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HTRF® package insert

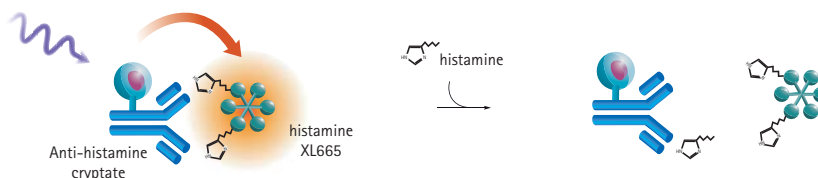
Document reference : 62HTMPEB rev06 (June 2009)

Packaging details :

	384-well low volume plate (20 µL)
62HTMPEB	1,000 tests

1. Assay description and intended use

This kit is intended for the direct quantitative determination of histamine in buffered solution or cell-culture supernatants. Its principle is based on HTRF® technology (Homogeneous Time-Resolved Fluorescence). The method is a competitive immunoassay between native histamine produced by cells and the histamine labeled with XL665. The tracer binding is visualized by a MAb anti-histamine labeled with Cryptate.



In order to improve the sensitivity of the assay, native histamine needs to be derivatized in the same way as the XL665 conjugated histamine so that the monoclonal antibody labeled with Cryptate will have similar affinity for both histamine molecules. This derivatization is carried out during the acylation step.

The specific signal (i.e. energy transfer) is inversely proportional to the concentration of acylated histamine in the calibrator or sample.

As for all other HTRF® assays, the calculation of the fluorescence ratio (665nm/620nm) eliminates possible photophysical interferences and allows the assay to be unaffected by the usual medium conditions (e.g. culture medium, serum, biotin, colored compounds).

2. Background

Histamine (β -imidazole ethylamine) is a biogenic amine generated by the enzymatic decarboxylation of histidine. It is produced by humans, plants and micro-organisms during normal metabolism. In humans, histamine is found in nearly all tissues and is mainly stored in the metachromatic granula of mast cells and in basophilic leukocytes. Histamine acts through four different surface receptors, H1, H2, H3, and the recently discovered H4. It is well established that H1 and H2 receptors belong to the GPCR family. The H3 receptor is also strongly suspected of belonging to the same family, although some conflicting results have left the question open.

Implication of histamine in allergy is well documented, but histamine is also found in some specific neurones, acts as a neurotransmitter and regulates sleep/wake cycles, hormonal secretion, cardiovascular control and thermo-regulation. Histamine is also involved in gastric acid secretion.

Histamine in allergy

Histamine acts predominantly on smooth muscles and blood vessels. It is responsible for the broncho-constriction occurring during anaphylactic shock. In vessels, its constrictive effects are limited to the venula, whereas arterioles are dilated. Furthermore, it causes a contraction of the cells of the vascular endothelium, increasing vascular permeability and allowing higher molecular substances to escape into tissues.

The histamine release from basophils during immediate type allergies is of clinical interest, as well as levels of histamine in biological fluids (plasma, urine) and cell culture supernatants after allergen challenge.

During the first challenge, IgE specific antibodies are produced and bind to their receptors on mast cells. At the second, production of IgE by plasma cells is no longer required as the allergen moves directly to the IgE already bound to mast cells, inducing histamine release from pre-formed stocks in granules.

3. Reagent preparation and stability

3.1. Supplied reagents and reconstitution

Allow the reagents to warm up to room temperature for at least 30 minutes before reconstitution.

Supplied reagents			Working solutions	
Anti histamine-Cryptate**	1 vial Lyophilized*	⇒	Add 5 mL of reconstitution buffer to each vial. Mix gently.	
Histamine-XL665	1 vial Lyophilized*	⇒		
Histamine calibrator. Concentrated Histamine	1 vial Lyophilized*	⇒	See label indications for reconstitution. Mix gently after reconstitution.	
Histamine control	1 vial lyophilized*	⇒		
Acylation reagent	1 vial lyophilized	⇒	See label indications for reconstitution. Mix gently after reconstitution.	
DMSO	1 vial of 1.2 mL			
Acylation buffer	1 vial of 1 mL			
Reconstitution Buffer 50mM Phosphate buffer, pH7.0, 0.8M KF	1 vial See volume on the label			
Diluent 50 mM Phosphate buffer, pH 7.0, 0.2% BSA, 0.02% NaN ₃ , preservatives	1 vial of 20 mL			

* All reagents were lyophilized in 50 mM phosphate buffer, pH 7, containing protease free BSA and stabilizers.

** The Cryptate conjugate concentration was optimized in order to ensure an average counting of 40,000 cps at 620 nm (384-well low volume plate), using the PHERAstar Plus reference reader (BMG LABTECH).

Precaution : HTRF® reagent concentrations have been set for optimal assay performances. Note that any dilution or improper use of the XL665 and Cryptate-conjugates will impair the assay's quality.

3.2. Reagent storage and stability

All reagents should be stored at 2-8°C until reconstituted. Under proper storage conditions, they are stable until the expiry date indicated on the labels.

Reconstituted reagents are stable for one week at 4°C. They can be refrozen (at -80°C) and thawed once only.

4. Assay protocol

4.1. Calibration curve preparation

Reconstitute the maximum calibrator according to the indications printed on the label and follow the dilution sequence shown in the following table to constitute the calibration curve. Dilution must be carried out with the diluent supplied.

