H3K27ME3 CELLULAR ASSAY

10,000 TESTS

PROTOCOL

For research use only.
Not for use in therapeutic or diagnostic procedures.

Storage temperature: ≤-60°C.

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Product information:

Packaging details :

	384-well low volume plate (20 µL)
62KC3PAD	10,000 tests

Document reference : 62KC3PAD rev04 (Sep. 2020)

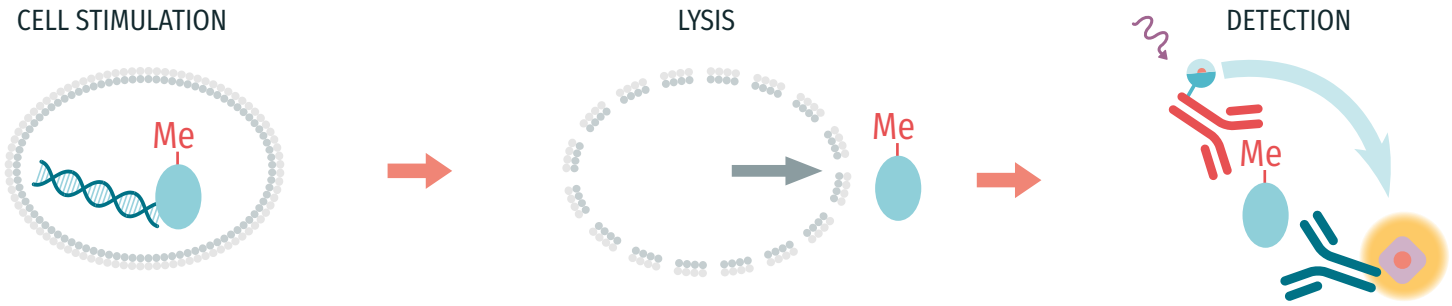
1. Assay description

This EPIgeneous™ H3K27Me3 cellular assay is intended for the simple, rapid and direct detection of endogenous levels of H3K27Me3 mark in cells. The trimethylation of Lysine 27 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 H3K27Me3, thereby generating FRET. The specific signal modulates positively in proportion to trimethylation on Lysine 27.

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 H3K27Me3 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 H3K27Me3 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.

