HTRF® G9A HISTONE H3K9 MONO-METHYLATION ASSAY (me0 → me1)

TECHNICAL NOTE

ABSTRACT G9a Histone H3K9 mono-methylation assay measures the monomethylation of a biotinylated histone H3(1-21) peptide at lysine 9.

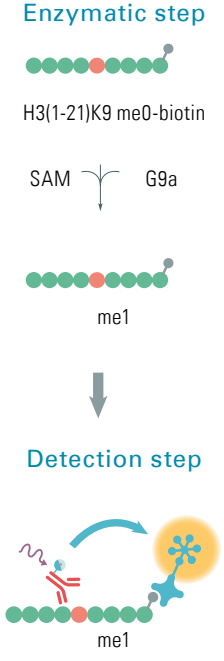
The HTRF G9a Histone H3K9 monomethylation assay uses a H3(1-21) lysine 9 un-methylated biotinylated peptide (substrate), a Eu3+-cryptate labeled anti-H3K9 me1 detection antibody and XL665-conjugated Streptavidin (SA-XL665).

The assay is performed in a single well and run in two steps: the enzymatic step and the detection step. HTRF signal is proportional to the concentration of monomethylated H3(1-21) peptide. The assays within this technical note were performed in a 384-well plate in a 20 µL final volume.

Enzyme	G9a
Substrate	H3(1-21)K9 me0-biotin ARTKQTARKSTGGKAPRKQ-LA-GG-K(Biotin)
Detection Antibody	Anti-H3K9 me1-Eu(K)

G9A HISTONE H3K9 MONO-METHYLATION ASSAY AND REAGENTS

H3K9 me1-Eu(K) Ab.	Cisbio Bioassays	# 61KB1KAE
Streptavidin XL-665	Cisbio Bioassays	# 610SAXLA
Detection buffer	Cisbio Bioassays	# 62SDBRDD
G9a	BPS Bioscience	# 51000
Histone H3(1-21) lysine 9 un-methylated biotinylated peptide	AnaSpec	# 61702
S-(5'-Adenosyl)-L-methionine chloride (SAM)	Sigma	# A7007
UNC0646	R&D Systems	# 4342
BIX01294	R&D Systems	# 3364
Sinefungin	Sigma	# S8559
S-(5?-Adenosyl)-L-homocysteine (SAH)	Sigma	# A9384
Enzymatic buffer	50 mM Tris-HCl, pH 8.8, 10 mM NaCl, 4 mM DTT, 0.01% Tween20	



Data shown on this application note has been obtained using Greiner # 784075, 384-well white microplates. For more information on the white plates we recommend, please visit http://www.htrf.com/htrf-technology/microplate-recommendations.

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ASSAY PROTOCOL

ENZYMATIC STEP

- Prepare working solutions of enzyme, peptide substrate, cofactors and inhibitor in enzymatic buffer just prior to use.
- Add to a 384-well small volume plate in the following order:
  - 4 µL of inhibitor (2.5X) or enzymatic buffer
  - 2 µL of G9a enzyme (5X)
  - Incubate for 5 min at room temperature
  - 4 µL of H3(1-21)K9 me0-biotin peptide/ SAM pre-mixture (2.5X)
- Cover the plate with a plate sealer and incubate at room temperature.

DETECTION STEP

- Prepare detection mixture containing the anti-H3K9 me1-Eu(K) 2X according to the product datasheet recommended final concentration and SA-XL665 at 10 nM in detection buffer. Final concentration of 5 nM for SA-XL665 corresponds to 0.25X the final concentration of peptide substrate.
- Add 10 µL of detection mixture (2X) to the plate.
- Cover the plate with a plate sealer and incubate 1h at room temperature.
- Remove plate sealer and read fluorescence emission at 665nm and 620nm wavelengths on an HTRF compatible reader.

