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Dual-label time-resolved fluoroimmunoassay for simultaneous measurement of human epidermal growth factor receptor 2 and human epididymis protein 4 in serum

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Abstract

In this study, a novel dual-label time-resolved fluoroimmunoassay (TRFIA) is described for simultaneous quantification of human epidermal growth factor receptor 2 (HER-2) and human epididymis protein 4 (HE4) in serum to screen gynecologic cancers. A double-antibody sandwich TRFIA was introduced with europium and samarium chelates to simultaneously detect the concentrations of HER-2 and HE4. Under optimal conditions, the proposed method exhibited wide linear ranges for HER-2 of 0.07–500 ng ml⁻¹ and for HE4 of 0.32–1000 pmol l⁻¹ with the average coefficient of variation below 10%. The specificity was satisfied through determining the other common tumor markers. The recovery rates were 94.5% and 96.6% on average for HER-2 and HE4, respectively. Good correlations were observed in clinical samples between developed method and commercial chemiluminescence immunoassay kits. The results demonstrated that dual-label TRFIA for HER-2 and HE4 was rapid and precise, and therefore could have a promising use in large sample detection for gynecological cancer screening.

Keywords: Human epidermal growth factor receptor 2, Human epididymis protein 4, Time-resolved fluoroimmunoassay, Gynecological cancer, Simultaneous detection

Introduction

Women's health is related not only to themselves but also to their families. In recent decades, the cancer incidence rate in female has risen significantly by 2.2% per year in China (Chen et al. 2016). Among all gynecological cancers, the mortality rates of breast cancer, cervical cancer, ovarian cancer, and uterine cancer have been increasing year by year (Bray et al. 2018; Chen et al. 2016; Fan et al. 2014). In

developed countries like the USA, breast cancer and uterine cancer rank the first and fourth in the incidence of gynecological tumors (Siegel et al. 2016). On account of economic development and increasing urbanization in our province, Jiangsu women have high mortalities of breast cancer, ovarian cancer, and uterine cancer (Han et al. 2017). However, the World Health Organization has pointed out one third of cancer deaths could be prevented. So, cancer screening has gradually become an important part of physical examination, in which serological detection based on tumor markers (TMs) has become indispensable with its rapidness, effectiveness, and simplicity. The newly discovered TM can give a big push to improve the targeting and accuracy of tumor screening and diagnostic efficiency. Thus, the research on novel TMs is very important for clinical medicine.

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Since human epidermal growth factor receptor 2 (HER-2, also named ERBB2) and human epididymis secretory protein 4 (HE4, also known as WAP four-disulfide core domain protein 2) are abnormally expressed in gynecological tumor tissues, they can be used as TMs in serological determination (Laidi et al. 2016; Simmons et al. 2013; Tchou et al. 2015). One biomarker is HER-2 for breast cancer. Overpressed HER-2 amplified by 17q12 has a strong relationship with tumor generation and transformation, through the high-grade-like pathway characterized by loss of 13q and gain of chromosomal region 11q13 (Harbeck et al. 2019). The oncogene HER-2, encoding HER-2 receptor tyrosine kinase from the epidermal growth factor receptor family, is overactive and amplified in tumor cells. Overpressed HER-2 is associated with poor prognosis in the absence of systemic therapy. Therefore, HER-2 detection plays a pivotal role in the treatment choice, prognosis evaluation, and efficacy prediction of HER-2-positive breast cancer patients (Waks and Winer 2019). Compared to cancer antigen (CA) 153, serum HER-2 is a strong independent prognostic factor for survival after relapse in metastatic breast cancer (Fehm et al. 2004). The other TM studied was HE4, a special biomarker for ovarian cancer and endometrial cancer. HE4 is associated with cancer cell adhesion, migration, and tumor growth. It could activate the MAPK and FOCAL adhesion signaling pathways, promoting ovarian cancer cell invasion and metastasis in ovarian cancer progression (Lu et al. 2012; Zhuang et al. 2014). This TM is negative in normal ovarian epithelium, and low in normal, benign tumor and adjacent tissues, but high in cancer (Ferraro et al. 2013; Ferraro et al. 2015; Simmons et al. 2013). Moreover, HE4 promised a better sensitivity than CA 125 in the detection of early-stage ovarian cancer. Thus, the national institutes of health regarded it as an important tool to screen patients with pelvic mass for benign and malignant diseases (Simmons et al. 2013).

Since HER-2 and HE4 have been considered as useful predictors in diagnosis of gynecologic cancers (Ferraro et al. 2013; Sorensen et al. 2009; Tchou et al. 2015), a quantitative and reliable method is urgent to detect them in serum samples. In our previous studies, a sensitive and wide-range TRFIA technology was used to measure serum HER-2 and HE4, respectively (Fan et al. 2018; Zhang et al.

2017). Until now, few papers have focused on the combined determination of above biomarkers for gynecological cancer screening. In the present work, a novel immunoassay was developed and evaluated for simultaneous measurement of HER-2 and HE4 in serum, by dual-label TRFIA with europium and samarium chelates.

Results

The assay principle

The dual-label assay was based on a double-antibody sandwich model, with coating monoclonal antibodies against HER-2 and HE4 immobilized on the microtiter plates. After adding the analytes and lanthanide-labeled antibodies, the immune reaction was taken place in the reagents with moderate shake. Through the wash step, the aimed immune complex was reserved on the solid phase. Finally, the enhancement solution, as a key reagent, was used for dissociating the lanthanide ions and amplifying the fluorescence signals, which were positively correlated with the concentrations of HER-2 and HE4 in the samples. The protocol of the assay is indicated in Fig. 1.

