Practical Laboratory Medicine 34 (2023) e00308

Contents lists available at ScienceDirect

Practical Laboratory Medicine

journal homepage: www.elsevier.com/locate/plabm

Development of an automated chemiluminescent immunoassay for cancer antigen 72–4 and the evaluation of its analytical performance

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ARTICLE INFO

Keywords:
CA 72–4
ARCHITECT
Chemiluminescent microparticle immunoassay
Gastric cancer marker
Ovarian cancer marker

ABSTRACT

Objectives: Cancer antigen (CA) 72–4 assay is widely used for monitoring gastric and ovarian cancers. The antigen is a mucin-like, tumor-associated glycoprotein known as TAG-72. It has been identified and characterized using two different monoclonal antibodies, CC49 and B72.3, which recognize its glycochain epitopes, Galβ(1–3) sialyl-Tn and sialyl-Tn antigens, respectively. This study describes the quantitative analytical performance of a newly developed CA 72–4 assay, ARCHITECT CA 72–4.

Design: and Methods: The ARCHITECT CA 72–4 assay was developed using the ARCHITECT i2000SRs and three ARCHITECT i1000SRs. The assay performance was evaluated based on guidance from CLSI (Clinical and Laboratory Standards Institute) and correlation against Elecsys CA 72–4.

Results: In the total precision study, the minimum coefficient of variation (CV) for Control/Panel samples over 4 U/mL was 1.1%. The measuring interval was from 0.95 to 200 U/mL with good linearity; and limits of blank (LoB), detection (LoD), and quantitation (LoQ) were 0.09, 0.18, and 0.95 U/mL, respectively. High dose hook effect; differences among specimen tube types; and interference of common drugs, potential cross-reactants, and endogenous substances were not observed. Significantly, this assay has high biotin tolerance at 4875 mg/mL and correlates well with the Elecys CA 72–4 assay (correlation coefficient: 0.95).

Conclusions: ARCHITECT CA 72–4 is a highly sensitive and precise assay for CA 72-4 measurement in human sera and plasma.

Abbreviations: CA 72–4, cancer antigen 72–4; CEA, carcinoembryonic antigen; CI, confidence interval; CLIA, chemiluminescence immunoassay; CLSI, Clinical and Laboratory Standards Institute; CV, coefficient of variation; ECLIA, electrochemiluminescence immunoassay; ESD, extreme studentized deviate; IVD, in vitro diagnostic; LoB, limit of blank; LoD, limit of detection; LoQ, limit of quantitation; TAG-72, tumor-associated glycoprotein 72.

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https://doi.org/10.1016/j.plabm.2023.e00308
Received 1 August 2022; Received in revised form 29 December 2022; Accepted 6 January 2023
Available online 14 January 2023

1. Introduction

Cancer antigen 72–4 (CA 72–4) assay is an immunoassay to detect tumor-associated glycoprotein 72 (TAG-72), which was discovered as an antigen expressed in certain tumor cell populations [1–3]. The antigen was identified as a 220–400 kDa mucin-type glycoprotein [1,4]. Antibody B72.3 was initially prepared using membrane-enriched cell extracts from breast tumors metastasized to the liver and has been shown to react with TAG-72 [3]. CC49 is an antibody specific to TAG-72. The CC49 and B72.3 antibodies recognize Galβ(1–3) sialyl-Tn and sialyl-Tn glycochain epitopes of TAG-72, respectively [5,6]. Interestingly, CA 72–4 content increases in the serum or plasma of patients with malignant gastric, ovarian, colorectal, and pancreatic cancers and is used to monitor patients with gastrointestinal and mucinous ovarian carcinomas [1,7–9].

A blood test can provide clinicians with information about the signs of various illnesses. Since the results of blood tests enhance diagnostic accuracy, it is essential for clinical decision-making that the diagnostic assays show accurate measurements. Until the 1940s, specimens were tested visually and manually. Each test was time-consuming, and test accuracy depended on the technician's skill level. In the 1950s, full-scale inspection automation began with the advent of automated analyzers from American manufacturers. The advances in automation have alleviated the problems of both accuracy and speed. Today, a considerable number of specimens are brought into laboratories in medical institutions (hospitals and clinical laboratory centers) nationwide and tested within 24 h.

Immunochemical methods are used routinely in medical laboratories. Electrochemiluminescence immunoassay (ECLIA) and chemiluminescence immunoassay (CLIA) are popularly used in automated analyzers due to their high sensitivity, low background, and operational simplicity. However, both methods are limited by the susceptibility of the immunochemical reaction to various interferences, such as autologous antibodies in serum, heterophilic antibodies against animal IgG, cross-reactions, hemolysis, hyperlipidemia, and hyperbilirubinemia.

Elecsys CA 72–4 is widely used for the quantitative determination of CA 72–4 in human serum/plasma. It is a sandwich immunoassay using electrochemiluminescence-based detection technology and employs the streptavidin-biotin system with streptavidin-coated paramagnetic beads along with biotinylated CC49 capture and ruthenylated B72.3 tracer antibodies [10]. Biotin in serum/plasma can influence assay results due to interference with the streptavidin (avidin)-biotin system [11,12].

Here, we developed a new assay for CA 72–4, which employs two antibodies, B72.3 and CC49, and acridinium as the tracer instead of the streptavidin-biotin system. We evaluated the quantitative analytical performance of the fully automated immunoassay for CA 72–4 on ARCHITECT i systems.

2. Materials and methods

ARCHITECT CA 72–4 is a one-step, sandwich immunoassay format that captures analytes with mouse monoclonal anti-CA 72-4 CC49 antibody-coated paramagnetic microparticles. In this study, the CA 72–4 analyte-microparticle complex was detected with an acridinium-labeled conjugate prepared from mouse monoclonal anti-CA 72–4 antibody B72.3. The reaction mixture was then exposed to onboard trigger reagents containing peroxide at alkaline pH for luminescence in proportion to the CA 72–4 concentration. ARCHITECT CA 72-4 calibrators ranged from 0 to 300 U/mL (0, 5, 15, 50, 150, and 300 U/mL) with an effective measurement range of 0.95–200.00 U/mL. A 1:3 auto-dilution was used to extend the measurable range to 600 U/mL. ARCHITECT CA 72–4 control concentrations were 7, 35, and 150 U/mL. Heterophile antibodies, rheumatoid factor, blocking agents, and murine antibodies of different isotypes were included to minimize the risk of interference from human anti-mouse antibodies. The fully automated assay was completed within 29 min, with a throughput of 200 tests/h.

