



6042 Cornerstone Court West, Suite B
San Diego, CA 92121
Tel: 1.858.829.3082
Fax: 1.858.481.8694
Email: info@bpsbioscience.com

Assay Report

Histone Demethylases (HDM) Inhibitor Assays Enzymatic Study of Compounds from Client



6042 Cornerstone Court West, Suite B
San Diego, CA 92121
Tel: 1.858.829.3082
Fax: 1.858.481.8694
Email: info@bpsbioscience.com

Client_HDM_Year Month Day

HDM Inhibitor Assays

Study Sponsor: Client

Attention:

Address:

Study Director: Henry Zhu, Ph.D.

Testing Facility: BPS Bioscience Inc.
6042 Cornerstone Court West, Ste. B
San Diego, CA 92121
USA

Study Period:

Report Version: 1

Report Date: Month Day, Year



6042 Cornerstone Court West, Suite B
San Diego, CA 92121
Tel: 1.858.829.3082
Fax: 1.858.481.8694
Email: info@bpsbioscience.com

<h2>Study Director</h2>

Scientist

Date

Henry Zhu, Ph.D.
President

Date



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San Diego, CA 92121
Tel: 1.858.829.3082
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Email: info@bpsbioscience.com

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1. Purpose of the Study

The purpose of the study is to determine the effects of compounds from client on the enzymatic activities of recombinant human JMJD2A, JMJD2B, JMJD2C, JMJD2D, JMJD2E, JMJD3, JMJD1A, LSD1, Jarid1A, Jarid1B, Jarid1C, JHDM1D, JMJD1B, FBXL10, FBXL11, PHF8, and UTX using an in vitro enzymatic assay.



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2. Materials and Methods

2.1 Materials

GSK-LSD1 is purchased from Sigma-Aldrich (St. Louis, MO, Catalog number SML1072).

2,4-Pyridine dicarboxylic acid is purchased from Acros Organics (New Jersey, Catalog number 101860010).

JIB-04 is purchased from Sigma-Aldrich (St. Louis, MO, Catalog number SML0808).

GSK J1 is purchased from Tocris (Bristol, United Kingdom, MO, Catalog number 4593).

AlphaLISA anti-mIgG acceptor beads from PerkinElmer (Santa Clara, CA, Catalog number AL105C).

AlphaLISA anti-rIgG acceptor beads from PerkinElmer (Santa Clara, CA, Catalog number AL104C).

AlphaScreen Streptavidin-conjugated donor beads from PerkinElmer (Santa Clara, CA, Catalog number 6760002).

Primary antibody 2 from BPS (Catalog number 52140B1).

Primary antibody 5 from BPS (Catalog number 52140E).

Primary antibody 6 from BPS (Catalog number 52140F).

Primary antibody 10 from BPS (Catalog number 52140J).

Primary antibody 16 from BPS (Catalog number 52140P).

Primary antibody 17-3 from BPS (Catalog number 52140Q3).

Biotinylated histone H3 peptide substrate (JMJD2's) (BPS).

Biotinylated histone H3 peptide substrate (Jarid's) (BPS).

Biotinylated histone H3 peptide substrate (LSD1) (BPS).

Biotinylated histone H3 peptide substrate (JMJD3 and UTX) (BPS).

Biotinylated histone H3 peptide substrate (JMJD1A, JMJD1B, and JHDM1D) (BPS).

Biotinylated histone H3 peptide substrate (FBXL10 and FBXL11) (BPS).

Biotinylated histone H3 peptide substrate (PHF8) (BPS).

JMJD2A Assay Buffer from BPS.

JMJD2B Assay Buffer from BPS.

JMJD2C Assay Buffer from BPS.

JMJD2D Assay Buffer from BPS.

JMJD2E Assay Buffer from BPS.

JMJD3 Assay Buffer from BPS.

JMJD1A Assay Buffer from BPS.

JMJD1B Assay Buffer from BPS.

LSD1 Assay Buffer 2 from BPS.

Jarid1A Assay Buffer from BPS.

Jarid1B Assay Buffer from BPS.

Jarid1C Assay Buffer from BPS.



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JHDM1D Assay Buffer from BPS.
 FBXL10 Assay Buffer from BPS.
 FBXL11 Assay Buffer from BPS.
 UTX Assay Buffer from BPS.
 PHF8 Assay Buffer from BPS.
 4x Detection buffer from BPS (Catalog number 52301).

2.2 Compounds

The test compounds are supplied by Client.

Compound I.D.	Compound Supplied	Stock Concentration	Dissolving Solvent	Test Range (µM)
GSK-J1* (Control Jarid's, JMJD3, UTX)	Powder	10mM	DMSO	10 - 0.0003
GSK-LSD1* (Control LSD1)	Powder	35mM	H ₂ O	10 - 0.0003
JIB-04* (Control JMJD1A, JMJD1B, PHF8)	Powder	10mM	DMSO	100 – 0.003
2,4-pyridine dicarboxylic acid* (Control JMJD2's, FBXL10, FBXL11, and JHDM1D)	Powder	10mM	DMSO	100 – 0.003

*Reference compounds.

