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DEVELOPMENT AND VALIDATION OF THE MAGNETIC IMMOBILIZATION TO DETERMINE PAPP-A IN HUMAN SERUM

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Abstract. The novel magnetic method used to immobilize the primary antibody in 96-well polystyrene plates was investigated to determine the pregnancy-associated plasma protein-A (PAPP-A), one of the most important protein markers in pregnancy. The optimal conditions for the immobilization are 20 µL magnetic nanoparticles (0.72 mg/mL), 2µL primary antibody (30 µg/mL), and a one-step process. The reaction was implemented in 40 minutes and 30 ºC. The calibration curve was established and the linear range shows up to 2090 mU/L. The LOQ and LOD are 7.2 and 24.0 mU/L, respectively. The reaction is non-specific for other pregnancy hormones such as hCG and aFP even at a high level. The Passing & Bablok regression showed the linear relationship and the agreement between new and reference methods, y = a (95% CI)x + b(95% CI), a = 1.00 (0.971 to 1.019), b = 22.07 (-73.104 to 117.244), R^2 = 0.999, p<0.001. The Bland-Altman plot also showed the high concordant. The new method can be used to determine PAPP-A in the serum sample with mild conditions, simple and time-saving reaction, high sensitivity and selectivity, and comparable results with the commercial method.

Keywords: PAPP-A, magnetic immobilization, one-step process.

I. Introduction

Pregnancy-associated plasma protein-A (PAPP-A) is a metalloprotease, produced and secreted by the placental syncytiotrophoblast, the protein level increases from 5 weeks of gestation and continuously rises along with the age of the fetus. The main PAPP-A function is to release insulin-like growth factor (IGF) from its binding protein (IGF-binding protein). Therefore, the PAPP-A plays a critical role in placental invasion, placental development, and maintenance of placental functions [1]-[3]. The level of PAPP-A, especially in the first trimester was widely investigated and used as a biomarker for many pregnancy complications. For instance, low serum PAPP-A is associated with

fetal growth restriction, fetal loss, intrauterine fetal demise, pre-eclampsia, gestational diabetes, and T21 trisomy [1]-[2]. In addition, the low serum PAPP-A is also associated with short stature in offspring and postpartum de-novo maternal diabetes mellitus [4]. Otherwise, the high serum PAPP-A level is related to a high risk of placenta accrete [5], [6]. A small quantity of PAPPA can be found in other tissues such as the breast, kidneys, bone marrow, and colon, abnormal elevated PAPP-A serum in non-pregnancy is a marker for relapse and prognostic clinical outcomes in malignant cancer such as breast cancer, cardiac disease, and end-stage renal disease [2], [7]-[9].

Quantification PAPP-A is mainly based on the immunoassay. In the assay on 96-well plates, the primary antibody is permanently immobilized by adsorption onto the well surfaces [10]. The step is time-consuming and needs to be prepared before the quantification reaction. This study aims to develop and validate the novel method for primary antibody immobilization by magnetic to be used for immunoassay reaction quantification of the PAPP-A in serum samples. In brief, the primary antibody is labelled by biotin (Ab-biotin), and the secondary antibody, which has a different epitope compared with the primary antibody is labelled with the fluorophore to obtain a fluorescent signal. The primary antibodies, PAPP-As and secondary antibodies freely interact within the solution. To wash non-specific binding, the immunocomplex is transiently immobilized through the interaction of Ab-biotin and magnetic nanoparticles or beads labelled streptavidin (MNP-S) in the presence of a magnet. Finally, the fluorescence intensity is obtained to quantify the amount of PAPP-A in the samples (Figure 1).

