AUTHOR'S COMMENTS ABOUT REVIEW (ROUND 1)

We wish to express our appreciation regarding your useful comments, which have helped us in improving the paper significantly.

Comment 1:

From a practical standpoint, the reactions between CMO and AFB1, as well as the oximation of AFB1 and its binding to BSA, are influenced by multiple factors, leading to unstable reaction yields. Moreover, the exact number of AFB1 molecules bound to BSA remains uncertain, resulting in poor correlation between the final detection signal and the AFB1 concentration in the sample.

Response:

Thank you for your kind comment. In this study, we studied the lateral flow immunoassay on a spiked sample prepared by adding a known AFB1 mycotoxin concentration to the food sample. So we can optimize the number of moles of BSA attached to AFB1.

To determine the appropriate molar ratio of AFB1-CMO to BSA, five molar ratios of AFB1-CMO and BSA were investigated including: 6:1, 8:1, 10:1, 12:1, and 14:1. The binding efficiency of AFB1 and BSA was examined by the dot-blot technique.

The dot-blot technique were performed as follows:

- Spot 2 µl of AFB1-BSA at 5 molar ratios as examined above onto the nitrocellulose membrane (marking small antigen areas). Let the membrane dry, block non-specific sites by 2% Glycine solution, 3% sucrose, and 1% Tween 20 in Borate pH 7.2
- Drop the anti-AFB1 antibody gold nanoparticles complex onto the marked area.
- Wash three times with 10 mM Borate pH 7.2.

Evaluation of results: The darkest signal intensity indicates the best binding efficiency

Results of AFB1-CMO and BSA molar ratio investigation

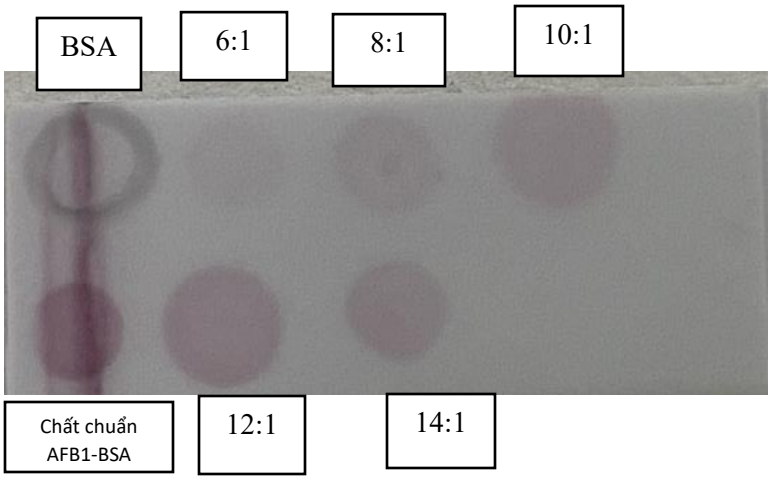


Figure 1. Dot-blot analysis results for the AFB1-CMO and BSA conjugation reaction with molar ratios of: 6:1; 8:1; 10:1; 12:1; and 14:1. AFB1-BSA is the standard.

The maximum binding efficiency in our study showed that: 12 mol of AFB1-CMO was enough to conjugate with 1 mol of BSA

After successfully binding AFB1 and BSA, we dropped the sample onto Lateral flow immunoassay to detect the mycotoxin AFB1 in the sample.

In the future, we might detect the mean of the AFB1 concentration in corn and rice samples in Vietnam according to the routine inspection of the Ministry of Health; then, we can calculate the concentration of BSA added to the sample.

Comment 2:

Compounds containing ketone or aldehyde groups in the samples may react with CMO, thereby affecting the oximation of AFB1.

