.4 nM cGMP. These give 3300, 33, 11, 3.7, 1.2, 0.41, and 0.14 nM (200, 2, 0.67, 0.22, 0.074, 0.025, and 0.008 pmol) final concentrations in the assay. <ol style="list-style-type: none"> 1. Add 100 μL of the 30 μM cGMP calibrator to 200 μL of cGMP Assay Buffer. This is the 10,000 nM stock calibrator. 2. Add 10 μL of the 10,000 nM stock calibrator to 990 μL of cGMP Assay Buffer. This is the 100 nM stock calibrator. 3. In cGMP Assay Buffer, serially dilute the 100 nM stock calibrator in a 3-fold fashion five times to produce the 33, 11, 3.7, 1.2, and 0.4 nM cGMP stock calibrators. 20 μL of each calibrator are required per replicate. Prepare 100 μL of each (ample for three replicates). As a 0 nM (zero dose) calibrator, use the cGMP Assay Buffer itself.

* On dilution of the 10X Wash Buffer concentrate, the reagent contains 0.02 M Tris, 150 mM NaCl, 0.05% Tween™ 20, and 0.05% Proclin™ 200 (pH 7.4).

Preparing the Plate

For ease of analysis and calculation, the assay plate contains the calibrators, controls, and samples as outlined below. Use the suggested template (Table 5) and run the samples in triplicate.

Note: The order of addition of each component to the wells and the incubation time are important. Mix samples and all reagents gently but thoroughly before use. All assay wells will be prepared by dispensing the assay components in the following order:

- 1) Calibrator, control, or sample
- 2) Rabbit anti-cGMP Antibody
- 3) HRP-cGMP Conjugate

Table 5: To prepare the assay plate:

Step	Action																																																																																
1	<p>Designing your assay plate (plate map):</p> <p>We suggest preparing the plate according to the template below. If you are using SoftMax® Pro to analyze your results, you may set up a template before or after reading the plate. If you set up the plate before, you can print out a template to help in preparing your plate.</p> <table style="margin-left: auto; margin-right: auto; border-collapse: collapse;"> <tr> <td></td> <td colspan="9" style="text-align: center;">Column</td> </tr> <tr> <td></td> <td style="text-align: center;">1</td> <td style="text-align: center;">2</td> <td style="text-align: center;">3</td> <td style="text-align: center;">4</td> <td style="text-align: center;">5</td> <td style="text-align: center;">6</td> <td style="text-align: center;">7</td> <td style="text-align: center;">8</td> <td style="text-align: center;">9</td> </tr> <tr> <td style="text-align: center;">Row</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td style="text-align: center;">A</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td style="text-align: center;">B</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td style="text-align: center;">C</td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> <td></td> </tr> <tr> <td></td> <td style="text-align: center;">control</td> <td style="text-align: center;">3300 nM (200 pmol)</td> <td style="text-align: center;">33 nM (2 pmol)</td> <td style="text-align: center;">11 nM (0.66 pmol)</td> <td style="text-align: center;">3.7 nM (0.22 pmol)</td> <td style="text-align: center;">1.2 nM (0.074 pmol)</td> <td style="text-align: center;">0.41 nM (0.025 pmol)</td> <td style="text-align: center;">0.14 nM (0.008 pmol)</td> <td style="text-align: center;">0 nM (buffer only)</td> </tr> <tr> <td></td> <td colspan="9" style="text-align: center; border: 1px solid black;">Rows D – P and all of columns 10-24: Samples for analysis</td> </tr> </table>		Column										1	2	3	4	5	6	7	8	9	Row										A										B										C											control	3300 nM (200 pmol)	33 nM (2 pmol)	11 nM (0.66 pmol)	3.7 nM (0.22 pmol)	1.2 nM (0.074 pmol)	0.41 nM (0.025 pmol)	0.14 nM (0.008 pmol)	0 nM (buffer only)		Rows D – P and all of columns 10-24: Samples for analysis								
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2	Add 20 µL of the appropriate concentration of Calibrator working solution (see step 5 from “Preparing the Reagents on page 4, Table 4) to columns 2 through 9 (rows A – C). Column 1 is designated here for optional controls. For example, one may wish to use a zero dose calibrator with no Rabbit anti-cGMP Antibody to evaluate background.																																																																																
3	Place 20 µL of samples to be analyzed in appropriate wells (e.g. Columns 1 – 9, rows D – P, and columns 10 – 24). See Appendix A for cell stimulation and lysis protocols.																																																																																
4	Add 20 µL of reconstituted Rabbit anti-cGMP Antibody to all wells except any reserved for no antibody controls. If these are to be performed, add 20 µL assay buffer in place of antibody.																																																																																

