R&D, Administration and Europe Office
In Vitro Technologies - HTRF®/Bioassays
Phone : +33 (0)4 66 79 67 05
Fax : +33 (0)4 66 79 19 20
E-mail : bioassays@cisbiointernational.fr
Japan Office
Sceti Medical Labo K.K.
Phone : +81-(0)3-5510-2932
Fax : +81-(0)3-5510-0134
E-mail: reagent@scetimedilabo.co.jp
USA Office
CIS-US, Inc.
Phone : 888-963-4567
Fax : 781-275-2634
E-mail : htrfsales@cisusinc.com

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cAMP HiRange

For in vitro research use only
Storage temperature : 2-8°C

www.htrf.com

HTRF® package insert
General information

Document reference : 62AM6PEB rev02 (May 2007)

Packaging details :

	384-well low volume plate (20 µL)
62AM6PEB	1,000 tests

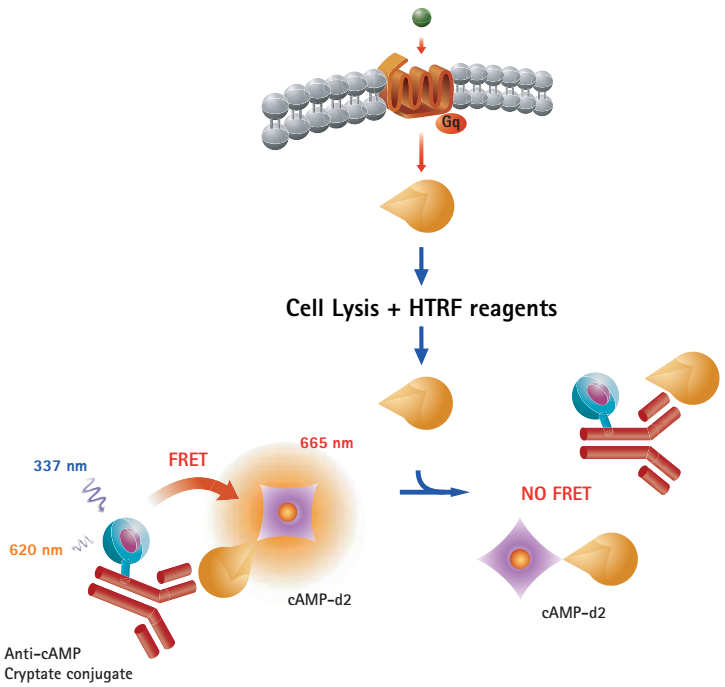
1. Assay description and intended use

This kit is intended for the direct quantitative determination of cyclic AMP. The assay conditions have been optimized in order to reach a high signal-to-noise and to enable cAMP assessment either on suspended or on adherent cells.

Its principle is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). The method is a competitive immunoassay between native cAMP produced by cells and the cAMP labeled with the dye d2. The tracer binding is visualized by a Mab anti-cAMP labeled with Cryptate.

The specific signal (i.e. energy transfer) is inversely proportional to the concentration of cAMP in the standard or sample.

As for all other HTRF® assays, the calculation of the fluorescence ratio (665 nm/620 nm) eliminates possible photophysical interferences and allows the assay to be unaffected by the usual medium conditions (e.g. culture medium, serum, biotin, colored compounds).



2. Background

Cyclic AMP (cyclic adenosine 3',5'-monophosphate, MW 351.2) is one of the most important intracellular mediators.

Its concentration in cells can be increased upon binding of many hormones to their receptors. The most studied pathway consists in the release of αsubunit GTP-binding proteins following ligand-receptor interaction, which in turn activates or inhibits the ATP/cAMP conversion function of adenylate cyclase. cAMP is then involved in many complex regulatory processes such as protein kinase activation or ion channel gating.

Given this large involvement in cell regulation, cAMP quantification has been of considerable interest in the exploration of cell physiology and dysfunction.

The cAMP HiRange kit allows the measurement of agonist and antagonist effects on Gαs and Gαi coupled receptors in different cell lines.

3. Reagent preparation and stability

3.1. Supplied reagents

Allow the reagents to warm up to room temperature for at least 30 mins before reconstitution.