Response:

Thank you for your kind comment. We acknowledge that compounds with ketone or aldehyde groups in cereal samples could theoretically compete with AFB1 during oximation with CMO. However, our methodology includes critical controls to address this issue:

1. **Sample Pre-treatment**: Prior to derivatization, the sample underwent rigorous extraction and purification using methanol.
2. **Excess CMO**: An excess of CMO was used to ensure complete derivatization of AFB1, even in the presence of competing compounds. However, excessive CMO may interfere with LFIA performance, while insufficient CMO may fail to fully react with AFB1 and competing compounds. Common ketones or aldehydes in cereals are present at trace levels, such as:

Hexanal 0.15-10.40 mg/kg (Chitsamphandhvej, 2008). A headspace solid phase microextraction method for using to monitor hexanal and heptanal content in food samples. Agriculture and Natural Resources, 42(5), 206-212.)

2-Heptanone: 0.1- 1.5 mg/kg (Maga, J. A. 1978). Cereal volatiles, a review. Journal of Agricultural and Food Chemistry, 26(1), 175-178)

Truong Quoc Phong et at (2018) reported that the optimal ratio of AFB1:CMO for efficient conjugation of Aflatoxin B1 with BSA was 1:2. Vietnam Journal of Science and Technology 56 (4A) (2018) 190-198. Therefore, we chose this ratio for our studies.

With the spiked samples, we added 100 µl AFB1 (1mg/ml) and the optimal amount of CMO (200 µl, 3 mg/ml); then, the mixture was incubated for 30 minutes at 80-100^{0}C.

Comment 3:

High concentrations of BSA (20 mg/mL) were utilized in the synthesis of AFB1-BSA conjugates. In cases where AFB1 levels in the sample are low (ng/mL), a significant portion of BSA remains unbound to AFB1. Those unbound BSA will bind to the BSA antibodies on the magnetic nanoparticles, leading to interference in the detection process.

Response:

Thank you very much for your kind comment. We had made some mistakes during writing the manuscript because we did not specify the volume of BSA added to the sample. With the spiked sample (as I mentioned above) added with AFB1 being 200 µg, the volume of BSA (20 mg/ml) added to the sample was 100 µl. In case of BSA excess, the BSA will be bound to the anti-AFB1 antibody-magnetic particle; then this complex will drift to the absorbent pad. This complex will not retained on the test line because of no AFB1 mycotoxin in the sample. Therefore, the magnetic beads-anti-BSA antibody complex might not interfere with the test strip's performance.

Comment 4:

The entire sample processing procedure is intricate, time-consuming, and demands a high level of technical proficiency from users, thereby diminishing the advantages of LFIA as a simple and user-friendly rapid detection method.

Response:

We understand that the entire sample processing process is complicated, but to increase the sensitivity of the test strip, we have tried to produce the LFIA sandwich. Vietnam has a climate of high temperature and high humidity, so mold grows and produces AFB1 mycotoxin. Methods to detect AFB1 mycotoxin,

such as high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) cannot be performed in mountainous areas, remote areas, border areas, or in the field in Vietnam. Because those approaches still require laborious, time-consuming, complex, and expensive detection processes, as well as trained personnel.

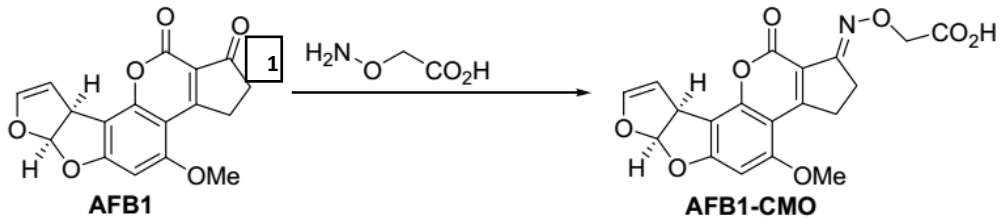
Therefore, the lateral flow immunoassay (LFIA) method is used as a rapid, low-cost method with high levels of accuracy, reliability, and short assay times. The test strip is used to screen for AFB1 mycotoxins that exceed the allowable standard. And these techniques are performed by health workers at district and commune health centers. The development of sandwich LFIA can reduce the limits of detection (LOD), and we can detect samples with AFB1 concentrations exceeding the allowable standard.

