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1 # Quantitative Detection of β_2-adrenergic agonists with Fluorescence
2 # Quenching by Immunochromatographic Assay

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Abstract: β_2-adrenergic agonists are banned in China and other areas in the world. In this study, a novel method was developed to quantitatively detect β_2-adrenergic agonists. The clorprenaline (CLP)-bovine serum albumin (BSA) conjugation is mixed with BSA-fluorescent microspheres (FMs) complex and sprays on nitrocellulose membrane as the test line; the goat-anti-mouse antibody is mixed with BSA-fluorescent microspheres (FMs) complex and sprays on nitrocellulose membrane as the control line. If the target molecule is absent in the sample, colloidal gold-monoclonal antibody will bind to the CLP-BSA conjugation coated on the test line, the colloidal gold quenches the fluorescent microspheres, so that no fluorescent signal develops in the test line, indicating a negative result. The target molecule present in the sample at a cutoff level or higher binds to the colloidal gold-monoclonal antibody in the ELISA well. The colloidal gold-monoclonal antibody (Au-mAb) does not bind to the CLP-BSA conjugation coated on the test line. The fluorescent signal developed in the test line indicates a positive result. The limit of detection (LOD) of the immunochromatographic assay test strip was 0.12 ng/mL when the antibody amount was 0.8 μg/mL with detection time at 15 min. The immunochromatographic assay test strip could simultaneously detect five β_2-adrenergic agonists, including clorprenaline, bambuterol, terbutaline, clenbuterol, and salbutamol. When spiked swine urine samples (5.0 ng/mL and 10.0 ng/mL) were tested by the novel immunoassay, the recovery was 39.00±3.0 and 32.00±2.0, respectively.

Key words: β_2-adrenergic agonists; immunochromatographic assay; fluorescence quenching.

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1. Introduction

β_2-adrenergic agonists were once used for the treatment of asthma[1] but now forbidden to be used as lean meat-boosting feed additive because of their side effect to human beings, including anxiety, palpitations, headaches, nausea, and tremor.[2] Clenbuterol (CLEN) is a typical lean meat-boosting feed additive that can promote animal muscular mass growth and decrease fat accumulation.[3] Clorprenaline (CLP),[4] bambuterol (BAM), terbutaline (TER),[5] and salbutamol (SAL)[6] belong to the β_2-adrenergic agonist family and are banned in China.

Detecting these β_2-adrenergic agonists using methods such as capillary zone electrophoresis,[7] high-pressure liquid chromatography,[8] and near-infrared spectroscopy[9] are time-consuming and need expensive instruments and complicated operation. Lateral flow assay is rapid, inexpensive, and user-friendly. The method based on colloidal gold lateral flow assay to quantitatively detect CLP has been established in our laboratory.[10]

Colloidal gold and fluorescent microspheres are widely used as labels in lateral flow assay to separately detect target materials.[11–13] In this study, a novel method to quantitatively detect β_2-adrenergic agonists was developed using immunochromatographic assay based on fluorescence quenching.

2. Materials and methods

2.1 Materials

Clorprenaline (CLP), bambuterol (BAM), terbutaline (TER), and salbutamol

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(SAL), bovine serum albumin (BSA), ractopamine (RAC), phosphate buffer saline (PBS, 0.01 M), 2-morpholinoethanesulfonic acid (MES), and goat anti-mouse antibody were obtained from Sigma (St. Louis, MO). CLP-BSA conjugate antigen and anti-β2-adrenergic agonist monoclonal antibody were provided by Zodolabs Biotech Co., Ltd (Jiangxi, China). Hydrogen tetrachloroaurate trihydrate ($HAuCl_4 \cdot 3H_2O$) was obtained from Aldrich (Milwaukee, WI). Fluorescein isothiocyanate fluorescent microspheres (FMs) (diameter = 175 nm; excitation wavelength = 470 nm; emission wavelength = 525 nm; COOH = 443 μeq/g) were obtained from Merck (Darmstadt, Germany). The sample pad, nitrocellulose membrane, and absorbent pad were purchased from Millipore, Inc. (Bedford, MA). All solvents and other chemicals were of analytical reagent grade. The BioDot XYZ platform, which combines motion control with the BioJet Quanti3050k dispenser and AirJet Quanti3050k dispenser, was acquired from BioDot (Irvine, CA). The fluorescent microsphere immunochromatographic test strip (FM-ICTS) reader (excitation wavelength = 470 nm; emission wavelength = 520 nm) and handheld reader were from Shanghai Huguo Science Instrument Co., Ltd (Shanghai, China).