2. Assay principle



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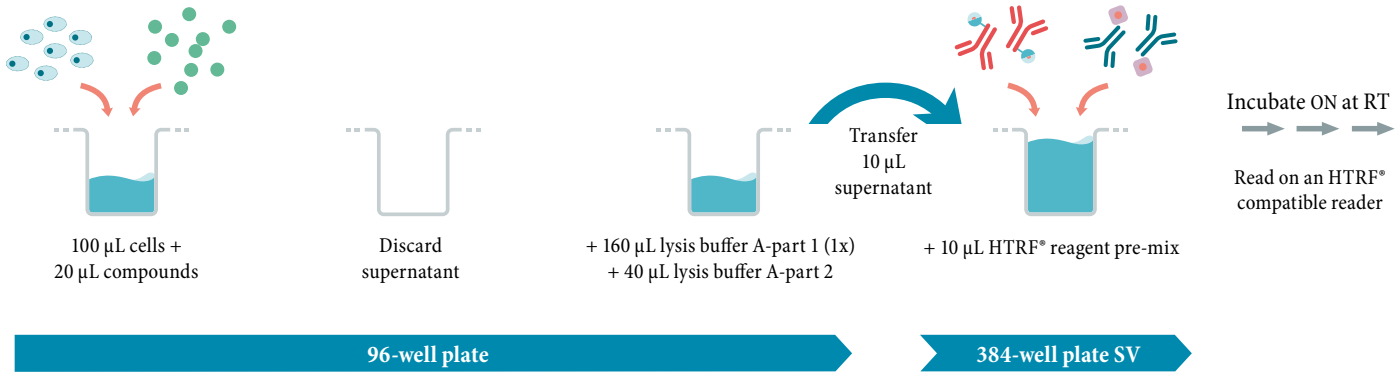
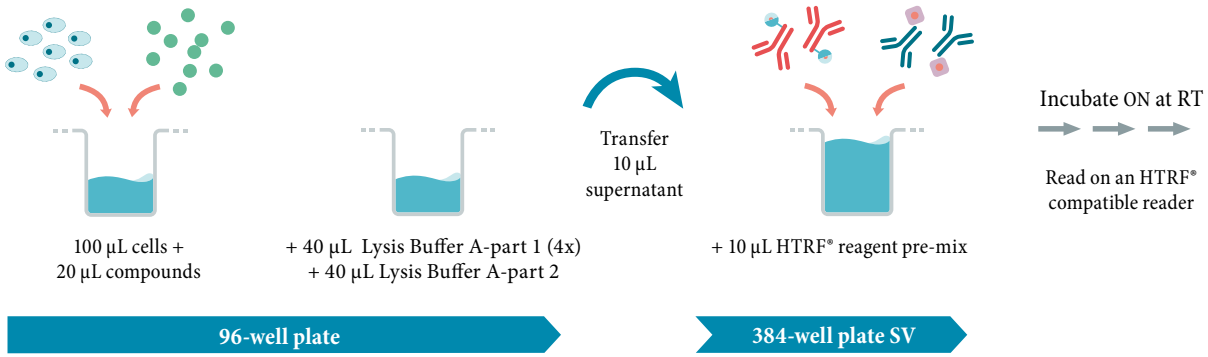
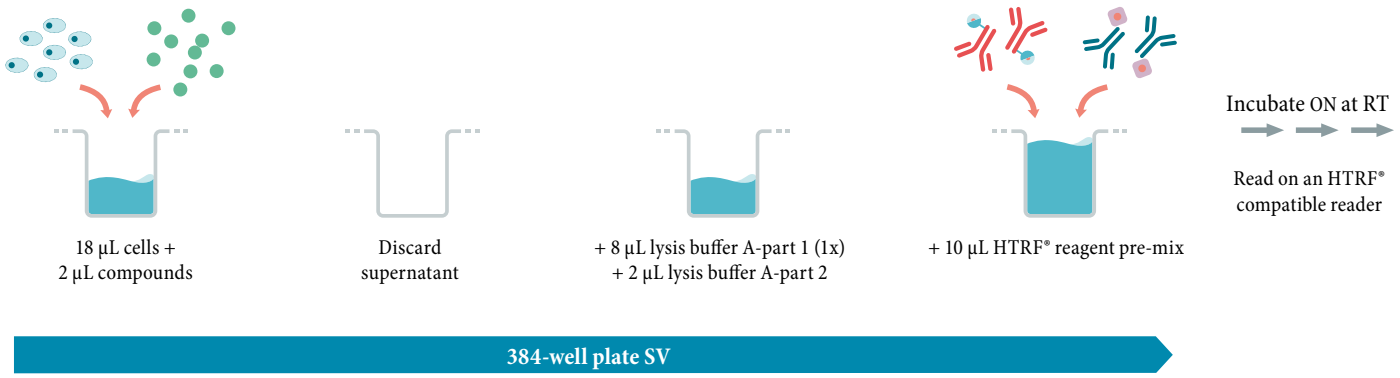
➥ *Two-plate assay protocol for adherent cells*



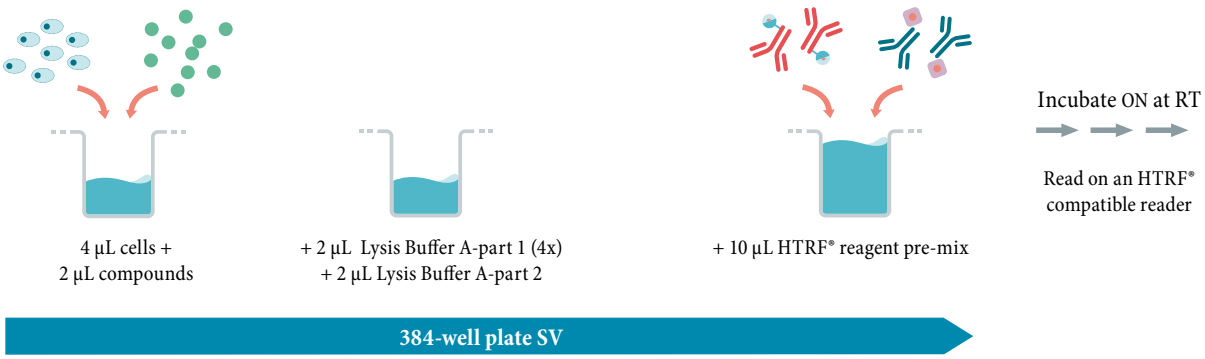
➥ *Two-plate assay protocol for suspension cells or adherent cells kept in medium for lysis*



