

Performance evaluation of CA242 by flow fluorescence assay

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Abstract

Carbohydrate antigen 24–2 (CA24–2) is usually used as a biomarker for the diagnosis of pancreatic cancer and colorectal cancer. Currently, a new quantitative assay kit for CA242 by flow fluorometry assay (FFA) was developed by Shanghai Tellgen Cooperation Co. Ltd. China. Therefore, we conducted the performance evaluation for it.

According to the “Guiding principles on performance analysis of diagnostic reagents in vitro” and “American association of clinical laboratory standardization guidelines EP15-A2”, the accuracy, precision, linear range, reportable range, biological reference interval verification, carry-over contamination rate, anti-interference capability and cross reaction of the assay kit used in TESMI F3999-Luminex200 automatic immunoassay system were evaluated. In addition, the assay kit was performed in parallel to CanAg kit (CanAg Diagnostics Products Beijing Co., Ltd.) to analyze the correlation between the 2 kits.

The bias of accuracy of the new assay kit was less than 12.5% and the coefficient of variations (CVs) of precision were all less than 10.0%. The linear range of CA242 concentration of the testing kit was between 3.46 U/ml and 434.76 U/ml and the reportable range was 6.00 to 535.13 U/ml. The CA242 reference interval 0.00 to 20.00 U/ml was suitable for use in laboratory. The carry-over contamination rate was –0.14%. Correlation analysis showed a satisfactory relevance and consistency ($r = 0.982$, $P < .001$) between the new assay kit and CanAg kit, with a regression equation $Y = 1.0012X + 0.878$ ($R^2 = 0.9647$, $P < .001$). No statistically significant difference between serum samples without interferences and samples containing lipemia, bilirubin and hemoglobin. And no cross reaction existed between the assay kit and the other tumor markers, such as carbohydrate antigen 125 (CA125), alpha-fetoprotein (AFP), and cytokeratin-19 soluble fragment (CYFRA21-1).

The new CA242 quantitative assay kit possesses good detection performance when it is used in TESMI F3999-Luminex200 automatic immunoassay system, which can be used for the examination of CA242 in clinical practice.

Abbreviations: AFP = alpha-fetoprotein, CA125 = carbohydrate antigen 125, CA242 = carbohydrate antigen 242, CLSI = Clinical and Laboratory Standards Institute, CRC = colorectal cancer, CV = coefficient of variation, CYFRA21-1 = cytokeratin-19 soluble fragment, ELISA = enzyme-linked immunosorbent assay, FFA = flow fluorescence assay.

Keywords: assay kit, carbohydrate antigen 242, flow fluorescence assay, performance evaluation

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All data generated or analyzed during this study are included in this published article [and its supplementary information files]. The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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1. Introduction

Carbohydrate antigen 242 (CA242) is a sialylated carbohydrate antigen that can be detected on the surface of cells or in serum. Studies showed that the expression of CA242 was strongly associated with the clinicopathologic characteristics of many kinds of gastrointestinal malignant tumors, such as gastric cancer, colorectal cancer (CRC) and pancreatic cancer.^[1–3] Zhang, et al found that increased serum level of CA242 could be used as biomarker for the diagnosis of CRC and pancreatic cancer, in which the specificity of CA242 was higher than that of CA50 or CEA and combination examination of CEA with CA242 had a much higher sensitivity in CRC than either used alone.^[4,5] Thus, the measurement of CA242 is significant for cancer patients, particularly gastrointestinal cancers.

In the past, researchers usually used ELISA (enzyme-linked immunosorbent assay) to detect the activity of CA242, however, the activity of CA242 in serum or plasma was not stable, and the detection results of CA242 activity by ELISA were not accurate enough and time-consuming.^[6,7] In order to overcome the various shortcomings of CA242 activity detection by ELISA, currently, a new quantitative assay kit for CA242, which measured the serum concentration of CA242 by flow fluorometry assay (FFA), was developed by Shanghai Tellgen Cooperation Co. Ltd. China. And this quantitative assay kit was more sensitive and faster than the determination of CA242 activity by ELISA.

