Chapter 13

Screening of Transgenic Cotton Based on a Porous Silicon Biosensor

Liangliang Chen, Peng Li, Xiaoyi Lv, and Ji Ma

Abstract

Label-free porous silicon (PSi)-based biosensor enables the detection and identification of the protein in transgenic cotton. Changes in optical response signal in the presence of the target protein can be detected by Fourier transform infrared (FTIR) spectromicroscopy when binding of the target protein with its antibody is selectively captured on the PSi biosensor. Here we describe the transgenic protein (antifreeze protein, AFP), and compare with non-transgenic plants. Significant red shifts are observed for transgenic antifreeze protein cotton lines.

Key words Transgenic cotton, Antibody, Porous silicon, Protein biosensor

1 Introduction

Traditionally, Northern blotting and Western blotting are used for identifying the expression of a foreign gene in transgenic plants. However, these blotting methods are limited in screening the transgenic individuals at large scale in the field because they are time consuming, cost expensive, as well as technologically complex. Thus, a label-free, efficient, and low-cost method for foreign gene detection in field is necessary.

Biosensor includes biological molecular recognition element (receptor) and signal converter (transducer). When the molecules under test binds specifically to the receptor molecules, changes in light or heat can be detected by signal recognition device [1]. Porous silicon is an ideal base material for sensor with its features of very large internal surface area, low cost, full compatibility with existing integrated circuit, etc. [2, 3]. To use porous silicon to detect a target protein, the antibody of the target protein should firstly be fixed on the internal surface of the porous to generate a porous protein sensor. When the target protein specifically binds to the antibody in the protein sensor, the effective refractive index of the porous silicon will increase [2]. The

Baohong Zhang (ed.), *Transgenic Cotton: Methods and Protocols*, Methods in Molecular Biology, vol. 1902,
https://doi.org/10.1007/978-1-4939-8952-2_13,

generated interference spectrum on the surface of the sample that is illuminated could be transformed into FTIR spectra in Fourier transform infrared spectroscopy (FTIR). By analyzing the changes of the shift in the interference value before and after the binding of the target protein, the quantitative and qualitative determination to the target protein can be implemented.

Label-free porous silicon (PSi) biosensors represent one promising platform for rapid and efficient detection of biomolecules, including proteins [4], oligonucleotides [5], and other small molecules [6]. The response optical signal of PSi biosensors is generated by changes in the refractive index due to affinity-binding events of biomolecules. Furthermore, detection signals are directly generated as reporters without additional labels [4]. Psi biosensors have many advantages including ease of sample preparation, label free, and low cost as well as high efficiency for simultaneous detection of a large number of samples [7]. Here, a novel label-free porous silicon-based biosensor was developed for the identification of transgenic frost-resistant cotton at the protein level [8]. Changes in the optical response signal were detected when target AFPs were selectively captured by the porous sensor with the use of AFP-antibody probes. Compared with the negligible spectral shift for non-transgenic cotton protein, varying degrees of red shifts were observed for all of the transgenic cotton lines. This result provides the foundation for the large-scale and rapid detection of transgenic cotton in the field.

2 Materials

Prepare all solutions using ultrapure water (prepared by purifying deionized water, to attain a sensitivity of 18 M Ω-cm at 25 °C) and analytical grade reagents. Prepare and store all reagents at room temperature (unless indicated otherwise). Prepare all reagents that have irritating smell in fume hood. Diligently follow all waste disposal regulations when disposing waste materials.

2.1 Single Layer Porous Silicon (PSi)

1. Monocrystalline silicon wafer: n-type (100) silicon, resistivity $\rho = 0.02–0.03\ \Omega$ cm, thickness 380 ± 10 μm (obtained from Tianjin institute of semiconductors, China).
2. Electrochemical etching solution: volume ratio of hydrofluoric acid (HF):ethanol = 1:3. Add 50 mL of HF (48%) to 150 mL ethanol (98%) in a 500 mL graduated cylinder (*see* **Note 1**).
3. 30% Hydrogen peroxide solution: purchased from Zhiyuan Chemical reagent, Tianjin, China (*see* **Note 2**).
4. Nitrogen gas.
5. Plastic tray.