Table 5: To prepare the assay plate:

5	Place plate on shaker for 5 minutes or gently agitate by hand to ensure mixing.
6	Add 20 μ L reconstituted HRP-cGMP Conjugate to every well except those reserved for no antibody controls.
7	Mix well and allow to incubate 2 hours at room temperature.
8	Aspirate plate contents and wash 4 times with wash buffer, 80 μ L/well with each wash.
9	<p>Prepare Stoplight Red Substrate: Dilute 220 μL of 100X stock Stoplight Red Substrate into 22 mL of Substrate Buffer, then add 25 μL of 3% H_2O_2 (880 mM) to bring the final concentration to 0.0034% (1 mM) H_2O_2.</p> <p>Important: The reconstituted substrate solution is sensitive to light. To insure optimal performance we recommend preparing a fresh stock solution and adding directly to assay plate within 60 min. At all times keep substrate protected from light.</p>
10	Add 50 μ L Stoplight Red substrate to every well, minimizing the time between starting and finishing. Cover the plate and leave at room temperature for at least 10 minutes, shielded from light.
11	Read the fluorescence intensity of the plate on an appropriate instrument (see below for settings). Plates may generally be read anytime between 10 minutes and 24 hours.

Analyst Parameter Settings **Reading the Plate in Analyst HTS Assay Detection System**

The following instrument settings are recommended when using 384-well plates with a 50 μ L volume in Analyst HTS systems:

Table 6: Fluorescence intensity settings for Analyst HTS Detection System:

Parameter	Setting
Mode	Fluorescence Intensity
Excitation filters	530-25 nm
Emission filters	590-20 nm
Dichroic mirror	50 / 50 beamsplitter
Z-height	3 mm

Attenuator	Medium
Integration time	50,000 μ sec
Lamp	Continuous
Readings per well	One
PMT setup	Smart Read+ (sensitivity = 2)
Units	Counts / sec

Reading the Plate in the Gemini XS Detection System

The following instrument settings are recommended when using 96-well plates with a Gemini XS system:

Table 7: Fluorescence intensity settings for GEMINI XS Detection System

Parameter	Setting
Instrument Settings	Endpoint
Mode	Fluorescence (RFUs)
Excitation	530 nm
Emission	590 nm
Cutoff	570 nm
Sensitivity	6
PMT	Auto
Auto Calibrate	ON

Data Analysis

Analyzing the Calibration Data

The average intensity values (y-axis) can be plotted against calibrator concentration (x-axis) to create a calibration or standard curve. Unknown samples can be interpolated using this curve to calculate cGMP doses in sample. We recommend using SoftMax Pro software for accurate curve fitting and interpolation of data from MDC instruments. Figure 2 shows a SoftMax Pro template and calibration curve for the cGMP assay when read on the Analyst™.