Anti cAMP - Cryptate	1 vial, lyophilized
cAMP-d2	1vial, lyophilized
cAMP standard. Concentrated free cAMP	1 vial, lyophilized
cAMP control. Free cAMP assay control	1 vial, lyophilized
Conjugate & lysis buffer	1 vial of 13 mL
Diluent	1 vial of 20 mL

For reagent reconstitution, refer to attached protocols.

3.2. Reagent storage and stability

	Storage	Stability
Supplied reagents	4°C until reconstitution	Until expiry date indicated on the labels
Stock solutions	4°C	1 week
	frozen (-20°C)	May be frozen and thawed twice
Working solutions of conjugates	4°C Do not freeze	24 hours

4. Assay protocols

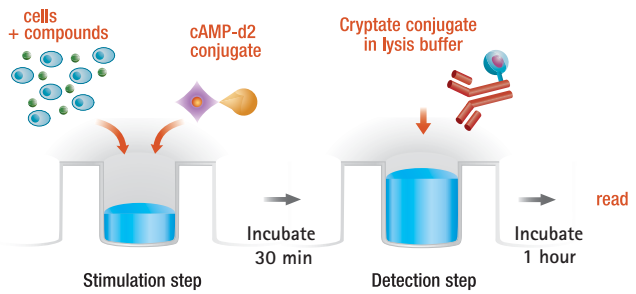
The cAMP HiRange kit offers the possibility to follow two different dispensing protocols, a one step protocol or a two step protocol after cell stimulation. The choice of the protocol will depend on your automatization constraints and HTS needs.

The procedures for reagent reconstitution, standard curve and cell based assay are described for each protocol in 2 separate attached sheets (appendix 1 and 2).

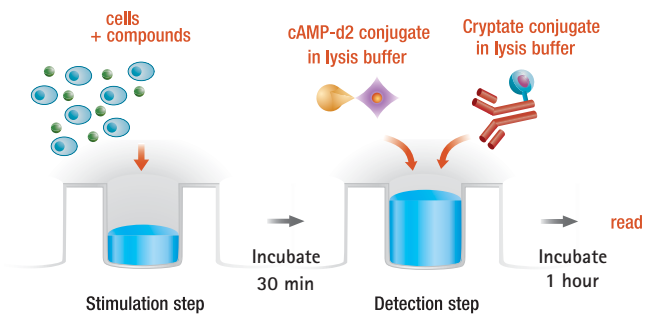
Both protocols include:

1- cell stimulation followed by an incubation (actual incubation depends on cell type, most commonly 30 min are necessary).
2- detection with HTRF® reagents followed by 1 hour incubation at room temperature before reading.

1 step protocol after cell stimulation



2 steps protocol after cell stimulation



5. Data reduction

Results are calculated from the 665nm / 620nm ratio and expressed in Delta F. An example of data reduction is given in the table below (readout on RUBYstar). This data should not be substituted for results obtained in the laboratory. Draw up the standard curve by plotting delta F% versus cAMP concentration as shown in the graph below.

	A (665nm)	B (620nm)	Ratio (1)	Mean Ratio (2)	CV % (3)	Delta R	Delta F % (4)
Negative control	1637 1708	39172 40637	418 420	419	0.4		
[standard] nM final							
0 Positive control	50385 49656	36880 37174	13662 13358	13510	1.6	13091	3123
0.17	47524 49934	37355 38737	12722 12891	12806	0.9	12387	2956
0.68	47080 47986	38779 39058	12141 12286	12213	0.8	11794	2814
2.73	43533 43958	39131 39774	11125 11052	11088	0.5	10669	2546
10.94	34061 33850	39644 39910	8592 8482	8537	0.9	8118	1937
43.75	21801 20828	43546 39012	5006 5339	5173	4.5	4754	1134
175	9191 9807	42880 44137	2143 2222	2183	2.5	1764	421
700	4081 4014	43641 42807	935 938	936	0.2	517	123
2,800 (maximum standard)	2298 2250	44439 44247	517 509	513	1.2	94	22
cAMP control	30368 31174	40379 41088	7521 7587	7554	0.6	7135	1702

