MANUAL

Technology: HTRF® Epigenetics

HTRF EPIgeneous™ H3K4Me2 cellular assay

Part number	62KA2PAE	62KA2PAD
Test size	500 tests	10,000 tests

Storage: ≤-60°C

Version: 05 **Date:** January 2024

ASSAY PRINCIPLE

This EPIgeneous™ H3K4Me2 cellular assay is intended for the simple, rapid and direct detection of endogenous levels of H3K4Me2 mark in cells. The demethylation of Lysine 4 on histone H3 is detected in a sandwich assay format using 2 different specific antibodies, one labelled with Eu Cryptate (donor) and the second with d2 (acceptor).

When the dyes are in close proximity, the excitation of the donor with a light source (laser or flash lamp) triggers a Fluorescence Resonance Energy Transfer (FRET) towards the acceptor, which in turn fluoresces at a specific wavelength (665 nm). One reagent binds to Histone H3 and the other binds to H3K4Me2, thereby generating FRET. The specific signal modulates positively in proportion to dimethylation on Lysine 4.

The assay can be run under a two-plate assay protocol, where cells are plated (stimulated) and lysed in the same culture plate and then transferred to the assay plate for the detection of H3K4Me2 by HTRF® reagents. This protocol enables the cells' viability and confluence to be monitored. It can also be further streamlined to a one-plate assay protocol. Detection of H3K4Me2 with HTRF® reagents is performed in a single plate used for plating, stimulation and detection. No washing steps are required. This protocol, HTS designed, allows miniaturization while maintaining HTRF® quality.

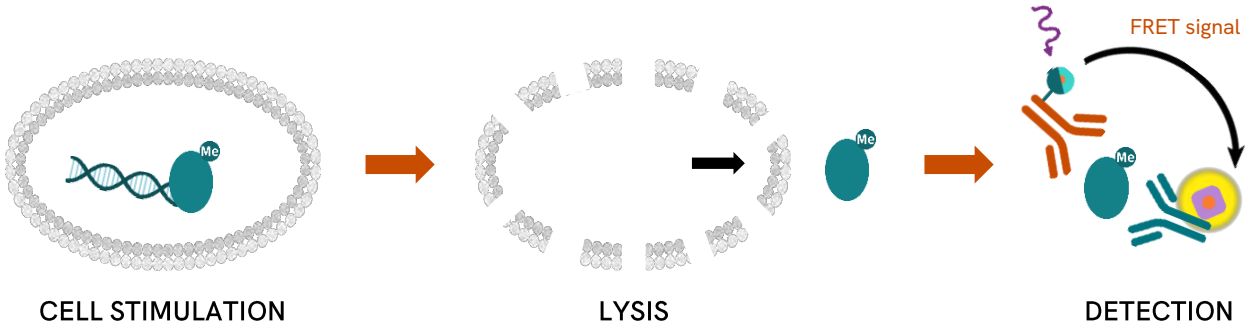
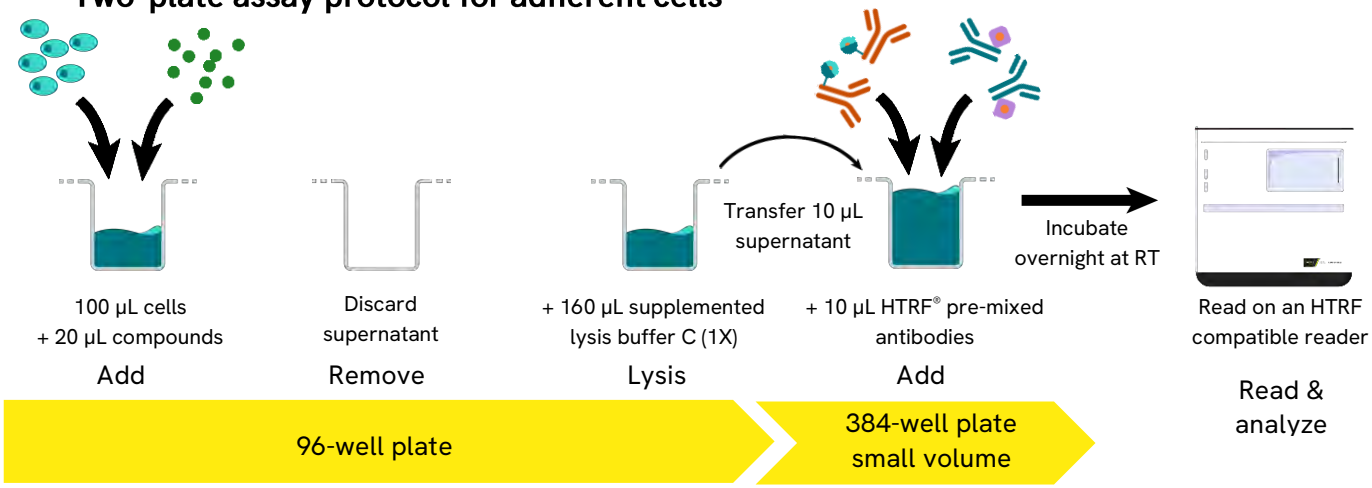