2.3 Experimental Conditions

2.3.1 Enzymes and Substrates

Assay	Catalog #	Enzyme Lot #	Enzyme Used (ng) / Reaction	Substrate
JMJD2A	50123	141216	100	Biotinylated histone H3 peptide
JMJD2B	50111	120810-G	100	Biotinylated histone H3 peptide
JMJD2C	50105	121107	100	Biotinylated histone H3 peptide
JMJD2D	50117	140711-G1	100	Biotinylated histone H3 peptide
JMJD2E	50118	120730	100	Biotinylated histone H3 peptide
JMJD3	50115	120827	18	Biotinylated histone H3 peptide



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JMJD1A	50130	160329	40	Biotinylated histone H3 peptide
LSD1	50103	130806-D	50	Biotinylated histone H3 peptide
Jarid1A	50110	141009	100	Biotinylated histone H3 peptide
Jarid1B	50121	150817-3	100	Biotinylated histone H3 peptide
Jarid1C	50112	140709	50	Biotinylated histone H3 peptide
JHDM1D	50419	140925-1	32	Biotinylated histone H3 peptide
JMJD1B	50421	150122	300	Biotinylated histone H3 peptide
UTX	50116	140731-1	30	Biotinylated histone H3 peptide
FBXL10	50120	150728A	30	Biotinylated histone H3 peptide
FBXL11	50156	140813C	30	Biotinylated histone H3 peptide
PHF8	50131	150120-1	65	Biotinylated histone H3 peptide

2.3.2 Assay Conditions

All of the enzymatic reactions were conducted in duplicate at room temperature for 60 minutes in a 10 µl mixture containing assay buffer, histone H3 peptide substrate, demethylase enzyme, and the test compound (Section 2.2). These 10 µl reactions were carried out in wells of 384-well Optiplate (PerkinElmer). The serial dilution of the compounds was first performed in 100% DMSO with the highest concentration at 1mM. Each intermediate compound dilution (in 100% DMSO) will then get directly diluted 30x fold into assay buffer for 3.3x conc (DMSO). Enzyme only and blank only wells have a final DMSO concentration of 1%. From this intermediate step, 3 µl of compound is added to 4 µl of demethylase enzyme dilution is incubated for 30 minutes at room temperature. After this incubation, 3 µl of peptide substrate is added. The final DMSO concentration is 1%.

After enzymatic reactions, 5 µl of anti-Mouse Acceptor beads (PerkinElmer, diluted 1:500 with 1x detection buffer) or 5 µl of anti-Rabbit Acceptor beads (PerkinElmer, diluted 1:500 with 1x detection buffer) and 5 µl of Primary antibody (BPS, diluted 1:200 with 1x detection buffer) were added to the reaction mix. After brief shaking, plate was incubated for 30 minutes.

Finally, 10 µl of AlphaScreen Streptavidin-conjugated donor beads (Perkin, diluted 1:125 with 1x detection buffer) were added.

In 30 minutes, the samples were measured in AlphaScreen microplate reader (EnSpire Alpha 2390 Multilabel Reader, PerkinElmer).



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2.3.3 Data Analysis

Enzyme activity assays were performed in duplicates at each concentration. The A-screen intensity data were analyzed and compared. In the absence of the compound, the intensity in each data set was defined as 100% activity. In the absence of enzyme, the intensity in each data set was defined as 0% activity.

The values of % activity versus a series of compound concentrations were then plotted using non-linear regression analysis of Sigmoidal dose-response curve generated with the equation $Y=B+(T-B)/1+10^{((\text{LogEC}_{50}-X)\times\text{Hill Slope})}$, where Y=percent activity, B=minimum percent activity, T=maximum percent activity, X= logarithm of compound and Hill Slope=slope factor or Hill coefficient. The IC₅₀ value was determined by the concentration causing a half-maximal percent activity.

3. Assay Results

3.1. Summary of the Inhibitory Effects of the Compounds on Individual HDM Activities

The IC₅₀ of the compounds is summarized in Table 3.1. If the IC₅₀ is higher than XμM, the percentage inhibition of the compound at XμM is calculated.

Table 3.1 Inhibitory Effects of the Compounds on HDM Activities (IC₅₀)

HDMs	IC ₅₀ (μM) or Percentage Inhibition			
	2,4-Pyridine Dicarboxylic Acid	GSK-J1	GSK-LSD1	JIB-04
JMJD2A	0.21			
JMJD2B	0.22			
JMJD2C	0.17			
JMJD2D	0.20			
JMJD2E	0.38			
JMJD3		0.020		
UTX		0.036		
JMJD1A				1.8
JMJD1B				3.0
JHDM1D				0.53
PHF8				1.8



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LSD1			0.012	
Jarid1A		0.23		
Jarid1B		0.021		
Jarid1C		0.024		
FBXL10	1.8			
FBXL11	1.1			

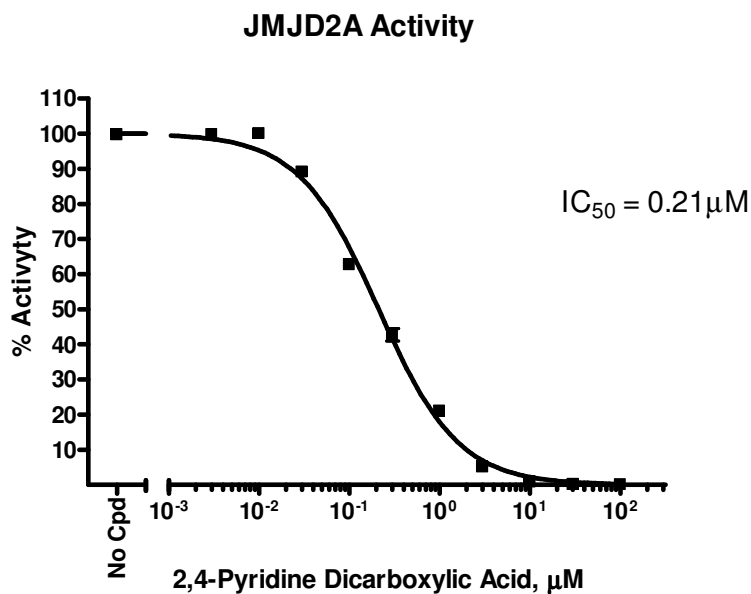
3.2. Results of the Effects of the Compounds on Individual HDM Activity

3.2.1. JMJD2A

3.2.1.1. 2,4-Pyridine Dicarboxylic Acid

Table 3.2.1.1. Data for the Effect of 2,4-Pyridine Dicarboxylic Acid on JMJD2A Activity

