

## RESEARCH ARTICLE

# The p16<sup>INK4a</sup> Antibody Immobilization Method for Immonosensor Application

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### Abstract

**Background:** The p16<sup>INK4a</sup> is a protein that expressed in Liquid-based cervical cytology specimens and has been proved link to cervical cancer. The p16<sup>INK4a</sup> could be detection by piezoelectric immunosensor and the immobilization of the p16<sup>INK4a</sup> antibody influence the sensitivity of the piezoelectric immunosensor. **Materials and Methods:** 5μL mouse polyclonal antibody against p16<sup>INK4a</sup> was bound onto the surface of immonosensor through two methods. (directly immobilized method; protein A method). Absorb of the p16<sup>INK4a</sup> antibody on the surface of immonosensor caused a shift in the resonant frequency of the immunosensor and The frequency changes recorded showed a better reproducibility. The activity of the immobilization antibody with the directly method and protein A method was tested with p16<sup>INK4a</sup> antigen. **Results:** The resonant frequency for different antibody immobilization methods were different, and the sensitivity for p16<sup>INK4a</sup> detection also different. **Conclusions:** The protein A method was found to be much more better than the directly method for the immobilization of the p16<sup>INK4a</sup> antibody on the gold electrode of the quartz crystal for cervical lesion detection. The Protein A method created more reproducible and stable immobilization antibody layers with p16<sup>INK4a</sup> antigen.

**Keywords:** p16<sup>INK4a</sup> - cervical cancer - protein A - antibody immobilization

*Asian Pac J Cancer Prev*, 16 (12), 5115-5118

### Introduction

The cervical cancer is one of the most common cancers in developing Asian countries (Razak et al., 2013). The wide application of cytologic screening programs is a effective way in reducing cervical cancer incidence and mortality (Sahebali et al., 2006; Darragh et al., 2012; Eralp et al., 2013). Cervical screening using a Papanicolaou (Pap) test or liquid-based cytology to detect abnormal cells that may develop into cancer. If abnormal cells are found, women are invited to have a colposcopy. During a colposcopic inspection, biopsies can be taken and abnormal areas can be treated and prevent them from developing into cervical cancer. But, conventional cytologic diagnosis is often hampered by high false-negative rates compared with biopsy and interobserver variability (Bergeron et al., 2010). Another question arose as to whether all women with minimally abnormal tests needed such an expensive and uncomfortable procedure. We should find another ancillary test that could better separate those women who required colposcopy from those who did not. The use of biomarkers in both cervical cytology and histology has demonstrated the ability to overcome both false-positive and false-negative results, leading to improved positive predictive value of cervical screening results

Human papillomavirus (HPV) is the most common sexually transmitted infection (STI), nearly all sexually active men and women get it at some point in their lives.

Most HPV infections are subclinical and regress in two years; however, in Some people subclinical infections will become clinical and may cause benign papillomas (such as warts or squamous cell papilloma), or persistent infection with “high-risk” HPV types may progress to precancerous lesions and eventually into invasive cancer of the cervix, vulva, vagina, penis, oropharynx and anus (Ekalaksananan et al., 2006; Jing et al., 2013). Detection of molecular alterations caused by HPV in host cells could potentially be used as an adjunct to cytological screening. Among these candidate biomarkers, only p16<sup>INK4a</sup>, one of the cyclin-dependent kinase inhibitors, has sufficient evidence on which to make recommendations to apply to HPV-associated precancer lesions (Eleuterio et al., 2009).

Previous studies suggested that amid most of the cancers, p16<sup>INK4a</sup> protein is in under-expression while amid the cervical cancer, the p16<sup>INK4a</sup> is over-expressed, and closely related to HPV infection and cervical cancer development (Ishikawa et al., 2006). Ishikawa also found that in the high-risk HPV the p16<sup>INK4a</sup> positive expression rates reached 32.4%, 82.1%, 93.2% and 100%, it is obviously that all the HR-HPV specimens showed protein expression and the expression rate increased with the lesion progression. So, p16<sup>INK4a</sup> could be a biomarker to predict the outcomes of cervical lesions.

Immunosensor are device which utilize antigen-antibody reactions for detecting target analytes. It could

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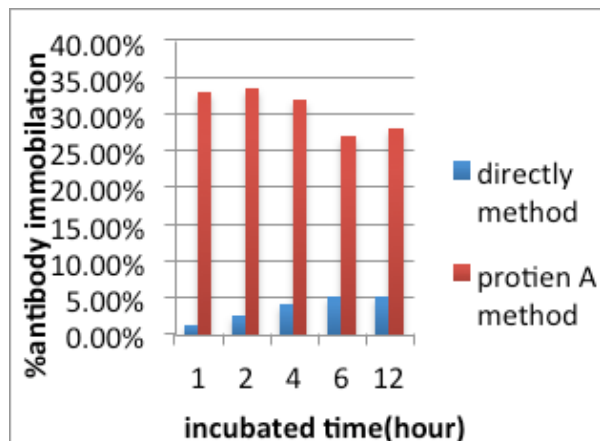
be used for clinical diagnose if the correct biomarker is choose. The quartz crystal microbalance (QCM) has been widely used as highly sensitive immunosensor because of the piezoelectricity property. The QCM commonly configure with electrodes on both sides of a thin disk AT-cut quartz which had nearly zero frequency drift with temperature around room temperature. In the late 1950s ,Sauerbrey found the relationship between resonant frequency and mass deposit onto surface of quartz in gas phase .Since the work of Sauerbrey, the quartz crystal microbalance (QCM) has been widely used to monitor slight mass change (nanogram) in many research area such as biology, physics, medicine and so on. The relationship of frequency to mass can be described as the Sauerbrey's equation (Sauerbrey et al., 1956).

