Bridge-It®

cAMP all in one Fluorescence Assay

Cat. No. 122938: 384 measurements (384-well microplate)
Cat. No. 122939: 384x2 measurements (384-well microplate)
Cat. No. 122940: 384x3 measurements (384-well microplate)

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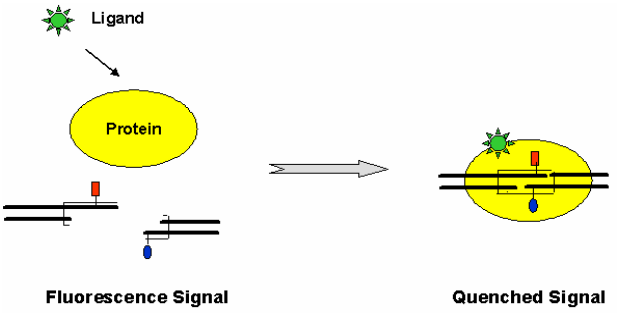
Introduction

The common property of all sequence-specific DNA binding proteins is their ability to bind with high affinity and specificity to a DNA duplex containing a unique nucleotide sequence, i.e., the DNA binding site for the protein. Mediomics' assay platform relies on this common characteristic. A DNA duplex containing the sequence-specific DNA binding site for a given target protein is split into two DNA "half-site" duplexes. These single-stranded extensions are short enough so that in the absence of the target protein little spontaneous re-association occurs. When the target protein is present, however, its high affinity for the full-length DNA sequence will drive the re-association of the two half-site DNA duplexes. This re-association can be sensitively detected by incorporating appropriate fluorescence probes into each of the two DNA half-sites. The presence of the DNA binding protein will be detected as a quenching of the fluorescence signal. A simple variation of this basic platform design allows a DNA binding protein to function as a sensitive biosensor for its specific ligand as shown schematically below:



Bridge-It® Cyclic AMP Assay Principle

Adenosine-3',5'-cyclic monophosphate (cAMP) is an important second messenger which is involved in the modulation of numerous biological processes. The measurement of cAMP is especially important in new drug discovery since cAMP levels are closely related to the activity of one of the major targets for new drug discovery - the G protein-coupled receptors (GPCR). Mediomics fluorescence cAMP assay method for determining the concentrations of cAMP is based on the assay platform design described above. Basically CAP, a bacterial DNA binding protein whose DNA binding activity depends on the presence of cAMP, is used as a highly specific biosensor for measuring cAMP levels.

Bridge-It® cAMP all in one Assay: Reagents and Storage Requirements

The quantity of reagents provided in an assay kit depends on the total number of measurements (wells) for the specific kit size being used (see Ordering information for available kit sizes). Store Bridge-It® cAMP all in one kits at -20ºC until use. **Thaw only the appropriate number of tubes needed for use.** Each tube of cAMP all in one Assay Solution contains reagent adequate for performing fifty (50) measurements using a 384-well black polypropylene microplate. The assay kit reagents retain their activity for at least one year when stored frozen at –20°C.

Tube Cap Color	Assay Reagent	ml/tube	Storage Conditions
Green	10 µM cAMP stock standard in H_20	0.25	Maybe thawed and refrozen up to five times.
Orange	2X Assay Solution A	0.4	Once thawed store at 4ºC. **DO NOT RE-FREEZE.** May be refrigerated for up to 1 week.
Clear	10X Lysis Buffer	0.1	Once mixed with 2X Assay Solution A, store under same storage conditions as Assay Solution. May be re-frozen prior to mixing.
Blue	Buffer B*	1.0	Once thawed store at 4ºC.

***Note:** Buffer B may be used for diluting cAMP standards and test samples.
384-well black polypropylene microplates can be obtained from Mediomics.

Reagent <u>not</u> included in the kit:

10X KRB-IBMX Buffer: 10X Krebs-Ringers Bicarbonate Buffer containing 7.5 mM the phosphodiesterase inhibitor IBMX (10X KRB-IMBX buffer) may be obtained from Mediomics (Catalog # 122937). KRB-IMBX buffer is recommended for either attached or suspended cells during chemical stimulation (e.g., forskolin).