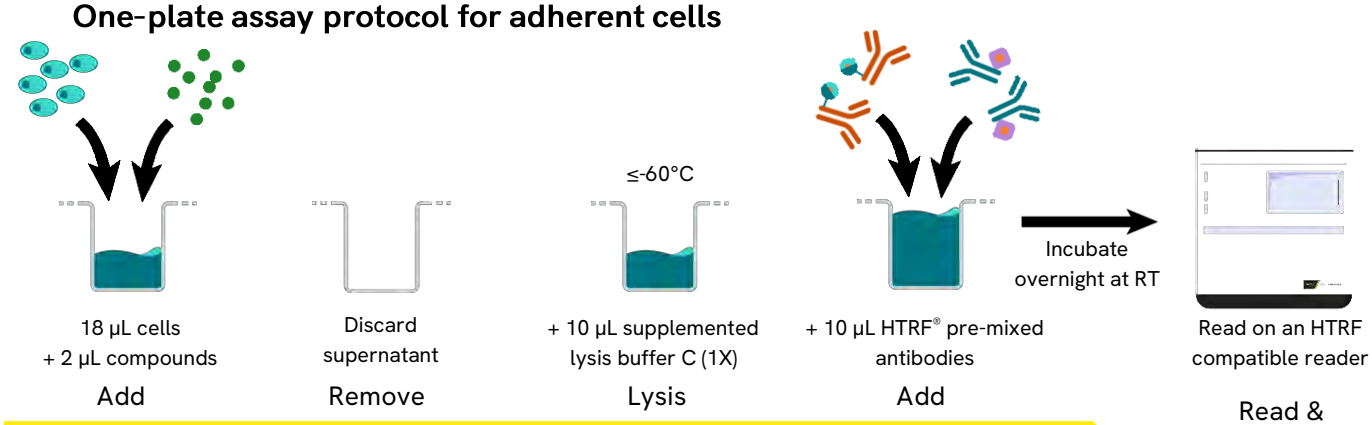
Figure 1: Principle of HTRF EPIgeneous™ H3K4Me2 cellular assay