2.2 Preparation of colloidal gold solution

One milliliter of $HAuCl_4 \cdot 3H_2O$ stock solution (1% wt/vol) was added to 99 mL of ultrapure water and heated to boiling point. Then, 1.3 mL of sodium citrate solution (freshly prepared, 1%) was added to the gold solution under constant stirring. When the color of the mixture turned red, the mixture was continued to be boiled and stirred for another 10 min. The colloidal gold solution was then cooled at room temperature

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4 81 and stored at 4 °C.
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6 82 **2.3 Preparation of colloidal gold-monoclonal antibody**
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9 83 A total of 2 mL of colloidal gold solution was adjusted to pH 6.0 using 0.2 M
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11 84 K_2CO_3. Afterward, 0.2 mL of monoclonal antibody solution was added dropwise to
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14 85 the colloidal gold solution to a final concentration of 0.8 μg/mL with gentle stirring
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16 86 for 60 min. Then, 0.2 mL of polyethylene glycol 20000 solution (1%) was added to
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19 87 the solution and stirred for 30 min. Afterward, 0.2 mL of BSA (10% wt/vol) was
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21 88 added for further blocking for 30 min. The resulting solution was centrifuged at 8000
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24 89 r/m at 4 °C for 30 min. The supernatant was discarded to remove the unreacted free
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26 90 antibody, and the resulting precipitate was redissolved in 200 μL of dilution buffer.
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29 91 **2.4 Preparation of BSA-fluorescent microspheres complex**
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31 92 Thirty microliter of fluorescent microspheres (10 mg/mL) was added to 6 mL of
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34 93 MES buffer (pH = 6.0, 0.05M) and gently stirred for mixture. Then, 600 μL of BSA
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36 94 solution (10%) was added to this mixture and stirred for blocking for 1 h. The mixture
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39 95 was centrifuged at 11000 r/min at 4 °C for 20 min. The supernatant was discarded,
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41 96 and the resulting precipitate was redissolved in 600 μL of dilution buffer.
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44 97 **2.5 Preparation of the immunochromatographic test strips**
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46 98 The sample pad was pretreated with 50 mM borate buffer (pH 7.4; containing 1%
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49 99 BSA, 0.5 % Tween-20, and 0.05% sodium azide). After which, the pad was dried at
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51 100 60 °C for 2 h. The CLP-BSA conjugation (5.83 mg/mL) and goat anti-mouse antibody
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54 101 (5.5 mg/mL) were mixed with BSA-fluorescent microsphere complex and diluted
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56 102 with PBS (0.01 M) to final concentrations of 0.5 and 0.7 mg/mL, respectively. The
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103 two mixtures were then applied to the test and control lines on the nitrocellulose
104 membrane and dried at 30 °C for 4 h. The nitrocellulose membrane, absorption pad,
105 and pretreated sample pad were assembled as the test strip.

106 **2.6 Optimization of the antibody amount**

107 The antibody amount (0.5, 0.8, 1.0, 1.2, and 1.5 μg/mL) was added dropwise to
108 the colloidal gold solution to form colloidal gold-monoclonal antibody. Positive (3
109 ng/mL) and negative samples (0 ng/mL) were detected to optimize the antibody
110 amount.

111 **2.7 Immunological kinetic analysis of the test strip**

112 The kinetic curve of the immunochromatographic test strip was obtained as
113 follows: 100 μL of sample (0 and 10 ng/mL) was pipetted into the ELISA well, in
114 which 1 μL of colloidal gold-monoclonal antibody was added and incubated for 3 min.
115 The mixture was added to the sample pad of the test strip. After 1 min of incubation,
116 the strip was detected using the FM-ICTS reader. The signal of the test and line was
117 recorded every 1 min for 20 min.