Cell Preparation and General Notes for the Bridge-It® all in one Assay Method

Cell samples obtained from either cultured adherent or suspension cells may be analyzed for cAMP. We provide data obtained using HEK 293 cells plated at a concentration of ~5,000 cells / well for stimulated with increasing concentrations of forskolin (0-200 µM). Stimulation by forskolin is not required in all applications, however, it is included in the method presented below. The cells may be processed either directly in the wells of the microtiter plate or processed in tubes and then transferred into the wells of a black polypropylene microplate. In the following procedures, the samples are described for direct placement into microplate wells. It is recommended that all sample preparation be carried out in the presence of a phosphodiesterase inhibitor in order to minimize degradation of cAMP in the test samples. A TECAN, SPECTRAFluor PLUS plate reader is used in our laboratory for fluorescent signal detection but any fluorescent plate reader or spectrofluorometer capable of measuring fluorescein may be used.

General Notes regarding the Bridge-It® cAMP all in one Assay Method

The following precautions are recommended to help optimize assay performance:

- Avoid bubbling as much as possible. Pipette slowly to allow for complete recovery and transfer of assay solutions.

- The assay may be carried out in Eppendorf tubes prior to transfer to wells of a black 384-microtitre plate for final analysis. After addition of the cAMP all in one Assay Solution to Eppendorf tubes, immediately vortex for ~1 second at medium speed. Samples may also be incubated in the tubes for 25-30 minutes in the dark prior to transfer into a black polypropylene microplate for immediate reading. This allows for recovery of all solution that may coat the tubes.

- When using Eppendorf tubes, 18 µl may be transferred into the appropriate well of a 384-well black polypropylene microplate. This allows for consistency in volume. The transfer of 18 µl / well versus 20 µl / well when assayed directly in the microtiter plate will not effect assay sensitivity. It is recommended that the volume used for standards and unknowns should however, be the same.

- You may gently tap the microplate on the counter top to remove any trapped bubbles. Large bubbles may be easily broken with the tip of a small gauge needle (i.e., 27 gauge) while placing your fingertip or a syringe on the other end of the needle to avoid loss of volume by capillary action

- Because of the small number of cells used in the assay, it is difficult to see the cell pellet following centrifugation in the Eppendorf tubes. It is recommended, therefore, that all of the tubes be placed into the centrifuge in the same orientation (i.e., place the top hinge of the tube in the same direction) in order to know where the cell pellet is located after centrifugation. This will make it easier to remove the supernatant without disturbing the cell pellet.

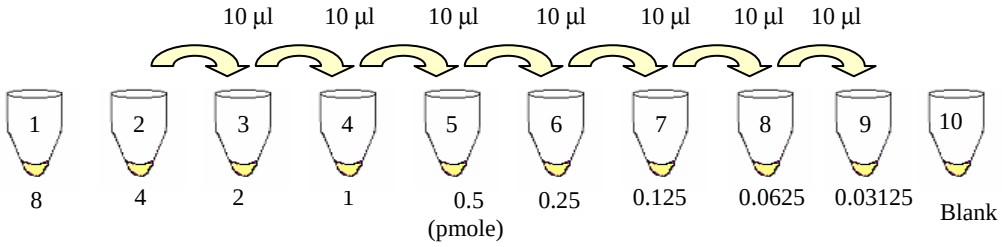
Preparation of cAMP all in one Assay Solution

After thawing, the 10X lysis buffer (clear cap tube) solution may appear turbid. This turbidity will disappear when the solution warms to room temperature and is vortexed. The 10X lysis buffer may be centrifuged to allow for complete recovery. Add the contents of one tube of 10X lysis buffer (clear cap tube) into one tube of 2X Solution A (orange cap tube) and mix immediately by pipetting up and down several times to prepare one tube of cAMP all in one Assay Solution. To avoid confusion, place a cAMP all in one Assay Solution label (provided with the kit) onto each of the newly prepared Assay Solution tubes. **If more than one tube of all in one Assay Solution is to be used, the contents of all such tubes should be mixed together prior to use to insure reagent consistency.** Keep on ice until ready for use.

Bridge-It® cAMP all in one Standard Curve

It is recommended that the cAMP standard curve be prepared in Buffer B or under similar conditions of the test samples. If a different buffer from Buffer B or KRB-IMBX is used, a test to determine the effect of that buffer on the assay performance should be conducted before using it in the assay. The standard curve may also be directly added to un-stimulated cells (see Figure 2).