Calibrator	Calibrator concentration (nM)	Preparation
Cal 8	400	200 µL of stock solution + 800 µL of diluent
Cal 7	200	500 µL cal 8 + 500 µL diluent
Cal 6	100	500 µL cal 7 + 500 µL diluent
Cal 5	50	500 µL cal 6 + 500 µL diluent
Cal 4	25	500 µL cal 5 + 500 µL diluent
Cal 3	12.5	500 µL cal 4 + 500 µL diluent
Cal 2	6.25	500 µL cal 3 + 500 µL diluent
Cal 1	3.12	500 µL cal 2 + 500 µL diluent

4.2. Sample preparation

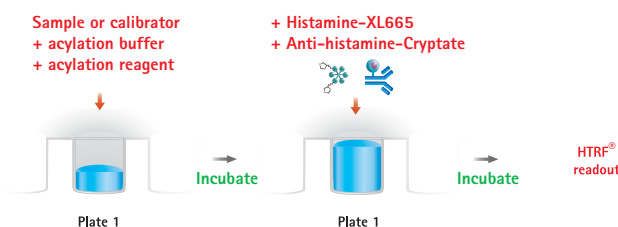
Both standard curve and sample preparation must be made using the same medium, i.e. when samples are prepared in a specific buffer (e.g. Phosphate buffer plus BSA or Triton), the same buffer should be used for the preparation of the standard curve. Consecutive dilutions should be made within the 3 to 400 nM range (working solution).

4.3. Assay protocol for 384-well low volume plate (20 µL)

- Dispense the reagents in the following order :
 - 9 µL sample or calibrator *
 - 0.5 µL acylation buffer
 - 0.5 µL acylation reagent
- Incubate at room temperature for 15 minutes
- Dispense the following reagents :
 - 5 µL Histamine-XL665 **
 - 5 µL Anti-histamine-Cryptate
- Cover the plate with a plate sealer and incubate at room temperature for 3 hours.
- Remove the plate sealer and read on a compatible HTRF® reader (more information about compatible reader at www.htrf.com/readers)

*For positive control, replace the first reagent by 9 µL of diluent.

**For negative control, replace the first reagent by 9 µL of diluent and Histamine-XL665 by 5 µL of reconstitution buffer.



4.4. Data reduction and example of a calibration curve

Results are calculated from the 665nm / 620nm ratio and expressed in Delta F. An example of data reduction is given in the table below (readout on PHERAstar Plus). This data should not be substituted for results obtained in the laboratory.

	A (665nm)	B (620nm)	Ratio (1)	Mean Ratio (2)	CV % (3)	Delta F % (4)
Negative control	1402 1592	35840 41148	391 387	389	1	
[calibrator] nM						
0	25359 26955	35879 38914	7068 6927	6997	1	1699
3.125	24972 26667	39292 39971	6355 6672	6514	3	1574
6.25	22836 24237	36649 39777	6231 6093	6162	2	1484
12.5	19620 21750	36945 39181	5311 5551	5431	3	1296
25	17543 18022	40306 40388	4352 4462	4407	2	1033
50	13052 13537	37691 38517	3463 3515	3489	1	797
100	9742 10013	38164 41201	2553 2430	2491	3	540
200	6479 6574	39280 41591	1649 1581	1615	3	315
400	4361 4740	38966 42071	1119 1127	1123	0	189
Control	15426 15304	37704 37866	4091 4042	4066	1	945

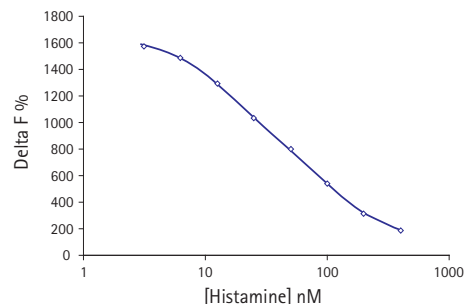
$$1. \text{Ratio} = \frac{A_{665\text{nm}}}{B_{620\text{nm}}} \times 10^4$$

$$2. \text{Mean Ratio} = \frac{\sum \text{ratios}}{2}$$

$$3. \text{CV} = \frac{\text{Std deviation}}{\text{Mean ratio}} \times 100$$

$$4. \text{Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{\text{neg}}}{\text{Ratio}_{\text{neg}}} \times 100$$

(Ratio_{neg} = negative control)



* The assay control validates the accuracy of the calibration curve. The concentration deduced from delta F obtained should fall into the concentration range indicated on the label of the vial.

5. Assay characteristics

The table summarizes the characteristics of the assay relative to the detection limit (histamine concentration corresponding to the «dose of mean zero - 2SD») and the EC50 (histamine concentration which allows the displacement of 50% of binding). This data has been obtained using the PHERAstar Plus reference reader (BMG LABTECH). Assay performances are presented after a 3 hour incubation at room temperature.

	Detection limit	EC50
Incubation 3 hours at RT	1.8 nM	43 nM

6. Cross reactivity

Acylated histamine 100%

Acylated L-histidine <0.0067%

Acylated serotonin <0.0067%

7. Assay flexibility and miniaturization

When used as suggested, the kit will provide sufficient reagents for 1,000 tests using a 384- well low volume plate in 20 µL final assay volume (HTRF® packaged basis).

To move to 96 half-well plate with 100 µL final volume, while retaining assay performances, 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.

Plate references : 96 half-well plate (Costar # 3694 or equivalent), 384-well low volume plate (Greiner # 784076).

Assay components	Assay format	
	384-well low volume (20 µL)	96 half-well (100 µL)
Sample	9 µL	45 µL
Acylation buffer	0.5 µL	2.5 µL
Acylation reagent	0.5 µL	2.5 µL
XL665 conjugate	5 µL	25 µL
Cryptate conjugate	5 µL	25 µL
	1,000 tests	200 tests