118 **2.8 Establishment of the quantitative calibration curve of clorprenaline**

119 The test strip was prepared for an initial signal (T_0) before adding the sample.
120 The PBS was spiked with CLP at concentrations of 0, 1.0, 3.0, 5.0, 7.0, 10.0, 15.0,
121 20.0, and 30.0 ng/mL. A total of 100 μL of the solution was added into the ELISA
122 plate well, in which 1 μL of the colloidal gold-monoclonal antibody was added. After
123 3 min of incubation, the mixture was added to the sample pad of the test strip. After
124 15 min, the strip was read for second signal (T_1). The calibration curve was

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125 constructed by plotting the $T_0/(T_1-T_0)$ ratio as the ordinate (Y) and the CLP
126 concentrations (c) as the abscissa (X). Information of the linear regression equation
127 was established for quantitative analysis.

128 **2.9 Cross-reactivity experiment:**

129 The PBS was spiked with CLP, BAM, TER, CLEN, SAL, and RAC at a
130 concentration of 20 ng/mL. The test strips were read before and after adding the
131 samples for 15 min. All experiments were performed with three replications.

132 **2.10 Recovery experiment:**

133 Swine urine sample, which was confirmed to be negative of CLP, BAM, TER,
134 CLEN, and SAL, was spiked with CLP at concentrations of 5, and 10 ng/mL. The
135 spiked samples were detected with the test strips in triplicate.

136

137 **3. Results and discussion**

138 **3.1 Colloidal gold characterization and method principle**

139 β_2-adrenergic agonists are small molecules with single antigenic determinants.
140 Competitive inhibition formats are typically used when testing these targets, which
141 cannot simultaneously bind to two antibodies. In this “turn off” mode, if the target is
142 present in the sample at a cutoff level or higher, it will bind to antibodies, so that no
143 line develops in the test region (T line), which indicates a positive result.

144 In this study, a kind of “turn on” mode, in which the concentration of the target
145 molecule is proportional to the signal value, was developed for sensitivity detection
146 (Fig. 1). The CLP-BSA conjugation and goat anti-mouse antibody are mixed with

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4 147 BSA-fluorescent microsphere complex and sprays on the nitrocellulose membrane as
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6 148 test and control line, respectively. If the target molecule is absent in the sample, the
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9 149 colloidal gold-monoclonal antibody binds to the CLP-BSA conjugation coated on the
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11 150 test line. The colloidal gold quenches the fluorescent microspheres so that no
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14 151 fluorescent signal is developed in the test line, indicating a negative result. The target
15
16 152 molecule present in the sample at a cutoff level or higher binds to the colloidal
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19 153 gold-monoclonal antibody in the ELISA well. The colloidal gold-monoclonal
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21 154 antibody does not bind to the CLP-BSA conjugation coated on the test line. The
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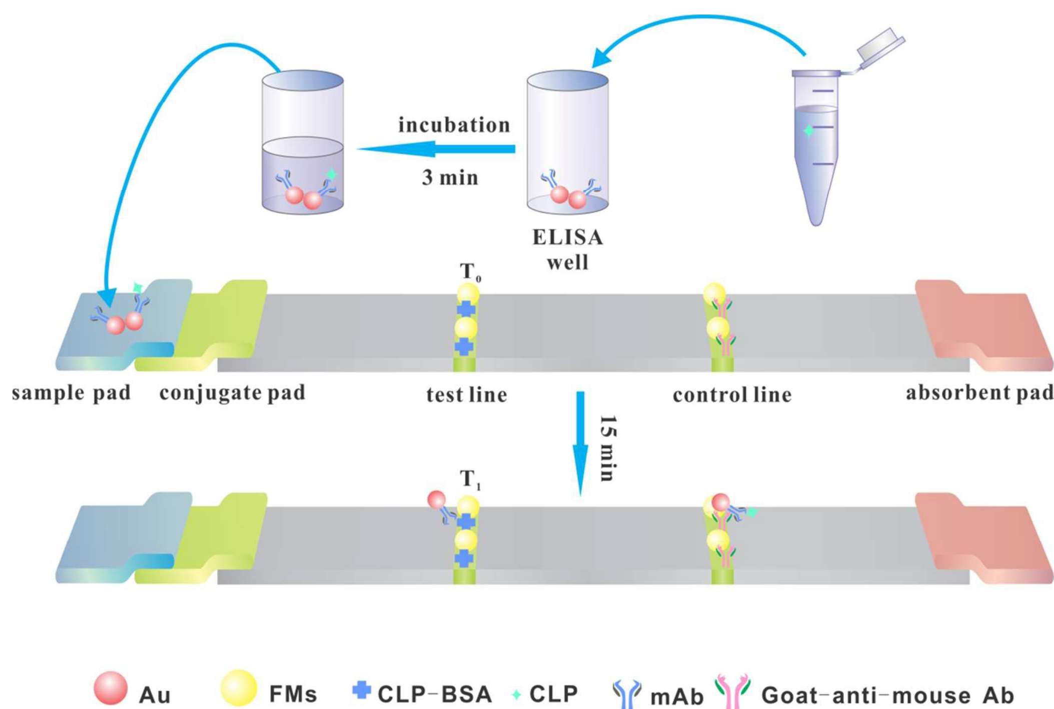
24 155 fluorescent signal developed in the test line indicates a positive result.