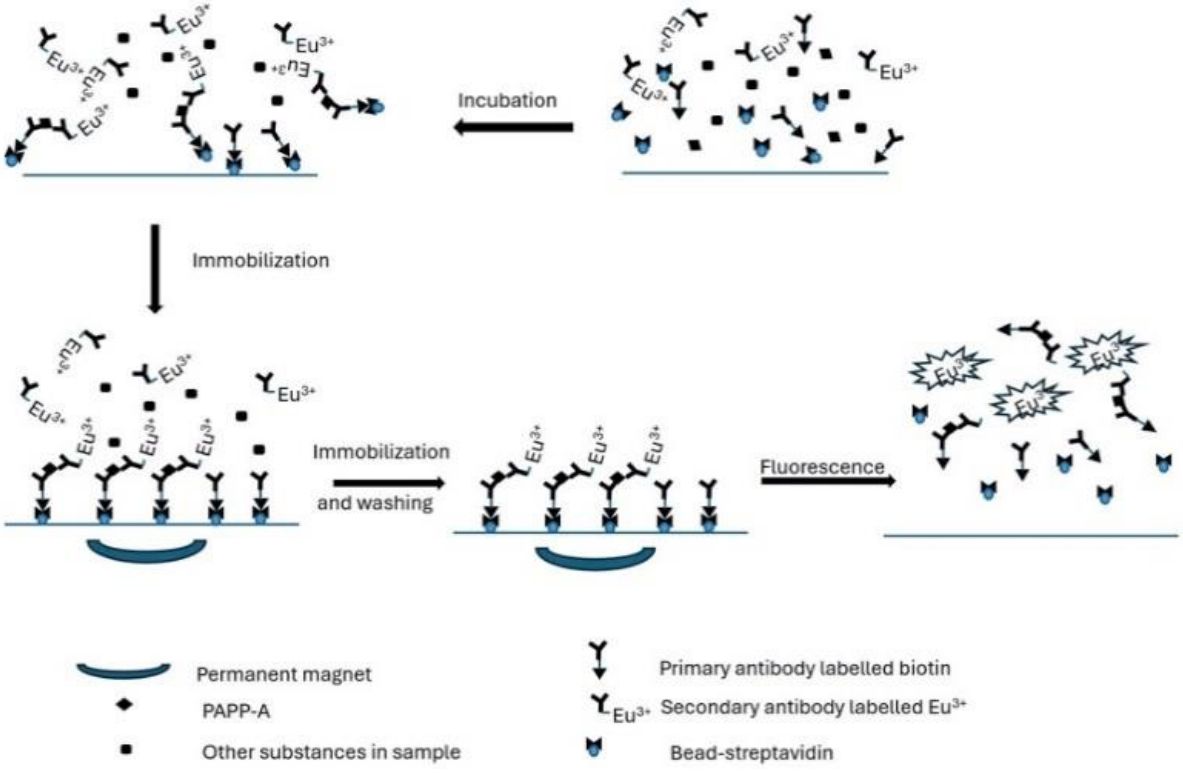

Figure 1. The schematic of magnetic immobilization immunoassay reaction

2. Content

2.1. Experiments

**** Materials and equipment***

Bead or magnetic nanoparticles-streptavidin (MNP-S) (0.72 mg/mL, Roche, USA), primary antibody labelled biotin (Ab-biotin, 30 µg/mL, Perkin Elmer, Finland), secondary antibody labelled europium (Ab-DTTA-Eu^{3+}, Perkin Elmer, Finland), 1-(2-naphthoyl)-3,3,3-trifluoroacetylacetone (NTAA) (Perkin Elmer, Finland), PAPP-A standard solutions (0, 9.9, 39.7, 205, 820, 2090 mU/L) (Perkin Elmer, Finland), were within their expiration date from … The standard 0 was a pseudo serum sample or blank sample. The washing solution (0.9 % NaCl, 0.005% NaN_3, 0.05% tween 20, 0.050M Tris-HCl, pH 7.8) was freshly prepared before using. The first trimester pregnancy serum samples were provided by Chemedic Vietnam JSC. with the permission of patients and were used only for scientific purposes.