PROTOCOL AT A GLANCE

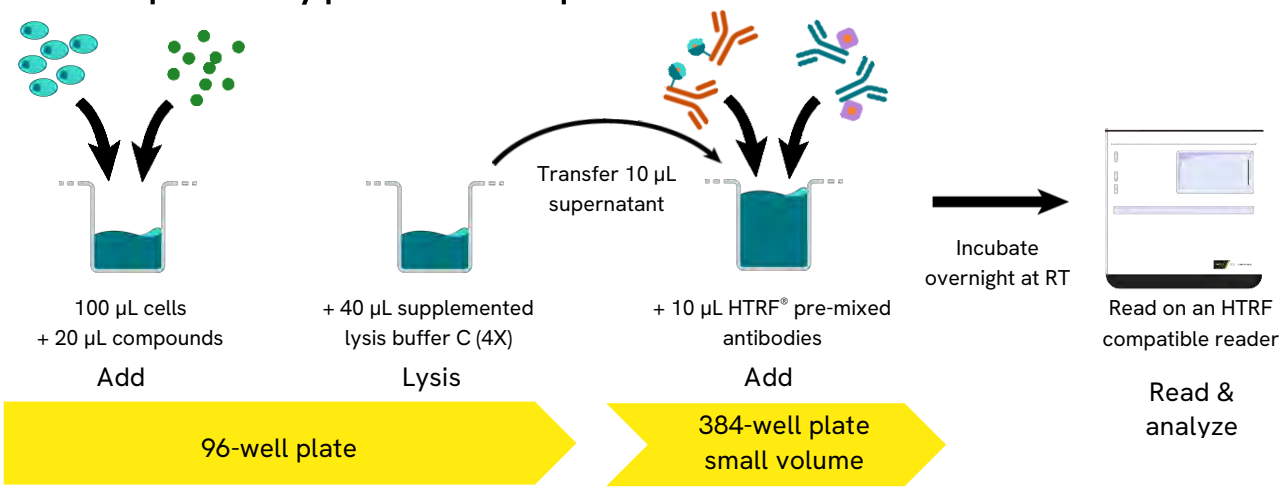
Two-plate assay protocol for adherent cells



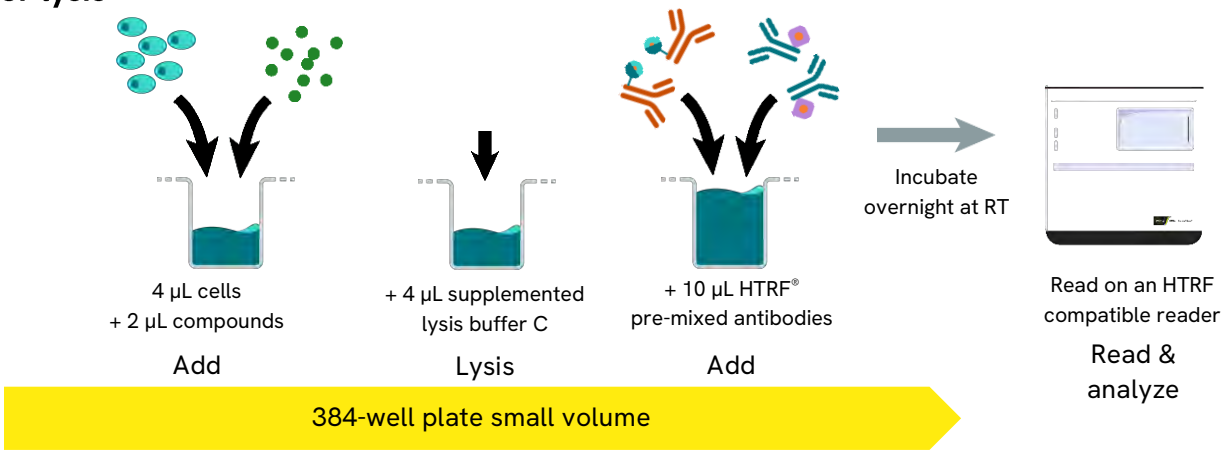
One-plate assay protocol for adherent cells



Two-plate assay protocol for suspension or adherent cells



One-plate assay protocol for suspension cells and adherent cells kept in medium for lysis