For a new quantitative assay kit, quality verification is an important way to ensure the reliability of the kit. Quality verification and quality control are designed to ensure the accuracy of each test result. At present, laboratories usually carry out internal laboratory quality control and external laboratory quality control regularly in their daily work, and the results are statistically analyzed and evaluated to ensure the quality of laboratory test results.^[8] In order to ensure that the kit can meet the performance required for the detection of CA242 concentration in clinical serum samples, according to the “Guiding principles on performance analysis of diagnostic reagents in vitro,” we conducted the performance evaluation for it. In this study, the accuracy, precision, linear range, reportable range, biological reference interval, contamination carrying rate, anti-interference capability and cross reaction of the assay kit used in TESMI F3999-Luminex200 automatic immunoassay system were evaluated. In addition, the assay kit was performed in parallel to CanAg kit (CanAg Diagnostics Products Beijing Co. Ltd.) to analyze the correlation between the 2 kits.

2. Materials and methods

2.1. Sample collection

All the samples analyzed in this study were collected from the patients with gastrointestinal cancers (gastric cancer, colorectal cancer and pancreatic cancer, total 56 cases), benign diseases (gastritis, colitis and pancreatitis, total 50 cases) and the healthy people (162 cases) from January 2019 to June 2020. The serum samples were separated by centrifugation (2200g, 10 minutes) and stored at -30°C until used. The study followed the principles of the Declaration of Helsinki and was approved by the Ethics Committee of Xiamen Humanity Hospital Fujian Medical University. Written informed consents were obtained from the guardians of the patients before the study began.

2.2. Reagents and instruments

1. Testing kit: the quantitative assay kit for CA242 by FFA which was developed by Shanghai Tellgen Cooperation Co. Ltd. China.
2. Comparative kit: CanAg kit for CA242 by ELISA which was developed by CanAg Diagnostics Products Beijing Co. Ltd. China.
3. Interferential substances: hemoglobin, bilirubin, triglyceride, rheumatoid factor.
4. Cross-reaction substances: carbohydrate antigen 125 (CA125), alpha-fetoprotein (AFP), and cytokeratin-19 soluble fragment (CYFRA 21-1).
5. TESMI F3999-Luminex200 automatic immunoassay system which was developed by Shanghai Tellgen Cooperation Co. Ltd. China.

2.3. Performance validation

Performance of the accuracy, precision, linear range, reportable range, biological reference interval, contamination carrying rate, anti-interference capability, cross reaction and method comparison of the assay kit were validated according to the “Guiding principles on performance analysis of diagnostic reagents in vitro,” which was formulated by the China Food and Drug Administration and mainly referenced to the Clinical and

Laboratory Standards Institute (CLSI) documents.^[9] Specifically, accuracy was verified according to CLSI EP9-A2,^[10] precision was verified according to CLSI EP5-A,^[11] linear range and reportable range were verified according to CLSI EP6-A,^[12] method comparison was verified according to CLSI EP9-A2,^[13] and anti-interference capability was verified according to CLSI EP7-A.^[14]

2.3.1. Accuracy. This procedure followed the EP9-A2 guideline published by CLSI.^[10] Accuracy was evaluated by comparing the bias between the instrumental concentration and the theoretical concentration of the calibration solutions of known concentrations. In this study, 2 calibration solutions of known concentrations were prepared, the first solution was the high concentration calibration solution and the second 1 was the low concentration calibration solution. Both of the 2 solutions were detected 3 times and the average concentrations were calculated. Then the bias of the high concentration and low concentration calibration solutions between instrumental concentration and theoretical concentration was calculated, respectively. Bias (%) = [(instrumental concentration - theoretical concentration)/theoretical concentration] * 100%. When the bias between the instrumental concentration and theoretical concentration was no more than 12.5%, the accuracy would be accepted.

2.3.2. Precision. According to the EP5-A guideline published by CLSI,^[11] precision was evaluated by calculating the coefficients of variations (CVs) of intra-assay and inter-assay. The experiments were performed using 2 concentrations (low concentration and concentration) of serum samples, which were analyzed twenty times, respectively. CV (%) = (Standard deviation/average) * 100%. When the CV was no more than 10.0%, the precision would be accepted.

