20-Hydroxyecdysone Competitive ELISA Kit

Catalog Number EIAHYD (96 tests), EIAHYDX10 (10 x 96 tests)

Pub. No. MAN0018781 **Rev** C.0

Note: For safety and biohazard guidelines, see the "Safety" appendix in the *ELISA Technical Guide* (Pub. no. MAN0006706). Read the Safety Data Sheets (SDSs) and follow the handling instructions. Wear appropriate protective eyewear, clothing, and gloves.

Product description

The 20-Hydroxyecdysone ELISA Kit is designed to detect and quantify the level of 20-hydroxyecdysone (20E) in tissue extracts from hemolymph, plants, or anthropods.

20-hydroxyecdysone is part of a steroid hormone family that is found in arthropods, and widely distributed in plant species. Of the many ecdysteroids, 20-hydroxyecdysone is the most functionally active and widely distributed in arthropods.

Contents and storage

Kit and components are shipped at –20°C. Upon receipt, store the kit at –20°C. Once open, store the kit at 4°C and use within 2 weeks.

Components	Quantity (96 tests)	Quantity (10 x 96 tests)
Coated Clear 96-well plate; strip-well plate coated with goat anti-rabbit IgG	1 plate	10 plates
20-Hydroxyecdysone Standard; 2,500 ng/mL	40 µL	10 x 40 µL
20-Hydroxyecdysone Antibody	3 mL	10 x 3 mL
20-Hydroxyecdysone Conjugate	3 mL	10 x 3 mL
Assay Buffer Concentrate (5X)	28 mL	10 x 28 mL
Wash Buffer Concentrate (20X)	30 mL	2 x 125 mL
TMB (Tetramethylbenzidine) Substrate	11 mL	10 x 11 mL
Stop Solution; contains 1 M HCl, **CAUSTIC**	5 mL	1 x 50 mL
Plate Sealer	1	10

Materials required but not supplied

- Distilled or deionized water
- Microtiter plate reader with software capable of measurement at or near 450 nm (preferably with correction between 570 nm and 590 nm)
- Plate washer–automated or manual (squirt bottle, manifold dispenser, or equivalent)
- Calibrated adjustable precision pipettes and glass or plastic tubes for diluting solution
- Methanol (for hemolymph samples)

Procedural guidelines

IMPORTANT! Reagents are lot-specific. Do not mix or interchange different reagent lots from various kit lots.

- Review the **Procedural guidelines** and **Plate washing directions** in the *ELISA Technical Guide* available at **thermofisher.com**.
- Allow reagents to reach room temperature before use. Mix to redissolve any precipitated salts.
- Samples should be considered as potentially infectious and all items which contact the samples as potentially contaminated.
- Ensure all buffers used for samples are azide free.

Prepare 1X Wash Buffer

1. Dilute Wash Buffer Concentrate 1:20 by adding 1 part Concentrate to 19 parts of deionized or distilled water. Label as 1X Wash Buffer.
2. Store the Concentrate and 1X Wash Buffer in the refrigerator. Use the diluted buffer within 3 months.

Prepare 1X Assay Buffer

1. Dilute Assay Buffer Concentrate 1:5 by adding 1 part of the concentrate to 4 parts of deionized or distilled water.
2. Store the concentrate and 1X Assay Buffer in the refrigerator. 1X Assay Buffer is stable at 4°C for 3 months.

Sample preparation guidelines

- This kit utilizes a peroxidase-based readout system. Buffers containing sodium azide will inhibit color production from the enzyme. Make sure all buffers used for samples are azide free.
- Samples may need to be extracted depending on source. A study of the scientific literature to determine suitable methods of isolating 20-Hydroxyecdysone from organisms and plants is recommended.

Prepare hemolymph samples

Sample concentrations should be within the range of the standard curve. Because conditions may vary, each investigator should determine the optimal dilution for each application.

1. Take one part of aqueous sample and add 3 volumes of chilled methanol (75% of total volume), then vortex for 30 seconds.
2. Centrifuge the solution at 10,000 rpm for 10 minutes at 4°C.
3. Draw supernatant out carefully without disturbing the pellet, then dry the pellet completely using a centrifugal concentrator at 30°C for 2–3 hours.
4. Store the dried powder/pellet at –20°C until assayed, or dissolve in a minimum of 125 µL 1X Assay Buffer to run immediately.

Determine extraction efficiency

Perform an extraction of hemolymph samples with methanol prior to testing to remove matrix effects. Additional information can be found in *Endocrine Manual for the Reproductive Assessment of Domestic and Non-Domestic Species* (CRC Endocrine Laboratory, 2005).

