# ASSAY PROTOCOL

## Bridge-It® cAMP designer Fluorescence Assay

### (96-well microplate format)

**Mediomics, LLC**
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St. Louis, Missouri 63124 USA

| | |
|---|---|
| Ordering: | 1-800-292-4808, Direction Code 2068<br>1-314-971-3028 |
| Tech Support: | 1-800-292-4808, Direction Code 2688<br>1-314-971-3026 |
| Fax: | 1-314-997-2422 |
| Web: | www.mediomics.com |

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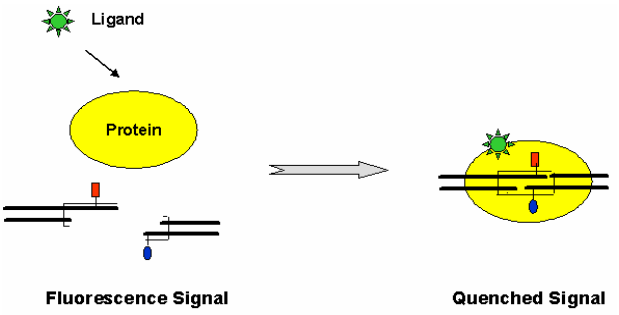
# Introduction

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 designer 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 designer Assay Reagents and Storage Requirements

The quantity of reagents depends on the number of cAMP measurements per kit. Store the Bridge-It® cAMP designer fluorescence assay kit at -20ºC until use. **Thaw only the appropriate number of tubes needed for use.** Each tube of cAMP designer Assay Solution A contains reagent adequate for performing 10 cAMP measurements using a black 96-well microplate. The 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 $H_2O$ | 0.25 | Maybe thawed and re-frozen up to five times. |
| Orange | Assay Solution A | 1.0 | Once thawed store at 4ºC. May be refrigerated for up to 1 week. **DO NOT RE-FREEZE.** |
| Clear | 10X Lysis Buffer | 0.10 | Once thawed store at 4ºC. May be refrigerated for up to 1 week. **DO NOT RE-FREEZE.** |
| Blue | Buffer B[1] | 1.0 | Once thawed store at 4ºC. May be refrigerated for up to 1 week. **DO NOT RE-FREEZE.** |
| Yellow | 10X KRB-IBMX Buffer[2] | 1.5 | After thawing and 1:10 dilution in double distilled water, store at 4°C |

[1]**Notes:**
Buffer B may be used for diluting cAMP standards and test samples.
96-well black polypropylene microplates may be purchased from Mediomics, LLC.

[2]**10X KRB-IBMX Buffer** (Catalog #122937 / 1.5 ml/tube) containing 7.5mM phosphodiesterase inhibitor IBMX may also be purchased separately from Mediomics. Use of this buffer is recommended with either attached or suspended cells during chemical stimulation (e.g., forskolin stimulation) to inhibit degradation of cAMP.

# Bridge-It® cAMP designer Assay - Cell Preparation and General Notes

Cell samples obtained from either cultured adherent or suspension cells may be analyzed for cAMP. We tested HEK 293 cells at a concentration of ~25,000 cells/well in 96-well black polypropylene microplates stimulated with increasing concentrations of forskolin (0-200 μM). Different numbers of cells/well and different cell types may be used depending upon particular needs. Stimulation by forskolin is not required for all cell types and applications. However, it is included in the method presented below using HEK-293 cells. **Additional information is presented in the attached flowcharts for suspended and attached cells.** It is recommended that sample preparation be carried out in the presence of a phosphodiesterase inhibitor such as IBMX in order to prevent the degradation of cAMP. A TECAN, SPECTRAFluor PLUS plate reader was used in our laboratory to detect the fluorescent signal, but any fluorescent plate reader or spectrofluorometer capable of measuring fluorescein may be used.