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157 **Fig. 1** Flow chart of immunochromatographic assay based on fluorescence quenching

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159 The fluorescence quenching phenomena in homogeneous phase were commonly

160 believed to be caused by fluorescence resonance energy transfer (FRET).[14, 15] The

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161 donor and the acceptor are indispensable in the FRET system. The emission spectrum
162 of donor can overlap the absorption spectrum of acceptor. The distance between the
163 donor and acceptor molecules should be in a range of 2-9 nm.[16] The fluorescence
164 quenching phenomena in test strips were also attributed to FRET in the heterogeneous
165 phase.[17]

166 In this study, colloidal gold has a strong and wide absorption peak at the
167 wavelength range from 461 nm to 600 nm (Fig. 2). The absorbance peak of colloidal
168 gold-monoclonal antibody was blue shifted compared to colloidal gold because the
169 size of colloidal gold monoclonal antibody was bigger than that of colloidal gold.

170 The excitation and emission wavelength of the fluorescent microspheres is 470
171 and 525 nm, respectively, whereas that of FM-reader is 470 nm and 520 nm,
172 respectively. In Fig. 3A, the fluorescent signal of the test line on test strip was zero, as
173 read by the FM-ICTS reader, because the fluorescent signal was completely quenched
174 by the colloidal gold. In Fig. 3B, the fluorescent signal of the test line on test strip
175 could be observed by the naked eye using a handheld reader. The results indicated that
176 fluorescence quenching was decreased with the wide range of excitation wavelength
177 of the handheld reader.

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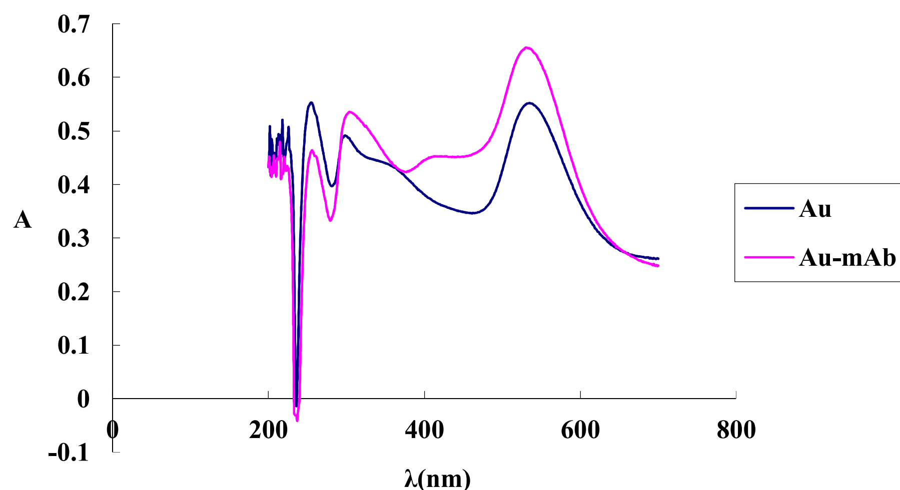

