

HTRF[®] Hyaluronic Acid (HA) Assay

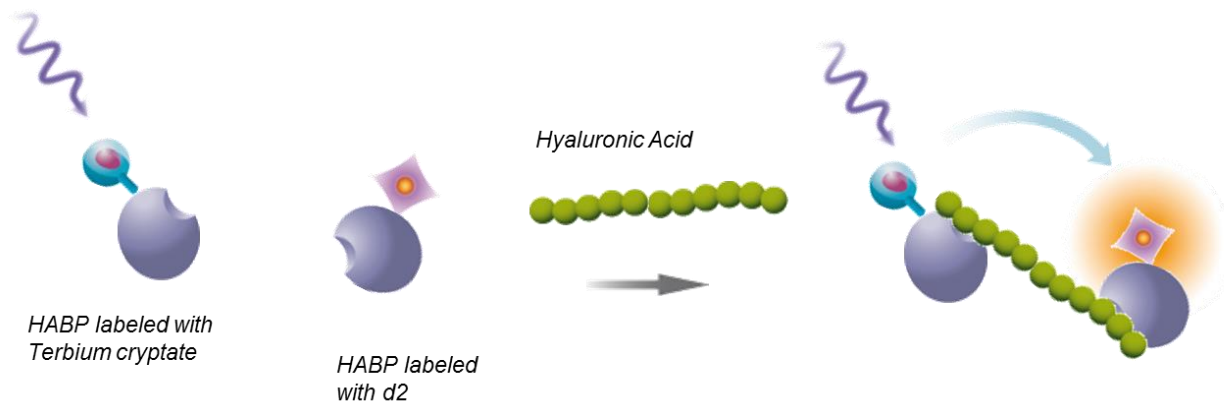
10,000 tests

Section 1

Assay Description:

This kit is intended for the determination of levels of Hyaluronic Acid (HA) in cell supernatant. The principle of this kit is based on HTRF technology (Homogeneous Time-Resolved Fluorescence). As shown in Figure 1, HA is detected in a sandwich HTRF assay using Hyaluronic Acid binding protein (HABP), one labeled with a Terbium-cryptate donor and the other with a d2 acceptor dye. The specific FRET signal is directly proportional to the amount of HA in the sample.

Figure 1: Diagram of the HTRF HA detection assay



Section 2

Reagent Description

| Components | Quantity / Storage | Stock Solutions | Number of Tests | Volume |
|-------------------------|-----------------------------|-----------------|-----------------|---------|
| HABP-d2* | 1 vial / Frozen | 50X | 10,000 | 1,000uL |
| HABP-Tb* | 1 vial / Frozen | 50X | 10,000 | 1,000uL |
| HA Standard* | 1 vial/ Frozen | 500 ug/mL | | 100uL |
| Dilution Buffer | 1 bottle / Frozen or 4°C | 1X | | 20mL |
| Binding Buffer | 1 bottle / Frozen | 4X | | 25 mL |
| Detection Buffer | 1 bottle / Frozen or 4°C | 1X | | 105 mL |

Reagent Handling

*Store labeled proteins at -80°C.

Make aliquots of labeled proteins and store at -80°C, to avoid numerous freeze/thaw cycles.



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Section 3

Working Solution Preparation

Solution A

HABP-d2

Make a 50 fold dilution.

For example, add 2450uL of *detection buffer* to 50uL of HABP-d2 stock solution.

Solution B

HABP-Tb

Make a 50 fold dilution.

For example, add 2450uL of *detection buffer* to 50uL of HABP-Tb stock solution.

Standard Dilutions

Using **HA Standard** stock solution (500ug/mL), prepare the following dilutions:

Dilution 1: Prepare a 10-fold dilution using stock solution.

For example: add 50uL of stock solution to 450uL of Dilution Buffer (50ug/mL).

Then prepare the following serial dilutions:

Table 1. HA Standard Curve Dilutions

| Standard # | uL of Diluent | uL of Standard# | Standard Concentration (ng/mL) |
|------------|---------------|-------------------------|--------------------------------|
| 1 | 980 | 20 of Dilution 1 | 1000 |
| 2 | 200 | 200 of #1 | 500 |
| 3 | 200 | 200 of #2 | 250 |
| 4 | 200 | 200 of #3 | 125 |
| 5 | 200 | 200 of #4 | 62.5 |
| 6 | 200 | 200 of #5 | 31.25 |
| 7 | 200 | 200 of #6 | 15.62 |
| 8 | 200 | 200 of #7 | 7.8 |
| 9 | 200 | 200 of #8 | 3.9 |
| 10 | 200 | 0 | 0 |

Standard #10 is the assay negative control.

Binding Buffer:

Dilute 2 fold the Binding Buffer stock solution with distilled water (e.g. take 1mL of Binding Buffer stock solution and add it to 1mL of distilled water). Mix gently.



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Section 4

Assay protocol for 384-well low volume, WHITE plate (e.g. Greiner part# 784075), 20uL final volume

- Dispense the reagents in the following order:
 - 5uL of standard or cell supernatant to be tested
 - 5uL of Binding Buffer 2X
 - 5uL of **HABP-d2** (Solution **A**)
 - 5uL of **HABP-Tb** (Solution **B**)
- Cover the plate with a plate sealer and incubate overnight at RT or 4°C for better performances
- Remove the plate sealer and read on a compatible HTRF reader (information on HTRF readers at www.htrf.com/readers)

Perform the following assay controls:

Buffer control: To make sure that buffers are not contaminated by cryptate and do not generate any background fluorescence.

