Molecular Devices

Product Insert

CatchPoint™ Cyclic-GMP Fluorescent Assay Kit (Bulk Kit)

Product # R8075

Quantity: 960, 120 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.

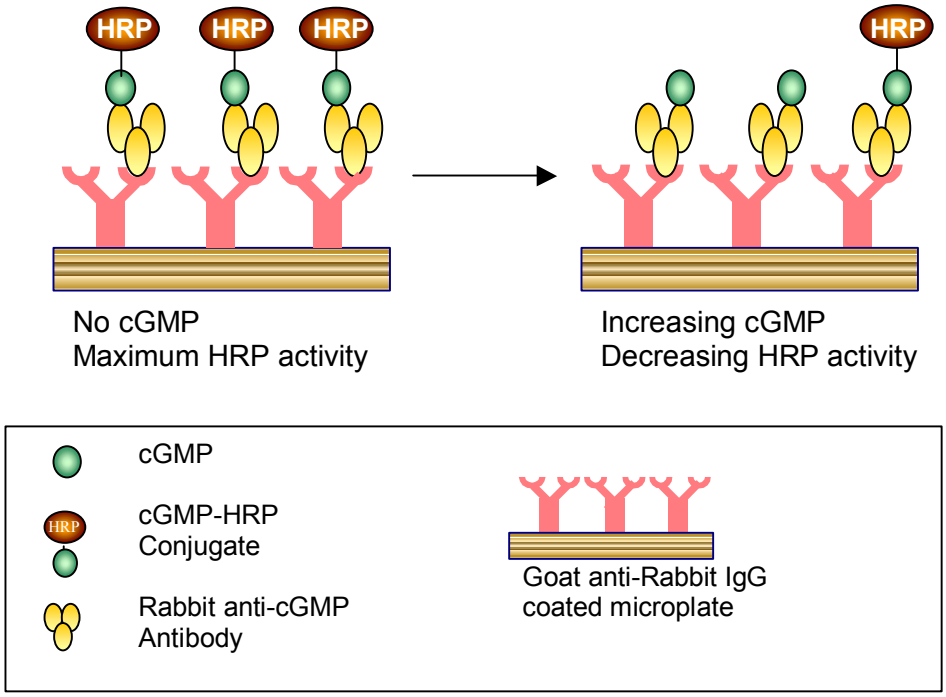

Figure 1. Principle of the CatchPoint cGMP assay system

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 R8075) contents

Reagent	Quantity	Description
96-well black plates (clear bottom)	10 plates	96-well plate coated with Goat anti-Rabbit IgG. Plates are pre-blocked and ready to use.
cGMP Assay Buffer	950 mL	
Rabbit anti-cGMP Antibody	2 vials	Antibody, lyophilized
cGMP Calibrator	2 vials	30 μM 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	950 mL	
Cell Lysis Buffer	450 mL	
Stoplight Red ™ Substrate	2 x 900 μL	100X Substrate in DMSO
Substrate Buffer	950 mL	

When the working concentrations of the above reagents are used as suggested, each kit provides sufficient reagents for ten 96-well microplates with 120 μL 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-cGMP, cGMP Calibrator, and HRP-cGMP 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_2O_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™ GT - Analyst™ HT	 MDC P/N 0200-6003 or 0200-6004 MDC P/N 0200-6043 or 0200-6044
Gemini XPS	MDC P/N Gemini XPS Spectrophotometer
Gemini EM	MDC P/N Gemini EM Spectrofluorometer
SpectraMax® M2	MDC P/N M2
SpectraMax® M5	MDC P/N M5
FlexStation™	MDC P/N FlexStationII Scanning Fluorometer
Embla 96/384™ plate washer	MDC P/N 0200-3948
AquaMax DW4 with 96-well	MDC P/N AquaMax DW4 Liquid Handling System

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CatchPoint cGMP Fluorescent Assay Kit Experimental Protocol

Preparing the Reagents

The following protocol provides sufficient reagents to run five 96-well plates, each with 16 calibrators, 2 controls, and up to 78 samples.