The following precautions are recommended to optimize assay performance and reproducibility:

- Avoid bubbling as much as possible.
- Pipette slowly to avoid bubbling and allow complete recovery and transfer of assay solutions.
- After addition of the cAMP designer Assay Solution to the Eppendorf tubes and vortexing, any remaining bubbles may be easily broken by manually flicking the tubes. The samples may also be incubated in the tubes for 25-30 minutes covered in the dark prior to transfer into the wells of a 96-well black polypropylene microplate and fluorescence reading. This allows for complete recovery of all solution that may coat the tubes.
- After the samples have been transferred into the 96-well black polypropylene microplate, gently tap the microplate on the counter top to release 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 on the other end of the needle to avoid loss of volume through capillary action.
- Because of the small number of cells used in the assay, it is difficult to visualize the cell pellet after centrifugation of the Eppendorf tubes. It is recommended that all of the Eppendorf tubes be placed into the centrifuge in the exact same orientation (i.e., place all of the tubes in the centrifuge holders so that the tube top hinges all are located in the same orientation in their holders) so that the cell pellets also all have an identical orientation in the tubes. Knowing the cell pellet orientation will make it much easier to remove the supernatants from the tubes without disturbing the cells.

## Preparation of 1X KRB-IBMX Buffer

**Product Description:**

The 10X KRB-IBMX buffer concentrate (yellow cap tube - 1.5ml/kit) provided with the kit must be diluted 1:10 to prepare the 1X KRB-IBMX buffer needed for use with cells. The 1X KRB-IBMX buffer provides adherent and suspension target cells with (a) the balanced salt solution and essential inorganic ions required to maintain appropriate pH and osmotic balance, and, (b) a phosphodiesterase enzyme inhibitor (i.e., IBMX) that is capable of effectively blocking the degradation the cAMP

produced by target cells. Use of 1X KRB-IBMX buffer solution is recommended for the preparation of target cells intended for cAMP analysis using the Bridge-It® cAMP designer fluorescent assay.

**Preparation of 1X KRB-IBMX Buffer:**

1. Each yellow capped tube contains 1.5 ml of frozen 10X KRB-IBMX buffer concentrate.
2. Thaw the 1.5 ml of 10X KRB-IBMX buffer by placing the tube into a 37°C water bath for a few minutes (the thawed 10X buffer concentrate is turbid in appearance).
3. Gently mix the tube and then transfer the full 1.5 ml volume into a tube containing 13.5 ml of room temperature sterile double distilled water (1:10 dilution). In this way, a total of 15 ml of 1X KRB-IBMX buffer is prepared from each 1.5 ml tube of thawed 10X KRB-IBMX buffer concentrate.
4. Tightly cap and incubate the 1X KRB-IBMX buffer-containing tube(s) at 37°C for 5 minutes and then vortex them in order to remove any remaining turbidity.

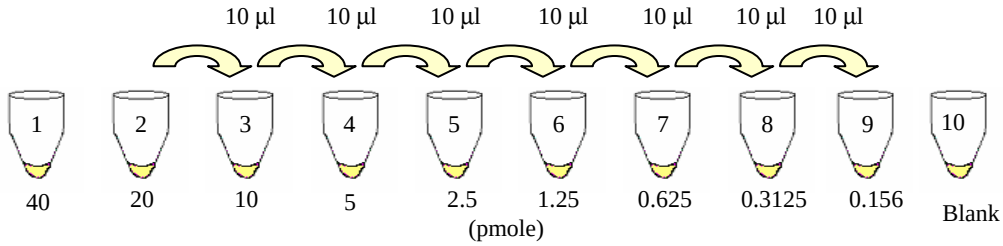
## Preparation of Bridge-It® cAMP designer Assay Solution

After thawing, the 10X lysis buffer solution (clear cap tube) may appear turbid. This turbidity will disappear after the 10X buffer solution is warmed to room temperature (~ 25°C) and gently mixed. Prepare only the number of cAMP designer Assay Solution tubes needed to perform the experiment. Add the contents of one 10X lysis buffer to one tube Assay Solution A (orange cap tube) in order to prepare one tube of cAMP designer Assay Solution. To avoid confusion, place a cAMP designer Assay Solution label (labels are provided with the kit) onto each tube containing freshly prepared cAMP designer Assay Solution. Mix the tube gently. It is recommended that if more than one tube of cAMP designer Assay Solution is prepared for use in an experiment, the contents of all the cAMP designer Assay Solution tubes be pooled and mixed together before use. Keep the cAMP designer Assay Solution tubes on ice until ready for use.

## Bridge-It® cAMP designer Assay Standards

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 assay performance should be conducted before using it in the assay. Please note that if the samples are extracted and dried prior to addition of the cAMP designer Assay Solution, the cAMP standards should be prepared in Eppendorf tubes as described below and then dried.

