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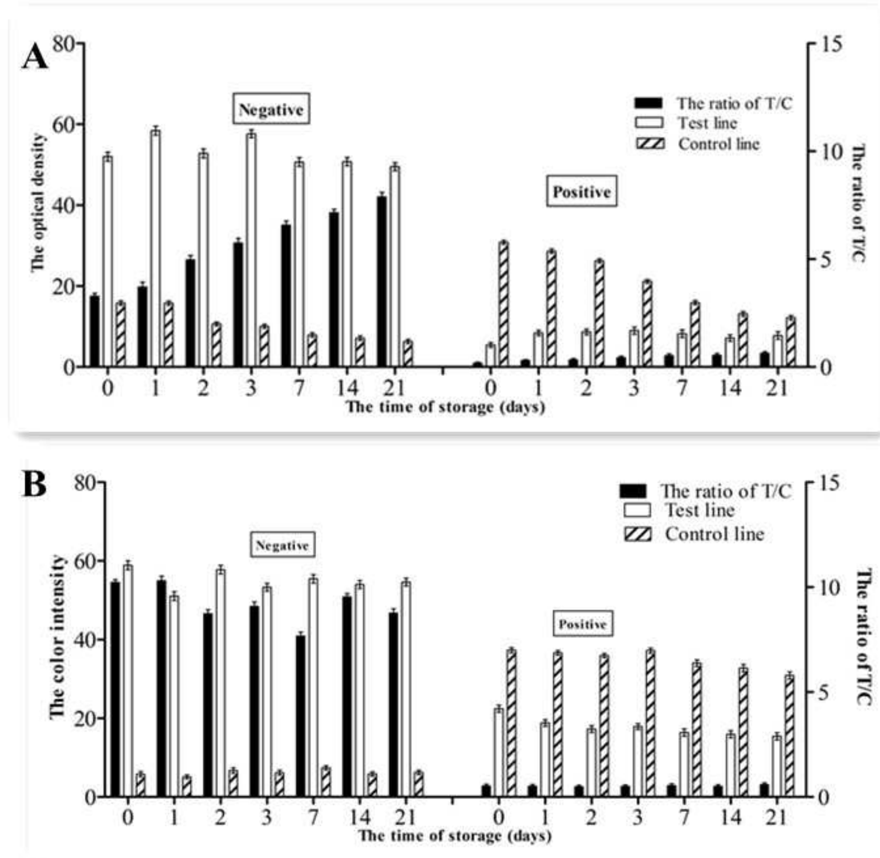

A)The stability of traditional test strips
B)The stability of the test strips after improved

The stability of lateral-flow immunochromatographic assay for quantitative detection of clenbuterol was studied and improved.

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Improvement of the Stability of Immunochromatographic Assay for the Quantitative Detection of Clenbuterol in Swine Urine

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Running title: **Improvement of the Stability of Immunochromatographic Assay**

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Abstract

Clenbuterol is banned as a feed additive in China and in other countries. Lateral-flow immunochromatographic assay can be applied in the quantitative detection of clenbuterol. Our group has previously developed an immunochromatographic assay to detect clenbuterol in swine urine rapidly and quantitatively. This method was based on the ratio of the color intensity of a test line to that of a control line (T/C) to offset the matrix effects of samples and diminish variations among different strips. In this study, the stability of this method was successfully improved and verified by an accelerated aging test that involved storage at 60°C for three weeks. Results showed that the control line was the main factor affecting the strip stability. To improve the stability of the test strip, we mixed goat anti-mouse antibody spotted on the control line with WellChampion, Antibody Enhancer, and Protein StabilPLUS. Alterations in the T/C ratio were evaluated by negative and positive swine urine samples. Stability was effectively improved stability by adding WellChampion. Furthermore, the newly prepared strips showed satisfactory stability by drying the nitrocellulose membrane at 60°C for one day.