1. Prepare a 20-hydroxyecdysone solution of known concentration in 1X Assay Buffer.
2. Spike one aliquot of your sample with a volume of the steroid solution in 1X Assay Buffer (control spike) and one aliquot of sample with the same volume of 1X Assay Buffer (control sample).
3. Extract samples and Controls with chilled methanol as described in "Prepare hemolymph samples".
4. Compare the concentration of the steroid measured in the extracted control (control spike-control sample) with the concentration of the steroid measured before extraction (steroid solution of known concentration).

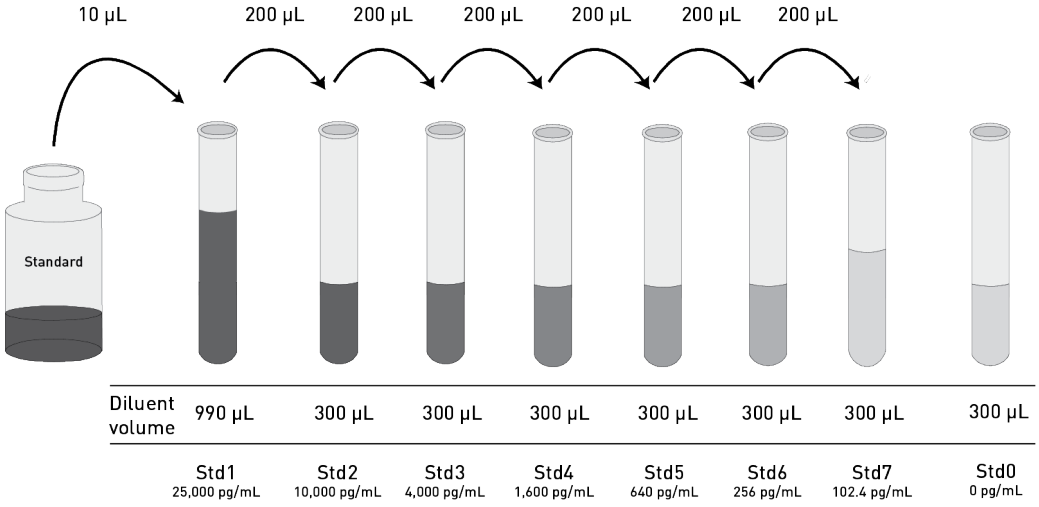
Dilute standards

Note: Use glass or plastic tubes for diluting standards.

The 20-Hydroxyecdysone Standard contains an organic solvent. Pipette the standard up and down several times to wet the pipet tip before transfer to ensure that volumes are accurate.

1. Add 10 µL 20-Hydroxyecdysone Standard to one tube containing 990 µL 1X Assay Buffer and label as 25,000 pg/mL 20E.
2. Add 300 µL 1X Assay Buffer to each of 7 tubes labeled as follows: 10,000, 4,000, 1,600, 640, 256, 102.4, and 0 pg/mL 20E.
3. Make serial dilutions of the standard as described below in the dilution diagram. Mix by vortexing between steps.

Use the standards within 2 hours of preparation. Discard any remaining reconstituted standard.

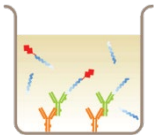


Perform ELISA (Total assay time: 2.5 hours)

IMPORTANT! Perform a standard curve with each assay.

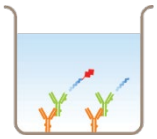
Allow all components to reach room temperature before use. Mix all liquid reagents prior to use.

Determine the number of 8-well strips required for the assay. Insert the strips in the frames for use. Re-bag any unused strips and frames, and store desiccated at 2°C to 8°C for future use. The silica pack in the bag keeps the plate dry, and turns from blue to pink if the bag is not properly sealed.



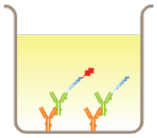
Bind antigen

a. Add 50 µL standards or samples to the appropriate wells.
b. Add 75 µL 1X Assay Buffer to wells for detecting non-specific binding (NSB).
c. Add 50 µL 1X Assay Buffer to wells for detecting maximum binding (B0 or zero standard).
d. Add 25 µL 20-Hydroxyecdysone Conjugate to each well.
e. Add 25 µL Hydroxyecdysone Antibody to each well except NSB wells.
f. Tap the side of the plate to mix, then cover the plate with a plate sealer.
g. Shake at room temperature for 2 hours. If the plate is not shaken, signals bound will be ~20% lower.
h. Thoroughly aspirate the solution and wash wells 4 times with 300 µL of 1X Wash Buffer.



Add chromogen

a. Add 100 µL TMB Substrate to each well. The substrate solution will begin to turn blue.
b. Incubate for 30 minutes at room temperature.
   Note: TMB should not touch aluminum foil or other metals.