2.3.3. Linear range verification. Following the EP6-A guideline published by the CLSI,^[12] the concentrations of L-Series standard solutions (L1-L5, known concentrations of 15.84 U/ml, 120.34 U/ml, 224.83 U/ml, 329.33 U/ml and 433.82 U/ml, respectively) were detected by the testing kit. All raw fluorescence intensity data were converted into corresponding concentrations through the calibration curve and a scatter diagram was made (X: theoretical concentration, Y: instrumental concentration). Then, multiple regression models were applied to analyze the linear range.

2.3.4. Reportable range. Following the EP6-A guideline published by the CLSI,^[12] the testing kit was used to repeatedly detect 2 serum samples with different concentrations for 10 times, respectively. The lower limit of the reportable range was defined as the lowest measured concentration of the serum sample. The higher limit of the reportable range was defined as the restore concentration of the high concentration serum sample. In order to obtain the higher limit of the reportable range, 5 × and 10 × dilutions of the high concentration serum sample were evaluated at the same time. Bias (%) = [(restore concentration - theoretical concentration)/theoretical concentration] * 100%. When the bias of the restore concentration and the theoretical concentration differed within $\pm 10\%$, the higher limit of the reportable range would be accepted.

2.3.5. Biological reference interval verification. In this study, 20 healthy people were randomly selected and the serum CA242 concentrations were detected. If there was no more than 1 result beyond the reference interval, the reference interval used by the laboratory is valid.

2.3.6. Carry-over contamination rate. Carry-over contamination rate mainly reflects the degree of contamination between samples. By measuring the high concentration sample 3 times (H1, H2, H3) and low concentration sample 3 times (L1, L2, L3), the carry-over contamination rate was calculated: carry-over contamination rate (%) = $(L1 - L3)/(H3 - L3) * 100\%$. When the carry-over contamination rate was not higher than 3%, the carry-over contamination rate would be accepted.

2.3.7. Method comparison. Following the EP9-A2 guideline published by the CLSI,^[10] comparison of methods from different manufacturers was carried out. The level of serum tumor marker CA242 was parallelly evaluated by the testing kit and comparative kit in this study with 106 patients and 162 healthy people, to analyze the correlation between the 2 kits.

2.3.8. Anti-interference reaction. This procedure followed the EP7-A2 guideline published by CLSI.^[13] In this study, firstly, serum samples with different CA242 concentrations were examined by the testing kit. Secondly, the serum samples were spiked with interfering substances (including lipemia, bilirubin and hemoglobin), and the lipemia-, bilirubin- and hemoglobin-containing samples were then examined by the testing kit. Finally, the interference effects of lipemia, bilirubin and hemoglobin on the accuracy of testing kit were evaluated by calculating the bias of the test results of the before and after addition of interference factors. Bias (%) = $[(\text{interference concentration} - \text{original concentration})/\text{original concentration}] * 100\%$. Interference was considered significant when the bias (Bias %) was greater than $\pm 10\%$.

2.3.9. Cross reaction. CA242 calibration solution of 0.00 U/ml (recorded as negative) was utilized in the experiments to determine the present of cross reaction in the kit. CA125, AFP, and CYFRA 21-1 were spiked individually into the serum samples. All the substances and CA242 concentrations of the samples were assayed. When the CA242 concentration of the sample spiked with CA125, AFP, or CYFRA 21-1 was less than the effective detection sensitivity, it was considered that no cross reaction existed.

2.4. Statistical analysis

All statistical analysis was performed by SPSS 25.0 software.

3. Results

3.1. Accuracy

The bias of the high concentration calibration solution and low concentration calibration solution between instrumental concentration and theoretical concentration were -3.84% and -9.59% , respectively, which were both less than 12.5%

Table 1

Evaluation of accuracy.

Sample	Instrumental concentration	Theoretical concentration	Bias (%)
Low CA242 concentration (U/ml)	54.52	60.30	-9.59%
High CA242 concentration (U/ml)	209.16	217.51	-3.84%

Bias (%) = $[(\text{instrumental concentration} - \text{theoretical concentration})/\text{theoretical concentration}] * 100\%$.

according to the EP9-A2 guideline published by CLSI,^[10] indicating that the accuracy of the testing kit could be accepted (Table 1).

