

Sandwich Enzyme-linked Immunosorbent Assay (ELISA) Analysis of Plant Cell Wall Glycan Connections

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[Abstract] Sandwich ELISA is a highly sensitive method that can be used to determine if two epitopes are part of the same macromolecule or supramolecular complex. In the case of plant cell wall glycans, it can reveal the existence of inter-polymers linkages, leading to better understanding of overall cell wall architectures. This development of a conventional sandwich ELISA protocol uses a carbohydrate-binding module (CBM), a small protein domain found in some carbohydrate catalysing or activating enzymes, and rat monoclonal antibodies (mAbs) which can be combined in the same ELISA plate without risk of cross reaction; the secondary anti-rat HRP antibody being only able to bind to the rat mAb and not the CBM (Figure 1). This protocol was developed and modified in the Prof. J. Paul Knox lab at the University of Leeds.

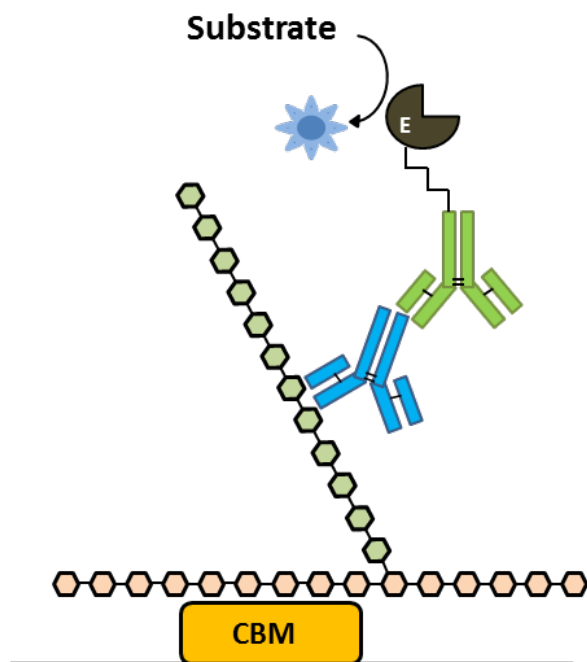


Figure 1. Sandwich ELISA analysis of complex glycans

Materials and Reagents

1. Cell wall extracts (or purified cell wall polymers)

Notes:

- a. *Reagents for cell wall extracts are 50 mM cyclohexanediamine tetraacetic acid (CDTA) (Sigma-Aldrich, catalog number: D1383) or 4 M KOH/1% (w/v) NaBH₄ (Sigma-Aldrich, catalog number: 452882)*
 - b. *Cell wall extraction protocol using 4 M KOH/1% (w/v) NaBH₄ is described in Cid et al. (2010). This extraction will disrupt and release most cell wall glycans.*
 - c. *CDTA is a chelating agent which is used to specifically extract the pectin fraction from cell walls. The same protocol can be followed using 50 mM CDTA instead of 4 M KOH (no neutralizing step necessary).*
2. Skimmed milk powder (Marvel Original) [used at 5% (w/v) in 1x PBS]
3. Purified recombinant carbohydrate-binding module (CBM)

Note: A protocol for CBM expression and purification can be found in Lee et al. (2013).
4. Primary monoclonal antibody (PlantProbes, www.plantprobes.net)
5. Secondary antibody (depending on the origin of the primary antibody used)
 - a. Anti-rat IgG-horseradish peroxidase (HRP)-conjugated (Sigma-Aldrich, catalog number: A9542)
 - b. Anti-mouse IgG-horseradish peroxidase (HRP)-conjugated (Sigma-Aldrich, catalog number: A6782)
6. 10 mg/ml tetramethyl benzidine (Sigma-Aldrich, catalog number: T2885)
7. 1 M sodium acetate buffer (pH 6)
8. 6% H₂O₂ (VWR International, catalog number: 2858175C)
9. 2.5 M H₂SO₄ (VWR International, catalog number: 191675A)
10. Tween 20 (Sigma-Aldrich, catalog number: P2287) (optional)
11. 1x phosphate-buffered saline (PBS) (pH7) (see Recipes)
12. HRP developing solution (see Recipes)

Equipment

1. 96-well surface treated ELISA microtitre plates (Thermo Fisher Scientific, Nunc-Immuno Maxisorp, catalog number: 442404)
2. Microtitre plate reader (450 nm absorbance) (Thermo Fisher Scientific, Multiskan™ FC Microplate Photometer)
3. Multi-channel pipette (if available, not critical)
4. pH meter

Procedure

1. Coat the microtitre plate using 0.5 to 1 µg/ml CBM in 1x PBS, 100 µl per well at 4 °C overnight.

Note: The amount of CBM necessary to obtain a good signal will vary depending of its affinity to the substrate. Trials may be necessary to find the appropriate dilution.