2.1. ARCHITECT CA 72–4 assay protocol

The following procedures were automatically performed using the ARCHITECT instruments, i2000SR and i1000SR (Abbott Laboratories; Lake County, IL, USA). First, 50 μL conjugate solution, 50 μL microparticle solution, and 25 μL sample were mixed and incubated for 25 min at 37 °C. Then, after a wash step, the pre-trigger/trigger reagent was added, and the luminescent signal was read. For the 1:3 auto-dilution, the wash buffer provided with the instrument was used to dilute the sample.

2.2. CA 72–4 antigen and concentration assignment

Purified CA 72–4 antigen for preparing ARCHITECT CA 72-4 calibrators, controls, and panels was obtained from Fitzgerald Industries International (North Acton, MA, USA).

2.3. ARCHITECT CA 72-4 calibrators, controls, and panels

ARCHITECT CA 72-4 calibrators and controls were prepared by spiking purified CA 72–4 into a buffer-based diluent, whereas ARCHITECT CA 72-4 panels were prepared by spiking CA 72–4 into heat inactivated CA 72–4 negative human serum pool.

The results of ARCHITECT CA 72-4 calibrators were assigned using specimens measured by the Elecsys CA 72–4 assay (Roche diagnostics, Rotkreuz, Switzerland), as there is no International Standard for CA 72–4.

2.4. Magnetic microparticles coated with anti-CA 72–4 antibody

Anti-CA 72–4 antibody CC49 was coated on carboxylated magnetic microparticle (Polymer Laboratories; Church Stretton, UK) surface with *N*-ethyl-*N*′-(3-dimethylaminopropyl)carbodiimide (Sigma-Aldrich, St. Louis, MO, USA) as a coupling reagent.

2.5. Anti-CA 72–4 conjugate

Anti-CA 72-4 B72.3 antibody was conjugated with acridinium (Abbott Laboratories; Lake County, IL, USA). The conjugate was purified by Superdex 200 pg (Cytiva; Tokyo, Japan) using AKTA (Cytiva).

2.6. Specimens

Specimens used in this study were obtained from ProMeDx (Norton, MA, USA), Biobank (Tokyo, Japan), iSpecimens (Lexington, MA, USA), BioIVT (Westbury, NY, USA), MRN diagnostics (Frankin, MA, USA), Biomex (Heidelberg, Germany), Slieagen (Austin, TX, USA), and BocaBiolistics (Pompano Beach, FL, USA). The specimens were collected under a protocol approved by the institutional review board and stored at −20 °C or colder until use.

2.7. Evaluation methods

2.7.1. Total precision

Total precision was evaluated based on the guidance from CLSI (Clinical and Laboratory Standards Institute) protocol EP05-A3 using ARCHITECT CA 72–4 controls and panels (Table 1). Panel LoQ, Panel L, Panel M, Control L, and Control M were tested twice per day for 20 days using three reagent kit lots and five instruments. Panel H and Control H were tested for 5 days using three reagent kit lots and six instruments in duplicate.

2.7.2. Lower limits of measurement

Lower limits of measurement were set based on the guidance from CLSI EP17-A2 (Table 2). Two ARCHITECT i2000SR and three ARCHITECT i1000SR instruments were tested using three ARCHITECT CA 72–4 reagent kit lots over a minimum of three days. The limit of blank (LoB) represents the 95th percentile from n ≥ 60 zero-analyte sample replicates. The limit of quantitation (LoQ) is the lowest concentration with a maximum allowable precision [20% coefficient of variation (CV)] and was determined from n ≥ 60 low-analyte sample replicates. The limit of detection (LoD) represents the lowest concentration at which the analyte can be detected with 95% probability based on n ≥ 60 low-analyte sample replicates and is defined by the formula:

$$LoD = LoB + c_p SD_L$$

where SD_L is the pooled SD and

$$c_p = \frac{1.645}{\left[1 - \left\{\frac{1}{4}(L–J)\right\}\right]}$$

where L is the total number of the low-analyte sample results (i.e., replicates) for a given level, J is the number of low-analyte samples, and L-J represents the degrees of freedom in the estimated SD_L.

2.7.3. Dilution linearity

Dilution linearity was determined based on the guidance from CLSI EP06-A (Fig. 1A). The samples were prepared by diluting six native high-titer serum specimens to 233–260 U/mL with serum and then serially diluting them with the ARCHITECT CA 72-4 calibrator-A (0.00 U/mL). The observed diluted concentrations for specimens were subjected to regression analysis.

The linearity in ARCHITECT CA 72–4 and Elecsys CA 72–4 were compared using 12 additional native high-titer serum specimens (Fig. 1B). Elecsys CA 72–4 was tested on the Cobas 8000 e801 module (Roche, Mannheim, Germany). Correlations between expected and observed values were calculated as follows:

$$expected\ value = \frac{\text{observed value of the most diluted sample}}{\text{dilution factor}}$$

2.7.4. Hook effect

The hook effect was evaluated using samples containing various CA 72–4 concentrations up to approximately 24000 U/mL. The 24000 U/mL sample was prepared by spiking the purified CA 72–4 antigen into ARCHITECT calibrator-A. The lower concentration samples were prepared by a serial two-fold dilution of a 24000 U/mL sample with ARCHITECT calibrator-A. The CA 72–4 concentrations within the measuring interval were determined considering the dilution factor and ARCHITECT CA 72–4 assay.

