

# Establishment of a novel immunoassay system for rapid detection of 2,4-dichlorophenoxyacetic acid residues based on magnetic-fluorescent probes

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**Abstract:** A novel immunoassay system based on magnetic-fluorescent probes was established to detect 2,4-dichlorophenoxyacetic acid (2,4-D) residue in liquid system in food and agricultural products. The composites of anti-2,4-D antibody bound to  $\text{Fe}_3\text{O}_4@\text{SiO}_2\text{-NH}_2$  was employed as the solid phase as well as magnetic probe. The composites composed of 2,4-D-OVA labeled with  $\text{CdTe}@\text{SiO}_2\text{-NH}_2$  as the fluorescent probe was used to produce fluorescent signal. 2,4-D and its fluorescent probe competed binding the antibody on the surface of the magnetic probe. The optimization of 2,4-D-OVA dosage, coupling pH and reaction time in preparing the fluorescent probe were investigated. It showed that in the synthesis of fluorescent probe 8.2 was the optimal pH, 70 min was the optimal coupling time, 500  $\mu\text{L}$  amount of 2,4-D-OVA. The standard curve was obtained with the concentration of 2,4-D and the maximum fluorescence intensity. The detection limit of the assay was gotten and it was  $3.55 \times 10^{-8}$ . One reaction step and one washing step were needed. The assay significantly shortened the testing time and amplified the detection signal compared with classic ELISA.

**Key words:** immunoassay; fluorescence probe; magnetic probe; 2,4-D; quantum dots

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## 1 Introduction

2,4-Dichlorophenoxyacetic acid is one of the most widely used herbicides and plant growth regulator in agriculture, forestry and right-of-way applications. 2,4-D has been used and monitored for several decades. It has been reported it concerned with the occurrence of cancer in humans<sup>[1]</sup>, endocrine-disrupting activities<sup>[2]</sup>, acute congestion and degenerative changes in central nerve system<sup>[3]</sup>. Presently, an increasing number of countries strictly limited the residues of 2,4-D in grain, fruit and vegetables. Thus, it is essential to develop a more efficient method for detection of 2,4-D.

Several methods have been described for the determination of 2,4-D, such as Thin-layer chromatography,

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chromatography (GC), high performance liquid chromatography (HPLC), gas chromatography mass spectrometry (GC/MS) and liquid chromatography mass spectrometry (HPLC/MS) [4-7]. However, they are always complicated operation, time-consuming, even require costly and bulky instrumentation. Immunoassay is a common useful assay for biochemical analysis. The strong, specific binding of an antibody to its antigen has been widely exploited in biochemical studies, sensor design, clinical diagnostics, environmental monitoring, and food safety.

Semiconductor quantum dots (QDs) exhibit a lot of unique optical properties compared with organic fluorescent dyes such as sharp emission band with broad excitation, size-controlled tunable maximum wavelength of emission, higher fluorescence intensity, and strong resistance to photo bleaching, which can be exploited in biological imaging and bio-conjugation [8-12]. Magnetic nanoparticles (NPs) show some special magnetic properties including superparamagnetism, lower Curie temperature, and higher susceptibility, which have been widely used in magnetic storage media, catalysis, magnetic separation, and biomedicine. Composite nanoparticles of  $\text{Fe}_3\text{O}_4@ \text{SiO}_2$  and  $\text{CdTe}@ \text{SiO}_2$  can enhance the biocompatibility and hydrophilicity of single nanoparticles, thus they can be used as powerful tools for biochemical analysis and sensitive detection in environmental safety.

In this research, a new immunoassay system based on magnetic-fluorescent probes dedicated to 2,4-D rapid determination was systemically investigated. The antibody, labeled with  $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-NH}_2$  which serves as solid phase, is employed as magnetic probe. The 2,4-D-OVA, bound with  $\text{CdTe}@ \text{SiO}_2\text{-NH}_2$  is used as fluorescent probe. 2,4-D standard solution and fluorescent probe competed for the same antibody active sites. Furthermore, the optimization of dosage of 2,4-D-OVA, coupling pH and reaction time in preparation of the fluorescent probe were also studied.