Fig. 2 UV-Vis absorption spectrogram of Au and Au-mAb

λ: the absorption wavelengths; A: the value of absorption

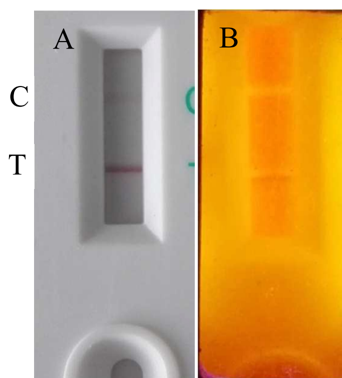

Fig. 3 Different test strip results using FM-ICTS and handheld readers

A: fluorescent signal of the test line on test strip was zero, as read by the FM-ICTS reader;

B: the fluorescent signal of the test line on test strip could be observed by the naked eye using a handheld reader.

3.2 Optimization of the amount of monoclonal antibody

The amount of monoclonal antibody is an important factor for test strip sensitivity. Monoclonal antibodies measuring 0.5, 0.8, 1.0, 1.2, and 1.5 μg/mL were

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studied to prepare the immunochromatographic assay test strip with positive (3 ng/mL) and negative sample (0 ng/mL). The results (Fig. 4) showed that when the amount of monoclonal antibody in the test strip was 0.5 μg/mL, the fluorescent signal of the test line was 340 and 360 when the sample was 0 and 3 ng/mL, respectively. These two fluorescent signals were too close. When the amount of monoclonal antibody in the test strip was 0.8 μg/mL, the fluorescent signal of test line was 60 and 130 when the sample was 0 and 3 ng/mL, respectively. These two fluorescent signals were differentiable. When the amount of monoclonal antibody in the test strip was 1.0, 1.2, and 1.5 μg/mL, the fluorescent signal of test line was too low, even zero. Therefore, the amount of monoclonal antibody was selected at 0.8 μg/mL.

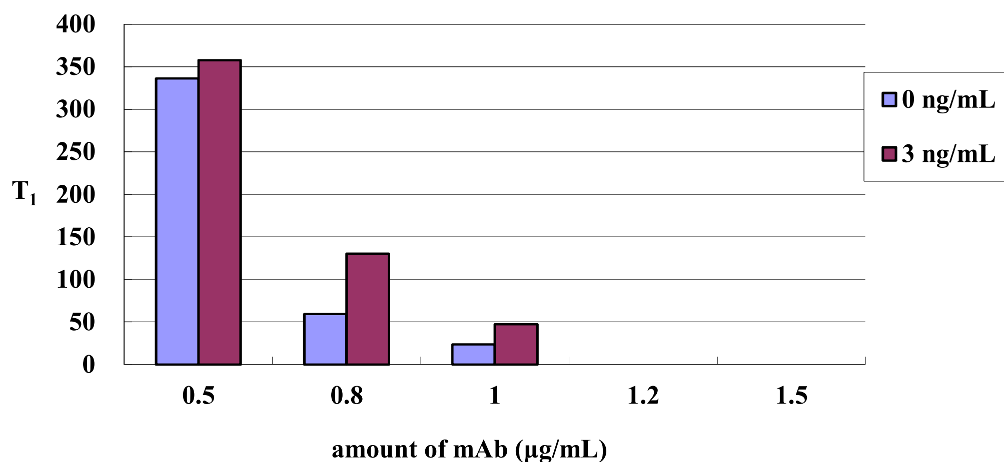

Fig. 4 Amount of labeled antibody

3.3 Immunological kinetics analysis of the test strip

One hundred microliter of positive (10 ng/mL) and negative sample (0 ng/mL) were pipetted into the ELISA well. The mixture was added to the sample pad of test strip after incubating with 1 μL of colloidal gold-monoclonal antibody for 3 min. The