2,4-Pyridine Dicarboxylic Acid [μ M]	JMJD2A Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	96595	97093	99	100
0.003	95699	97966	99	101
0.01	96576	97713	99	101
0.03	87245	85965	90	88
0.1	62395	59905	64	61
0.3	43696	40248	45	41
1	21248	20608	21	21
3	5878	5463	5	5
10	1563	1597	1	1
30	836	843	0	0
100	800	764	0	0
Background	722	783	0	0

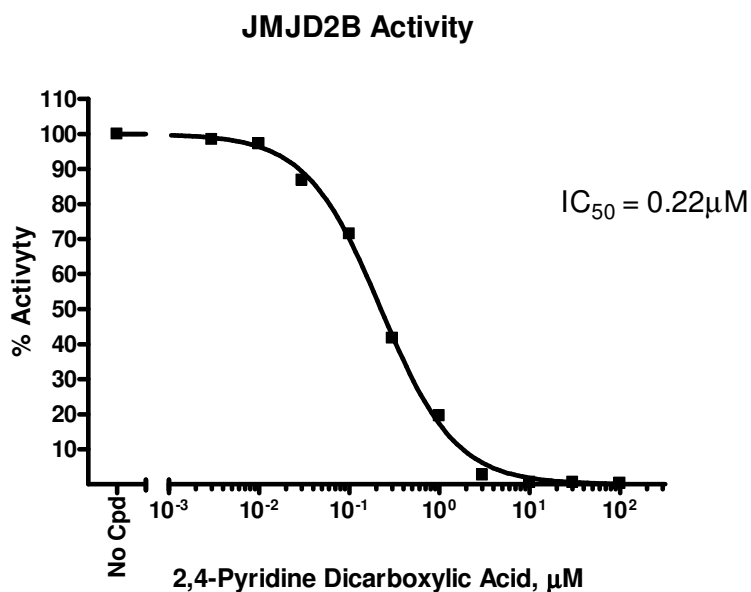


3.2.2. JMJD2B

3.2.2.1. 2,4-Pyridine Dicarboxylic Acid

Table 3.2.2.1. Data for the Effect of 2,4-Pyridine Dicarboxylic Acid on JMJD2B Activity

2,4-Pyridine Dicaboxylic Acid [μ M]	JMJD2B Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	108711	108359	100	100
0.003	106041	107755	98	99
0.01	106389	104718	98	96
0.03	95645	92970	88	86
0.1	78244	77381	72	71
0.3	44935	46509	41	42
1	21224	22620	19	20
3	3897	3551	3	2
10	1425	1508	1	1
30	1476	1413	1	0
100	1192	1036	0	0
Background	861	889	0	0

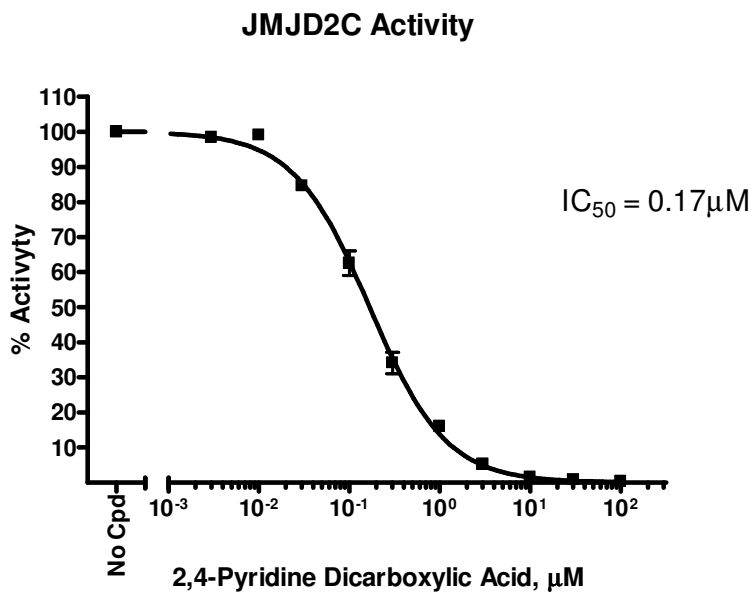


3.2.3. JMJD2C

3.2.3.1. 2,4-Pyridine Dicarboxylic Acid

Table 3.2.3.1. Data for the Effect of 2,4-Pyridine Dicarboxylic Acid on JMJD2C Activity

2,4-Pyridine Dicaboxylic Acid [μ M]	JMJD2C Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	70854	71928	99	101
0.003	70259	70363	98	99
0.01	70305	71262	98	100
0.03	59514	61659	83	86
0.1	47602	42709	66	59
0.3	27323	23046	37	31
1	12573	12472	16	16
3	5381	4557	6	5
10	2367	2334	2	2
30	1731	1957	1	1
100	1547	1434	0	0
Background	1311	1223	0	0