## 2 Materials and methods

### 2.1 Reagents and instruments

Ethanol 2,4-D, 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC · HCl, 98.5%), N-Hydroxy succinimide (NHS, 98%), 3-aminopropyltriethoxysilane (APTS) (98%), ammonium hydroxide, Tris (hydroxymethyl) aminomethane (Tris), HCl, 3-(2-aminoethylaminol) propylmethyldimethoxysilane (AEAPS), tetraethyl orthosilicate (TEOS), Bovine serum albumin (BSA) and other reagents were of analytical grade and purchased from Aladdin. Red CdTe QDs ( $\lambda_{\text{em}} = 634 \text{ nm}$ ), anti-2,4-D polyclonal antibody (The antibody titer by ELISA method was 1:12 000,  $\text{IC}_{50}$  was 50 ng/mL) and complete antigen (2,4-D-OVA) were produced in our laboratory. Double distilled water was used through the process. Fluorescence spectrophotometer was from Varian company (America), 318k high-speed refrigerated centrifuge was from Sigma (America), KQ-250 ultrasonic cleaner was from Kunshan Ultrasonic Instruments Co., Ltd (China), RH KT/C Magnetic stirrers was from IKA (Germany), DHP-9082 cultural incubator with constant temperature was from Shanghai Yiheng Scientific instruments Ltd (China).

### 2.2 Preparation of 2,4-D magnetic probe

#### 2.2.1 Synthesis of $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-NH}_2$ composite particles

$\text{Fe}_3\text{O}_4@ \text{Organic Layer complex microspheres}$  were firstly synthesized by thermal decomposition method. Single magnetic microspheres coated with 30 nm of silica shell could be obtained with the reverse micro-emulsion system. Then the  $\text{Fe}_3\text{O}_4@ \text{SiO}_2$  magnetic composites were modified with AEAPS and  $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-NH}_2$  composite particles were prepared [17].

#### 2.2.2 Immobilization of anti-2,4-D antibody on $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-NH}_2$ composite particles

3.0 mg of  $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-NH}_2$  composite particles was adequately dissolved in 3.0 mL of phosphate-buff-

ered saline ( PBS pH = 8.2 0.2 mol/L) ,then the supernatant was discarded by means of magnetic separation. The pH value of the solution was adjusted twice by Tris-HCl buffer solution ( pH = 8.2 50 mmol/L) . Subsequently 200  $\mu$ L of anti-2,4-D antibody was added into the mixture and incubated at 37 $^{\circ}$ C for 30 min. After separating magnetically and washing with PBS for two times ,excess functional groups on Fe<sub>3</sub>O<sub>4</sub>@ SiO<sub>2</sub>-NH<sub>2</sub> composite was blocked by buffer ( PBS of 3% BSA) and then stored at 4 $^{\circ}$ C overnight. The product was magnetically separated from free antibody and washed with PBST ( PBS of 0.05% Tween-20) for three times. Finally the sample was re-suspended boric acid buffer solution ( pH = 8.2 0.2 mol/L) and stored at 4 $^{\circ}$ C before used ( Scheme 1b) .

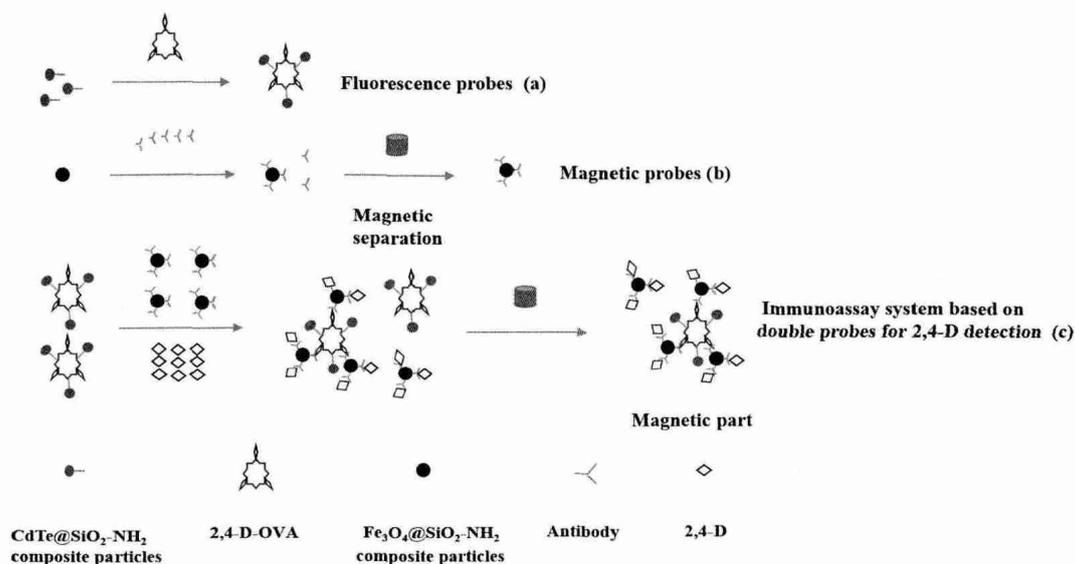
### 2.3 Preparation of 2,4-D fluorescent probe

