Supplementary materials

Development of a Time-Resolved Fluorescence Microsphere Immunochromatographic Assay for Simultaneous Quantitative Detection of Urinary Estrone-3-Glucuronide and Pregnanediol-3-Glucuronide

Yu Yi[1],Xiangnan He [1], Jianfeng Mei[1], Bailong Wang[2]， Shujiang Wu[2]， Guoqing Ying[1*],

[1]College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou 310014, China

[2]Biotest Biotech Co., Ltd, Hangzhou 310014, China

*Corresponding author

Address for correspondence

Dr. Yu Yi

E-mail address: yiyu1106@zjut.edu.cn

College of Pharmaceutical Science, Zhejiang University of Technology, Hangzhou 310014, China

Yu Yi, yiyu1106@zjut.edu.cn

Xiangnan He: 2064952466@qq.com

Jianfeng Mei： mrion@zjut.edu.cn

Bailong Wang： erik.wang@biotests.com.cn

Shujiang Wu： wade.wu@biotests.com.cn

Guoqing Ying*： gqying@zjut.edu.cn

Materials and methods

Apparatus

The following equipment was utilized in this study: the HGS101 continuous dispensing platform from AUTOKUN Technology Co., Ltd. (Hangzhou, China); FIC-S100 dry fluorescence immunoassay analyzer from Helmen Precision Instruments Co., Ltd. (Suzhou, China); GA-RDS-001 gold conjugate dispenser from Greatly-Auto Co., Ltd. (Hangzhou, China); WRF-QG001 high-speed strip cutter from Werfen Equipment Co., Ltd. (Jiaxing, China); SM-900D ultrasonic homogenizer from Shunmayq Co., Ltd. (Nanjing, China); H2500R high-speed refrigerated centrifuge from Hunan Xiangyi Laboratory Instrument Development Co., Ltd. (Changsha, China); pH meter from Mettler-Toledo Instruments Co., Ltd. (Shanghai, China); Infinite F50 microplate reader from Tecan Ltd. (Shanghai, China).

Distribution of pregnancy urine samples

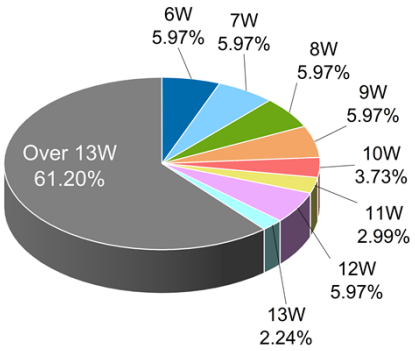

Fig. S1 Distribution plot of pregnancy urine samples

Optimization of key parameters

Particle Size of Europium (III) fluorescent microspheres（EuFMs）

EuFMs of varying particle sizes (200 nm and 300 nm) were pre-washed and conjugated with E1-3-G-mAb or PdG-mAb in equimolar ratios to prepare EuFM-mAb conjugates. The influence of particle size on assay performance was evaluated using E1-3-G and PdG linear reference standards.

Antibody concentration during Conjugation

Antibody concentration critically influences conjugation efficiency. Insufficient antibody levels reduce labeling yield, whereas excessive concentrations may provoke steric hindrance or nonspecific adsorption, thereby compromising conjugate performance.[1] To optimize this, E1-3-G and PdG antibodies at concentrations of 50, 100, and 150 μg mL^{-1} were conjugated with fixed

quantities of EuFMs. The impact of antibody concentration on assay functionality was analyzed using PdG and E1-3-G linear calibration standards.

pH of the Reaction Solution

Hydrophobic EuFMs are prone to aggregation under conditions of high particle concentration, surface charge neutralization, or elevated electrolyte levels.[2] . To enhance dispersion stability, the pH of the 0.05 M MES reaction buffer was systematically adjusted to 6.0, 7.0, 8.0, and 9.0. EuFM-mAb conjugates were prepared at each pH, and optimal dispersion was determined by analyzing their performance with E1-3-G and PdG standards.

Reaction Time Optimization for Immunochromatographic Strips

Positive samples (10 μg mL^{-1} PdG and 1800 ng mL^{-1} E1-3-G) and negative urine matrices were tested. Fluorescence intensities of the T1 and T2 lines, along with T/C values (T1/C and T2/C), were recorded at 5, 10, 15, 20, 25, and 30 minutes post-reaction. Immunokinetic curves were plotted with reaction time (X-axis), T-line fluorescence intensity (left Y-axis), and T/C values (right Y-axis). Fluorescence scanning profiles of negative urine samples at varying time points were also analyzed to assess reaction time.