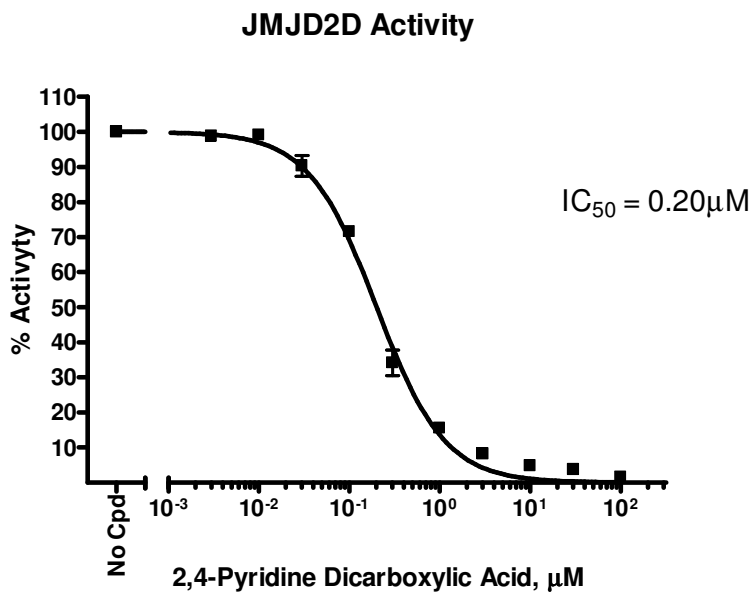


3.2.4. JMJD2D

3.2.4.1. 2,4-Pyridine Dicarboxylic Acid

Table 3.2.4.1. Data for the Effect of 2,4-Pyridine Dicarboxylic Acid on JMJD2D Activity

2,4-Pyridine Dicaboxylic Acid [μ M]	JMJD2D Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	9725	9775	100	100
0.003	9583	9727	98	100
0.01	9811	9545	101	98
0.03	8700	9191	87	93
0.1	7381	7404	71	72
0.3	4598	3990	38	30
1	2837	2661	17	14
3	2163	2128	8	8
10	1960	1764	6	4
30	1731	1812	3	4
100	1595	1588	2	1
Background	1449	1484	0	0

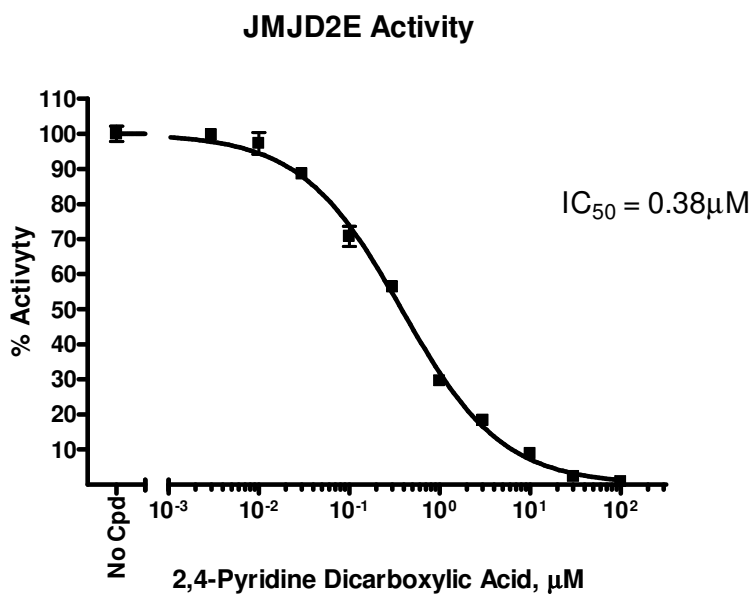


3.2.5. JMJD2E

3.2.5.1. 2,4-Pyridine Dicarboxylic Acid

Table 3.2.5.1. Data for the Effect of 2,4-Pyridine Dicarboxylic Acid on JMJD2E Activity

2,4-Pyridine Dicaboxylic Acid [μ M]	JMJD2E Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	16310	15631	102	98
0.003	16187	15658	101	98
0.01	16026	15062	100	94
0.03	14323	14011	90	88
0.1	10928	11828	68	74
0.3	8981	9223	56	57
1	4625	5143	28	31
3	3026	3189	18	19
10	1644	1582	9	9
30	581	588	2	2
100	395	319	1	1
Background	205	243	0	0

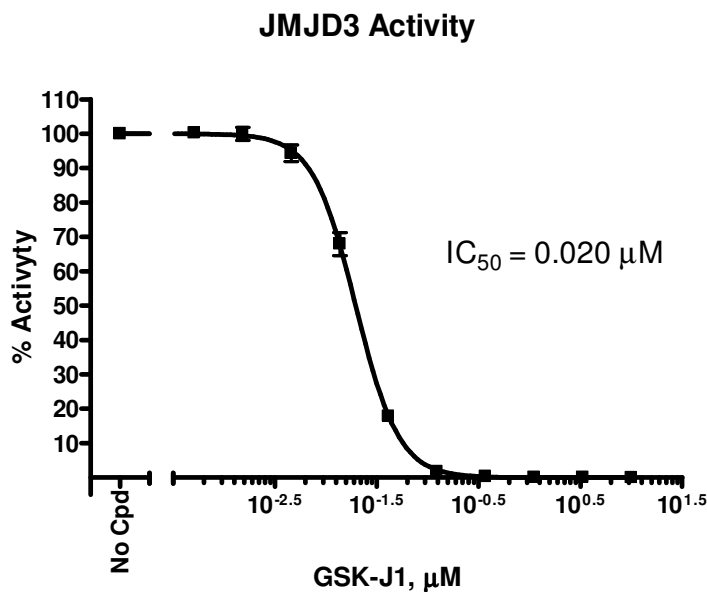


3.2.6. JMJD3

3.2.6.1. GSK-J1

Table 3.2.6.1. Data for the Effect of GSK-J1 on JMJD3 Activity

GSK-J1 [μ M]	JMJD3 Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	158864	162231	99	101
0.0003	160649	161125	100	100
0.001	157428	163426	98	102
0.003	147539	155362	92	97
0.01	114497	103810	71	65
0.03	30144	27828	19	17
0.1	3648	2781	2	2
0.3	698	1066	0	0
1	478	627	0	0
3	462	482	0	0
10	391	332	0	0
Background	297	426	0	0



