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Development of a lateral-flow immunochromatographic strip for Rapid and Quantitative Detection of small molecule compounds
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1   **TITLE:**

2   **Development of a Lateral Flow Immunochromatographic Strip for Rapid and Quantitative**

3   **Detection of Small Molecule Compounds**

4

5   **AUTHORS AND AFFILIATIONS:**

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7

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13

14 **KEYWORDS:**

15 lateral flow immunoassay; colloidal gold; monoclonal antibody; artificial antigen; small

16 molecular compound; strip

17

18 **ABSTRACT:**

19 Membrane-based lateral flow immunochromatographic strips (ICSs) are useful tools for low-cost

20 self-diagnosis and have been efficiently applied to toxin, physiological index and clinical

21 biomarker detection. In this protocol, we provide a detailed description of the steps to develop

22 a rapid, sensitive and quantitative lateral-flow immunoassay (using AuNPs as a marker and

23 mAbs as a probe). The procedure describes the preparation and characterization of colloidal

24 gold, synthesis of the AuNP-mAb conjugate, assembly of the immunochromatographic strip, and

25 methodological investigation of the assay. The results showed that the final strips can be further

26 utilized for the rapid and convenient self-diagnosis of a small molecule, which may provide an

27 alternative tool in the rapid and precise analysis of physiological and biological indices.

28

29 **INTRODUCTION:**

30 Membrane-based lateral flow immunochromatographic strips (ICSs) are useful tools for low-cost

31 and rapid detection. The nitrocellulose membrane as the carrier and colloidal gold as markers of

32 immune chromatography rapid diagnostic reagents are the most commonly used POCT (point of

33 care testing) method, and the testing scope of the project is wider. From their original

34 application in monitoring during pregnancy, their use has been extended to monitor blood

35 coagulation state[1-2], myocardial injury[3], veterinary medicine[4], pesticide residues[5], infectious

36 diseases[6] and drug concentrations. More types of samples can be assessed, including urine,

37 saliva, whole blood, serum and other body fluids[7-8].

38

39 In recent years, numerous novel assays have been developed for detecting biomarkers in the

40 diagnosis of disorders, including HPLC, UPLC, LC–MS and ELISA, which are sensitive and accurate,

41 credible and specific. However, these methods require sophisticated instrumentation, complex

42 preprocessing and time-consuming treatments[9]. Hence, developing a more rapid and
43 convenient point-of-care diagnostic strategy for the self- and real-time detection of medicinal
44 active compounds is urgent[10-11].

46 The popularity of ICSs, especially for common tests, is driven by their ease of use, as they do not
47 require professionals or elaborate instrumental setups[12]. In other words, people who do not
48 have special training can operate strips or self-tests[13]. The results of the test can be obtained in
49 5 minutes, which means it can be used for site inspections[14]. Moreover, according to our
50 calculations, the cost of strips could be lower than 1 RMB[15], which means that the tests are
51 inexpensive to promote[16]. Hence, the ICS is a relatively accurate, simple, and inexpensive
52 disposable device. ICSs based on colloidal gold[17-18] are also applied in rapid COVID-19
53 detection.

55 The principle of ICS can be divided into sandwich ICS and competitive ICS. **Figure 1A** is a
56 schematic diagram of the sandwich ICS, which is mainly used for detecting macromolecular
57 substances such as proteins, including tumor markers, inflammatory factors, and human
58 chorionic gonadotropin (HCG, early pregnancy antigen). In this method, paired antibodies
59 targeted at different epitopes of the antigen are used, and the capture antibody is dried on the
60 NC membrane as a test line. Labeled antibody is dried on the conjugate pad, and secondary
61 antibody is used as the control line.

63 **Figure 1B** is a schematic diagram of competitive ICS, which is mainly used to detect small
64 molecule substances (MWCO < 2000 Da). The coating antigen is fixed on the NC membrane as a
65 test line, and the labeled antibody is dried on the conjugate pad. During detection, the sample
66 and labeled antibody flow through the detection line under capillary action, and the coated
67 antigen competitively binds free antigen in the sample and develops a red color on the
68 detection line.

70 Recently, we described the procedure of monoclonal antibody generation against natural
71 products[19]. In this work, we developed a novel lateral flow immunoassay based on the prepared
72 anti-SSD mA[20] for rapid, on-site detection. The results indicate that the
73 immunochromatography assay is an indispensable and convenient tool for detecting natural
74 product-derived compounds.

76 [Figure 1 here]

78 **PROTOCOL:**

80 All of the procedures performed in this study were approved by the Ethics Review Committee at
81 the Beijing University of Chinese Medicine (approval number 2017BZYYL00120).

