MANUAL

Technology: HTRF® Epigenetics

HTRF EPIgeneous™ binding domain kit A

Part number	62BDAPEG	62BDAPEH
Test size	500 tests	10,000 tests

Storage: ≤-16°C

Version: 03 **Date:** January 2024

ASSAY PRINCIPLE

The EPIgeneous™ Binding Domain Kit A is designed to measure the interaction between binding domain protein and modified lysine residues of the N- terminal tails of histones. This enables rapid characterization of the reaction inhibitors in a high throughput format.

Two packaging sizes are available.

As shown below, the interaction between a GST-tagged reader and a biotinylated peptide containing target acetylated or methylated lysine is detected using an anti-GST antibody coupled to Cryptate (donor) and a red acceptor-labeled streptavidin (SA) that binds with high affinity to the peptide substrate via the biotin moiety (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). The specific signal modulates positively in proportion to the protein/peptide interaction.

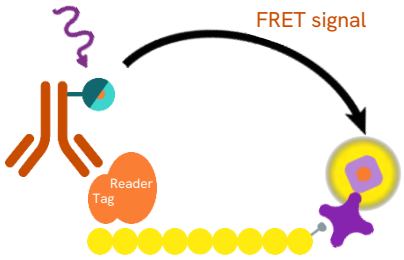
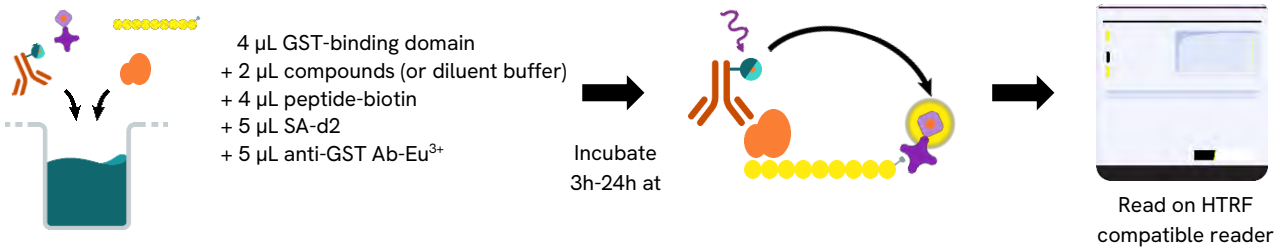


Figure 1: Principle of HTRF EPIgeneous™ binding domain kit A

EPIgeneous™ Binding Domain Kit A has already been validated on BRD2(1) - BRD3(1) - BRD4(1) - CBX1

ASSAY PROTOCOL FOR 384W-LOW VOLUME PLATE (20 µL)



Set up your reader for Eu^{3+}-Cryptate and read the fluorescence emission at two different wavelengths (665nm and 620nm) on a compatible HTRF® reader.

For HTRF certified reader

For more information about HTRF® compatible readers and for set-up recommendations, please visit our website.

Purchase separately

96-well or 384-well small volume (SV) detection microplates - For more information about microplate recommendations, please visit our website.

	PEPTIDE TITRATION		TEST OF INHIBITORS		
	Positive signal	**Negative control**	**Inhibitor test**	**Positive control**	**Negative control**
Protein, GST-tag	4 µL	-	4 µL	4 µL	
Compound	-	-	2 µL		
Diluent Buffer (supplemented with DMSO)	2 µL	6 µL		2 µL	6 µL
Peptide-Biotin	4 µL		4 µL		
Streptavidin-d2	5 µL		5 µL		
GST-Eu^{3+}-Cryptate antibody	5 µL		5 µL		

Seal the plate and incubate at RT (see table §4 for incubation time).
Results are calculated from the 665nm and 620nm fluorescence signals and expressed in HTRF ratio :

HTRF Ratio = (665nm/620nm) x 104.

KIT DESCRIPTION

KIT COMPONENTS	STORAGE	500 TESTS			10,000 TESTS			STOCK SOLUTION CONCENTRATION
		TUBES DESCRIPTION		VIALS	TUBES DESCRIPTION		VIALS	
Binding Domain Diluent Buffer (Ref #62DLBDDF, 10.000 tests)	2-8°C		white cap	1 vial 20 mL		white cap	1 vial 200 mL	Ready to use
Binding Domain Detection Buffer #1 (Ref #62DB1FDG, 10.000 tests)	2-8°C		red cap	1 vial 20 mL		red cap	1 vial 130 mL	Ready to use
Streptavidin-d2 reagent*	≤-16°C		blue cap	1 vial 150 µL		purple cap	1 vial 1.8 mL	15 µM
GST-Eu^{3+}-Cryptate Antibody	≤-16°C		red cap	1 vial 50 µL		orange cap	1 vial 1 mL	50X

*Amount of reagent provided is sufficient for the associated number of tests using 1µM biotinylated peptide (including 5 peptide titrations for optimization experiment).
Additional reagents needed but not provided: compound, peptide-biotin and GST-Binding domain are not supplied in the kit. Please refer to table in section 4 for recommended products and associated providers.