2.7.5. Recovery

Recovery was evaluated by comparing samples spiked with CA 72-4 high titer serum and divided into 12 individual specimens and

Table 1
Total precision.

			i2000SR					
			Instrument1					Instrument2
Sample (Target U/mL)	Total precision		Lot 1 (100Test kit)	Lot 2 (100Test kit)	Lot 1 (500Test kit)	Lot 2 (500Test kit)	Lot 3 (500Test kit)	Lot 2 (100Test kit)
Control L (7.00)	Mean (U/mL)	**7.08**	7.01	7.02	7.18	7.02	7.16	7.12
	SD	**0.17**	0.14	0.15	0.13	0.13	0.12	0.16
	CV	**2.3%**	2.0%	2.1%	1.8%	1.9%	1.7%	2.3%
Control M (35.00)	Mean (U/mL)	**35.15**	35.05	35.17	35.60	34.92	35.50	34.69
	SD	**0.76**	0.60	0.54	0.53	0.55	0.54	0.87
	CV	**2.2%**	1.7%	1.5%	1.5%	1.6%	1.5%	2.5%
Control H (150.00)	Mean (U/mL)	**149.16**						
	SD	**2.65**						
	CV	**1.8%**						
Panel LoQ (1.00)	Mean (U/mL)	**0.83**	0.77	0.81	0.79	0.82	0.91	0.85
	SD	**0.06**	0.05	0.06	0.05	0.06	0.05	0.07
	CV	**7.1%**	6.3%	7.0%	6.3%	7.2%	5.0%	8.2%
Panel L (1.00)	Mean (U/mL)	**6.07**	5.74	6.17	5.85	6.17	6.13	6.27
	SD	**0.16**	0.16	0.16	0.14	0.15	0.13	0.18
	CV	**2.7%**	2.7%	2.6%	2.4%	2.4%	2.1%	2.8%
Panel M (75.00)	Mean (U/mL)	**71.99**	68.89	73.94	69.95	73.25	72.58	72.82
	SD	**1.57**	1.02	2.32	1.28	1.10	1.08	1.62
	CV	**2.2%**	1.5%	3.1%	1.8%	1.5%	1.5%	2.2%
Panel H (150.00)	Mean (U/mL)	**148.19**						
	SD	**2.57**						
	CV	**1.7%**						

CV: Coefficienct of variation, SD: Standard deviation.
Control: Controls were samples used to evaluate assay calibration. They were prepared by spiking a commercially available antigen into a buffer-based diluent also used in ARCHITECT CA 72–4 assay Calibrators. The titers of Control L (7.00 U/mL), M (35.00 U/mL), and H (150.00 U/mL) were set based on the medians between CalB (5.00 U/mL) and CalC (15.0 mL), CalC and CalD (50.00 U/ml), and CalD and CalF (300 U/ml), respectively. ARCHITECT CA 72–4 assay recommends testing a single sample of each control level once every 24 h. **Panel:** Panels are samples that imitate actual samples for reagent development. They were prepared by spiking a commercially available antigen into a normal human serum pool consisting of CA 72–4 negative serum. The titers of panel LoQ (1.0 U/mL), M (75.00 U/mL), H (150.00 U/mL), and L (7.00 U/mL) were set based on the limit of detection (0.95 U/mL), median value (100 U/mL), and upper value (200 U/mL) of measuring intervals for ARCHITECT CA 72–4 assay and the cut-off value in Elecsys CA 72–4 assay (6.9 U/mL).

a pooled serum specimen. Recovery or percent recovery was calculated as follows:

$$recovery = [\{(\text{CA } 72-4 \text{ concentration of individual specimen spiking CA } 72-4) - (\text{CA } 72 - 4 \text{ concentration of the individual specimen spiking diluent})\} - \{(CA\ 72-4\ concentration\ of\ the\ pooled\ serum\ spiking\ CA\ 72-4) - (CA\ 72-4\ concentration\ of\ the\ pooled\ serum\ spiking\ diluent)\}]$$

$$(\%\, recovery) = 100 \times \left[\frac{recovery}{\{(CA\ 72-4\ concentration\ of\ the\ pooled\ serum\ spiking\ CA\ 72-4) - (CA\ 72-4\ concentration\ of\ the\ pooled\ serum\ spiking\ diluent)\}}\right]$$

2.7.6. Assay interference

Assay interferences were performed based on the guidelines from CLSI EP07-A2 (Table 3). Each endogenous substance, potential cross reactant, and drug was tested in 12 replicates at two CA 72–4 concentrates (4.00 and 10.0 U/mL) prepared by spiking a high titer CA 72-4 specimen into a pooled serum specimen.

2.7.7. Within-assay specimen carryover

Within-assay specimen carryover was evaluated by alternately testing CA 72–4 negative solution (ARCHITECT CA 72-4 Calibrator diluent) and a high titer 15,000 U/mL CA 72-4 solution (Fitzgerald Industries International, MA, USA). The change of the CA 72-4 level in the negative solution was calculated.

2.7.8. Manual vs. automated dilutions

Manual vs. automated dilution was evaluated by calculating the percentage difference of instrument-diluted specimens containing more than 200.00 U/mL CA 72–4 (1:3 on the ARCHITECT system) compared with manually diluted specimens.

2.7.9. Reagent onboard stability study

Reagent onboard stability studies were conducted with one reagent lot of two different size codes (100 and 500 test kit) using two