The equipment used included a permanent magnet (6x4x2 cm)**,** a thermal incubator (Perkin Elmer, Finland), a plate shaker incubator (Perkin Elmer, Finland), 96-well polystyrene plates (Corning, USA), a fluorescence reader Victor-2D (Perkin Elmer, Finland).

**** Development and validation method***

- Development method:

Magnetic immobilization primary antibody. The procedure was investigated by two methods.

In the one-step assay method, MNP-S, Ab-biotin, PAPP-A, and Ab-DTTA-Eu^{3+} were simultaneously incubated in 96-well plates. In step 1, the reaction mixture, consisting of 20 µL of MNP-S, 2 µL of Ab-biotin, 10 µL of PAPP-A standard or sample, and 10 µL of Ab-DTTA-Eu^{3+}, was vortexed for 15 seconds and then incubated in a shaking thermos reactor at 800 rpm. The concentrations of MNP-S and Ab-biotin, along with the incubation time and temperature, were optimized for this reaction. In Step 2, the reaction product was immobilized onto a permanent magnet for 3 minutes and washed three times with 200 µL of washing solution in total. In step 3, NTAA solution was added to the reaction well, and the fluorescence signal was measured within 5 minutes.

In the stepwise method, MNP-S and Ab-biotin were first incubated together (Step 1a). This was followed by immobilization using a permanent magnet, with subsequent washing performed three times (Step 1b). Next, PAPP-A and Ab-DTTA-Eu^3+ were added to the reaction, and the mixture was continuously incubated (Step 1c). Steps 2 and 3 were performed as described in the one-step method.

The reactions were performed with a blank sample and spiked samples at two PAPP-A levels (250 and 2090 mU/L). The scheme of reaction is described in Figure 1.

The effect of time to reaction: The time of reaction was investigated in 20, 30, 40, 60, and 120 minutes, other conditions are fixed.

The effect of temperature on reaction: The temperature incubation was investigated at 20, 25, 30, and 35ºC, other conditions were fixed.

The effect of MNP-S concentration on reaction: The concentration of MNP-S was investigated at 5, 10, 15, 20, and 25 µL, other conditions were fixed.

The effect of Ab-biotin concentration on reaction: The concentration of ab-biotin was investigated at 2, 4, 6, 8, and 10 µL, other conditions were fixed.

All reactions were performed at the 2090 mU/L PAPP-A concentration.

- *Validation method:*

+ *The repeatability:* The reaction with 2090 mU/L PAPP-A concentration was repeated five times to assess repeatability.

+ *The specificity*: The blank samples spiked with hCG, and AFP (9930 U/L and 1010 U/L, respectively) were measured to determine the reaction cross-activity.

+ *The calibration curve instruction*: Six standard samples (0 – 2090 mU/L) were used to construct the calibration curve.

+ *Low detection (LOD) and low quantification (LOQ) estimation*: The LOD and LOQ were estimated as the average intensity fluorescence signal plus 3 standard deviations of 10 blanks and the average intensity fluorescence signal plus 10 standard deviations of blanks, respectively.

Comparison with reference method: Twelve pregnancy serum samples were measured using the new method and the reference method (PAPP-A DELFIA assay, Perkin Elmer, Finland). The results obtained from the developed method were compared with those from the reference method by using the Passing & Bablok regression ($y = a$ (95% CI)$x + b$(95% CI)) to determine the correlation and linear regression. The Bland-Altman plot describes the agreement between the two methods [11].

2.2. Results and discussion

**** Development method***

- *Magnetic immobilization primary antibody*:

With the blank sample, at the 0 mU/L concentration of PAPP-A, the fluorescence signal of the one-step method is slightly lower than the step-wise method. However, at 205 and 2090 mU/L concentrations, the one-step method is higher than the step-wise method (Figure 2). The immobilization efficiency is a critical factor in the reaction, as it directly influences the ability to capture PAPP-A in the sample. The results demonstrated that the one-step method exhibited lower background noise and higher fluorescence signals at both low and high concentrations. Furthermore, the one-step procedure is simpler and more time-efficient compared to traditional or alternative enzyme-linked immunosorbent assays (ELISA), which typically involve three steps and the use of pre-coated primary antibodies.