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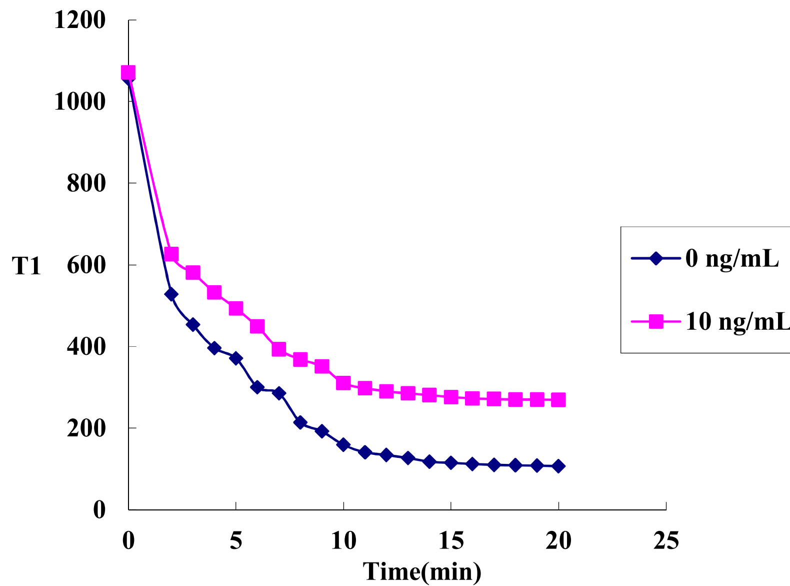
206 strip was then detected using an FM-ICTS reader. The signal of the test and line was
207 recorded every 1 min for 20 min. The fluorescent signal intensity decreased from
208 1070 at 0 min and remained constant after 15 min (Fig. 5).



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210 **Fig. 5** Immunoreaction dynamics of the test line with different CLP concentrations

211 Time: the reading time; T_1: the absorption value of test line

212 **3.4 Establishment of the quantitative calibration curve**

213 Serially spiked samples were detected using the immunochromatographic assay
214 test strip to establish the calibration curve, as shown in Fig. 6. The calibration curve
215 was constructed by plotting the $T_0/(T_1\text{-}T_0)$ ratio as the ordinate (Y) and the CLP
216 concentrations (c) as the abscissa (X). The curve exhibited good linearity in the range
217 of 3.0-30.0 ng/mL (R^2=0.9985), and the coefficient of variation for each concentration
218 is less than 5%. The limit of detection (LOD) was calculated by analyzing 20 negative
219 samples. The calculated LOD using the mean of the results of the negative signals
220 plus threefold standard deviation was 0.12 ng/mL.[18]

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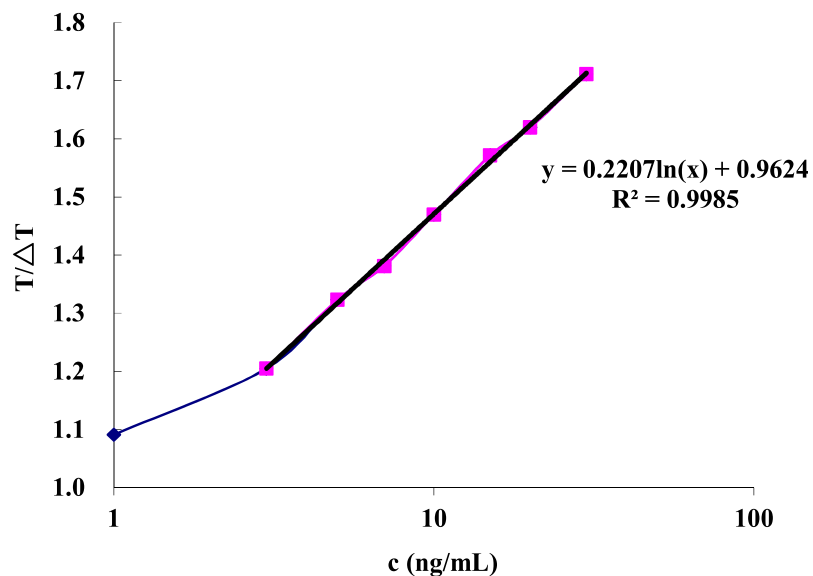

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25 222 **Fig. 6** Quantitative calibration curve

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28 223 $T/\Delta T$: $T_0/(T_1-T_0)$; c: CLP concentrations

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30 224 **3.5 Cross-reactive experiment**

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33 225 CLP, BAM, TER, CLEN, SAL, and RAC were detected to evaluate the