Characterization of labeled antibodies

As the first eluent peak was collected for Eu-HER-2, the concentration of antibody protein was detected by UV-spectrometer. The level of Eu was estimated according to the Eu standard in the labeling kit. Hence, the label rate of Eu-HER-2 was from dividing Eu amount by the protein amount, so was the rate of Sm-HE4. In the assay, there were 6.9 Eu ions per HER-2 antibody and 7.4 Sm ions per HE4 antibody on average.

Assay optimization

The concentrations of coating and labeling antibodies were estimated for better sensitivity and wider liner range by detecting HER-2 and HE4 standards. Under optimal conditions, the fluorescent counting of standard curve was high enough, when the antibody-antigen complex on the plate was almost saturate. As plotted in Fig. 2, $2\mu\text{g ml}^{-1}$ of anti-HER-2 antibody and $2\mu\text{g ml}^{-1}$ of anti-HE4 antibody were selected for immobilization on the plate. For low background and

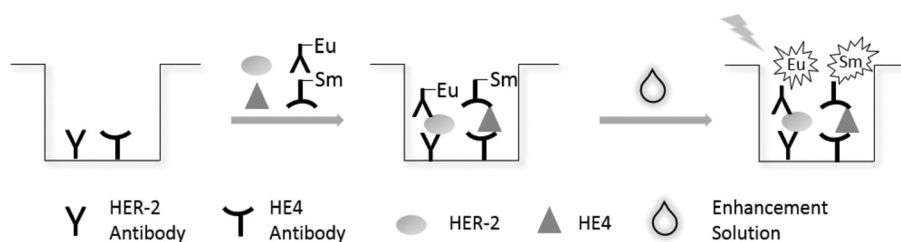


Fig. 1 Schematic procedure for dual-label time-resolved fluoroimmunoassay for HER-2 and HE4

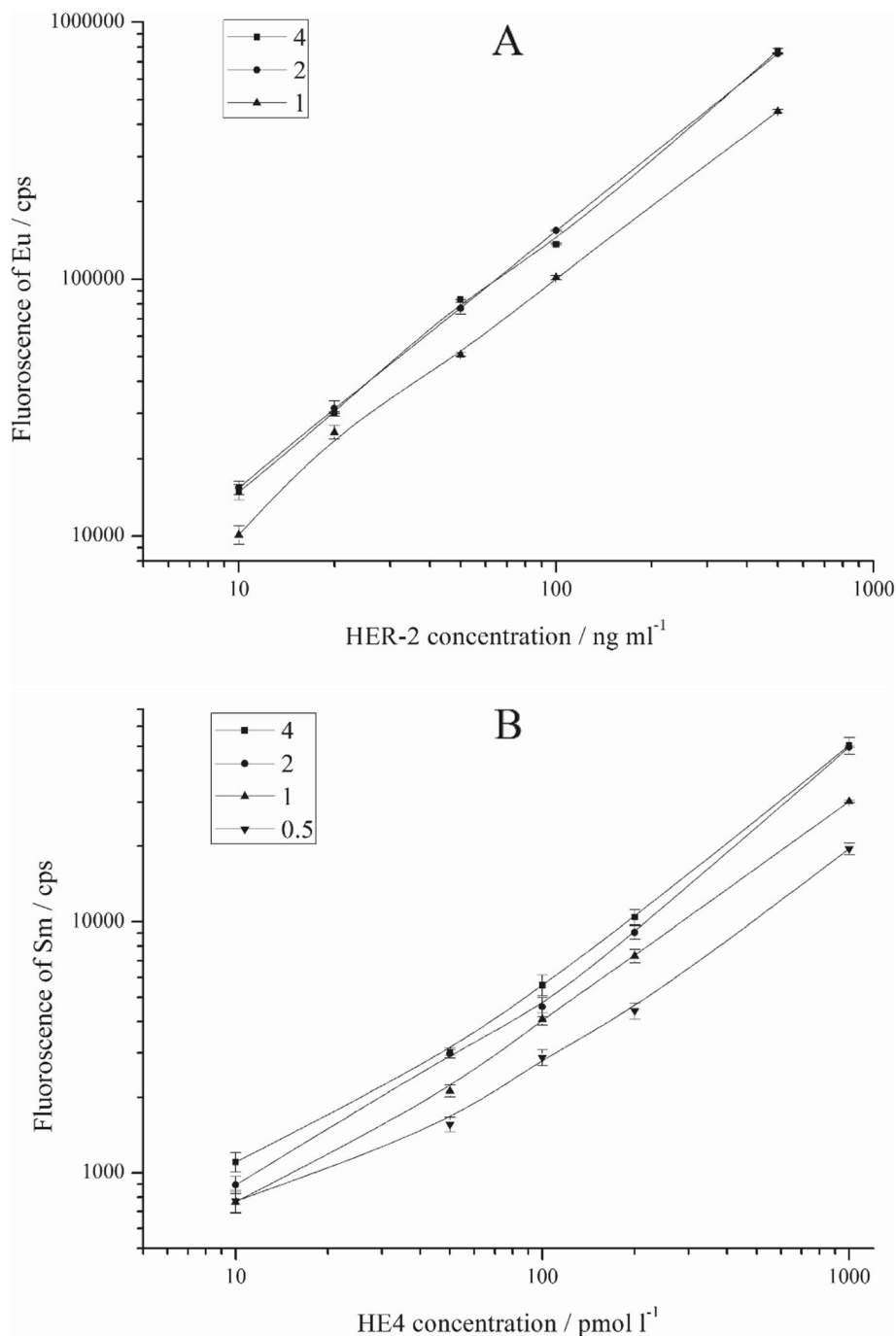


Fig. 2 Reaction curves for different concentrations of coating monoclonal antibodies. **a** HER-2 curves. **b** HE4 curves

high fluorescent signal, dilution rates of labeled antibodies were assessed and the effect is shown in Fig. 3. It indicated that 1:100 was the most suitable ratio for both Sm-HE4 and Eu-HER-2 in the assay.