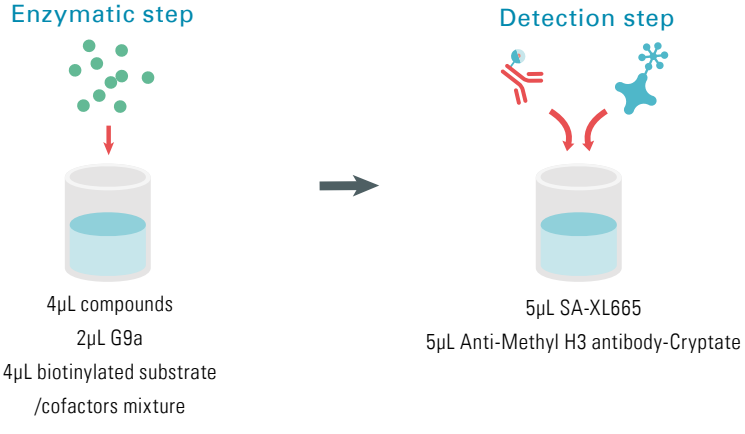
HTRF Ratio = (665nm/620nm)X10^4

Delta Ratio = Sample Ratio - Ratio negative

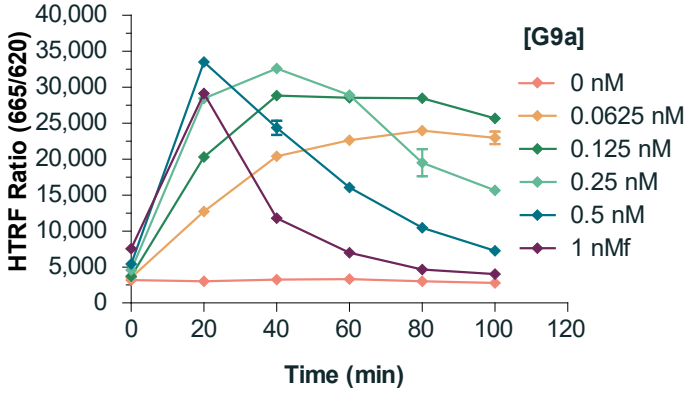
Delta F% = (Delta Ratio/Ratio Negative) X100

DISTRIBUTION: ENZYME INHIBITION STUDY

	ENZYMATIC STEP				DETECTION STEP	
	ENZYMATIC BUFFER	INHIBITOR	G9A	COFACTOR/ SUBSTRATE MIXTURE	CRYPTATE-Ab	SA-XL 665
SAMPLE	-	4 µL	2 µL	4 µL	5 µL	5 µL
POSITIVE CONTROL	4 µL	-	2 µL	4 µL	5 µL	5 µL
NEGATIVE CONTROL	6 µL	-	-	4 µL	5 µL	5 µL

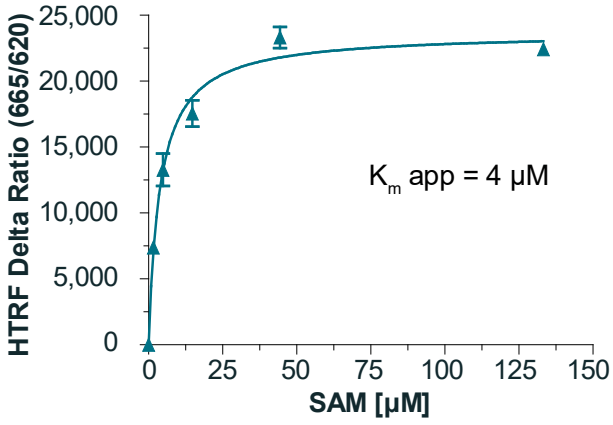


1. TIME COURSE AND ENZYME TITRATION



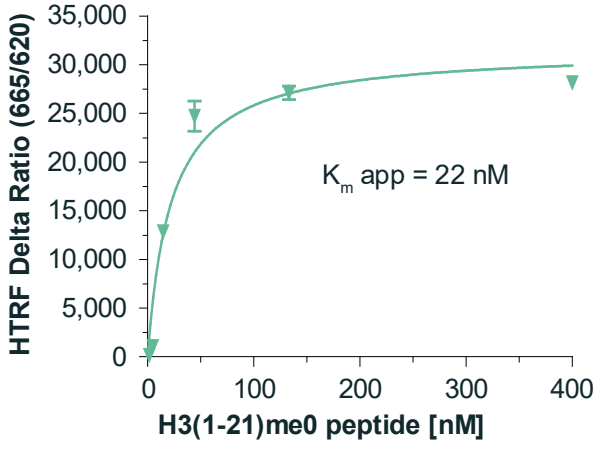
This step allows the optimal enzyme concentration and enzyme reaction time to be determined. Human recombinant G9a was serially diluted to the concentrations indicated in the figure (0.063, 0.13, 0.25, 0.5, 1 nM), and the assay was carried out with 80 nM biotinylated H3K9(1-21) me0 peptide substrate and 200 µM SAM. Enzyme kinetics depends on the G9a specific activity and substrate concentrations. The enzymatic reaction was carried out at RT and then stopped by adding H3K9me1-K Ab and SA-XL665 (detection reagents) after each time point (20, 40, 60, 80, 100 min). For further experiments, 80 nM peptide substrate, a reaction time of 40min at RT, and 0.12 nM enzyme were selected. The decrease of signal at highest concentrations and incubation times is due to the fact that the enzyme can further methylate the peptide while our antibody is specific for the monomethylated state.