1. Dilute the 10 µM cAMP standard (green cap tube) 1:2.5 using Buffer B (blue cap tube) for a final concentration of 4 µM cAMP. Prepare 10 Eppendorf tubes. Add 10 µl of Buffer B to tubes 2-10. Add 10 µl of the 4 µM (40 pmole) cAMP stock to tubes 1 and 2. Mix the contents of tube 2 by pipetting up and down 5 times. Transfer 10 µl from tube 2 into tube 3. Continue serially diluting the cAMP standard through tube 9. After mixing, discard 10 µl from tube 9. All tubes should contain a final volume of 10 µl of cAMP standard solution.




**Note:** cAMP standards may be diluted directly in the microtiter plate. Manual mixing of sample with cAMP designer Assay Solution must be done gently to avoid bubbling.

2. Prepare fresh new cAMP designer Assay Solution as described earlier (page 5). Keep the cAMP designer Assay Solution tube(s) on ice until ready for use.

3. Add **90 µl** of the cAMP designer Assay Solution to each fresh Eppendorf tube and vortex slightly to mix and remove any bubbles.
**Note:** If the unknown samples were ethanol extracted and dried, you may prepare the cAMP samples as shown above in Eppendorf tubes and dry them using a Speed Vac (medium heat). Re-suspend the dried cAMP standards using **100 µl** cAMP designer Assay Solution. This should not result in any significant change in assay sensitivity (see Figure 2).

4. Carefully transfer **90 µl** of each tube into the appropriate well of a 96-well black polypropylene microplate.

5. Cover plate with tinfoil to avoid evaporation and exposure to light. Incubate the microplate at room temperature for 30 minutes.

6. Read fluorescence intensity with a fluorescence plate reader (settings: excitation ~480-485 nm; emission ~520-535 nm).

## Determination of cAMP in Cell Pellets

1. Cells (adherent or suspension) are harvested, washed in buffered saline solution, and brought up to the desired cell concentration in a serum-free buffer in Eppendorf tubes (for example: ~25,000 HEK 293 cells in 50 µl 1X KRB-IBMX buffer/tube). It is recommended that the cells be allowed to equilibrate for ~15 minutes at room temperature before stimulation.

2. OPTIONAL: Cell stimulation (for example, forskolin treatment for 15-30 min at 25ºC while rotating tubes).

3. Centrifuge tubes at ~12,000g for 5 minutes. Discard supernatants.

***cAMP Assay:***

4. Add 100 µl of the cAMP designer Assay Solution to the cell pellet. Vortex ~1 second.

5. Carefully transfer 90 µl of each tube into the appropriate well of a 96-well black polypropylene microplate.

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

7. Read fluorescence intensity with a fluorescence plate reader (settings: excitation ~480-485 nm; emission ~520-535nm).

## Determination of cAMP in Attached Cells

1. Cells are plated at the desired concentration and incubated for an appropriate time (e.g., plate 25,000 HEK 293 cells in 50-100µl medium/well in a 96-well polystyrene tissue culture microtiter and allow the cells to attach to the wells overnight).

2. Carefully remove medium from wells. Gently wash the attached cells with 100µl serum-free buffered saline being careful not to disturb the cell layer.

3. OPTIONAL: Remove buffered saline solution from wells. Add a serum-free buffer (50 µl of 1X KRB-IBMX buffer/well). Forskolin stimulate the attached cells for 15-30 min at 25ºC while rotating plate. It is recommended that the cells be allowed to equilibrate for ~15 minutes at room temperature (~ 25°C) before stimulation.

**Bridge-It® cAMP designer Fluorescence Assay:**

4. Remove buffer from attached cells in the wells of the 96-well culture microplate. Add 100 µl of the cAMP designer Assay Solution to each well.

5. Cover microplate with tinfoil to avoid exposure to light. Place the microplate place on a rotator and gently mix for 30 minutes at room temperature (~ 25ºC).

6. Gently stir and scrape the bottom of each well with a pipette tip and transfer 100 µl of the wells into the corresponding well of a 96-well black polypropylene microplate.

7. Read fluorescence intensity with a fluorescence plate reader (settings: excitation ~480-485 nm; emission ~520-535nm).

## Determination of cAMP Following Ethanol Extraction Of Cells in Suspension

1. Cells (adherent or suspension) are harvested, washed in a buffered saline solution and brought up to the desired cell concentration in a serum-free buffer in Eppendorf tubes (e.g., 25,000 HEK 293 cells in 50µl of 1X KRB-IBMX buffer/tube). It is recommended that the cells be allowed to equilibrate for ~15 minutes at room temperature before stimulation.