3.2. Precision

The intra-assay CV(s) of low CA242 concentration and high CA242 concentration were 2.97% and 1.08%, and the inter-assay CV(s) of low CA242 concentration and high CA242 concentration were 1.10% and 1.01%, respectively, which were all less than the acceptable range of no more than 10.0% according to the EP5-A guideline published by CLSI,^[11] indicating that the precision of the testing kit was perfect (Table 2).

3.3. Linear range verification

The concentrations of L-Series standard solutions (L1-L5, known concentrations of 15.84 U/ml, 120.34 U/ml, 224.83 U/ml, 329.33 U/ml, and 433.82 U/ml, respectively) were 15.84 U/ml, 125.79 U/ml, 201.48 U/ml, 329.75 U/ml, and 433.82 U/ml by the testing kit, respectively. And the scatter diagram of instrumental concentration and theoretical concentration was shown in Figure 1. The linear range of CA242 concentration of the testing kit was between 3.46 U/ml and 434.76 U/ml.

3.4. Reportable range

The lower limit of the reportable range was 6.00 U/ml, which was the lowest measured concentration of the serum sample. The bias of the 2 high-concentration serum samples were 7.39% and 4.13%, which were both within $\pm 10\%$ according to the EP6-A guideline published by the CLSI,^[11] indicating that the higher limit of the reportable range was 535.13 U/ml. Therefore, the reportable range was 6.00 to 535.13 U/ml (Table 3).

3.5. Biological reference interval verification

The CA242 concentrations of the 20 healthy people ranged from 2.67 U/ml to 15.68 U/ml, which were all within the reference interval of 0.00 to 20.00 U/ml, indicating that this reference interval was suitable for use in laboratory. The scatter diagram of

Table 2

Evaluation of precision.

	Intra-assay precision		Inter-assay imprecision	
	Low CA242 concentration (U/ml)	High CA242 concentration (U/ml)	Low CA242 concentration (U/ml)	High CA242 concentration (U/ml)
AVERAGE	9.51	225.70	12.57	204.70
STDEV	0.28	2.44	0.13	2.06
CV	2.97%	1.08%	1.10%	1.01%

CV (%) = (Standard deviation/average), CV = coefficient of variation, SD = standard deviation. *100%.

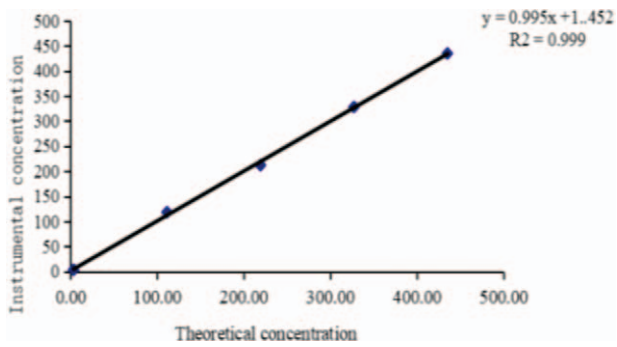


Figure 1. The concentrations of L-Series standard solutions were 15.84 U/ml, 125.79 U/ml, 201.48 U/ml, 329.75 U/ml and 433.82 U/ml by the testing kit, respectively. And the linear range of CA242 concentration of the testing kit was between 3.46 U/ml and 434.76 U/ml.

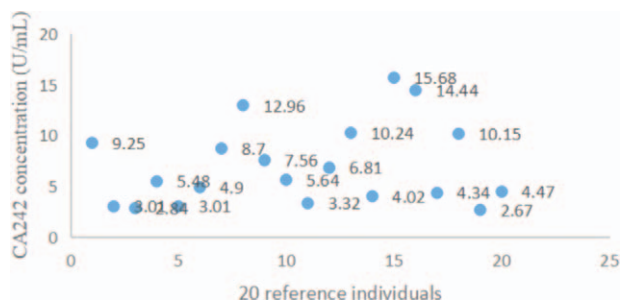


Figure 2. The CA242 concentrations of the 20 healthy people ranged from 2.67 U/ml to 15.68 U/ml, which were all within the reference interval of 0.00 to 20.00 U/ml, indicating that this reference interval was suitable for use in laboratory.

the CA242 concentrations of the 20 healthy people was shown in Figure 2.