1. Dilute the 10 µM cAMP standard (green cap) 1:12.5 using Buffer B (blue cap) for a final concentration of 0.8 µM cAMP. Prepare 10 Eppendorf tubes. Add 10 µl of Buffer B to tubes 2-10. Add 10 µl of the 0.8 µM (8 pmol) cAMP stock to tubes 1 and 2. Mix the contents of tube 2 by pipetting up and down 5 times and then transfer 10 µl from tube 2 into tube 3. Continue serially diluting the cAMP standards through tube 9. After mixing the contents of tube 9, discard 10 µl from tube 9. All cAMP standard assay tubes should contain a final total volume of 10 µl.



Note: cAMP standards may be serially diluted in the wells of the 384-well microplate. If this is done, care should be taken during mixing to avoid bubbling by gently pipetting up and down ~3-5 times.

2. Prepare cAMP all in one Assay Solution as described previously (page 5) and keep on ice until ready for use.

3. Add 10 µl of the cAMP all in one Assay Solution to each tube and vortex immediately ~1 second at medium speed. If the standard curve is prepared directly in the wells of the 384-well microtiter plate, mix by gently pipetting the solution up and down ~ 4 times.

4. If Eppendorf tubes are used, 18 µl from each tube may be transferred into the appropriate well of a 384-well black polypropylene microplate to ensure consistency in volume. The transfer of 18 µl / well versus 20 µl / well when assayed directly in the microtiter plate will not effect assay sensitivity. It is recommended that the volume used for standards and unknowns should however, be the same.

5. Cover the microplate with tinfoil to avoid evaporation and exposure to light. Incubate the covered microplate at room temperature (~25°C) for 30 minutes.

6. Read fluorescence intensity (excitation ~480 nm, emission ~520 nm) with a fluorescence plate reader.

Determination of cAMP in Suspension Cells

1. Cells are harvested, washed in 37ºC warmed buffered saline solution, brought up to the desired cell concentration in a warmed serum-free buffer containing a phosphodiesterase inhibitor and aliquoted into 384-well black polypropylene microplate wells (for example: ~5,000 HEK 293 cells in 10 µl KRB-IBMX buffer/384-well).

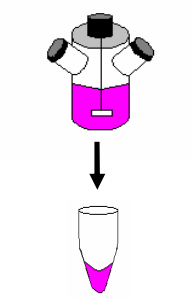
2. To stimulate cells to produce cAMP, an agonist was added to each well (e.g., 0-200 µM forskolin) and incubated for 15-30 minutes at room temperature while rotating. It is recommended that the cells be allowed to equilibrate in the KRB-IBMX buffer for 15 minutes at room temperature before the addition of forskolin.

cAMP Assay:

3. To lyse cells and start the cAMP assay, add 10 µl of the cAMP all in one Assay Solution to each of the wells of the 384-well black polypropylene microplate containing cells and mix immediately by gently pipetting up and down 4-times.

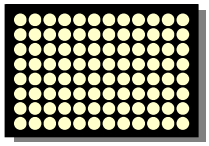
4. Cover the microplate to avoid evaporation and exposure to light. Incubate the microplate at room temperature (~25°C) for 30 minutes.

5. Read fluorescence intensity (excitation ~480 nm, emission ~520 nm) with a fluorescence plate reader.

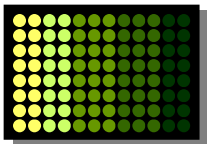


Cells suspended in medium. Wash, re-suspend in KRB-IBMX buffer (~5x10^5/ml). Incubate 15 min (~25ºC)

Add ~5,000 cells in 10µl / well
Add forskolin. Incubate for 15-30 min (~25ºC) while rotating



Add 10 µl all in one Assay Solution / well
Mix. Incubate 30 min (~25ºC) while rotating



Read fluorescent signal

Determination of cAMP in Attached Cells

1. Cells were grown to approximately 80% confluency, trypsinized and harvested by centrifugation. The cell suspension was then washed in 37°C warmed PBS and brought up to the desired cell concentration in a warmed serum-free buffer containing a phosphodiesterase inhibitor (1X KRBG-IBMX).

2. Aliquote cells into 384-well black polypropylene microplate wells (for example: ~5,000 HEK 293 cells in 10 µl KRB-IBMX buffer/384-well).