VALIDATION

Reader	Incubation time before reading	Recommended peptide-biotin concentration (final in the well)	DMSO Tolerance (%)	Recommended provider with associated reference	
				Peptide-biotin	GST-reader domain
BRD4(1)	3h – 24h	0 DMSO – 2 nM 0.1 – 1% DMSO – 5 nM	0 – 2%	[Lys(5,8,12,16)Ac]-H4(1-21)-biotin peptide Anaspec #64989	Reaction Biology Corp. / Revvity #RD-11-157
BRD3(1)	3h – 24h	0 – 1% DMSO – 3 nM	0 – 1%		Reaction Biology Corp. / Revvity #RD-11-163
BRD2(1)	3h – 24h	0 – 1% DMSO – 3 nM	0 – 1%		Reaction Biology Corp. / Revvity #RD-11-156
CBX1	3h – 24h	0 – 1% DMSO – 3 nM	0 – 4%	[Lys(9)Me3] H3(1-21)-biotin peptide Anaspec #64360	BPS Bioscience #55009

REAGENT PREPARATION

Allow the stock solution to thaw at room temperature.

- We recommend centrifuging the vials gently after thawing before pipetting the stock solutions.
- Mix the solution gently by pipetting several times.
- In order to avoid repeated freeze and thaw cycles, stock solution can be divided into aliquots and frozen for additional use.

When the GST-binding domain of interest has already been validated, inhibitor titration experiments can be performed directly using the recommended conditions (kit and peptide-biotin concentration) described in the section §4 table or obtained with the discovery kit. If the assay specifications obtained under these recommended conditions do not meet your requirements, the assay window or sensitivity can be further optimized by performing peptide-biotin titration.

Inhibitor IC_{50} determination

	Buffer used for diluting:
Binding Domain Diluent Buffer	Compound Peptide-Biotin GST-Binding domain
Detection Buffer #1	Streptavidin-d2 reagent GST- Eu^{3+}-Cryptate antibody

Preliminary remarks:

- Compound, peptide-biotin and GST-Binding domain are not supplied in the kit. Please refer to table in section 4 for recommended products and associated providers.
- If the protein studied is not referenced in the table, Discovery kit (#62BDDPEG) can be used in order to select the best binding kit.
- See section §2 for dispensing protocol.

Compound

Prepare supplemented Binding Domain Diluent Buffer with DMSO to get a constant percentage throughout the inhibitor titration.

Dilute the compound in supplemented Binding Domain Diluent Buffer to get a 10X working solution depending on the final concentration in the well.

Remark: DMSO may act as an inhibitor of the interaction between the GST-binding domain and the biotinylated peptide. This can lead to a decrease of the assay window as DMSO % increases. We therefore recommend the use of compatible percentage of DMSO.

Peptide-biotin

We recommend preparing a peptide-biotin stock solution at 350 µM in H_2O, 10%DMSO then aliquote it and store at ≤-16°C.

Prepare the peptide-biotin at optimal concentration in Binding Domain Diluent Buffer to get a 5X working solution depending on the final optimal concentration in the well. If necessary, refer to concentration determined when performing peptide-biotin titration.

Streptavidin-d2 reagent

The peptide-biotin/ streptavidin-acceptor ratio must be equal to 8/1 final in the well (e.g. Peptide-biotin determined at 4 nM final in the well, SA-d2 must be used at 0.5 nM final in the well).

Prepare the SA-d2 solution in Detection Buffer #1 to get a 4X working solution depending on the final optimal concentration in the well.

GST-Eu^{3+} cryptate antibody

Dilute the conjugate 50-fold with Detection Buffer #1 to obtain the working solution ready to be dispensed.

This kit provides sufficient reagent for 500 tests using a 384-well low volume plate in 20 µL final assay volume.

GST-tagged binding domain

We recommend using the GST-tagged binding domain at 5 nM final concentration in the well (20 µL assay volume).

Prepare the working solution at 5X depending on the final concentration in the well in Binding Domain diluent buffer (here 25 nM).

Peptide-biotin titration (OPTIONAL)

In the case of the assay specifications obtained with the recommended conditions do not meet requirements, one can further optimize the assay window or sensitivity by performing peptide-biotin titration.

Remark: DMSO is known to be an inhibitor of some binding domain / histone peptide interactions. Then it can decrease the HTRF signal (hence assay window) and increase apparent Kd of the binding domain / histone peptide-biotin. We therefore recommend performing peptide-biotin titrations with the same percentage of DMSO than used in the inhibitor titration.