83   **1.   Preparation and Characterization of Colloidal Gold**

84

85   NOTE: For colloidal gold synthesis, as colloidal gold is easily adsorbed on the inner wall of the

86   vessel and is prone to precipitation by impurities, the vessel for synthesis and storage of

87   colloidal gold should be thoroughly cleaned and soaked in acid (40 mL of distilled water, 360 mL

88   of concentrated sulfuric acid, 20 g of potassium dichromate) or subjected to surface passivation

89   treatment. A citric acid reduction method was used to synthesize colloidal gold.

90

91   1.1 Turn on the magnetic stirrer and place the flask (250 mL) on the mixer.

92

93   1.2 Prepare 4% gold chloride acid solution and 1% sodium citrate solution, respectively.

94

95   1.3 Add 120 mL of distilled water to a round bottom flask and heat it to boiling on a thermostatic

96   magnetic stirrer.

97

98   1.4 Keep boiling, and quickly add 0.5 mL of 4% chloroauric acid and 5 mL of 1% sodium citrate.

99

100   1.5 Observe the color of the solution. The pale yellow chloroauric acid solution turns wine red

101   within a few minutes.

102

103   1.6 Continue heating for 10 minutes, until the solution changes from colorless to transparent

104   wine red.

105

106   1.7 Turn off the power of the thermostatic magnetic mixer, cool to room temperature, and move

107   the mixture to a clean bottle. Store it at 4 °C.

108

109   1.8 Determine the size and morphology of the AuNPs by ultraviolet spectroscopy and TEM

110   imaging.

111

112   NOTE: Different sizes of colloid gold particles for various applications can be obtained by

113   changing the proportion of citrate sodium and chloroauric acid.

114

115   **2. Synthesis of AuNPs-mAb Conjugate**

116

117   NOTE: Since antibodies bind to colloidal gold by electrostatic adsorption, charges on the surface

118   of proteins and colloidal gold directly affect the binding intensity; therefore, the buffer pH value

119   is an important factor affecting the stability of the antibody-colloidal gold conjugate. SSD and

120   anti-SSD mAbs are used as examples in this protocol.

121

122   2.1 Evaluation of coupling pH

123

124   2.1.1 Add 100 µL of NaCl solution (10% m/v) into eight tubes.

125

126   2.1.2 Adjust the AuNP solutions at pH 5, 6, 7, 8, 9, 10, 11 and 12 with 0.1 M K_2CO_3.

127

128   2.1.3 Add 100 µL of colloidal gold solution (pH adjusted 5–12) to eight tubes containing NaCl.

129

130   2.1.4 Allow the solutions to stand for several minutes after blending. Observe the color change
131   of each tube solution, and record the tube that remains red.

132

133   2.1.5 Choose the pH value with the least addition of K_2CO_3 and stable solution color as the
134   optimum pH value for preparing AuNP-mAb conjugates.

135

136   NOTE: Do not use a pH meter because the probe may be destroyed by AuNPs.

137

138   2.2 Evaluation of antibody amount

139

140   2.2.1 Add 100 µL of NaCl solution (10% m/v) to eight tubes.

141

142   2.2.2 Add 100 µL of colloidal gold solution with optimum pH to each tube.

143

144   2.2.3 Add the monoclonal antibody solutions (protein concentration 0.1 mg/mL-3.2 mg/mL) to
145   the abovementioned eight tubes.

146

147   2.2.4 Allow the solutions to stand for several minutes after blending. Observe the color change
148   of each tube solution, and record the tube that remains red.

149

150   2.2.5 Choose the antibody amount with the lowest concentration of mAb and stable solution
151   color as the optimum mAb amount for preparing AuNP-mAb conjugates.

152

153   2.3 Prepare the resuspension buffer: add 1 M Tris-HCl (pH 8.8), 1% (w/v) BSA, 0.5% (v/v) Tween
154   20 and 1% (v/v) PEG 20000 and blend well.

155

156   2.4 Synthesis of AuNP-mAb conjugate

157

158   2.4.1 Take 10 mL of colloidal gold solution and use 0.1 M K_2CO_3 to adjust the solution to the
159   optimal pH value.

160

161   2.4.2 Slowly add SSD mAbs at appropriate concentrations and shake at room temperature for 30
162   min.

163

164 2.4.3 Centrifuge the mixture at 83 x g (1,000 rpm) for 10 min at 4 °C. Remove the precipitate,
165 which contains impurities or precipitated colloidal gold.

166

167 2.4.4 Centrifuge the mixture at 8,330 x g (10,000 rpm) for 30 min at 4 °C. Discard the
168 supernatant, and the precipitate is the colloidal gold-mAb conjugate.

