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ORIGINAL ARTICLE

Development of a lateral flow immunoassays-based method for the screening of ractopamine in foods and evaluation of the optimal strategy in combination of screening and confirmatory tests

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Abstract

Ractopamine has been authorized as a feed additive and permitted in animal husbandry. With the establishment of the regulation to limit the concentration of ractopamine, a rapid screening method for ractopamine is urgently needed. Additionally, how to combine the screening and confirmatory tests of ractopamine is also critical to maximizing the efficiency of testing. Here, we developed a lateral flow immunoassays-based method for the screening of ractopamine in foods and proposed a cost-benefit analysis approach to optimize cost allocation between screening and confirmatory tests. After verifying the analytical and clinical performances of the screening method, a mathematical model was established to calculate the screening and confirmatory test results with various parameter settings, such as cost allocation, false-negative tolerance, and total budget size. The developed immunoassay-based screening test could successfully distinguish gravy samples with ractopamine levels over and lower than maximum residue limits (MRL). The area under curve (AUC) value of receiver operating characteristic (ROC) curve is 0.99. For the cost-benefit analysis, mathematical simulation indicated that when the samples are allocated to screening and confirmatory tests at the optimized cost allocation, the number of confirmed positive samples can increase by 26 times compared to the scenarios entirely relying on confirmatory testing. While conventional wisdom considers that screening should be carried out at low false-negative rates, such as 0.1%, our results indicated that the cutoff value of a screening test with a 20% false-negative rate at MRL could capture the maximum number of confirmed positive samples at a limited budget. Our work indicated that the participation of the screening method in ractopamine analysis and optimized cost allocation between screening and confirmatory tests could enhance the efficiency in detecting the positive samples, which provides a rational basis for decision-making in food safety enforcement for public health.

Keywords: Competitive colloidal gold-based lateral flow competitive immunoassay, Cost-benefit analysis, Ractopamine, Screening and confirmatory tests, Simulation

1. Introduction

The quality and safety of food have become considerable issues during the past few decades. According to the "WHO estimates of the global burden of foodborne diseases" published by the WHO Foodborne Disease Burden Epidemiology Reference Group (FERG) in 2015 [1], 32 diseases induced by 31 foodborne hazards caused illness in approximately 600 million people and the death of 420,000 people. Ractopamine, a β-adrenergic agonist (β-agonist) with phenolic group substituent, was authorized as feed additive to promote lean muscle in 1990 in the USA [2]. Because the intake of ractopamine-treated meat may pose health risks such as cardiovascular effects and potential genitourinary

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toxicity [3,4], 120 countries have set the maximum residue limits (MRL) of ractopamine [5]. According to the Taiwan Food and Drug Administration (TFDA)'s regulation, ractopamine is prohibited from applying as a feed additive for locally produced pork and beef. In 2007 and 2021, Taiwan lifted the ban on importing ractopamine-contained beef and pork, respectively, and set up the MRL at 0.01 ppm (mg/kg) for muscle tissues [6].

With the increasing demand for food safety, governments have legislated to ensure food safety. In most countries, regulatory agencies have developed analytical methods to identify, monitor, and assess foodborne hazards. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) is widely recognized as the gold standard for food analysis owing to adequate certainty in the qualitative analysis of chemicals and sufficient sensitivity for quantitative analysis [7]. Although LC-MS/MS provides a huge advantage in food analysis, the full procedures for LC-MS/MS analysis are costly in time, instrument, chemical standards, and personnel training [8].

Screening tests can rapidly analyze many samples and detect targets at the level of interest in the available cost [8,9]. Because the screening test cannot provide identical accuracy as confirmatory analysis due to technical limitations, employing confirmatory analysis after the screening test was utilized to increase both efficiency and accuracy for the detection of the positive sample (Fig. 1). The concept of combining screening and confirmatory tests has been adopted in numerous types of analyses. For instance, diverse portable biosensors are available for the diagnosis of SARS-CoV-2 to support time-consuming and labor-intensive quantitative real-time polymerase chain reaction (qRT-PCR) [10–14]. In Taiwan, the analysis of controlled drugs in urine is also divided into preliminary and confirmation analyses using competitive immunoassays and LC-MS/MS, respectively [15]. Although the benefit of screening tests has been recognized as a supporting method to reduce the cost of LC-MS/MS in food analysis [8], the developed screening and confirmatory tests for food analysis are still limited.

Here, we developed a compatible colloidal gold-based lateral flow competitive immunoassays-based rapid detection device (RDD) for the determination of ractopamine in pork samples. The analytical and clinical performances of the developed method was evaluated. Additionally, we also investigated the optimal strategy to combine screening and confirmatory tests, such as LC-MS/MS, in ractopamine analysis. The screening test is typically designed with high false positive, and a minimum false negative due to the false positive sample could be further confirmed by a confirmatory test [8,16,17]. However, for surveying contaminants in food, the high false-positive rate also represents the waste of the cost for confirmatory analysis [18]. Therefore, how to divide the limited budget into screening and confirmatory tests to detect the maximize the number of positive samples needs further investigation. In this work, a cost-benefit analysis [19,20] was performed to investigate the optimal cost allocation between screening and confirmatory tests within a given total budget. Furthermore, two pivotal variables, thresholds of screening tests and total budgets, were also investigated in the cost-benefit analysis to maximize the number of positive samples. A model of screening and confirmatory tests was established to mathematically generate the measurement results in different cost allocation schemes and thresholds.

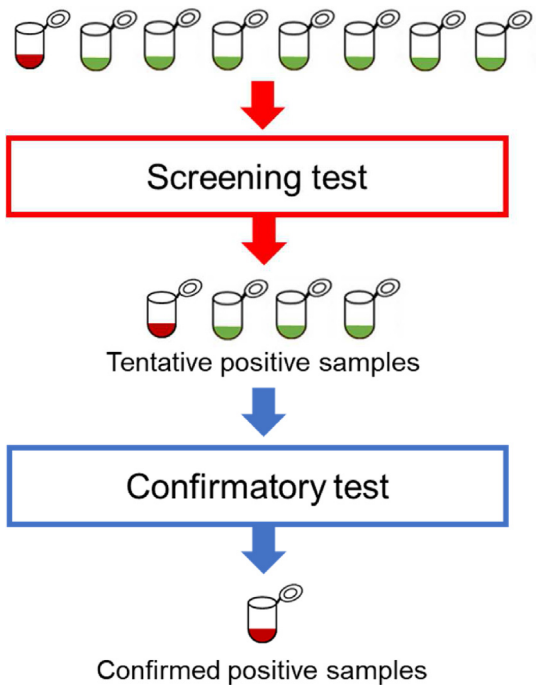

Fig. 1. The concept of combining screening and confirmatory testing. The red vial symbolizes a true-positive sample, and the green vials are negative samples. Although the screening test may provide false results due to its inferior analytical performance (represented by the green vials in the screening test results), the confirmatory test can confirm the screening test result with high accuracy. In addition, due to the adoption of the screening test, the overall analysis throughput is improved.