#### 2.3.1 Synthesis of CdTe@ SiO<sub>2</sub>-NH<sub>2</sub> composite particles

The red CdTe nanocrystals ( Em = 634 nm) prepared under 105 $^{\circ}$ C was used to synthesis CdTe@ SiO<sub>2</sub>-NH<sub>2</sub> composite particles. 12 mL of CdTe QDs was added in 72 ml of ethanol and 15.2 mL of double distilled water. After dispersing adequately ,the mixtures were added by 8 mL of ammonium hydroxide and stirred for 10 min. Subsequently ,1 mL of TEOS was added in and stirred for 30 min. Finally 40  $\mu$ L of APTS was introduced under vigorous stirring for 4 h. The resulting product was centrifuged at 12 000 r  $\cdot$  min<sup>-1</sup> for 3 min and washed with double distilled water and ethanol for three times ,respectively. The sample was redissolved in double distilled water as a stock solution<sup>[16]</sup> .

#### 2.3.2 Conjugation of 2,4-D-OVA to CdTe@ SiO<sub>2</sub>-NH<sub>2</sub>

1.0 mL of CdTe@ SiO<sub>2</sub>-NH<sub>2</sub> solution was added into 10.0 mL of boric acid buffer ( 0.2 mol/L) with dispersing adequately. 4 mg of EDC  $\cdot$  HCl and 3 mg of NHS were dissolved in the mixtures and incubated at 37 $^{\circ}$ C for 10 min. Then 2,4-D-OVA was added into the mixtures and stirred for about 1 h. After centrifuging at 5 000 r  $\cdot$  min<sup>-1</sup> for 3 min ,the precipitation ( CdTe@ SiO<sub>2</sub>-NH<sub>2</sub>-2,4-D-OVA) was dispersed in boric acid buffer ( Scheme 1a) .



Scheme 1 (a) Synthesis of fluorescence probe; (b) Synthesis of magnetic probe;  
(c) The immunoassay system based on magnetic-fluorescent probes for 2,4-D detection

### 2.4 Optimization of reaction parameters in preparation of fluorescent probe

Several parameters were investigated systematically in order to synthesis the fluorescent probe with optimal

conditions, including the dosage of 2,4-D-OVA, pH value of boric acid buffer and coupling time.

### 2.5 Establishment of the 2,4-D immunoassay system based on magnetic-fluorescent probes

300  $\mu\text{L}$  of fluorescent probe was added into 2,4-D standard solution of different concentrations and dispersed adequately. Subsequently, 1.0 mL of magnetic probe was added into the mixture and incubated at 37°C for 40 min. 2,4-D standard solution and fluorescent probes competed binding the antibody on the surface of the magnetic probes. After magnetic separation, the magnetic part was washed three times with boric acid buffer solution to remove the residual fluorescent probe. Finally, the product was re-suspended in Boric acid buffer solution (0.2 mol/L, pH 8.20) and the fluorescent signal was monitored by the fluorescence photometer. Establishment of the immunoassay system was presented in Scheme 1c.