➥ *One-plate assay protocol for adherent cells*



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



3. HTRF reagents

		Nb of vials	Volume per vial	Storage
H3K27Me3 Eu Cryptate Antibody (stock solution 50x)	red cap	1	1 mL	≤-16°C
H3 d2 Antibody (H3K27Me3 kit) (stock solution 50x)	blue cap	1	1 mL	≤-16°C
H3K27Me3 Control Lysate (ready to use)	green cap	3	150µL	≤-60°C
EPIgeneous™ Lysis buffer A-part 1 (stock solution (4x))	White cap	3	170 mL	≤-16°C
EPIgeneous™ Lysis buffer A-part 2 (ready to use)	Red cap	3	170 mL	≤-16°C
Cellular Histone Detection Buffer (ready to use)	White cap	1	120 mL	≤-16°C

4. 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.

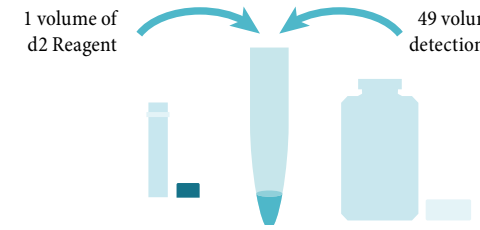
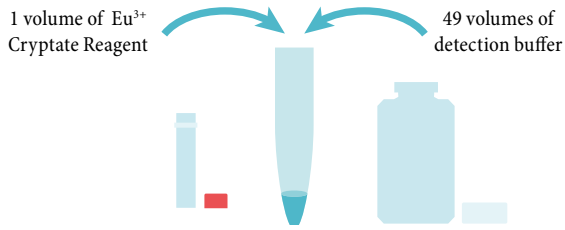
5. Reagent preparation

Allow all reagents to thaw before use.
We recommend centrifuging the vials gently after thawing, before pipeting 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.

5.1. 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 the detection buffer. In practice:

H3 d2 Antibody (H3K27Me3 kit)	H3K27Me3 Eu Cryptate Antibody
	

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).

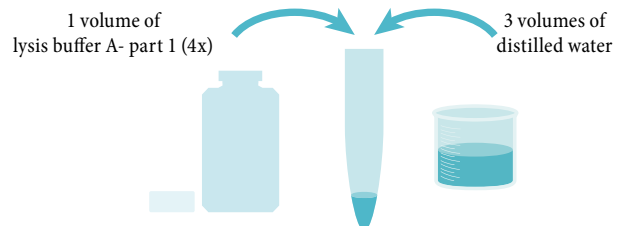
5.2. Preparation of lysis buffer

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.
Be aware that whatever the protocol chosen, two buffers are necessary for the lysis step.

➥ *Lysis buffer A-part 1 (1x) for two-plate and one-plate assay protocols on adherent cells*

Prepare the required amount of lysis buffer A-part 1 (1x) before running the assay.
Determine the amount of lysis buffer A-part 1 needed for the experiment.
- For the two-plate assay protocol, each well requires 160 µL of lysis buffer A-part - 1 (1x).
- For the one-plate assay protocol, each well requires 8µL of Lysis buffer A-part 1 (1x).

In practice:

Preparation of Lysis buffer A-part 1 (1x)

Dilute the lysis buffer A-part 1 (4x) 4-fold with distilled water e.g. take 1.25 mL of lysis buffer (4x) and add it to 3.75 mL of distilled water. Mix gently.

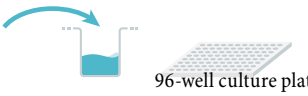
➥ *Lysis buffer A-part 1 (4x) for two-plate & one-plate assay protocols for suspension cells and adherent cells kept in medium for lysis*

For these assays, the buffer is ready to use.
Determine the amount of lysis buffer A-part 1 (4x) needed for the experiment.
- For the two-plate assay protocol on suspension cells or homogeneous assay with adherent cells, each well requires 40 µL of lysis buffer A-part 1 (4x).
- For the one-plate assay protocol, each well requires 2 µL of lysis buffer A-part 1 (4x).

➥ *Lysis buffer A-part 2 for two-plate & one-plate assay protocols on suspension and adherent cells*

Lysis buffer A-part 2 is used for all protocols, and is ready to use.