KIT DESCRIPTION

KIT COMPONENTS	STORAGE	500 TESTS			10,000 TESTS			STOCK SOLUTION CONCENTRATION
		TUBES DESCRIPTION		VIALS	TUBES DESCRIPTION		VIALS	
H3K4Me2 Eu-Cryptate antibody	≤-16°C		red cap	1 vial 50 µL		red cap	1 vial 1 mL	50X
H3 d2 antibody (H3K4Me2 kit)	≤-16°C		blue cap	1 vial 50 µL		blue cap	1 vial 1 mL	50X
H3K4Me2 control lysate Spare part #62KA2TDA	≤-60°C		green cap	1 vial 150 µL		green cap	3 vials 150 µL	ready to use
EPIgeneous™ lysis buffer C-part 1 Spare part #62EL3FDD/H	≤-16°C		red cap	1 vial 25 mL		red cap	3 vials 170 mL	4X
EPIgeneous™ lysis buffer C-part 2 Spare part #62EL3FDD/H	≤-16°C		purple cap	1 vial 1 mL		white cap	1 vial 20 mL	100X
Cellular histone detection buffer	≤-16°C		transparent cap	1 vial 6 mL		white cap	1 vial 120 mL	ready to use

STORAGE STABILITY

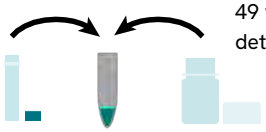
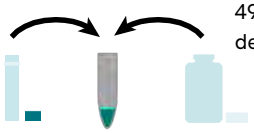
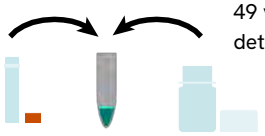
All reagents should be stored frozen until used.
To avoid freeze/thaw cycles, aliquot stock solutions into disposable plastic vials for storage at ≤-16°C.
Thawed lysis and detection buffers can be stored at 2-8°C.

REAGENT PREPARATION

Allow all reagents to thaw before use.
We recommend centrifuging the vials gently after thawing before pipetting the stock solutions.
Prepare the working solutions from stock solutions by following the instructions below.
The control lysate is only provided as an internal assay control to check the quality of the results obtained.
The window between control lysate and negative control should be greater than 2.

Preparation of reagent working solutions

HTRF® reagent concentrations have been set for optimal assay performances.
Note that any dilution or improper use of the d2 and Eu Cryptate-reagents will impair the assay's quality.
Dilute the reagents with detection buffer. In practice:

500 TESTS KIT	10,000 TESTS KIT
H3 d2 Antibody (H3K4Me2 kit)	
1 volume of d2 reagent  49 volumes detection buffer	1 volume of d2 reagent  49 volumes of detection buffer
Dilute the frozen stock solution 50-fold with the detection buffer: e.g. add 2,450 µL of detection buffer to the 50 µL of reagent stock solution (in a different vial).	Dilute the frozen stock solution 50-fold with the detection buffer: e.g. add 2,450 µL of detection buffer to the 50 µL of reagent stock solution (in a different vial).
H3K4Me2 Eu-Cryptate Antibody	
1 volume of Eu^{3+}-Cryptate reagent  49 volumes detection buffer	1 volume of Eu^{3+}-Cryptate reagent  49 volumes of detection buffer
Dilute the frozen stock solution 50-fold with the detection buffer: e.g. add 2,450 µL of detection buffer to the 50 µL of reagent stock solution (in a different vial).	Dilute the frozen stock solution 50-fold with the detection buffer: e.g. add 2,450 µL of detection buffer to the 50 µL of reagent stock solution (in a different vial).

Preparation of lysis buffer

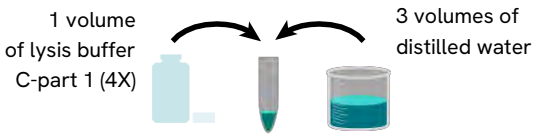
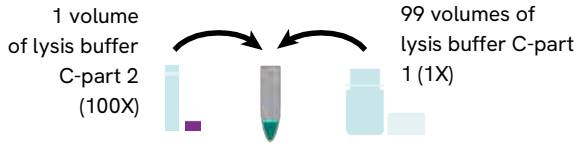
Make sure to use the appropriate lysis buffer depending on the chosen protocol's specifications.
Make sure that lysate has been generated by using the kit reagents.