## 3 Results and Discussion

### 3.1 Optimization of the dosage of 2,4-D-OVA in preparation of fluorescent probe

As described in Section 2.3.2, 2,4-D-OVA at different dosage (100, 300, 500, 700  $\mu\text{L}$ ) was conjugated with CdTe@SiO<sub>2</sub>-NH<sub>2</sub> composite particles. Fluorescent signal was monitored by the fluorescence photometer to determine the maximum dosage of combination.

Figure 1 illustrated the fluorescence spectra of the fluorescent probes in different dosage of 2,4-D-OVA. As the dosage of 2,4-D-OVA increasing, the fluorescence intensity of the fluorescent probe gradually enhanced and subsequently remained stable when the dosage of 2,4-D-OVA arrived at 500  $\mu\text{L}$ . Hence, 500  $\mu\text{L}$  amount of 2,4-D-OVA was employed in the synthesis of fluorescent probe.

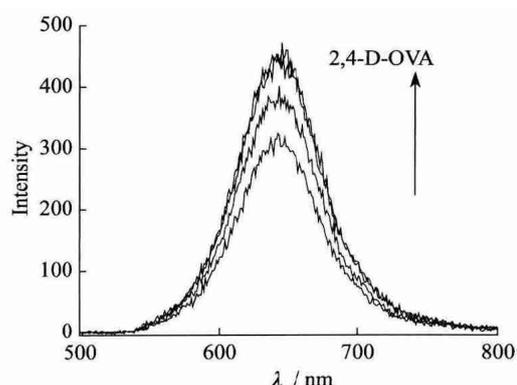


Figure 1 Fluorescence spectra of CdTe@SiO<sub>2</sub>-NH<sub>2</sub> in different dosage of 2,4-D-OVA (from bottom to top: 100, 300, 500, 700  $\mu\text{L}$ )

### 3.2 Optimization of PH value in preparation of fluorescent probe

In order to obtain the optimal fluorescent signal, different pH value of boric acid buffer (7.1, 7.4, 7.8, 8.2, 8.6, 9.0) were applied in the conjugation of 2,4-D-OVA to CdTe@SiO<sub>2</sub>-NH<sub>2</sub>.

Figure 2 showed the fluorescent intensity of CdTe@SiO<sub>2</sub>-NH<sub>2</sub> and fluorescent probe (CdTe@SiO<sub>2</sub>-NH<sub>2</sub>-2,4-D-OVA) in different pH. The effect of pH on the fluorescent intensity of CdTe@SiO<sub>2</sub>-NH<sub>2</sub> is comparatively small while the fluorescent intensity of CdTe@SiO<sub>2</sub>-NH<sub>2</sub>-2,4-D-OVA is easily affected. The fluorescent intensity of CdTe@SiO<sub>2</sub>-NH<sub>2</sub>-2,4-D-OVA reaches a maximum when the pH is 8.2, which is almost close to the fluorescent intensity of CdTe@SiO<sub>2</sub>-NH<sub>2</sub>. Thus, 8.2 is the optimal pH in the synthesis of fluorescent probe.

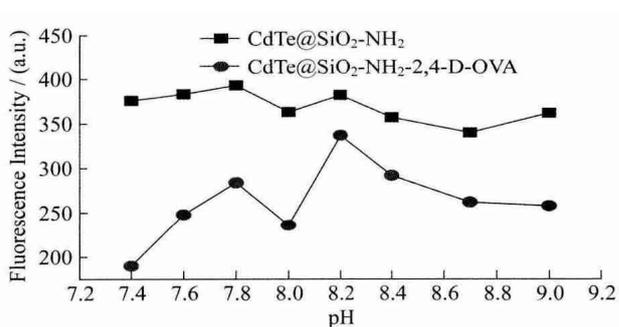


Figure 2 Effect of different pH value on the fluorescent intensity of CdTe@SiO<sub>2</sub>-NH<sub>2</sub> and CdTe@SiO<sub>2</sub>-NH<sub>2</sub>-2,4-D-OVA fluorescent probe

### 3.3 Optimization of coupling time in preparation of fluorescent probe

Effect of coupling time on the fluorescent intensity of fluorescent probe was examined and optimized. 30, 40, 50, 60, 70, 80 min were applied in the conjugation from 2,4-D-OVA to CdTe@SiO<sub>2</sub>-NH<sub>2</sub> (Figure 3). The

fluorescent intensity of fluorescent probe reached a maximum when reaction time arrived at 70 min. So that 70 min was the optimal coupling time for preparation of 2,4-D fluorescent probe.