169

170 2.4.5 Add the resuspension buffer to dissociate the precipitation.

171

172 **3. Assembly of the Strip**

173

174 NOTE: For later flow immunoassays, the selection and pretreatment of membrane material will
175 directly affect the test, which should be investigated. The immunochromatographic strip
176 consists of a sample pad, a conjugate pad, a nitrocellulose (NC) membrane, an absorbent pad
177 and a PVC board (**Figure 1**). The membrane material should be checked and evaluated by
178 stereomicroscopy to eliminate inhomogeneity.

179

180 3.1. Paste the NC membrane on a PVC board 2 cm apart from the edge of the suction end of the
181 board.

182

183 3.2 Add SSD-BSA (2 mg/mL) dropwise to the NC membrane (2 cm apart from the upper edge) as
184 a test line (1 mm wide), and add goat anti-mouse IgG (1.5 mg/mL) dropwise on the NC
185 membrane (2 cm apart from the lower edge) as a control line (1 mm wide). Control the amount
186 of protein added.

187

188 3.3 Attach the absorbent pad to the PVC sheet above the NC membrane and overlap it with the
189 NC membrane by 2 mm.

190

191 3.4 Submerge the glass fiber membrane into the AuNPs-mAb conjugate solution. Dry the wet
192 membrane in an incubator at 37 °C.

193

194 3.5 Trim the glass fiber membrane to 5 cm long and 2 cm wide and use it as a conjugate pad.

195

196 3.6 Paste the pretreated conjugate pad under the NC membrane. The length of overlap with the
197 NC membrane should be 0.1 cm.

198

199 NOTE: The glass fiber membrane has a strong ability to bind and release proteins.

200

201 3.7 Trim the fusion 3 membrane to 1.8 cm long and 3.5 cm wide and use it as the sample pad.

202

203 3.8 Paste the sample pad on the PVC board and overlap it with the conjugate pad by 2 mm.

204

205 3.9 Cut the assembled paper board into 3.5-mm-wide strips using a cutting machine and
206 compact it using a batch lamination system.
207
208 3.10 Finally, place the test strips into the shell, seal them in an aluminum foil bag containing
209 desiccant, and store them away from light. The ICSs are now assembled.
210
211 NOTE: The above is the laboratory procedure. In production, gold spraying equipment and
212 cross-membrane instruments are used to spray gold and make the T and C lines.
213
214 **4. Quantitative Detection**
215
216 4.1 Drop 50 µL of sample solution onto the sample hole to observe the chromatography
217 process.
218
219 NOTE: As a result of the capillary action driven by the absorbent pad, the sample solution
220 migrates to the other end of the strip. When the sample solution reaches the conjugate pad,
221 the SSD (antigen) in the sample reacts with the AuNPs-mAb preloaded on the pad. When the
222 solution migrates and reaches the T-line, the AuNPs-mAb without SSD can be selectively
223 captured by SSD-BSA (antigen-carrier protein conjugate), showing as a red color on the T-line.
224 Then, the solution migrates to the C line, where the AuNPs-mAb are captured by goat
225 anti-mouse IgG in the region, thus showing as a red color on the C line.
226
227 4.2 Analyze the strips with a portable strip reader. The machine can provide the ratio of the test
228 line to the control line (T/C).
229
230 4.3 Evaluate the specificity, sensitivity, repeatability and stability of the ICS test.
231
232 NOTE: Under qualitative detection, one red line indicates a positive result (control line). Two
233 red lines indicate a negative result (test and control lines). If the control line is not present, the
234 test is considered invalid.
235
236 **REPRESENTATIVE RESULTS:**
237 **Characterization of colloidal gold**
238 The prepared colloidal gold solutions were claret red. TEM analyses were used to determine
239 the morphology and shape of AuNPs (**Figure 2A-D**). **Figure 2A** and **Figure 2B** reveal that the
240 particles are polyhedral in shape and uniformly distributed. The average diameter of AuNPs was
241 found to be approximately 14 nm (**Figure 2C**). A high-resolution TEM (HRTEM) image of AuNPs
242 is shown in **Figure 2D** and **Figure 2E**. The HRTEM image taken from an individual AuNP shows a
243 continuous fringe pattern with a spacing of 0.117 nm. The UV-vis absorption peaks of the five
244 gold colloidal solutions were 518 nm, 521 nm, 524 nm, 534 nm and 540 nm, which showed that
245 the particle size increased slightly with decreasing sodium citrate (**Figure 2F**).