Keywords: lateral flow immunochromatographic assay; quantitative detection; stability; clenbuterol

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1. Introduction

Clenbuterol (CLE), which belongs to the β–agonist family, is currently used as a bronchodilator to treat asthma in humans and as a tocolytic agent in veterinary medicine. However, CLE has been used illegally at higher dosages to promote animal muscular mass development and decrease fat accumulation. CLE is banned as a feed additive in China and in other countries. In China, more than 1000 people developed illnesses in Guangdong Province in 2001 after they consumed contaminated swine liver and heart. A person died in Guangdong Province on 19 March 2006, and is the first CLE-related death worldwide. Approximately 300 individuals were also poisoned in Shanghai on September 15, 2006.[1] Shuanghui Group, China's largest meat processor apologized on March 17, 2011 after an illegal additive was allegedly found in meat products that were manufactured by an affiliate of the company.[2]

Various analytical methods used to determine of CLE in different biological matrices have been described. Quantification and confirmation have been performed using methods based on liquid chromatography coupled with mass spectrometry[3-5] and gas chromatography coupled with mass spectrometry.[6] Enzyme linked immunosorbent assay (ELISA) is considered as one of the most sensitive detection systems for this compound and has been used to screen this substance.[7-10]

Lateral-flow immunochromatographic assay (LFIA) based on colloidal gold, which is a rapid and sensitive detection technology, has provided the latest advancements in rapid detection.[11] In LFIA, an antibody reacts with an antigen via an immunochromatographic procedure.[1] LFIA exhibits four benefits, namely, user-friendly

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format, availability of test result after a short period of time, long-term stability under various climates, and relatively low cost.[1] These characteristics contribute to the suitability of LFIA for on-site testing by untrained personnel.[12]

However, traditional LFIA exhibits some disadvantages. This method is neither sensitive nor can be used to quantify CLE accurately. In addition, varied results are obtained because findings are assessed by different individuals, in this test, the intensity of colored lines on test strips is evaluated using the naked eye, thereby yielding varied results.[13] Hence, studies on the use of LFIA for quantitative detection with instrumental measurement have been performed. In our previous study, LFIA was developed to detect CLE in swine urine quantitatively in 10 min. The quantitative detection system for CLE was developed on the basis of the concept in which the ratio of the color intensity of the test line (A_T) to that of the control line (A_C) was used. The values of A_T and A_C were obtained by the given instrumental measurement. Differences among strips and the matrix effects can be offset using the ratio of A_T/A_C.[14] A quantitative method based on T/C has also been developed to detect many targets in food and other matrices.[15-18]

LFIA is a competitive binding immunoassay to detect CLE. In this method, A_T is negatively proportional to the amount of analytes present in the samples. In qualitative detection, A_C is not important, and only a red line should appear. In quantitative detection, A_C is an important component. In theory, the stability of the strips will affect the T/C ratio. Colloidal gold-based strips, which are commercial products, are transported to different places for 1 or 2 d in China. The temperature in a delivery truck or in a train is often >60°C in summer. Under extremely high temperature conditions, the stability of test

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strips decreases rapidly because antigens and antibodies in the strip are seriously affected by high temperatures. To obtain accurate detection results, researchers should consider the stability of strips. In the current study, the stability of LFIA to detect CLE quantitatively was determined. To our knowledge, this study is the first to report the stability of LFIA in quantitative detection.

2. Experimental

2.1 Materials

Reagents

CLE hydrochloride and goat anti-mouse antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA). Colloidal gold solution and anti-CLE monoclonal antibody were produced in our laboratory. Sample pad, conjugate pad, nitrocellulose (NC) membrane, and absorbent pad were purchased from Millipore (Bedford, MA, USA). WellChampion, Antibody Enhancer, and Protein StabilPLUS were purchased from KEM-EN-TEC Diagnostics A/S (Taastrup, Denmark). All solvents and other chemicals were of analytical reagent grade.

Equipment

Our strip reader was purchased from Skannex Biotech Co., Ltd. (Changzhou, China). A BioDot XYZ platform equipped with a motion controller, and BioJet Quanti3050k and AirJet Quanti3050k dispensers to dispense solution were supplied by BioDot (Irvine, CA). A vacuum drying oven was purchased from Shanghai Sumsung Laboratory Instrument Co., Ltd. (Shanghai, China). An automatic programmable cutter was purchased from Shanghai Jinbiao Biotechnology Co., Ltd. (Shanghai, China).

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104 *Swine urine samples*

105 A total of 40 swine urine samples that did not contain CLE, as validated by liquid
106 chromatography–tandem mass spectrometry (LC–MS)/MS, were collected from Jiangxi
107 Province. The samples were mixed as a negative sample. A portion of the mixture sample
108 was spiked with CLE at concentrations of 0.5, 1.0, 2.0, 3.0, 4.0, 5.0, and 7.0 μg/l.
109 Considering that 5.0 μg/l is the limit level in many areas in China, 5.0μg/l was chosen as
110 the positive CLE concentration to evaluate in the accelerated aging test.