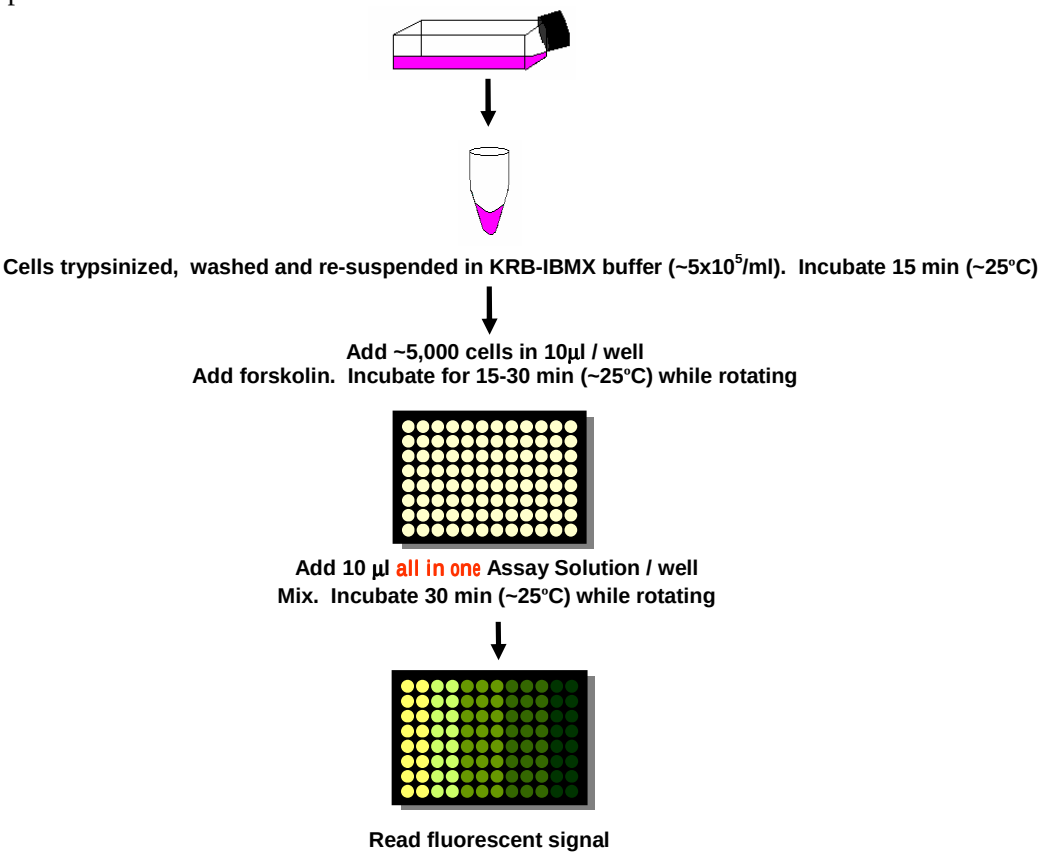
3. To stimulate cells to produce cAMP, an agonist was added to each well (e.g., 0-200 µM forskolin) and incubated for 15-30 minutes at room temperature while rotating. It is recommended that the cells be allowed to equilibrate in the KRB-IBMX buffer for 15 minutes at room temperature before the addition of forskolin.

cAMP Assay:

4. To lyse the cells and start the cAMP assay, add 10 µl of the cAMP all in one Assay Solution to each of the wells of the 384-well black polypropylene microplate containing cells and mix immediately by gently pipetting up and down 4-times.

5. Cover the microplate to avoid evaporation and exposure to light. Incubate the microplate at room temperature (~25°C) for 30 minutes.

6. Read fluorescence intensity (excitation ~480 nm, emission ~520 nm) with a fluorescence plate reader.



Direct Determination of cAMP in Liquid Samples

The cAMP all in one assay is designed to use 50% volume of clarified liquid test samples added directly to the cAMP all in one Assay Solution. If the sample cAMP concentration is greater than the recommended standard curve, then the sample may be diluted in Buffer B prior to addition to the cAMP all in one Assay Solution. The mixture is then incubated for 30 minutes at room temperature (~25°C) and read fluorescence intensity (excitation ~480 nm, emission ~520 nm) with a fluorescence plate reader.

Bridge-It® cAMP all in one Assay - Data Analysis

There are two possible ways of analyzing the fluorescent intensity readouts from the assay.

1. Raw fluorescence intensity may be used as a signal inversely proportional to cAMP concentration (y axis is linear, x axis is common log).

