



HNE-histidine STRESS ELISA Kit

ALX-850-322-KI01

2 x 96 wells

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For laboratory use only. Not for human or diagnostic use.

Introduction

HNE-histidine FINE ELISA kit is an indirect enzyme immunoassay (EIA), which measures protein-bound HNE as HNE-histidine adducts. The kit will measure human cell lysate samples.

Test Principle

Samples containing HNE-histidine adducts are coated onto a microplate. HNE-histidine specific mouse monoclonal antibodies detect HNE-histidine conjugates. Secondary peroxidase labeled anti-mouse antibody is used to detect mouse monoclonal antibody. After addition of substrate and stop solution the amount of HNE-histidine conjugate is determined. The standard curve demonstrates a direct relationship between Optical Density (OD) and HNE-histidine concentration: i.e., the higher the OD the higher the HNE-histidine concentration in the sample.

Application

HNE-histidine STRESS ELISA kit is designed to measure the amount of HNE-histidine conjugate in human cell culture lysates induced by strong oxidative stress. Do not use serum free cell culture supernatants! Tissue homogenates are not tested. There are enough reagents included in this kit for two 96-well immuno-assay plates. Second plate should be used to determine unspecific (background) values for each sample. Running duplicate wells for samples and standards is recommended.

Kit materials

1. ELISA Plate: 2x
2. Washing Buffer, 10x concentrated, 2x 100 ml
3. Standards, 8x (at -80°C), 200 μl each
4. Solution A - Monoclonal antibody, 0.75 ml
5. Solution B, H_2O_2 , 5 ml
6. Solution C - HRP conjugated secondary antibody, 1.5 ml
7. Solution D - TMB stain, 1 ml
8. Stop Solution, H_2SO_4 , 12 ml
9. R_1 , 10x concentrated, 5 ml
10. R_2 diluent, 100 ml
11. R_2 powder, 5 g
12. R_3 , 10x concentrated, 10 ml
13. R_4 diluent, 90 ml
14. R_4 powder, 2.7 g
15. R_5 , 10x concentrated, 14 ml

Do not open reagents before use! Reagents are sterile and could be contaminated. See chapter "Preparation of Reagents" and "Reagents mixtures".

Materials required but not supplied

1. Multi-channel or repeating pipettes
2. Plate shaker (optional)
3. 96-well microplate Reader with 450 nm/620 nm filter
4. Mechanical vortex
5. Adhesive plate cover or parafilm

Precautions

The instructions provided have been designed to optimize the kit's performance. Deviation from the instructions may result in suboptimal performance of the kit and the failure to produce accurate data. Read manual carefully before starting.

Preparation of Reagents

1. Washing Buffer

Salt crystals can be present in concentrated solution, but it will dissolve after bringing to room temperature and mixing or diluting. Add the entire content of the 10X Washing Buffer concentrate to appropriate container, dilute with deionized water. Stir to homogeneity.

2. R₁

Add the entire content of the 10X R₁ concentrate to appropriate container, dilute with deionized water. Stir to homogeneity.

3. R₂

Add the entire content of the R₂ powder in R₂ diluent. Stir overnight to homogeneity at room temperature. Solution will remain turbid.

3. R₃

Add the entire content of the 10X R₃ concentrate to appropriate container, dilute with deionized water. Stir to homogeneity.

4. R₄

Add the entire content of the R₄ powder in R₄ diluent. Stir overnight to homogeneity at room temperature. Solution will remain turbid.

5. R₅

Add the entire content of the 10X R₅ concentrate to appropriate container, dilute with deionized water. Stir to homogeneity.

All diluted reagents are stable at +4°C for 24 hours and could be prepared in advance. For prolonged storage diluted reagents can be stored at -20°C. Repeated freeze-thaw cycles up to three times are possible. Do not freeze solutions A, B, C and D; solutions are stable at +4°C after opening. Solution D could be solid at +4°C, but it will thaw after bringing to room temperature. Mix it well before use.