111 2.2 Preparation of colloidal gold-labeled anti-CLE monoclonal antibody

112 Colloidal gold with an average diameter of 30 nm was used to conjugate the monoclonal
113 antibody. Approximately 10 ml of colloidal gold solution was adjusted to pH 6.0 with 0.2
114 M K_2CO_3. As the solution was gently stirred, 1 ml of anti-CLE monoclonal antibody
115 solution was added dropwise to the colloidal gold solution to obtain a final concentration
116 of 2.5 μg/ml. The resulting mixture was also stirred gently for 1 h at room temperature.
117 Bovine serum albumin (BSA, 10%, w/v; 1 ml) was added to block this solution. After
118 stirring gently for another 30 min, we centrifuged the mixture at 8000 r/min for 30 min at
119 4°C to remove unlabeled, free antibodies. The precipitate was then dissolved in 1 ml of
120 dilution buffer.

121 2.3 Preparation of immunochromatographic test strips

122 The sample pad was treated with 50 mM borate buffer (pH 7.4, containing 1% BSA, 0.5%
123 Tween-20, and 0.05% sodium azide) and dried for 2 h at 60°C. CLE-BSA conjugation
124 (0.8 mg/ml) was spotted on the test line, and the goat anti-mouse antibody (0.4 mg/ml)
125 was spotted on the control line. The spotting density of test and control lines was 0.75

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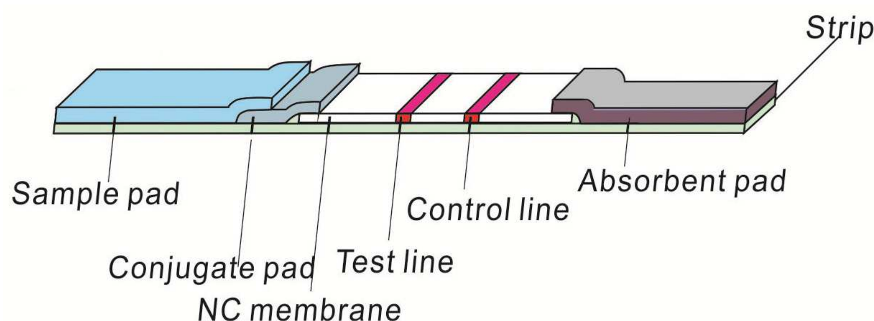
126 μl/cm, and the interval between test and control lines was 0.6 cm. The prepared NC
127 membranes were dried at 37°C for 12 h. An absorption pad was used without treatment. A
128 colloidal gold probe was applied to an untreated glass-fiber membrane and completely
129 dried at 35°C with a vacuum dryer for 2.5 h. Sample pad, conjugation pad, NC membrane,
130 and absorption pad were assembled, cut into strips (0.40 cm × 6 cm) by using an
131 automatic programmable cutter, and packaged in foil bags containing desiccant gel (Fig.
132 1).



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134 **Fig. 1** Schematic of the test strip used in this study.

135 2.4 Assay test procedure

136 Swine urine sample (100 μl) without pretreatment was pipetted into a sample well of the
137 strip. After 10 min, the strip was scanned with a strip reader. A_T, A_C, and T/C ratio were
138 recorded. CLE concentrations were measured according to the standard curve set in the
139 strip reader. The standard curve was developed by plotting the T/C ratio against the
140 logarithm concentration of CLE. Each spiked concentration of the standard solutions was
141 repeated four times.

142 2.5 Accelerated aging test at 60°C

143 The prepared strips were placed in a drying oven at 60°C for three weeks. A_T, A_C, and T/C
144 ratio were recorded using the strip reader for 1, 2, 3, 7, 14, and 21 d

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145 2.6 Improvement of stability of the strips by using agents

146 The goat anti-mouse antibody was mixed with WellChampion, Antibody Enhancer, and
147 Protein StabilPLUS. The concentration of each of these agents was 5% (v/v). The goat
148 anti-mouse antibody mixtures were spotted on the control line of the NC membrane. The
149 accelerated aging test of the prepared strips was performed as described previously.

150 2.7 Improvement of strip preparation by modifying the drying condition

151 The prepared NC membranes spotted with an optimal protective agent on the control line
152 were not dried at 60°C for 24 h. The other conditions were the same as those described
153 previously. The newly prepared strips were presented as described previously to evaluate
154 stability.