6. Two-plate assay protocol step by step

	For adherent cells	For suspension cells and adherent cells kept in medium for lysis	
	GENERAL LAB WORK PRIOR USING CISBIO KIT		
1	Plate 100 µL of cells in a 96-well tissue-culture treated plate in appropriate growth medium. Incubate 3 to 4h at 37°C in CO_2 atmosphere in order to allow cell adhesion. Optimization of cell seeding densities is required.	Plate 100 µL of cells in a 96-well tissue-culture treated plate, in appropriate growth medium. Optimization of cell seeding densities is required.	 96-well culture plate 96-well culture plate
2	Dispense 20 µL of compound (6X), diluted in cell culture medium. Incubate the cells with the compound for the required time. Note that concentration above 0.5% DMSO will impair assay performances. We recommend a time course study to determine the optimal incubation time.		 96-well culture plate 96-well culture plate
3	***Carefully remove supernatant using aspiration.*** Be careful not to touch the cell layer. 	***Do not remove supernatant*** 	
	H3K27ME3 DETECTION USING CISBIO KIT		
4	Immediately add 160 µL of lysis buffer A-part 1 (1x) and incubate for 45 minutes at room temperature under shaking. After incubation add 40 µL of Lysis Buffer A-part 2.	After desired incubation, add 40 µL of lysis buffer A-part 1 (4x) and incubate for 45 minutes at room temperature under shaking. After incubation add 40 µL of Lysis Buffer A-part 2.	 96-well culture plate
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.		 96-well culture plate 384-well SV 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 10µL of this pre-mix is added. Cover the plate with a plate sealer. Incubate ON at room temperature. Read the fluorescence emission at two different wavelengths (665 nm and 620 nm) on an HTRF®-compatible reader.		 384-well SV plate
For more information about HTRF® compatible readers and for set-up recommendations, please visit our website at: www.cisbio.com/content/htrf-microplate-readers			

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

	Non stimulated cells	Stimulated cells	Control lysate	Blank control*	Negative control**	
Prior using Cisbio kit						
Cells	10 µL	10 µL	-	10 µL	-	-
Cell culture Medium	-	-	-	-	-	6 µL
Using Cisbio kit						
Control lysate	-	-	10 µL	-	-	-
Lysis buffer A-part1 (4x)	-	-	-	-	-	2 µL
Lysis buffer A-part1 (1x)	-	-	-	-	8 µL	-
Lysis buffer A-part 2	-	-	-	-	2 µL	2 µL
Cellular Histone Detection Buffer	-	-	-	5 µL	-	-
H3 d2 Antibody (H3K27Me3 kit)	5 µL	5 µL	5 µL	-	5 µL	5 µL
H3K27Me3 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

7. One-plate assay protocol step by step

	For adherent cells	For suspension cells and adherent cells kept in medium for lysis	
	GENERAL LAB WORK PRIOR USING CISBIO KIT: CELL PREPARATION		
1	Plate 18 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. 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.	 96-well culture plate
2	Dispense 2 µL of compound (10x) diluted in appropriate growth medium.	Dispense 2 µL of compound (3x) diluted in appropriate growth medium.	 96-well culture plate
	Incubate the cells with compound for required time. Evaporation can be problematic with cells cultured in micro-titer plates for a long incubation (overnight and more). This evaporation issue must be managed carefully. Note that concentration above 0.5% DMSO will impair assay performances. We recommend a time course study to determine the optimal incubation time We recommend checking the evaporation issue during incubation		
3	***Carefully remove supernatant using aspiration.*** Be careful not to touch the cell layer. 	***Do not remove supernatant*** 	
	H3K27ME3 DETECTION USING CISBIO KIT		
4	Add 8 µL of lysis buffer A-part 1 (1x) and incubate for 45 minutes at room temperature. After incubation, add 2 µL of lysis Buffer A-part 2 Use the appropriate lysis buffer.	Add 2 µL of lysis buffer A-part 1 (4x) and incubate for 45 minutes at room temperature. After incubation add 2 µL of lysis Buffer A-part 2 Use the appropriate lysis buffer.	 384-well SV plate
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 ON at room temperature. Read the fluorescence emission at two different wavelengths (665 nm and 620 nm) on an HTRF®-compatible reader.		 384-well SV plate
	For more information about HTRF® compatible readers and for set-up recommendations, please visit our website at: www.cisbio.com/content/htrf-microplate-readers		