Cryptate control: To check the Cryptate signal at 620 nm.

Negative control: Combination of donor and acceptor in the presence of appropriate buffer without sample (HA). This control is used for the calculation of delta F.

Table 2: Reagent dispensing for HA assay and controls

| | <i>Buffer control</i> | <i>Cryptate control</i> | <i>Negative control</i> | <i>Sample</i> |
|-------------------------|-----------------------|-------------------------|-------------------------|---------------|
| Diluent Only | 5uL | 5uL | 5uL | |
| Cell Supernatant | | | | 5uL |
| Binding Buffer | 5uL | 5uL | 5uL | 5uL |
| Detection Buffer | 10uL | 5uL | | |
| HABP-d2 | | | 5uL | 5uL |
| HABP-Tb | | 5uL | 5uL | 5uL |



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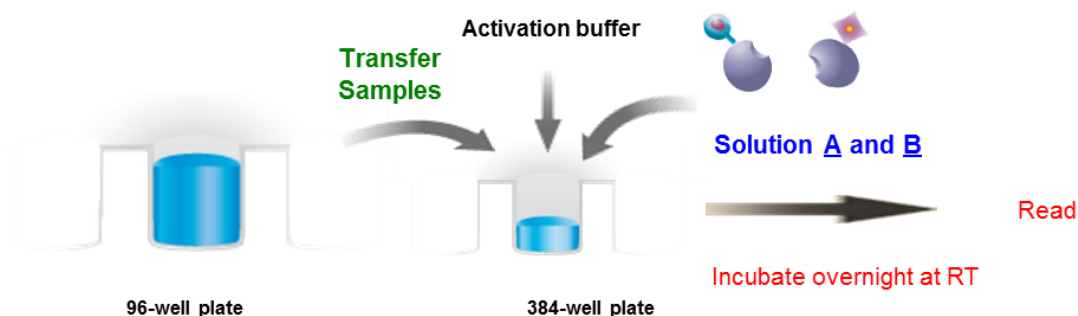


Figure 2. HTRF HA Assay Protocol

Results

The table below is representative of data obtained after incubation overnight at room temperature. The standard curve covers a range of 3.9ng/mL to 1,000ng/mL of HA. Incubation at 4°C will improve assay performances.

Table 3: Typical results after overnight at RT

| HA [ng/mL] | A(665nm) | A(620nm) | Ratio (1) | Delta F% (2) |
|------------|----------|----------|-----------|--------------|
| 1000 | 16921 | 11548 | 14655 | 429% |
| 500 | 14682 | 11755 | 12488 | 351% |
| 250 | 11804 | 12534 | 9418 | 240% |
| 125 | 8903 | 12290 | 7245 | 162% |
| 62.5 | 6857 | 12630 | 5429 | 96% |
| 31.25 | 5433 | 12749 | 4262 | 54% |
| 15.6 | 4637 | 13026 | 3560 | 29% |
| 7.8 | 4057 | 12753 | 3181 | 15% |
| 3.9 | 3892 | 12832 | 3033 | 10% |
| 0 | 3838 | 13857 | 2770 | 0% |



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

$$2. \text{ Delta F} = \frac{\text{Calibrator or sample Ratio} - \text{Ratio}_{\text{neg}}}{\text{Ratio}_{\text{neg}}} \times 100$$

(Ratio_{neg} = negative control)

Ratio: The ratio is calculated for each well individually and multiplied by a factor of 10,000 for easier data processing. The mean and standard deviation are calculated for replicates.

Delta F%: (DF%) is used for the comparison of day to day runs of the same assay. It reflects the signal to background fluorescence of the assay. The negative control is used as the internal assay control.

For more information regarding data reduction, please visit our website:

<http://www.htrf.com/htf-technology/ratio-and-data-reduction>



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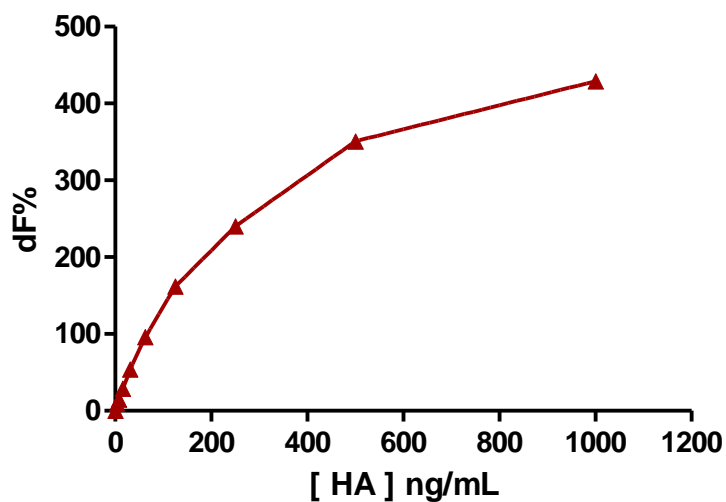
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Section 5

Typical results

Standard curve tested following the kit protocol. Reading performed on a PheraStar FS lamp (BMG Labtech).

Hyaluronic acid assay Overnight at RT - Pherastar FS lamp



For complete information regarding data reduction for HTRF assays, please visit our website at:

<http://www.htrf.com/htrf-technology/ratio-and-data-reduction>



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