HUMAN HMGB1 KITS

PROTOCOL

Part # 62HMGPEG & 62HMGPEH

Test size#: 500 tests (62HMGPEG) and 10,000 tests (62HMGPEH) - assay volume: 20 µL

Revision: 06-May 2020

Store at: -60°C or below (62HMGPEG); -60°C or below (62HMGPEH)

For research use only. Not for use in diagnostic procedures.

ASSAY PRINCIPLE

This kit is intended for the simple and rapid quantification of all forms of human HMGB1 in cell/tissue culture supernatants and offers a fast alternative to ELISA. The assay is compatible with human, mouse (rat to be checked) but not porcine. The bovine form is not detected, therefore the assay is compatible with FCS complemented media.

The detection principle of this kit is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). As shown in Figure 1, HMGB1 is detected in a sandwich assay by using anti HMGB1 antibody labeled with Europium cryptate (donor), and anti-HMGB1 antibody labeled with d2 (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). Signal intensity is proportional to the number of antigen-antibody complexes formed and therefore to the HMGB1 concentration.

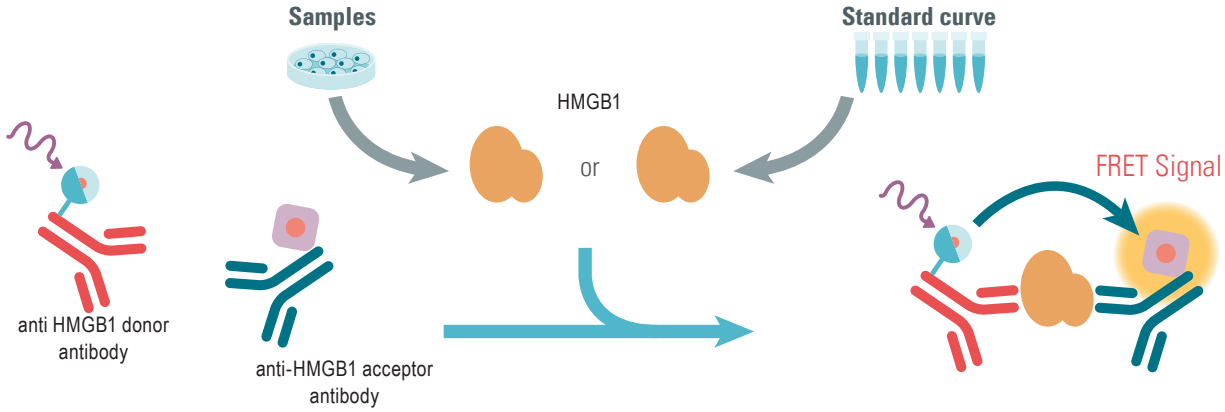
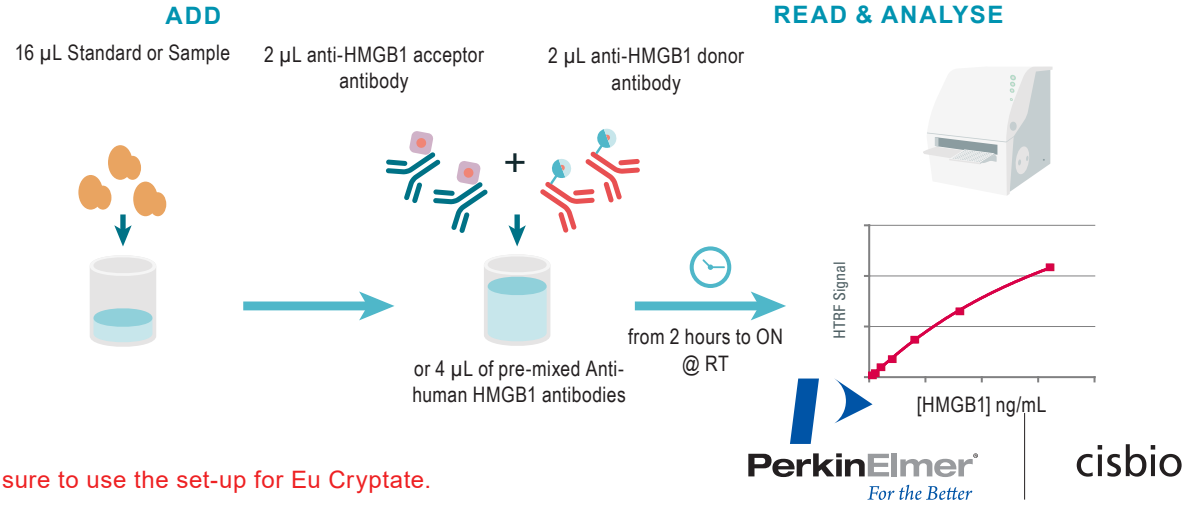


Figure 1: Principle of HTRF HMGB1 sandwich assay.