The GST-Binding domain concentration is fixed at 5nM final concentration, while the peptide-biotin is serially diluted.

For each peptide-biotin concentration, a negative control is performed by not adding the GST-reader protein to the wells. This negative control is used as non-specific signal to calculate the HTRF® delta ratio (= specific signal). This specific signal is proportional to the specific interaction measured between GST reader and biotin peptide.

The Kd value is determined from this experiment using one or two site specific binding regressions (saturation equations).

Peptide-biotin and SA-d2 reagent preparation

We recommend preparing a peptide-biotin stock solution at 350 µM in H_2O, 10% DMSO, then storing the aliquots at ≤-16°C.

We recommend a range of peptide-biotin concentrations from 2,000 nM to 0.1 nM final assay concentration (3 fold serial dilutions).

During the detection step, the peptide-biotin/streptavidin ratio must be kept constant at 8/1 during the detection step. The following table describes how to adjust the concentration of the SA-d2 reagent for each peptide-biotin concentration.

The background rises with increasing acceptor concentrations. It is then necessary to run a negative control (no adding of GST-tagged protein) for each SA-d2 concentration.

Dilute the Peptide-biotin in Binding Domain diluent buffer to get a 10 µM solution = Standard 10
Dilute the SA d2 reagent in Detection Buffer #1

Prepare the peptide-biotin serial dilutions as follows:

PEPTIDE-BIOTIN SERIAL DILUTION			
#	Final assay concentration in 20 µL	Working solution: 5X	
	nM	nM	preparation
Standard 10	2 000	10 000	150 µL Peptide-biot at 10µM
Standard 9	667	3 333	50 µL Standard 10 + 100 µL diluent buffer
Standard 8	222	1 111	50 µL Standard 9 + 100 µL diluent buffer
Standard 7	74	370	50 µL Standard 8 + 100 µL diluent buffer
Standard 6	25	123	50 µL Standard 7 + 100 µL diluent buffer
Standard 5	8.2	41	50 µL Standard 6 + 100 µL diluent buffer
Standard 4	2.7	14	50 µL Standard 5 + 100 µL diluent buffer
Standard 3	0.9	4.6	50 µL Standard 4 + 100 µL diluent buffer
Standard 2	0.3	1.5	50 µL Standard 3 + 100 µL diluent buffer
Standard 1	0.1	0.5	50 µL Standard 2 + 100 µL diluent buffer
Standard 0	0	0	100 µL diluent buffer

Prepare the SA-d2 serial dilutions as follows:

SA-d2 SERIAL DILUTION			
#	Final assay concentration in 20 µL	Working solution: 5X	
	nM	nM	preparation
Sol 10	250	1,000	
Sol 9	83	333	50 µL Sol 10 + 100 µL detection buffer #1
Sol 8	28	111	50 µL Sol 9 + 100 µL detection buffer #1
Sol 7	9.3	37.0	50 µL Sol 8 + 100 µL detection buffer #1
Sol 6	3.1	12.3	50 µL Sol 7 + 100 µL detection buffer #1
Sol 5	1.0	4.1	50 µL Sol 6 + 100 µL detection buffer #1
Sol 4	0.3	1.4	50 µL Sol 5 + 100 µL detection buffer #1
Sol 3	0.11	0.46	50 µL Sol 4 + 100 µL detection buffer #1
Sol 2	0.038	0.152	50 µL Sol 3 + 100 µL detection buffer #1
Sol 1	0.013	0.051	50 µL Sol 2 + 100 µL detection buffer #1
Sol 0	0	0	100 µL detection buffer #1

Anti-GST-Eu^{3+}-cryptate conjugate & GST-reader domain

In order to prepare the working solution, please refer to section § 5.1.

Note that the higher the peptide-biotin concentration, the higher the inhibitor IC50. The optimal peptide-biotin concentration can be selected by finding a compromise between assay window and assay sensitivity (i.e. select the lower peptide-biotin concentration that gives a robust assay window).

