Product Information

Background | this ELISA assay utilize the principle of competitive binding to measure the concentration of hormone in plant extracts.

1. The trans-zeatin riboside specific antibodies are pre-coated to the surface of the reaction wells.

2. The plant extract sample, containing an unknown amount of hormone, is mixed in the reaction well with a known amount of a tracer to react with a limited number of antibody binding sites in the reaction wells.

3. During incubation the hormone in the sample competes with the tracer for the antibody binding sites.

4. Unbound hormone, tracer and plant extract are washed out of the reaction wells.

5. Following substrate addition which reacts with a tracer bound to the antibody and produces a yellow-coloured product.

6. The absorbance of the sample is converted to concentration of hormone by means of a standard curve which is produced by simultaneously used standards along with the samples.

Important note | do not use reagents after expiry date. All reagents must be stored according to this instruction. Avoid repeated freeze-thawing. Pipetting of all samples and tracer is critical to the accuracy and reproducibility of the assay. It is important to include a standard curve together with each run in cases when all strips are not processed at the same time and all plates and buffers are equilibrated to 25°C.
Do NOT methylate cytokinin samples as this will significantly decrease the sensitivity of the assay.
Test is not valid unless Bo reads greater than 0.700 O.D. Increase substrate incubation time until the desired O.D. is obtained (but do not exceed an additional 60 min).
Pre-purification (removal of chlorophyll and lipids) is highly recommended for plant samples but there is no need in the case of certain samples like algae and bacteria. Analyses of several sample dilutions are recommended.

Storage instructions:
Reaction wells | very sensitive and must be stored at -20°C (stable for more than 6 months)
Tracer | store at -20°C (stable for more than 6
months). Diluted tracer is stable for 7 days at +4°C.
Standards | store at -20°C (stable for more than 6 months); stable for 7 days at +4°C.
Substrate | can be stored at +4°C or -20°C. Working solution is stable for 5 hours at +4°C

Kit components
Reaction Wells 5 pcs antibody coated and blocked (480 assays, 60 strips with 8 wells) **(1)**
Tracer, alkaline phosphatase, 20 – 50 µl **(reagent 2)**
Tracer Diluent 5 x concentrated stock: 250 mM TBS Tris, 10 mM NaCl, 1mM $MgCl_2$, pH 7.5 stock + 0.02 % NaN_3, **(reagent 3)**
Reaction and Wash Solution 10 x concentrated stock TBS + 0.02 % NaN_3 **(reagent 4)**
Stopping Reagent 2 x concentrated stock: 5 N KOH **(reagent 5)**
Substrate Diluent 10 x concentrated stock: 500 mM $NaHCO_3$ stock, pH 9.6+ 0.02 % NaN_3 **(reagent 6)**
Substrate 100 mg of p-nitrophenylphosphate **(reagent 7)**
Standards: 600 µl of each, 15.6 pmol, 7.8 pmol, 3.9 pmol, 1.95 pmol, 975 fmol, 488 fmol, 244 fmol, 122 fmol, 61 fmol, 30,5 fmol, 15,2 fmol, concentration/50 µl **(reagent 8)**
Your samples: plant or algae extract volume 50-250 µl

Assay development time: 4-5 hours
Sensitivity: around 50 fmol in 50 µl

1. **Preparation of diluted Tracer Solution (reagent 9)**
Prepare cytokinin Tracer Diluent (1 ml from concentrated tracer diluent stock **(reagent 3)** + 4 ml deionized water, confirm pH to be 7.5) then add 2-5 µl of a Tracer Stock Solution **(reagent 2;** check label**)**. Diluted tracer can be stored at 2-4° C for up to 2 days. Use always pre-incubated (25°C, min. 30 min) tracer solution for ELISAs.

2. **Preparation of standards**
Slowly thaw the standards **(reagent 8)** and leave them to shake for 1 min. Pre-incubation at 25°C for at least min. 30 min is necessary.