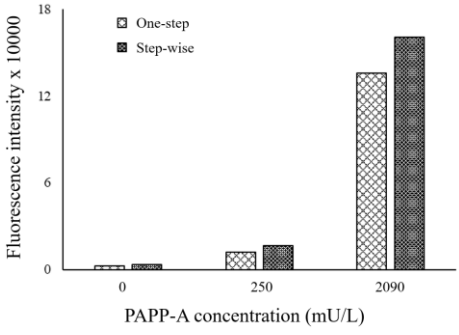

Figure 2. The one-step and step-wise fluorescence signal in different levels of PAPP-A

- *The effect of time on the reaction*:

The incubation time can affect the efficiency of binding immunocomplex. The fluorescence signal slightly increases when incubation time increases from 10 to 20 minutes, however, significantly increases at 30 minutes and continuously slightly increases at 40 minutes. The fluorescence signal remains unchanged and evenly extends the incubation time to 120 minutes (Figure 3). The incubation time of 40 minutes is appropriate for further reaction experiments. Instead of permanently immobilizing primary antibodies on solid surfaces to form immunocomplexes, allowing free reactions between primary antibodies, antigens, and secondary antibodies in solution offers significant time advantages. This approach results in a reaction time considerably shorter than that of traditional or alternative ELISA immunoassays, which typically took 1.5 to 2 hours, or even up to 3 hours. [12].

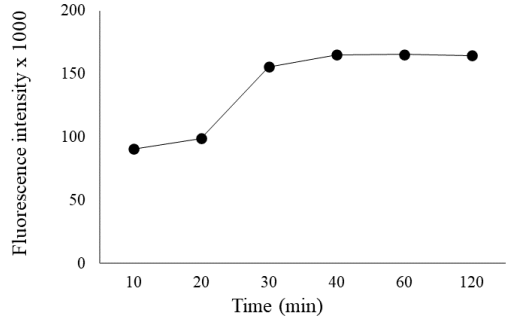

Figure 3. The effect of incubation time on fluorescence signal

- *The effect of temperature on the reaction:*

The increased incubation reaction can accelerate the reaction rate. However, high temperatures may cause instability in the protein and antibodies. The fluorescence signal increases significantly when incubated from 20 ºC to 25 ºC and slightly increases (approximately 7%) at 30 ºC and remains the intensity signal until 35 ºC (Figure 4). The optimal temperature can be archived at 30 ºC. This range temperature is also widely used on the immunoassay such as traditional or alternative ELISA immunoassay, electrochemical luminescence, or chemiluminescent microparticle immunoassay [13].

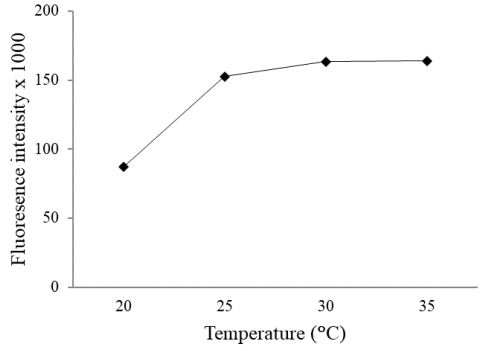

Figure 4. The effect of temperature on the fluorescence signal

- *The effect of MNP-S concentration on reaction*:

The concentration of beads, when directly immobilized in the magnetic field, can significantly affect the fluorescence intensity. The signal increases with the concentration

of MNP-S, ranging from 5 to 20 µL (0.78 mg/mL) or 3.6 to 14.4 µg of beads. However, when the concentration of MNP-S is increased to 25 µL or 18.0 µg of beads, the fluorescence intensity remains unchanged. (Figure 5). The 20 µL (0.78 mg/mL) bead is suitable to get the optimal intensity signal and save the reagent.