Table 4: Preparation of reagents

Step	Action
1	Reconstitute one vial of 30 μM cGMP Calibrator: Add 5 mL of cGMP Assay Buffer 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 to one of the vials for a 10X stock. Mix well to ensure dissolution of all contents. Transfer the entire contents of the vial to a 100 mL graduated cylinder by repeated washings with assay buffer. Adjust the final volume to 100 mL with assay buffer and mix thoroughly. The diluted HRP-cGMP conjugate is ready for use in the assay. Store on ice or at 4°C.
3	Reconstitute one vial of Rabbit anti-cGMP Antibody: Add 10 mL of cGMP Assay Buffer to one of the vials for a 10X stock. Mix well to ensure dissolution of all contents. Transfer the entire contents of the vial to a 100 mL graduated cylinder by repeated washings with assay buffer. Adjust the final volume to 100 mL with assay buffer and mix thoroughly. The diluted Rabbit anti-cGMP conjugate is ready for use in the assay. Store on ice or at 4°C.
4	Dilute 10X Wash Concentrate 10-fold in deionized water, i.e. for one assay plate dilute 500 mL of 10X Wash Concentrate in 4500 mL of deionized water.*
5	Prepare calibrators: The 30 μM 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 (400, 4.0, 1.3, 0.44, 0.15, 0.049, and 0.016 pmol) final concentrations in the assay. 1. Add 500 μL of the 30 μM cGMP calibrator to 1000 μL of cGMP Assay Buffer. This is the 10,000 nM stock calibrator. 2. Add 20 μL of the 10,000 nM stock calibrator to 1980 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. 40 μL of each calibrator are required per replicate. Prepare 1000 μL of each (ample for 10 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 duplicate.

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:

<table>
<tr><th>Step</th><th>Action</th></tr>
<tr><td>1</td><td>Designing your assay plate (plate map):

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.

<table>
<tr><th></th><th>Column 1</th><th>2</th><th>3</th></tr>
<tr><td>A</td><td>3300 nM
(400 pmol)</td><td>3300 nM
(400 pmol)</td><td>Control 1</td></tr>
<tr><td>B</td><td>33 nM
(4 pmol)</td><td>33 nM
(4 pmol)</td><td>Control 2</td></tr>
<tr><td>C</td><td>11 nM
(1.3 pmol)</td><td>11 nM
(1.3 pmol)</td><td rowspan="7">Column 3 (C – H) and all of columns 4 - 12: Samples for analysis</td></tr>
<tr><td>D</td><td>3.7 nM
(0.44 pmol)</td><td>3.7 nM
(0.44 pmol)</td></tr>
<tr><td>E</td><td>1.2 nM
(0.15 nM)</td><td>1.2 nM
(0.15 nM)</td></tr>
<tr><td>F</td><td>0.41 nM
(0.049 pmol)</td><td>0.41 nM
(0.049 pmol)</td></tr>
<tr><td>G</td><td>0.14 nM
(0.016 pmol)</td><td>0.14 nM
(0.016 pmol)</td></tr>
<tr><td>H</td><td>0 nM
(buffer only)</td><td>0 nM
(buffer only)</td></tr>
</table>
</td></tr>
<tr><td>2</td><td>Add 40 µL of the appropriate concentration of Calibrator working solution (see step 5 from “Preparing the Reagents” on Table 4, page 4) to columns 1 and 2 (rows A – H). Column 3 (A & B) 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.</td></tr>
</table>

Table 5: To prepare the assay plate:

3	Place 40 µL of samples to be analyzed in appropriate wells [e.g. Column 3 (C – H), and columns 4 – 12]. See Appendix A for cell stimulation and lysis protocols.
4	Add 40 µL of reconstituted Rabbit anti-cGMP Antibody to all wells except any reserved for no antibody controls. If these are to be performed, add 40 µL assay buffer in place of antibody.
5	Place plate on shaker for 5 minutes or gently agitate by hand to ensure mixing.
6	Add 40 µL reconstituted HRP-cGMP Conjugate to every well.
7	Mix well and allow to incubate 2 hours at room temperature.
8	Aspirate plate contents and wash 4 times with 300 µL wash buffer for each wash.
9	Prepare Stoplight Red Substrate: Dilute 600 µL of 100X stock Stoplight Red Substrate into 60 mL of Substrate Buffer, then add 68 µL of 3% H_20_2 (880 mM) to bring the final concentration to 0.0034% (1 mM) H_20_2. 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.
10	Add 100 µL 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. For optimal performance you may want to briefly mix the plate prior to reading.