PROTOCOL AT A GLANCE



Make sure to use the set-up for Eu Cryptate.

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MATERIALS PROVIDED:

KIT COMPONENTS	500 TESTS * CAT # 62HMGPEG	10,000 TESTS * CAT # 62HMGPEH
HMGB1 Standard Frozen	1 vial - 150 µL 250 ng/mL	2 vials - 150 µL 250 ng/mL
HMGB1 Eu Cryptate Antibody	1 vial - 20 µL Frozen - 50X	1 vial - 0.4 mL Frozen - 50X
HMGB1 d2 Antibody	1 vial - 20 µL Frozen - 50X	1 vial - 0.4 mL Frozen - 50X
Diluent #5 ** 5X	1 vial 2 mL	1 vial 10 mL
Detection buffer *** ready-to-use	2 vials 1.5 mL Detection Buffer #3	1 vial 50 mL Detection Buffer #3

* When used as advised, the two available kit sizes will provide sufficient reagents for 500 tests and 10,000 tests respectively in 20 µL final volume..
Assay volumes can be adjusted proportionally to run the assay in 96 or 1536 well microplates.
** Medium like cell culture medium can be an alternative to the diluent.
*** The Detection buffer is used to prepare working solutions of acceptor and donor reagents.

PURCHASE SEPARATELY:

- HTRF®-Certified Reader. Make sure the setup for Eu Cryptate is used.

For a list of HTRF-compatible readers and set-up recommendations, please visit www.cisbio.com/compatible-readers

- Small volume (SV) detection microplates - .

For more information about microplate recommendations, please visit our website at: cisbio.com/microplates-recommendations

STORAGE AND STABILITY

Store the kit at -60°C or below.

Under proper storage conditions, reagents are stable until the expiry date indicated on the label. Diluent and detection buffer are shipped frozen, but can be stored at 2-8°C in your premises.

Reagents

If lyophilized, reconstituted reagents, antibodies, and standard stock solutions may be frozen and thawed only once. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below .

Volume of Human HMGB1 standard aliquots should not be under 10 µL.

Thawed diluent and detection buffer can be stored at 2-8°C in your premises.

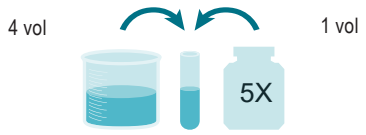
REAGENT PREPARATION

BEFORE YOU BEGIN:

- It is very important to prepare reagents in the specified buffers. The use of an incorrect diluent may affect reagent stability and assay results.
- Thaw the frozen reagents at room temperature, allow them to warm up to room temperature for at least 30 mins before use
- Before use, allow Diluent and Detection buffer to warm up at room temperature and homogenize them with a vortex.
- It is recommended to filter buffers.
- Antibody solutions must be prepared in individual vials and can be mixed prior to dispensing.
- HMGB1 standards (for standard curve) must be prepared in diluent or in the same medium as the samples.

TAKE CARE TO PREPARE STOCK AND WORKING SOLUTIONS ACCORDING TO THE DIRECTIONS FOR THE KIT SIZE YOU HAVE PURCHASED.

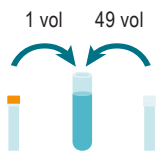
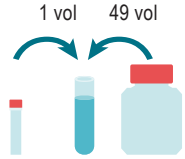
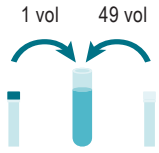
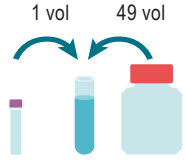
TO PREPARE REAGENT STOCK SOLUTIONS:

500 TESTS KIT - 62HMGPEG		10,000 TESTS KIT - 62HMGPEH
Anti-HMGB1 Eu Cryptate antibody		
Thaw the HMGB1 Eu Cryptate antibody . Mix gently. This 50X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.		Thaw the HMGB1 Eu Cryptate antibody . Mix gently. This 50X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.
Anti-HMGB1 d2 antibody		
Thaw the HMGB1 d2 antibody . Mix gently. This 50X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.		Thaw the HMGB1 d2 antibody . Mix gently. This 50X stock solution can be frozen and stored at -16°C or below. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solutions into disposable plastic vials for storage at -16°C or below.
HMGB1 Standard		
Thaw the Human HMGB1 standard stock solution (250 ng/mL) at RT. Mix gently. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solution into disposable plastic vials for storage at -20°C or below.		Thaw the Human HMGB1 standard stock solution (250 ng/mL) at RT. Mix gently. To avoid freeze/thaw cycles, it is recommended to dispense remaining stock solution into disposable plastic vials for storage at -20°C or below.
Diluent		
Dilute 5-fold the 5 X diluent #5 with distilled water: homogenize the 5 X diluent #5 with a vortex and add 1 volume of stock solution in 4 volumes of distilled water (e.g., 1 mL of diluent + 4 mL of distilled water). Mix gently after dilution. This 1X diluent can be frozen and stored at -60°C or below.		Dilute 5-fold the 5 X diluent #5 with distilled water: homogenize the 5 X diluent #5 with a vortex and add 1 volume of stock solution in 4 volumes of distilled water (e.g., 10 mL of diluent + 40 mL of distilled water). Mix gently after dilution. This 1X diluent can be frozen and stored at -60°C or below.
Detection buffer		
The Detection buffer is ready-to-use.		The Detection buffer is ready-to-use.

TO PREPARE ANTIBODY WORKING SOLUTIONS:

Each well requires 2 µL of HMGB1-Eu Cryptate Antibody and 2 µL of HMGB1-d2 Antibody.

Prepare the two antibody solutions in separate vials.

500 TESTS KIT - 62HMGPEG		10,000 TESTS KIT - 62HMGPEH
HMGB1 Eu Cryptate antibody		
Dilute 50-fold the 50X stock solution (thawed reagent) of human HMGB1 Eu Cryptate antibody with Detection buffer #3: add 1 volume of Eu Cryptate antibody stock solution in 49 volumes of detection buffer (e.g. 20 µL of Eu Cryptate antibody stock solution + 980 µL of detection buffer).		 Dilute 50-fold the 50X stock solution (thawed reagent) of human HMGB1 Eu Cryptate antibody with Detection buffer #3: add 1 volume of Eu Cryptate antibody stock solution in 49 volumes of detection buffer (e.g. 0.4 mL of Eu Cryptate antibody stock solution + 19.6 mL of detection buffer).
HMGB1 d2 antibody		
Dilute 50-fold the 50X stock solution (thawed reagent) of human HMGB1 d2 antibody with Detection buffer #3: add 1 volume of d2 antibody stock solution in 49 volumes of detection buffer (e.g. 20 µL of d2-antibody stock solution + 980 µL of detection buffer).		 Dilute 50-fold the 50X stock solution (thawed reagent) of human HMGB1 d2 antibody with Detection buffer #3: add 1 volume of d2 antibody stock solution in 49 volumes of detection buffer (e.g. 0.4 mL of d2 antibody stock solution + 19.6 mL of detection buffer).
Antibody mix		
It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Cryptate antibody solution (e.g. 1 mL of d2 antibody + 1 mL of Cryptate antibody).		It is possible to pre-mix the two ready-to-use antibody solutions just prior to dispensing the reagents by adding 1 volume of d2 antibody solution to 1 volume of Cryptate antibody solution (e.g. 20 mL of d2 antibody + 20 mL of Cryptate antibody).

TO PREPARE STANDARD WORKING SOLUTIONS:

- Each well requires 16 µL of standard.
- Dilute the standard stock solution serially with diluent #5 (1X) or in the medium used for the preparation of the samples.
- If culture medium is used to dilute the standard, we recommend to supplement it with serum (2 to 10%) or BSA (0.2 to 1%) in order to avoid HMGB1 sticking to assay plates.
- In order to check for a potential interference effect from your own assay buffer when using the assay for the first time, we highly recommend the parallel preparation of a standard curve in your own supplemented cell culture medium and in diluent #5 (1X).
- In order to counteract any standard sticking, we recommend changing tips between each dilution.