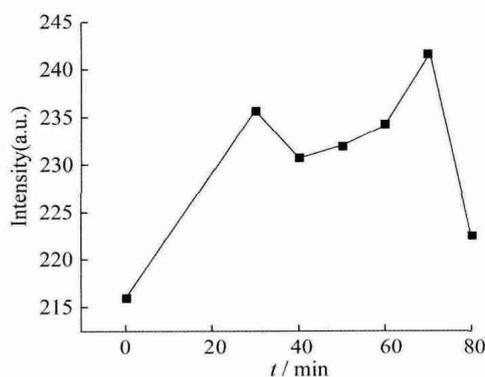


Figure 3 Effect of different coupling time on fluorescent intensity of fluorescent probe

### 3.4 Characterization of the immunoassay based on magnetic-fluorescent probes

The composites by the anti-2,4-D antibody labeled with  $\text{Fe}_3\text{O}_4@ \text{SiO}_2\text{-NH}_2$  served as solid phase, was employed as magnetic probe. The 2,4-D-OVA composite bonded with  $\text{CdTe}@ \text{SiO}_2\text{-NH}_2$  was employed as fluorescent probe. 2,4-D standard solution and fluorescent probe competed binding the antibody on the surface of the magnetic probe. The complex of 2,4-D magnetic probe and magnetic-fluorescent probe were formed, respectively. Only the complex of magnetic-fluorescent probe could produce a fluorescent signal which could indicate the presence of 2,4-D. If there was no 2,4-D in the detection system, the complex of magnetic-fluorescent probe was formed and in this case, the fluorescent intensity reaches a maximum (Figure 4A). Otherwise 2,4-D would compete with the fluorescent probe, which would weaken the fluorescent signal. In addition, the higher concentration of 2,4-D, the weaker intensity of fluorescent signal would be (Figure 3B-F).

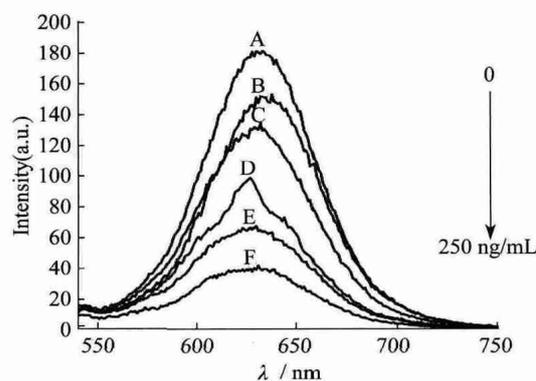


Figure 4 The fluorescent spectra of 2,4-D standard solution concentration of 0, 50, 100, 150, 200, 250 ng/mL for the immunoassay based on magnetic-fluorescent probes

For the immunoassay based on magnetic-fluorescent probes, the standard curve was obtained with the concentration of 2,4-D as the abscissa and the maximum fluorescence intensity as the ordinate (Figure 5). The regression equation was  $y = -0.5703x + 182.3714$  ( $R^2 = 0.99659$ ), which indicated that the fluorescent intensity was inversely proportional to the concentration of 2,4-D standard solution. However, deviation of the result would occur when the fluorescent signal approaches the maximum or the minimum. Therefore, the effective value should range from 10% ~ 90% of the maximum. The detection limits (LOD) of 2,4-D in this assay was  $35.5 \times 10^{-9}$  based on the curve.

Only about 1.0 h was needed to finish the procedure of the immunoassay based on magnetic-fluorescent probes with one reaction step and one washing step. However, to fulfill the procedure of the traditional ELISA, four reaction steps and three washing steps are needed and moreover more than 2.0 h were needed. Comparing with the flat solid phase, the easy separated and re-dispersed nature allowed for a "in solution" reaction, which also significantly shortened the reaction time and amplified the detection signal. Meanwhile, the LOD of 2,4-D

ELISA kit is  $5 \times 10^{-8}$  and that of 2,4-D colloidal gold strip is  $2.1 \times 10^{-5}$  in the market. It indicated the assay was also feasible.

