BromoMELT™

Cat # BRD-Kit-01

For 320 reactions

Instruction Manual

For Research Purposes only

Description

The BromoMELT™ assay kit is designed for profiling compound binding to a collection of human bromodomain proteins (Fig 1). BromoMELT™ is a thermal stability assay (TSA), where heat-induced protein denaturation exposes hydrophobic surfaces that interact with a dye, thereby increasing its fluorescence. A plot of fluorescence versus the gradually increasing temperature is analyzed to obtain a melting temperature (Tm), represented by an inflection point of the curve. Interaction between the protein and a ligand increases protein stability, leading to an increase in Tm.

	2	3	4	5	6	7	8	9	10	11
A	Buffer blank	BRD2-1 (His)	BRD2-2 (His)	BRD2-Tndm (His)	ATAD2 (GST)	ATAD2 (His)	ATAD2B (His)	TAF1-1 (His)	TAF1-2 (GST)	TAF1-2 (His)
B	CECR2 (GST)	BRD3-1 (His)	BRD3-2 (His)	BRD3-Tndm (His)	BRD1 (His)	BRD7 (GST)	BRD9 (His)	TAF1L-1 (GST)	TAF1L-1 (His)	TAF1L-2 (His)
C	BPTF-[PHD-BRD] (His)	BRD4-1 (His)	BRD4-2 (His)	BRD4-Tndm (His)	BRPF1a (His)	BRPF1b (His)	BRPF3 (His)	TAF1L-Tndm	TAF1-Tndm (His)	PHIP-Tndm (GST)
D	BPTF-[PHD-BRD](GST)	BRD4-1 (GST)	BRD4-2 (GST)	BRDT-2 (GST)	BAZ2A (HIS)	BAZ2B (His)	SP100 (GST)	ZMYND8[PHD-BRD]-GST	PB1-1 (GST)	PB1-2 (His)
E	BPTF-[BRD](His)	BRDT-1 (His)	BRDT-2 (His)	BRDT-Tndm (His)	SP100 (His)	SP110c (GST)	SP140 (His)	PB1-3 (His)	PB1-4 (His)	PB1-5 (His)
F	KAT2A (His)	Buffer blank	BAZ1B (His)	BRD8-1 (tagless)	SP140L (His)	TRIM24 (His)	MLL-[PHD-BRD]-his	PB1-6 (GST)	SMARCA2a (GST)	SMARCA2a (HIS)
G	KAT2B (His)	BRD8-1 (his)	BRWD1-2 (His)	BRWD3-2 (GST)	TRIM33a (GST)	TRIM33a (His)	TRIM28 (GST)	SMARCA2b (GST)	SMARCA4 (GST)	SMARCA4 (His)
H	KAT2B (GST)	CREBBP (His)	EP300 (His)	PHIP-2 (His)	TRIM33b (His)	TRIM66 (His)	TRIM28 (His)	BAZ1A (His)	ASH1L-[BRD](GST)	GST alone
	Subfamily 1	Subfamily 2	Subfamily 3		Subfamily 4	Subfamily 5	Subfamily 6	Subfamily 7	Subfamily 8	

Figure 1. BromoMELT components. Concentrations of individual bromodomains vary and have been optimized for each protein to provide sufficient signal in the TSA assay. Each protein is supplied as a 10x stock.

Kit Components

All kit components are shipped on dry ice. Upon receipt, store individual items as shown in the table below. **Avoid freeze thaw cycles for protein components**. The reagents included in the kit are sufficient to perform four assays per protein with a total of 320 main reactions and 6 pre-test reactions.

Component	Quantity	Storage temperature
10x Protein stock plate, 10 µL of each protein	1	-80°C
Protein dilution buffer, 2.5x concentrated solution	1.2 ml	-20°C
Reaction buffer, 5x concentrated solution	1.1 ml	-20°C
5000x SYPRO® Orange	8 µL	-20°C
Control compound mix	5 µL	-20°C
100% DMSO	10 µL	-20°C
384 well qPCR plate	1	RT
clear seal, large	1	RT
Empty 96 well plate for reaction mix dispensing	1	RT
96 well dispensing guide	1	RT
Optional pre-test:		
5x pre-test protein	30 µL	-80°C
clear seal, small	1	RT

Components required but not supplied

Centrifuge with plate-compatible baskets

Real time PCR/qPCR instrument

Multichannel micropipettor (to dispense 12 µL aliquots)

400x Stock(s) of Test Compound in DMSO

Pre-test protocol (optional):

If you do not perform TSA assays routinely, perform a pre-test run to ensure that the instrument is set up correctly and acceptable melt curves are obtained.