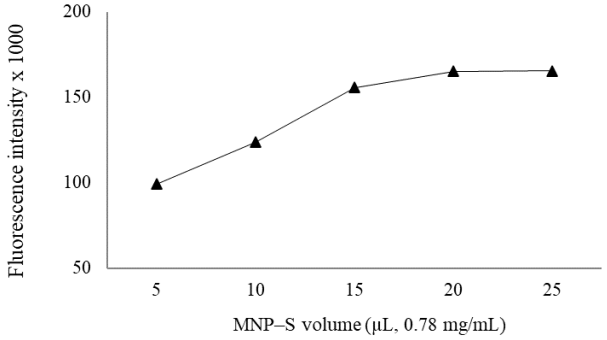

Figure 5. The effect of MNP-S concentration on the fluorescence signal

- *The effect of Ab-biotin concentration on reaction*:

The fluorescence intensity shows minimal variation (< 2%) when the primary antibody concentration is changed from 2 µL to 10 µL (60 ng to 300 ng) (Table 1). Therefore, the optimal concentration of Ab-biotin for the reaction is 2 µL (30 µg/mL).

Table 1. The effect of Ab-biotin concentrations on the fluorescence signal

Ab-biotin (µL, 30 µg/mL)	Fluorescence intensity	% Deviation (compare to minimum intensity signal)
2	164894	0.890
4	163437	0
6	164254	0.005
8	165620	1.340
10	166682	1.990

**** Validation method***

- *The repeatability*:

The repeatability was evaluated at the PAPP-A level of 2090 mU/L, which is in the range of PAPP-A in the first trimester pregnancy serum samples. The standard deviation (SD) and coefficients of variation (CV) of the fluorescence intensity signal are 1233.82 and 0.75%, respectively (Table 2). A low CV (< 10%) is typically considered satisfactory and indicates a high precision of the reaction [12].

Table 2. The repeatability of the novel method

Fluorescence intensity (5 replication)	164098/163667/164874/166309/166849
Average	165159
SD	1233.82
CV (%)	0.75

- *The specificity*:

The blank sample (free PAPP-A) and the high-concentration spike samples of hCG and AFP did not show significant differentiation (Figure 6). The results showed that the reaction is a non-specific or non-cross activity with other pregnancies.

- *The calibration curve instruction*:

The calibration curve was optimized by the least-square method to fit the y = ax + b curve. The linear range shows up to 2090 mU/L. The statistical values are a = 76.61, s_a = 1.93, b = 4325.5, s_b = 1948, s_y = 3391.5, R^2 = 0.9980 (Figure 7).

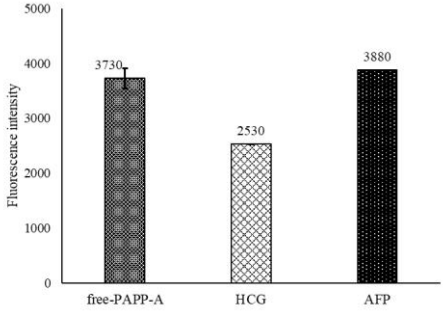

Figure 6. The specificity of the novel reaction

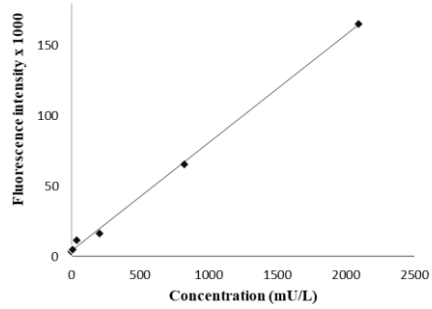

Figure 7. The calibration curve

- *The LOD and LOQ estimation:*

The LOD and LOQ of the methods were determined by measuring the blank samples to be 7.2 and 24.0 mU/L. The LOD and LOQ defined using the standard deviation of the calibration curve were 5.6 and 18.6 mU/L. These values from the two methods are in agreement. This LOQ demonstrates sufficient sensitivity to detect extremely low levels of PAPP-A, below 0.4 times the median, approximately 330 mU/L at the 10th week of gestation. Additionally, the PAPP-A threshold value can be used to assess prognostic outcomes in myocardial infarction [14]-[15].