2. OPTIONAL: Forskolin stimulation (15-30 min at ~25ºC) while rotating the tubes.

3. Add an appropriate amount of ice-cold 100% ethanol to cell suspension in order to obtain a final concentration of 70% ethanol (e.g., 50µl cells + 120µl 100% ethanol).

4. Incubate tubes on ice for 15 minutes. Vortex for ~ 1 second.

5. Centrifuge tubes for 10 minutes at ~12,000g and transfer the supernatants containing the cAMP into fresh Eppendorf tubes.

6. Dry samples in Speed Vac (medium heat). If not used immediately, dried samples may be stored frozen at -20ºC until needed.

**Bridge-It® cAMP** designer **Fluorescence Assay:**

7. Add 100 µl of the cAMP designer Assay Solution to each Eppendorf tube. Vortex ~1 second.

8. Carefully transfer 100 µl of the Eppendorf tubes into the appropriate wells of a 96-well black polypropylene microplate.

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

10. Read fluorescence intensity with a fluorescence plate reader (settings: excitation ~480-485nm; emission ~520-535nm).

## Determination of cAMP Following Ethanol Extraction Of Attached Cells

1. Cells are plated at a desired concentration for appropriate period of time (e.g., plate 25,000 HEK 293 cells in 50-100µl medium/well in a 96-well polystyrene tissue culture microtiter plate and allow to attach overnight).

2. gently remove medium. Wash attached cells with serum-free buffered saline being careful not to disturb the attached cell layer.

3. OPTIONAL: Remove buffered saline from the wells. Add a serum free buffer (e.g., 50µl of KRB-IBMX buffer/well). Forskolin stimulate cells for 15-30 min at 25ºC while rotating the microplate. It is recommended that the cells equilibrate for 15 minutes at room temperature (~ 25°C) before stimulation.

4. Remove buffer from the attached cells. Add 50µl to 100µl of ice-cold 70% ethanol to each well of the microplate.

5. Incubate microplate on ice for 15 minutes.

6. Gently pipette the contents of each well up and down about 5-times. You may scrape the bottom of the wells with the end of the pipette tip. Transfer the lysed cell suspension into an Eppendorf tube. Vortex tube for ~ 1 second.

7. Centrifuge tubes for 10 minutes at ~12,000g and transfer the supernatants containing the cAMP to fresh Eppendorf tubes.

8. Dry samples in Speed Vac (medium heat). If not used immediately, dried samples can be stored frozen at -20ºC until ready for use.

**Bridge-It® cAMP designer Fluorescence Assay*:***

9. Add 100 µl of the cAMP designer Assay Solution to each Eppendorf tube containing dried sample. Vortex ~1 second.

10. Transfer 90 µl of each Eppendorf tube to the corresponding well of a 96-well black polypropylene microplate.

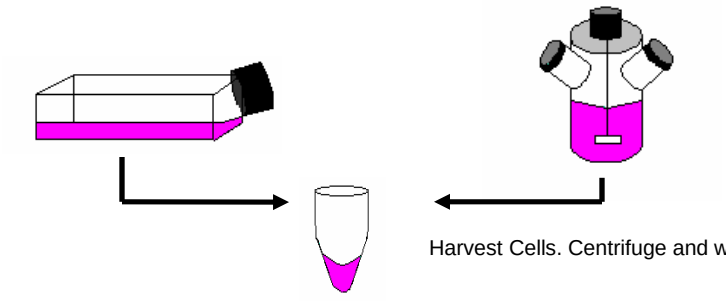
11. Cover the microplate with tinfoil to avoid evaporation and exposure to light. Incubate microplate at room temperature (~25°C) for 30 minutes.

12. Read fluorescence intensity with a fluorescence plate reader (settings: excitation ~480-485nm; emission ~520-535nm).

## Direct Determination of cAMP in Liquid Samples

If sample extraction and drying is not required, it is possible to use the assay to determine cAMP levels in liquid samples (e.g., clarified urine). Up to 20% by volume of such liquid sample may be added directly to the Bridge-It® cAMP designer Assay Solution. Incubate the mixture for 30 minutes at room temperature (~25°C) and read fluorescence intensity with a fluorescence plate reader (settings: excitation ~480-485nm; emission ~520-535nm).

# Bridge-It® cAMP designer Assay - Flowchart for Cells in Suspension



Harvest Cells. Centrifuge and wash with buffered saline solution.