155 2.8 Statistical analysis

156 Statistical significance was evaluated by ANOVA and F test. $P < 0.05$ was considered
157 statistically significant.

158 **3. Results and discussion**

159 3.1 Estimating the stability of strips in the accelerated aging test

160 The standard curve was obtained by plotting the T/C ratio against the logarithm
161 concentration of CLE (Fig. 2). The prepared strips were stored at 60°C for three weeks.
162 The stability of strips was evaluated using negative (0 μg/l) and positive (5.0 μg/l)
163 samples (Fig. 3). A_T showed relative stability from 1 d to 21 d. However, A_C decreased
164 throughout the experiment and declined by > 60% after three weeks. The T/C ratio of the
165 strips in negative and positive samples doubled after 21 d. According to the standard
166 curve (Fig. 2), the measured values of the positive samples were 5.602 ± 0.006, 4.225 ±

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0.047, 3.880 ± 0.037, 3.072 ± 0.024, 2.450 ± 0.038, 2.294 ± 0.014, and 1.829 ± 0.076 at 0, 1, 2, 3, 7, 14, and 21 d, respectively. The strips showed very low stability. This result can be ascribed to the decline of A_C, which is the main factor influencing the stability of strips.

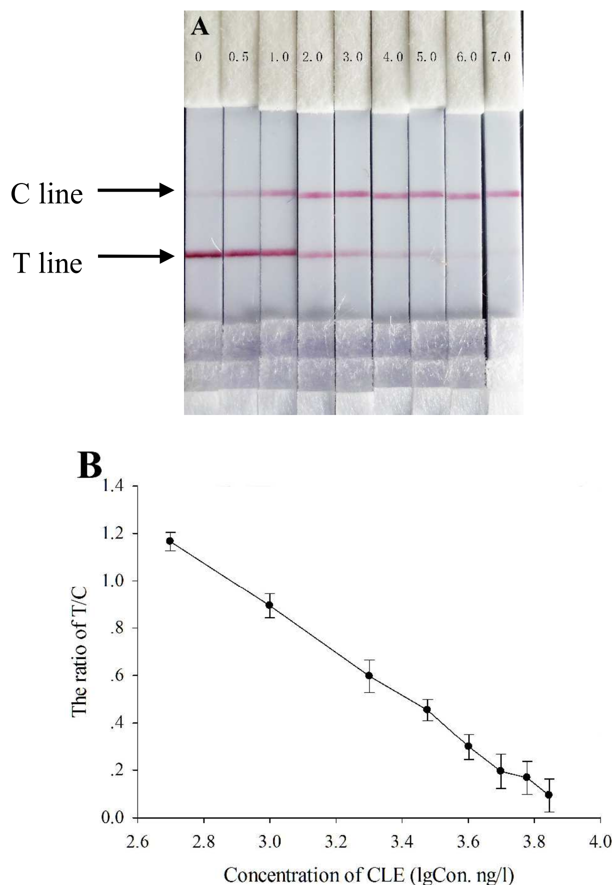

Fig. 2 Calibration curve of CLE with a series of concentrations at 0.5 1.0, 2.0, 3.0, 4.0, 5.0, 6.0, and 7.0 μg/l. A) Image of the change in color intensity with concentration. B) Calibration curve is plotted by T/C ratio against logarithm concentration of CLE [$Y = -0.4110\ \mathrm{Ln}(X) + 3.7243$, $R^2 = 0.9979$] of the test strips.

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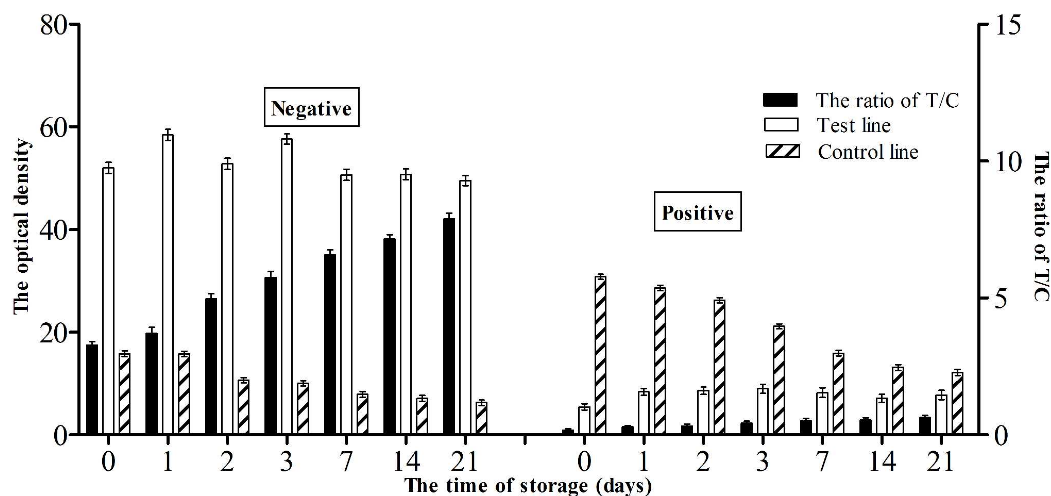