2. Materials and methods

2.1. Chemicals, food samples and the device of competitive colloidal gold-based lateral flow competitive immunoassays

Ractopamine standard was purchased from Sigma Aldrich (St. Louis, MO, USA). Competitive colloidal gold-based lateral flow immunochromatographic was purchased from Kanyoku Corporation (Taipei, Taiwan). Six meat samples used for the RDD analysis were obtained from a local butcher shop in Tainan, Taiwan. Before the RDD analysis, the ractopamine residue in all meat samples was confirmed by LC-MS/MS analysis. The ractopamine residue of each sample was lower than the limit of detection (LOD, 1 μg/kg). The basic principle of competitive lateral flow immunochromatography is described in Supporting Information Fig. S1 [https://www.jfda-online.com/journal/vol31/iss2/7/]. In brief, the immunoassays contain the antigen-binding sites of ractopamine and colored colloidal gold-antibody. When the ractopamine contained sample droplets flow through the NC membrane, ractopamine will compete with colloidal gold-antibody for the antigen-binding sites, which will cause the color of the T-line fade.

2.2. Sample preparation

Meat samples were homogenized by scissors. The mixed minced meat sample was separated into six replicate samples of 25 g each. For extraction, 25 mL of deionized water was added to the six minced meat samples and incubated at 90 °C for 15 min. After incubation, six samples were centrifuged at 10,000×*g* for 30 min, and 15 mL of the supernatant of each sample was collected and spiked with ractopamine standard.

2.3. Analytical and clinical validation of competitive colloidal gold-based lateral flow competitive immunoassays-based screening test method

The calculation of analytical and clinical performances were based on Microsoft Excel 2019 (Redmond, WA, USA). Four analytical performances (linearity, precision, recovery, and LOD) of the lateral flow competitive immunoassays-based screening test were investigated in this study. The meat samples spiked ractopamine standard with concentration levels of 0, 2.5, 5, 7.5, 10, and 20 μg/kg were repeated 51 times, respectively. After 5 min of reaction, measurement results were recorded by a digital camera and transferred to digitized information by ImageJ [21]. One of the repetitions from each concentration level was used to generate the standard curve. The standard curve of ractopamine was obtained when the test line (T-line)/control line (C-line) (T/C ratio) was plotted against the logarithm of six concentrations of ractopamine-spiked meat samples. The LOD was defined as 10% inhibition of the T/C ratio of the 0 μg/kg sample [22] which was determined as the concentration level corresponded to 10% T/C ratio of 0 μg/kg sample using the calibration curve. For the assessment of the precision, the coefficient of variation (CV) of the digitized T/C ratio of each concentration with 50 repetitions was reported as the precision of the proposed testing. The recovery was determined by calculating the difference between the spiked concentration and the concentration estimated by the standard curve.

Five clinical performances (sensitivity, specificity, false-positive rate, false-negative rate, and receiver operating characteristic (ROC) curve) of the lateral flow competitive immunoassays-based screening test method were estimated by the simulation results of a screening test based on the reported ractopamine residue distribution in pork sample. A survey of ractopamine residue in the Midwest US retail market [23] was adopted for generating the distribution of ractopamine residue in pork. To simulate the proximate distribution of ractopamine residue in countries restricting the use of ractopamine, the distribution was further divided by 7 to reach the positive rate of simulated samples of approximately two thousandths. The measurement results at eight different cutoff value settings (eight thresholds with false-negative rates of 0.1%, 1%, 5%, 10%, 20%, 30%, 40%, and 50% at 10 μg/kg-spiked samples) were calculated and further generated the corresponding sensitivity, specificity, false-positive rate, and false-negative rate, respectively. The ROC curve of the lateral flow competitive immunoassays-based screening test was illustrated by the sensitivity and specificity data from 8 cutoff values.

2.4. Development of the model of screening and confirmatory tests for the evaluation of the optimal combination strategy

The calculation of screening and confirmatory tests was based on Microsoft Excel 2019 (Redmond, WA, USA). The model of screening and confirmatory tests was developed to estimate the optimal combination strategy between screening and confirmatory tests. A hypothetical procedure of screening and confirmatory tests is illustrated in

Fig. 2. In the proposed procedure, the total budget (*TB*) for the analysis was first allocated to the screening test and confirmatory test, where *S*% is the allocation ratio of the screening test and $(1-S\%)$ is the allocation ratio of the confirmatory test. Each test can only analyze the samples by the acquired budget in the following testing. After determining the cost allocation, samples are first analyzed by the screening test. The number of screening test-analyzed samples is determined by the given allocation of the budget. Two situations would appear after the screening analysis: one is the budget for a confirmatory test that can afford or beyond to confirm that all the positive samples passed the screening test, and the other is the budget for a confirmatory test that cannot pay for testing all the positive samples from the screening test. For the first situation, a confirmatory test would analyze all the positive samples from the screening test and directly test the samples without the screening results using the remaining budget. For the situation in which the confirmatory test cannot afford all the positive samples to pass the screening test, the confirmatory test only confirms part of the screening test-passed samples by the given budget for the confirmatory test. The number of detected positive samples during the entire procedure is defined as the benefit of the test. The enhancement of detected positive samples in a fixed budget also represent the increase of efficiency in testing.

The mathematical model of screening and confirmatory tests can be interpreted by Eq. (1):

$$TPN = SCPN + DCPN \tag{1}$$

where *TPN* is the total number of positive samples detected by the proposed procedure, *SCPN* is the number of positive samples that pass both screening and confirmatory tests, and *DCPN* is the number of positive samples directly tested by confirmatory test using remaining budget. The SCPN in Eq. (1) is defined in Eq. (2) and Eq. (3):

$$SCPN = \left(\frac{S\% * TB}{C_{screening}} * P_{screening}\right) * P_{confirmatory} \tag{2}$$

where,

$$\left(\frac{(1-S\%) * TB}{C_{confirmatory}}\right) \geq \left(\frac{S\% * TB}{C_{screening}} * P_{screening}\right)$$

$$SCPN = \left(\frac{(1-S\%) * TB}{C_{confirmatory}}\right) * P_{confirmatory} \tag{3}$$

where,

$$\left(\frac{(1-S\%) * TB}{C_{confirmatory}}\right) < \left(\frac{S\% * TB}{C_{screening}} * P_{screening}\right)$$