A recommended standard dilution procedure is listed and illustrated below:

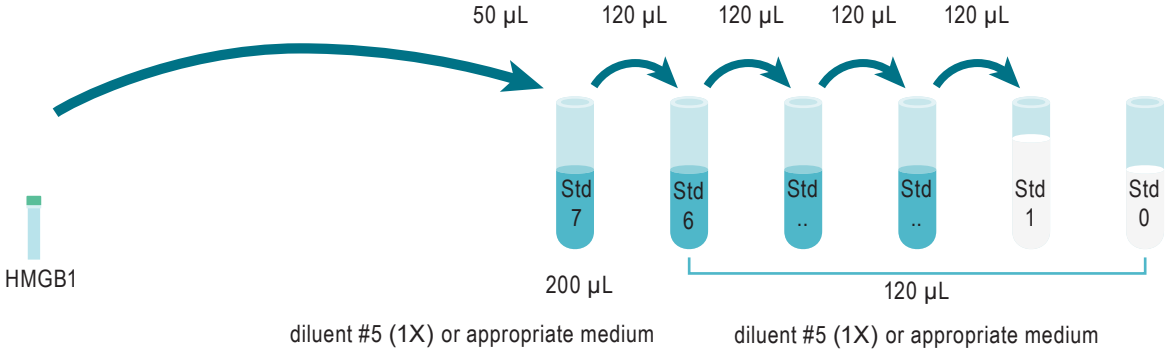
Dilute the standard stock solution 5-fold with diluent; this yields the Standard Max solution (50 ng/mL)

Dilute the standard stock solution 5-fold with diluent #5 (1X) to prepare high standard (Std 7): e.g. take 50 µL of standard stock solution and add it to 200 µL of diluent #5 (1X). Mix gently.

Use the high standard (Std 7) to prepare the standard curve using 1/2 serial dilutions as follows:

- Dispense 120 µL of diluent #5 (1X) in each vial from Std 6 to Std 0.
- Add 120 µL of standard to 120 µL of diluent #5 (1X), mix gently and repeat the 1/2 serial dilution to make standard solutions: std6, std5, std4, std3, std2, std1.

This will create 7 standards for the analyte. Std 0 (Negative control) is diluent #5 (1X) or appropriate culture medium alone.



STANDARD	SERIAL DILUTIONS	HUMAN HMGB1 WORKING SOLUTIONS (ng/mL)
Standard Stock solution	Thawed stock solution	250
Standard 7	50 µL Standard Solution stock + 200 µL diluent (1X)	50
Standard 6	120 µL standard 7 + 120 µL Diluent (1X)	25
Standard 5	120 µL standard 6 +120 µL Diluent (1X)	12.5
Standard 4	120 µL standard 5 + 120 µL Diluent (1X)	6.25
Standard 3	120 µL standard 4 + 120 µL Diluent (1X)	3.1
Standard 2	120 µL standard 3 + 120 µL Diluent (1X)	1.6
Standard 1	120 µL standard 2 + 120 µL Diluent (1X)	0.8
Standard 0	120 µL Diluent (1X)	0

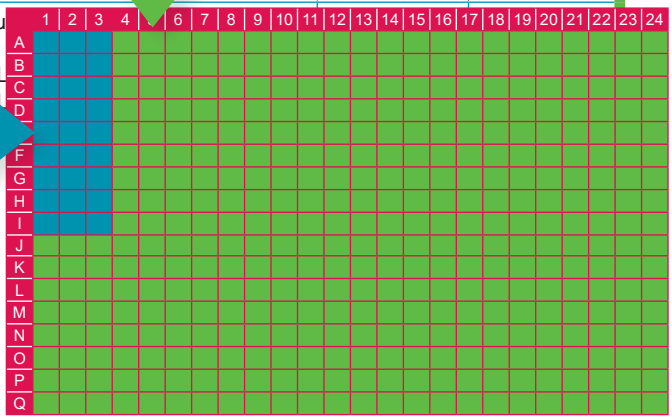
TO PREPARE SAMPLES:

- Each well requires 16 µL of sample.
- Just after their collection, put the samples at 4°C and test them immediately. For later use, samples should be dispensed into disposable plastic vials and stored at -60°C or below. Avoid multiple freeze/thaw cycles.
- Cell supernatants must be prepared using a culture medium supplemented with serum (2 to 10%) or BSA (0.2 to 1%) to avoid HMGB1 sticking to culture vessels.
- Samples with a concentration above the highest standard (Std 7) must be diluted in your appropriate sample medium, prepared, as recommended above.
- In order to measure human HMGB1 in cell lysates, cells must be lyzed with Lysis Buffer #3 (1X) for 30 min at RT under gentle shaking. Please note that the 4X stock solution of Lysis Buffer #3 must be ordered separately (Ref# 64KL3FDF, 130 mL) and 4-fold diluted with distilled water before use.