Analyst Parameter Settings

Reading the Plate in Analyst HTS Assay Detection System

The following instrument settings are recommended when using 96-well plates with a 100 μL volume in Analyst HTS systems:

Table 6: Fluorescence intensity settings for Analyst HT or GT 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 μsec
Lamp	Continuous
Readings per well	One
PMT setup	Smart Read+ (sensitivity = 2)
Units	Counts / sec

Reading the Plate in the Gemini XS, Gemini XPS, Gemini EM, SpectraMax® M2 and SpectraMax® M5 Detection System

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

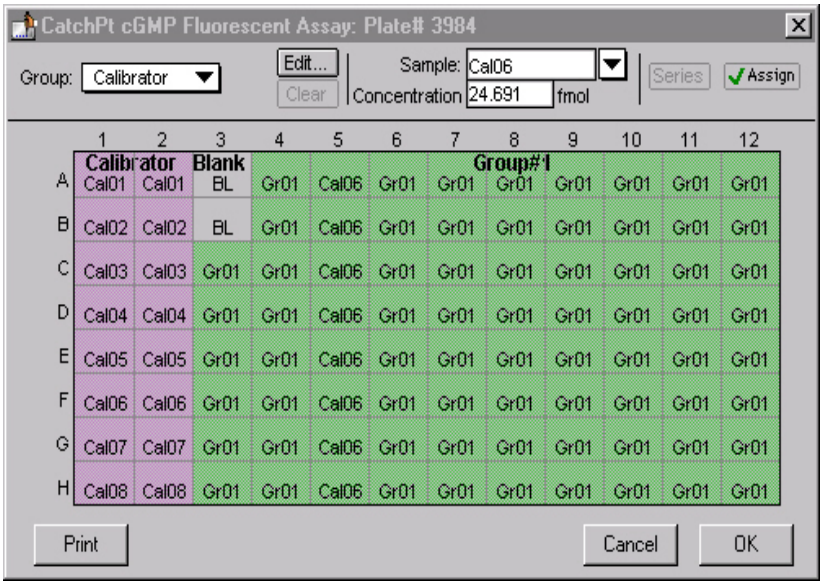
Table 7: Fluorescence intensity settings for GEMINI XS, Gemini XPS, Gemini EM, SpectraMax® M2 and SpectraMax® M5 Detection System

Parameter	Setting
Read Mode	Endpoint
Read Type	Fluorescence (RFUs)
Excitation	530 nm
Emission	590 nm
Cutoff	570 nm
Sensitivity	6
PMT	Auto
AutoCalibrate	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 the samples. 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™.