The kinetic characteristics of the dual-label assay were determined by incubating standards for 30, 60, 120, and 180 min at room temperature with continuous shaking. The result is shown in Fig. 4. Maximum signals for

HER-2 and HE4 were obtained after 120 min incubation. Therefore, 120 min was chosen for the reaction time of the assay.

Calibration and reproducibility

A series of HER-2 and HE4 standards was detected in triple by dual-label TRFIA in the optimized situations. The standard concentration and fluorescence

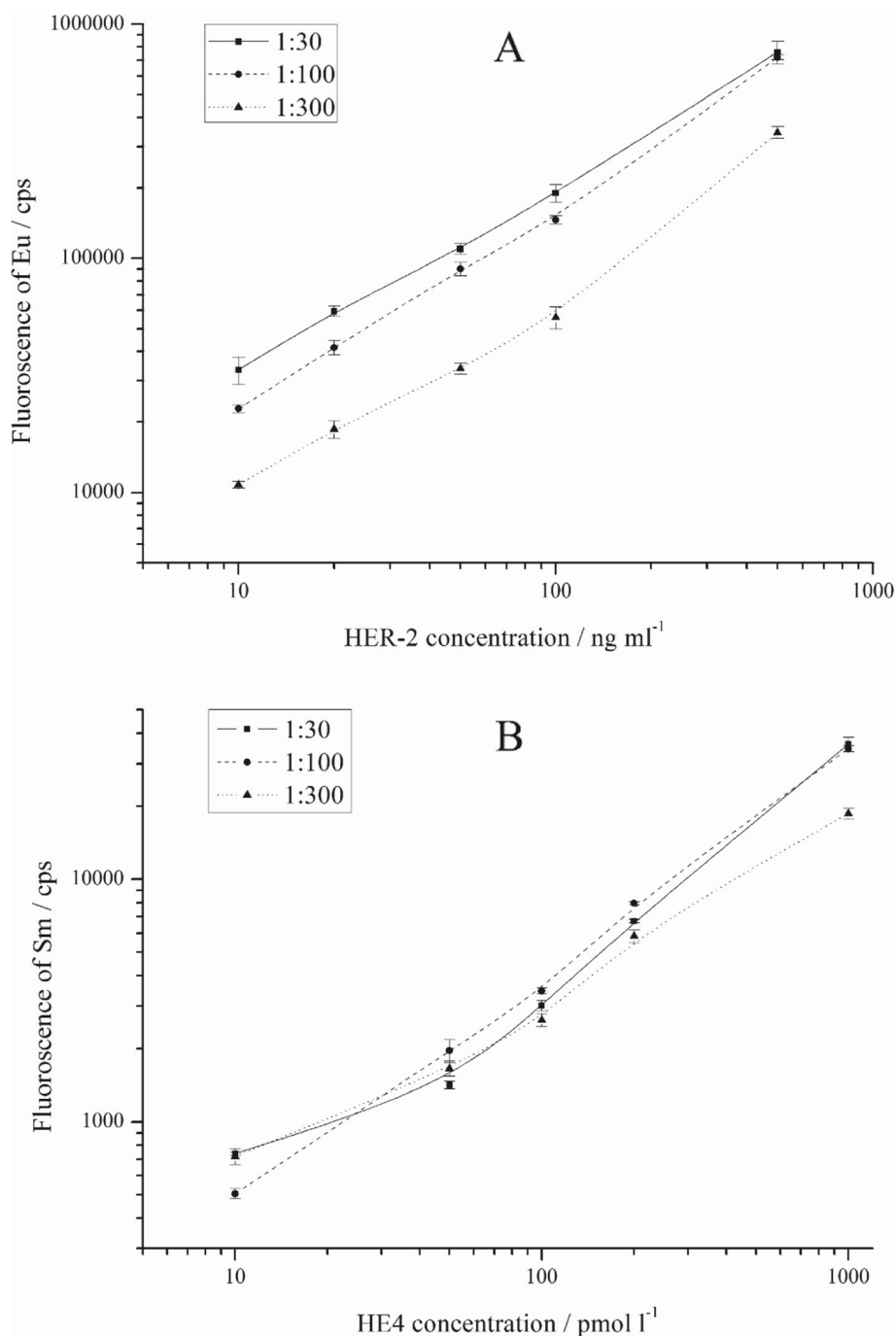


Fig. 3 Reaction curves for dilutions of labeled antibodies. **a** HER-2 curves. **b** HE4 curves

counting were transformed logarithmically, and the linear equations of the assay are illustrated in Fig. 5. The sensitivity was 0.07 ng ml^{-1} for HER-2 and 0.32 pmol l^{-1} for HE4, calculated from the concentration corresponding to the mean plus two standard deviations (SD, $n = 10$) of the blank standard fluorescence. The R values of the equations were both above 0.99 (0.9987 for HER-2 and 0.9893 for HE4),

which meant the assay offers linear working ranges of $0.07\text{--}500 \text{ ng ml}^{-1}$ and $0.32\text{--}1000 \text{ pmol l}^{-1}$ for HER-2 and HE4 determination, respectively. The figure also indicated the coefficients of variation (CV) were 1.5–11.8% for HER-2 and 1.4–12.1% for HE4. The average draft rates were both under 10%, standing for the good repeatability of the dual-label immunoassay.