2.2 Porous Silicon Activation

1. Silicon alkylation solution: water:methanol volume ratio 1:1 containing 5%APTES (3-aminopropyltriethoxysilane). Mix 9 mL methanol to 10 mL deionized water. Add 1 mL of APTES to the alcohol water mixture.
2. PBS containing 5% glutaraldehyde: add 1 mL of glutaraldehyde to 19 mL of PBS buffer solution (*see* **Note 3**).
3. Plastic tray.
4. Glass container.

2.3 Immobilization of Antibody

1. Phosphate buffered saline tween-20 (PBST): PBS containing tween-20. Add 1 mL of Tween-20 to 500 mL of PBS, adjust pH to 7.0–7.2 with 1 N HCl. Store at 4 °C for no more than 1 week (*see* **Note 4**).
2. Antibody of anti-antifreeze protein MpAFP698: Prepared as reported [8].
3. 3%BSA: weigh 3 g BSA and transfer to the cylinder. Add PBS to a volume of 100 mL. Mix and adjust pH to 7.0–7.2 with 1 N HCl. Store at 4 °C for no more than 1 week.
4. Plastic 18-well box.

2.4 Immunodetection of Antigen Protein

1. His-MpAFP698 protein: a recombinant antifreeze protein from the desert beetle *Microdera punctipennis* as reported [9]. Concentration 1 mg/mL.
2. Total protein from wild-type cotton: prepared by using plant total protein extraction kit (CWBIO, Beijing, China) containing 15 μL protease inhibitor. Protein concentration 1 mg/mL (*see* **Note 5**).
3. Total protein from the transgenic cottons: prepared from Kanamycin-resistant transgenic cotton plants by using plant total protein extraction kit (CWBIO, Beijing, China) containing 15 μL protease inhibitor. Protein concentration 1 mg/mL (*see* **Notes 5** and **6**).
4. Fourier transform infrared spectroscopy (FTIR, Brucker VERTEX70).

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

1. Cut the monocrystalline silicon wafer to a size of 2 cm × 2 cm. Immerse one piece of a silicon wafer into 15 mL electrochemical etching solution in the etching device (Fig. 1) (*see* **Note 7**). The device is connected to a computer controlled by