i2000SR								i1000SR		
Instrument2	Instrument3				Instrument4		Instrument5	Instrument 6	Instrument 7	Instrument 8
Lot 3 (100Test kit)	Lot 1 (100Test kit)	Lot 2 (100Test kit)	Lot 1 (500Test kit)	Lot 2 (500Test kit)	Lot 2 (100Test kit)	Lot 3 (100Test kit)	Lot 3 (500Test kit)	Lot 1 (100Test kit)	Lot 2 (100Test kit)	Lot 3 (100Test kit)
7.08								7.08	7.01	7.11
0.21								0.24	0.16	0.18
3.0%								3.4%	2.3%	2.6%
35.24								35.12	35.05	35.14
0.80								1.19	0.69	1.00
2.3%								3.4%	2.0%	2.8%
	150.13	148.34	149.28	148.5	148.91	149.78	150.35	149.3	148.27	153.22
	2.41	2.05	2.93	2.08	2.98	3.24	2.31	4.40	2.97	3.53
	1.6%	1.4%	2.0%	1.4%	2.0%	2.2%	1.5%	2.9%	2.0%	2.3%
0.91								0.74	0.80	0.88
0.06								0.06	0.06	0.07
6.1%								8.4%	7.9%	8.0%
6.08								5.86	6.14	6.25
0.17								0.21	0.12	0.21
2.9%								3.6%	2.0%	3.3%
72.51								69.85	73.03	73.08
1.74								2.06	1.35	1.59
2.4%								2.9%	1.9%	2.2%
	147.37	148.38	147.08	148.76	147.75	149.78		145.73	146.73	149.26
	3.33	2.06	1.48	1.68	2.94	3.24		3.61	2.00	3.19
	2.3%	1.4%	1.0%	1.1%	2.0%	2.2%		2.5%	1.4%	2.1%

instruments, two ARCHITECT controls (L and M), and three panels (L, M, and LoQ). A calibration curve was established on the initial day and used to determine the CA 72–4 concentrations in each sample with reagents stored at 2–8 °C off the instrument (Control) and onboard the instrument (Test) for 30 days. The baseline was calculated as the mean result on the first day. Concentration of each sample was evaluated for trends over time in control and test reagents. Calibration curve storage was calculated from the control reagents tested for onboard stability. The concentrations of each sample at each time point from the calibration performed at the initiation of the study were within the control range. The onboard sample stabilities were evaluated by comparing the samples assayed immediately and 3 h after setting onboard. The percent difference between both conditions was calculated.

2.7.10. Tube type study

Seven tube types used to collect serum or plasma (standard serum tube, serum separator tube, dipotassium EDTA tube, tripotassium EDTA, lithium heparin, disodium EDTA, and sodium heparin) were evaluated using spiked high titer native specimen or 1, 7, or 35 U/mL purified antigen (Fitzgerald 30-AC23). The 95% confidence interval (CI) difference from serum plain tube for each tube type was evaluated. Serum or plasma was collected and stored for 24 h at 15–30 °C after the draw on the cells/clot, for 7 days or more at 2–8 °C on the cells/clot, and 7 days at 2–8 °C off the cells/clot followed by one or more freeze/thaw cycle. The 95% CI of the shift from the initial was evaluated.

2.7.11. Correlation study between ARCHITECT CA 72–4 assay and Elecsys CA 72–4 assay

Elecsys CA 72–4 assay was used as a comparison method in this study and tested on the Cobas. The samples used in this study were chosen from purchased carcinoembryonic antigen (CEA) sera with the following criteria:

- sufficient volume of sample for this assay and
- CA 72–4 concentration was within the measuring interval of ELECYS or ARCHITECT CA 72–4 assay.

The CA 72–4 concentrations in the samples were determined by ELECYS and ARCHITECT CA 72–4 assays performed in duplicate. A single Cobas analyzer was used as the platform in Elecsys CA 72–4 assay with one assay regent lot, whereas two i2000SR and two i1000SR analyzers were used as the platform in ARCHITECT CA 72–4 assay with three different assay reagent lots. The slope was analyzed using Weighted–Deming method, and the correlation was evaluated using Pearson's method.

2.7.12. Statistical analysis

Statistical analyses were performed using Analyse-it version 4.80.8 (Analyse-it Software Ltd, Leeds, United Kingdom). Compliance with STARD guidelines is shown in Supplementary Table 1.

Table 2
Lower limits of measurement.

Reagent Kit Lot	i2000SR				i1000SR		
	Instrument1		Instrument2		Instrument3	Instrument4	Instrument5
	Lot 1	Lot 2	Lot 1	Lot 3	Lot 1	Lot 2	Lot 3
LoB (U/mL)	0.08	0.03	0.09	0.02	0.09	0.07	0.04
LoD (U/mL)	0.14	0.13	0.16	0.08	0.18	0.17	0.13
LoQ (U/mL)	0.18	0.35	0.35	0.16	0.36	0.95	0.34

Abbreviations: LoB, Limit of blank; LoD, limit of detection; LoQ, limit of quantification.

3. Results

The total precisions were 1.7–3.4, 1.5–3.4, 1.4–2.9, 2.0–3.6, 1.5–3.1, and 1.0–2.5% CV for Control L, Control M, Control H, Panel L, Panel M, and Panel H, respectively, and 0.05–0.07 U/mL SD for Panel LoQ (Table 1).

The lower limit of measurement study showed that LoB, LoD, and LoQ were 0.09, 0.18, and 0.95 U/mL, respectively (Table 2).

The results of dilution linearity study using specimens spiked with CA 72–4 concentrations across the measuring interval are shown in Fig. 1. The largest difference of the best fit polynomial curve from linear regression ranged from −6.8 to 5.4% (Fig. 1A, Specimens