Suspend cells to 5 x $10^5$ / ml in 1X KRB-IBMX. Incubate for ~15 min (~25ºC)
Add 50µl cells (~25,000 cells) / Eppendorf tube

Add forskolin to tubes and incubate 15-30 min at ~25ºC while rotating

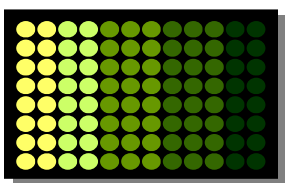
**Ethanol Extraction**

Add cold 100% ETOH to 70% final concentration

Incubate 15 min on ice. Vortex.
Centrifuge 10 min, 12,000 g
Transfer supernatant to new tube and dry

Add 100 µl designer Assay Solution to tube
Vortex. Incubate 30 min (~25ºC)

Transfer to a black 96-well microplate. Read fluorescence

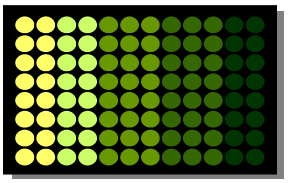


**designer Assay Solution Lysis**

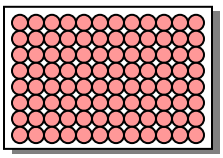
Centrifuge 5 min, 12,000 g
Discard supernatant

Add 100 µl designer Assay Solution to pellets
Vortex. Incubate 30 min (~25ºC)

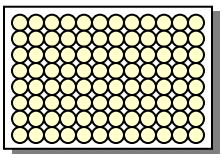
Transfer to a black 96-well microplate. Read fluorescence



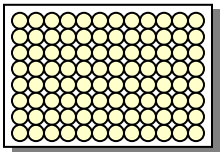
# Bridge-It® cAMP designer Assay - Flowchart for Attached Cells



Remove media. Wash wells carefully with 100 µl buffered saline.



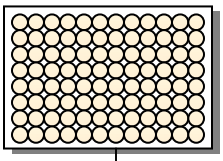
Remove saline wash. Add 50 µl 1X KRB-IBMX/well. Incubate for ~15 min (min (~25ºC). Add forskolin.
Incubate while rotating for 15-30 min at ~25°C



Remove KRB-IBMX / forskolin solution

**Ethanol Extraction**

Add 50-100 µl cold 70% ETOH to wells
Incubate 15 min on ice

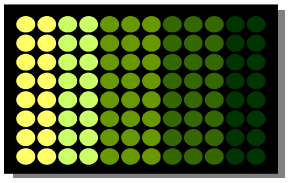


Transfer well contents to Eppendorf tubes Vortex

Centrifuge 10 min, 12,000 g
Transfer supernatant to new tube and dry

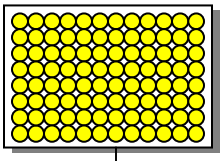
Add 100 µl designer Assay Solution to tube
Vortex. Incubate 30 min (~25ºC)

Transfer to a black 96-well microplate
Read fluorescence signal

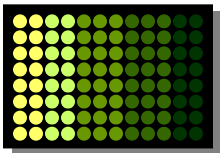


**designer Assay Solution Lysis**

Add 100 µl designer Assay Solution / well
Incubate 30 minutes at ~25°C while rotating



Transfer to a black 96-well microplate
Read fluorescence signal



## Bridge-It® cAMP designer 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 (x axis is common log, y axis is linear).

2. Relative fluorescence change 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 or sample: $RF = (F_0 - F)/F_0$
   Use of RF values are highly reproducible for the same cAMP concentrations and do not depend greatly on the instrument used to read fluorescence.

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

## Bridge-It® cAMP designer Assay Performance

# Bridge-It® cAMP designer Assay - Standard Curves

The following cAMP standard curves were prepared using the Bridge-It® cAMP designer cAMP assay. Figure 1 shows the standard curve prepared in Buffer B with the fluorescence signal read after 30 minutes, 1 hour and 20 hours after addition of cAMP designer Assay Solution. The standard errors were from duplicate measurements. Figure 2 shows a comparison of standard curves where cAMP standards were dried and re-suspended in 100µl cAMP designer Assay Solution and cAMP standards that were diluted in 10µl of Buffer B with 90µl of cAMP designer Assay Solution. There was no significant difference between the curves (single points).