246

247   [Figure 2 here]

248

249   **Evaluation of the dipsticks**

250   **Sensitivity of the ICS**

251   To determine the sensitivity of ICS, immunochromatographic strips were used to detect a
252   variety of different concentrations of SSD (150,000, 60,000, 12,000, 2400, 480, and 96 ng/mL)
253   standard samples (**Figure 3A**). Double-distilled water was used as the control. In the
254   quantitative experiment, the strips were scanned by a JY1502GS portable ICS reader (**Figure
255   3B-C**). The linear regression equation was y = −0.113ln(x) + 1.5451, with a correlation
256   coefficient (R2) of 0.983 (**Figure 3D**). It displayed good linearity at 96 ng/mL to 150 µg/mL. The
257   IC50 value was 10.39 µg/mL. In the quantitative experiment, the optimal test time was
258   suggested to be 10 min.

259

260   [Figure 3 here]

261

262   **Specificity of the ICS**

263   The specificity of the ICS was tested by evaluating the cross-reactivity with SSD-similar
264   compounds. **Table 1** shows the cross-reactivity of ICS with SSD-related compounds. It can be
265   clearly seen that the ICS only had low cross-reactivity with SSa and had no cross-reaction with
266   other compounds, including SSc, SSb1, or SSb2 (**Table 1**), indicating that the prepared ICS has
267   high specificity.

268

269   [Table 1 here]

270

271   **Recovery rate**

272   As shown in **Table 2**, the average recovery rate was 102.05% (mean ± SD, n = 3). On the basis of
273   its accuracy and consistency, this assay was sufficiently reliable for the determination of SSD in
274   biological samples.

275

276   [Table 2 here]

277

278   **Stability analysis of the ICS assay**

279   To evaluate the stability of the ICS, the test strips stored for 8 and 16 weeks were tested and
280   compared with the newly prepared strips. **Table 3** shows that the results of the stored and
281   newly prepared strips for the negative and positive samples were essentially unchanged,
282   indicating that the ICS can be stored at room temperature for at least several months and that it
283   is suitable for large-scale promotion and application.

284

285   [Table 3 here]

286

Figure 1 Schematic diagram of the immunochromatography assay (**A**) Sandwich immunochromatographic test strips. (**B**) Indirect competitive immune chromatographic test strips. This figure has been modified from Zhang *et al.*, 2018[21].

Figure 2 Characterization of colloidal gold. (**A**) TEM image of AuNPs (100,000× magnification). (**B**) TEM image of AuNPs (500,000× magnification). (**C**) The size distribution of AuNPs. As seen in the TEM image, the diameters of the AuNPs ranged from 10 nm to 18 nm, with an average diameter of approximately 14 nm. (**D, E**) High-resolution TEM (HRTEM) image of AuNPs. The HRTEM image taken from an individual AuNP shows a continuous fringe pattern with a spacing of 0.117 nm. (**F**) UV-vis absorption spectra of different AuNPs ranging from 10 nm to 18 nm. This figure has been modified from Zhang *et al.*, 2018[21].

Figure 3 Characterization of the lateral-flow immunoassay for SSD. (**A**) Photographs of results for standard solutions containing different concentrations of SSD assayed using the ICS. (**B**) Photograph of the matched colloidal gold scan. (**C**) The intensity patterns of the test and control lines scanned by the colloidal gold quantitative instrument. (**D**) Standard curve of icELISA for SSD determination using the ICS. The regression equation is $y = -0.113\ln(x) + 1.5451$, with a correlation coefficient (R^2) of 0.983. This figure has been modified from Zhang *et al.*, 2018[21].

Table 1. Cross-reactivity of compounds measured by ICS and icELISA
The specificity of the ICS was evaluated by ICS (**a**) and icELISA (**b**). This table has been modified from Zhang *et al.*, 2018[21].

Table 2. Recovery rate of SSD
Data are the mean $\pm$ SD from triplicate samples at each spiked concentration of SSD. The percentage of recovery was calculated as follows: recovery (%) = measured amount/amount × 100%. This table has been modified from Zhang *et al.*, 2018[21].

Table 3. Variations among ICS used for the analysis of SSD
[a]The values indicate coefficients of variance for triplicate samples on 3 different strips used 1 day after manufacture. [b]The values indicate coefficients of variance for triplicate samples on 3 different strips used after being stored for 4 weeks. [c]The values indicate coefficients of variance for triplicate samples on 3 different strips used after being stored for 8 weeks. This table has been modified from Zhang *et al.*, 2018[21].