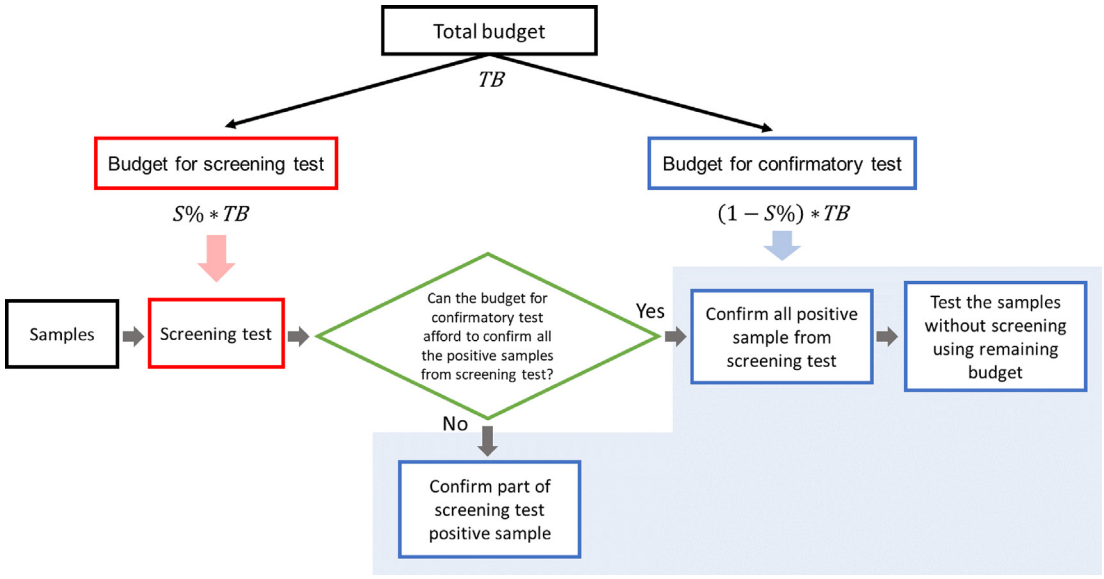

*Fig. 2. Schematic illustration of the hypothetical screening and confirmatory test procedure. The total budget (TB) for the analysis was first allocated to screening tests (S%*TB) and confirmatory tests ((1-S%) *TB). Based on the limited budget for two tests, the operation of the confirmatory tests was divided into two possible routes. If the allocation of budget for confirmatory analysis cannot test all positive samples from the screening tests (result of conditional block after the screening test is "No"), only part of the positive samples would be confirmed by confirmatory test. When the confirmatory test can afford or beyond to analyze all positive samples from the screening test (result of conditional block is "Yes"), all positive samples that passed the screening test would be tested by the confirmatory test. The remaining budget of the confirmatory test was further used to analyze samples without the results of the screening test.*

The description of parameters were listed in Table 1. Eq. (2) and Eq. (3) represent two situations described in the hypothetical procedure of screening and confirmatory tests. Eq. (2) is utilized when the maximum number of samples that can be analyzed by confirmatory test $\left(\frac{(1-S\%)*TB}{C_{confirmatory}}\right)$ is larger than the number of positive samples that passed the screening test $\left(\frac{S\%*TB}{C_{screening}}*P_{screening}\right)$. In this situation, all positive samples that passed the screening test can be confirmed by the confirmatory test. On the other hand, a confirmatory test can only confirm that part of the samples passed the screening test based on the given budget. The number of confirmed samples is only associated with the maximum number of samples that can be analyzed by confirmatory testing.

The *DCPN* in Eq. (1) is defined in Eq. (4) and Eq. (5):

$$DCPN=\left[\left(\frac{(1-S\%)*TB}{C_{confirmatory}}\right)-\left(\frac{S\%*TB}{C_{screening}}*P_{screening}\right)\right]*TP \quad (4)$$

where,

$$\left(\frac{(1-S\%)*TB}{C_{confirmatory}}\right)<\left(\frac{S\%*TB}{C_{screening}}*P_{screening}\right)$$

$$DCPN=0 \quad (5)$$

where,

$$\left(\frac{(1-S\%)*TB}{C_{confirmatory}}\right)<\left(\frac{S\%*TB}{C_{screening}}*P_{screening}\right)$$

The *DCPN* is a positive number only when the maximum number of samples that can be analyzed by confirmatory testing is larger than the number of positive samples that pass the screening test because the proposed screening and confirmatory test procedure only allows the confirmatory test to directly analyze the samples without passing the screening test using the leftover budget. The value of *DCPN* was related to the remaining capacity of the confirmatory test $\left[\left(\frac{(1-S\%)*TB}{C_{confirmatory}}\right)-\left(\frac{S\%*TB}{C_{screening}}*P_{screening}\right)\right]$ and the positive rate when the confirmatory test analyzed the sample without the screening results (*TP*).

In this model, both *SCPN* and *DCPN* are related to the allocation ratio of the screening test (*S*%), which indicated that *S*% is the essential variable affecting the number of detected positive samples. In addition to *S*%, the other six parameters (Table 1) which necessary for the establishment of the model were determined based on the case of screening and confirmatory tests.

3. Results

3.1. Validation of competitive colloidal gold-based lateral flow competitive immunoassays-based screening test method

The gravy samples spiked with ractopamine in final concentrations equal to 0, 2.5, 5, 7.5, 10, and 20 μg/kg were tested by lateral flow competitive immunoassays and scanned the response of T-line and C-line by camera (Fig. 3A). The complete images of the RDD analysis results of all ractopamine-spiked samples are shown in Fig. S4~S9 [https://www.jfda-online.com/journal/vol31/iss2/7/]. It was shown that the color of T-line was progressively faded with the increasing concentration of ractopamine. The standard curve of the competitive colloidal gold-based lateral flow competitive immunoassays was illustrated in Fig. 3B. Because the curve with original concentration scale cannot fit the polynomial, the standard curve of the method was transferred so that T/C ratio was plotted against the ten-based logarithm of a series concentration of ractopamine (Fig. 3C). The R^2 value (0.9864) showed the standard curve of the method was usable. The LOD of method was evaluated as 0.18 μg/kg based on 10% inhibition of the T/C ratio of the 0 μg/kg sample. The precision and recovery of the method in 2.5, 5, 7.5, 10, and 20 μg/kg were listed in Table 2. The precision which over 17% indicated that a depressed false-negative value in the setting of cutoff value would accompany a high false-positive

Table 1. Six parameters for the generation of the simulation model of screening and confirmatory tests in the case of ractopamine residue analysis.