3.6. Carry-over contamination rate

The 3 test results of the high concentration sample were 305.52 U/ml, 292.39 U/ml and 309.16 U/ml, and the 3 test results of the low concentration sample were 15.64 U/ml, 15.16 U/ml, and 16.04 U/ml. Therefore, the carry-over contamination rate was -0.14%, which was much lower than 3%, indicating that the carry-over contamination rate could be accepted (Table 4).

3.7. Method comparison

The concentration of the 268 research objects was 13.63 (5.05, 26.33) U/ml detected by the new assay kit and 15.05 (5.03, 28.49) U/ml detected by CanAg kit. Correlation analysis showed

Table 3

Upper limit of reportable range.

	H1 (U/ml)	H2 (U/ml)
Average concentration	114.94	55.72
Diluent folds	5	10
Restore concentration	574.70	557.20
Theoretical concentration	535.13	535.13
Bias	7.39%	4.13%

Bias (%) = [(restore concentration - theoretical concentration)/theoretical concentration] * 100%.

Table 4

Carry-over contamination rate.

	H1	H2	H3
High CA242 concentration (U/ml)	305.52	292.39	309.16
Low CA242 concentration (U/ml)	15.64	15.16	16.04
Carry-over contamination rate	-0.14%		

Carry-over contamination rate (%) = (L1 - L3) / (H3 - L3) * 100%.

a satisfactory relevance and consistency ($r=0.982$, $P<.001$) between the new assay kit and CanAg kit, with a regression equation $Y=1.0012X$ to 0.878 ($R^2=0.9647$, $P<.001$) (Fig. 3).

3.8. Anti-interference reaction

Analysis of interfering substances revealed that all the bias were within $\pm 10\%$ according to the EP7-A2 guideline published by CLSI,^[13] indicating that no statistically significant difference between serum samples without interferences and samples containing lipemia, bilirubin and hemoglobin (Table 5).

3.9. Cross reaction

After adding 1000 U/ml CA125, 800 ng/ml AFP or 200 ng/ml cytokeratin-19 soluble fragment (CYFRA21-1) to the calibration solution with a CA242 concentration of 0.00 U/ml, the detection results of CA242 were 1.50 U/ml, 3.90 U/ml and 1.30 U/ml, respectively, which were all less than the effective detection sensitivity (6.00 U/ml), suggesting that no cross reaction existed between the assay kit and the other tumor markers, such as CA125, AFP, and CYFRA21-1 (Table 6).

4. Discussion

As a potential tumor-associated biomarker, it was reported that the expression of CA242 was significantly increased in the serum of patients with gastrointestinal malignant tumors, while the level of CA242 in the serum of patients with benign diseases was very low.^[14] CA242 has been considered as a good tumor marker for pancreatic cancer and colon cancer, and compared with CA199, CA242 had a better specificity and diagnostic efficiency.^[15] Thus, the concentration of CA242 in serum could be used for the screening of gastrointestinal malignancies and the monitoring of treatment effects.

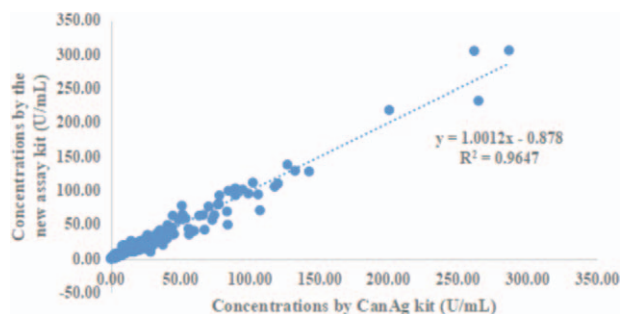


Figure 3. The concentration of the 268 research objects was 13.63 (5.05, 26.33) U/ml detected by the new assay kit and 15.05 (5.03, 28.49) U/ml detected by CanAg kit. Correlation analysis showed a satisfactory relevance and consistency ($r=0.982$, $P<.001$) between the new assay kit and CanAg kit, with a regression equation $Y=1.0012X$ to 0.878 ($R^2=0.9647$, $P<.001$).