Reagents mixtures

Prepare reagents mixtures immediately before use! Mixtures are not stable and can not be stored! Do not mix all amounts of reagents at once, but calculate the amount needed for analysis.

Mix 1

Add 10 μ l of solution A per 1 ml of R₃

Mix 2

Add 50 μ l of solution B per 1 ml of R₄

Mix 3

Add 10 μ l of solution C per 1 ml of R₃

Mix 4

Add 15 μ l of solution D and 0,2 μ l of solution B per 1 ml of R₅

HNE-histidine Standards

After defreezing the standards should be kept for 30 min. at +4°C before further use. Vortex and mix vigorously before use to achieve homogeneity. Repeated freeze-thaw cycles are not recommended.

Preparation of Samples

It is recommended to test each sample in duplicate. Cell samples should be washed with PBS, and cell pellet stored at -80°C. Within two weeks of freezing, cell samples should be lysed in cold lysis buffer. Add 400 μ l of lysis buffer per 1×10^6 cells. Keep samples on ice for 60 minutes and vortex them every 10 minutes. Total cell lysate should be used for the assay. Use immediately after lysis.

Lysis buffer: 150 mM NaCl
200 mM Na₂HPO₄
10 mM TRIS-HCl
6 M guanidine hydrochloride
adjust pH to 8.0

Storage of Kit Components

Maintain the unopened kit at 2-8°C or -80°C (for those labeled as such) until expiration date indicated on the label. After opening, treat reagents as described under "Preparation of Reagents".

Assay procedure

Run 2 plates in parallel! The second plate will be used to eliminate sample background values. Treat the plates the same except in step No. 6. where different reagents for plate 1 and plate 2 should be used. If needed, only one plate at the time could be used; in such case split the plate in the half so one half of the plate should be used for sample analysis and the other for sample background reading.

1. Add 200 µl of R₁ (see *Preparation of Reagents* section) to all wells that will be used.
2. Add 20 µl of samples or standards carefully in the centre of the well. Be careful not to make air bubbles. After adding all samples and standards, tap gently on the sides of the plate so that they are distributed equally on the bottom of the well. It is recommended that standards and samples be run in duplicate. Also, run two plates in parallel with the same samples (one for sample reading, the other for background reading). Incubate for 4,5 hours at +4°C covered with adhesive plate cover.
3. Decant the content of the plate. Fill vigorously each well with ddH₂O. Decant the content of the plate. Pat dry onto paper towels.
4. Add 400 µl of R₂ (see *Preparation of Reagents* section) to all wells and incubate for 3 hours at room temperature.
5. Decant the content of the plate. Fill vigorously each well with washing buffer. Decant the content of the plate. Pat dry onto paper towels.
6. Add 200 µl of Mix 1 per well (see *Preparation of Reagents* and *Reagents mixtures* sections) to the first plate. To the second plate add 200 µl of R₃. Incubate overnight at +4°C covered with an adhesive plate cover.
7. Decant the content of the plate. Pat dry onto paper towels. Fill vigorously each well with 400 µl of washing buffer. Wait for 2 minutes. Decant the content of the plate. Pat dry onto paper towels. Repeat the procedure for 6 more times for a total of 7 washes. Occasionally, tap gently on the side of the plates to improve washing procedure.
8. Add 400 µl of Mix 2 per well (see *Preparation of Reagents* and *Reagents mixtures* section) to all plates. Incubate 30 minutes at room temperature.
9. Decant the content of the plate. Pat dry onto paper towels. Fill vigorously each well with 400 µl of washing buffer. Wait for 2 minutes. Decant the content of the plate. Pat dry onto paper towels. Repeat the procedure for 6 more times for a total of 7 washes.
10. Add 200 µl of Mix 3 (see *Preparation of Reagents* and *Reagents mixtures* section) to all wells. Incubate for 1 hour at room temperature covered with an adhesive plate cover.
11. Decant the content of the plate. Pat dry onto paper towels. Fill vigorously each well with 400 µl of washing buffer. Wait for 2 minutes. Decant the content of the plate. Pat dry onto paper towels. Repeat the procedure for 6 more times for a total of 7 washes.
12. Fill vigorously each well with 400 µl of R₅ (see *Preparation of Reagents* section). Decant the content of the plate. Pat dry onto paper towels.
13. Add 200 µl of Mix 4 (see *Preparation of Reagents* and *Reagents mixtures* section) to all wells. Mix 4 should be cold, taken immediately from +4°C. Incubate 5 min for ELISA STRESS at room temperature. Tap gently every minute during incubation period with the palm of the hand on the both sides of the plate so color is equally distributed in the well or if you see that developed color has precipitated on the bottom of the well. If sample color is not well developed, prolonged incubation will be needed. Check blue color development and proceed to the step 14 when satisfied.
14. Add 50 µl of stop reagent into each well. The blue color will change to yellow. Shake it if necessary to achieve color homogeneity and wait for 5 minutes.
15. Measure the absorbance at plate reader at 450/620 nm. Subtract the obtained values in second plate from the first plate. The background values (second plate) are usually very low. CAUTION: Bubbles in the wells will cause inaccurate readings. Ensure that all bubbles are removed prior to taking the absorbance reading.