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178 **Fig. 3** Variations in the color intensity of the test line, the control line, and the T/C ratio with storage

179 time at 60°C. Variations were tested by negative (0 μg/l) and positive (5.0 μg/l) samples; NC

180 membranes were dried at 37°C for 12 h

181 3.2 Evaluation of the three agents on the stability of strips

182 The goat anti-mouse antibody on the control line was mixed with WellChampion,

183 Antibody Enhancer, or Protein StabilPLUS. The stability of strips was evaluated using

184 negative (0 μg/l) and positive (5.0 μg/l) samples. A_T, A_C, and T/C ratio are presented in

185 Fig. 4. The changes in T/C ratio (Figs. 4B and 4C) indicated that Antibody Enhancer and

186 Protein StabilPLUS were unable to maintain the stability of the control line; A_C decreased

187 gradually in storage, particularly in the positive sample. After mixing Antibody Enhancer

188 with the goat anti-mouse antibody, we found that the measured values of the positive

189 sample were 4.392 ± 0.086, 1.774 ± 0.103, 1.369 ± 0.008, 0.846 ± 0.004, and 0.599 ±

190 0.141 at 0, 1, 7, 14, and 21 d, respectively. After adding Protein StabilPLUS to the goat

191 anti-mouse antibody, we obtained the following measured values of the positive sample:

192 5.095 ± 0.039, 3.276 ± 0.221, 1.992 ± 0.133, 2.086 ± 0.072, and 1.213 ± 0.069 at 0, 1, 7,

193 14, and 21 d, respectively. After adding WellChampion, we obtained the following

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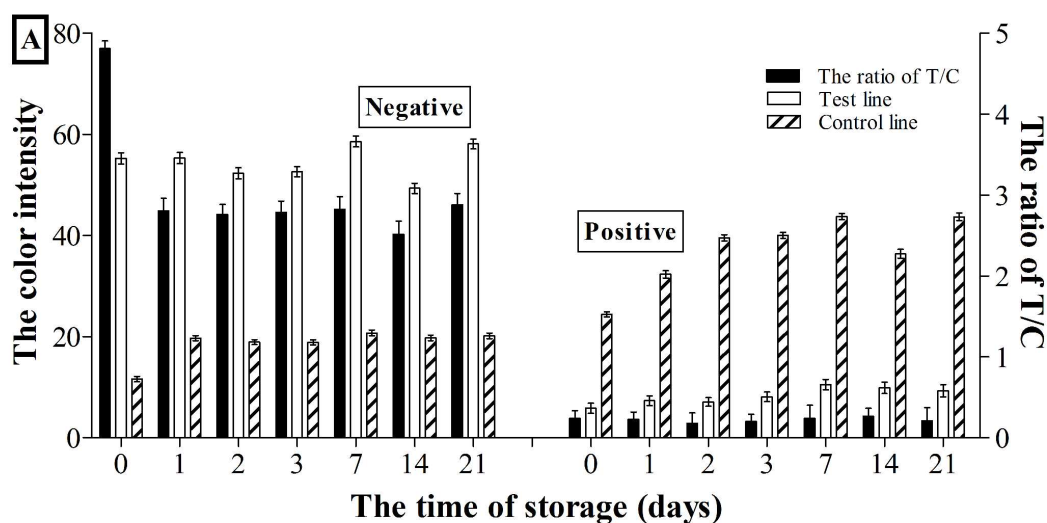
194 measured values of the positive samples: 4.511 ± 0.033. 4.673 ± 0.014, 5.330 ± 0.060,
195 5.349 ± 0.019, 5.009 ± 0.018, 4.589 ± 0.033, 4.736 ± 0.020 at 0, 1, 7, 14, and 21 d,
196 respectively. Compared with the spiked concentration, the measured values were accurate
197 with average recovery ranging between 90% and 107% without significant alteration;
198 hence, the test strip showed relative stability. A_T, A_C, and T/C ratio of the positive samples
199 did not change significantly (Fig. 4A). The measured values were close to those of the
200 spiked concentration. In the detection of the negative sample, the reaction on the NC
201 membrane was activated at 60□ from 0 to 1 day, leading to an increase in A_C and a
202 decrease in T/C ratio. The strip showed satisfactory stability after 1 d. WellChampion is a
203 new generation reagent that remarkably speeds up plate coating and stabilization in
204 ELISA. WellChampion can prevent degradation, denaturation, and leaching; this reagent
205 also improves assay sensitivity (optical density, OD) and precision over the plate. In our
206 study, WellChampion could be used to improve the stability of test strips.