1. Thaw pre-test protein on ice. Once thawed, hand-mix gently and briefly spin the tube in a centrifuge to collect the contents on the bottom.
2. Prepare 200 µL of 1x reaction buffer.
3. Prepare 50 µL of 50x solution of SYPRO® Orange (Life Technologies) from 5000x stock in 1x reaction buffer.
4. Pipette 3 µL of 5x pre-test protein solution into 6 wells in columns 1-2 (A) **or** 23-24 (B) as show below.

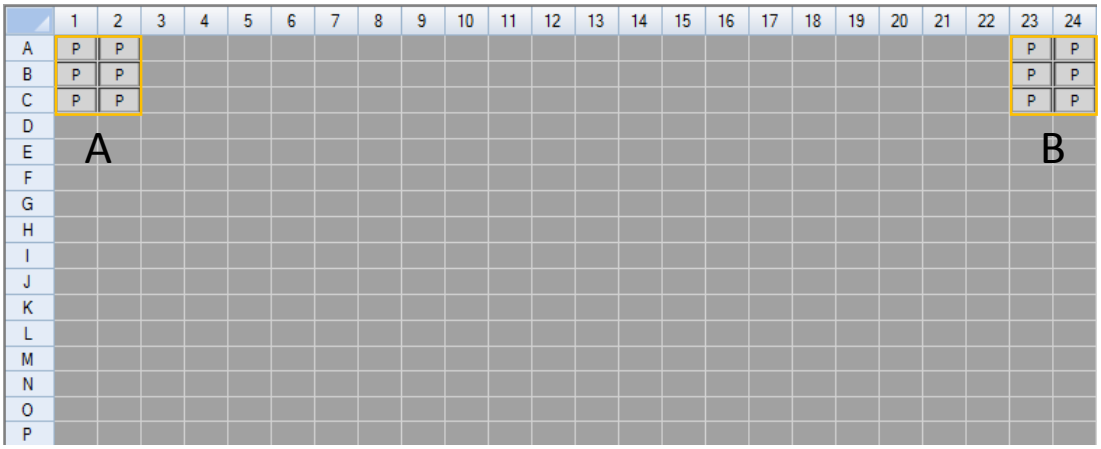


Figure 2. Pre-test plate set-up

NOTE: The plate can be heated several times without affecting performance.

5. Mix reaction buffer with 50x SYPRO® Orange stock and pipette 12 µL into wells containing the pre-test protein.

Volume in µL

Component	Mix/well	Mix/8 wells
1x Buffer	10.5	94.5
50x SYPRO® Orange	1.5	13.5
Total:	12	108

6. Seal reaction wells using a small seal (provided) and run the TSA assay protocol.

Suggested qPCR protocol:

Temperature Settings:

Hold at 25°C for 10 sec

Melt: Increase temperature from 25°C to 80°C in 0.5°C increments, holding each temperature for 30 sec

Fluorescence Readings:

Select appropriate channel according to the instrument being used:

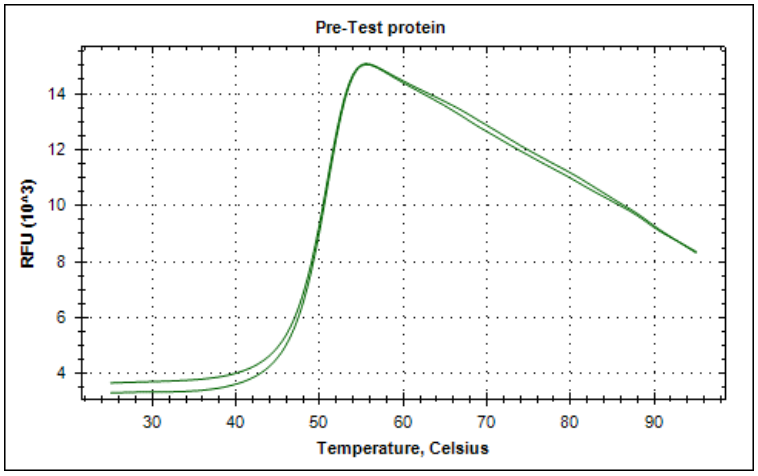
Excitation wavelength range: 450-490 nm

Emission wavelength range: 560-580 nm

Read fluorescence after each 0.5°C step

Expected result:

The figure below shows the expected melt curve for the pre-test protein. The absolute values for Relative Fluorescence Units (RFU) will likely vary depending on the instrument used, but the signal is expected to be several fold above the background with T_m of 51°C.



Main Reaction Protocol

1. Thaw protein stock plate on ice.
2. Prepare 2-3 ml of 1x protein dilution buffer by mixing 2.5x stock with dH_2O.
3. Prepare 5x protein solutions: Dispense 10 µL of 1x protein dilution buffer to each well in columns 2-11 of protein stock plate. Mix gently by pipetting.
4. Spin the plate for 1 min at 1000 rpm and, if not using immediately, return to ice.
5. Dispense 3 µL of each bromodomain into four adjacent wells of 384 well RT-PCR plate as shown below. Spin the plate for 1 min and place on ice.

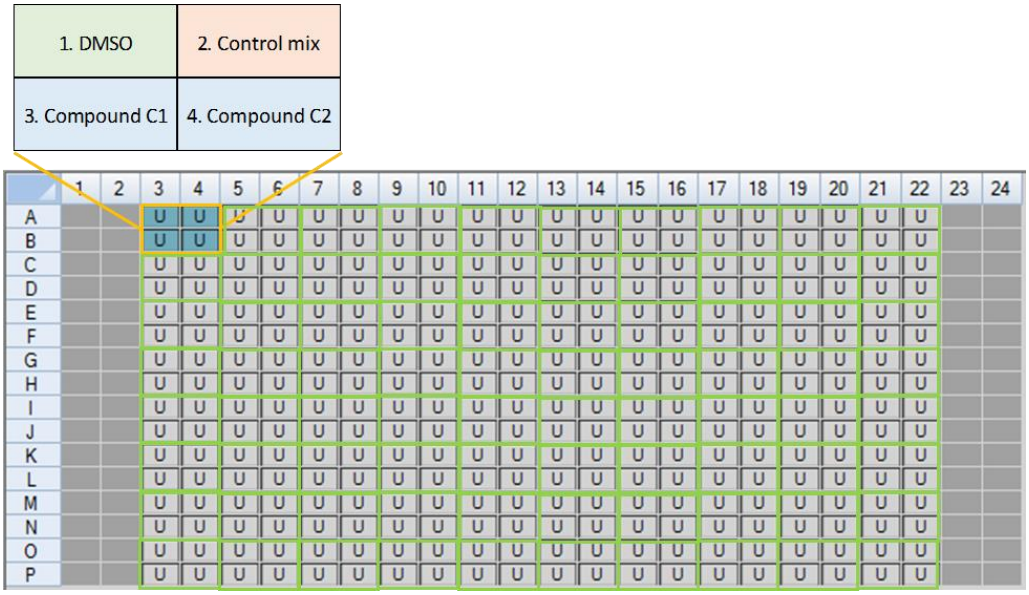


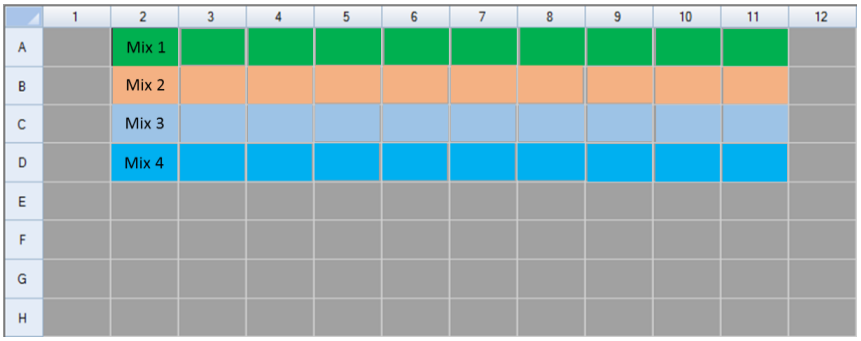
Figure 3: Main reaction plate set-up.