The calibration of the working calibrator

Theoretical concentrations of E1-3-G and PdG working calibrators were serially diluted within the detection ranges specified by the Cayman PdG and E1-3-G ELISA kits. Following measurement, back-calculated concentrations were compared to theoretical values via linear regression analysis to quantify deviations. As illustrated in Figures S2a and S2b, the adjusted coefficient of determination (adj. R^2) exceeded 0.99 for both analytes, with regression slopes >0.95 and slope 0.90–1.10, satisfying the predefined acceptance criteria.

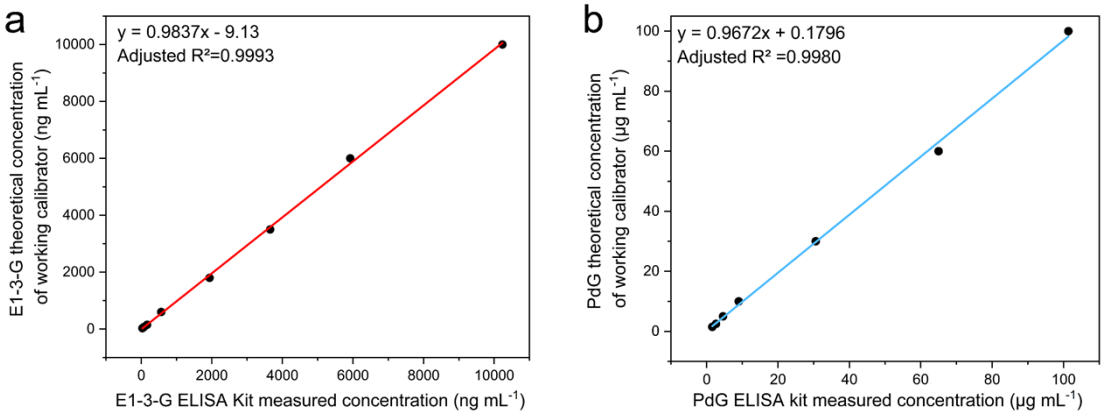

Fig. S2 The calibration of the working calibrator: (a) E1-3-G; (b) PdG.

Method for Specificity and Interference Detection

Cross-reactive substances were diluted in a negative urine matrix to the concentrations

specified in Tables S1 and S2 (Supporting Information). These diluted substances were subsequently mixed with E1-3-G reference standards (6000 ng mL^{-1} and 150 ng mL^{-1}) or PdG reference standards (100 μg mL^{-1} and 10 μg mL^{-1}) at a 1:9 volumetric ratio (substance:reference standard). In parallel, high- and low-concentration blank controls were prepared by mixing the negative urine matrix with the same E1-3-G or PdG reference standards at an identical 1:9 ratio. Relative deviations between the test samples (containing cross-reactive substances and reference standards) and their corresponding blank controls were calculated. A substance was classified as non-cross-reactive if the relative deviations for both high- and low-concentration mixtures remained within ±15%.

Table S1 The concentration of cross-reacting substances added to the reference standards.

Structurally related compounds	The concentration of cross-reacting substances in E1-3-G（μg mL^{-1}）	The concentration of cross-reacting substances in PdG（μg mL^{-1}）
Corticosterone	100	100
Cortisol	100	100
17 α-OH P	100	100
Testosterone	100	50
Pregnenolone	100	50
Estriol	50	10
Pregnanediol	100	100
DHEA Sulfate	100	50
Estrone	1	100
Progesterone	100	100
Estradiol	50	100
Aldosterone	10	10

Table S2 The concentration of interferents Added to reference standards.