➥ *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 Cisbio kit	Cells	18 µL	18 µL	-	18 µL	-
	Cell culture Medium	2 µL	-	-	2 µL	-
	Compound(s)	-	2 µL	-	-	-
		Discarded volume				
Using Cisbio kit	Control lysate			10 µL		
	Lysis buffer A-part 1 (1x)	8 µL	8 µL	-	8 µL	8 µL
	Lysis buffer A-part 2	2 µL	2 µL	-	2 µL	2 µL
	Cellular Histone Detection Buffer	-	-	-	5 µL	-
	H3 d2 Antibody (H3K27Me3 kit)	5 µL	5 µL	5 µL	-	5 µL
	H3K27Me3 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 Cisbio kit	Cells	4 µL	4 µL	-	4 µL	-
	Compound(s)	-	2 µL	-	-	-
	Cell culture Medium	2 µL	-	-	2 µL	6 µL
Using Cisbio kit	Control lysate	-	-	10 µL	-	-
	Lysis buffer A-part 1 (4x)	2 µL	2 µL	-	2 µL	2µL
	Lysis buffer A-part 2	2 µL	2 µL	-	2 µL	2 µL
	Cellular Histone Detection Buffer	-	-	-	5 µL	-
	H3 d2 Antibody (H3K27Me3 kit)	5 µL	5 µL	5 µL	-	5 µL
	H3K27Me3 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

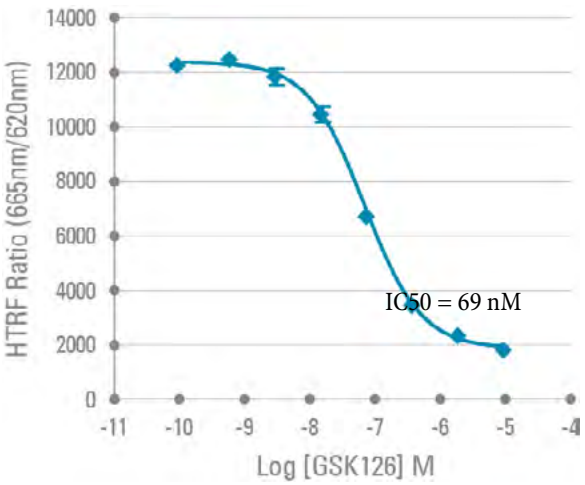
* Blank control is used to check the Cryptate signal at 620 nm. ** Negative control is used to check the Non specific signal.

8. Data reduction

This data should be considered as an example only. Results may vary from one HTRF compatible reader to another. The curves are drawn up by plotting HTRF Ratio versus the log [compound] concentration.

Results on SU-DHL-6 cells (20,000 cells/well), using the two-plate assay protocol for suspension cells. Cells were stimulated with GSK126 for 72h before lysis step, following the previously-described protocol

[GSK 126] nM	Log [GSK126] M	HTRF Ratio (1)	CV% (2)
10000	-5	1810	4%
2000	-5.7	2339	4%
400	-6.4	3453	6%
80	-7.1	6715	1%
16	-7.8	10449	3%
3.2	-8.5	11838	3%
0.64	-9.2	12470	1%
0.00	-10	12255	2%



HTRF Ratio (1)	$\frac{\text{Signal}_{665nm}}{\text{Signal}_{620nm}} \times 10^4$	HTRF Ratio must be calculated for each individual well.
CV% (2)	$\frac{\text{Standard deviation}}{\text{Mean ratio}} \times 100$	The mean and standard deviation can then be worked out from ratio replicates.
For more information about data reduction, please visit our website at: www.cisbio.com/content/signal-treatment-and-analysis		

9. 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. (Low base sv-384-plate Greiner # 788073, lid 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 in consequence (adjusted with Mix lysis Buffer A, 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 at: www.cisbio.com/content/htrf-microplate-readers
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

To obtain additional information or support, please contact your technical support team (htrfservices@cisbio.com)..

FOR MORE INFORMATION
Europe and other countries +33(0)466-796-705 U.S. and Canada 1-888-963-4567 China +86-21-5018-9880
Japan +81-(0)43-306-8712 Visit www.cisbio.com to find a list of our regional distributors

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