HTRF® JARID1C HISTONE H3K4 MONO-DEMETHYLATION ASSAY (me3 → me2)

TECHNICAL NOTE

ABSTRACT JARID1C Histone H3K4 mono-demethylation assay measures the demethylation of a biotinylated histone H3(1-21) peptide at lysine 4.

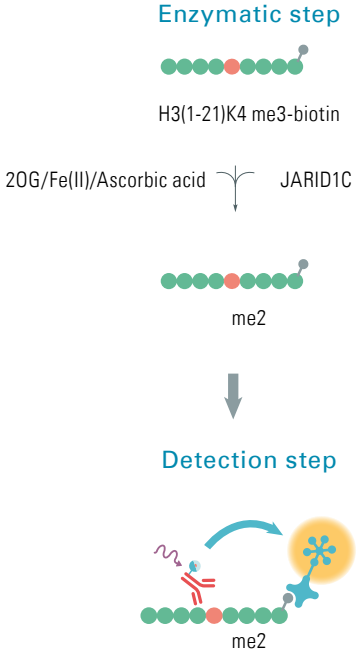
The HTRF JARID1C Histone H3K4 demethylation assay uses a H3(1-21) lysine 4 tri-methylated biotinylated peptide (substrate), a Eu3+-cryptate labeled anti-H3K4 me2 detection antibody and XL665-conjugated Streptavidin (SA-XL665).

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 demethylated H3(1-21) peptide. The assays within this technical note were performed in a 384-well plate in a 20 µL final volume.

Enzyme	JARID1C
Substrate	H3(1-21)K4 me3-biotin ART-K(Me3)-QTARKSTGG-KAPRKQLA-GGK(Biotin)
Detection Antibody	Anti-H3K4 me2-Eu(K)

JARID1C HISTONE H3K4 MONO-DEMETHYLATION ASSAY AND REAGENTS

H3K4 me2-Eu(K) Ab.	Cisbio Bioassays	# 61KA2KAE
Streptavidin XL-665	Cisbio Bioassays	# 610SAXLA
Detection buffer	Cisbio Bioassays	# 62SDBRDD
JARID1C	BPS Bioscience	# 50112
Histone H3(1-21) lysine 4 tri-methylated biotinylated peptide	AnaSpec	# 64357
Ammonium iron(II) sulfate hexahydrate (Fe(II))	Sigma	# F3754
L-ascorbic acid (Asc)	Sigma	# A5960
alpha-ketoglutaric acid disodium salt (2OG)	Sigma	# K3752
N-Oxalylglycine	Sigma	# O9390
Enzymatic buffer	50 mM MES pH6.5, 50 mM NaCl, 0.01% tween20 plus 2mM ascorbic acid	



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 JARID1C enzyme (5X)
  - Incubate for 5 min at room temperature
  - 4 µL of H3(1-21)K4 me3-biotin peptide/ 2OG/Fe(II) pre-mixture (2.5X)
- Cover the plate with a plate sealer and incubate at room temperature.

DETECTION STEP

- Prepare detection mixture containing the anti-H3K4 me2-Eu(K) 2X according to the product datasheet recommended final concentration and SA-XL665 at 25 nM in detection buffer. Final concentration of 12.5 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.