Supplemented lysis buffer C (1x) for two-plate & one-plate assay protocols for adherent cells
Determine the amount of supplemented lysis buffer C (1x) needed for the experiment.

- For the one-plate assay protocol, each well requires 10 µL of supplemented lysis buffer C (1x).
- For the two-plate assay protocol, each well requires 160 µL of supplemented lysis buffer C (1x).

Prepare a lysis buffer C-part 1 solution (1x).
Then dilute 100-fold the lysis buffer C-part 2 with the freshly prepared lysis buffer C-part 1.

In practice:

500 TESTS KIT	10,000 TESTS KIT
Preparation of lysis buffer C-part 1 (1x)	
	
Dilute the lysis buffer C-part 1 (4x) 4-fold with distilled water to prepare lysis buffer (1x). E.g. take 1.25 mL of lysis buffer (4x) and add it to 3.75 mL of distilled water. Mix gently.	
Preparation of supplemented lysis buffer C (1x)	
	
Dilute the Lysis Buffer C-part 2 (100x) 100-fold with Lysis buffer C-part 1 (1x). E.g. take 50 µL of Lysis Buffer C-part 2 and add it to 4.95 mL of Lysis buffer C-part 1 (1x). Mix gently.	

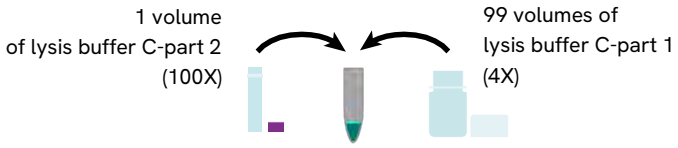
Supplemented lysis buffer C (4x) for two-plate and one-plate assay protocols for suspension cells and adherent cells kept in medium for lysis

Determine the amount of supplemented lysis buffer C (4x) needed for the experiment.

- For the one-plate assay protocol, each well requires 4 µL of supplemented lysis buffer C (1x).
- For the two-plate assay protocol, each well requires 40 µL of supplemented lysis buffer C (1x).

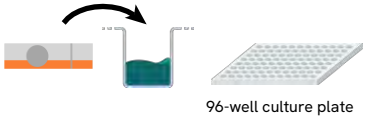
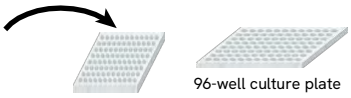
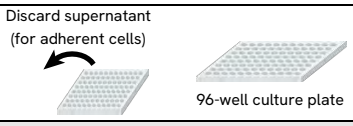
Dilute the lysis buffer C-part 2 (100x) 100-fold with this lysis buffer C-part 1 (4x).

In practice:

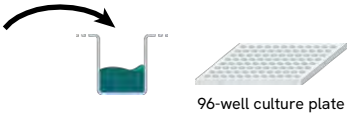
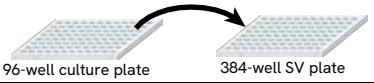
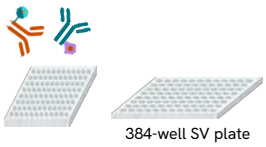
500 TESTS KIT	10,000 TESTS KIT
Preparation of supplemented lysis buffer C (4x)	
	
Dilute the Lysis Buffer C-part 2 (100x) 100-fold with Lysis buffer C-part 1 (4x). E.g. take 50 µL of Lysis Buffer C-part 2 and add it to 4.95 mL of Lysis buffer C-part 1 (4x). Mix gently.	

TWO-PLATE ASSAY PROTOCOL STEP BY STEP

GENERAL LAB WORK PRIOR USING HTRF KIT: CELLS PREPARATION

	FOR ADHERENT CELLS	FOR SUSPENSION CELLS AND ADHERENT CELLS KEPT IN MEDIUM FOR LYSIS	
1	Plate 100 µL of cells in 96-well tissue-culture treated plate in appropriate growth medium and incubate 3 to 4h, at 37°C in CO_2 atmosphere in order to allow cell adhesion.	Plate 100 µL of cells in 96-well tissue-culture treated plate, in appropriate growth medium.	
	Optimization of cell seeding densities is required.		
2	Dispense 20 µL of compound (6x), diluted in cell culture medium. Incubate the cells with the compound for the required time. We recommend a time course study to determine the optimal incubation time.		
3	Remove carefully supernatant using aspiration. Be careful not to touch the cell layer.	Do not remove supernatant.	