Add stop solution

Add 50 µL Stop Solution to each well. Tap side of the plate gently to mix. The solution in the wells changes from blue to yellow.

Read the plate and generate the standard curve

1. Read the absorbance at 450 nm. Read the plate within 10 minutes after adding the Stop Solution.
2. Average the duplicate Optical Density (OD) values for each standard and sample. Use curve-fitting software to generate the standard curve. A four-parameter algorithm provides the best standard curve fit. Optimally, the background absorbance may be subtracted from all data points, including standards, unknowns, and controls, prior to plotting.
3. Calculate the concentrations for unknown samples and controls from the %B/B0 curve. Multiply value(s) obtained for sample(s) by the appropriate factor to correct for the sample dilution.
   Note: Dilute samples producing signals greater than that of the highest standard in 1X Assay Buffer and reanalyze. Multiply the concentration by the appropriate dilution factor.

Performance characteristics

Standard curve (examples)

The following data were obtained for the standards over the range of 0–25,000 pg/mL 20-hydroxyecdysone (20E).
Note: 100 pg/mL of 20E is equivalent to 208.06 pM.

20E Standard (pg/mL)	Net OD (450 nm)	%B/B0
25,000	0.139	17.2
10,000	0.243	30.1
4,000	0.376	46.6
1,600	0.532	66
640	0.651	80.7
256	0.720	89.2
102.4	0.762	94.5
0	0.807	100

Note: The NSB gave a Mean OD value of 0.081.

Intra-assay precision

Three spiked hemolymph samples diluted with 1X Assay Buffer were assayed in replicates of 20 to determine precision within an assay.

Parameters	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	4,623	2,707	1,997
%CV	8.9	10.6	10.2

CV = Coefficient of Variation

Inter-assay precision

Three spiked hemolymph samples diluted with 1X Assay Buffer were assayed in duplicate in 18 assay runs by multiple operators to determine precision between assays.

Parameters	Sample 1	Sample 2	Sample 3
Mean (pg/mL)	4,522	2,706	1,867
%CV	9.1	9.6	7.8

CV = Coefficient of Variation

Performance characteristics, continued

Expected values

This assay has been tested with extracted Dungeness crab and crayfish hemolymph samples. In Dungeness crab, the concentration of ecdysones are low during intermolt (~20 ng/mL) and maximal during premolt (~2000 ng/mL). In crayfish, the ecdysone level can increase to ~60 ng/mL during natural molts and ~80 ng/mL during induced molts.

Sample	Range
Dungeness crab (intermolt) n=3	30.7– 34.0 ng/mL
Crayfish hemolymph (induced molt) n=4	Undetectable to 57.5 pg/mL
Crayfish hemolymph (natural molt) n=3	Undetectable to 42.4 pg/mL

Linearity of dilution

Linearity was determined using two spiked hemolymph samples diluted 1:20, one with a low 20E concentration (1,478 pg/mL) and one with a higher concentration (9,256 pg/mL). The samples were mixed in the ratios given below. The measured 20E concentrations were compared to the expected values based on the ratios used.

High sample	Low sample	Expected Conc. (pg/mL)	Observed Conc. (pg/mL)	% Recovery
80%	80%	8,473	7,701	110
60%	40%	6,639	6,145	108
40%	60%	5,014	4,589	109
20%	80%	3,187	3,034	105
			Mean recovery	**108%**

Sensitivity

The analytical sensitivity of 20-hydroxyecdysone is 197.8 pg/mL. This was determined by adding two standard deviations to the mean O.D. obtained when the zero and Std7 was assayed 20 times, and calculating the corresponding concentration.

Specificity

The following cross reactants were tested in the assay and calculated at the 50% binding point.

Steroid	Cross-reactivity %
20-Hydroxyecdysone	100
Makisterone A (MAK A)	5.90
Ecdysone	0.71
Ponasterone A (PON A)	0.61
β-Estradiol	0.09
Testosterone	0.05
Corticosterone	0.04
Cortisol	0.04
7-Dehydrocholesterol	0.02

Limited product warranty

Life Technologies Corporation and/or its affiliate(s) warrant their products as set forth in the Life Technologies' General Terms and Conditions of Sale found on Life Technologies' website at **www.thermofisher.com/us/en/home/global/terms-and-conditions.html**. If you have any questions, please contact Life Technologies at **www.thermofisher.com/support**.

Catalog Number

Batch code


Temperature limitation


Use by


Manufacturer


Consult instructions for use


Caution, consult accompanying documents

Manufacturer: Life Technologies Corporation | 7335 Executive Way | Frederick, MD 21704 | USA

The information in this guide is subject to change without notice.