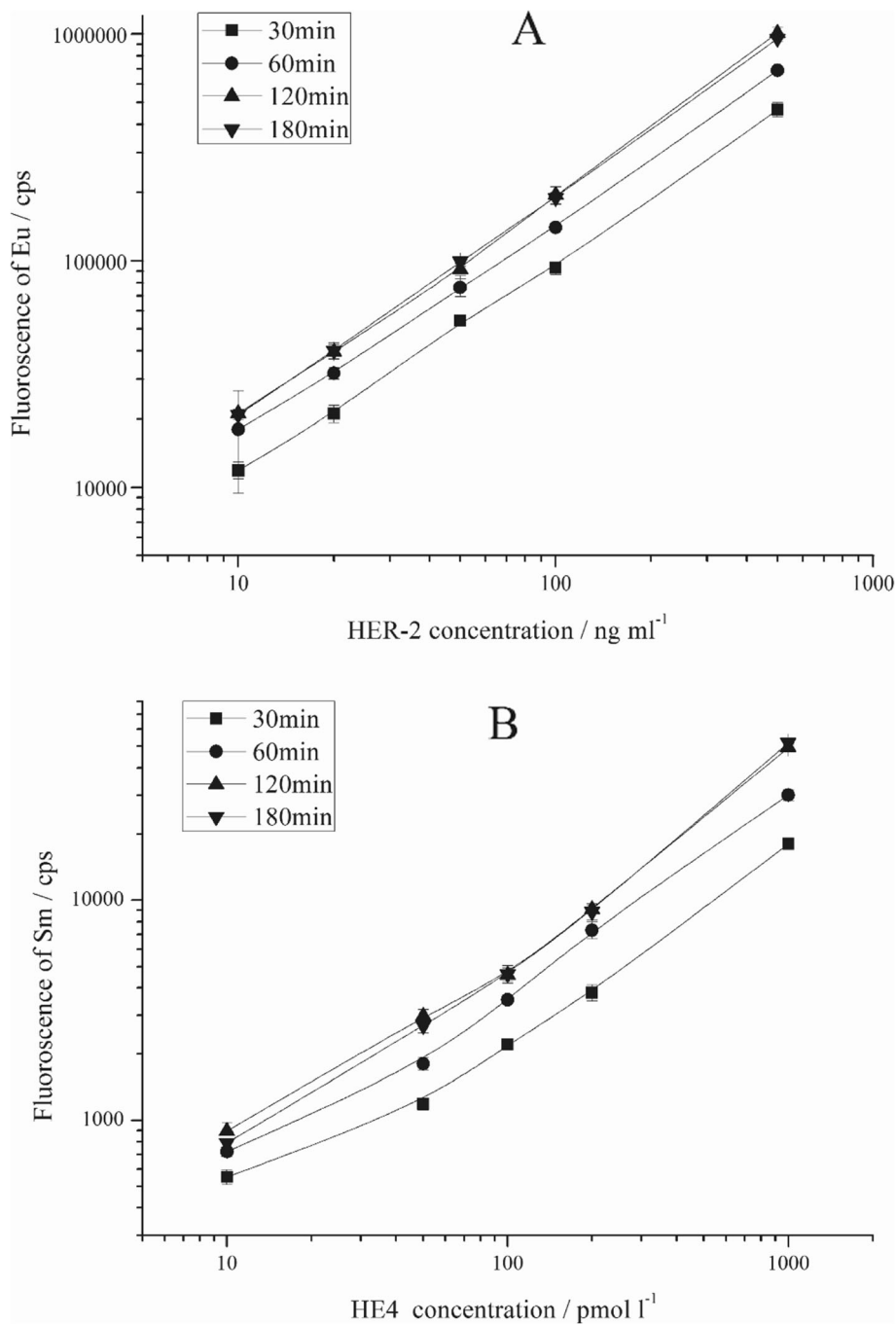


Fig. 4 Effect of reaction times for HER-2 (a) and HE4 (b) TRFIA

Specificity and recovery

To evaluate the specificity of monoclonal antibodies used in the method, various tumor markers in high levels were determined by the proposed assay. These interferences were of purified antigens, namely, alpha fetoprotein (AFP), carcino-embryonic antigen (CEA), total-prostatic specific antigen (T-PSA), CA199, CA 242, CA 153, CA 125, and neuron specific enolase (NSE). Each substance was

monitored twice by the proposed method, and the cross-reactivity rates are listed in Table 1. The data showed that the monoclonal antibodies used in the assay had less interference to the common tumor markers and presented high specificity for HER-2 and HE4 antigen.

To verify the analytical recovery of the proposed method, three samples from healthy women were collected and spiked with different concentrations of HER-2