ASSAY PROTOCOL

	Standard (Std 0 - Std 7)	Samples
Step 1	Dispense 16 µL of each HMGB1 standard (Std 0 - Std 7) into each standard well	Dispense 16 µL of each sample into each sample well
Step 2	Add 2 µL of HMGB1 d2 antibody working solution to all wells	
Step 3	Add 2 µL of HMGB1 Eu Cryptate antibody working solution to all wells	
Step 4	Seal the plate and incubate from 2 hours to ON @ RT	
Step 5	Remove the plate sealer and read on an HTRF® compatible reader	

	1	2	3	4	5	6
A	16 µL Std 0 (Negative control) 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well A1	Repeat Well A1	16 µL Sample 1 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well A4	Repeat Well A4
B	16 µL Std 1 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well B1	Repeat Well B1	16 µL Sample 2 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well B4	Repeat Well B4
C	16 µL Std 2 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well C1	Repeat Well C1	16 µL Sample 3 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well C4	Repeat Well C4
D	16 µL Std ... 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well D1	Repeat Well D1	16 µL Sample ... 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well D4	Repeat Well D4
E	16 µLStd ... 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well E1	Repeat Well E1	16 µL Sample ... 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well E4	Repeat Well E4
F	16 µL Std ... 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well F1	Repeat Well F1	16 µL Sample ... 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well F4	Repeat Well F4
G	16 µL Std ... 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well G1	Repeat Well G1	16 µL Sample ... 2 µL HMGB1-d2 2 µL HMGB1-Eu C	Repeat Well G4	Repeat Well G4
H	16 µL Std ... 2 µL HMGB1-d2 2 µL HMGB1-Eu Cryptate	Repeat Well H1	Repeat Well H1	16 µ 2 µL		



DATA REDUCTION

1. Calculate the ratio of the acceptor and donor emission signals for each individual well.

$$\text{Ratio} = \frac{\text{Signal 665 nm}}{\text{Signal 620 nm}} \times 10^4$$

2. Calculate the % CVs. The mean and standard deviation can then be worked out from ratio replicates.

$$\text{CV (\%)} = \frac{\text{Standard deviation}}{\text{Mean Ratio}} \times 100$$

3. Calculate the delta ratio of the acceptor and donor emission signals for each individual well. The Standard 0 (Negative control) plays the role of an internal assay control.

$$\text{delta Ratio} = \text{Ratio Standard or sample} - \text{Ratio Standard 0}$$

For more information about data reduction, please visit http://www.cisbio.com/htrf-ratio-and-data-reduction

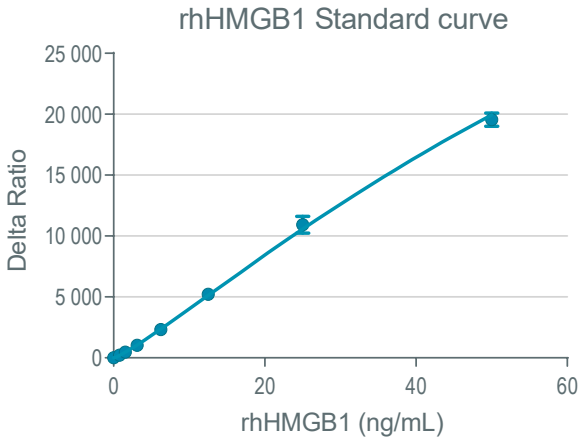
RESULTS

This data must not be substituted for the data obtained in the laboratory and should be considered only as an example.

Results may vary from one HTRF® compatible reader to another.

Standard curve fitting with the 4 Parameter Logistic (4PL) model (with $1/Y^2$ weighting):

	Ratio (1)	CV (2)	Delta Ratio
Standard 0 - Negative control	767	3%	0
Standard 1 - 0.8 ng/mL	989	8%	222
Standard 2 - 1.6 ng/mL	1233	5%	466
Standard 3 - 3.1 ng/mL	1801	2%	1034
Standard 4 - 6.25 ng/mL	3098	4%	2331
Standard 5 - 12.5 ng/mL	5988	3%	5221
Standard 6 - 25 ng/mL	11687	6%	10920
Standard 7 - 50 ng/mL	20327	3%	19560



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