Traditionally, AFB1 is detected based on competitive immunochromatography test strips. However, the limit of detection of competitive LFIA is high, and the color signal on the test line is weak, which makes us unable to evaluate samples that meet or exceed the allowable standard. The development of sandwich LFIA can reduce the limits of detection, and we can analyze AFB1 contamination in food samples exceeding the allowable standard.

In the future, if the test strip is applied in practice, we will put the sample processing process, the reagents, and the instruments into the test strip box. The steps in the sample processing process are fully optimized and expected as follows:

Step 1: Grind 1 gram of corn and rice sample and mix with 5 ml of methanol-PBS (7:3; v/v), then centrifuge at 1600 rpm for 5 minutes. Aspirate the careful supernatant solution. This is the sample extraction step.

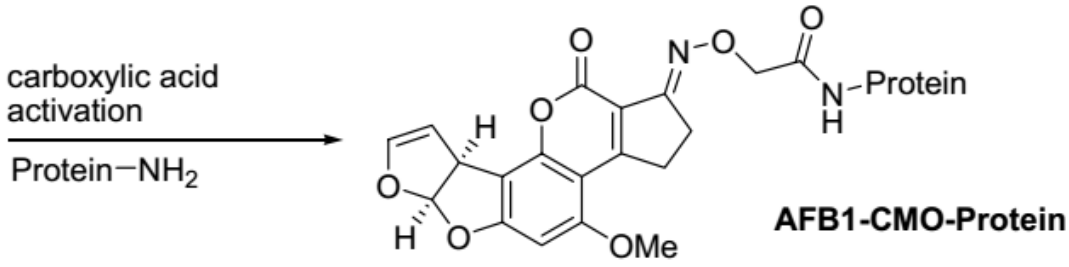
Step 2: Add 100 µl of pyridine to the prepared solution and incubate for 30 minutes at room temperature. (pyridine will attack the 1st position of AFB1 then CMO will replace pyridine to attack that position)



Step 3: Then add 200 µl CMO (3mg/ml) to the solution in step 2 and incubate for 30 minutes at 80-100^{0}C to create the functional group - COOH. Then transfer the solution to the eppendort tube.

Step 4: Activate the -COOH radical in step 3 with 100 µl EDC (10mM) and 100 µl NHS (10mM) for 30 minutes

Step 5: Slowly add 50 µl BSA (20mg/ml) to the solution in step 4 and incubate for 1 hour at room temperature



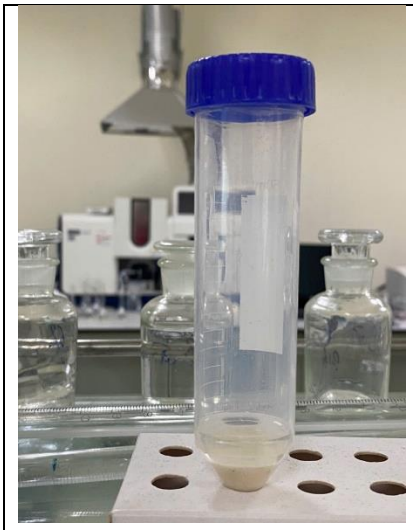
Step 6: Take 50 µl of the prepared sample solution and mix it with 50 µl of running buffer (15mM pH 7.4 PBS comprising 0.6% glycine, and 1% Tween 20), the solution is then analyzed by LFIA.