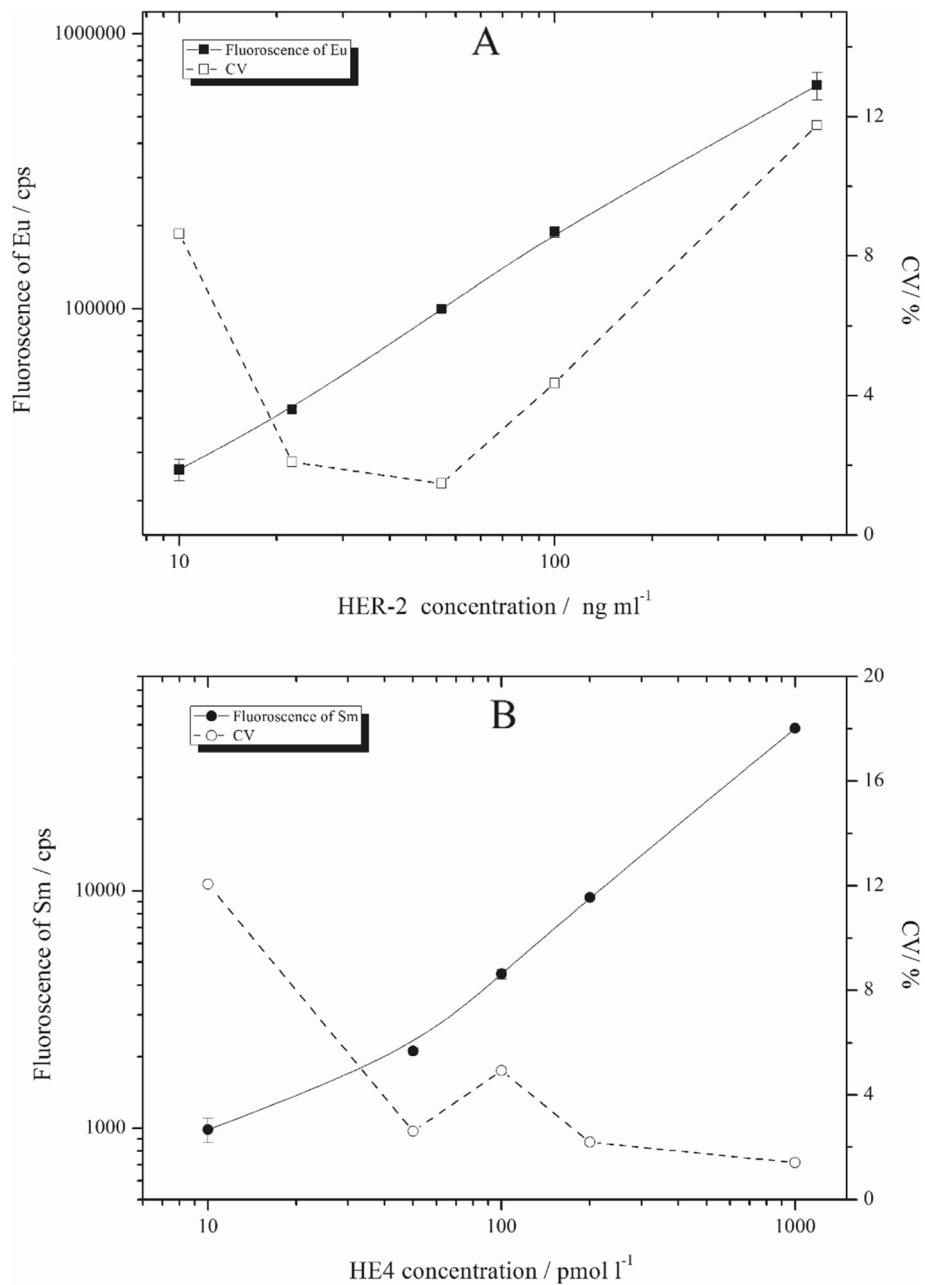


Fig. 5 Standard curves for HER-2 (a) and HE4 (b) measurement by dual-label TRFIA

or HE4. The original as well as added sera were determined by dual-label TRFIA. From the results in Table 2, the recovery rates for HER-2 and HE4 were 94.5% and 96.6%, respectively, and the average CV were 5.0% and 4.7%, respectively. It indicated that the developed assay had a satisfied recovery and potential application.

Clinical consistency

Correlation analysis for clinical application was investigated by detecting 46 serum specimens in which 20 samples were collected from healthy volunteers. Results in Fig. 6 are

obtained from the developed dual-label TRFIA and commercial chemiluminescence immune assay (CLA) kits and compared by linear regression analysis and paired *T* test. The CLA detection was carried out by automatic chemiluminescence instruments. With *R* of 0.955 for HE4 and 0.978 for HER-2, the linear equation was fitted to $y = 0.9175x + 3.2268$ or $y = 0.8614x + 2.5237$, respectively, where *x* stood for the concentration from the CLA kit and *y* stood for that from the proposed method. The two-tailed *P* values were 0.304 for HE4 and 0.347 for HER-2, which meant there were no significant differences between the

Table 1 The cross-reactivity of developed HER-2 and HE4 assay

Interfering substance	Concentration	HE4/pmol l ⁻¹		HER-2/ng ml ⁻¹	
		Observed/pmol l ⁻¹	Cross-reactivity (%)	Observed/ng ml ⁻¹	Cross-reactivity (%)
AFP	1000 U ml ⁻¹	2.77	0.28	2.80	0.28
CEA	500 ng ml ⁻¹	0.29	0.06	0.00	0.00
T-PSA	100 ng ml ⁻¹	2.75	2.75	0.00	0.00
CA242	200 U ml ⁻¹	4.91	2.46	0.00	0.00
CA153	35 U ml ⁻¹	0.00	0.00	0.00	0.00
CA199	300 U ml ⁻¹	4.03	1.34	1.94	0.65
NSE	350 ng ml ⁻¹	0.00	0.00	1.02	0.29
CA125	600 U ml ⁻¹	0.00	0.00	0.00	0.00
HER-2	500 ng ml ⁻¹	0.00	0.00	500.00	100.00
HE4	2000 pmol l ⁻¹	1949.00	97.45	0.30	0.02

two methods in HER-2 and HE4 detection. These data suggested that dual-label TRFIA could have a potential application for simultaneous detection of serum HER-2 and HE4.