Description of parameters	Code names	Value	Unit
Total budget	*TB*	100,000	U.S. Dollar
Cost per screening test	$C_{screening}$	4	U.S. Dollar
Cost per confirmatory test	$C_{confirmatory}$	100	U.S. Dollar
True positive rate of food with the chemical exceeding the MRL[a]	*TP*	0.177	%
Positive rate of screening test	$P_{screening}$	Non-fixed[b]	%
Positive rate of confirmatory test	$P_{confirmatory}$	Non-fixed[b]	%

[a] MRL: maximum residue limits.
[b] Corresponding to the thresholds of the screening test, which listed in Table 2

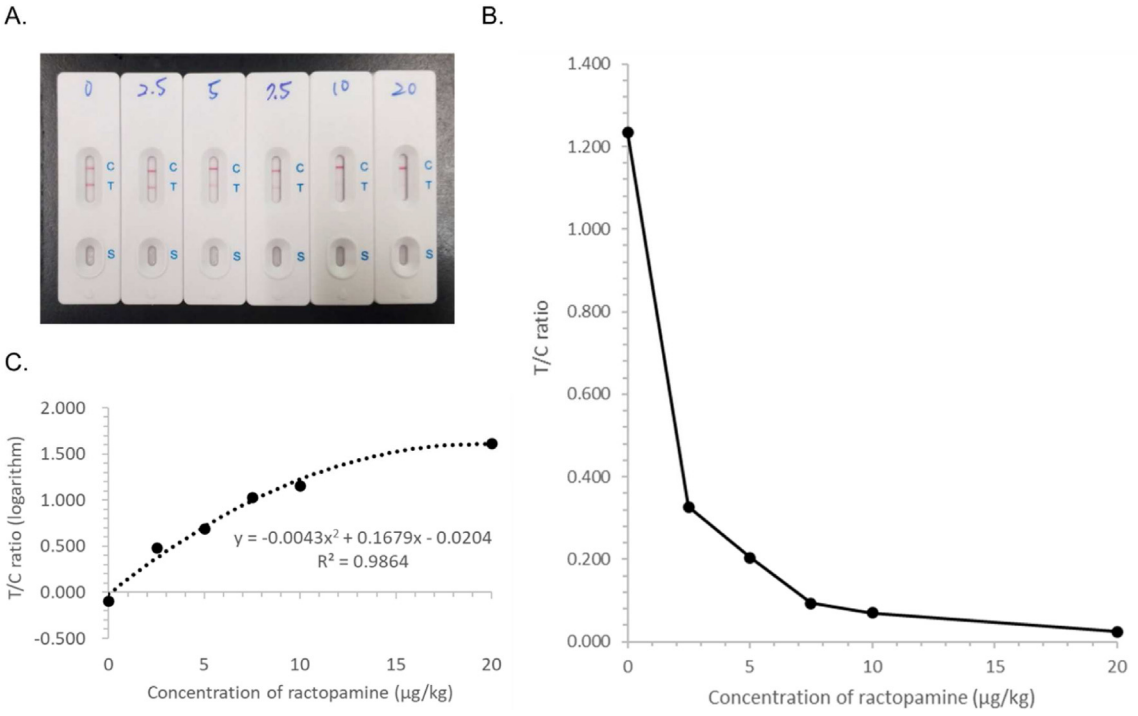

Fig. 3. The standard curve of the competitive colloidal gold-based lateral flow competitive immunoassays. (A) picture of lateral flow competitive immunoassays test results of 6 ractopamine standard spiked-samples. (B) the standard curve in original concentration scale, each concentration point has only one test result. (C) the standard curve in ten-based logarithm concentration scale.

rate, which was identical to the theoretical RDD performance [8].

The sensitivity, specificity, false-positive rate, and false-negative rate at eight different cutoff value settings (0.1%, 1%, 5%, 10%, 20%, 30%, 40%, and 50% at 10 μg/kg-spiked samples) were listed in Table 3, and the corresponding ROC curve was illustrated in Fig. 4. The high specificity value among eight cutoff value settings can attributed to the low positive rate in population (0.177%). The area under curve (AUC, 0.99) indicated the competitive colloidal gold-based lateral flow competitive immunoassays-based screening test method can distinguish between samples with ractopamine level over and lower then MRL (10 μg/kg).

Table 2. Detection level, recovery, and precision of lateral flow immunoassays-based screening method.

Spiked level (μg/kg)	Detection level (μg/kg)[a]	Recovery (%)[a]	Precision (%)
2.5	1.99 ± 0.42	79.53 ± 16.97	21.33
5	4.54 ± 0.89	90.84 ± 17.79	19.58
7.5	6.90 ± 1.66	92.04 ± 22.12	24.04
10	9.04 ± 1.88	91.42 ± 20.38	20.85
20	17.00 ± 3.05	85.02 ± 15.27	17.96

[a] Mean ± standard deviation.

3.2. Evaluation of the optimal strategy in combination of screening and confirmatory tests for ractopamine analysis

For the estimation of the optimal combination strategy between screening and confirmatory tests. Six necessary parameters of the mathematic model were acquired and used to establish the testing model. Three of six parameters, total budget (*TB*), cost per screening test ($C_{screening}$) and cost per confirmatory test ($C_{confirmatory}$), were defined as 100,000, 4, and 100 U.S. dollar (Table 1) based on the pricing information of Kanyoku Corporation and Core Facility Center of National Cheng Kung University. The other three parameters, true positive rate of food with the chemical exceeding the MRL, positive rate of screening test, and positive rate of confirmatory test were calculated based on the data of market survey. Although the data from survey research can give the most accurate information, a survey of ractopamine residue over the entire market would be both impractical and expensive to implement in a short time. In this study, we simulated the ractopamine residue distribution in pork samples based on the data reported in publication [23]. The generated distribution of ractopamine residue in samples and

Table 3. Sensitivity, specificity, false positive rate, and false negative rate of lateral flow immunoassays-based screening method in eight threshold settings.

Thresholds of the screening test[a]	Sensitivity (%)	Specificity (%)	False positive rate (%)	False negative rate (%)
0.170, 0.1%	100.0	93.5	6.5	0.0
0.150, 1.0%	99.4	95.5	4.5	0.6
0.130, 5.0%	93.2	97.2	2.8	6.8
0.120, 10.0%	88.1	97.9	2.1	11.9
0.110, 20.0%	80.2	98.5	1.5	19.8
0.100, 30.0%	70.1	98.8	1.2	29.9
0.095, 40.0%	54.2	99.0	1.0	45.8
0.090, 50.0%	52.5	99.2	0.8	47.5

[a] Value of threshold (unit: T/C ratio), estimated false-negative rates at 10 μg/kg-spiked samples.

the performance of lateral flow competitive immunoassays were used to calculate these three-market survey-related parameters. Section 3.3 to 3.5 and Fig. 5 depicts the detailed calculation process of the three parameters.

3.3. Threshold value determination in the screening test

According to the regulation of the TFDA, the MRL of ractopamine is set at 0.01 mg/kg (10 μg/kg) for muscle tissues [24]. The distribution of the analysis results of the RDD from 10 μg/kg-spiked ractopamine samples (Fig. 5B) indicated that the measurement uncertainty needs to be considered in the threshold determination. Most of the thresholds of screening tests were stipulated with a high false-positive rate and the minimum false-negative rate [8] because false-positive samples can be excluded in subsequent confirmatory analyses. However, a high false-positive rate represents a greater number of samples requiring further confirmation. In the case of fixed analysis costs, a high false-positive rate may reduce the number of positive samples detected by confirmatory analysis. In our study, eight screening test threshold values were set and used to optimized threshold setting in screening analysis. The histogram of T/C ratio distribution of 10 μg/kg-spiked ractopamine samples was fit to Student's *t*-distribution and calculated the threshold values with one-tailed *p* values equal to 0.1%, 1%, 5%, 10%, 20%, 30%, 40% and 50% (Fig. 6), which represented T/C ratios of determined thresholds were 0.170, 0.150, 0.130, 0.120, 0.110, 0.100, 0.095 and 0.090, respectively. The one-tailed *p* values are also represented the endured false-negative rates of each threshold.