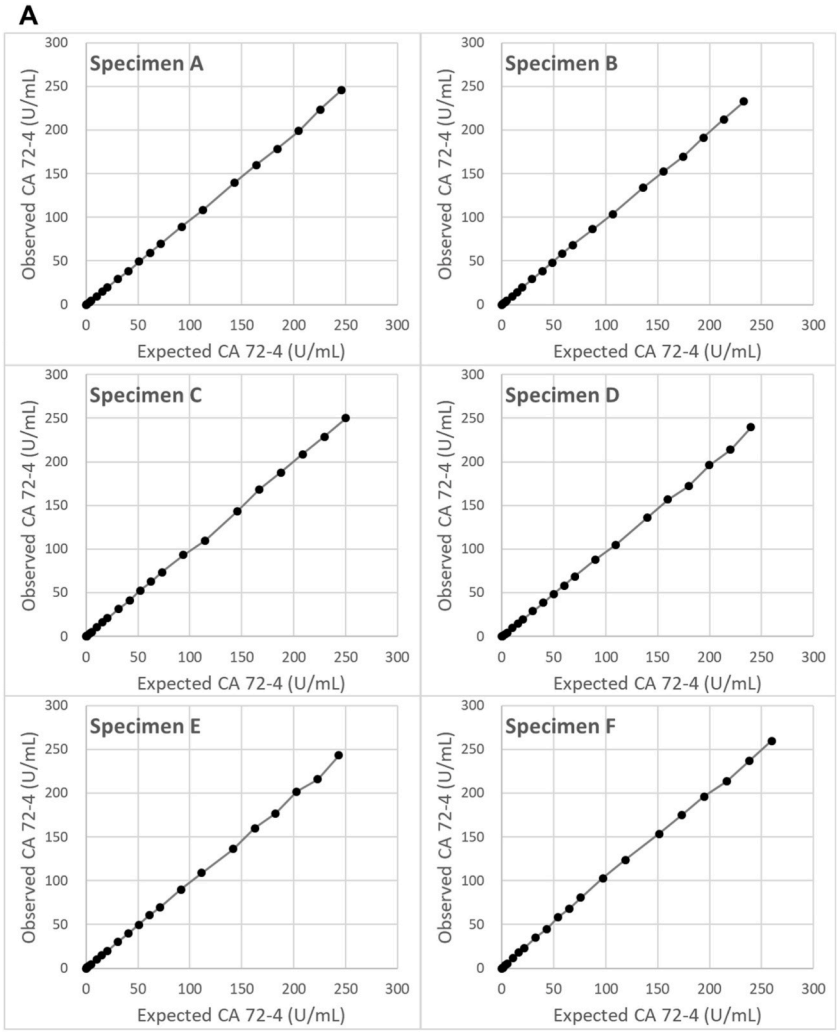
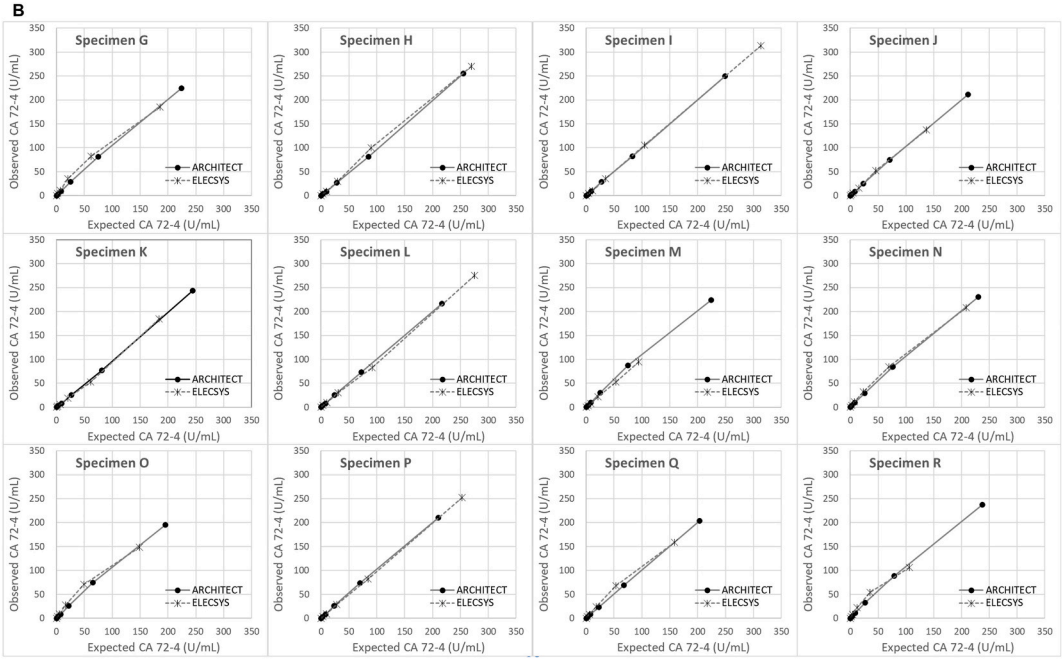


Fig. 1. Evaluation of dilution linearity and hook effect. (A) Dilution linearities of six high-dose specimens on ARCHITECT i2000SR were evaluated across the measurement range. All six specimens showed good linearity on ARCHITECT. (B) Dilution linearities of 12 specimens within measurement range were evaluated on ARCHITECT i2000SR and ELECSYS. (C) High-dose hook effect using purified antigen was evaluated on ARCHITECT i2000SR.



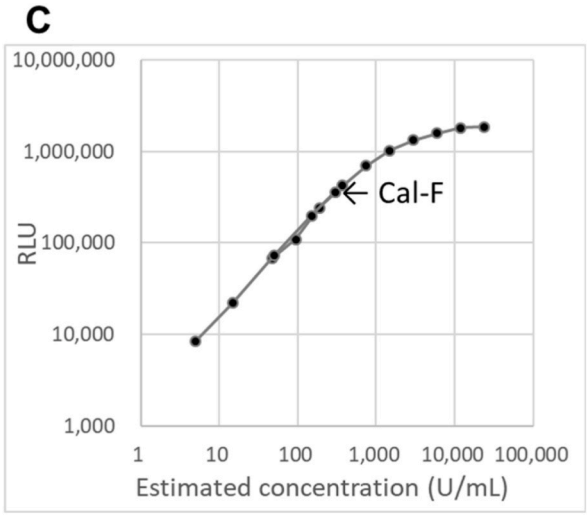


Fig. 1. (*continued*).

A–F). Moreover, we compared the linearity between ARCHITECT CA 72–4 and Elecsys CA 72–4 using 12 individual specimens (Fig. 1B, Specimens G–R). The correlation between the expected and observed values of Specimens G–R on ARCHITECT was 1.00, while that for Specimens G–R on Elecsys was 0.99, 1.00, 1.00, 1.00, 1.00, 1.00, 1.00, 0.99, 0.98, 1.00, 0.99, and 0.97, respectively. The hook effect on ARCHITECT was not observed until CA 72–4 concentration reached 24,000 U/mL (Fig. 1C).