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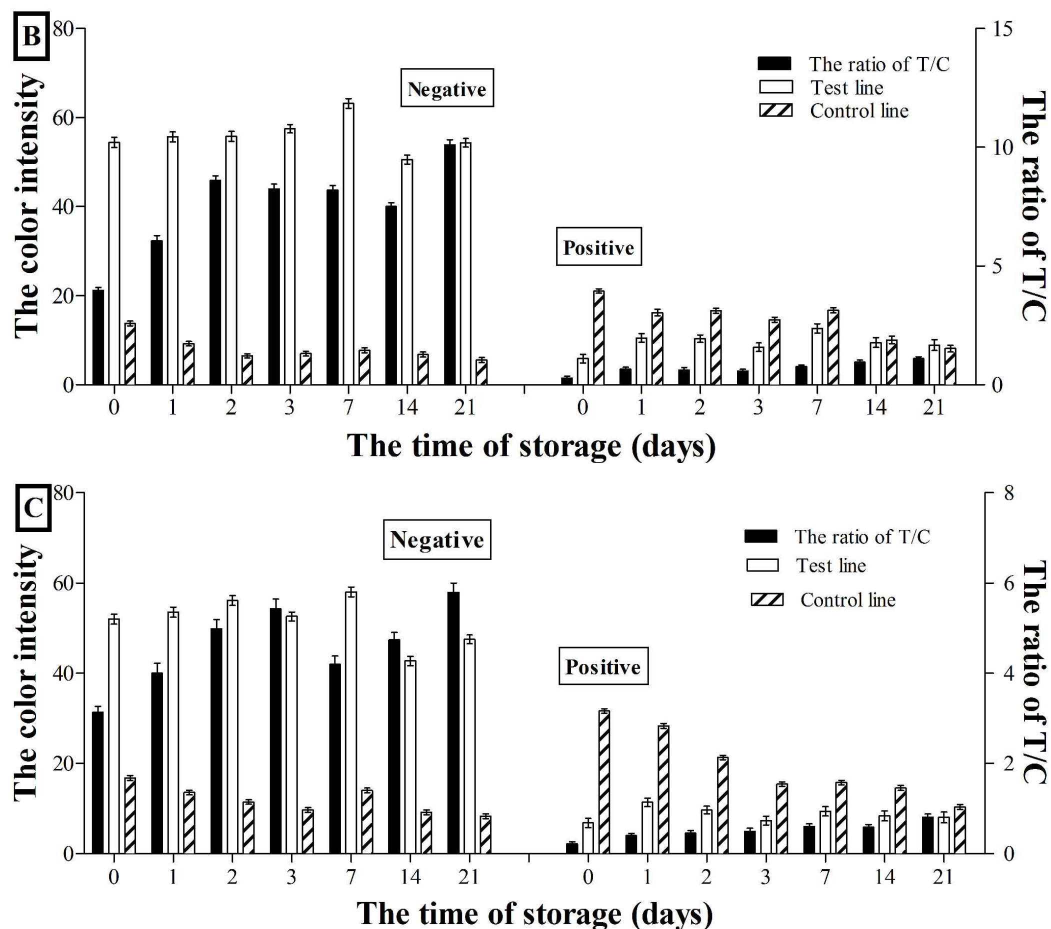

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210 **Fig. 4** Color intensity of test line, control line, and T/C ratio varying with storage time at 60 °C. Goat

211 anti-mouse antibody on the control line was mixed with WellChampion (A), Antibody Enhancer (B),

212 and Protein StabilPLUS (C). Variations were tested using negative (0 μg/l) and positive (5.0 μg/l)

213 samples; NC membranes were dried at 37°C for 12 h.