2. Relative fluorescence change or percent quenching can be calculated from raw fluorescence values using the following formula where RF = Relative Fluorescence, F_0 = fluorescent intensity of the blank or buffer control) and F = fluorescence of cAMP: $RF = (F_0 - F)/F_0$
   Use of RF values are highly reproducible for the same cAMP concentrations and do not depend greatly on the instrument used to read fluorescence. An RF of 1.0 is equal to 100% quenching.

   The software program "Sigma Plot" was used to analyze and graph the data obtained from the Bridge-It® cAMP all in one assay. Data were converted to RF values and plotted as a sigmoidal, 4 parameter graph (x axis as common log, y as linear).

3. For calculation of the **total** amount of cAMP in unknown samples, the cAMP value must be multiplied by the dilution factor of 2.

Bridge-It® cAMP all in one Assay - Assay Performance

Bridge-It® cAMP all in one Standard Curves

The following cAMP standard curves are representative of those prepared using the Bridge-It® cAMP all in one cAMP assay. **Note:** Definition of RF (% Quenching) is on page 11. Figure 1 shows a standard curve prepared in Buffer B in a 384-well plate with the fluorescence signal read after 30 minutes, 1 hour and 2 hours after addition of all in one Assay Solution. Figure 2 represents a standard curve prepared with and without cells in the 384-well format.

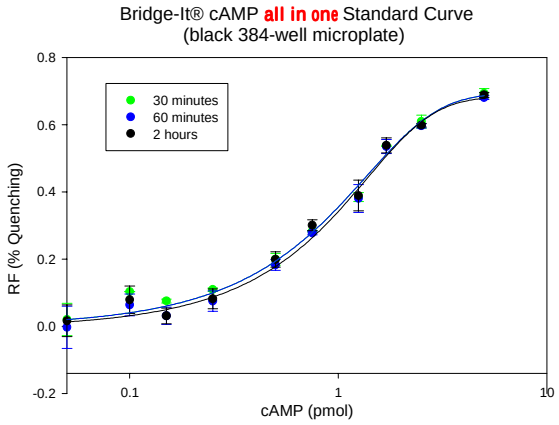


Figure 1

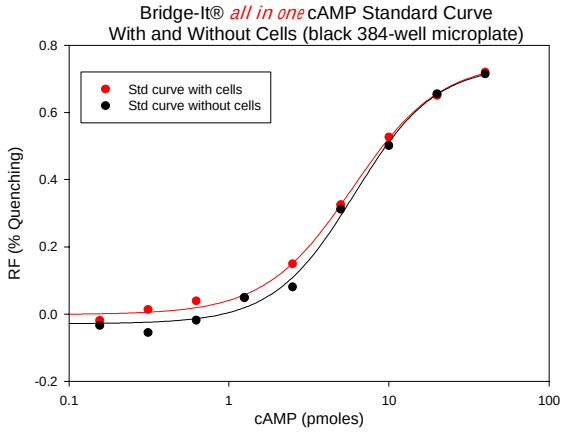


Figure 2

Bridge-It® cAMP all in one Cell Studies

The following figures show the results of stimulated HEK 293 cells. The assays were run in 384-well microplates (Fig. 3). The standard errors were from duplicate measurements and the assay was performed as previously outlined. Figure 4 shows the response of various amounts of HEK 293 cells in suspension to forskolin. The assay was performed according to 384-well microplate protocol.

