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# ELISA-based quantitative detection of peptides in plant tissue

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**We use this protocol and it is working**

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## Abstract

Phytocytokines play a critical role in the fine tuning of plant immunity and development. The final bioactive peptide form can vary from 1-5 kDa and is usually expressed in low amounts, making them difficult to detect with conventional methods. Here, we present an ELISA-based assay that allows rapid and cost-effective detection of naturally released peptides in plant tissues. This protocol is optimized for Zip1, a 17-amino-acid phytocytokine derived from *Zea mays* that elicits salicylic acid signaling in maize leaves. Using a custom peptide antibody, we designed an experimental pipeline to achieve a specific, selective and sensitive recognition of Zip1 allowing its detection in complex biological samples. Using this protocol, we are able to quantify in leaf material native Zip1-containing peptides with a detection limit in the nanogram range. Our methodology has a three-day turn-over and allows the use of raw extract from plant tissue. This method can be adapted for the detection and quantification of a variety of plant signaling peptides.

## Materials

### EQUIPMENTS

- Tecan infinite M200 PRO (with Tecan i-control software)
- Multichannel Pipette (20 - 200µL)

### MATERIALS

- 96-well microtiter plate (CORNING COSTAR 9018; Thermofisher scientific, Ref: 44-2504-21)
- Adhesive film for Microplates (VWR, Article No. 391-0620)
- SuperSignal™ West Pico PLUS Chemiluminescence-Substrate (Thermofisher Scientific, Catalog Number: 34580)
- ZIP1 peptide (Sequence: QPWEGESELKLATQGASVRR, GenScript Biotech)
- Anti-ZIP1 (Sequence: EGESELKLATQGASVRR; Davids Biotechnologie, polyclonal antibody from rabbit)
- Anti-rabbit IgG, HRP-linked Antibody (Cell Signaling Technology, Category Number: 7074S)
- Paper towel

### BUFFERS

1) Bicarbonate Buffer
3.03g $Na_2CO_3$
6.0g $NaHCO_3$
1000ml $dH_2O$

2) ZIP1 Stocks for ELISA Standard Calibration Curve
In bicarbonate buffer, dilute the synthesized ZIP1 peptide to the following concentrations:
SL1: 20µg/ml
SL2: 10µg/ml
SL3: 5µg/ml
SL4: 0.2µg/ml
SL5: 0.1µg/ml
Store the ZIP1 stocks at -20°C.

3) PBS Buffer, pH 7.4
1.44 g $Na_2HPO_4$
0.2g KCl
0.246g $KH_2PO_4$
8.0g NaCl
1000ml $dH_2O$

4) Blocking Buffer
5% Skim Milk Powder dissolved in PBS Buffer, pH 7.4

5) Covering Agent

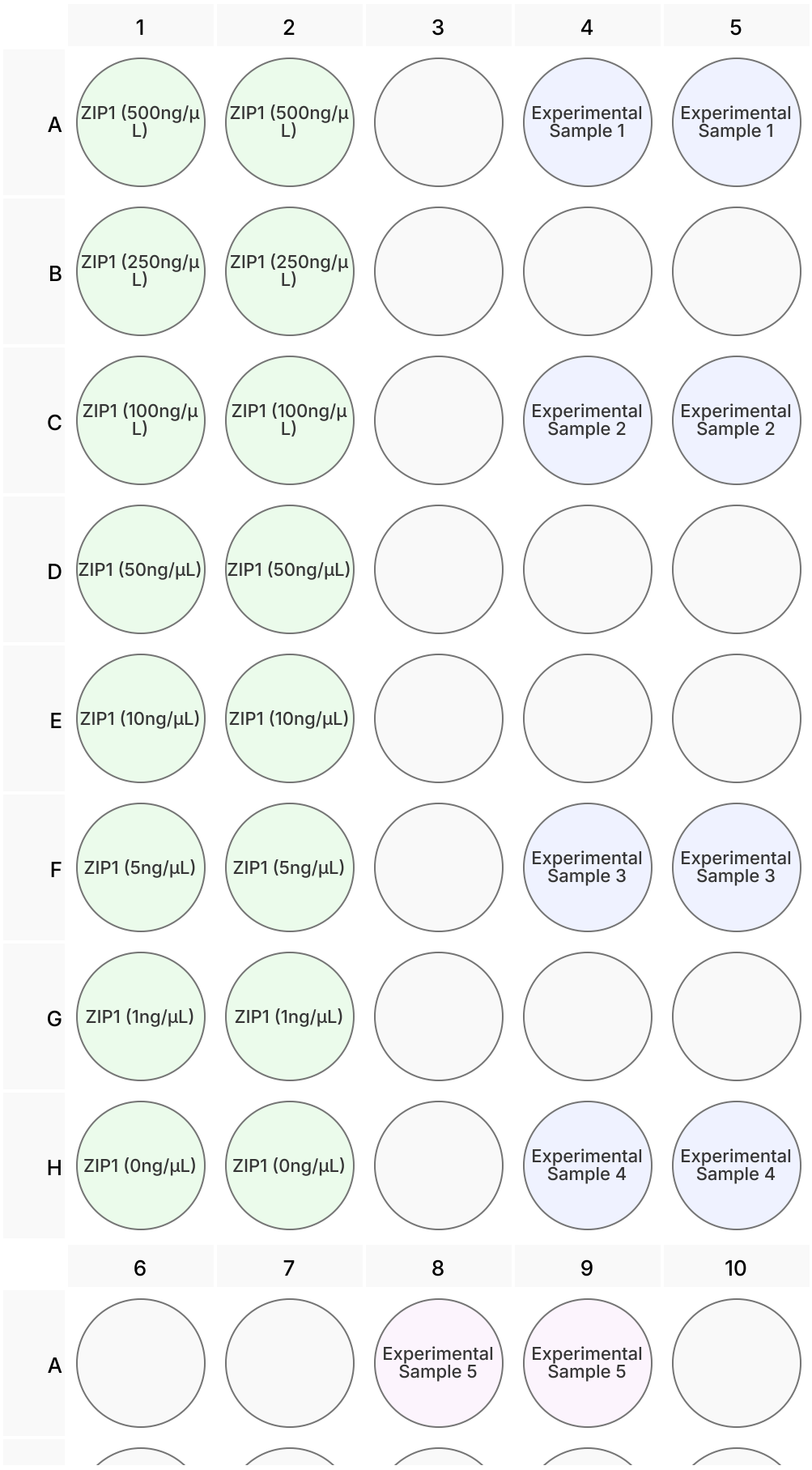
70% Ethanol
5% Bromophenol Blue
25% MilliQ $H_2O$