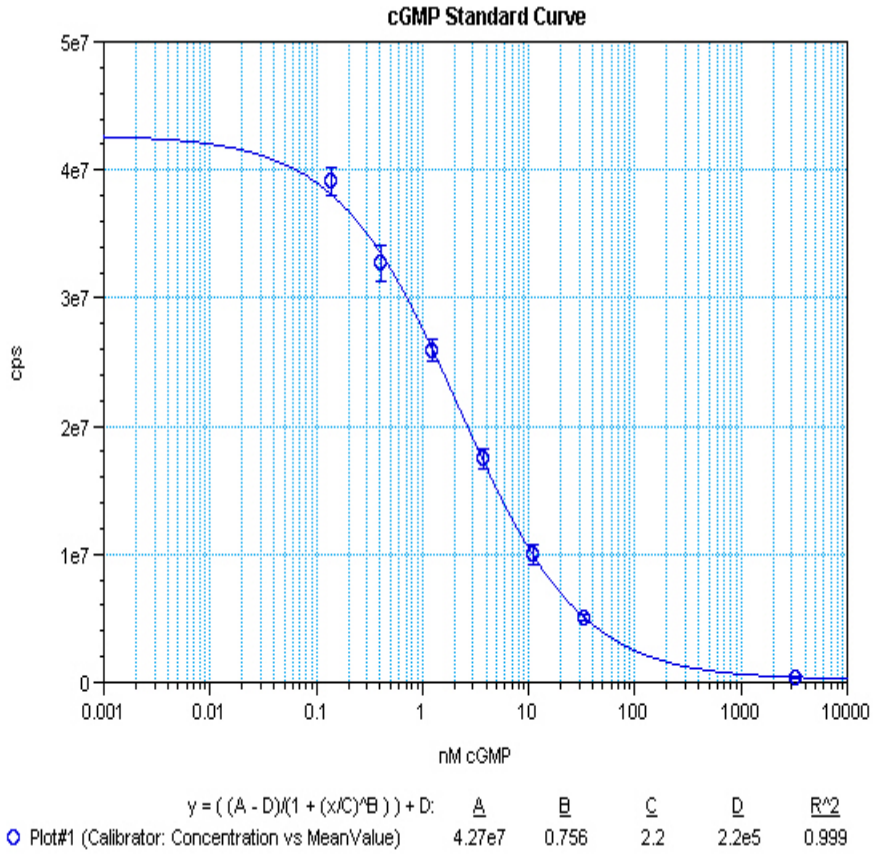


Figure 2. Calibration curve for the CatchPoint cyclic GMP Fluorescent Assay Kit, read on the Analyst system. Data was taken 60 minutes after addition of Stoplight Red substrate. Fluorescence intensity settings for Analyst HTS Detection System were as stated in Table 6. The EC_{50} value of the calibration curve, calculated as coefficient C using SoftMax Pro, was 2.2 nM (264 fmol).

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ADDITIONAL INFORMATION

The following typical information is based on data from an Analyst HT. Absolute readings may differ on other instruments, but relative values should be comparable.

Sensitivity (Typical values)

Minimum Detectable Concentration = 0.2 nM (24 fmol / well)
Maximum Measurable Concentration = 120 nM (14 pmol / well)
EC_{50} = 2.5 ± 0.6 nM (300 fmol)

Intra-assay precision

Intra-assay precision was calculated from measurements of 0 and 1.2 nM calibrator. The results for a single assay are shown below:

Signal intensity / background (for 0 nM calibrator) [§]	**%B/B$_o$* precision for 1.2 nM calibrator**
Signal intensity$_{Avg}$ / background = 290 Standard deviation (SD) = 12 %CV = 4 n = 23	%B/B$_o$ (mean) = 59 Standard deviation (SD) = 4 %CV = 7 n = 23

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

* $\%B/B_o = 100 \times (I_{1.2\,nM} - I_\infty) / (I_o - I_\infty)$

where $I_{1.2\,nM}$ = Signal intensity for 1.2 nM calibrator
I_o = 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/B$_o$ for 1.2 nM calibrator in 3 successive assays. The results are shown below:

Measurement	Data points	%B/B$_0$	SD	%CV
A	n = 2	58	5	8
B	n = 2	61	4	6
C	n = 23	59	4	7

Precision correlation

22 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	208470	7826	3.8
cal 02	33 nM	1994030	135129	6.8
cal 03	11 nM	4876559	202898	4.2
cal 04	3.7 nM	10220036	447378	4.4
cal 05	1.2 nM	17045496	808640	4.7
cal 06	0.41 nM	22045375	982043	4.5
cal 07	0.14 nM	24362726	1233519	5.1
cal 08	0 nM	28592964	1049992	3.7

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 μM
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 mM 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 x 10^5 cells/mL.

2) Incubate plated cells overnight at 37°C in a humidified atmosphere of 5% CO_2 : 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 40 µL per well neat or suitably diluted lysate in additional lysis buffer (see page 6, 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.

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