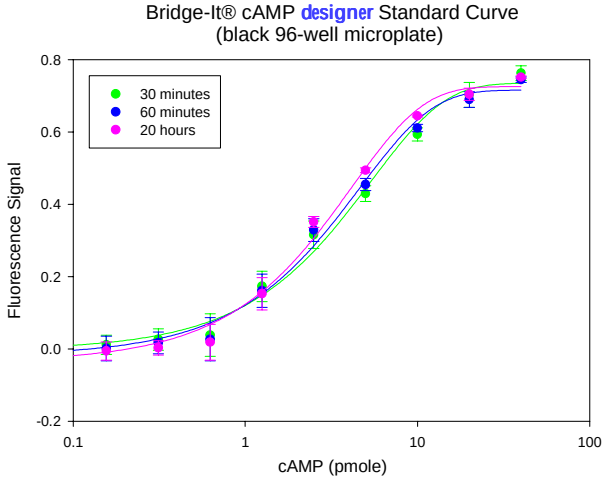



**Figure 1**

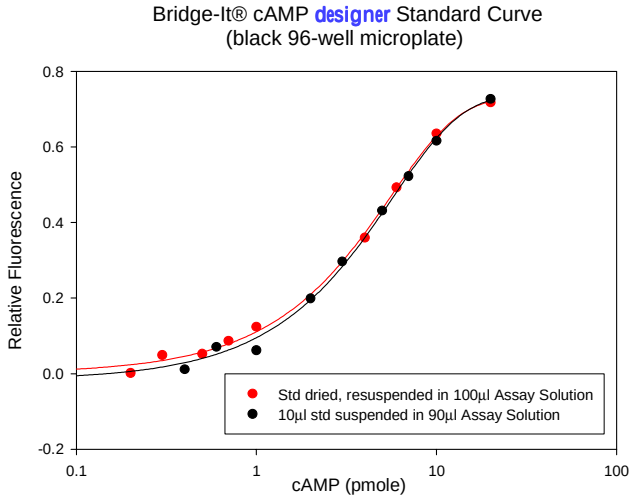



**Figure 2**

# Bridge-It® cAMP designer Assay - Cell Studies

The following figures show the results of two separate experiments with HEK 293 cells in suspension. Figure 3 shows duplicate samples extracted using cAMP designer Assay Solution as previously described. Figure 4 shows the comparison of lysis with the cAMP designer Assay Solution versus ethanol extraction. The standard errors were from duplicate measurements.

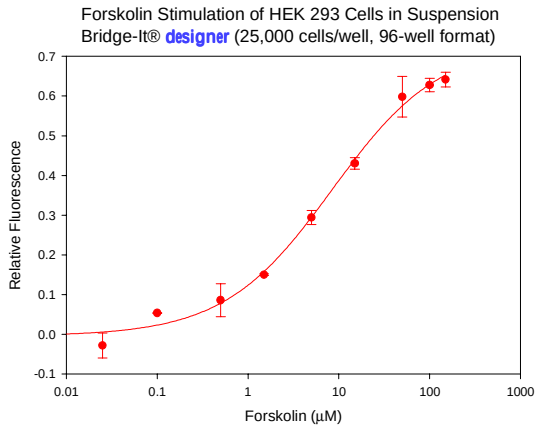



**Figure 3**

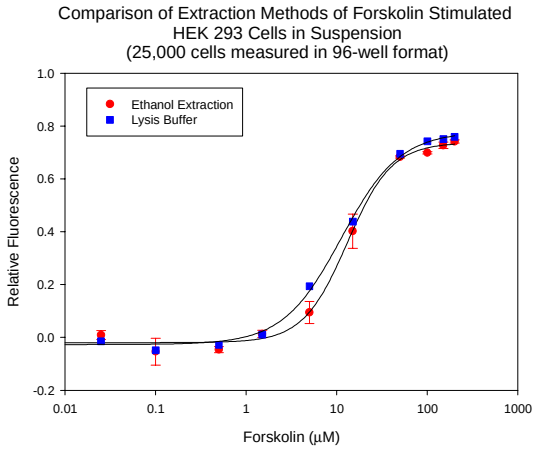



**Figure 4**

Figure 5 shows a comparison of forskolin-stimulated cAMP production in HEK 293 cells using 25,000 cells and 50,000 cells/well. Extraction was done using cAMP designer Assay Solution. The standard errors are of duplicate samples.