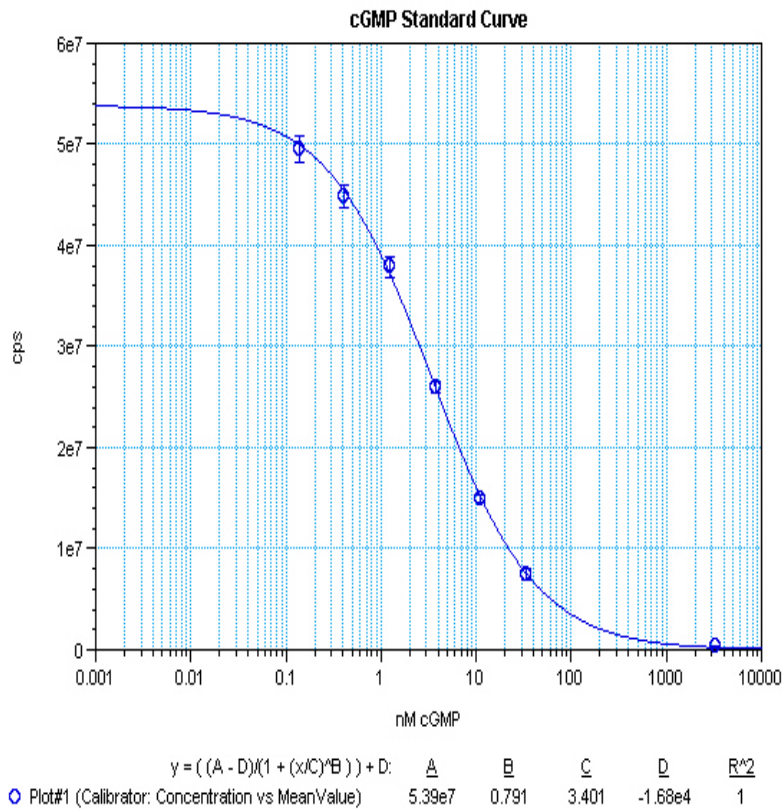
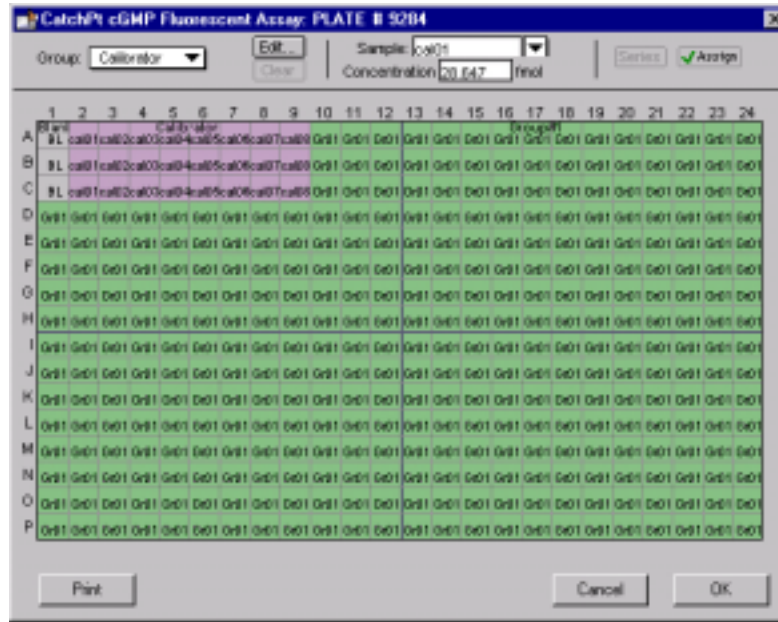


Figure 2. Calibration curve for the CatchPoint cyclic GMP Fluorescent Assay Kit, read on the Analyst system. Data was taken 60 minutes after addition of Stoplight Red substrate. Fluorescence intensity settings for Analyst HTS Detection System were as stated in Table 6. The EC₅₀ value of the calibration curve, calculated as coefficient C using SoftMax Pro, was 3.4 nM (204 fmol).

ADDITIONAL INFORMATION
Sensitivity (Typical values)

Minimum Detectable Concentration = 0.2 nM (12 fmol / well)
 Maximum Measurable Concentration = 500 nM (30 pmol / well)
 EC₅₀ = 2.9 ± 0.7 nM (170 fmol)

Intra-assay precision

Intra-assay precision was calculated from measurements of 0 and 3.7 nM calibrator. The results for a single assay are shown below:

Signal intensity / background (for 0 nM calibrator) §	%B/B₀* precision for 3.7 nM calibrator
Signal intensity _{Avg} / background = 336 Standard deviation (SD) = 10 %CV = 3.0 n = 64	%B/B ₀ (mean) = 45 Standard deviation (SD) = 2.5 %CV = 6.7 n = 64

§ Background taken as the signal intensity for wells in which no cGMP-HRP or Rabbit anti-cGMP Antibody are added

$$* \%B/B_0 = 100 \times (I_{3.7 \text{ nM}} - I_{\infty}) / (I_0 - I_{\infty})$$

where $I_{3.7 \text{ nM}}$ = Signal intensity for 3.7 nM calibrator
 I_0 = Signal intensity for 0 nM calibrator
 I_{∞} = Signal intensity for 3333 nM calibrator

Inter-assay precision (three assays performed on separate days)

Inter-assay precision was calculated from measurements of %B/B₀ for 3.7 nM calibrator in 3 successive assays. The results are shown below:

Measurement	Data points	%B/B ₀	SD	%CV
A	n = 8	40	2.1	5.2
B	n = 8	47	2.2	4.7
C	n = 8	48	2.9	6.0

Precision correlation

64 replicates of each of the standards were prepared and % coefficient of variation was determined for each concentration using SoftMax Pro.