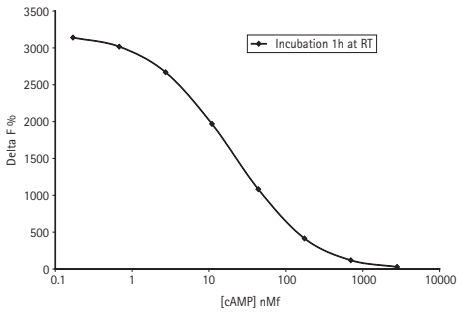
1. $\text{Ratio} = \frac{A_{665nm}}{B_{620nm}} \times 10^4$

2. $\text{Mean Ratio} = \frac{\Sigma \text{ratios}}{2}$

3. $\text{CV} = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$

4. $\text{Delta F} = \frac{\text{Standard or sample Ratio} - \text{Ratio}_{neg}}{\text{Ratio}_{neg}} \times 100$

(Ratio_{neg} = negative control)



Delta F% obtained for samples can be reported on the standard curve to deduce respective cAMP concentrations.

6. Assay characteristics

The table summarizes the characteristics of the assay relative to the EC50 (cAMP concentration which allows the displacement of 50% of binding) and the signal over background. This data has been obtained using the reference RUBYstar reader (BMG LABTECH).

	EC50	Signal to background
Incubation 1 hour at RT	< 25 nM	>25

Plate readout may be carried out several times within 24 hours. Determination of sample concentrations should be done with respect to a standard curve which followed the same incubation course.

7. Assay flexibility and miniaturization

When used as suggested, the kit will provide sufficient reagents for 1,000 tests using a 384- well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

To move to other plate formats (96 half-well or 1536-well) and final volumes (100 µL to less than 10 µL), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20 µL final assay volume. For instance, in the case of the 1536-well format in 10 µL final volume, half as much material per well is used, thereby allowing 2,000 tests to be run. The performances of the HTRF® assay remain the same whatever the level of miniaturization.

Example: The two step protocol assay

Assay components	Volume proportion	Assay format		
		1536-well (10 µL)	384-well low volume (20 µL)	96 half-well (100 µL)
Standard or cells	1 volume	2.5 µL	5 µL	25 µL
Diluent or test compound	1 volume	2.5 µL	5 µL	25 µL
d2 conjugate	1 volume	2.5 µL	5 µL	25 µL
Cryptate conjugate	1 volume	2.5 µL	5 µL	25 µL
	Bulk size	2,000 tests	1,000 tests	200 tests

Plate references : 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076), 1536-well (Greiner # 782086).

8. Cell density optimization

The cell density optimization is a key step in cyclic AMP kit handling. Typically, the level of cAMP produced by cells must fall within the linear range of the standard curve, e.g between A and B respectively (cf. figure 1).

The optimization consists of testing a wide range of cell concentrations (e.g. between 4,500 and 20,000 cells per well), in the presence or absence of a direct activator of the cell adenylate cyclase enzyme, such as forskolin. The addition of a phosphodiesterase inhibitor in cell dilution buffer is absolutely necessary (e.g. IBMX) in order to prevent cAMP degradation. The optimum cell density is the number of cells per well which leads to the highest signal amplitude obtained between the inactivated state (basal level of cAMP produced by cells) and the activated condition (in the presence of forskolin). Figure 2 represents an example of a cell density optimization experiment. In this example, the cell density selected for the following experiments with agonists and antagonists is 20,000 cells per well.

Figure 1

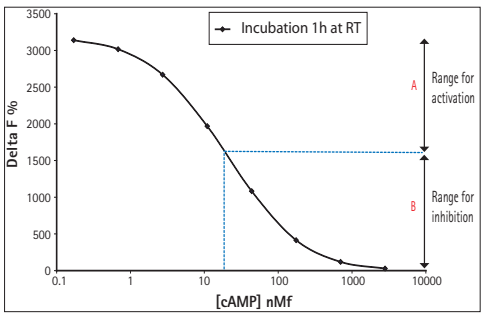
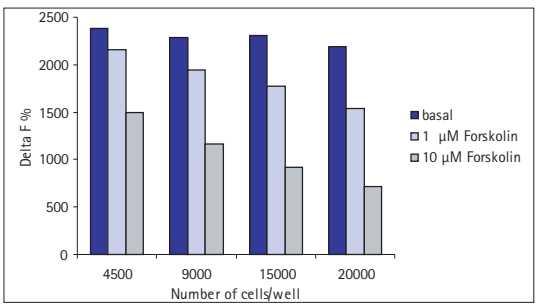


Figure 2



· Screening for agonist on Gαs coupled receptor

In the presence of an agonist, the secretion of cAMP will increase and a decrease of signal will be detected.