6. Prepare four tubes that will contain reaction buffer and SYPRO® Orange plus one of the following: a) DMSO, b) control compound mix c) 400x stock of test compound in DMSO for final concentration #1 (C1), d) 400x stock of test compound in DMSO for final concentration #2 (C2). You can either run 2 concentrations of your compound (10 and 25 µM for example) or a single concentration in duplicate (C1 = C2).
7. For each of the four tubes, combine 240 µL of 5x reaction buffer, 955 µL of deionized water, 1.5 µL 5000x SYPRO® Orange and 3.75 µL of DMSO or compound(s). Ensure that your compound stock concentration is at least 10 mM and that equivalent volumes of DMSO, Control mix and test compound in DMSO are added to reaction mixes. DMSO is known to weakly bind and stabilize bromodomains, so its effects will later need to be subtracted out when calculating a ΔTm value.

Volume in µL

Component	Mix 1	Mix 2	Mix 3	Mix 4
5x Buffer	240	240	240	240
5000x SYPRO® Orange	1.5	1.5	1.5	1.5
DMSO	3.75	x	x	x
Control Mix	x	3.75	x	x
400x C1 Test Compound	x	x	3.75	x
400x C2 Test Compound	x	x	x	3.75
water	955	955	955	955
Total	1200	1200	1200	1200

8. Dispense 118 µL of each mix into 10 wells of a single row of the empty 96 well plate (supplied).



9. Optional: If desired, use dispensing guide to isolate destination wells for each mix by fixing it in place using lab tape. To dispense Mix 1, align top left well of the guide with well A1 of the qPCR plate. Visible wells in columns 2-11 are destination wells for Mix 1 (Fig 4 A). For Mix 2, align top left well of guide with A2; Mix 3 – B1 and Mix 4 - B2

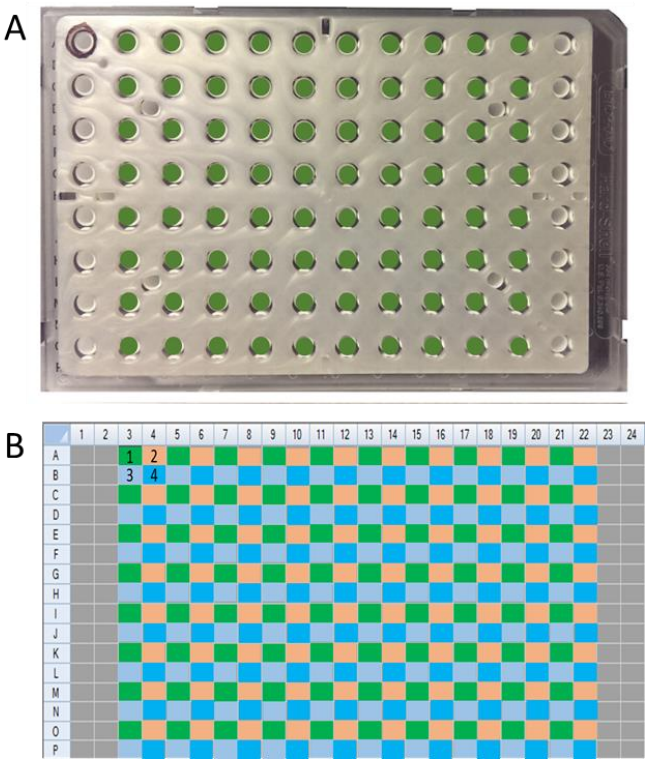


Figure 4. Destination wells for Mix 1 are isolated on the reaction plate by using the dispensing guide and are shown in green in (A). Destination wells for all four mixes are color coded in (B)

10. Dispense 12 µL of each reaction mix to the destination wells. Mixing is optional.
11. Repeat for all four reaction mixes.
12. Spin the plate for 1 minute at 1000 rpm
13. Seal the plate using provided clear seal. Take care not to touch the surface of the seal.
14. Run the TSA protocol.