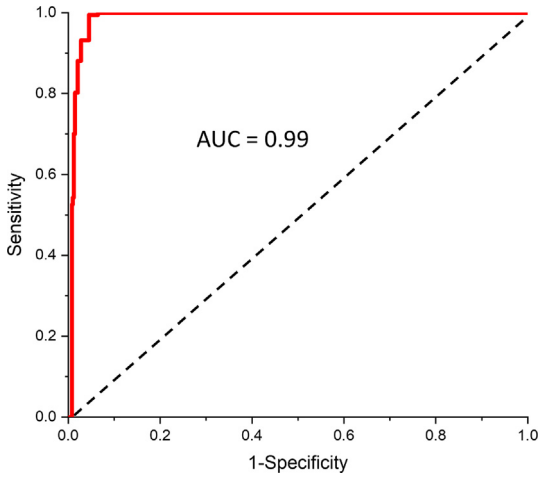

Fig. 4. The receiver operating characteristic (ROC) curve of the competitive colloidal gold-based lateral flow competitive immunoassays-based screening method. The simulated distribution of ractopamine residue in pork samples (as outlined in section 2.3) was used to generate the population data. The area under curve (AUC) is 0.99.

3.4. Calculation of the function of positive rate-ractopamine concentration association using polynomial curve fitting

Polynomial interpolation was performed to generate a linear function that fitted five positive rates from the experimental results and determined the positive rate-ractopamine concentration association of each screening test threshold value setting. For example, when the threshold of the screening test was 0.13 (5% false-negative rate at 10 μg/kg), the experimental positive rates in four ractopamine-spiked sample groups, 0 μg/kg, 2.5 μg/kg, 5 μg/kg, and 7.5 μg/kg, were 5.65×10^{-10}, 5.22×10^{-5}, 5.66×10^{-2}, and 5.33×10^{-1}. The relationship between the log-transformed (base 10) positive rates (y) and concentrations of ractopamine (x) can be represented by a 3rd degree polynomial function Eq. (6):

$$y = a*x^3 + b*x^2 + c*x + d \quad (6)$$

The given 3rd degree polynomial function has four coefficients, a, b, c, and d, which can exactly fit four log-transformed positive rate values (Fig. 5C). For example, the positive rate equation of the threshold 0.13 can be given as:

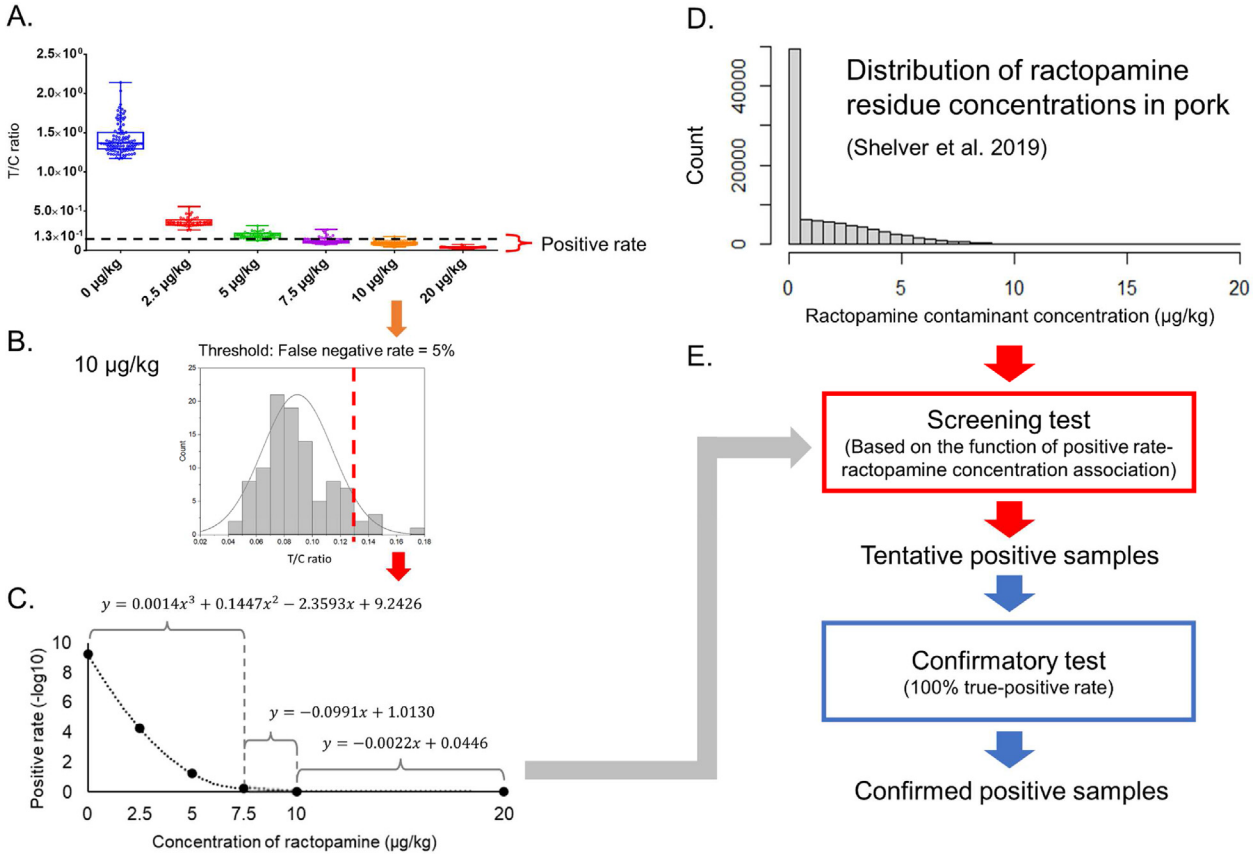

Fig. 5. Calculation of true positive rate of food with the chemical exceeding the MRL, positive rate of screening test, and positive rate of confirmatory test in the case of ractopamine residue analysis. (A) Scatter plot presenting the T/C ratios of all spiked ractopamine samples. (B) Frequency distribution histogram of T/C ratio of 10 μg/kg-spiked ractopamine samples. (C) The function of positive rate-ractopamine concentration association calculated with a cutoff at 0.13, which represents a 5% false-negative rate for 10 ppb. (D) Frequency distribution histogram of the simulated ractopamine residue concentration in the food matrix based on a publication and the calculation of the true positive rate. (E) Calculation of the positive rate of screening and confirmatory tests based on the ractopamine residue concentration distribution and positive rate-ractopamine concentration relationship of the screening test.

$$y = 0.0014 * x^3 + 0.1447 * x^2 - 2.3593 * x + 9.2462 \quad (7)$$

Due to Runge's phenomenon [25], we did not further estimate the relationship between the positive rate and the concentrations of ractopamine by higher-order polynomials in the concentration range of ractopamine from 7.5 μg/kg to 20 μg/kg. The relationship between the positive rate and spiked concentrations of ractopamine in the range from 7.5 μg/kg to 20 μg/kg was estimated by linear interpolation (Fig. 5C). For the assessment of the positive rate of the sample with ractopamine residue above 20 μg/kg, the positive rate of the screening test was applied to the positive experimental rate in the 20 μg/kg ractopamine-spiked sample groups.