· Screening for agonist on Gαi coupled receptor

When a decrease of cAMP is expected, the cells are preactivated by forskolin in order to raise cAMP production (1 µM in the example with 20,000 cells per well). In the presence of an agonist, the secretion of cAMP will decrease and an increase of signal will be detected.

IMPORTANT RECALLS

- ***HTRF® reagent working solutions must not be stored for longer than 24 hours at 4°C (prepare and use only as needed).***
- ***Proportional downsizing of assay components should be strictly applied for miniaturization (see miniaturization process section).***
- ***Never pre-mix the two HTRF® conjugates before dispensing in order to avoid a pre-kinetic equilibrium between the two components.***
- ***Readout should only be carried out with HTRF® compatible instruments.***

R&D, Administration and Europe Office
In Vitro Technologies - HTRF®/Bioassays
Phone : +33 (0)4 66 79 67 05
Fax : +33 (0)4 66 79 19 20
E-mail : bioassays@cisbiointernational.fr
Japan Office
Sceti Medical Labo K.K.
Phone : +81-(0)3-5510-2932
Fax : +81-(0)3-5510-0134
E-mail: reagent@scetimedilabo.co.jp
USA Office
CIS-US, Inc.
Phone : 888-963-4567
Fax : 781-275-2634
E-mail : htrfsales@cisusinc.com

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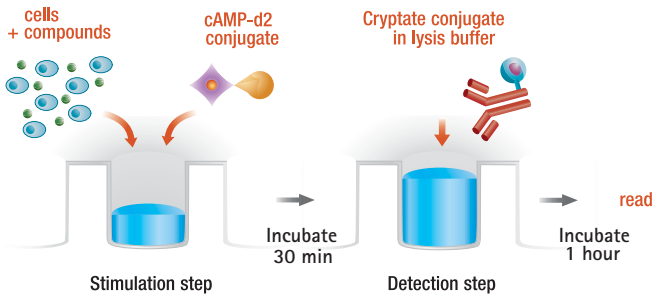
HTRF® package insert
Appendix 1: One step protocol

Document reference : 62AM6PEB rev02 (May 2007)

Packaging details :

	384-well low volume plate (20 µL)
62AM6PEB	1,000 tests



1. Reagent reconstitution

Allow the reagents to warm up to room temperature for at least 30 mins before reconstitution.

Supplied reagents	Reagent reconstitution (stock solutions)		Working solutions
anti cAMP - Cryptate 1 vial, lyophilized	Reconstitute with 1.1 mL of distilled water. Mix gently.	⇨	1/10 dilution in Conjugate & lysis buffer. Dilute 1 volume of reconstituted reagent in 9 volumes of conjugate & lysis buffer (e.g. for 1,000 tests: 1.1 mL of reconstituted reagent + 9.9 mL of Conjugate & lysis buffer).
cAMP-d2 1 vial, lyophilized	Reconstitute with 0.25 mL distilled water. Mix gently.	⇨	1/40 dilution in the cell preparation. *Dilute 1 volume of reconstituted reagent in 39 volumes of the cell preparation (e.g. add 0.150 mL of reconstituted cAMP-d2 to 5.85 mL of cell preparation). Keep 100 µL of cAMP-d2 stock solution for buffer mix preparation.**
cAMP standard. Concentrated free cAMP 1 vial, lyophilized	See indications on labels for reconstitution volume. Reconstitute each vial with buffer mix.** Mix gently after reconstitution	⇨	See standard curve preparation (section 2.1) for further dilution.
cAMP control. Free cAMP assay control 1 vial, lyophilized		⇨	To be used directly after reconstitution.

The Cryptate conjugate concentration was optimized in order to ensure an average counting of 40,000 cps at 620 nm (384-well low volume format), using the reference RUBYstar reader (BMG LABTECH).