Interferents	The concentration of interferents in E1-3-G	The concentration of interferents in E1-3-G
Albumin	50 mg mL^{-1}	50 mg mL^{-1}
Glucose	200 mg mL^{-1}	100 mg mL^{-1}
Urea	80 mg mL^{-1}	80 mg mL^{-1}
Creatinine	50 mg mL^{-1}	50 mg mL^{-1}
Vitamin C	40 mg mL^{-1}	20 mg mL^{-1}
HCG	100 iu mL^{-1}	100 iu mL^{-1}
LH	1000 miu mL^{-1}	1000 miu mL^{-1}
FSH	75 miu mL^{-1}	75 miu mL^{-1}
Ethyl alcohol	1%	1%

Extrapolation of shelf life using accelerated stability data calculated with *Arrhenius* Equation

According to *Arrhenius Equation* which relates chemical reaction rate ***(k)*** to the absolute temperature (***T)***:

$$d\,(\ln k)/dT = \Delta E_a/RT^2$$

E_a is the activation energy; and R is the universal gas constant.

Assuming E_a = approx. 19.5 Kcal/mol, the following table derived from the *Arrhenius Equation*, illustrates the estimated length of time at a particular storage temperature required for product to achieve a one-year shelf life.

Table S3 The required duration to predict a one-year shelf life at different temperatures.

Storage Temperature (°C)	Days Required for 1 Year Stability
90.4	0.8
85.2	1.2
80.2	1.8
74.9	2.7
70.1	4.0
65.0	6.0
60.1	9.2
55.1	14.6
50.1	23.0
45.0	37.5
40.1	64.4
37.0	91.0
30.1	193.0
25.1	343.7
22.1	494.8
20.1	617.7
15.1	1145.3
12.0	1688.4

According to Arrhenius Equation, 14.6days at 55 °C is equivalent to 1 year at room temperature.

Results

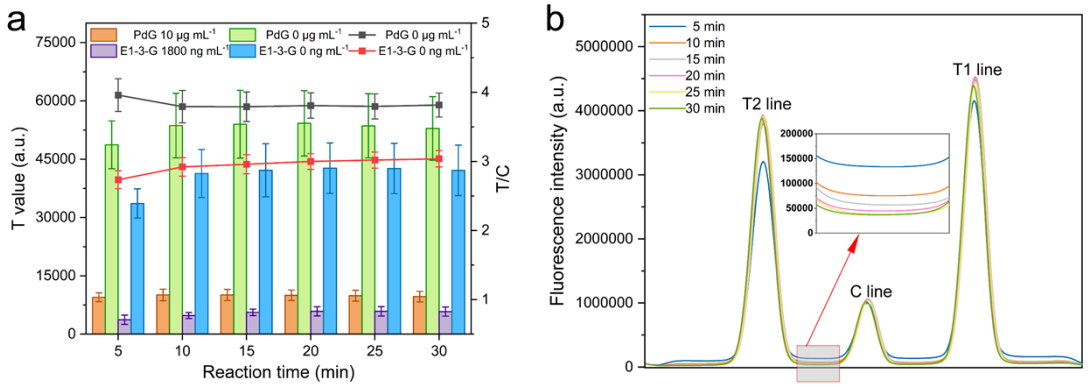

Fig. S3 Optimization of key parameters: (a) immunoassay kinetic curve; (b) fluorescence scanning curves at sequential time points.

Table S4 Impact of Different Urine pH on E1-3-G and PdG

Negative urine matrices pH	E1-3-G (ng mL^{-1})	E1-3-G measured concentration Mean±SD (ng mL^{-1})	Relative deviation (%)	PdG (μg mL^{-1})	PdG measured concentration Mean±SD (μg mL^{-1})	Relative deviation (%)
4.0	6000	6396.52±449.29	6.61	100	97.63±6.71	-2.37
	150	162.11±7.39	8.08	10	10.15±0.82	1.50
7.0	6000	6589.65±136.40	9.83	100	100.68±2.33	0.68
	150	156.32±8.30	4.22	10	10.19±1.03	1.90
9.0	6000	6577.19±260.66	9.62	100	95.49±2.16	-4.51
	150	163.50±8.26	9.00	10	9.31±0.35	-6.90

References

1. J. Liang, X. Li, B. Huang, Y. Pan, Z. Zhuang, Q. Ye, C. Peng, H. Deng, Y. Yi, B. Zhang, P. Chen and X. Chen, *Biosens. Bioelectron.*, 2024, **258**, 116357.
2. M. Majdinasab, M. Sheikh-Zeinoddin, S. Soleimanian-Zad, P. Li, Q. Zhang, X. Li, X. Tang and J. Li, *Food Control*, 2015, **47**, 126-134.