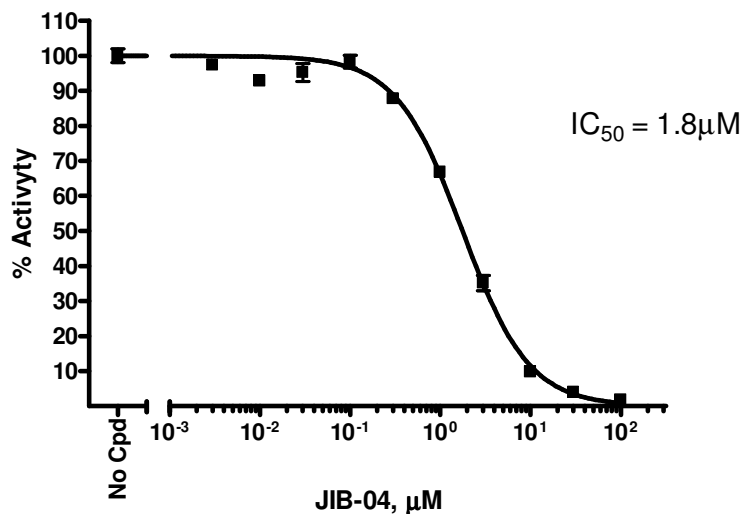
3.2.7. JMJD1A

3.2.7.1. JIB-04

Table 3.2.7.1. Data for the Effect of JIB-04 on JMJD1A Activity

JIB-04 [μ M]	JMJD1A Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	18753	18174	102	98
0.003	17985	18152	97	98
0.01	17382	17387	93	93
0.03	18133	17368	98	93
0.1	17932	18492	96	100
0.3	16818	16453	89	87
1	13501	13488	67	67
3	9106	8448	37	33
10	4776	5190	8	11
30	3943	4256	3	5
100	3824	3745	2	2
Background	3653	3378	1	-1

JMJD1A Activity



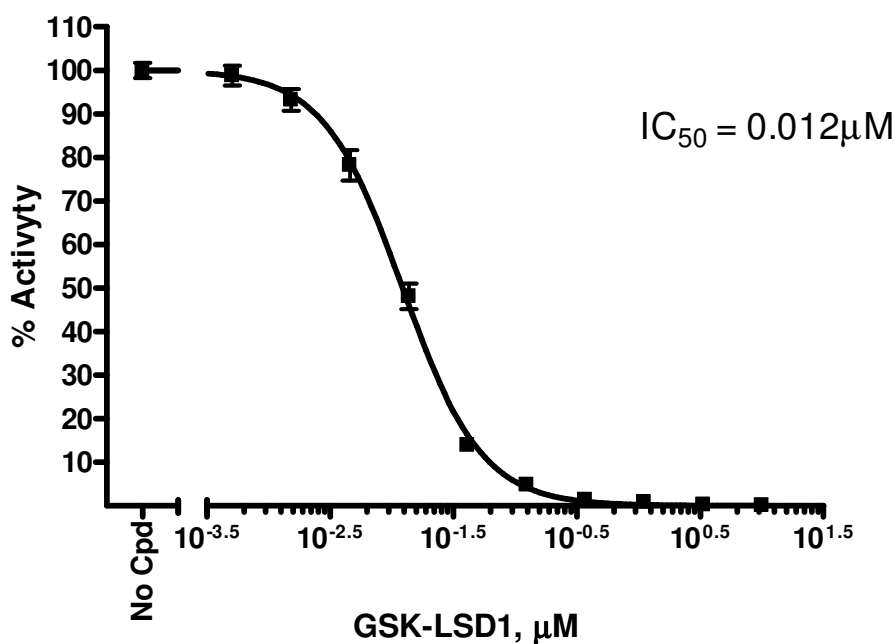
3.2.8. LSD1

3.2.8.1. GSK-LSD1

Table 3.2.8.1. Data for the Effect of GSK-LSD1 on LSD1 Activity

GSK-LSD1 [μ M]	LSD1 Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	29642	28615	102	98
0.0003	28134	29434	97	101
0.001	27892	26447	96	91
0.003	23847	21816	82	75
0.01	14962	13258	51	45
0.03	4213	4227	14	14
0.1	1564	1633	5	5
0.3	567	651	1	2
1	458	444	1	1
3	304	273	0	0
10	259	244	0	0
Background	200	220	0	0

LSD1 Activity

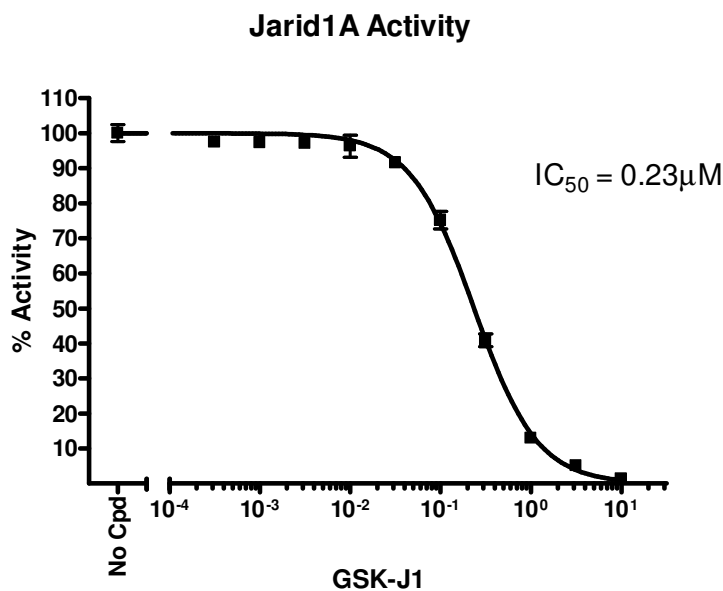


3.2.9. Jarid1A

3.2.9.1. GSK-J1

Table 3.2.9.1. Data for the Effect of GSK-J1 on Jarid1A Activity

GSK-J1 [μ M]	Jarid1A Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	10854	10354	102	98
0.0003	10468	10201	99	96
0.001	10391	10244	98	97
0.003	10130	10469	95	99
0.01	9894	10542	93	99
0.03	9837	9617	93	91
0.1	8283	7765	78	73
0.3	4262	4648	39	43
1	1531	1565	13	13
3	705	762	5	5
10	324	354	1	1
Background	170	230	0	0