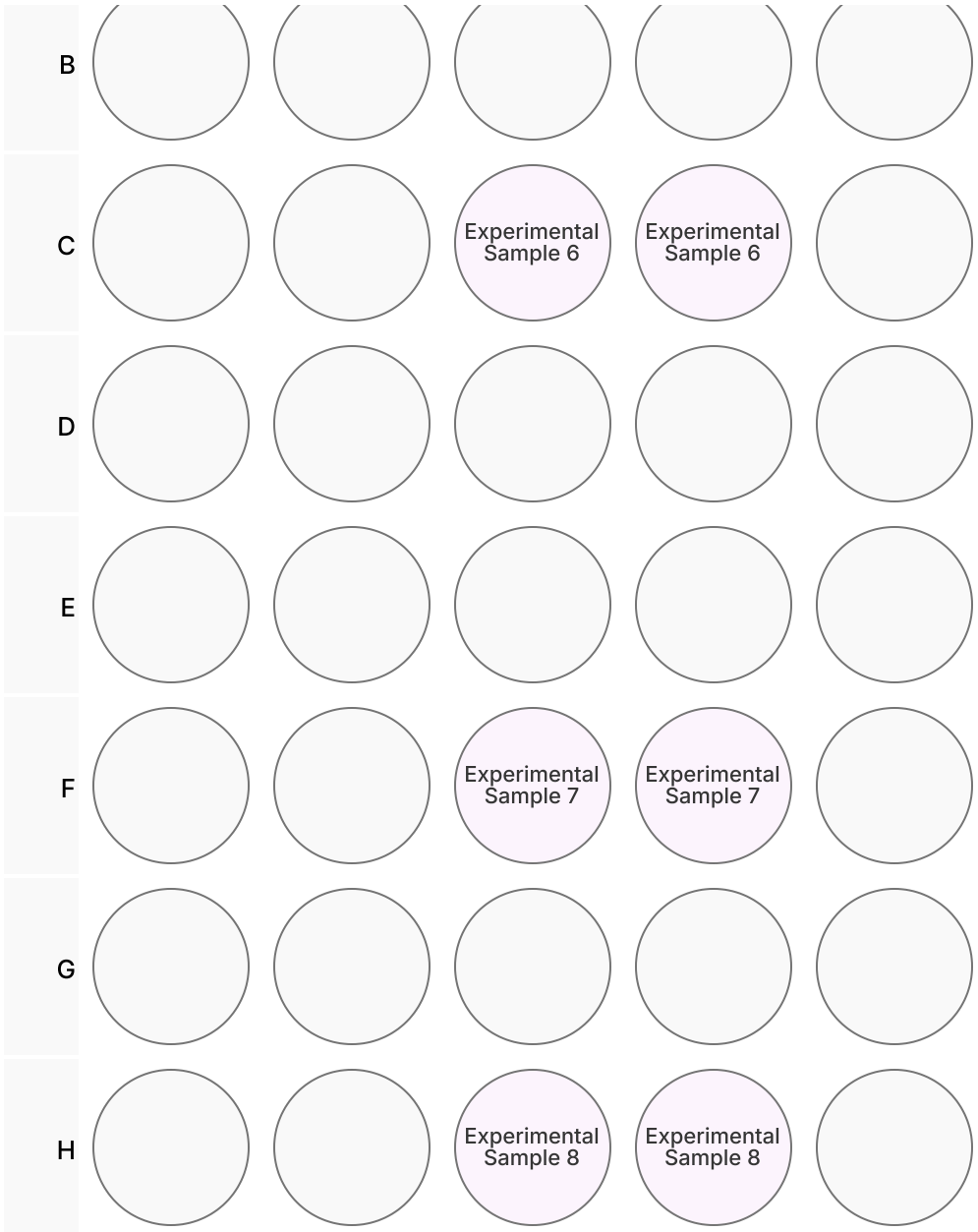
## Designing the 96-well Plate Layout

1 The following is an example of an ELISA 96-well microtiter plate layout.
The first two columns of the 96-well microtiter plate is used for the ZIP1 standard calibration curve. Remaining spaces is allocated to the experimental group.

It is recommended for a "barrier" of empty wells to be put between each experimental group duplicates.

Duplicates are recommended for every sample group. Whereby if space allows, triplicate will yield a higher (thus better) R-squared value for the standard calibration curve.


1
2
3
4
5
A
ZIP1 (500ng/µL)
ZIP1 (500ng/µL)
Experimental Sample 1
Experimental Sample 1
B
ZIP1 (250ng/µL)
ZIP1 (250ng/µL)
C
ZIP1 (100ng/µL)
ZIP1 (100ng/µL)
Experimental Sample 2
Experimental Sample 2
D
ZIP1 (50ng/µL)
ZIP1 (50ng/µL)
E
ZIP1 (10ng/µL)
ZIP1 (10ng/µL)
F
ZIP1 (5ng/µL)
ZIP1 (5ng/µL)
Experimental Sample 3
Experimental Sample 3
G
ZIP1 (1ng/µL)
ZIP1 (1ng/µL)
H
ZIP1 (0ng/µL)
ZIP1 (0ng/µL)
Experimental Sample 4
Experimental Sample 4
6
7
8
9
10
A
Experimental Sample 5
Experimental Sample 5


B
C
Experimental Sample 6
Experimental Sample 6
D
E
F
Experimental Sample 7
Experimental Sample 7
G
H
Experimental Sample 8