H3K4ME2 DETECTION USING HTRF KIT

	FOR ADHERENT CELLS	FOR SUSPENSION CELLS AND ADHERENT CELLS KEPT IN MEDIUM FOR LYSIS	
4	Immediately add 160 µL of supplemented lysis buffer C (1X) and incubate for 45 minutes at room temperature under shaking.	After desired incubation, add 40 µL of supplemented lysis buffer C (4x) and incubate for 45 minutes at room temperature under shaking.	
5	After homogenization by pipetting up and down, transfer 10 µL of cell lysate from the 96-well cell-culture plate to a 384-well small volume white plate.		
6	Add 5 µL of each HTRF reagent, prepared in the detection buffer. The 2 reagents can also be pre-mixed JUST PRIOR to dispensing, and 10 µL of this pre-mix is added. Cover the plate with a plate sealer. Incubate overnight at room temperature. Read the fluorescence emission at two different wavelengths (665 nm and 620 nm) on an HTRF®-compatible reader.		

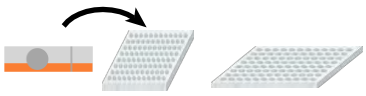
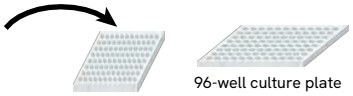
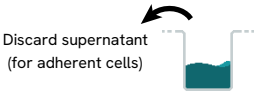
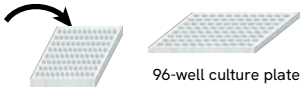
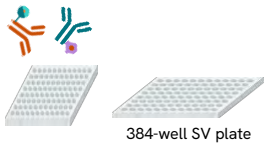
Two plate assay protocol in 20µL final volume after lysis step: standard protocol

	Non stimulated cells	Stimulated cells	Control lysate	Blank control*	Negative control**	
Prior using Revvity kit						
Cells	10 µL	10 µL	-	10 µL	-	-
Growth medium	-	-	-	-	-	6 µL
Using Revvity kit						
Control lysate			10 µL		-	
Supplemented lysis buffer C (1x) (depending on protocol used)	-	-	-	-	10 µL	-
Supplemented lysis buffer C (4x) (depending on protocol used)	-	-	-	-	-	4 µL
Cellular Histone Detection Buffer	-	-	-	5 µL		
H3 d2 Antibody (H3K4Me2 kit)	5 µL	5 µL	5 µL		5 µL	5 µL
H3K4Me2 Eu Cryptate Antibody	5 µL	5 µL	5 µL	5 µL	5 µL	5 µL
Total volume	20 µL	20 µL	20 µL	20 µL	20 µL	20 µL

* Blank control is used to check the Cryptate signal at 620 nm.