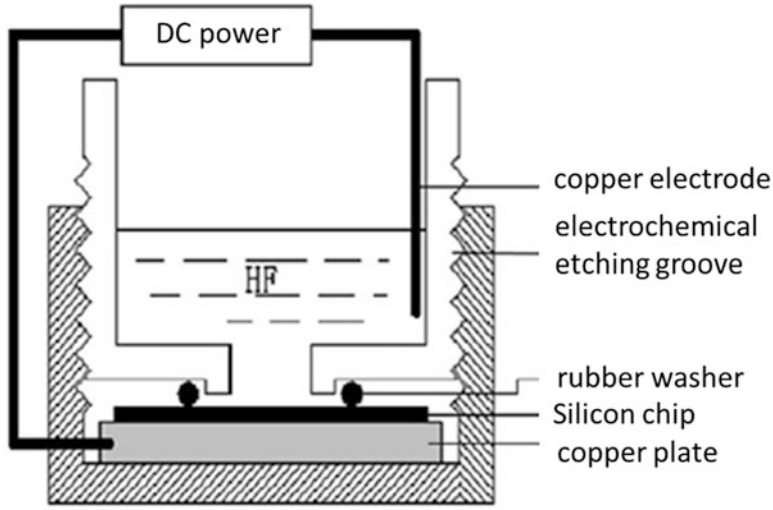

Fig. 1 Silicon etching device

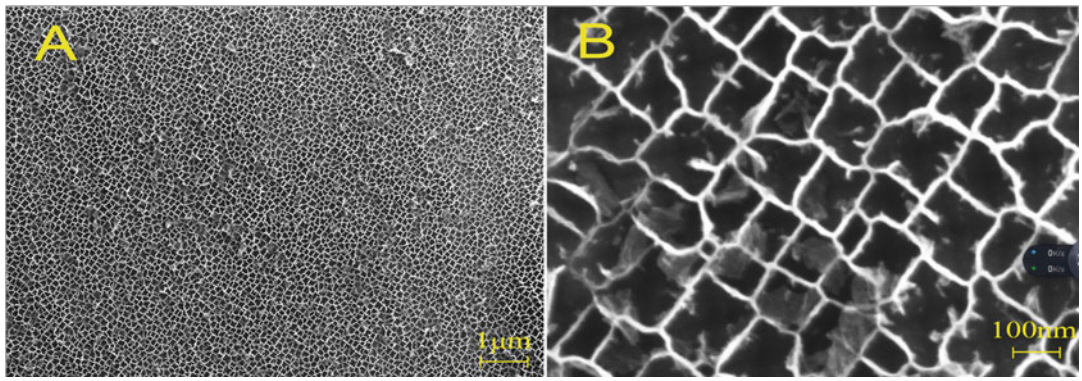

Fig. 2 Plan-view FESEM images of the Psi biosensor at 1 μm magnification (**a**) and at 100 nm magnification (**b**) [8]

3.1 Preparation of Single Layer Porous Silicon Thin Film Materials and Formation of Oxidative Film

Labview8.0 software. Etching time for each piece of silicon is 210 s with current density 60 mA/cm^2. Etch silicon wafers one by one.

2. Wash the etched silicon at least three times with deionized water (*see* **Note 8**). Dry the etched silicon in nitrogen drying cabinet. The quality of the porous silicon (PSi) chips is examined under field emission scanning electron microscopy (FESEM) (Fig. 2).
3. Put the freshly etched and washed PSi chips in array in a plastic tray. Add hydrogen peroxide solution to the chips (*see* **Note 9**). Put the tray at 55 °C incubator for 2 h.

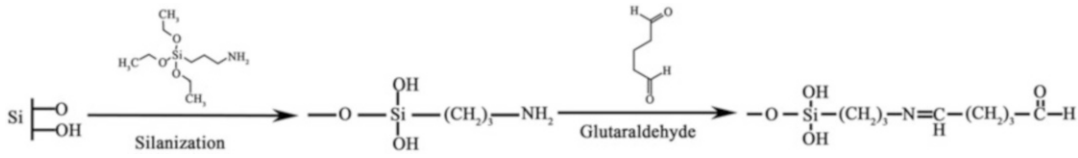

Fig. 3 Bio-functional process to PSi

3.2 Porous Silicon Chip Activation

The PSi bio-functional process consists of the following steps: oxidation, silanization, and glutaraldehyde treatment (Fig. 3).

1. Put the porous silicon chips in array in a plastic tray. Add silicon alkylation solution to the tray to immerse the chips. Soak the chips for 1 h for silicon alkylation (*see* **Note 10**).
2. Wash the silicon chips at least three times with deionized water (also *see* **Note 8**). Dry the chips in nitrogen drying cabinet. Transfer the chips in a glass tray, and put the tray in a vacuum drying oven at 100 °C for 10 min.
3. Soak the above chips in glutaraldehyde-PBS in a glass tray at 25 °C for 50 min (also *see* **Notes 3** and **10**).
4. Wash the silicon chips at least three times with deionized water (also *see* **Note 8**). Dry the chips in nitrogen drying cabinet.

3.3 Immobilization of Anti-Antifreeze Protein Antibody

1. Put one PSi chip in one well of a plastic 18-well box. In total, 18 chips can be treated once a time. Add 1 mL of PBST to each well to immerse the chips. Gently shake (10–20 r/min) the box on a shaker for 5 min.
2. Gently dry the chips with a hair drier blowing room temperature wind for 5 min.
3. Add 40 μL (drop by drop) of antibody of antifreeze protein (MpAFP698) onto one side of a chip to cover the chip surface (*see* **Note 11**).
4. Put the 18-well box containing chips at 37 °C for 2 h in an incubator (*see* **Note 12**).
5. Add 1 mL of PBST to each well to immerse the chips. Gently shake (10–20 r/min) the box on a shaker for 5 min. Repeat the PBST wash process three times to remove the uncoupled antibody.
6. Repeat **step 2**.
7. Add 1 mL of 3% BSA to each well to immerse the chips.
8. Put the 18-well box containing chips at 37 °C for 2 h in an incubator.