* The cell density is optimized following the indication of section 8.
**For buffer mix preparation, dilute 1 volume of cAMP-d2 stock solution in 39 volumes of cell culture media used for the cell preparation (e.g. add 80 µL of reconstituted cAMP-d2 to 3120 µL of cell culture media).
In the One Step Protocol reconstitute the cAMP standard and control with buffer mix following the reconstitution volume printed on the vials.

Conjugate working solutions must be prepared in distinct vials and dispensed separately.

Precaution : HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the cAMP-d2 and Cryptate-conjugates will impair the assay's quality.

2. Assay protocol

2.1. Standard curve preparation

Follow the dilution sequence shown in the following table to constitute the standard curve.

Standard 8 reconstitution and standard curve dilution must be carried out with buffer mix preparation. The standard curve covers an average range of 0.17-2800 nM (final concentration of cAMP per well).

Standard (Calibrator)	Preparation	cAMP working solution nMi	cAMP final concentration nMf
Std 8	Reconstituted reagent in the buffer mix	11200*	2800
Std 7	↳20 µL Std 8 + 60 µL buffer mix	2800	700
Std 6	↳20 µL Std 7 + 60 µL buffer mix	700	175
Std 5	↳20 µL Std 6 + 60 µL buffer mix	175	43.75
Std 4	↳20 µL Std 5 + 60 µL buffer mix	43.75	10.94
Std 3	↳20 µL Std 4 + 60 µL buffer mix	10.94	2.73
Std 2	↳20 µL Std 3 + 60 µL buffer mix	2.73	0.68
Std 1	↳20 µL Std 2 + 60 µL buffer mix	0.68	0.17
Std 0 (Positive control)	60 µL buffer mix	0	0

* [cAMP] is indicated on the label of the maximum standard.

2.2. Assay protocol for 384-well low volume plate (20 µL)

<table>
<tr><th colspan="3">Standard curve</th><th colspan="3">Cells</th></tr>
<tr><td>Negative control</td><td>Standard curve</td><td>Assay control</td><td>Cell negative control</td><td>Non stimulated cells</td><td>Stimulated cells</td></tr>
<tr><td>5 µL cell culture media</td><td>5 µL cAMP standard in buffer mix</td><td>5 µL cAMP control in buffer mix</td><td>5 µL cells without cAMP-d2</td><td colspan="2">5 µL cells cAMP-d2 mix</td></tr>
<tr><td colspan="3" rowspan="2">5 µL compound buffer</td><td colspan="2">5 µL compound buffer</td><td>5 µL test compound *</td></tr>
<tr><td colspan="3">Seal the plate and incubate for cell stimulation 30 min. at room temperature</td></tr>
<tr><td colspan="6">10 µL anti-cAMP Cryptate</td></tr>
<tr><td colspan="6">Seal the plate and leave to incubate at room temperature for 1 hour</td></tr>
</table>

Remove the plate sealer and read on a compatible HTRF® reader (more information about compatible reader at htrf-assays.com/readers).

* Do not use kit diluent for test compound dilution.

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Phone : +33 (0)4 66 79 67 05
Fax : +33 (0)4 66 79 19 20
E-mail : bioassays@cisbiointernational.fr
Japan Office
Sceti Medical Labo K.K.
Phone : +81-(0)3-5510-2932
Fax : +81-(0)3-5510-0134
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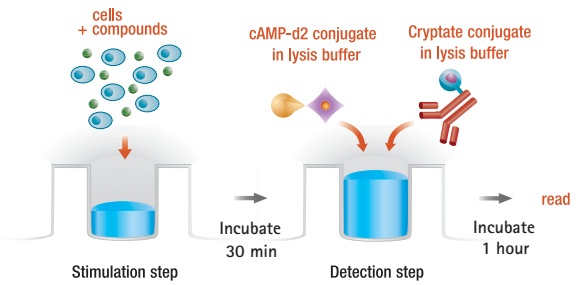
HTRF® package insert
Appendix 2: Two step protocol

Document reference : 62AM6PEB rev02 (May 2007)

Packaging details :

	384-well low volume plate (20 µL)
62AM6PEB	1,000 tests



1. Reagent reconstitution

Allow the reagents to warm up to room temperature for at least 30 mins before reconstitution.