Analytical Sensitivity

Sensitivity: 8.1 pmol/mg

Range of Detection: 0.143 nmol/mg to 1.714 nmol/mg

Intra-assay Variation: $\pm 7,3 \%$

Inter-assay Variation: $\pm 12 \%$

ELISA STRESS - spiking

expected values (nmol/mg)	measured values (nmol/mg)	recovery (%)
0.453	0.478	106
0.241	0.261	109

Recovery was calculated by addition of defined amount of HNE into cell lysate samples.

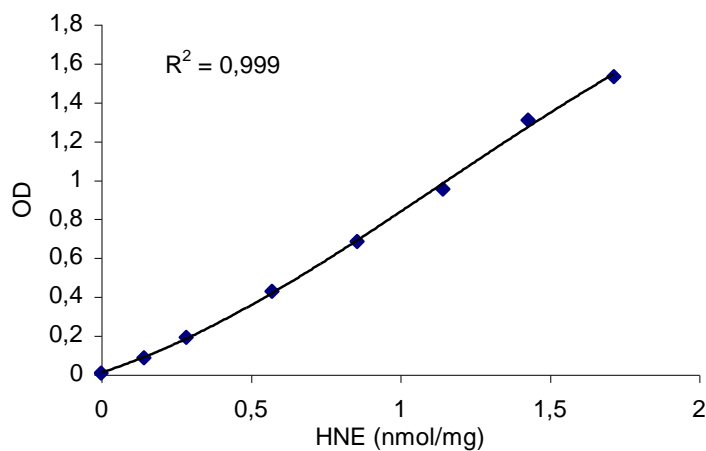
Calculation of Results

Results are expressed as nmol HNE/mg of protein. It is not necessary to determine amount of protein in a sample if described protocol for sample preparation is used. However, determine the amount of proteins in a sample if total amount of HNE-protein conjugates is required.

HNE-histidine STRESS ELISA Standard Curve

ELISA STRESS standards (nmol/mg)

S1	S2	S3	S4	S5	S6	S7	S8
0	0,143	0,286	0,571	0,857	1,143	1,429	1,714



References

1. Esterbauer, H., et al. Chemistry and biochemistry of 4-hydroxynonenal, malonaldehyde and related aldehydes. *Free Radic Biol Med*, 1991, 11:81-128.
2. Zarkovic, N. 4-Hydroxynonenal as a bioactive marker of pathophysiological processes. *Mol Asp Med*, 2003, 24:281-291.
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4. Borovic, S., et al. Differential sensitivity to 4-hydroxynonenal for normal and malignant mesenchymal cells. *Redox Rep*, 2007, 12:50-54.

ASSAY PROCEDURE SUMMARY