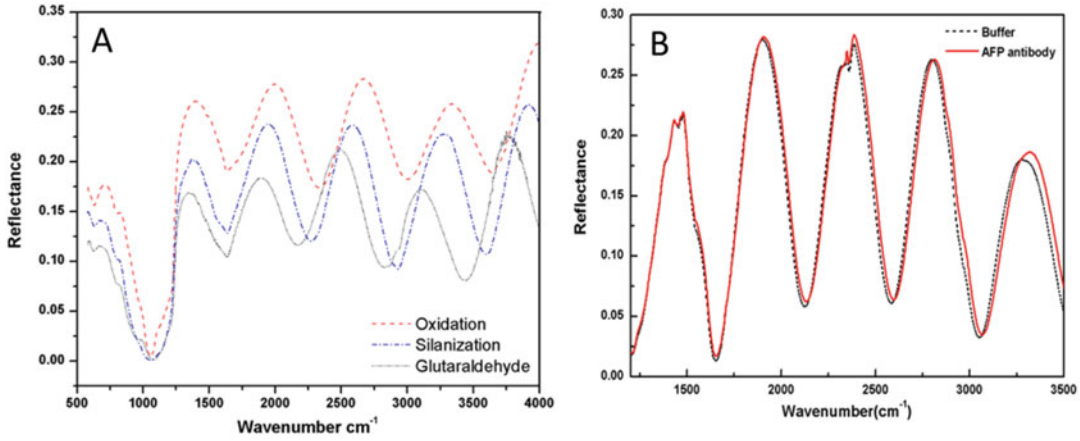

Fig. 4 Quality check of the PSi biosensor. (**a**) Reflection spectrum of the PSi chip after successive oxidation, silanization, and glutaraldehyde treatments. (**b**) No reflection shift upon exposure of the PSi chip to buffer solution [8]

9. Repeat **step 5**.
10. Repeat **step 2**.
11. Detect the infrared absorption spectrum of the PSi biosensor with Fourier transform infrared spectromicroscopy (FTIR) with a resolution of 0.4 cm^{-1}. The FTIR detection can be performed after each of the treatments on the PSi chips to check the quality of the PSi biosensor (Fig. 4).

3.4 Immuno-detection of Antifreeze Protein MpAFP698

Set three groups of samples: His-MpAFP698 protein (positive control), wild-type cotton protein (negative control), and protein samples from transgenic kanamycin-resistant cottons. The two control groups have three repeats, respectively. The number of the transgenic samples varies depending on the number of the kanamycin-resistant cotton plants.

1. Add 40 μL of protein solutions onto one chip drop by drop to cover the chip surface. After one group of the chips are finished, neatly put them in a 6-well container (*see* **Note 13**), and put the container in an incubator at 37 °C for 1 h.
2. Add 1 mL of PBST solution to each well to immerse the chip. Gently shake (10–20 r/min) the box on a shaker for 5 min, repeat the PBST wash process three times to remove the uncoupled antibody.
3. Gently dry the chips with a hair drier blowing room temperature wind for 5 min.
4. Repeat **step 3**.
5. Repeat **step 4**.

6. Add 1 mL of 3% BSA to each well to immerse the PSi chip. Put the chips at 37 °C for 2 h in an incubator.
7. Repeat **step 4**.
8. Detect the infrared absorption spectrum of the PSi biosensor with Fourier transform infrared spectromicroscopy (FTIR) with a resolution of 0.4 cm^{-1}. The process of the PSi biosensor detection is schematically shown in Fig. 5. Results of the infrared absorption spectrums of the PSi biosensor for the positive control (His-MpAFP698) and negative control (wild-type cotton) are shown in Fig. 6. Results of the infrared absorption spectrums of the PSi biosensor for the kanamycin-resistant transgenic cotton plants are shown in Fig. 7.