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35 226 cross-reactivity of the immunochromatographic assay test strip. The IC_{50} of CLP,

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38 227 BAM, TER, CLEN, SAL, and RAC was 4.30, 3.03. 4.75, 4.30, 4.25, and 252.94

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40 228 ng/mL, respectively (Table 1), indicates that CLP, BAM, TER, CLEN, and SAL could

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236 Table 1 Result of cross-reactivity of the immunochromatographic assay test strip

β2-adrenergic agonists	IC_{50} (ng/mL)	Cross-reactivity (%)
CLP	4.30	100
BAM	3.03	141.8
TER	4.75	90.5
CLEN	4.30	100
SAL	4.25	101.1
RAC	252.94	1.7

237

238 **3.6 Recovery experiment:**

239 When spiked swine urine samples (5.0 ng/mL and 10.0 ng/mL) were tested by
240 the novel immunoassay, the recovery was 39.00±3.0 and 32.00±2.0, respectively. The
241 influence of matrix effect could not be ignored when the method was applied in the
242 field for on-site detection of CLP in swine urine samples (Table 2).

243 Table.2 Recovery of CLP in swine urine samples (n=3)

Spiked concentration (ng/mL)	Measured concentration (ng/mL)	Recovery (%)
5.0	1.95±0.15	39.00±3.0
10.0	3.20±0.20	32.00±2.0

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245 **4 Conclusions**

246 In this study, a novel method was developed to quantitatively detect
247 β_2-adrenergic agonists. Colloidal gold was used to quench fluorescence signal coated

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on NC membrane. The immunochromatographic assay test strip could simultaneously detect CLP, BAM, TER, CLEN, and SAL.

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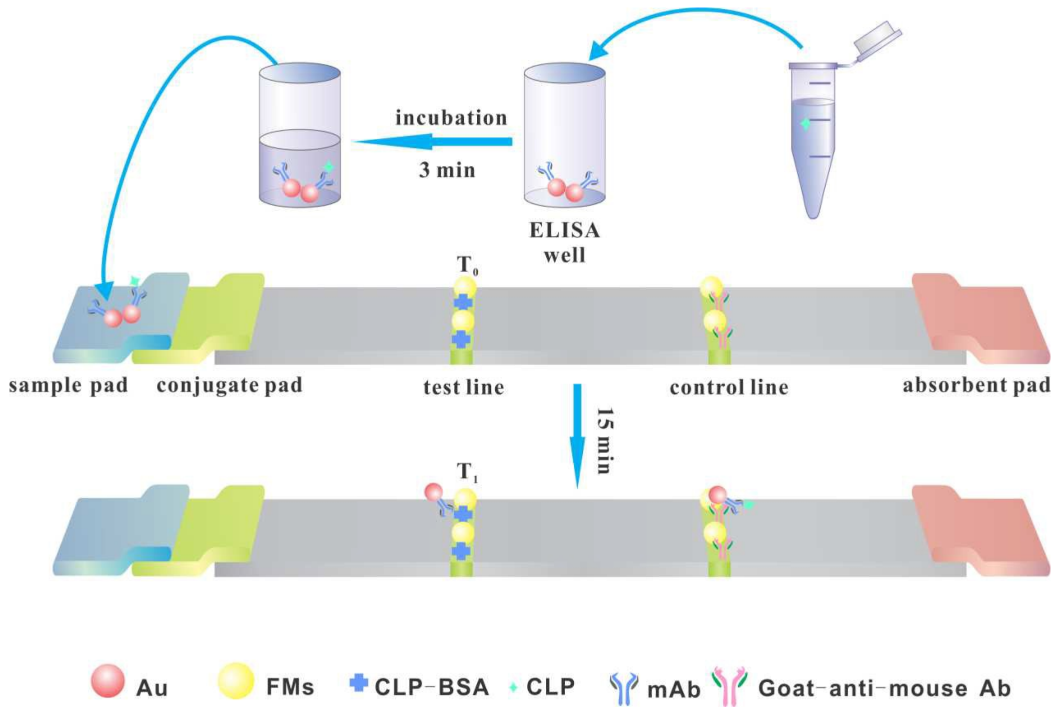

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