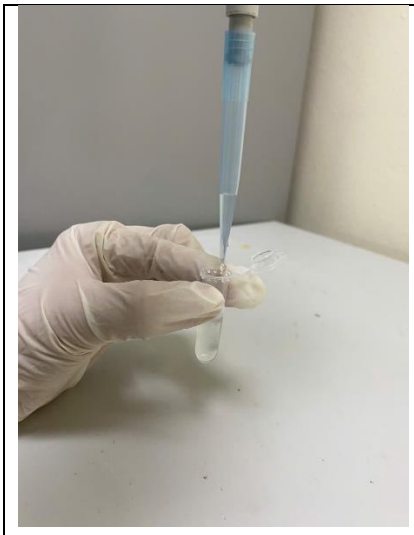
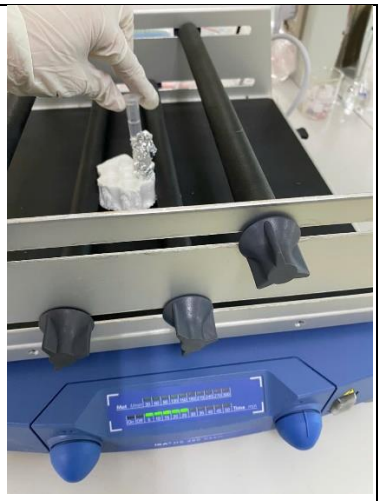
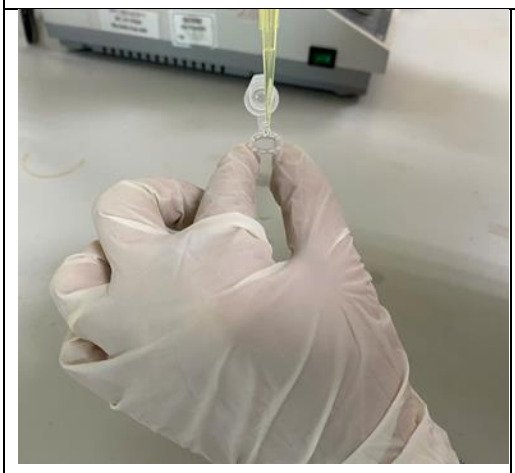
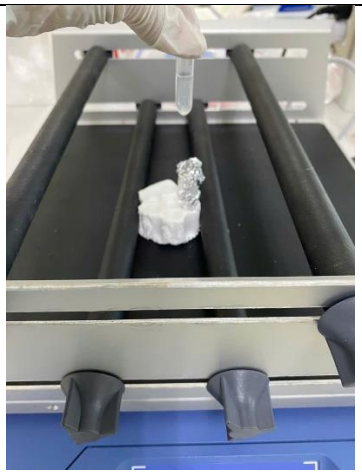
Read the result:

After 12 minutes, the test strip only shows 1 control line, this is a negative sample

After 12 minutes, the test strip only shows 2 lines on the test line and control line, this is a positive sample

Image of sample processing steps

		
Step 1: the sample extraction		Step 2: Add 100 µl of pyridine

<table>
<tr><td>  </td><td>  </td><td>  </td></tr>
<tr><td colspan="2">Step 3: Incubate 30 minutes at 80-100 ^{0}C with CMO</td><td>Step 4: Activate the –COOH by EDC and NHS</td></tr>
<tr><td>  </td><td>  </td><td></td></tr>
<tr><td colspan="2">Step 5: bond to BSA</td><td></td></tr>
</table>

We want to express our thankfulness to all of your detail comments in this present study.

RESPONSE TO REVIEWER ROUND 2

We wish to express our appreciation regarding your useful comments, which have helped us in improving the paper significantly.

Comment 1:

From a practical standpoint, the reactions between CMO and AFB1, as well as the oximation of AFB1 and its binding to BSA, are influenced by multiple factors, leading to unstable reaction yields. Moreover, the exact number of AFB1 molecules bound to BSA remains uncertain, resulting in poor correlation between the final detection signal and the AFB1 concentration in the sample.

Response:

Thank you for your kind comment. In this study, we studied the lateral flow immunoassay on a spiked sample prepared by adding a known AFB1 mycotoxin concentration to the food sample. So we can optimize the number of moles of BSA attached to AFB1.

To determine the appropriate molar ratio of AFB1-CMO to BSA, five molar ratios of AFB1-CMO and BSA were investigated including: 6:1, 8:1, 10:1, 12:1, and 14:1. The binding efficiency of AFB1 and BSA was examined by the dot-blot technique.