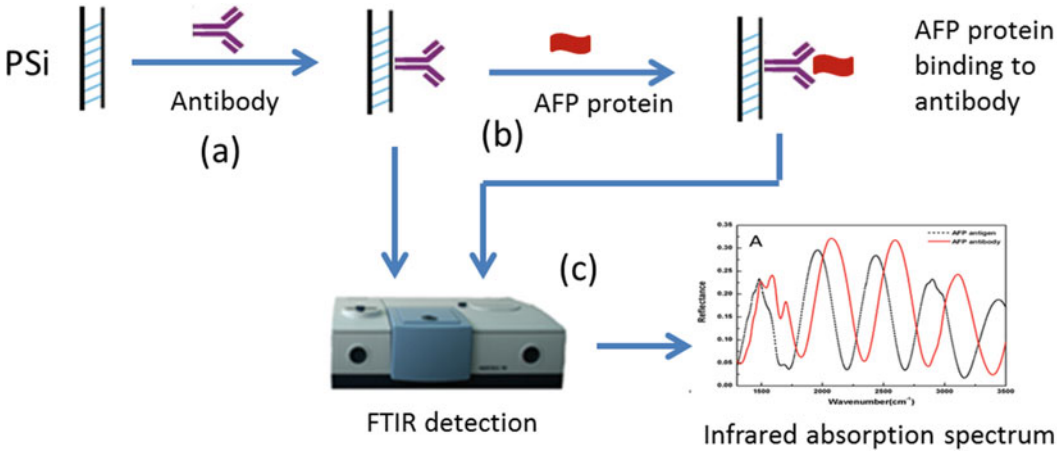

Fig. 5 Schematic illustration of the PSi biosensor for protein detection. Covalent binding of an antibody to the PSi chip (**a**), affinity binding of target (AFP) protein to the antibody (**b**), and reflectivity spectra detection (**c**)

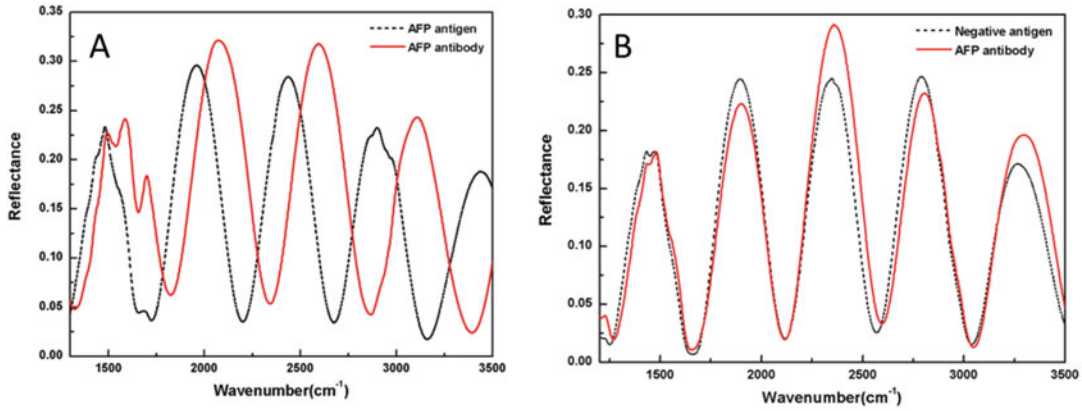

Fig. 6 Detection of the infrared absorption spectrum of the PSi biosensor. (**a**) Reflection shift of the PSi chip upon MpAFP antibody infiltration (solid line) and MpAFP binding (dashed line). (**b**) Negligible reflection shift of the PSi chip with MpAFP antibody after infiltration with negative antigen (non-transgenic cotton protein) [8]