** Negative control is used to check the non-specific signal.

ONE-PLATE ASSAY PROTOCOL STEP BY STEP

	GENERAL LAB WORK PRIOR USING HTRF KIT: CELLS PREPARATION		
	FOR ADHERENT CELLS	**FOR SUSPENSION CELLS AND ADHERENT CELLS KEPT IN MEDIUM FOR LYSIS**	
1	Plate 18 µL of cells in 384-well small volume tissue culture treated white plate in appropriate growth medium. Optimization of cell seeding densities is required. The use of tissue culture treated plate is mandatory.	Plate 4 µL of cells in a 384-well small volume tissue culture treated white plate in appropriate growth medium. Optimization of cell seeding densities is required.	
2	Dispense 2 µL of compound (10x) diluted in appropriate growth medium.	Dispense 2 µL of compound (3x) diluted in appropriate growth medium.	
	Incubate the cells with compound for required time. Evaporation can be problematic with cells cultured in microtiter plates for a long incubation (overnight and more). You absolutely must control this evaporation issue. We recommend a time course study to determine the optimal incubation time. We recommend checking the evaporation issue during incubation.		
3	Remove carefully supernatant using aspiration. Be careful not to touch the cell layer.	Do not remove supernatant.	
	H3K4ME2 DETECTION USING HTRF KIT		
	FOR ADHERENT CELLS	**FOR SUSPENSION CELLS AND ADHERENT CELLS KEPT IN MEDIUM FOR LYSIS**	
4	Add 10 µL of supplemented lysis buffer C (1x) and incubate for 45 minutes at room temperature. Use the appropriate lysis buffer.	Add 4 µL of supplemented lysis buffer C (4x) and incubate for 45 minutes at room temperature. Use the appropriate lysis buffer.	
5	Add 5 µL of each HTRF reagent, prepared in the detection buffer. The 2 reagents can also be pre-mixed JUST PRIOR to dispensing, and 10 µL of this pre-mix is added. Cover the plate with a plate sealer. Incubate overnight at room temperature. Read the fluorescence emission at two different wavelengths (665 nm and 620 nm) on an HTRF®-compatible reader.		

One-plate assay protocol in 20 µL final volume: standard protocol for adherent cells

	Non stimulated cells	Stimulated cells	Control lysate	Blank control*	Negative control**
Prior using Revvity kit					
Cells	18 µL	18 µL	-	18 µL	-
Growth medium	2 µL	-	-	2 µL	-
Compound(s)	-	2 µL	-	-	-
	discarded volume				
Using Revvity kit					
Control lysate	-	-	10 µL	-	-
Supplemented lysis buffer C (1x)	10 µL	10 µL	-	10 µL	10 µL
Cellular Histone Detection Buffer	-	-	-	5 µL	
H3 d2 Antibody (H3K4Me2 kit)	5 µL	5 µL	5 µL	-	5 µL
H3K4Me2 Eu Cryptate Antibody	5 µL	5 µL	5 µL	5 µL	5 µL
Total volume	20 µL	20 µL	20 µL	20 µL	20 µL

* Blank control is used to check the Cryptate signal at 620 nm.

** Negative control is used to check the non-specific signal.

One-plate assay protocol in 20 µL final volume: standard protocol for suspension cells and adherent cells kept in medium for lysis

	Non stimulated cells	Stimulated cells	Control lysate	Blank control*	Negative control**
Prior using Revvity kit					
Cells	4 µL	4 µL	-	4 µL	-
Growth medium	2 µL	-	-	2 µL	6 µL
Compound(s)	-	2 µL	-	-	-
Using Revvity kit					
Control lysate	-	-	10 µL	-	-
Supplemented lysis buffer C (4x)	4 µL	4 µL	-	4 µL	4 µL
Cellular Histone Detection Buffer	-	-	-	5 µL	
H3 d2 Antibody (H3K4Me2 kit)	5 µL	5 µL	5 µL	-	5 µL
H3K4Me2 Eu Cryptate Antibody	5 µL	5 µL	5 µL	5 µL	5 µL
Total volume	20 µL	20 µL	20 µL	20 µL	20 µL

* Blank control is used to check the Cryptate signal at 620 nm.