The dot-blot technique were performed as follows:

- Spot 2 µl of AFB1-BSA at 5 molar ratios as examined above onto the nitrocellulose membrane (marking small antigen areas). Let the membrane dry, block non-specific sites by 2% Glycine solution, 3% sucrose, and 1% Tween 20 in Borate pH 7.2

- Drop the anti-AFB1 antibody gold nanoparticles complex onto the marked area.

- Wash three times with 10 mM Borate pH 7.2.

Evaluation of results: The darkest signal intensity indicates the best binding efficiency

Results of AFB1-CMO and BSA molar ratio investigation

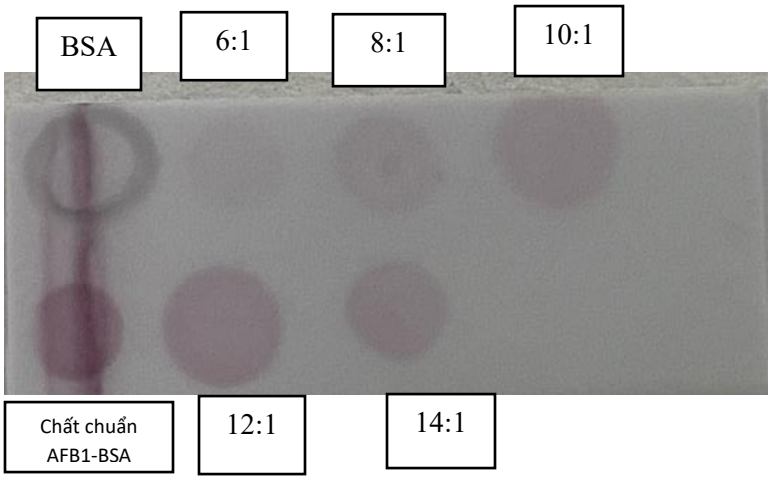


Figure 1. Dot-blot analysis results for the AFB1-CMO and BSA conjugation reaction with molar ratios of: 6:1; 8:1; 10:1; 12:1; and 14:1. AFB1-BSA is the standard.

The maximum binding efficiency in our study showed that: 12 mol of AFB1-CMO was enough to conjugate with 1 mol of BSA

After successfully binding AFB1 and BSA, we dropped the sample onto Lateral flow immunoassay to detect the mycotoxin AFB1 in the sample.

In the future, we might detect the mean of the AFB1 concentration in corn and rice samples in Vietnam according to the routine inspection of the Ministry of Health; then, we can calculate the concentration of BSA added to the sample.

After our explanation, we hope to receive your valuable comments from reviewer.

Comment 2:

Compounds containing ketone or aldehyde groups in the samples may react with CMO, thereby affecting the oximation of AFB1.

Response:

Thank you for your kind comment. We acknowledge that compounds with ketone or aldehyde groups in cereal samples could theoretically compete with AFB1 during oximation with CMO. However, our methodology includes critical controls to address this issue:

3. **Sample Pre-treatment**: Prior to derivatization, the sample underwent rigorous extraction and purification using methanol.
4. **Excess CMO**: An excess of CMO was used to ensure complete derivatization of AFB1, even in the presence of competing compounds. However, excessive CMO may interfere with LFIA performance, while insufficient CMO may fail to fully react with AFB1 and competing compounds. Common ketones or aldehydes in cereals are present at trace levels, such as:

Hexanal 0.15-10.40 mg/kg (Chitsamphandhvej, 2008). A headspace solid phase microextraction method for using to monitor hexanal and heptanal content in food samples. Agriculture and Natural Resources, 42(5), 206-212.)

2-Heptanone: 0.1- 1.5 mg/kg (Maga, J. A. 1978). Cereal volatiles, a review. Journal of Agricultural and Food Chemistry, 26(1), 175-178)

Truong Quoc Phong et at (2018) reported that the optimal ratio of AFB1:CMO for efficient conjugation of Aflatoxin B1 with BSA was 1:2. Vietnam Journal of Science and Technology 56 (4A) (2018) 190-198. Therefore, we chose this ratio for our studies.