Reference interval

The average levels of 32 controls were 15.34 ± 4.89 ng ml⁻¹ for serum HER-2 and 39.34 ± 15.73 pmol l⁻¹ for serum HE4. The reference intervals of HER-2 and HE4 of healthy women by the proposed method were 0–25.12 ng ml⁻¹ and 0–70.81 pmol l⁻¹ for HER-2 and HE4, respectively, calculated from mean +2SD. The gynecological cancer groups had higher concentrations of serum HER-2 and HE4 than healthy groups with limit cases in the study ($P < 0.05$). According to the cutoff values, the positive cases were obtained. The true positive rates were 28.6% (4/14) for HER-2 in breast cancer and 35.7% (5/14) for HE4 in ovarian cancer. In the breast cancer group, two

(14.3%) cases were only positive for HER-2 and three (21.4%) only for HE4, while two (14.3%) were both for HER-2 and HE4. In the ovarian cancer group, one (8.3%) case was only positive for HER-2 and four (33.3%) only for HE4, while one (8.3%) both for HER-2 and HE4. The results are listed in Table 3.

Discussion

TRFIA, as a kind of nonradioactive immunoassay, has been widely used in research and clinic with significant advantages (Fan et al. 2018; Huang et al. 2017; Mitrunen et al. 1995; Sheng et al. 2016; Zhang et al. 2017; Zhang et al. 2018). The unique properties of lanthanide render TRFIA to realize detecting two analytes in one single assay. With Eu, Sm, Tb, and Dy ions labeled, TRFIA can provide multiple detection with excellent signal-to-background ratio. Among all the lanthanides, Eu ion has been commonly used in TRFIA because of its higher

Table 2 The recoveries of serum samples spiked with HER-2 and HE4 by dual-label TRFIA

Sample	HER-2				HE4			
	Spiked/ng ml ⁻¹	Observed/ng ml ⁻¹ , $n = 3$	Recovery (%)	CV (%)	Spiked/pmol l ⁻¹	Observed/pmol l ⁻¹ , $n = 3$	Recovery (%)	CV (%)
Case 1	0	9.8			0	48.1		
	20	27.6	89	10.1	50	102.3	108.4	5.2
	50	57.5	95.4	3.4	100	142.1	94	7.1
	100	106.1	96.3	2.6	200	229.7	90.8	3.6
Case 2	0	16.0			0	31.0		
	20	34.9	94.5	7.3	50	80.2	98.4	2.9
	50	66.7	101.4	1.5	100	123.9	92.9	4.9
	100	109.6	93.6	5.2	200	237.6	103.3	2.8
Case 3	0	17.5			0	37.0		
	20	35.6	90.5	2.6	50	87.8	101.6	9.5
	50	63.4	91.8	3.0	100	127.3	90.3	2.6
	100	115.2	97.7	9.1	200	215.8	89.4	3.4
Average			94.5	5.0			96.6	4.7