- *Comparison with reference method*:

The PAPP-A concentrations in 12 first trimester serum samples were determined by the magnetic method and the PAPP-A DELFIA assay. The results are shown in Table 3.

Table 3. The PAPP-A concentrations determined by the two methods

No.	ID sample	PAPP-A concentration mU/L (The magnetic method)	PAPP-A concentration mU/L (The PAPP-A DELFIA assay)
1	148022	923,912	984,534
2	148912	1319,359	1256,082
3	145230	1845,8472	1822,535
4	237925	3234,124	3306,537
5	149469	656,132	608,876
6	149467	1004,766	1044,436
7	149468	3194,246	2917,348
8	147470	6665,4116	7147,315
9	149471	2247,445	2365,936
10	149473	5226,006	5536,49
11	149474	8359,069	7979,19
12	149476	1446,869	1460,154

The Passing Bablok regression equation is, y = *a* (95% CI)*x* + *b*(95% CI), R^2 = 0.999, p < 0.001 (Figure 8). The results showed a significant linear correlation between the new method and the reference method. Moreover, the coefficient *a* includes 1 (*a* = 1.00 (0.971 to 1.019) and *b* includes 0 (*b* = 22.07 (-73.104 to 117.244). Therefore, there is high agreement between the two methods. In addition, the Bland-Altman plot showed no significant difference between the two results, with all values falling within the mean ± 1.96 SD (Figure 9).

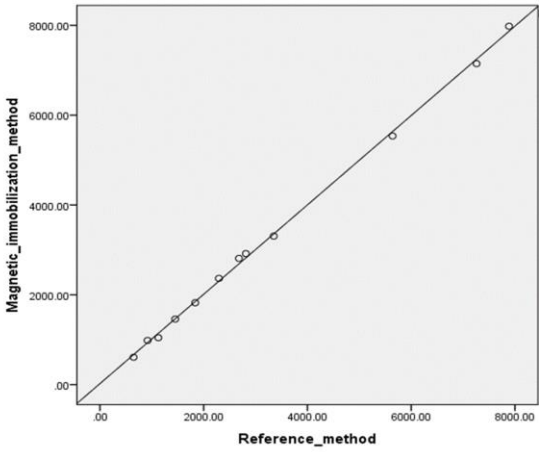

Figure 8. The Passing & Bablok regression

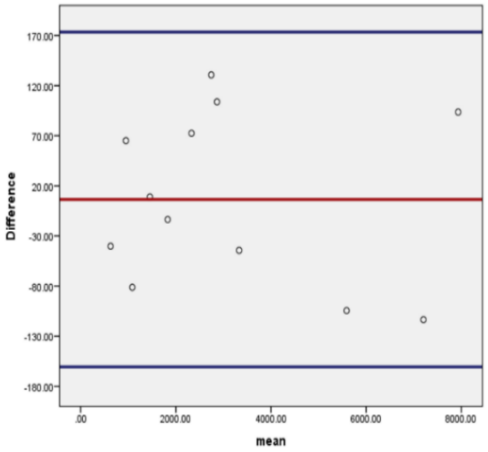

Figure 9. The Bland-Altman plot

3. Conclusions

A new method utilizing magnetically immobilized primary antibodies on 96-well polystyrene plates was developed for the quantification of PAPP-A in serum samples. The optimal reaction conditions were established, including one-step immobilization, incubation time and temperature, and reagent concentrations. The method was validated through repeatability, sensitivity, calibration curve, LOD/LOQ, and comparison with a reference method. This study presents a novel approach for determining PAPP-A in serum samples under mild conditions, with a simple, time-saving procedure, high sensitivity, selectivity, and results comparable to those obtained using commercial methods.

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