3.5. Calculation of the positive rate of the screening test and the positive rate of the confirmatory test

The distribution of ractopamine residue in samples (Fig. 5D, generation method was described in section 2.3) and the calculated functions of the positive rate-ractopamine concentration association were used to calculate the positive rate of screening test ($P_{screening}$) and confirmatory test ($P_{confirmatory}$) (Fig. 5E). For the positive rate of the screening test, the concentrations of ractopamine residue of each sample in the distribution were put into the function of positive rate-ractopamine concentration association and converted to the positive rate values. Each positive rate value was further used to generate the simulated screening test result with assigned probability using Microsoft Excel. The positive rate of the screening test was calculated by the following function Eq. (8):

$$P_{screening} = \frac{N_{positive,\ screening}}{N_{total}} \quad (8)$$

where $P_{screening}$ is the positive rate of the screening test, $N_{positive,\ screening}$ is the number of samples that pass the screening test in the Excel simulation, and

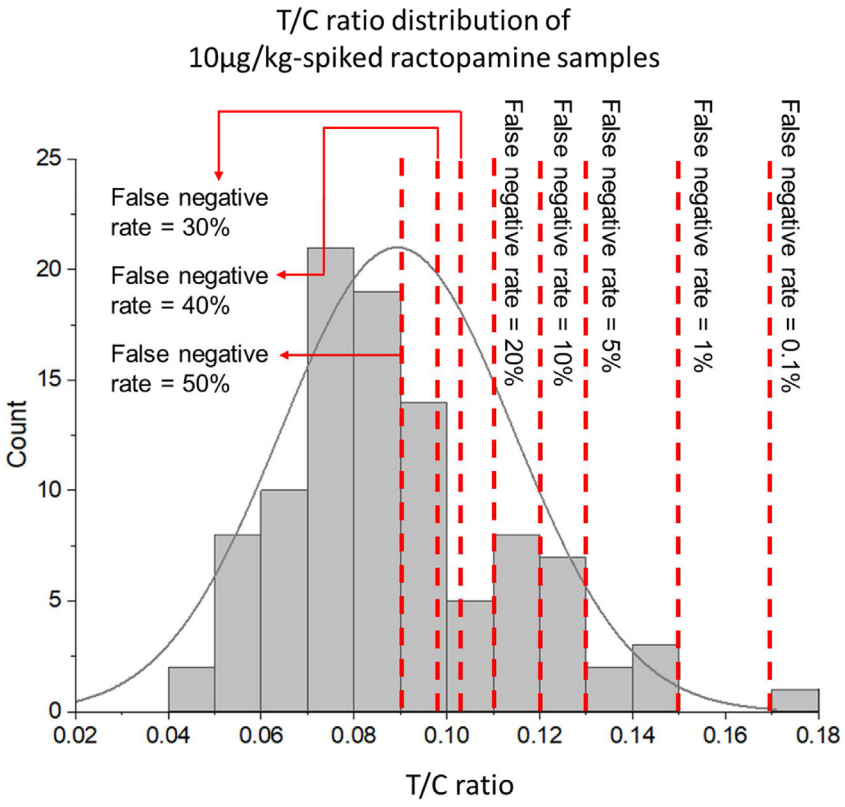

Fig. 6. Frequency distribution histogram of T/C ratio of 10 μg/kg-spiked ractopamine samples. The distribution indicates that the measurement uncertainty needs to be considered in the threshold determination of the screening test. The eight red lines, 0.170, 0.150, 0.130, 0.120, 0.110, 0.100, 0.095 and 0.090, in the figures represent the determined thresholds with estimated false-negative rates of 0.1%, 1%, 5%, 10%, 20%, 30%, 40% and 50%, respectively.

N_{total} is the number of all samples in the distribution, which is 100,000. Because each determined threshold value of the screening test can generate one positive rate-ractopamine concentration association function, eight positive rates of the screening test were calculated in our study.

The positive rate of the confirmatory test was calculated by Eq. (9):

$$P_{confirmatory} = \frac{N_{positive,\ confirmatory}}{N_{positive,\ screening}} \quad (9)$$

where $P_{confirmatory}$ is the positive rate of the confirmatory test and $N_{positive,\ confirmatory}$ is the number of samples that pass both screening and confirmatory tests. In this simulation, the performance of the confirmatory test referred to the LC-MS-based method, which was considered to have a 100% true positive rate [26]. Based on the 100% true positive rate, the $N_{positive,\ confirmatory}$ was equal to the number of true positive cases in the sample pass screening test. The relationships among the thresholds of the screening test, positive rate of the screening test, and positive rate of the confirmatory test are listed in Table 4.

3.6. Cost-benefit analysis to optimize the cost allocation between screening and confirmatory tests

Fig. 7 illustrates the cost-benefit analysis results of the screening and confirmatory tests for the detection of ractopamine residue. The eight polylines in Fig. 7A represent the number of confirmed positive samples with the setting of eight screening test thresholds. Each line illustrated the number of confirmed positive samples based on different cost allocations between screening and confirmatory tests. Detailed data on the eight polylines are provided in Supporting Information Tables S1 to S8 [https://www.jfda-online.com/journal/vol31/iss2/7/]. The cost-benefit analysis results show that all polylines have a peak representing the highest number of confirmed positive samples. Compared to the procedure without the screening tests, the point in Fig. 7A with a budget for screening tests (S%) = 0, employing screening tests can increase the number of confirmed positive samples from 1 to 26 at

Table 4. Eight thresholds of the screening test and corresponding test positive rates of screening test/confirmatory test in the case of ractopamine residue analysis. The positive rates of screening test and confirmatory test are expected values calculated based on mathematical simulation.