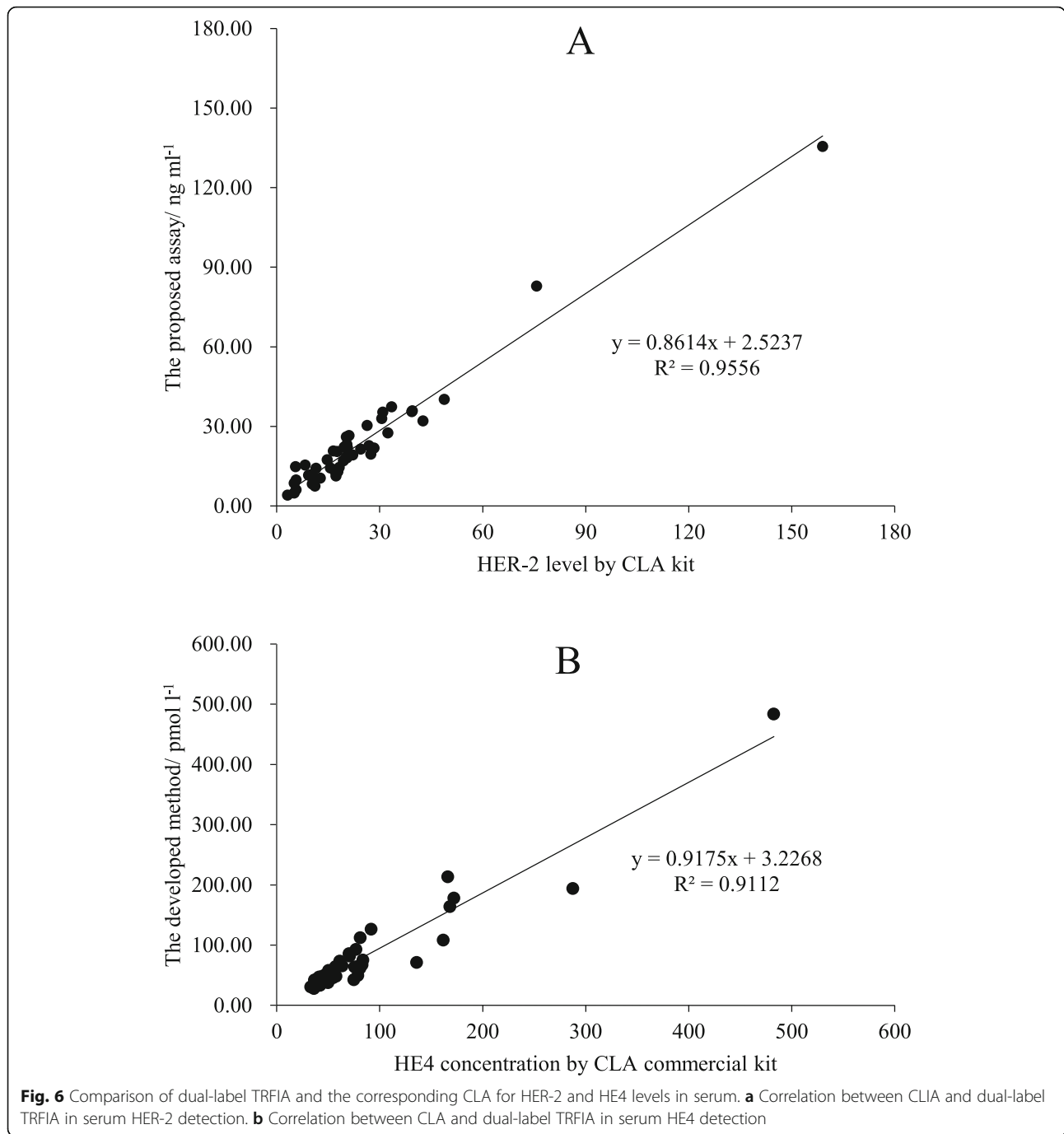


Table 3 The levels of serum HER-2 and HE4 by dual-label TRFIA in gynecological cancer cases and healthy volunteers

Diagnosis	Number	HER-2/ng ml ⁻¹	Positive cases	HE4/pmol l ⁻¹	Positive cases
Health controls	32	15.34 ± 4.89		39.34 ± 15.73	
Gynecological cancer	26	17.87 ± 9.94*	6	132.37 ± 204.70*	10
Breast cancer	14	19.87 ± 11.98*	4	59.86 ± 44.93*	5
Ovarian cancer	12	15.53 ± 6.63*	2	216.97 ± 279.90*	5

*P < 0.05

fluorescent intensity and lower background noise. In dual-label assays, Sm is often in pair with Eu (Huang et al. 2018; Sheng et al. 2016; Zhang et al. 2018). They have the same excitation wavelengths (340 nm) and use the same fluorescence-enhancement reagent (β -naphthoyl trifluoroacetone) to form fluorescent chelates, while they differ significantly in the emission wavelengths and their quench times. Since it only gives 1.5% of fluorescence intensity of Eu, Sm sets to be the second tracer in dual-label assays for the analyst required less sensitivity (Mitrunen et al. 1995). In previous studies, HE4 determination had a wider linear range and required less sensitive than HER-2 detection (Fan et al. 2018; Zhang et al. 2017). Therefore, Sm was conjugated against HE4 while Eu ion was against HER-2 to improve the assay limit. With this labeling combination, the proposed method had obtained working ranges by four orders of magnitude for HER-2 and HE4 detection, respectively. Thus, that satisfied sensitivity and accuracy of the method were acquired which would be helpful to distinguish cancer patients from healthy groups.

Comparing to the single assays, the developed dual-label TRFIA had several advantages. The one-step performance of the assay had simplified the operation and improved the diagnosing efficiency. Comparing to single TRFIA in previous studies (Fan et al. 2018; Zhang et al. 2017), the newly developed method combining HER-2 and HE4 determination in one test could shorten to about 2 h. Furthermore, the rapid and simultaneous detection obviously reduced the operation and system errors comparing to two separate analyses. In the study, the assay recoveries ranged within 10% and within-run CV was not above 5%, which proved the method had a high precision and was benefit for clinical usage. In addition, the proposed assay saved the cost by reducing the total amount of sample volume and reagents due to the detection of two biomarkers in one single test. Therefore, the method was of good precision while achieved time and cost savings, which may have a potential value of laboratory medicine.

Until now, immunohistochemistry and fluorescence in situ hybridization have been commonly applied for HER-2 detection in clinical analysis (Bonacho et al. 2019; Pertschuk et al. 1999). Although both of them could monitor HER-2 gene accurately, they required surgery to sample the tissue, which was invasive and not suitable for early screening or prognosis assessment. Since HER-2 protein could be shed by protease hydrolysis and released to circulation (Jeong et al. 2019), immunoassays for serum HER-2 have been reported using enzyme-linked immunosorbent assay (ELISA) and CLA (Fehm et al. 2004; Oyama et al. 2015). With instable signals and narrow working range, colorimetric ELISA is inaccurate and insensitive especially without proper automatic instruments. With the support of instruments and equipment, CLA is a rapid technology, though it

requires costly reagents and specific operation environment. HE4 detection also has above shortages by ELISA and CLA (Chudecka-Glaz et al. 2016; Zhou et al. 2016). Furthermore, HER-2 and HE4 have to be analyzed separately with two kits by ELISA or CLA. In this study, dual-label TRFIA provided strong and stable fluorescent signals and cost-saving reagents to detect serum HER-2 and HE4 simultaneously.

According to the cutoff values of the proposed method, the gynecological cancer groups had high levels of serum HER-2 and HE4. In this study, HER-2 not only was a biomarker for breast cancer, but also expressed in ovarian cancer with 14.3%, while HE4 protein was high not only in the ovarian cancer group, but also in breast cancer with 50% (5/10) of positive rate. Some cases were only positive for HER-2 in ovarian cancer, and some others were only for HE4 in breast cancer patients, which could make the combined and simultaneous detection of HER-2 and HE4 for gynecological cancers valuable.

Conclusion

Using TRFIA method with Eu and Sm ions, a novel detection of serum tumor markers for gynecological cancers, HER-2 and HE4, was well developed and evaluated. It provided two wide detectable ranges with high sensitivity, good precision, specificity, and accuracy. The simultaneous detection helped to reduce the cost and save performing time, which made it more welcome in clinical usage. Now with the automatic instruments, this high-throughput method becomes more convenient and is much more suitable for detecting large numbers of serum samples for breast and ovarian cancer. It could be a useful tool for the early diagnosis and mass screening of gynecological cancer patients. The clinical assessment of the method will be further discussed elsewhere.