$$\Delta f = -\frac{2f_0^2}{A(\mu_q \rho_q)^{1/2}} \Delta m \quad (1)$$

Where  $f_0$  is the operating frequency of the QCM,  $\Delta f$  is the observed frequency change (in Hz) caused by mass change.  $\mu_q$  and  $\rho_q$  are the shear modulus and density of the crystal respectively,  $A$  is the active electrode area, and  $\Delta m$  is the mass change on the surface of the crystal (in ng). in this study,  $f_0$  equal to 10MHz,  $\rho_q$  and  $\mu_q$  are equal to 2.65 g/cm<sup>3</sup> and 2.95N/m<sup>2</sup> respectively. numerical substitution in Sauerbrey's equation yields:

$$\Delta m = 2.213 \Delta f \quad (2)$$

Thus, if a substance with the weight of 1ng is homogeneously attached to the active area of crystal, a frequency change of 0.4518 Hz.



**Figure 1. The Yield for Antibody Immobilization on Surfaces of Quartz Crystals at Different time with Directly Method and Protein A Method**

**Table 1. The Frequency Shift Caused by Absorb of Antibody**

Crystal number	1	2	3	4	5	6	7	8	9	average
$\Delta f_1$ (Hz)	300	296	308	302	302	319	298	305	300	303
$\Delta f'_1$ (Hz)	1875	1762	1826	1895	1912	1937	1823	1917	1902	1876

**Table 2. The frequency shift caused by antibody-antigen reaction**

Crystal number	1	2	3	4	5	6	7	8	9	average
$\Delta f_2$ (Hz)	198	196	108	202	172	219	198	205	200	187.5
$\Delta f'_2$ (Hz)	920	904	895	901	903	890	900	907	902	902.45

Used the p16<sup>INK4a</sup> as a biomarker, the quartz crystal microbalance could be used as immnosensor for early cervical cancer detection. The immobilization of the p16<sup>INK4a</sup> antibody on the surface of QCM directly influence the sensitivity of the p16<sup>INK4a</sup> detection in supernatant of different clinical sample. It is very important to find a suitable immobilization method for p16<sup>INK4a</sup> antibody for early cervical cancer detection.

In this study, two methods for p16<sup>INK4a</sup> antibody immobilization were proposed. The effect of the immobilization methods on the resonant frequency of quartz crystal was observed and the activity of immobilized antibodies to p16<sup>INK4a</sup> antigen was tested.

## Materials and Methods

### Materials

Staphylococcal protein A (SPA) and blocking reagent bovine serum albumin (BSA) were purchased from Sigma Co. (U.S.A). Human P16<sup>INK4A</sup> full length protein (ab84075) was purchased from Abcam Co. (U.S.A). Antibodies to p16<sup>INK4a</sup> (mouse monoclonal antibody, clone 6H12) were purchased from Maixin Co. (Huzhou, China). Phosphate-buffered saline (PBS) was composed of 137 mM NaOH, 2.7 mM KCL, 8.0 mM Na2HPO4, and 1.5 mM KH2PO4 (pH 7.2). All the PZ crystals were AT-cut and They were purchased from Tongfang Guoxin Electronics co., Ltd (Beijing, China). The crystal (diameter: 8 mm) was placed between two gold electrodes, mounted in metal holder. An symmetric electrode pattern was used so that the upper electrode and the lower electrode had same radius (2.5mm). The quartz crystal was driven at its resonant frequency with a homemade oscillator circuit and frequency measurement was performed with Agilent 531323a , with a precision of 0.1 Hz at a gate time of 0.1s.

### Measurement procedures

In order to detection the p16<sup>INK4a</sup> antigen, the antibody for p16<sup>INK4a</sup> was immobilized on the surface of piezoelectric quartz crystal.

### Directly immobilization

In order to obtain a hydrophilic gold surface, the quartz crystal was dipped in NaOH and HCl for 10 minutes respectively, the concentration of the NaOH and HCl are all 1.2M. After this procedure, the quartz crystal was washed with dual-distilled water and ethanol twice, then drying at the room temperature. The fundamental

resonant frequency of the quartz crystal which record as  $F_0$  was measured by frequency counter. The 5  $\mu$ L (5mg/ml) p16<sup>INK4a</sup> antibody was directly dropped on the surface of cleaned quartz crystal by a 5- $\mu$ L syringe. The quartz crystal which applied by p16<sup>INK4a</sup> antibody on the surface was incubated over silica gel blue at 4°C. Different incubated time were performed (1hour, 2hours, 6 hours, 12hours) in order to get relationship between the yield for antibody immobilization to the incubated time. Finally, the crystal was dripped into PBS (PH7.2) solution and wash with distilled water twice, make the crystal air drying and the resonance frequency in gas phase ( $F_1$ ) was measured.