Experimental Sample 8
11
12
A
Experimental Sample 9
Experimental Sample 9
B
C
Experimental Sample 10
Experimental Sample 10

## Coating

30m

2 You can find the recipe to make ZIP1 SL stocks in the materials section. 15m

To create the ZIP1 standard calibration curve, refer to the table below to the specific volumes of bicarbonate buffer and SL stock required for each concentration.

The proportions of bicarbonate buffer to SL stock in the table below is designed for one well.

| | ZIP1 (ng/µL) | Bicarbonate Buffer (µL) | SL Stock Volume Added (µL) | Total Volume (µL) |
|---|---|---|---|---|
| | 500 | 47.5 | 2.5 (SL2) | 50 |
| | 250 | 47.5 | 2.5 (SL3) | 50 |
| | 100 | 49.0 | 1.0 (SL3) | 50 |
| | 50 | 37.5 | 12.5 (SL4) | 50 |
| | 10 | 47.5 | 2.5 (SL4) | 50 |
| | 5 | 47.5 | 2.5 (SL5) | 50 |
| | 1 | 49.5 | 0.5 (SL5) | 50 |
| | 0 | 50.0 | 0.0 | 50 |

Table 1. Proportion of bicarbonate buffer to different SL stock concentration to make the ZIP1 standard calibration curve.

3 Load 50µL of each of your samples to two separate wells for duplicates. 10m

Note

The samples' protein concentration should be normalised either to cells/mL (protoplast), to fresh weight (leaf samples), or to equal protein abundance with Bradford Assay.