Methods

Reagents and apparatus

Labeling kits containing Eu and Sm chelates were obtained from Perkin-Elmer (USA). The affinity purified HE4 antigen and anti-HE4 monoclonal antibodies for coating and labeling were purchased from Waston (China). The recombinant HER-2 antigen and anti-HER-2 monoclonal antibodies for immobilization and capture were obtained from Origene (China). Sepharose CL-6B was purchased from Pharmacia Company (USA). Microtiter plates were purchased from ThermoFisher (USA). Assay buffer, wash solution, and enhancement solution were supplied by Jiangyuan (China). The other reagents were analytically pure and supplied by Sinopharm Chemical Reagent (China). The HER-2/neu (CLA) was performed by ADVIA Centaur from Siemens (Germany). An ECL2010 electrochemical luminescence apparatus for HE4 determination was a product from Roche (Switzerland). The UV-spectrometer was a Bio-Rad

product (USA). The assay analyzer and fluorescence reader were using an AutoDELFIA₁₂₃₅ by Perkin-Elmer (USA).

Coating of the microtiter plates

Two kinds of monoclonal antibodies, anti-HER-2 and HE4, were mixed and immobilized on the plates. The coating antibodies were diluted to certain concentrations in coating buffer (0.05 M Na₂CO₃–NaHCO₃, pH 9.6) and added to the plates with 200 µl per well. After incubated overnight at 2–8 °C, the coated plates were patted dryly and then saturated for 2 h with 250 µl of coating buffer containing 3 g/l BSA. Later, the plates were aspirated and preserved at – 20 °C in sealed plastic bags.

Labeling of antibody

Eu and Sm chelates were used to label the detection antibodies, which were HER-2 antibody labeled with Eu and the antibody recognized HE4 with Sm. The labeling process was according to the manual of labeling kits. One microgram of HER-2 antibody was resolved in a solution containing 0.05 M Na₂CO₃–NaHCO₃ (pH 9.0), mixed with 0.2 mg Eu-DTTA, and reacted overnight at room temperature with continuous gentle shake. Eu-HER-2 antibody was separated from the mixture by gel filtration on a Sephadex G-50 column with elution buffer containing 0.05 M Tris-HCl (pH 7.8), 0.9% NaCl, and 0.1% preserver. The labeled HER-2 antibody was collected from the first peak of protein eluate. The labeling rate was calculated from the Eu standard. The labeling procedure of Sm-HE4 was the same as that of Eu-HER-2 with the exception that the amount of Eu chelate was replaced to 0.5 mg of Sm. Later, the labeled antibodies were diluted with elution buffer and stored at – 20 °C.

Preparation of standards

The purified antigen was used for preparing standard substance. The standard buffer was a kind of 0.05 M Tris-HCl solution (pH 7.8) containing 0.9% NaCl and 0.5% BSA with preserver and formulated in our laboratory. The standards were diluted HER-2 and HE4 antigens with standard buffer to a series of concentrations of 0/0, 10/10, 20/50, 50/100, 100/200, and 500/1000 ng ml⁻¹/pmol l⁻¹.

The assay procedure

The reagents were warmed up to room temperature before performance. In the laboratory, 100 µl of standards contained HE4 and HER-2 purified antigens or serum samples, and 100 µl of labeled antibodies diluted with assay buffer was added to the microtiter wells. After shaken mildly at 25 °C for a proper time, the plate was washed for six times to fully remove the unreacted substances, dripped with 200 µl of enhancement solution, and then vibrated for 5 min. The Eu and Sm fluorescence in

the plate was finally scanned, recorded, and shifted to the concentrations of serum HER-2 and HE4 by AutoDELFIA₁₂₃₅.

Serum samples

A total of 58 serum samples with 32 healthy controls, 14 breast cancer patients, and 12 ovarian cancer patients were provided by Jiangyin People's Hospital and Jiangyuan Hospital Affiliated to Jiangsu Institute of Nuclear Medicine. The samples had been stored at – 20 °C before use. The Ethical Committee of Jiangsu Institute of Nuclear Medicine approved the collection project.

Statistics

Means and SD were calculated by Microsoft Excel. The curves were plotted using OriginLab Origin (USA). *P* < 0.05 was considered statistically significant.

Abbreviations

CLA: Chemiluminescence immune assay; ELISA: Enzyme-linked immunosorbent assay; HE4: Human epididymis protein 4; HER-2: Human epidermal growth factor receptor 2; TM: Tumor marker; TRFIA: Time-resolved fluoroimmunoassay

Acknowledgements

Not applicable.

Authors' contributions

MX and ZH drafted the study and acquired the funding. YZa, KW, and YZo wrote the manuscript and analyzed the data. JF and TH performed the experiments. YS and BZ collected the samples and prepared the materials. JZ revised the manuscript and interpreted the data. All authors read and approved the final manuscript.

Funding

The study was funded by the Research Project of Jiangsu Provincial Commission of Health and Family Planning (H201637), Jiangsu Provincial Key Medical Discipline (ZDXKA2016017), Innovation Capacity Development Plan of Jiangsu Province (BM2018023), Wuxi Key Medical Talents (ZDRC006), Medical and Public Health Project of Wuxi Sci-Tech Development Fund (WX18JIAN027), and Jiangsu Provincial Key Talent Program for Maternal and Child Health (FRC201741).

Availability of data and materials

Data sharing not applicable to this article as no datasets were generated or analyzed during the current study.

Competing interests

The authors declare that they have no competing interests.

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Received: 16 October 2019 Accepted: 23 December 2019

Published online: 16 January 2020

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