#### Protein A immobilization

The cleared procedure for quartz crystal as described above. A 5  $\mu$ L staphylococcal protein A solution in PBS (pH 7.2) with concentration of the 1 mg/mL was evenly spread on the up side of the crystal for 20 min, then rinse with distilled water to remove the excessive SPA solution, make the quartz crystal air drying and the frequency of QCM which record as  $F_0'$  was measured. 5  $\mu$ L p16<sup>INK4a</sup> antibody solution with concentration of 5.0 mg/mL was applied to the crystal surface. the quartz crystal was incubated over silica gel blue at 4°C for different time (1hour, 2hours, 6 hours, 12hours), then dip the quartz crystal to PBS (PH7.2) solution and wash with distilled water twice, making air drying and measured the resonance frequency of quartz crystal and recorded as  $F_1'$ .

#### Yield for antibody immobilization

The mass on the crystal changed with the absorb of the antibody on the surface of quartz crystal. Because of the piezoelectric for quartz crystal, the resonant frequency of quartz crystals shifted. The Sauerbrey's equation give the relationship between shifted frequency to added mass. Then the yield for antibody immobilization was calculated as following equation:

$$\% \text{antibody immobilization} = \Delta m / m \% \quad (3)$$

Where  $\Delta m$  is the added mass caused by immobilized antibody,  $m$  is the mass for the antibody applied on the surface of quartz crystal. In this study, 5  $\mu$ L p16<sup>INK4a</sup> antibody solution with concentration of 5.0 mg/mL was applied on the crystals, so the  $m$  equal to 25  $\mu$ g.

## Results

#### Determination of incubated time

Nine quartz crystals were used in this study. All crystals were applied p16<sup>INK4a</sup> antibody through directly method and protein A method. After different incubated time, resonant frequency of crystals were measured. The frequency shift caused by absorb of antibody was calculated as equation4 and equation5:

$$\Delta f_1 = F_0 - F_1 \quad (4)$$

$$\Delta f_1' = F_0' - F_1' \quad (5)$$

Use the equation (2), the added mass caused by immobilized antibody ( $\Delta m$ ) was calculated, then the

%antibody immobilization was determination.

The relationship between %antibody immobilization to incubated time for different method were showed as figure1 :

Figure1 The yield for antibody immobilization on surfaces of quartz crystals at different time with directly method and protein A method.

As shown in Figure1, the best incubated time for directly method to get higher yield of p16<sup>INK4a</sup> antibody immobilization is 6hours. The best incubated time for protein method is 1hour.

#### The form of the antibody layer

For directly immobilization method, after 6hours incubated, the antibody layer was performed and the resonant frequency ( $F_1$ ) was measured. The frequency shift caused by absorb of antibody was calculated as equation4. For protein A immobilization method, after 1hours incubated, the resonant frequency ( $F_1'$ ) was measured. The frequency shift caused by absorb of antibody was calculated as equation5, The resonant frequency shift for all nine quartz crystals were showed as table1:

Using the equation2 and equation3, the yield of antibody immobilization for directly method and protein A method were 5.32% and 33.16% respectively.

#### The response for p16<sup>INK4a</sup> antigen

The antibody coated quartz crystals with directly method and protein A method were used to detect p16<sup>INK4a</sup> antigen. All the crystals were dipped into a stirred 2 mL PBS containing 1000 ng/mL of antigen (Human p16<sup>INK4a</sup> full length protein) for 30 min at room temperature. When the reaction between the immobilized antibody and p16<sup>INK4a</sup> was over, dip the quartz crystal to PBS (PH7.2) solution and wash with distilled water twice, making air drying and measured the resonance frequency of quartz crystal and recorded as  $F_2$ . The frequency shifts specially induced by antibody-antigen reaction for directly method and protein A method were calculated as equation (6) and equation (7) respectively :

$$\Delta f_2 = F_1 - F_2 \quad (6)$$

$$\Delta f_2' = F_1' - F_2' \quad (7)$$

The resonant frequency of all crystals changed. For directly method, the average frequency shift caused by p16<sup>INK4a</sup> (2ml 1000ng/ml) was 187.5Hz. It is obviously that only 414ng p16<sup>INK4a</sup> antigen was detection. For protein A method, the average frequency shift caused by p16<sup>INK4a</sup> (2ml 1000ng/ml) was 902.45Hz and 1994ng p16<sup>INK4a</sup> antigen was detection.

## Conclusion

The protein A method was found to be much more better than the directly method for the immobilization of the p16<sup>INK4a</sup> antibody on the gold electrode of the quartz crystal for cervical lesion detection. The Protein A method created more reproducible and stable immobilization antibody layers with p16<sup>INK4a</sup> antigen.

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