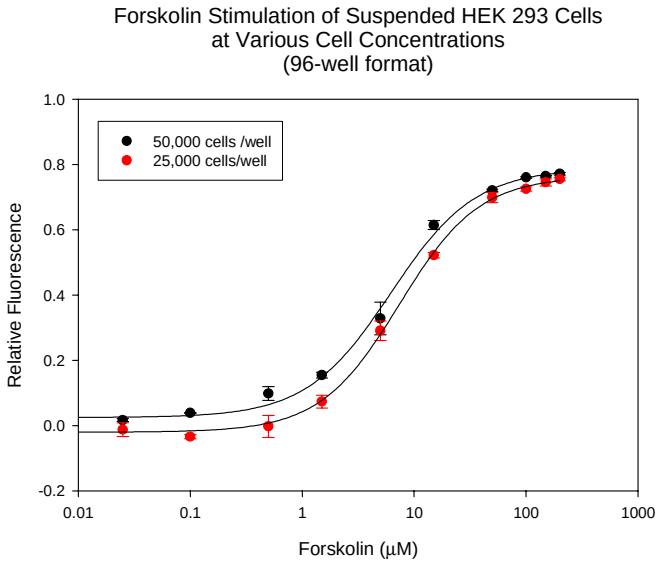



**Figure 5**

Figure 6 shows the stimulation and measurement of cAMP in adherent HEK 293 cells attached to the wells of a tissue culture microplate. Cell lysis was achieved using the Bridge-It® cAMP designer Assay Solution. Sample errors are from duplicate samples.

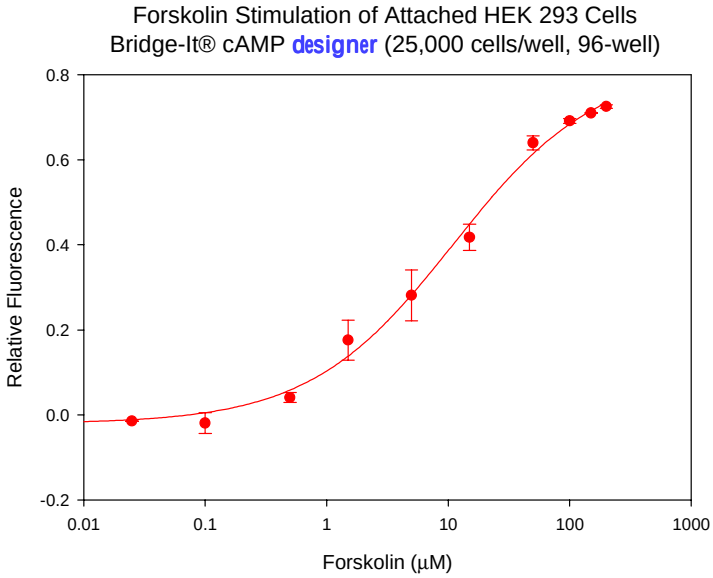
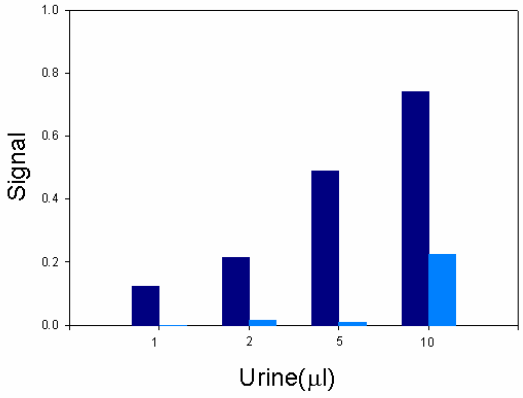



**Figure 6**

## Bridge-It® cAMP designer Assay - Urine Studies

Figure 7 illustrates the potential of using the Bridge-It® cAMP designer fluorescence assay for the detection of cAMP in urine samples. Small volumes of clarified urine were added directly to cAMP designer Assay Solution, incubated, and fluorescence intensity read.

**Figure 7** Detection of cAMP in urine. Light colored bars show the signal observed after treating the urine samples with phosphodiesterase.



# Bridge-It® cAMP designer Assay - Specificity Studies

Table 1 demonstrates the specificity of the Bridge-It® cAMP designer fluorescence assay using various related compounds. Samples were brought up to 0.1M concentration in water and adjusted to 200nM (20 pmole per 96-well) in Buffer B. The samples were then measured for cAMP activity.

**Table 1**

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

## References

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