The recovery study showed that the mean or median percent recoveries of two ARCHITECT CA 72–4 reagent kit lots were −0.37 and −0.31 U/mL for spiked sample under 4 U/mL and 92.2% and 95.6% for spiked sample over 4 U/mL.

The influences of the potential interfering substances, cross-reactants, and drugs are presented in Table 3. The 95% CI of interference of increased bilirubin, total protein, triglycerides, red blood cells and hemoglobin, and high total protein were between −6.0 and 8.4%. Moreover, the 95% CI of interference of potential cross-reactants and chemotherapeutic agents were between −6.0 and 4.5% and −10.0 and 5.1%, respectively.

The within-assay specimen carryover study showed that the upper limits of 95% CI of each mean of within-assay sample carryover evaluated using two instruments were 0.03 and 0.13 U/mL, respectively, the values were lower than the LoD (0.18 U/mL).

The manual vs. automated dilution study showed that the differences between 1:3 auto-dilution protocol results and manual results ranged from −1.7 to 8.6%.

The reagent onboard stability showed no significant changes from the baseline for up to 30 days. The percentage Shifts of Lower

Table 3
Interference.

(A) Endogenous substance

	CA 72–4 Level	Interference Concentration	Instrument1 (i2000SR)		Instrument2 (i1000SR)	
			%Difference lower limit of 95% CI	%Difference upper limit of 95% CI	%Difference lower limit of 95% CI	%Difference upper limit of 95% CI
Hemoglobin	4.00 U/mL	1197 mg/dL	−2.9%	6.3%	−4.6%	0.8%
	10.00 U/mL	1270 mg/dL	−3.5%	−0.9%	−2.3%	1.7%
Biotin	4.00 U/mL	4678 ng/mL	−5.2%	−1.7%	−5.3%	0.4%
	10.00 U/mL	4678 ng/mL	0.1%	2.9%	−1.7%	1.3%
Unconjugated Bilirubin	4.00 U/mL	60 mg/dL	−6.0%	0.8%	−2.9%	1.4%
	10.00 U/mL	60 mg/dL	−3.0%	1.0%	−2.2%	0.1%
Conjugated Bilirubin	4.00 U/mL	54 mg/dL	−0.9%	2.8%	−3.4%	0.5%
	10.00 U/mL	54 mg/dL	1.1%	4.6%	−3.4%	0.6%
Triglycelide	4.00 U/mL	3887 mg/dL	−5.6%	−1.1%	−3.2%	1.2%
	10.00 U/mL	3827 mg/dL	−0.5%	2.1%	−4.4%	−0.6%
Total Protein	4.00 U/mL	18 g/dL	2.8%	8.4%	−0.7%	5.3%
	10.00 U/mL	18 g/dL	−2.5%	3.6%	−1.3%	3.5%

(B) Potential cross reactant

Cross Reactant	CA 72-4 Level	Instrument1 (i2000SR)		Instrument2 (i1000SR)	
		%Difference lower limit of 95% CI	%Difference upper limit of 95% CI	%Difference lower limit of 95% CI	%Difference upper limit of 95% CI
150 ng/mL AFP	4.00 U/mL	−0.8%	2.3%	−2.9%	0.9%
	10.00 U/mL	0.1%	3.2%	−2.6%	1.4%
50 mIU/mL BhCG	4.00 U/mL	−2.0%	3.0%	−4.0%	1.5%
	10.00 U/mL	0.3%	2.5%	−1.3%	2.1%
1000 U/mL CA125	4.00 U/mL	−2.9%	1.4%	−1.9%	2.4%
	10.00 U/mL	−1.5%	3.0%	−2.5%	1.3%
800 U/mL CA15-3	4.00 U/mL	−6.0%	0.1%	−1.6%	4.1%
	10.00 U/mL	−1.1%	1.3%	−2.4%	1.0%
1200 U/mL CA19-9	4.00 U/mL	−2.9%	3.4%	−2.1%	4.5%
	10.00 U/mL	−0.9%	2.3%	−0.5%	3.1%
95 ng/mL CEA	4.00 U/mL	−3.3%	2.8%	−2.6%	1.1%
	10.00 U/mL	−1.5%	1.7%	−1.9%	1.9%
100 ng/mL CYFRA 21-1	4.00 U/mL	−1.1%	3.6%	−1.2%	3.1%
	10.00 U/mL	−2.0%	1.6%	−3.4%	1.1%

(C) Drug

Drug	CA 72-4 Level	Instrument1 (i2000SR)		Instrument2 (i1000SR)	
		%Difference lower limit of 95% CI	%Difference upper limit of 95% CI	%Difference lower limit of 95% CI	%Difference upper limit of 95% CI
165 μg/mL Cisplatin	4.00 U/mL	−1.4%	3.4%	−4.2%	1.8%
	10.00 U/mL	−0.3%	2.4%	−0.6%	3.3%
909 μg/mL Methotrexate	4.00 U/mL	−6.2%	−0.3%	−10.0%	0.3%
	10.00 U/mL	−4.7%	0.3%	−3.5%	0.7%
67 μg/mL Paclitaxel	4.00 U/mL	−1.0%	3.5%	−2.2%	2.2%

(*continued on next page*)

Table 3 (*continued*)