With the spiked samples, we added 100 µl AFB1 (1mg/ml) and the optimal amount of CMO (200 µl, 3 mg/ml); then, the mixture was incubated for 30 minutes at 80-100^{0}C.

Comment 3:

High concentrations of BSA (20 mg/mL) were utilized in the synthesis of AFB1-BSA conjugates. In cases where AFB1 levels in the sample are low (ng/mL), a significant portion of BSA remains unbound to AFB1. Those unbound BSA will bind to the BSA antibodies on the magnetic nanoparticles, leading to interference in the detection process.

Response:

Thank you very much for your kind comment. We had made some mistakes during writing the manuscript because we did not specify the volume of BSA added to the sample. With the spiked sample (as I mentioned above) added with AFB1 being 200 µg, the volume of BSA (20 mg/ml) added to the sample was 100 µl. In case of BSA excess, the BSA will be bound to the anti-AFB1 antibody-magnetic particle; then this complex will drift to the absorbent pad. This complex will not retained on the test line because of no AFB1 mycotoxin in the sample. Therefore, the magnetic beads-anti-BSA antibody complex might not interfere with the test strip's performance.

Comment 4:

The entire sample processing procedure is intricate, time-consuming, and demands a high level of technical proficiency from users, thereby diminishing the advantages of LFIA as a simple and user-friendly rapid detection method.

Response:

We understand that the entire sample processing process is complicated, but to increase the sensitivity of the test strip, we have tried to produce the LFIA sandwich. Vietnam has a climate of high temperature and high humidity, so mold grows and produces AFB1 mycotoxin. Methods to detect AFB1 mycotoxin, such as high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) cannot be performed in mountainous areas, remote areas, border areas, or in the field in Vietnam. Because those approaches still require laborious, time-consuming, complex, and expensive detection processes, as well as trained personnel.

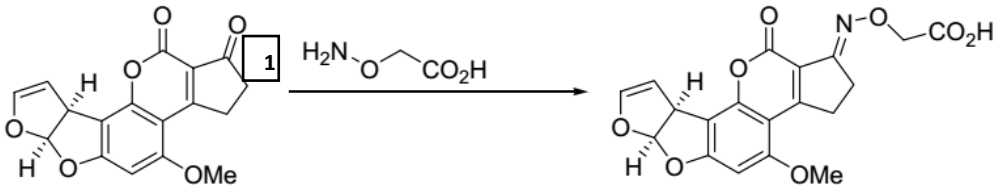
Therefore, the lateral flow immunoassay (LFIA) method is used as a rapid, low-cost method with high levels of accuracy, reliability, and short assay times. The test strip is used to screen for AFB1 mycotoxins that exceed the allowable standard. And these techniques are performed by health workers at district and commune health centers. The development of sandwich LFIA can reduce the limits of detection (LOD), and we can detect samples with AFB1 concentrations exceeding the allowable standard.

Traditionally, AFB1 is detected based on competitive immunochromatography test strips. However, the limit of detection of competitive LFIA is high, and the color signal on the test line is weak, which makes us unable to evaluate samples that meet or exceed the allowable standard. The development of sandwich LFIA can reduce the limits of detection, and we can analyze AFB1 contamination in food samples exceeding the allowable standard.

In the future, if the test strip is applied in practice, we will put the sample processing process, the reagents, and the instruments into the test strip box. The steps in the sample processing process are fully optimized and expected as follows:

Step 1: Grind 1 gram of corn and rice sample and mix with 5 ml of methanol-PBS (7:3; v/v), then centrifuge at 1600 rpm for 5 minutes. Aspirate the careful supernatant solution. This is the sample extraction step.