Table 5

Anti-interference reaction.

Interference substance	Interference substance concentration	Original concentration of CA242	Interference concentration of CA242	Bias (%)
Lipemia	200 mg/ml	53.7 U/ml	57.3 U/ml	6.8%
		195.7 U/ml	186.4 U/ml	4.7%
		521.5 U/ml	563.4 U/ml	8.0%
Bilirubin	10 mg/ml	53.7 U/ml	49.0 U/ml	8.8%
		195.7 U/ml	178.2 U/ml	9.0%
		521.5 U/ml	527.7 U/ml	1.2%
Hemoglobin	10 mg/ml	53.7 U/ml	54.6 U/ml	1.8%
		195.7 U/ml	204.4 U/ml	4.5%
		521.5 U/ml	571.7 U/ml	9.6%

Bias (%) = [(interference concentration - original concentration)/original concentration] * 100%.

At present, people mainly use ELISA to detect the activity of CA242, however, due to the instability of the activity of CA242, the results are often not accurate enough. In addition, the experiment operation by ELISA is very complicated and time-consuming.^[6,7] Therefore, compared with the CA242 activity test, the serum CA242 concentration test has more advantages. So, in this study, we conducted a performance verification analysis on a new CA242 quantitative assay kit, which detected the serum concentration of CA242 by FFA. This FFA assay kit was based on flow analysis and Luminex multifunction flow dot matrix, and obtained the concentration of CA242 by detecting the fluorescence intensity of the microsphere cross-linked antibody - CA242 antigen - phycoerythrin labeled paired antibody complex.^[16] The solid phase carrier of the kit was fluorescent microspheres. It could not only improve the sensitivity and speed of reaction, but also greatly improve the anti-interference ability of the test. In addition, it took about 40mins for the detection by this new assay kit, while it would take about 2 hours and a half for the detection by ELISA kit (CanAg kit), which could save about 2 hours.

Through analysis, we found that the accuracy and precision of the new assay kit were satisfactory as the bias of accuracy were all less than 12.5% and the CV(s) of precision were all less than 10.0%. The new assay kit had a good linearity at the CA242 concentration ranging from 3.46 to 434.76 U/ml with a reportable rang 6.00 to 535.13 U/ml, but only a high value sample with CA242 concentration of 535.13 U/ml was evaluated in this trial, higher concentration samples may be needed to further verify the upper limit of reportable range. According to the reference interval 0.00 to 20.00 U/ml given by the manufacturer, in our study, the CA242 concentrations of 20 healthy people were all within the reference interval, demonstrating that this reference interval was suitable for use in laboratory. Also, the consistency between the new assay kit and CanAg kit was very perfect, with a correlation coefficient of $r=0.982$ ($P<.001$), which was performed on 268 patients and healthy people. Therefore, this new assay kit could be used to detect the

concentrations of CA242 in clinical samples. In addition, the carry-over contamination rate was only -0.14%, no interference of lipemia, bilirubin and hemoglobin and no cross reaction of other tumor markers, such as CA125, AFP, and CYFRA21-1, was found with the detection of serum CA242 concentration, indicating that the detection of serum CA242 concentration by this new assay kit was rarely affected by other interference factors.

5. Conclusion

According to the results, the new quantitative assay kit for serum CA242 concentration by FFA developed by Shanghai Tellgen possesses excellent diagnostic performance and fulfills the requirements for clinical routine measurement, which can be promoted clinically.

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Table 6

Cross reaction of CA125, AFP, and CYFRA21-1.

Cross reaction substance	Cross reaction substance concentration	CA242 concentration
CA125	1000 U/ml	1.5 U/ml
AFP	800 ng/ml	3.9 U/ml
CYFRA21-1	200 ng/ml	1.3 U/ml

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