(C) Drug					
Drug	CA 72-4 Level	Instrument1 (i2000SR)		Instrument2 (i1000SR)	
		%Difference lower limit of 95% CI	%Difference upper limit of 95% CI	%Difference lower limit of 95% CI	%Difference upper limit of 95% CI
	10.00 U/mL	2.4%	4.4%	−0.3%	3.6%
40 μg/mL Doxorubicin	4.00 U/mL	−3.3%	2.2%	−2.8%	2.8%
	10.00 U/mL	−3.1%	1.8%	−1.0%	3.0%
500 μg/mL Carboplatin	4.00 U/mL	−5.0%	0.7%	−2.5%	3.1%
	10.00 U/mL	−2.7%	1.5%	−2.2%	1.4%
12 μg/mL Etoposide	4.00 U/mL	−3.4%	2.4%	−3.4%	2.4%
	10.00 U/mL	−1.3%	2.5%	−1.9%	2.3%
17.2 μg/mL Mitomycin C	4.00 U/mL	−2.2%	2.8%	−6.4%	−1.6%
	10.00 U/mL	−1.0%	3.0%	−1.4%	1.2%
1 μg/mL Docetaxel	4.00 U/mL	−3.2%	2.1%	−5.7%	−0.6%
	10.00 U/mL	−0.3%	2.7%	−4.3%	0.1%
2 μg/mL Epirubicin	4.00 U/mL	−4.0%	2.0%	−6.0%	1.3%
	10.00 U/mL	−3.0%	1.4%	−1.8%	1.8%
1000 μg/mL 5-Fluorouracil	4.00 U/mL	−1.5%	4.7%	−1.9%	2.5%
	10.00 U/mL	−2.4%	1.0%	−5.2%	0.2%
4.00 μmol/mL Tamoxifen	4.00 U/mL	−1.9%	2.3%	−2.1%	3.9%
	10.00 U/mL	−9.6%	5.1%	−4.6%	0.1%
1000 ng/mL Tegafur	4.00 U/mL	−4.2%	1.1%	−2.6%	0.9%
	10.00 U/mL	−3.5%	0.2%	−1.2%	2.2%
0.114 ng/mL Leucovorin	4.00 U/mL	−4.6%	1.9%	−5.3%	1.0%
	10.00 U/mL	−1.0%	2.6%	−0.4%	3.4%
20 μg/mL Irinotecan	4.00 U/mL	−1.9%	1.4%	−3.9%	2.5%
	10.00 U/mL	−1.8%	1.6%	−2.5%	1.3%

Abbreviations: CI, confidence interval; AFP, alpha-fetoprotein; BhCG, The β-subunit of human chorionic gonadotropin; CA, cancer antigen; CEA, carcinoembryonic antigen; CYFRA 21–1, cytokeratin 19 fragment; LoQ, limit of quantitation.

95% CL and Upper 95% CL for 0.77–70.92 U/mL samples ranged from −6.5 to 0.5% and from −2.1 to 6.4%, respectively. The calibration curve was stable until 30 days. The 95% CI of differences between samples assayed immediately, and 3 h after setting onboard were −3.3 to 3.0%.

The 95% CIs of the difference of each tube type from the plain serum tube were −0.16–0.04 U/mL for 1 U/mL sample, and −3.3–2.9% for 7 U/mL and 35 U/mL samples. For determining the stability in each tube type, 95% CIs of shift during sample storage in each tube type were −0.39–0.07 U/mL for samples under 4 U/mL, and −10.0–6.0% for samples over 4 U/mL.

A total of 392 CEA-positive sera were screened for the correlation study between Elecys and ARCHITECT CA 72–4 assays, and 177 sera were chosen (Fig. 2). Their CEA concentrations according to the vendor and CA 72–4 concentrations determined by ELECYS CA 72- were 7.6–9.9 ng/mL and 1.55–209.50 U/mL, respectively. The CA 72–4 concentrations of sera measured in the correlation of i2000SR and i1000SR to ELECYS CA 72–4 assay were between 1.06–176.33 and 1.10–179.36 U/mL, respectively. Two sera were omitted in the correlation study using i1000SR since the CA 72–4 concentrations measured with repeat assay in the study were <1.00 U/mL. The result showed that the slopes of the calibration curves were 0.93 and 0.92 for i2000SR and i1000SR, respectively, and the correlation coefficient was 0.95 for both platforms.

4. Discussion

CA 72–4 is used as a biomarker and CA 72–4 assays aid the therapeutic monitoring of gastric and ovarian cancers. Therefore, it should be reliable quantitative assay and compatible with on-market IVD products.

This study evaluated the analytical performance of newly developed ARCHITECT CA 72–4. The total precision of the assay showed 1.5–3.6% CV for >4 U/mL samples and 0.05–0.07 U/mL SD for <4 U/mL samples. The LoQ in the result with outlier by ESD test was <0.95 U/mL. Moreover, the 95% CIs of interference of endogenous substances, drugs, or potential cross-reactants were between −10.0% and 6.3%. These results showed acceptable reliability for interference. Testing blank samples after a high titer sample

indicated no sample carryover. ARCHITECT CA 72–4 assay showed good dilution linearity from 200 U/mL to the LoQ, and no high-dose hook effect was observed. ARCHITECT CA 72–4 assay showed acceptable performance with samples collected in various tubes, as shown by equivalency of the different anticoagulants compared to that of plain serum tubes and stability of the samples collected in various tubes under various conditions, including room temperature, 2–8 °C, and frozen. Moreover, reagent onboard stability for 30 d and sample stability for 3 h were good. The correlation study showed good agreement between Elecys and ARCHITECT CA 72–4 assays. The correlation coefficient was 0.95, and the slopes were 0.93 and 0.92 for i2000SR and i1000SR, respectively. Elecsys CA 72–4 assay is a widely assay for quantitative CA 72-4 determination in human serum/plasma worldwide, so ARCHITECT CA 72–4 was standardized against Elecsys CA 72–4. The result showed good agreement between both assays (Fig. 2).

ARCHITECT CA 72–4 assay can use two or more sample tube types, disodium EDTA, and sodium heparin. As the use of CA72-4 for cancer diagnosis, prognosis, and treatment is expanding, we believe that increasing the number of tube types that can be used and a high tolerance for biotin interference will reduce the burden on medical staff and their patients.