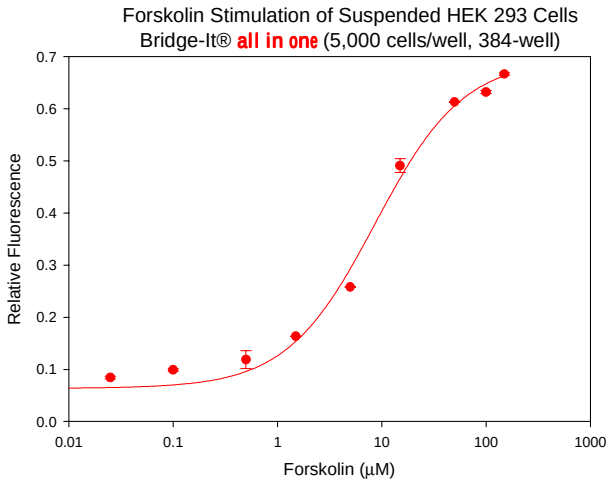


Figure 3

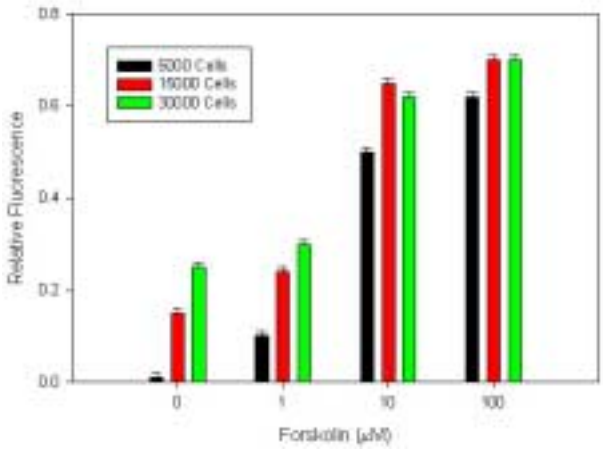


Figure 4

Bridge-It cAMP® all in one Urine Studies

Figure 5 illustrates the potential of using the Bridge-It® cAMP all in one assay for the detection of cAMP in urine samples. Small volumes of clarified urine were added directly to all in one Assay Solution, incubated, and fluorescence intensity read.

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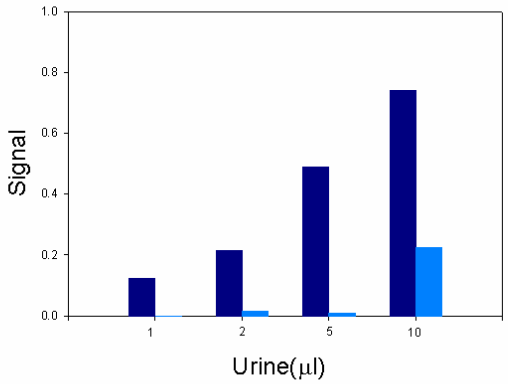
Fig. 5 Detection of cAMP in urine. Light colored bars show the signal observed after treating the urine samples with phosphodiesterase.



Specificity Studies of Bridge-It® cAMP Assay

Table 1 demonstrates the cross reactivity of the Bridge-It® cAMP assay for a number of related compounds. Samples were brought up to 0.1M concentration in water and adjusted to 200 nM (20 pmole) per 96-well in Buffer B and measured for cAMP activity.

Table 1

Compound	Cross Reactivity
cAMP	100%
ATP	0%
AMP	0%
cGMP	0%
GTP	0%
CTP	0%
GMP	0%

Bridge-It® References

1. Heyduk, T & Heyduk, E, Molecular beacons for detecting DNA binding proteins. *Nature Biotechnology* **20**, 171-176 (2002)
2. Heyduk, E, Fei, Y, & Heyduk, T, Homogeneous Fluorescence Assay for cyclic AMP, *Combinatorial Chemistry and High-Throughput Screening*, **6**, 183-194 (2003)
3. US Patent No. 6,544,746, Rapid Proximity-Based Assay for the Detection and Quantification of DNA-Binding Proteins.
4. Heyduk T, Knoll E, & Heyduk E, Molecular beacons for detecting DNA binding proteins: mechanism of action. *Anal Biochem* **316**, 1-10 (2003)

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Ordering Information

cAMP all in one **Assay Kits:**

Product Description[1]	Format	Measurements	Catalog No.	Kit Price[2]
Bridge-It® cAMP all in one Assay Kit (10X KRB-IBMX Buffer included)	384-well microplate	384	122938	$630.00
Bridge-It® cAMP all in one Assay Kit (10X KRB-IBMX Buffer included)	384-well microplate	768 (384 x 2)	122939	$1,150.00
Bridge-It® cAMP all in one Assay Kit (10X KRB-IBMX Buffer included)	384-well microplate	1152 (384 x 3)	122940	$1,610.00

10X KRB-IBMX Buffer:

Description[1]	Volume	Mediomics Cat. No.	Price per Kit[2]
10X KRB-IBMX Buffer	1.5 ml	122937	$8.00

384-well Black Microplate:

Description[1]	Mediomics Catalog No.	Price per Kit[2]
384-well round-bottom low volume non-binding surface black polystyrene microplate	163301	$9.50/microplate

[1]Bridge-It™ is a trademark of Mediomics, LLC.
[2]All prices are denominated in U.S. dollars. Shipping and handling cost will be applied. Prices shown may be changed without notice.

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