DISCUSSION
In this work, we present a protocol for the preparation of mAbs against natural product-derived small molecules. The essential steps and the matters needing attention in the procedure have been outlined, and we have demonstrated the utility of this protocol using the small molecule SSD as an example. Example spectra, TEM images, quantitative results and methodological investigations are shown in representative data. Hence, we have demonstrated that the

328 colloidal gold production, AuNP-mAb conjugation and strip assembly strategy presented here
329 result in the creation of a novel immunoassay that can be used to rapidly detect the target
330 molecule.

331

332 Immunochromatographic strips are easy to operate, and the reaction is very rapid. Only 50 μL of
333 sample is needed, and detection is complete within 5 minutes. In contrast, the ELISA method,
334 which is also an immunoassay, requires several hours, including multiple washes and long
335 incubations. Multiple washes and long incubation periods can effectively remove the
336 nonspecific reaction of the sample matrix to improve the signal-to-noise ratio and the sensitivity
337 by increasing the signal value. This means that the anti-interference ability and optimization
338 technology of tomographic paper are the key problems to be solved in this study. Technological
339 optimizations can be achieved in several ways. Select suitable materials for the sample pad and
340 conjugate pad. The length of the sample pad was increased appropriately to effectively improve
341 the pretreatment ability of the sample. High pH, high ionic strength and high buffering capacity
342 of material pretreatment can be used to avoid influencing factors such as the antigen–antibody
343 response of the pH value of the biological sample. As the conditional setting of "three high" can
344 interfere with the binding of antibodies, it should be considered comprehensively.

345

346 Moreover, it is also important to note that a monoclonal antibody (mAb) is the probe on the
347 strip. Therefore, this procedure is associated with the sensitivity of the mAb. The mAb we
348 applied was prepared in our previous work[17], and mAb production can be referenced in our
349 previous publication[19].

350

351 Colloidal gold-based immunochromatographic strips are widely used strips because they are
352 inexpensive and offer convenient detection. However, this method has its own limitations and
353 stability issues. The signal strength of AuNPs is lower than those of fluorescent dyes, colloidal
354 carbon and other markers, meaning that the sensitivity is limited. When exposed to air, the
355 AuNPs change from wine red to purple, which will cause a change in the gray value and affect
356 the quantitative detection. Hence, various modified immunochromatographic strips have
357 emerged, and we also prepared quantum dot-labeled fluorescent strips[22] and IgY-based
358 immunochromatographic strips[23], which have similar procedures for production.

359

360 Membrane-based immunoassays have been widely used in clinical indicators, infectious
361 diseases, pesticide residues and drug concentration monitoring. In this protocol, we focused on
362 the preparation of an AuNP-based method for small molecule natural products. This procedure
363 acts as a template for the development of various target immunoassays that can be effectively
364 utilized as analytical tools for the evaluation and quality control of natural products.

365

366 This work provides a detailed protocol for the development of lateral-flow immunoassays based
367 on immune chromatographic strips for use in the rapid, sensitive and quantitative detection of

small molecules. This procedure includes preparation of colloidal gold, synthesis of AuNP-mAb conjugates, assembly of the strip, and methodological investigation.

DISCLOSURES:

The authors have nothing to disclose.