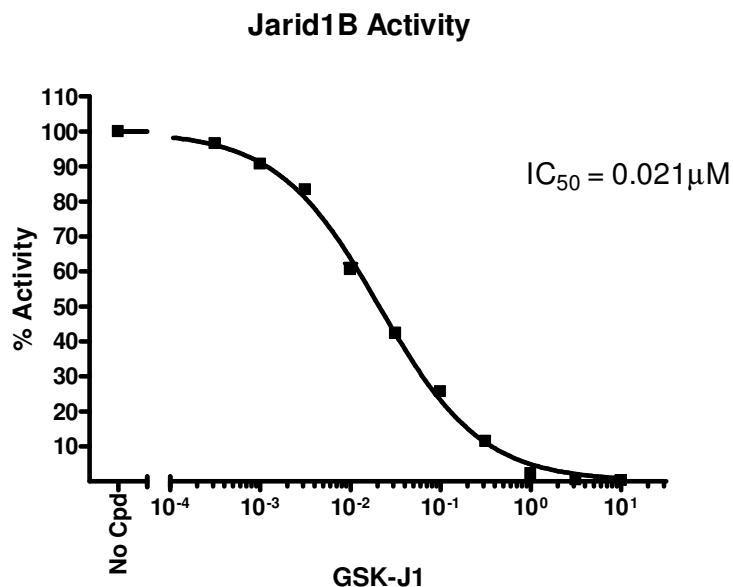


3.2.10. Jarid1B

3.2.10.1. GSK-J1

Table 3.2.10.1. Data for the Effect of the GSK-J1 on Jarid1B Activity

GSK-J1 [μ M]	Jarid1B Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	25870	26743	98	102
0.0003	25413	25403	97	97
0.001	23733	24028	90	91
0.003	21645	22275	82	85
0.01	16463	15574	62	59
0.03	10952	11586	41	44
0.1	6586	7229	24	27
0.3	3018	3365	11	12
1	889	699	3	2
3	286	363	0	1
10	280	263	0	0
Background	196	251	0	0

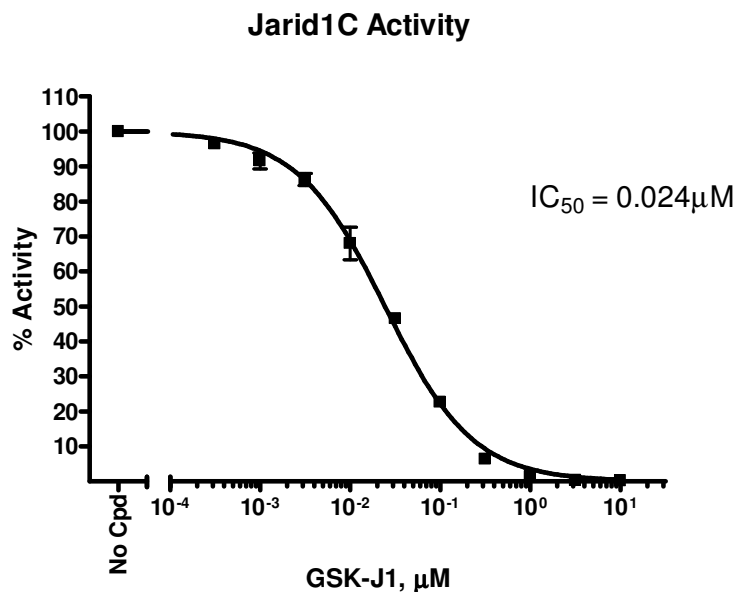


3.2.11. Jarid1C

3.2.11.1. GSK-J1

Table 3.2.11.1. Data for the Effect of GSK-J1 on Jarid1C Activity

GSK-J1 [μ M]	Jarid1C Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	33691	34048	99	101
0.0003	32836	32534	97	96
0.001	31818	30282	94	89
0.003	28661	29827	85	88
0.01	21500	24650	63	73
0.03	16147	15559	47	46
0.1	7744	7823	23	23
0.3	2310	2303	6	6
1	582	595	1	1
3	294	207	0	0
10	236	181	0	0
Background	146	166	0	0

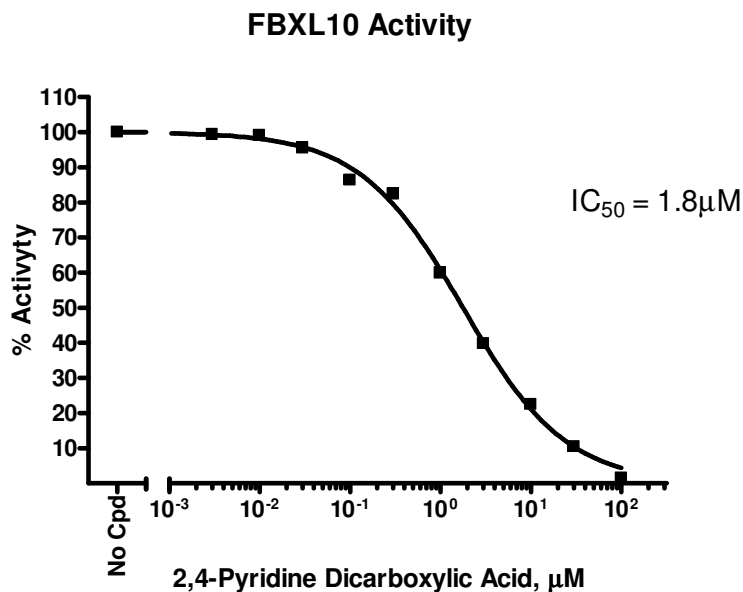


3.2.12. FBXL10

3.2.12.1. 2,4-Pyridine Dicarboxylic Acid

Table 3.2.12.1. Data for the Effect of 2,4-Pyridine Dicarboxylic Acid on FBXL10 Activity