Supplied reagents	Reagent reconstitution (stock solutions)		Working solutions
anti cAMP - Cryptate 1 vial, lyophilized	Reconstitute with1.1 mL of distilled water. Mix gently.	⇨	For each conjugate, make 1/5 dilution by diluting 1 volume of reconstituted reagent in 4 volumes of conjugate & lysis buffer (e.g. for 1,000 tests: 1.1 mL of reconstituted reagent + 4.4 mL of Conjugate & lysis buffer).
cAMP-d2 1 vial, lyophilized	Reconstitute with 2 mL of distilled water. Mix gently.		
cAMP standard. Concentrated free cAMP 1 vial, lyophilized	Reconstitute both vials with distilled water.* See indications on labels for reconstitution volume. Mix gently after reconstitution.	⇨	See standard curve preparation (section 2.1) for further dilution.
cAMP control. Free cAMP assay control 1 vial, lyophilized		⇨	To be used directly after reconstitution.

The Cryptate conjugate concentration was optimized in order to ensure an average counting of 40,000 cps at 620 nm (384-well low volume format), using the reference RUBYstar reader (BMG LABTECH).

*In the two step protocol reconstitute the cAMP standard and control with distilled water following the reconstitution volume printed on the vials.

Conjugate working solutions must be prepared in distinct vials and dispensed separately.

Precaution : HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the cAMP-d2 and Cryptate-conjugates will impair the assay's quality.

2. Assay protocol

2.1. Standard curve preparation

Follow the dilution sequence shown in the following table to constitute the standard curve. Dilution must be carried out with the diluent (or with cell culture media). The standard curve covers an average range of 0.17-2800 nM (final concentration of cAMP per well).

Standard (Calibrator)	Preparation	cAMP working solution nMi	cAMP final concentration nMf
Std 8	Reconstituted reagent with distilled water	11200*	2800
Std 7	↘50 µL Std 8 + 150 µL diluent	2800	700
Std 6	↘50 µL Std 7 + 150 µL diluent	700	175
Std 5	↘50 µL Std 6 + 150 µL diluent	175	43.75
Std 4	↘50 µL Std 5 + 150 µL diluent	43.75	10.94
Std 3	↘50 µL Std 4 + 150 µL diluent	10.94	2.73
Std 2	↘50 µL Std 3 + 150 µL diluent	2.73	0.68
Std 1	↘50 µL Std 2 + 150 µL diluent	0.68	0.17
Std 0 (Positive control)	200 µL diluent	0	0

* [cAMP] is indicated on the label of the maximum standard.

2.2. Assay protocol for 384-well low volume plate (20 µL)

Generation of the standard curve and controls and cell based assay:

<table>
<tr><th colspan="3">Standard curve</th><th colspan="3">Cells</th></tr>
<tr><td>Negative control</td><td>Standard curve</td><td>Assay control</td><td>Cell negative control</td><td>Non stimulated cells</td><td>Stimulated cells</td></tr>
<tr><td>5 µL diluent</td><td>5 µL cAMP standard</td><td>5 µL cAMP control</td><td colspan="3">5 µL cells</td></tr>
<tr><td colspan="3">5 µL compound buffer</td><td colspan="2">5 µL compound buffer</td><td>5 µL test compound*</td></tr>
<tr><td rowspan="2">5 µL conjugate & lysis buffer</td><td colspan="2" rowspan="2">5 µL cAMP-d2</td><td colspan="3">Seal the plate and Incubate for cell stimulation 30 min. at room temperature</td></tr>
<tr><td>5 µL conjugate & lysis buffer</td><td colspan="2">5 µL cAMP-d2</td></tr>
<tr><td colspan="6">5 µL anti cAMP-Cryptate</td></tr>
<tr><td colspan="6">Seal the plate and leave to incubate at room temperature for 1 hour</td></tr>
</table>

Remove the plate sealer and read on a compatible HTRF® reader (more information about compatible reader at htrf-assays.com/readers).

Note : alternatively, the procedure described above can be run in a "transfer mode". In this case, the steps of cell dispensing, incubation with the test compound and cell lysis are carried out in appropriate culture plates before transferring each cell lysate to the HTRF® assay plate. This protocol also allows further dilution of samples at the time of transfer.

*** Do not use kit diluent for test compound dilution.**