Step 2: Add 100 µl of pyridine to the prepared solution and incubate for 30 minutes at room temperature. (pyridine will attack the 1st position of AFB1 then CMO will replace pyridine to attack that position)

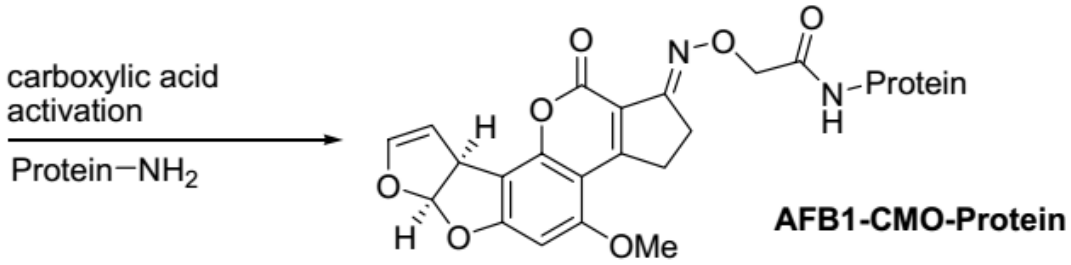


AFB1 **AFB1-CMO**

Step 3: Then add 200 µl CMO (3mg/ml) to the solution in step 2 and incubate for 30 minutes at 80-100^{0}C to create the functional group - COOH. Then transfer the solution to the eppendort tube.

Step 4: Activate the -COOH radical in step 3 with 100 µl EDC (10mM) and 100 µl NHS (10mM) for 30 minutes

Step 5: Slowly add 50 µl BSA (20mg/ml) to the solution in step 4 and incubate for 1 hour at room temperature



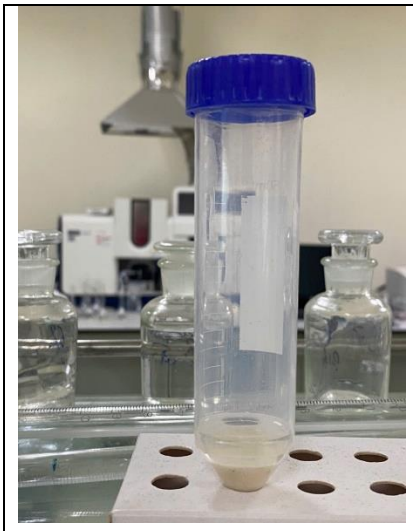
Step 6: Take 50 µl of the prepared sample solution and mix it with 50 µl of running buffer (15mM pH 7.4 PBS comprising 0.6% glycine, and 1% Tween 20), the solution is then analyzed by LFIA.

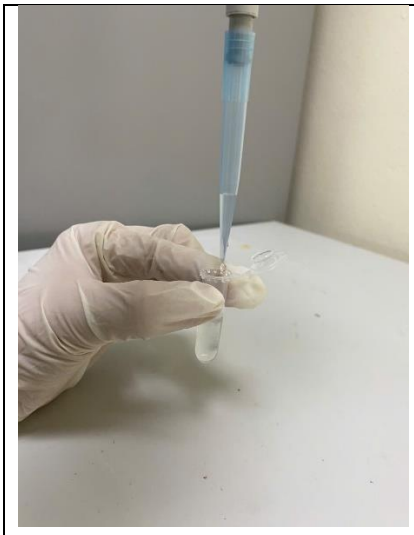
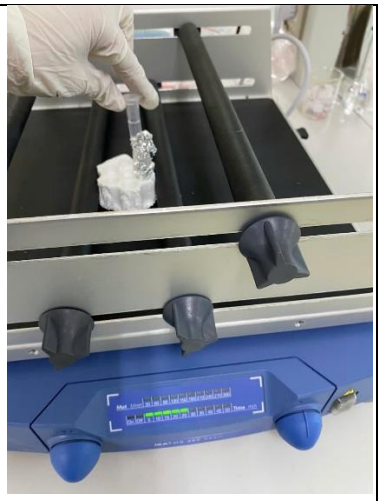
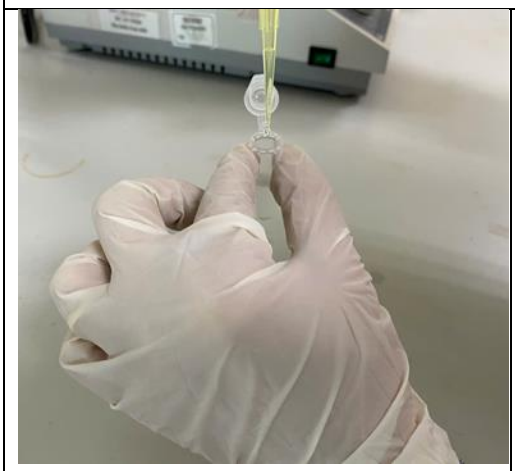
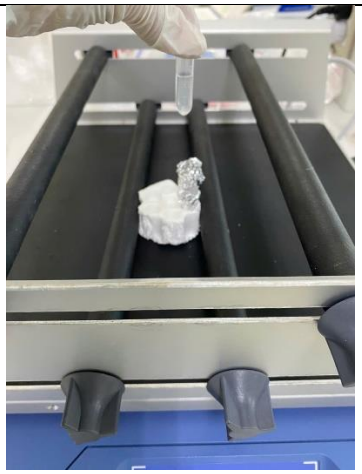
Read the result:

After 12 minutes, the test strip only shows 1 control line, this is a negative sample

After 12 minutes, the test strip only shows 2 lines on the test line and control line, this is a positive sample

Image of sample processing steps

<table>
<tr><td>  </td><td>  </td><td>  </td></tr>
<tr><td colspan="2">Step 1: the sample extraction</td><td>Step 2: Add 100 µl of pyridine</td></tr>
</table>

<table>
<tr><td>  </td><td>  </td><td>  </td></tr>
<tr><td colspan="2">Step 3: Incubate 30 minutes at 80-100 ^{0}C with CMO</td><td>Step 4: Activate the –COOH by EDC and NHS</td></tr>
<tr><td>  </td><td>  </td><td></td></tr>
<tr><td colspan="2">Step 5: bond to BSA</td><td></td></tr>
</table>

We want to express our thankfulness to all of your detail comments in this present study. After our explanation, we hope to receive your valuable comments from reviewer.

RESPONSE TO REVIEWER 2:

We wish to express our appreciation regarding your insightful comments, which have helped us in improving the paper significantly.

Comment 1:

Look like an objective (should be the “why” the research was done)

Response:

Thank you for your valuable comment and helpful advice. We had made some mistakes during writing the manuscript. We had corrected in the new version of manuscript.

Comment 2:

Wide

Must be concrete (and not to include Methods) in line with the paper title.

Response:

Thank you for your valuable comment and helpful advice.

Comment 3:

Should provide summarized details about the main issues of methods

Response:

Thank you for your valuable comment and helpful advice.

Comment 4:

Avoid using first person in scientific writing.

Response:

Thank you for your valuable comment and helpful advice. We had corrected in the new version of manuscript.

Comment 5:

"Fe_3O_4@Au; Lateral flow immunoassay" Verify at: DeCS – Descritores em Ciências da Saúde

Response:

Thank you for your advice. Base on your comment, we have changed the term "Fe_3O_4@Au; Lateral flow immunoassay" to "Magnetic nanoparticles; Immunochromatographic Assay" throughout the manuscript.

Comment 6:

Check format in author's guidelines for References

Response:

Thank you for your advice. We made references of the manuscript due to the requirement of the journal. We re-submitted you a new version with reference documents as directed.

Comment 7:

Is figure 1 and original graphic?

Otherwise the source should be quoted.

Response:

Thank you for your valuable comment and helpful advice. Figure 1 in our manuscript is the original image

Comment 8:

Consider change Table 1 orientation (counterclockwise).

See example below (will be easier for editors formatting). Should include columns heading.

Response:

Thank you for your valuable comment and helpful advice. Base on your comment, we have changed throughout the new manuscript.

Comment 9:

Should respond directly to objective. Avoid including results in conclusion. Just the qualitative generalization of results.

Response:

Thank you for your valuable comment and helpful advice.

Comment 10:

Data Availability Statement

Consider providing as a complementary file, the response for previous review (it clarifies author's work)

Response:

Thank you for your valuable comment and helpful advice. I would like to send the response for previous review.

After our explanation, we hope to receive your valuable comments about this part.