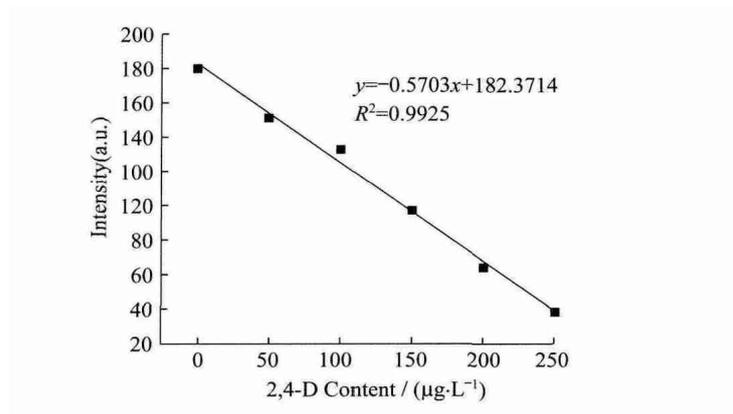


Figure 5 Calibration curve of the immunoassay based on magnetic-fluorescent probes for 2,4-D

## 4 Conclusion

In conclusion, this work established a novel immunoassay system for rapid detection of 2,4-D residues in liquid system. The composite of  $\text{Fe}_3\text{O}_4 @ \text{SiO}_2\text{-NH}_2$  labeled 2,4-D antibody served as a solid-phase carrier instead of ordinary coated microplate. The composite of  $\text{CdTe} @ \text{SiO}_2\text{-NH}_2$  bonded with 2,4-D-OVA was used to produce fluorescence signal as the fluorescence probe. It showed that 8.2 was the optimal pH, 70 min was the optimal coupling time, 500  $\mu\text{L}$  amount of 2,4-D-OVA in the synthesis of fluorescent probe. The standard curve was obtained with the concentration of 2,4-D and the maximum fluorescence intensity. The regression equation was gotten:  $y = -0.5703x + 182.3714$  ( $R^2 = 0.99659$ ). The detection limit of the assay was  $3.55 \times 10^{-8}$ . For the assay, one reaction step and one washing step were needed. The procedure could be finished within 1.0 h without complicated preparing procedures by competing reaction, and the detection limit is  $3.55 \times 10^{-8}$ . Moreover, the optimized parameters in preparation of fluorescent probe had been obtained. The results demonstrate that this immunoassay based on magnetic-fluorescent probes was a timesaving and feasible assay and would be a reliable tool for rapid detection of 2,4-D residues.

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## 基于磁性荧光双探针基础上的 2,4-二氯苯氧乙酸 残留的快速免疫检测体系的建立

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**摘要:** 建立了一个基于磁性荧光双探针基础上的免疫快速检测体系, 以实现液相中快速检测食品中 2,4-二氯苯氧乙酸(2,4-D) 残留。该体系将 2,4-D 抗体结合 Fe<sub>3</sub>O<sub>4</sub>@ SiO<sub>2</sub>-NH<sub>2</sub> 得到的复合物作为磁性探针和固相载体, 2,4-D-OVA 被标记 CdTe@ SiO<sub>2</sub>-NH<sub>2</sub> 作为荧光探针以产生荧光信号。通过荧光探针与磁性探针复合物与 2,4-D 抗体竞争结合实现免疫快速检测。探讨了荧光探针最佳优化条件, 在 pH 值 8.2, 2,4-D-OVA 加入量为 500 μL, 偶联时间为 70 min 时, 偶联得到的荧光信号最强。双探针检测后得到该检测体系最低检测限为 3.55 × 10<sup>-8</sup>。得到金磁、量子点荧光双探针免疫系统, 绘出标准曲线, 得到最低检测限达 3.55 × 10<sup>-8</sup>。该检测体系与传统 ELISA 方法相比, 可以大大缩短检测时间, 放大检测信号。

**关键词:** 免疫检测; 荧光探针; 磁性探针; 2,4-D; 量子点

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