2,4-Pyridine Dicarboxylic Acid [μ M]	FBXL10 Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	241417	243301	100	100
0.003	241990	239654	100	99
0.01	241315	238696	100	98
0.03	232649	230465	96	95
0.1	210661	208460	87	86
0.3	201649	198310	83	82
1	143631	147990	59	61
3	98987	96105	40	39
10	56382	55390	23	22
30	27619	26356	11	10
100	5033	5351	1	1
Background	1832	1761	0	0

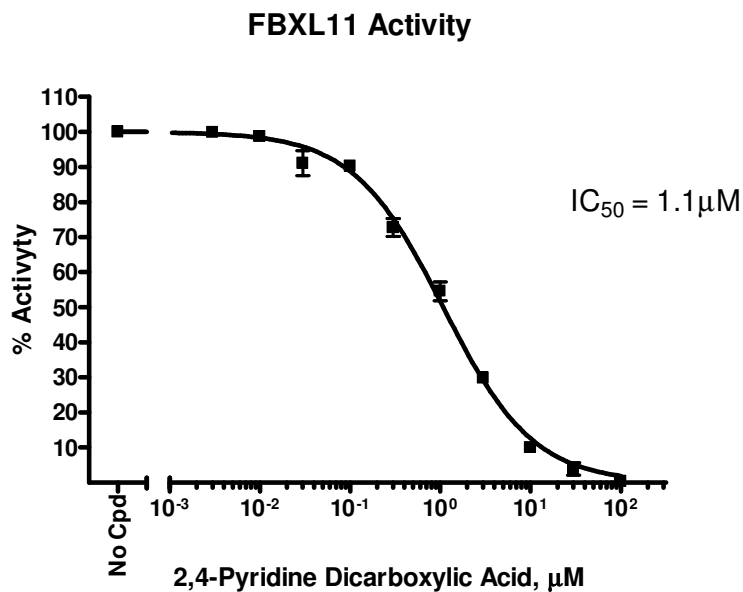


3.2.13. FBXL11

3.2.13.1. 2,4-Pyridine Dicarboxylic Acid

Table 3.2.13.1. Data for the Effect of 2,4-Pyridine Dicarboxylic Acid on FBXL11 Activity

2,4-Pyridine Dicarboxylic Acid [μ M]	FBXL11 Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	113635	112627	100	100
0.003	112108	113833	99	101
0.01	112179	111057	99	98
0.03	99109	107139	87	95
0.1	101611	102761	90	91
0.3	85374	79749	75	70
1	65183	59172	57	52
3	35784	33028	31	29
10	11815	12492	10	10
30	7318	3359	6	2
100	1383	1300	0	0
Background	919	1051	0	0

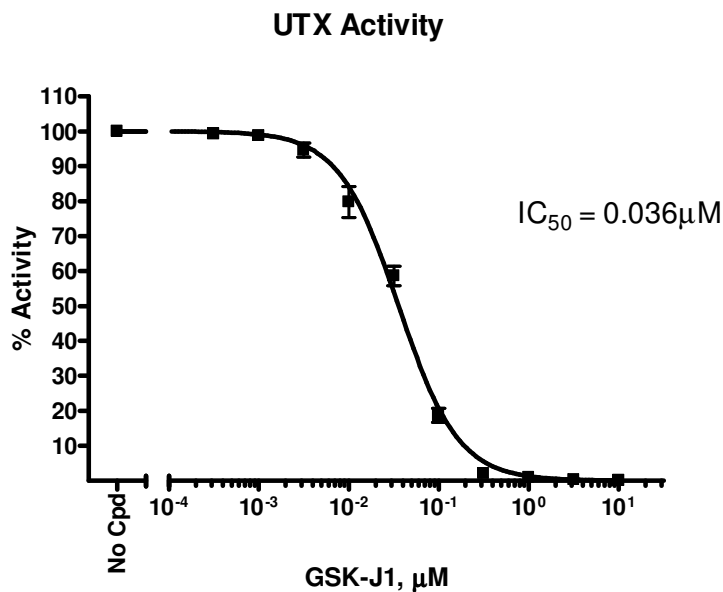


3.2.14. **UTX**

3.2.14.1. **GSK-J1**

Table 3.2.14.1. Data for the Effect of GSK-J1 on UTX Activity

GSK-J1 [μ M]	UTX Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	105489	106446	100	100
0.0003	105291	105033	99	99
0.001	105443	103774	100	98
0.003	102387	98235	97	93
0.01	89370	79961	84	75
0.03	65227	59417	61	56
0.1	22354	18120	21	17
0.3	2752	2564	2	2
1	1377	1611	1	1
3	799	775	0	0
10	615	587	0	0
Background	552	531	0	0