Some studies have reported that CA 72–4 is a good serum biomarker clinically used for diagnosing various cancer types, including digestive tract cancers (esophageal, gastric, and colorectal carcinomas), ovarian cancer, and non-small cell lung cancer. However, Xu et al. reported that diagnosis using CA 72–4 is still open to some debate as it is highly expressed in not only tumor tissues (such as gastric cancer, colorectal cancer, and ovarian cancer) but also normal tissues (such as endometrium in the secretory phase and transitional mucosa of the colon) [13]. CA 72–4 can generate false positive results for gastric cancer detection because the CA 72-4 levels in gout patient sera are even higher than those in gastric cancer patient sera. Zhang et al. collected and analyzed the clinical results of serum CA72-4 from 38,526 individuals, including healthy individuals and individuals with various non-neoplastic diseases and cancers. They reported that patients with gout (23.7 U/mL) and gouty arthritis (31.45 U/mL) had significantly higher average serum CA72-4 levels than those with cancers ($P < 0.0001$), with the average CA72-4 level in gastric cancer patients being 7.73 U/mL [14]. Another study reported abnormal serum CA72-4 level elevation in gout patients treated with colchicine [15]. Moreover, a recent prospective study on Chinese patients showed that the positive predictive value of CA72-4 (31.58%) was insufficient to establish gastric cancer diagnosis, indicating that the accuracy of CA72-4 is not good enough to meet the clinical demand [16]. Collectively, these observations highlight that more efforts are required to establish CA 72–4 as a reliable tumor diagnostic biomarker.

Herein, we report that ARCHITECT CA 72-4 has a high tolerance to biotin interference and can be used to test samples collected in various sample tube types. Thus, precise data can be generated, which may decrease stress on patients and staff working in a laboratory. Therefore, our new assay would promote CA 72-4 diagnostics in clinical research. ARCHITECT CA 72–4 was developed as a dedicated reagent for the ARCHITECT analyzer, and the optimized measuring conditions can be installed into the analyzer through the Abbott Link or CD-ROM, enabling even small clinical and diagnostic laboratories to get fast and reliable assay results. ARCHITECT CA 72–4 assay is a convenient, precise, and accurate automated method for CA 72–4 quantification in both serum and plasma. We are yet

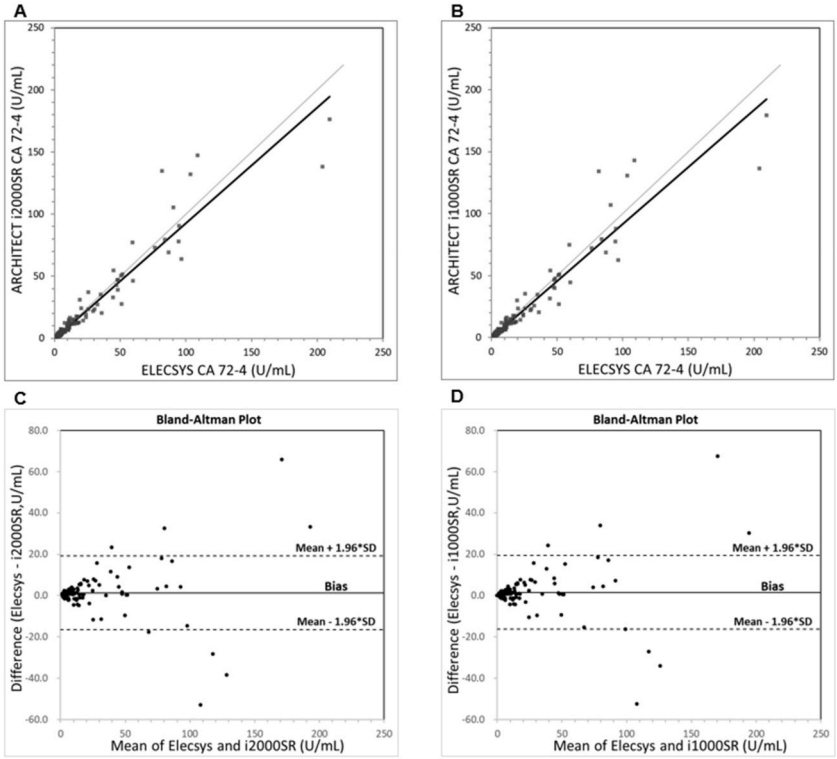


Fig. 2. Correlation between ARCHITECT CA 72–4 assay on (A, C) i2000SR or (B, D) i1000SR and Elecsys CA 72–4 assay.

to assess the diagnostic sensitivity or specificity of the ARCHITECT CA 72–4. Our next study will collect clinical data to evaluate the specificity of ARCHITECT CA 72–4.

Author statement

Fusamitsu Yanagihara: Conceptualization, Methodology, Validation, Writing - Original Draft. **Hideaki Okura:** Investigation. **Hisashi Ichikawa:** Investigation, Writing - Review & Editing. **Takuma Shirakawa:** Investigation. **You Pan:** Investigation. **Bailin Tu:** Investigation. **Zhihong Lin:** Investigation. **Ryan Bonn:** Investigation. **Sridevi Kurella:** Methodology, Validation. **Beth Schodin:** Validation, Supervision. **Toru Yoshimura:** Conceptualization, Methodology, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Funding

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Toru Yoshimura reports a relationship with Abbott Japan Co Ltd that includes: employment and equity or stocks. Fusamitsu Yanagihara reports a relationship with Abbott Japan Co Ltd that includes: employment and equity or stocks. Hideaki Okura reports a relationship with Abbott Japan Co Ltd that includes: employment and equity or stocks. Hisashi Ichikawa reports a relationship with Abbott Japan Co Ltd that includes: employment and equity or stocks. Takuma Shirakawa reports a relationship with Abbott Japan Co Ltd that includes: employment and equity or stocks. You Pan reports a relationship with Abbott Laboratories that includes: employment and equity or stocks. Bailin Tu reports a relationship with Abbott Laboratories that includes: employment and equity or stocks. Zhihong Lin reports a relationship with Abbott Laboratories that includes: employment and equity or stocks. Ryan Bonn reports a relationship with Abbott Laboratories that includes: employment and equity or stocks. Sridevi Kurella reports a relationship with Abbott Laboratories that includes: employment and equity or stocks. Beth Schodin reports a relationship with Abbott Laboratories that includes: employment and equity or stocks.

Data availability

The data that has been used is confidential.

Acknowledgments

The authors are grateful to the assay development teams at Abbott Japan, Denka, and Abbott Laboratories for their efforts in the assay development and support for evaluating the ARCHITECT CA 72–4 assay.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.plabm.2023.e00308.

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