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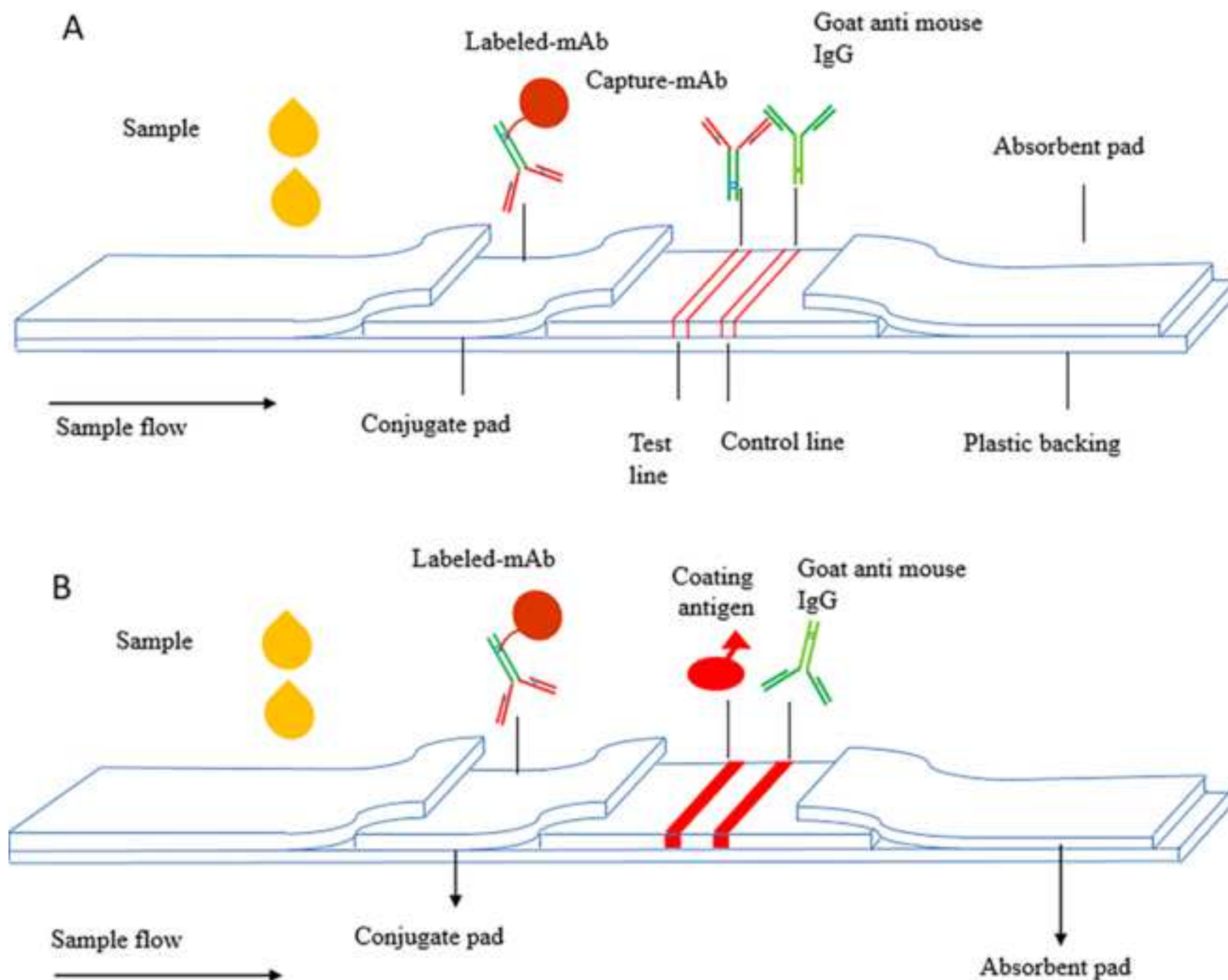424   *Science Edition*. **42** (2020).

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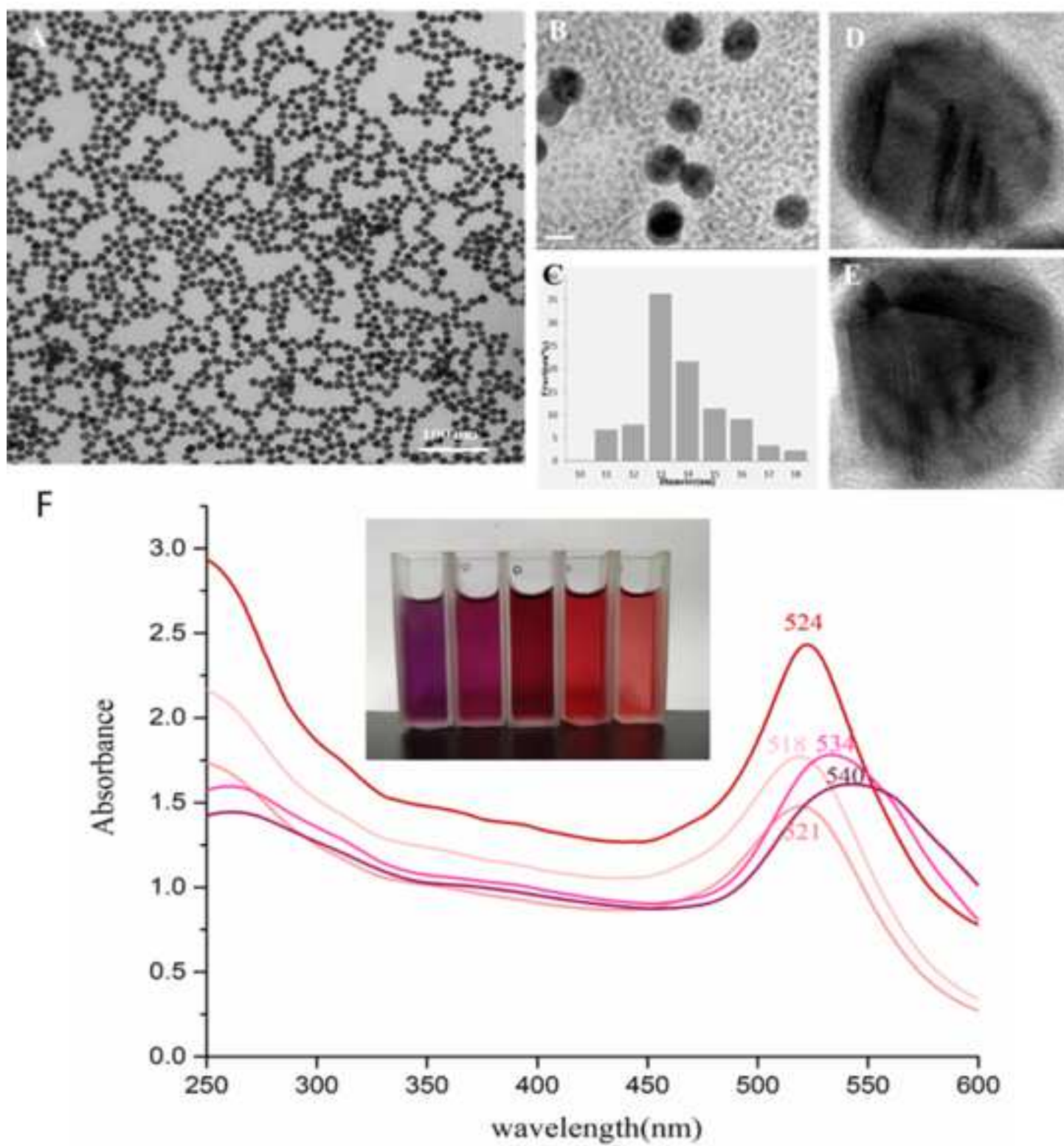426   *Visualized Experiments.* e57116 (2019).