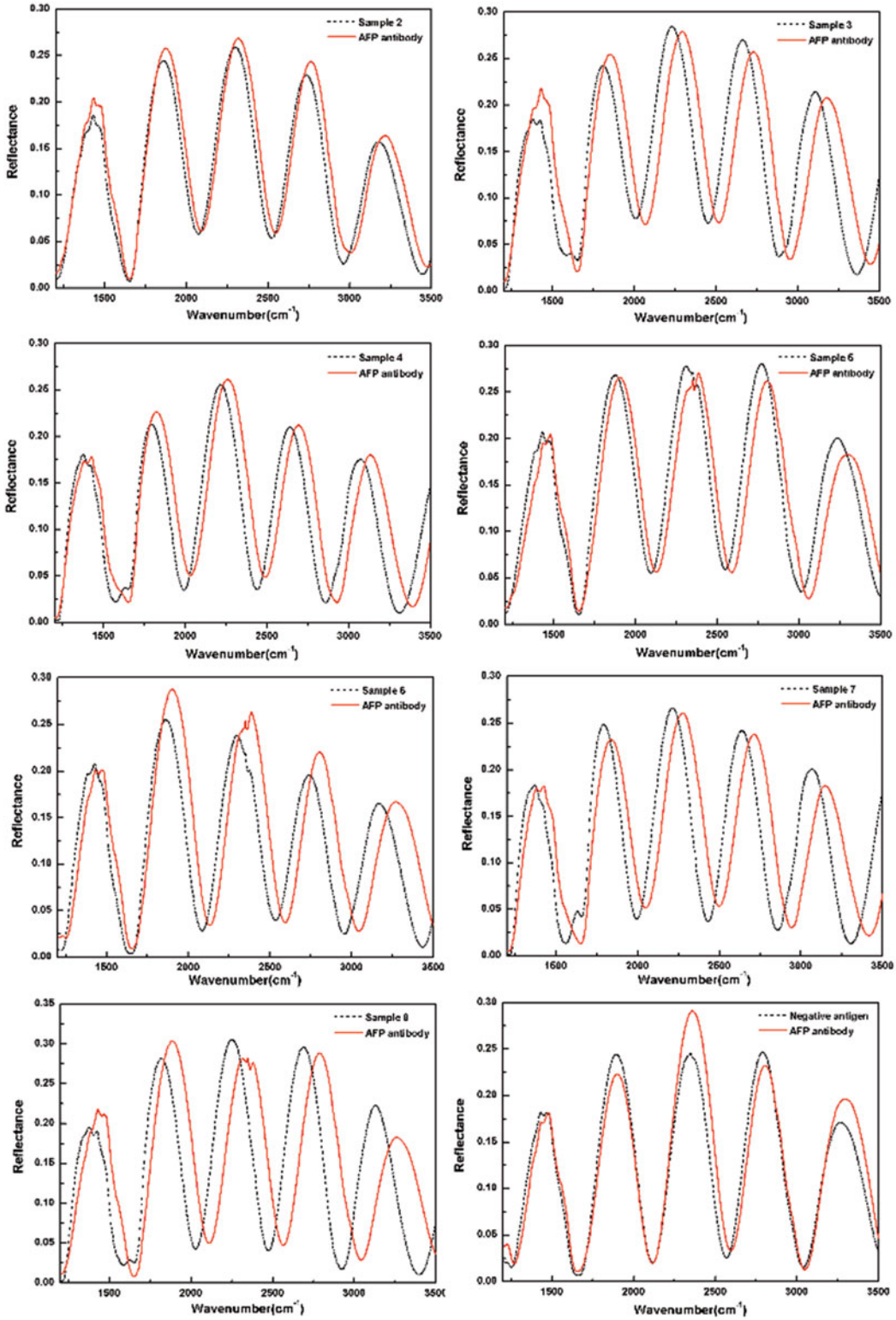

Fig. 7 Infrared absorption spectrums of the PSi biosensor for lines of transgenic cotton plants [8]