2. SAM TITRATION



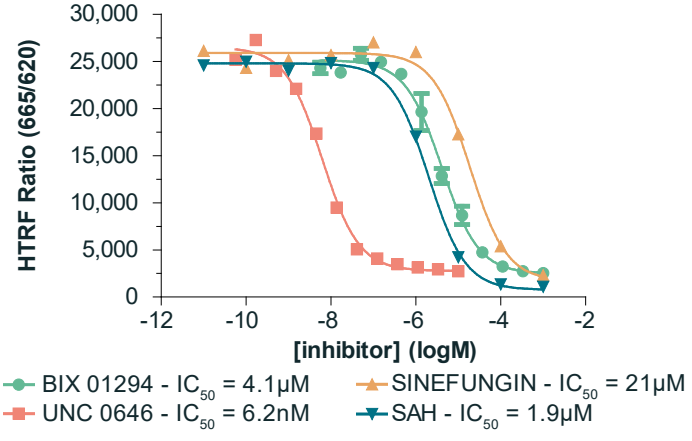
This step allows the determination of Km for SAM. The Km value was determined with 0.12 nM G9a and 80 nM biotinylated H3K9(1-21)me0 substrate in the enzymatic step. We recommend testing SAM concentrations ranging from 150 µM to 0.5 µM (serial dilutions). A negative control is performed by removing the SAM and peptide from the wells. This negative control is used as non specific signal to calculate the HTRF delta ratio (hence specific signal). The enzyme reaction was stopped by adding the detection reagents at the optimal incubation period (RT, 40 min). The 4 µM Km value for SAM was determined from this experiment using a Michaelis-Menten plot.

3. PEPTIDE TITRATION



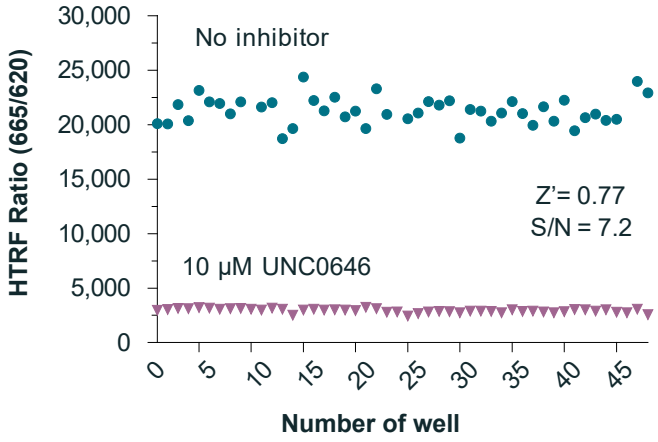
This step allows the determination of Km for peptide. The Km value was determined with 0.12 nM G9a and 200 µM SAM in the enzymatic step. We recommend testing biotinylated H3K9(1-21)me0 substrate concentrations ranging from 400 nM to 2 nM (serial dilutions). The streptavidin XL-665 concentration varie according to the peptide concentration keeping constant the ratio of 1/4 (Streptavidin XL-665 / peptide). For each concentration of peptide and streptavidin XL-665, a negative control is performed by removing the SAM and peptide from the wells. This negative control is used as non specific signal to calculate the HTRF delta ratio (hence specific signal). The enzyme reaction was stopped by adding the detection reagents at the optimal incubation period (RT, 40 min). The 22 nM Km value for peptide was determined from this experiment using a Michaelis-Menten plot.

4. ENZYME INHIBITION



G9a H3K9 monomethylation inhibitor assay was validated by measuring activity of known inhibitors. This assay was performed using 15µM SAM and 0.12 nM G9a. Serial dilutions of inhibitors were pre-incubated for 5 min with G9a. Enzymatic reaction was initiated by the addition of 40 nM biotinylated H3 (1-21) me0 peptide substrate plus 15 µM SAM. The enzyme reaction was stopped with the detection reagents after 40 min incubation at RT. IC50 values were calculated from inhibition curves.

5. Z' FACTOR DETERMINATION



The robustness of the assay was proven by performing a Z' determination with UNC0646 at IC100 (10 µM). The enzyme reaction was carried out with 0.12 nM G9a, 15 µM SAM and 40 nM H3K9(1-21)me0 substrate for 40 min at RT. Z' of 0.77 shows the robustness of the assay.

For more information, please visit us at www.htrf.com/epigenetic-toolbox-reagents

RELATED ARTICLES

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High-Throughput, Homogeneous Histone Demethylase JARID1A, and JARID1C Enzymatic applications with HTRF Technology.
Adachi K, Tokuda C, Roux T, Trinquet E, Degorce F - Miptec 2013, Basel, Switzerland.

High-Throughput, Homogeneous Histone H3 Methyltransferase, (HMT) and Demethylase (HDM) Enzyme Assays using HTRF®, Technology: G9a H3K-27dimethylation assay example.
Roux T, Adachi K, Tokuda C, Verdi J, Junique S, Trinquet E, Gonzalez-Moya A, Degorce F - SLAS 2013, Orlando, USA.

High-Throughput, Homogeneous Histone H3 Methyltransferase (HMT) and Demethylase (HDM) Enzyme Assays using HTRF Technology.
Adachi K, Tokuda C, Chevallier F, Roux T, Gonzalez-Moya A, Degorce F. - Discovery on Target 2012, Boston, MA, USA.

Development of a panel of HTRF assay reagents for epigenetic targets.
Chevallier F, Jean A, Raynaldy D, Romier M, Servent F, Tokuda C, Adachi K. - Miptec 2011, Basel, Switzerland.

Development of G9a (Histone H3K9 methyltransferase) assay using HTRF technology.
Adachi K, Tokuda C, Chevallier F, Preaudat M. - SBS 2011, Orlando, USA.

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