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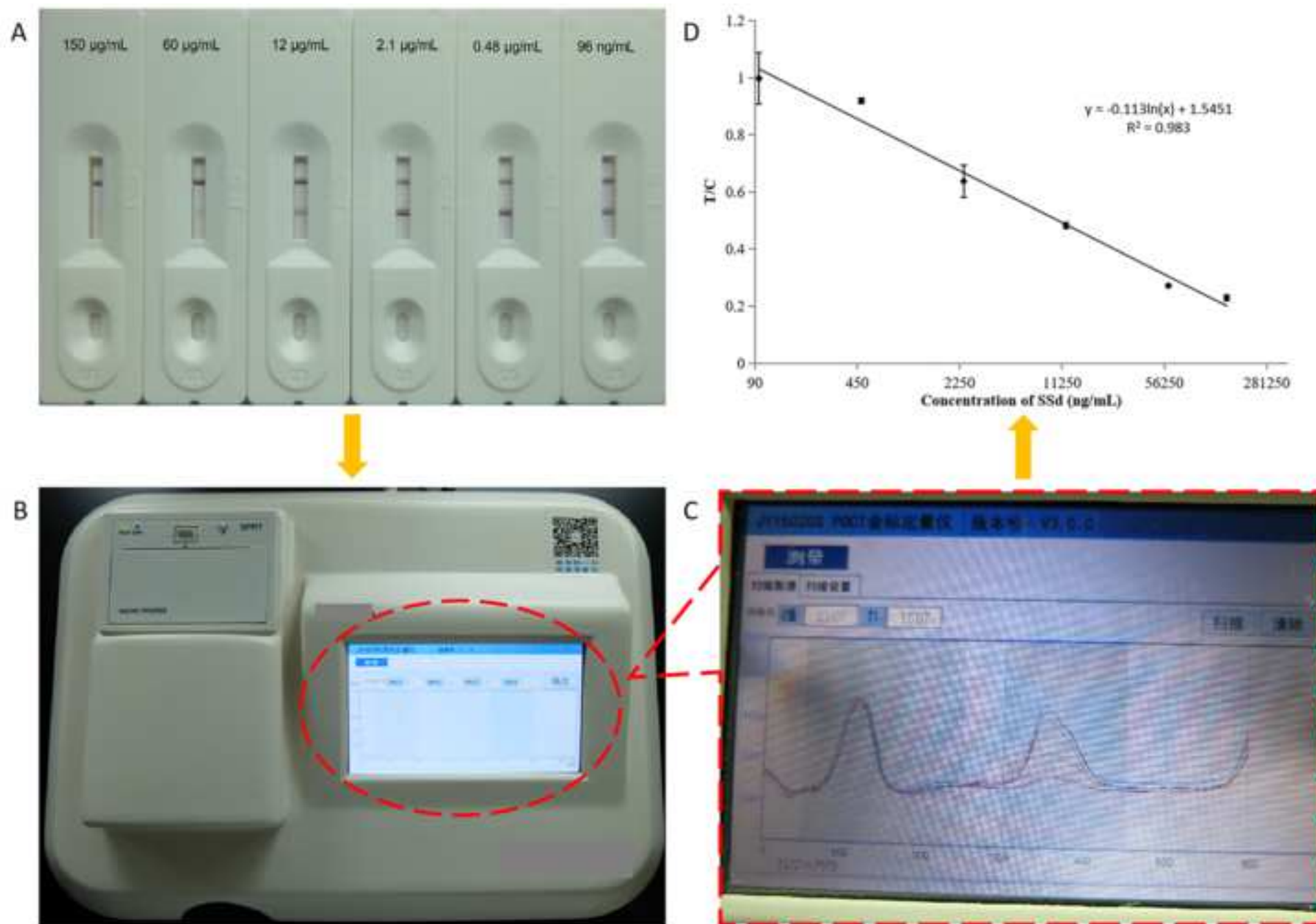429   *Planta Medica*. **82**, 432–439 (2016).