4 Notes

1. Hydrofluoric acid (HF) is toxic and has strong irritating smell. Make solutions in fume hood. HF is corrosive; it should be kept in airtight plastic bottles.
2. Hydrogen peroxide is a strong oxidant, and care should be exercised to avoid skin contact.
3. Glutaraldehyde has a pungent odor; it has a strong stimulating effect on eyes, skin, and mucous membranes. Wear a mask and make solutions in fume hood. Care should be exercised to avoid skin contact.
4. It is better to make the phosphate buffered saline Tween-20 (PBST) freshly before use.
5. Pick up cotton tender leaf and wrap with foil. Mark the sample, and immediately put it in liquid nitrogen (If the cotton is planted in laboratory, keeping leaf sample in liquid nitrogen is not required). Precool mortars at −20°C freezer. Weigh 0.3–0.5 g cotton leaf, and cut up, put in the precold mortar. Add 3 mL of protein extraction solution, and a small amount of silica sand. Rapidly grind the leaf into homogenate. During grinding, the mortar should be put in the ice. Transfer the homogenate into a 15 mL centrifuge tube. Use 5 mL extraction solution (divided into two times) to wash the mortar to let the remained homogenate into the centrifuge tube. Centrifuge at 10,000 r/min at 4 °C for 20 min. The supernatant is the soluble protein extracts. To avoid contamination, one mortar only used for one sample.
6. It is better that the transgenic cotton be checked primarily by kanamycin resistance in field before the PSi biosensor detection so as to avoid too much work.
7. Wear a mask to carefully and slowly add 200 mL of the HF solution into the groove. Silicon wafer is fragile. Carefully put one silicon wafer in the copper plate to avoid any injury.
8. Prepare three plastic trays containing deionized water. Carefully take out the chip with a pair of forceps, and rapidly put it in the first tray. Rinse the chip and transfer to the next tray, rinse again. Transfer it to another tray and rinse. Fresh deionized water is required for the wash of each chip.
9. To make a complete oxidation, avoid the chips floating. We make the liquid surface just immerse the chips. The chips should not be stacked together.
10. To ensure a complete alkylation, avoid the chips floating. Let the liquid surface just immerse the chips. The chips should not be stacked together.

11. When dropping the antibody solution onto the chip, be careful to let the entire surface to be covered. It is easy to identify the side that has been covered with antibody as it becomes smooth.
12. After all the chips have finished the antibody immobilization, put them in an incubator at 37 °C for 2 h.
13. To avoid samples confusion and contamination in the successive washing steps, it is better to put the chips of the same group in one container, and mark them clearly on the outside wall of the container.

Acknowledgment

This work was supported by NSFC grant (No. 31060292) and a key grant from the Ministry of Agriculture of China (No. 2009ZX08005-022B).

References

1. Wu BL, Zhang GM, Gao CG, Shuang SM (2004) Application and development of biosensors. China Biotechnol 24:65–69
2. Lin VS, Motesharei K, Dancil KP, Sailor MJ, Ghadiri MR (1997) A porous silicon-based optical interferometric biosensor. Science 278:840
3. Meskini O, Abdelghani A, Tlili A, Mgaieth R, Jaffrezicrenault N, Martelet C (2007) Porous silicon as functionalized material for immunosensor application. Talanta 71:1430–1433
4. Ouyang H, DeLouise LA, Miller BL, Fauchet PM (2007) Label-free quantitative detection of protein using macroporous silicon photonic bandgap biosensors. Anal Chem 79:1502–1506
5. Rong G, Najmaie A, Sipe JE, Weiss SM (2008) Nanoscale porous silicon waveguide for label-free DNA sensing. Biosens Bioelectron 23:1572–1576
6. Alvarez SD, Derfus AM, Schwartz MP, Bhatia SN, Sailor MJ (2009) The compatibility of hepatocytes with chemically modified porous silicon with reference to in vitro biosensors. Biomaterials 30:26
7. And DL, Miller BL (2005) Enzyme immobilization in porous silicon: quantitative analysis of the kinetic parameters for glutathione-s-transferases. Anal Chem 77:1950–1956
8. Chen LL, Li P, Zhao J, Li J, Lv XY, Jia ZH, Ji M (2014) Screening of transgenic frost-resistant cotton using a porous silicon biosensing platform. Cryo Letters 35:70–76
9. Qiu LM, Wang Y, Wang J, Zhang FC, Ma J (2010) Expression of biologically active recombinant antifreeze protein his-mpafp149 from the desert beetle (*Microdera punctipennis dzungarica*) in *Escherichia coli*. Mol Biol Rep 37:1725–1732