4 Seal the microtiter plate with an adhesive film. Press on the film to ensure a firm seal for each well across the plate. 15s

4.1 Hold the plate at one end, and tap gently on the other end for even mixing of the samples. 5s

5 Rest the microtiter plate on a flat surface Overnight at 4 °C 16h

## Washing 15m

6 The following steps should be done beside a sink.

7 Remove all of the liquid in the microtiter plate in one strong swinging motion. 10s

7.1 Stack some paper towel on a flat surface, flip the microtiter plate and tap it repeatedly on the paper towel to fully remove any remaining liquid. 20s

8 Using a multichannel pipette, add 200 µL of PBS buffer to all wells. 3m

8.1 Hold the plate at one end, and tap gently on the other end to ensure the PBS buffer covers the bottom of all wells 5s

8.2 Remove all of the liquid in the microtiter plate in one strong swinging motion. 10s

8.3 Flip the microtiter plate and tap it repeatedly on clean paper towel to fully remove any remaining liquid. 20s

8.4 go to step #8 10m
Repeat this for a total of three times.

## Blocking 5m

9 Using a multichannel pipette, add 200 µL of blocking buffer to all wells. 3m

10 Seal the microtiter plate with an adhesive film. Press on the film to ensure a firm seal for each well across the plate. 15s

10.1 Hold the plate at one end, and tap gently on the other end for even mixing of the samples. 5s

11 Rest the microtiter plate on a flat surface for 02:00:00 at Room temperature 2h

## Primary Antibody Incubation 30m

12 go to step #6 15m
Complete the washing step.

13 Add 100 µL of primary antibody (a-ZIP1, concentration of 1:7µg/mL, dissolved in blocking buffer) to each wells with samples. 5m

14 Add 200 µL of blocking buffer to the rest of the wells. 5m

15 Seal the microtiter plate with an adhesive film. Press on the film to ensure a firm seal for each well across the plate. 15s

15.1 Hold the plate at one end, and tap gently on the other end for even mixing of the samples. 5s

16 Rest the microtiter plate on a flat surface Overnight at 4 °C 16h

## Secondary Antibody Incubation 30m

17 go to step #6 15m
Complete the washing step.

18 Add 100 µL of the secondary antibody (a-rabbit, concentration of 1:1000 , dissolved in blocking buffer) to each wells with samples. 5m

19 Add 200 µL of blocking buffer to the rest of the wells. 5m

20 Seal the microtiter plate with an adhesive film. Press on the film to ensure a firm seal for each well across the plate. 15s

20.1 Hold the plate at one end, and tap gently on the other end for even mixing of the samples. 5s

21 Rest the microtiter plate on a flat surface for 02:00:00 at Room temperature 2h

## Detection 40m

22 go to step #6 15m
Complete the washing step.

23 Add 50 µL of SuperSignal™ West Pico PLUS Chemiluminescence-Substrate to each well with samples. 5m

24 Put the microtiter plate into the Tecan infinite M200 PRO machine, ready for detection. 10s

24.1 Open the "Tecan i-control" software, associated with the Tecan infinite M200 PRO machine. 2m

We will use the software available "MycoAlert Assay" template from the "Luminescence" category for detection.

Under "Plate definition", select "Thermofisher Scientific Transparent 96-well"

Under "Part of Plate", select all the wells with samples within for measurement plus an empty well in addition.

24.2 Click "Start" to begin the detection. 5m
Save the excel file after detection has been completed.

Note

Make sure you save the excel file after measurement is completed, before you begin the second round of detection or else the data will be lost.

25 Take the microtiter plate out and add 100 µL of covering agent to rest of the well 5m

Note

Take extra caution when adding the covering agent to the rest of the plate as the high percentage of ethanol makes the covering agent a volatile liquid that is prone to dripping during pipetting.

26 Put the microtiter plate into the Tecan infinite M200 PRO machine, ready for detection. 10s

26.1 We will use the same settings as previously used.

For reference:
We will use the software available "MycoAlert Assay" template from the "Luminescence" category for detection.

Under "Plate definition", select "Thermofisher Scientific Transparent 96-well"

Under "Part of Plate", select all the wells with samples within for measurement plus an empty well in addition.

26.2 Click "Start" to begin the detection.
Save the excel file after detection has been completed. 5m

## Data Analysis 15m

27 In the detection step, we took two separate measurements in case of the covering agent accidentally drips into any of the sample wells. If the measurements with covering agent is done properly, we will take that data set for further analysis as the covering agent helps eliminate background noise.

28 On excel, calculate the average RLU of every duplicates from the standard calibration curve and sample wells. 5m

28.1 Take the average and deduct the RLU value from the empty well. 2m

29 Plot a scatter plot of the ZIP1 concentration (ng/µL) against the RLU value for your ZIP1 standard calibration curve. 5m

29.1 From "Chart Elements", select "Trendline" then select "More options" to open "Trendline Options" 1m

29.2 Under "Trendline Options", select "Linear" for the trendline, select "Display equation on plot", and select "Display R squared value on plot" 1m

> Note
>
> A R squared value of 0.95 and above is recommended for good practice.

30 Using the displayed equation on plot, you can calculate the concentration of ZIP1 present in your sample wells. 1m

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