Suggested qPCR protocol:

Temperature Settings:

Hold at 25°C for 10 sec

Melt: Increase temperature from 25°C to 80°C in 0.5°C increments, holding each temperature for 30 sec

Fluorescence Readings:

Select appropriate channel according to the instrument being used:

Excitation wavelength range: 450-490 nm

Emission wavelength range: 560-580 nm

Read fluorescence after each 0.5°C step

Suggested Data Analysis.

Determining Tm's

Using your instrument's software, extract the inflection point of each melt curve. Each of these will represent the protein's melting temperature (Tm) under a particular assay condition (e.g. only DMSO present or test compound (added in DMSO) at concentration #1 (C1) etc.).

Alternatively, Tm's may be determined from the raw fluorescence vs. temperature data, by means of a straightforward spreadsheet analysis. (See Fig. 5).

1. Using spreadsheet software, paste your data as shown in Figure 5A (Column A – temperature; Column B – RFU)
2. Starting in cell C6, enter the formula [=SLOPE(B2:B6,A2:A6)]. Copy to the end of the dataset.
3. Starting in cell D6, enter the formula [=Average(A2:A6)]. Copy to the end of the dataset.
4. The highest value of the slope in column C will correspond to Tm.
5. Alternatively, a plot of average T vs Slope will show the Tm as a highest point of the curve.

A

	A	B	C	D
1	Temperature	RFU	SLOPE	Average T
2	25.00	4894.61		
3	25.50	4838.87		
4	26.00	4783.13		
5	26.50	4727.39		
6	27.00	4671.65	-111.47844	26.00
7	27.50	4615.91	-111.47844	26.50
8	28.00	4560.17	-111.47844	27.00
9	28.50	4501.45	-112.67141	27.50
10	29.00	4442.72	-114.46328	28.00
11	...	...	...	...
...	...	...	...	...

B

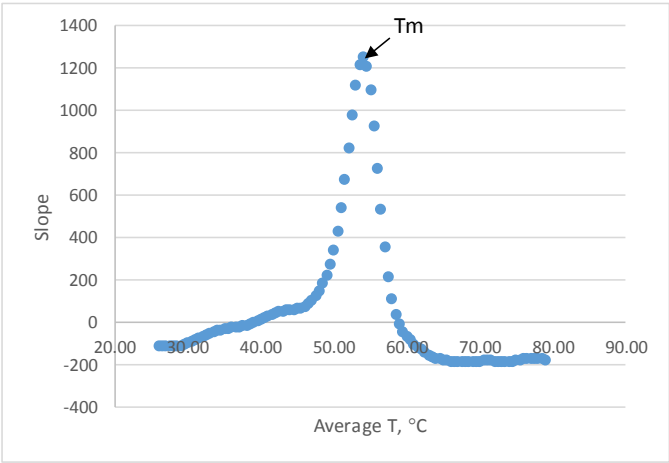


Figure 5. Manual Tm calculation set up (A). Representative plot of Average T vs Slope (B) showing Tm as the highest point of the curve.

Determining ΔTm's

DMSO itself is a weak bromodomain ligand. Since the control mix and test compounds are added as solutions in DMSO, a true stabilizing effect due to compound binding is reflected by a Tm increase over and above the Tm in the presence of DMSO alone. Therefore, for each protein, subtract Tm (DMSO) from the Tm's of each of the other three reactions (control inhibitor mix, C1 and C2). The results will represent the ΔTm for the control mix and for the two concentrations of your compound (C1 & C2). An increase in protein melting temperature (positive ΔTm) correlates with the binding of compound to the protein and with the increased stabilization against heat denaturation conferred by that binding.

Note: The control mix will not produce a significant positive ΔTm for some bromodomains. The mix is designed to target as many bromodomains as possible. However, for some bromodomains, potent ligands have not been identified yet.