3. **Preparation of Reaction and Wash Solutions**
For coupling reaction prepare Reaction Solution **(reagent 10)** by using 2 ml from concentrated stock **(reagent 4)** added to 8 ml of deionized water, pH 7.5, per each plate.
To prepare Washing Solution **(reagent 11)** use 2 ml from concentrated stock **(reagent 4)** added to 98 ml of deionized water.

4. **Plate preparation**
Remove the plate or chosen strips **(1)** from refrigerator, add 150 µl of diluted Reaction Solution **(reagent 10)** to each well for 60 minutes to equilibrate at room temperature (25°C as recommended). After 1 h incubation discard all solutions from the plate and remove excess of Reaction Solution by patching strips dry on paper towels (wall).
For blank wells add 150 µl diluted Reaction Solution only **(reagent 10)**.

Add 50 µl of chosen standards (**reagent 8**) in duplicates. Minimum five standard points in duplicates are recommended for the standard curve. For example 244 fmol, 488 fmol, 975 fmol, 1.95 pmol and 3.9 pmol is final standard concentration for each well. Leave two wells without any standard. They will serve as a positive control, called **Bo** (containing 100 µl of diluted Reaction Solution **(reagent 10) +** 50 µl of diluted Tracer Solution, prepared in point 1, **reagent 9**).

Add 50 µl of your samples dissolved in Reaction Solution **(reagent 10)** to chosen wells and then add 50 µl of diluted cytokinin tracer solution to all wells except to blank wells (**reagent 9**).
Only Blank wells are left free of Tracer Solution and contain 150 µl diluted Reaction Solution only **(reagent 10)**.

Example of layout of ELISA plate (each box represents 2 wells)

BLANK, 150 µl diluted Reaction Solution only **(UB)**	488 fmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution
No standard, 100 µl diluted Reaction Solution + 50 µl diluted Tracer Solution **(Bo)**	244 fmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution
15.6 pmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	122 fmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution
7.8 pmol pmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	61 fmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution
3.9 pmol pmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	30.5 fmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution
1.95 pmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	15.2 fmol, 50 µl standard + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution	50 µl sample + 50 µl diluted Reaction Solution + 50 µl diluted Tracer Solution
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6. Mix thoroughly on a shaker.

7. Cover the wells with plate sealer.

8. Incubate sealed wells at room temperature (25°C as recommended) for 1 hour. No shaking is necessary.

9. After 1 h incubation discard all solutions from the plate.

10. Wash wells by adding 200 µl of diluted Wash Solution **(reagent 11)** to each well. Decant wash mixture from the wells. Repeat this step 3 more times for a total of 4 washes and remove excess of Wash Solution by patching strips dry on paper towels (wall).

During 1 hour incubation mentioned above, prepare substrate solution **(reagent 7)**. For one plate 20 mg of substrate needs to be dissolved in 20 ml diluted Substrate Diluent **(reagent 6)** using 2 ml from concentrated stock and adding to 18 ml of deionized water, pH 9.6. Recommended temperature of the Substrate Solution is 25°C.

12. Add 150 µl of the Substrate Solution to all wells including blank, Bo, standards and samples.
Important: Activity of a tracer can be decreased by leaving wells without solution for too long period of time.

13. Cover wells with plate sealer.

14. Incubate at 25°C for 60 minutes.

15. Remove plates from incubator and add 50 µl diluted Stopping Reagent **(reagent 5)** by using 3 ml from concentrated stock and adding it to 3 ml of deionized water, mixing well. Wait for 5 minutes.

16. Read colour absorbance at 405 nm against Blank wells.

17. Record the optical densities.

Calculations
1. Record the optical densities.
2. Average the optical densities in duplicate standards or samples.

3. Calculate the % Binding of each standard point or sample by the following:

% Binding (B%) = Standard or Sample (O.D.)/ Bo O.D. x 100

Bo = 100 µl Reaction Solution + 50 µl Tracer = 100% Binding

4. Plot the % Binding versus the log of total concentration (fmol or pmol in 50 µl) of **standards** and draw the Best fit curve. Semi-log paper can be used for convenience.

5. The sample concentration is determined by extrapolation of the sample % Binding from the best-fit standard curve. Note: Linear curve can be drawn using a log-logit function.