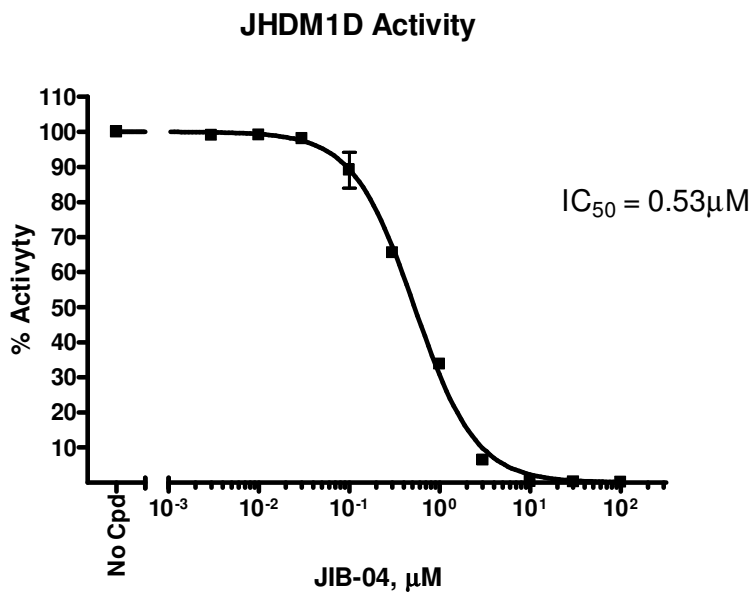


3.2.15. JHDM1D

3.2.15.1. JIB-04

Table 3.2.15.1. Data for the Effect of JIB-04 on JHDM1D Activity

JIB-04 [μ M]	JHDM1D Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	228170	227897	100	100
0.003	225472	226216	99	99
0.01	226772	225373	99	99
0.03	224620	222741	98	98
0.1	215050	192060	94	84
0.3	152635	148643	66	65
1	78481	80086	33	34
3	17327	18220	6	7
10	4119	4114	0	0
30	3626	3933	0	0
100	3248	3983	0	0
Background	3585	3049	0	0

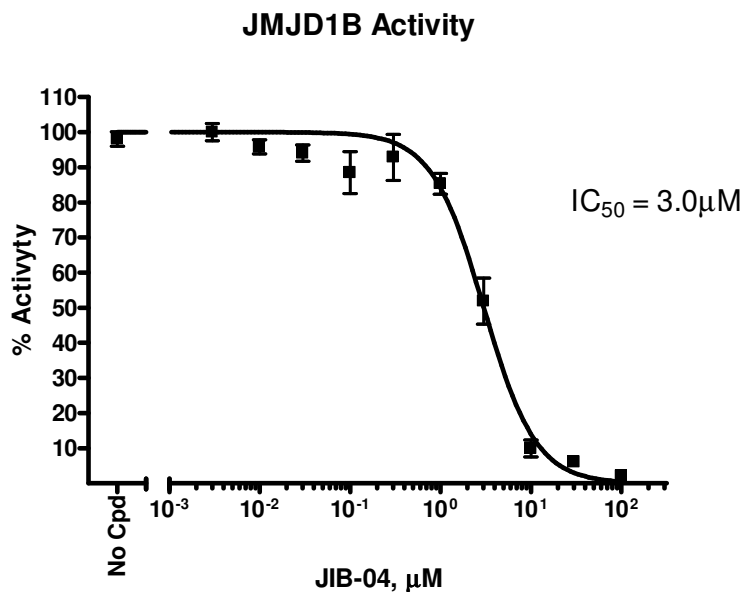


3.2.16. JMJD1B

3.2.16.1. JIB-04

Table 3.2.16.1. Data for the Effect of JIB-04 on JMJD1B Activity

JIB-04 [μ M]	JMJD1B Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	3616	3760	98	102
0.003	3840	3669	104	99
0.01	3540	3678	96	100
0.03	3467	3630	94	98
0.1	3561	3149	96	84
0.3	3735	3278	101	88
1	3347	3142	90	84
3	1859	2311	46	60
10	545	713	8	13
30	541	454	8	5
100	368	344	2	2
Background	199	369	0	0

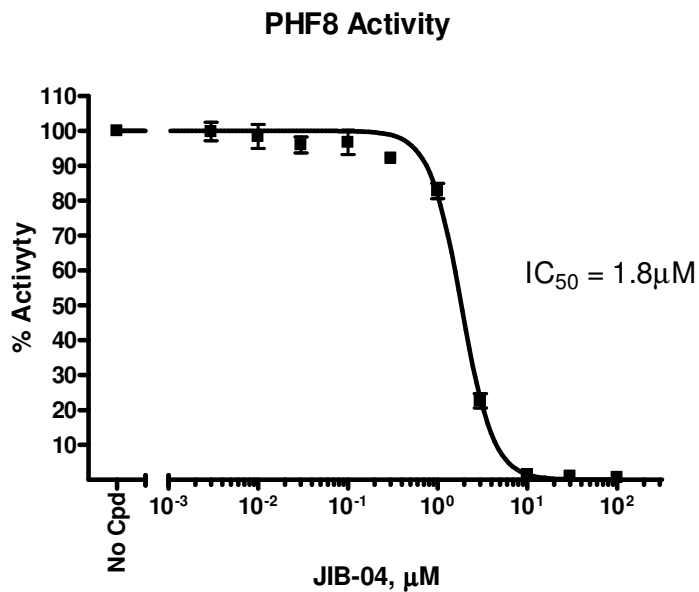


3.2.17. PHF8

3.2.17.1. JIB-04

Table 3.2.17.1. Data for the Effect of JIB-04 on PHF8 Activity

JIB-04 [μ M]	PHF8 Activity (A-counts)		% Activity	
	Repeat1	Repeat2	Repeat1	Repeat2
No Compound	28046	27150	102	98
0.003	28261	26823	102	97
0.01	26241	28079	95	102
0.03	25899	27129	94	98
0.1	25784	27649	93	100
0.3	25138	25847	91	93
1	22387	23572	81	85
3	6346	7446	21	25
10	1171	1228	1	1
30	1115	1135	1	1
100	1101	921	1	0
Background	811	880	0	0





6042 Cornerstone Court West, Suite B
San Diego, CA 92121
Tel: 1.858.829.3082
Fax: 1.858.481.8694
Email: info@bpsbioscience.com

4. Quality Assurance Statement

I certify that the results presented in this report were generated using the materials and methods mentioned and that these results reflect the Raw Data.

Henry Zhu, Ph.D.
President

Date