Thresholds of the screening test[a]	Positive rate of screening test ($P_{screening}$, %)	Positive rate of confirmatory test ($P_{confirmatory}$, %)
0.170, 0.1	7.08	2.50
0.150, 1.0	4.68	3.78
0.130, 5.0	3.00	5.64
0.120, 10.0	2.33	7.00
0.110, 20.0	1.67	9.17
0.100, 30.0	1.29	10.32
0.095, 40.0	1.05	10.81
0.090, 50.0	0.80	11.94

[a] Value of threshold (unit: T/C ratio), estimated false-negative rates at 10 μg/kg-spiked samples (%).

maximum. Fig. 7B illustrates the optimal cost allocations between screening and confirmatory tests at different screening test thresholds. The optimal S% increases with increasing false-negative rates of the screening test threshold at the MRL.

Fig. 7C demonstrates the relationship between different screening test thresholds and the maximum number of confirmed positive samples. The curve indicates that the threshold of the screening test with a 20% false-negative rate at MRL can detect the highest number of positive samples at the optimal cost allocation. As the false-negative rate of the screening test moves away from 20%, the number of detected positive samples decreases. This result indicates that tolerating some false-negative rates in screening analysis can obtain the highest benefit, even though a higher false-negative rate means that the screening test ignores part of the positive samples.

4. Discussion

The results of the cost-benefit analysis indicated that the involvement of screening tests and optimizing the cost allocation between screening and confirmatory tests are essential for increasing the number of detected positive samples in the analysis of ractopamine residue. Fig. 7A reveals that an optimal cost allocation occurs in the budget-limited screening and confirmatory tests when all the tentative positive samples tested by the screening test can be confirmed by the confirmatory test (the decision point of Fig. 2 is "yes" and $DCPN = 0$). If the cost allocation for screening is too high (S% higher than the optimal cost allocation), the confirmatory test can confirm only part of the tentative positive samples (the decision point of Fig. 2 is "no"), which wastes the budget on screening tests. In contrast, an S% lower than the optimal point indicates that the confirmatory tests can confirm more than the number of tentative positive samples (the decision point of Fig. 2 is "yes" and $DCPN \neq 0$). In

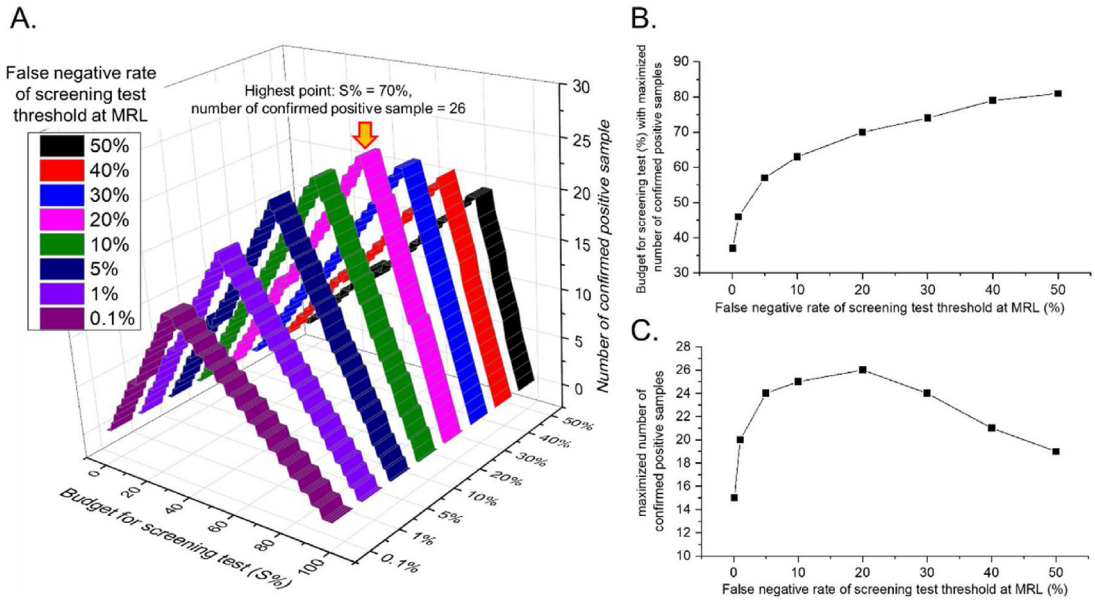

Fig. 7. Cost-benefit analysis result of the screening and confirmatory tests based on mathematical simulation. (A) Cost-benefit analysis curves of false-negative rates of screening test at MRL is 0.1%, 1%, 5% 10%, 20%, 30%, 40% and 50%; (B) cost allocation for screening test (S%) with the maximum number of confirmed positive samples at different screening test thresholds, which increases with the false-negative rate of screening test threshold at maximum residue limits (MRL); (C) number of confirmed positive samples at optimal screening test cost allocation. The screening test threshold with a 20% false-negative rate at MRL has the maximum number of confirmed positive samples (N = 26) compared with other results.

this situation, a confirmatory test needs to further analyze the samples without a screening test, which has a lower expected positive rate. For instance, when the threshold of the screening test is set at a 5% false-negative rate, the expected positive rate of the tentative positive samples is 5.42%, which is thirty times higher than the positive rate of all samples, 0.18%. The low expected positive rate wastes the budget on confirmatory tests. According to Fig. 7B, the optimal cost allocation between screening and confirmatory tests changes with different thresholds. The increasing false-negative rate of screening test threshold enhances the number of tentative positive samples passing the screening test, which causes the ratio of cost for confirmatory tests to increase synchronously to confirm all the tentative positive samples from the screening test exactly.

The cost-benefit analysis indicates that an optimal threshold setting for screening tests also exists. In our analysis, the optimal threshold of the screening test was noted at 0.11, which has a 20% false-negative rate at 10 μg/kg spiked samples (Fig. 7C). This analysis result conflicts with the typically designed screening test with a high false-positive rate and minimum false-negative rate [8]. Fig. 8 illustrates that the main reason to limit the number of detected positive samples at the screening test cutoff value's false-negative rate lower or higher than 20% is different. The slopes of the increasing proportion of polylines are similar among the results from the 0.1%, 1%, 5%, and 10% false-negative rates (Fig. 8A). The difference in the maximum number of confirmed positive samples is decided by the location of the inflection point, which indicates that the main reason to limit the number of detected positive samples is the cost allocation for the screening test. With a lower false-negative rate in the screening test, many false-positive samples are sent to the confirmatory analysis pipeline, which conversely limits the budget for the screening tests because the optimal cost allocation appears at the point where all the tentative positive samples from the screening tests can be confirmed by confirmatory tests. On the other hand, the analysis results of false-negative rates higher than 20% reveal that the main reason to reduce the maximum number of confirmed positive samples is the decreasing slope of the increasing line (Fig. 8B), which indicates that the main reason to limit the number of detected positive samples is the decrease in the analytical performance of the screening test. For instance, the positive rates of the screening test with the 20% and 50% false-negative rates of the screening test threshold at the MRL are 1.7% and 0.8%, respectively. However, the true-positive rates of the screening tests with the same thresholds are 9.2% and 11.9%, respectively. The increase in the true-positive rate is not enough to offset the decrease in the number of confirmed positive samples due to the reduction in the positive rates of the screening tests ($0.8/1.7 \approx 0.5$, $11.9/9.2 \approx 1.3$, the 30% increase in the positive rate in the confirmatory test cannot compensate for the halving performance of the screening test).