2. Wash the plate 3 times using tap water or 1x PBS 0.1 % (v/v) Tween 20. Shake the plate dry.

Note: We routinely used tap water but one might prefer using 1x PBS 0.1 % (v/v) Tween 20. The drying step is critical as any remaining water/buffer would dilute the next reagent. Make sure the plates are fully dried after each washing step.

3. Block the plate for 2 h using 5% (w/v) milk powder in 1x PBS, 200 µl per well at room temperature.

4. Wash the plate 3 times using tap water or 1x PBS 0.1 % (v/v) Tween 20. Shake the plate dry.

5. Incubate for 90 min with a cell wall extract or test glycan diluted in 5% (w/v) milk/1x PBS, 100 µl per well at room temperature.

Note: Normal ELISA analysis can be used to determine which dilution of the extract gives the best signal. However, the amount of CBM used will determine the amount of glycan that can be immobilised. Therefore it is better to use more concentrated sample rather than less.

6. Wash the plate 6 times using tap water or 1x PBS 0.1 % (v/v) Tween 20. Shake the plate dry.

7. Incubate with a primary antibody appropriately diluted in 5% (w/v) milk/1x PBS, 100 µl per well for 1 h at room temperature.

Note: Use 10x dilution of hybridoma cell culture supernatants if working with PlantProbes monoclonal antibodies.

8. Wash the plate 6 times using tap water or 1x PBS 0.1 % (v/v) Tween 20. Shake the plate dry.

9. Incubate with secondary HRP-conjugated antibody diluted 1:1,000 in 5% (w/v) milk/1x PBS, 100 µl per well for 1 h at room temperature.

10. Wash the plate 9 times using tap water or 1x PBS 0.1 % (v/v) Tween 20. Shake the plate dry.

Note: Plates should be thoroughly washed at this step to ensure no false positive signals due to non-specifically bound secondary antibody/HRP.

11. Develop the plate using 150 µl of substrate solution per well to detect secondary antibody binding. Let the plates develop for 5 to 10 min. Stop the reaction by adding 50 µl of 2.5 M sulphuric acid.

Note: As developing the plate is a time dependent process it is important to ensure that all comparable samples are developed for the same time.

12. To collect the data, read the plates at 450 nm using the microtitre plate reader.

Notes

1. Make sure all experimental combinations in the microtitre plate wells are assayed in triplicates.
2. A control with no CBM (1x PBS) needs to be included in every experiment. This should remain blank if the plates are properly blocked. In case of a signal in no CBM wells increase the % of milk powder, block for longer or use >3% Bovine Serum Albumin in place of milk powder.

Recipes

1. 1x phosphate-buffered saline (PBS) (pH 7) (1 L)
 - a. To prepare this buffer you need to add NaH_2PO_4 to 0.6712 moles/L and Na_2HPO_4 to 0.3288 moles/L.
 - b. The amount of acid and base is calculated using the Henderson-Hasselbalch equation $[\text{pH} = \text{pKa} + \log (\text{Base})/(\text{Acid})]$.
 - c. Prepare just under a litre of the solution using the correct amounts of NaH_2PO_4 and Na_2HPO_4 in dH_2O .
 - d. Check the pH using a pH meter and adjust slightly if necessary using phosphoric acid or sodium hydroxide.
 - e. Once the pH is adjusted, bring the volume to 1 L.
2. HRP developing solution (50 ml)

Mix 45 ml of dH_2O with 5 ml of 1 M sodium acetate buffer (pH 6)

Add 500 µl of tetramethyl benzidine

At the last minute add 50 µl of 6% H_2O_2

Note: Adding hydrogen peroxide starts the reaction. It is therefore important to add it at the last minute to avoid high background signal.

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