FAQ / TROUBLESHOOTING

Can I use a HIS-tag binding domain protein?	• The EPIgeneous™ Binding Domain Kits are compatible with GST tagged proteins but not HIS tagged proteins. • The comparison between HIS and GST tags has been done on some proteins using these kit conditions or anti 6HIS specific Ab. HIS tag requires a significantly higher amount of protein than GST tag to get a positive and robust signal. Moreover, even with a high amount of protein, HIS tag displays lower assay windows than GST tag. This is why we strongly recommend using GST tag proteins. • If GST tag protein is not available, please contact your technical support team for recommendations on reagent references and protocol using HIS tag.
Can I use an untagged binding domain protein?	• The EPIgeneous™ Binding Domain Kits are compatible with GST tagged proteins only. • If GST tag protein is not available, the anti GST antibody can be replaced by an antibody directly recognizing the binding domain. Please contact your technical support team for recommendations on reagent references and protocol.
Can I use a different binding domain protein than the one listed in the section §4 table?	• The section §4 table lists the providers and references of proteins that have been used for assay optimization. Another source of GST tagged protein can be used. • Note that the quality of the GST tagged protein solution will impact data quality. For example, free GST contamination will trap the antibody and then quench the HTRF signal. Then if the results are not good enough when testing a new protein source, we recommend testing the validated source (section §4 table) as positive control.
Can I use a different buffer than the Diluent or Detection Buffer provided in the kit?	• The buffers provided in the kit have been optimized to give good results. We recommend using these buffers and do not guarantee data quality if these buffers are changed. • Note that the Detection Buffer #1 contains 0.4M KF which is mandatory for europium cryptate usage.
Can I use DMSO% above the range given in the section §4 table?	• The range of DMSO% given enables inhibitor titrations with a robust signal in biological relevant conditions (i.e. avoiding saturation of peptide-biotin). • Therefore we do not recommend using higher DMSO% but if necessary, assay conditions can be optimized with the required DMSO% following the protocol given in section §5.2. As described, increasing the peptide-biotin concentration can help recover assay window while increasing DMSO%. However, note that the higher the peptide-biotin concentration, the lower the sensitivity for inhibitor titration.
There is no positive signal while testing a binding domain protein that is on the validated list.	• From one EPIgeneous binding kit to another, the HTRF donor fluorophore varies (Europium or Terbium). As reader set up also varies depending on the HTRF donor, please check reader set up to ensure that the appropriate one is used. For more information about HTRF® compatible readers and for set-up recommendations, please visit our website at. • Use the recommended plates: 384-well low volume plate (Greiner #784075) with 20µl final volume. • See section §5.2 in order to further optimize the assay window. • The quality of the GST tagged protein solution will impact data quality. For example, free GST contamination will trap the antibody and then quench the HTRF signal. Please refer to the table in section §4 to find the provider and reference of the validated proteins. • The detection step requires a biotin moiety on the peptide to bind the streptavidin-acceptor. Ensure that the peptide is biotinylated. • If DMSO is used then we recommend checking the signal without DMSO to identify if this is a problem of DMSO competition on the binding domain protein / peptide-biotin interaction. If this is the case, then we recommend titrating DMSO to determine the tolerance limit.
The assay window is good but there is a lack of sensitivity while performing inhibitor titrations. How can we improve the assay sensitivity?	• See section §5.2 in order to further optimize the assay sensitivity. • As DMSO is a competitive inhibitor of some binding domain proteins, decreasing the DMSO percentage or replacing it with ethylene glycol or ethanol can help to increase sensitivity.
The signal obtained in inhibitor titration is lower than the one obtained in peptide-biotin titration during optimization step.	• Peptides are sticky reagents and so the concentration can be slightly different between the peptide-biotin titration while serially diluting compared to inhibitor titration while applying a direct dilution. This can therefore lead to a difference in assay window between the two conditions.
The assay window (or signal) varies from one experiment to another. How can I improve reproducibility?	• We recommend reading the plate at the same incubation time for each experiment. • We recommend following exactly the same protocol for reagent preparation and dispensing of each experiment. • Peptide-biotins play an important role in the assay signal. They are sticky reagents and so the final concentration in the well can vary slightly depending on the way they are prepared. We therefore recommend following exactly the same protocol (dilution factors and steps) for each experiment. • The concentration of the streptavidin-acceptor stock solution is quite high which enables flexibility from one binding to another. However it sometimes requires a high dilution factor to obtain the working solution (when peptide-biotin concentration is low). In this case, in order to ensure good day to day reproducibility, we

	recommend making an intermediate solution to avoid pipetting low volumes (< 5 µl). Use exactly the same protocol for each experiment. • Batch to batch stock solutions of peptide-biotin and protein can lead to slight signal variations. We recommend using aliquots of the same stock solutions.
Assay miniaturization	• To move to other plate formats (96 half-well or 1536-well) and final volumes (100 µL to less than 10 µL), the volume of each assay component is simply proportionally adjusted in order to maintain the reagent concentrations as for the 20 µL final assay volume. • Make sure you have a final assay volume which is compatible with the plate used. For more information about recommended volumes for each plate, please visit our website. • Whatever the plate format, we recommend using white plates. • Note that the assay conditions have been optimized using 384-well low volume plates (Greiner #784075) with • 20µl final volume. Moving to other plate formats may require slight optimization regarding the peptide-biotin • concentration (see section §5.2).

REACH European regulations and compliance

This product contains material of biologic origin. Use for research purposes only. Do not use in humans or for diagnostic purposes.
The purchaser assumes all risk and responsibility concerning reception, handling and storage.

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