Sample	Concentration	Mean Value	SD	%CV
cal 01	3300 nM	355589	12636	3.6
cal 02	33 nM	6074562	195698	3.2
cal 03	11 nM	12356885	363567	2.9
cal 04	3.7 nM	21756260	676599	3.1
cal 05	1.2 nM	31679740	1001241	3.2
cal 06	0.41 nM	38084456	1242885	3.3
cal 07	0.14 nM	43128761	1155135	2.7
cal 08	0 nM	48143029	1445187	3.0

Appendix A: Preparing cell lysates for the cGMP Assay

The protocol below illustrates an application of the CatchPoint cGMP Fluorescent Assay. The protocol has been developed for use with adherent cells stimulated with atrial natriuretic peptide (ANP). The procedure may be modified for various hormones and stimulators, such as acetylcholine, insulin, serotonin, and nitric oxide.

For best results each cell line should be evaluated to determine the optimal concentration of cells per well, incubation times, and concentration of guanylate cyclase agonists or antagonists.

Use this protocol as a guide to help optimize the assay.

Reagents

Reagent	Stock Concentration
cGMP stimulator atrial natriuretic peptide, dissolved in phosphate buffered saline (PBS)	300 μ M
Phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine (IBMX) dissolved in DMSO	800 mM

Buffers

Buffer	Source
Krebs-Ringer Bicarbonate Buffer (KRBG) containing 10 mM Glucose (pH 7.4)	Sigma Chemical Company (P/N K4002) Note: 15 nM sodium bicarbonate is not included with this product and must be added separately.
Lysis Buffer (pH 7.3)	Molecular Devices Corporation (included in CatchPoint cGMP Fluorescent Assay Kit)
Phosphate Buffered Saline, PBS	Invitrogen™ Life Technologies (P/N 10010-023)

Assay Protocol

Adherent Cells

The following has been used for the adherent cell lines RFL-6 and CHO-K1 :

- 1) Culture cells (1 mL / well) in standard 12-well microtiter plates (tissue culture grade) with cell concentration at $0.75 - 2.5 \times 10^5$ cells/mL.
- 2) Incubate plated cells overnight at 37°C in a humidified atmosphere of 5% CO₂ : 95% air.
- 3) Gently aspirate media and slowly add 400 µL of Pre-stimulation Buffer (pH 7.4).

Pre-stimulation Buffer (containing 0.75 mM IBMX in KRBG Buffer; make fresh on day of experiment)
10 ml of KRBG Buffer (pH 7.4)
9.4 µL of 800 mM IBMX

Note: Once IBMX has been added to the KRBG, mix vigorously to obtain a homogeneous solution.

- 4) Incubate for 10 min at room temperature.
- 5) Add 200 µL of 3X ANP or PBS (negative control). Gently mix and incubate at 37°C for 15 minutes.

ANP, 3X (500 nM final concentration)
2.0 mL PBS (pH 7.4) containing
10 µL of 300 µM ANP (1.5 µM stock)

- 6) Add 200 µL of lysis buffer to each well. Agitate cells to facilitate cell lysis. This can be achieved by shaking the plate on a plate shaker for 10 min after adding the lysis reagent.
- 7) If desired, carry out microscopic evaluation using Trypan Blue to check if cells have lysed (1:1 ratio). Cell membrane may still be visible after cell lysis. Lysed cells are now ready for use and should be immediately processed in the cGMP Fluorescent Assay Kit. Use 20 µL per well neat or suitably diluted lysate in additional lysis buffer (see page 5, Table 5, Step 3 of the Product Insert).
- 8) Proceed with the remainder of the procedure in Table 5.

Cells in Suspension

For non-adherent cells, we recommend centrifugation of the cells from culture medium and suspension of the pellet in Pre-stimulation Buffer (pH 7.4). Add 1 mL (12 well plate) of cell suspension to each well of the plate. It is recommended that you then centrifuge the plates at 1000 rpm for up to 4 min (brake off). Proceed with step 3 (shown above) for adherent cells.

Sales Offices

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