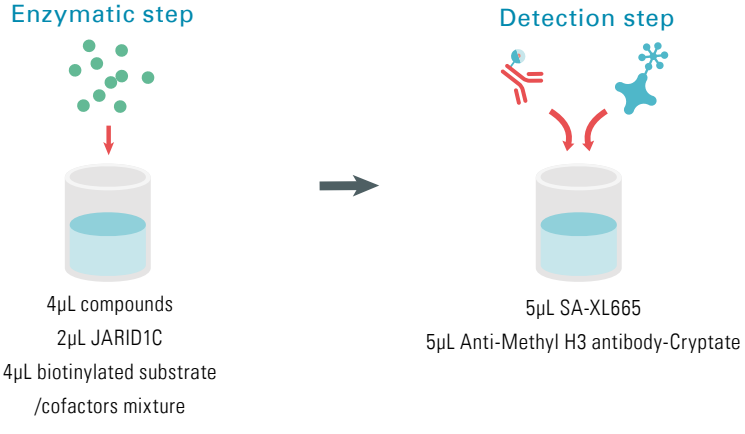
HTRF Ratio = (665nm/620nm)X10^4

Delta Ratio = Sample Ratio - Ratio negative

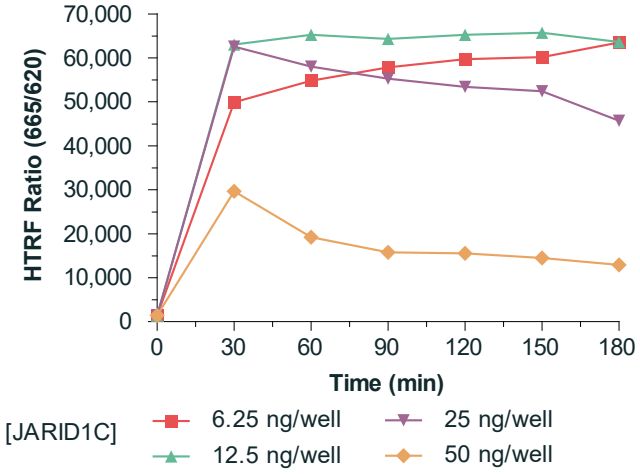
Delta F% = (Delta Ratio/Ratio Negative) X100

DISTRIBUTION: ENZYME INHIBITION STUDY

	ENZYMATIC STEP				DETECTION STEP	
	ENZYMATIC BUFFER	INHIBITOR	JARID1C	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

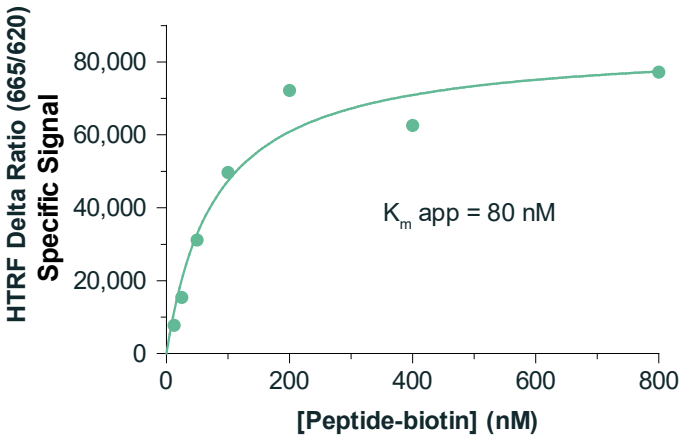


1. TIME COURSE AND ENZYME TITRATION



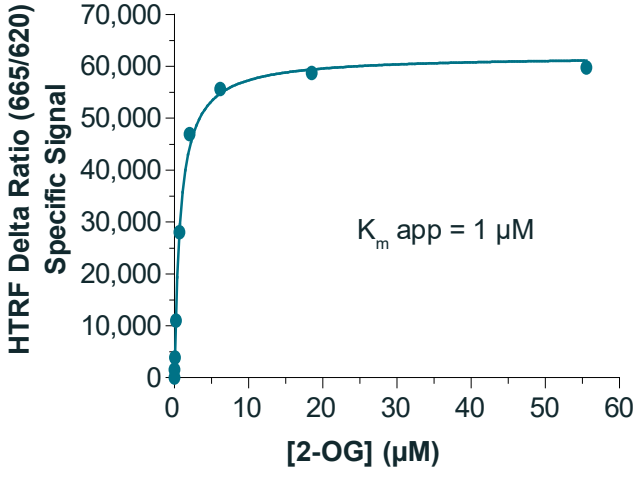
This step allows the optimal enzyme concentration and enzyme reaction time to be determined. Human recombinant JARID1C was serially diluted to the concentrations indicated in the figure (6.25, 12.5, 25, 50 ng/well), and the assay was carried out with 100 nM biotinylated H3K4(1-21) me3 peptide substrate, 1 mM 2-OG and 50µM Fe(II). Enzyme kinetics depends on the JARID1C specific activity and substrate concentrations. The enzymatic reaction was carried out at RT and then stopped by adding H3K4me2-K Ab and SA-XL665 (detection reagents) after each time point (0, 30, 60, 90, 120, 150, 180 min). For further experiments a reaction time of 30min at RT, and 10 ng/well enzyme were selected.

2. PEPTIDE TITRATION



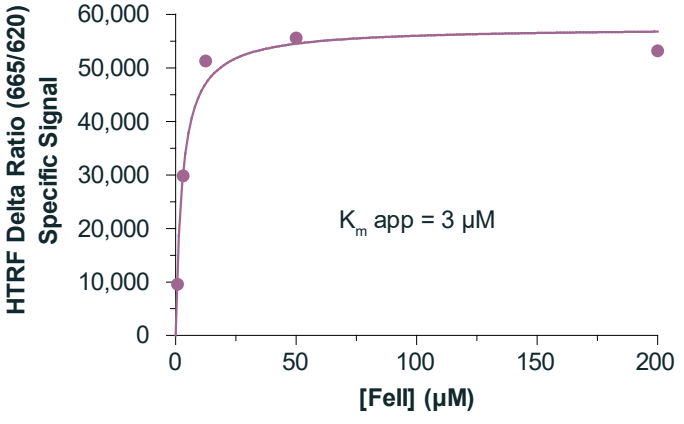
This step allows the determination of apparent Km for peptide. The Km value was determined with 10 ng/well JARID1C, 50µM Fe(II) and 1 mM 2-OG in the enzymatic step. We recommend testing biotinylated H3K4(1-21)me3 substrate concentrations ranging from 500 nM to 1 nM (serial dilutions). The streptavidin 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 2-OG 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, 30 min). The 80 nM apparent Km value for peptide was determined from this experiment using a Michaelis-Menten plot.