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435   using specific egg yolk antibodies. *Artificial Cells, Nanomedicine, and Biotechnology*. **1** (2017).

436

A

Sample

Labeled-mAb

Capture-mAb

Goat anti mouse IgG

Absorbent pad

Sample flow

Conjugate pad

Test line

Control line

Plastic backing

B

Sample

Labeled-mAb

Coating antigen

Goat anti mouse IgG

Sample flow

Conjugate pad

Absorbent pad

Samples	[a]ICS (%)	[b]icELISA (%)
SSd	100	100
SSa	4.30%	4.97%
SSb1	<0.09	<0.09
SSb2	<0.09	<0.09
SSc	<0.09	<0.09

SSd concentration (ng/mL)	SSd concentration established by the test system (ng/mL)	Recovery (%)
100	135.72 ± 61.97	135.72 ± 61.97
1000	926.59 ± 114.24	92.66 ± 11.42
10000	11128.16 ± 745.75	111.28 ± 14.12

SSd (ng/mL)	RSD %		
	1 day[a]	4 weeks[b]	8 weeks[c]
125	2.41	3.11	3.51
250	2.52	4	3.52
500	2.44	3	5.2
1000	3.12	2.71	4.5

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Table of Materials
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Title: Development of lateral-flow immunochromatographic strip for Rapid and Quantitative Detection of small molecule compounds

Dear editor，

Thank you very much for your reviewing and replying to our manuscript (JoVE62754R1). We would like to express our sincere gratitude to the editor and reviewers for their constructive and positive comments. We replied point by point to the comments, revised the manuscript and video accordingly and the changes were highlighted in red in the revised manuscript. We sincerely hope this revised manuscript is more acceptable for publication.

Thank you again and all the reviewers for the kind advice and look forward to hearing from you soon.

Yours sincerely,

Huihua Qu

Editorial comments:

Changes to be made by the Author(s):
1. Please employ professional copyediting services as the language in the manuscript is not publication grade.
Thank you for your comments, the manuscript has been edited by Elsevier language editing service. The language editing certificate have been attached.
2. Please provide reprint permissions for the figures and tables.
Thank you for your kind reminder, the figures is from *molecules* which is an open-access journal. According to MDPI open access information and policy, "Everyone is free to re-use the published material if proper citation of the original publication is given". As we have cited the original articles, the figures are not need reprint permissions.

Changes to be made by the Author(s) regarding the video:
1. Please change the title in the video to match the written manuscript: Development of a Lateral Flow Immunochromatographic Strip for Rapid and Quantitative Detection

of Small Molecule Compounds

Thank you for your kind reminder, we have modified them.

2. Please ensure that the protocol subheaders in the video match the protocol subheaders in the written manuscript. Use American English (characterization instead of characterisation)

Thank you for your kind reminder, we have modified them.

3. Video Editing Content:
- 03:50 Please remove a glitch happening between the shots.
- 05:08 - 05:13 Please remove dead air.
- Please use some image or video for conclusion section. Do not complete the section just by showing the chapter title card. Using of image or video on conclusion will be more effective for the audience.
- Please use cross dissolve on hard cut for making the video look smooth."

Thank you for your kind reminder, we have modified them.

4. Audio Editing and Pacing:
- Audio levels is low. Please increase the audio level and ensure audio level peaks average around -9 dB.

Thank you for your kind reminder, we have modified them.

Once done please ensure that the revised video is no more than 15 min in length. Please upload the revised file to https://www.jove.com/account/file-uploader?src=19140018

We have uploaded the revised video named JOVE62754R2.