214 3.3 Improvement of strip preparation by modifying the drying condition

215 Considering the results of the accelerated aging test described in previous sections, we

216 found that NC membrane can be affected by high temperatures. Hence, a new pattern to

217 prepare strips was developed using the following procedures. The goat anti-mouse

218 antibody (0.4 mg/ml) was mixed with 5% WellChampion and spotted on the control line

219 on the NC membrane. The prepared membrane was dried at 60°C for 24 h instead of 37°C

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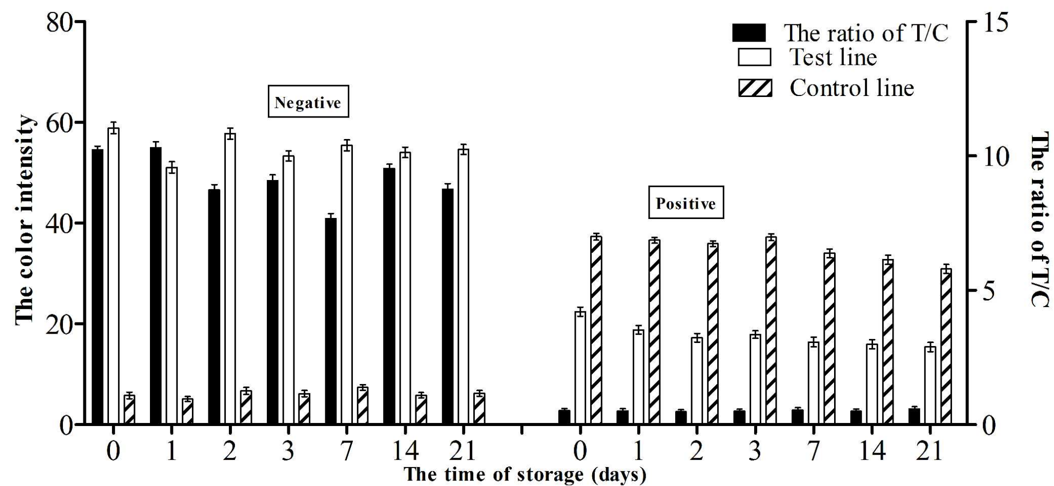
220 for 12 h. To verify the stability of the newly prepared strips, we stored the strips at 60°C 221 for three weeks. The A_C and T/C ratio did not vary significantly in the accelerated aging 222 test at a storage temperature of 60°C for three weeks (Fig. 5), and the corresponding 223 measured values (Table 1) did not show a significant change from the spiked 224 concentration of CLE (average recovery was between 93% and 105%).



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226 **Fig. 5** Color intensity of the test line, the control line, and the T/C ratio varying with storage time at 227 60°C. Variations were tested using negative (0 μg/l) and positive (5.0 μg/l) samples; NC membranes 228 were dried at 60°C for 24 h.

229 **Table 1** Measured values of a spiked sample in the evaluation of the stability of newly prepared strips 230 (*n* = 4)

Time (days)	0	1	2	3	7	14	21
Spiked concentration (μg/l)	5.00	5.00	5.00	5.00	5.00	5.00	5.00
Measured value (μg/l)	5.000 ± 0.064	5.038 ± 0.023	5.25 ± 0.021	5.138 ± 0.008	4.873 ± 0.018	5.094 ± 0.125	4.628 ± 0.026

231 **Note:** Strips were prepared with WellChampion, and NC membranes were dried at 60°C for 24 h.

232

233 **4. Conclusions**

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The results of the accelerated aging test showed that the stability of strips was related to the goat anti-mouse antibody on the control line. Amogn the three protective agents, WellChampion could be applied to improve the stability of strips used to detect CLE residues. The goat anti-mouse antibody (0.4 mg/ml) mixed with 5% WellChampion was spotted on the control line on the NC membrane. Compared with the traditional strips, the prepared NC membrane was dried at 60°C for 24 h instead of 37°C for 12 h. The newly prepared strips used to detect CLE quantitatively showed satisfactory stability at 60°C for three weeks. The stability of LFIA to detect CLE quantitatively was improved successfully.

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