** Negative control is used to check the non-specific signal.

DATA REDUCTION & INTERPRETATION

1) Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2) Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

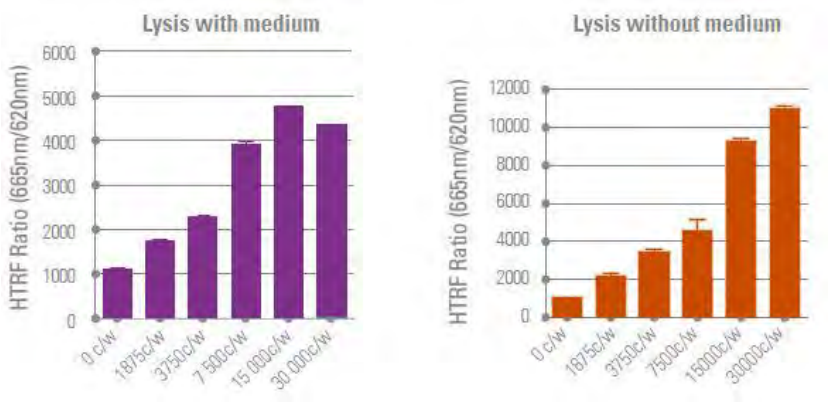
For more information about data reduction, please visit our website.

RESULTS

This data should only be considered as an example. Results may vary from one HTRF compatible reader to another. The histograms are drawn up by plotting HTRF Ratio versus the number of cells seeded in 96-well plate.

Results on HeLa cells (various number of cells per well), using the two plate protocol for lysis with or without medium.
Cells were incubated 24 hours before lysis step according to the previously described protocol (left panel, lysis with medium, right panel lysis without medium).

	Lysis with medium		Lysis without medium	
	HTRF Ratio (1)	CV% (2)	HTRF Ratio (1)	CV% (2)
0 c/w	1 113	2%	1 044	1%
1,875 c/w	1 757	1%	2 200	5%
3,750 c/w	2 294	1%	3 454	3%
7,500 c/w	3 898	2%	4 562	13%
15,000 c/w	4 772	0%	9 276	2%
30,000 c/w	4 315	1%	11 030	1%



HOW TO IMPROVE YOUR ASSAY PERFORMANCE

Depending on compound incubation time, medium evaporation can occur in microtiter plate (sv-384-plate)	A sterile breathable sealing membrane plus special lid can be placed on the plate. Sterile warm PBS can be added to all outer and unused wells in addition to special lids. (Greiner # 691 161). Check the evaporation issue during incubation.
Parameters such as cell density, stimulation time and lysis incubation time should be optimized for each cell line used.	The assay can be used for many adherent and non-adherent cell types, including transfected cell lines and primary cells. However, specific lysine mark methylation can vary from one cell line to another. Be sure to first determine the best cell concentration that is suited to the required stimulation time. Too high or low cell numbers can affect detection of methyl mark. Be carefull of hook effect. Some compounds are described as active after long incubation time. Stimulation time needs to be optimized.
EPIgeneous Total H3 normalization assay	The two-plate assay protocol recommends the use of 10 µL of lysate per well, whereas the 96-well cell culture microplate would use 160 µL or 200 µL of lysis buffer per well (depeding on lysis buffer used). Therefore, a typical cell lysate can also be assayed for total H3 level (using EPIgeneous Total H3 cellular assay) in order to measure the compound effect on Total Histone H3 level. Depending on the cell line used for the assay, Total H3 kit might be more or less sensitive compared to specific methyl mark kit. Please adapt in consequence the volume of cell lysate transferred adjusted with lysis Buffer (see protocol), or adapt the number of cells seeded.
Fluorescence reading	Using an inappropriate set-up may seriously impair the results. For information about HTRF compatible readers and for set-up recommendations, please visit our website.
Using adherent cells, allow time for your cells to recover after plating	Allow cells to recover by plating them at least 3-4 hours before starting the pharmacological treatment.
Limit the DMSO percentage given with compounds	Please, note that concentration above 0.5% DMSO (during compound treatment) will impair assay performances.
Generation of lysates	Ensure that the lysates used for the assay have been generated by using the HTRF lysis buffer provided in the kit.

REACH European regulations and compliance

This product contains material of biologic origin. Use for research purposes only. Do not use in humans or for diagnostic purposes. The purchaser assumes all risk and responsibility concerning reception, handling and storage.

Manufactured by Cisbio Bioassays - Parc Marcel Boiteux - 30200 Codolet – FRANCE

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