3. 2-OG TITRATION



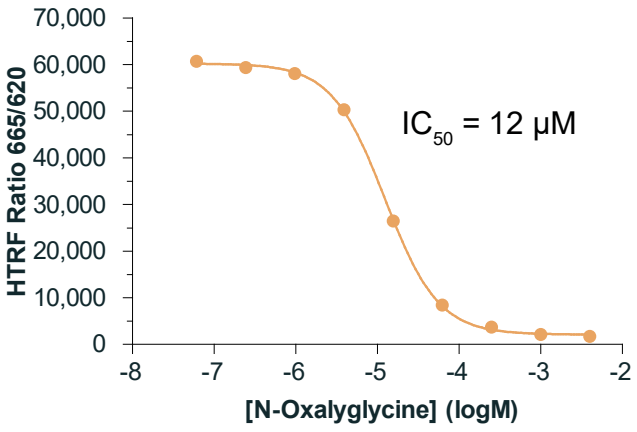
This step allows the determination of apparent Km for 2-OG. The Km value was determined with 10 ng/well JARID1C, 100 nM biotinylated H3K4(1-21)me3 substrate and 50 µM Fe(II) in the enzymatic step. We recommend testing 2-OG concentrations ranging from 50 µM to 0.01 µM (serial dilutions). A negative control is performed by removing the 2-OG 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, 30 min). The 1 µM apparent Km value for 2-OG was determined from this experiment using a Michaelis-Menten plot.

4. FE(II) TITRATION



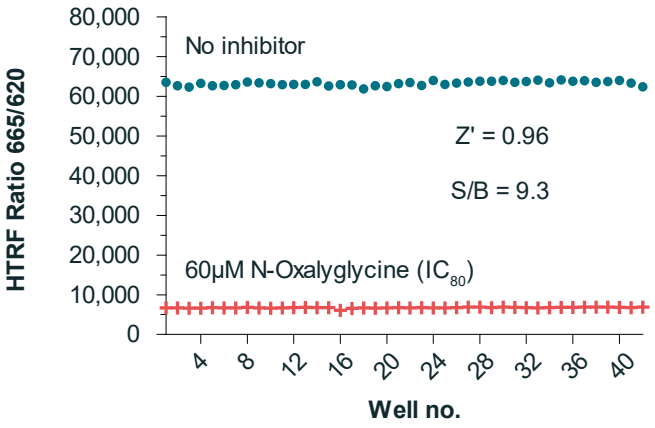
This step allows the determination of apparent Km for Fe(II). The Km value was determined with 10ng/well JARID1C, 100 nM biotinylated H3K4(1-21)me3 substrate and 1 mM 2-OG in the enzymatic step. We recommend testing Fe(II) concentrations ranging from 100 µM to 0.1 µM (serial dilutions). A negative control is performed by removing the Fe(II) 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, 30 min). The 3 µM Km value for Fe(II) was determined from this experiment using a Michaelis-Menten plot.

5. ENZYME INHIBITION



JARID1C H3K4 demethylation inhibitor assay was validated by measuring activity of known inhibitor. This assay was performed using 2µM 2-OG, 50 µM Fe(II) and 10 ng/well JARID1C. Serial dilutions of inhibitors were pre-incubated for 5 min with JARID1C. Enzymatic reaction was initiated by the addition of 100 nM biotinylated H3 (1-21) K4me3 peptide substrate plus 2 µM 2-OG. The enzyme reaction was stopped with the detection reagents after 30 min incubation at RT. IC50 values were calculated from inhibition curves.

6. Z' FACTOR DETERMINATION



The robustness of the assay was proven by performing a Z' determination with N-Oxalyglycine at IC80 (60 µM). The enzyme reaction was carried out with 10 ng/well JARID1C, 2 µM 2-OG, 50 µM Fe(II) and 100 nM H3K4(1-21)me3 substrate for 30 min at RT. Z' of 0.96 shows the robustness of the assay.

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Adachi K, Tokuda C, Chevallier F, Roux T, Gonzalez-Moya A, Degorce F. - Discovery on Target 2012, Boston, MA, USA.

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Chevallier F, Jean A, Raynaldy D, Romier M, Servent F, Tokuda C, Adachi K. - Miptec 2011, Basel, Switzerland.

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