To further understand the effect of the limited budget in the screening and confirmatory analysis, we simulate the analysis result with a total budget from 10,000 to 10,000,000 U.S. dollars. The cost allocation of the screening test selects the optimized value of each threshold, and the maximum number of samples is 100,000. When the budget for screening or confirmatory tests can analyze the number of samples that exceed the maximum number, the test can only test the maximum

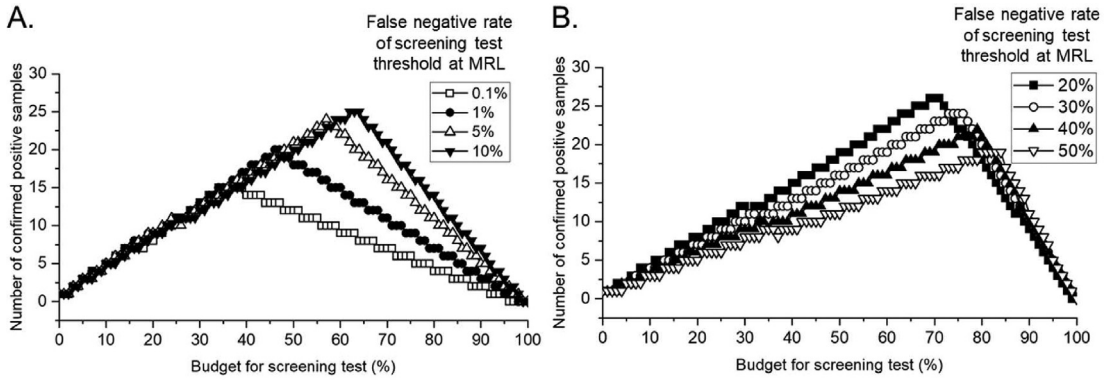

Fig. 8. Two different reasons limit the number of detected positive samples at the screening test cutoff value's false-negative rate lower or higher than 20% in the simulated cost-benefit analysis result. (A) Cost-benefit analysis curves with screening test threshold's false-negative rates at MRL are 0.1%, 1%, 5%, and 10%, the number of detected positive samples are limited due to the location of the inflection point; (B) cost-benefit analysis curves with screening test threshold's false-negative rates at maximum residue limits (MRL) are 20%, 30%, 40% and 50%, the number of detected positive samples are limited by the slope of the increasing line.

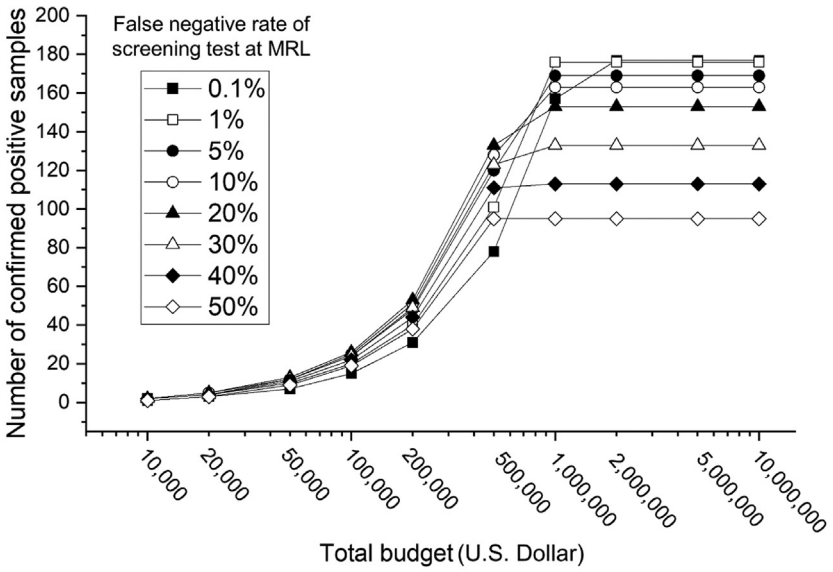

Fig. 9. Number of confirmed positive samples in different total budgets (x-axis) and screening thresholds (each line) in the simulated cost-benefit analysis result. The cost allocation of the screening test (S%) selects the optimized value of each threshold, and the maximum number of samples is 100,000. MRL: maximum residue limits.

number of samples, and the remaining cost is wasted. The simulated result is illustrated in Fig. 9. The result showed that only the result from the screening threshold set at a 0.01% false-negative rate at 10 μg/kg spiked samples could confirm all positive samples (100,000*0.177% = 177) when the total budget was over 2,000,000 U.S. Dollars; the other screening thresholds would be lost some of the positive samples. The simulated result with a 20% false-negative rate screening threshold has the highest number of confirmed positive samples until the total budget is equal to or over 1,000,000 U.S. dollars. These results indicated that when the budget for screening tests cannot afford to screen all the samples in the population, tolerating some false-negative rate in screening tests has the highest benefit in screening and confirmatory tests of additives and contaminants in foods. The benefit of a low false-negative rate only occurs when the budget for screening analysis can test all the samples in the population, and the following confirmatory tests can confirm all tentative positive samples from the screening test, which is difficult to happen in reality.

5. Conclusion

In this study, we developed a lateral flow immunoassays-based method for the screening of ractopamine in foods and estimated the optimal combination strategy between screening and confirmatory tests in the case of ractopamine analysis. The developed immunoassay-based screening test successfully distinguishes gravy samples with ractopamine levels over and lower than MRL. The cost-benefit analysis indicated that the optimal cost allocation is located on the point that all positive samples filtered by screening test can be analyzed by confirmatory test, which can increase the number of confirmed positive samples by 26 times at maximum compared to other cost allocations. Additionally, the cutoff value of the screening test also influences the number of detected samples that exceeded the ractopamine residue. The bearing of a certain false-negative rate can catch more positive samples when the budget is limited. In our simulation, the screening test threshold with a 20% false-negative rate at MRL can confirm the maximum number of verified positive samples when the budget was limited to 100,000 U.S. dollars. The present study demonstrated the cost-benefit analysis of screening and confirmatory tests for detecting ractopamine residue at a fixed cost, which provided a scientific basis for governments to make decisions on food safety enforcement.

Conflict of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Yuan-Chih Chen: Methodology, Data Curation, Formal analysis, Writing - Original Draft, Writing - Review & Editing, Visualization. Jen-Yi Hsu: Writing - Original Draft, Investigation, Data Curation. Chien-Sheng Chen: Writing - Original Draft, Investigation. Yi-Ting Chen: Writing - Original Draft, Investigation, Data Curation. Pao-Chi Liao: Conceptualization, Writing - Review & Editing, Supervision.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.38212/2224-6614.3458.

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