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Fetal and Neonatal Alloimmune Thrombocytopenia: evidence based screening

Winkelhorst, D.

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Chapter 7

Fast and low-cost direct ELISA for high-throughput serological HPA-1a typing

Dian Winkelhorst

Leendert Porcelijn

Eva Muizelaar

Gonda Oldert

Elly Huiskes

C. Ellen van der Schoot

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Abstract

Background. Fetal and neonatal alloimmune thrombocytopenia (FNAIT) is caused by maternal alloantibodies against fetal human platelet antigens (HPAs), mostly caused byanti-HPA-1a. Population-based screening for FNAIT is still a topic of debate. Logistically and financially, the major challenge for implementation is the typing of pregnant women to recognize the 2% HPA-1a-negative women. Therefore, there is need for a high-throughput and low-cost HPA-1a-typing assay.

Study design and methods. A sandwich ELISA was developed, using a monoclonal anti-GPIIIa as coating antibody and horseradish-peroxidase-conjugated recombinant anti-HPA-1a, as detecting antibody. The ELISA results were compared to an allelic discrimination PCR-assay. In phase I, samples from unselected consecutive pregnant women were tested with both assays. Phase II was part of a prospective screening study in pregnancy and genotyping was restricted to samples with an, arbitrary set, OD < 0.500.

Results. The ELISA was optimized to require no additional handling(swirling or spinning) of stored tubes. During phase I, 506 samples were tested. In phase II, another 62,171 consecutive samples were phenotyped, with supportive genotyping in 1,902. In total 1,585 HPA-1a negative and 823 HPA-1a positive women were genotyped. The assay reached 100% sensitivity with a cut-off OD from 0.160, corresponding with a 99.9% specificity and a false-HPA-1a negative rate of 0.03.

Conclusion. A high throughput, low-cost and reliable HPA-1a phenotyping assay was developed which can be used in population-based screening to select samples for testing of presence of anti-HPA-1a. Because plasma from tubes of 3 – 6 day-old samples can be used, this assay is applicable to settings with suboptimal conditions.

Introduction

Fetal and neonatal alloimmune thrombocytopenia (FNAIT) can occur after maternal alloimmunization against foreign, paternally-derived human platelet antigens (HPAs) on fetal cells. Clinical consequences of FNAIT range from an asymptomatic thrombocytopenia to a severe internal organ hemorrhage, such as intracranial hemorrhages (ICHs) or even perinatal death. Antenatal treatment with weekly intravenous immunoglobulin (IVIg) infusions can effectively prevent these bleeding complications. However, in current practice without population-based screening, it is solely applicable in pregnancies with known alloimmunization, recognized in previous pregnancies.[1] Generally, FNAIT is considered the platelet counterpart for hemolytic disease of the fetus and newborn (HDFN) caused by red blood cell (RBC) alloimmunization. Whereas anti-RhD is the predominant cause of (severe) HDFN, alloimmunization against HPA-1a is the major cause of bleeding complications in FNAIT, accountable for approximately 80% of cases.[2,3] In the Caucasian pregnant population, 2.1% is HPA-1a negative, of which 10% will produce alloantibodies against HPA-1a (1 in 500 pregnancies), leading to a severe thrombocytopenia in approximately 1 in 1500 pregnancies.[4] The introduction of a screening and prevention program for HDFN caused by anti-RhD, has further decreased morbidity and fatal complications of anti-RhD-mediated HDFN hardly ever occur anymore in the Western world.[5,6] Its pathophysiologic similarities to HDFN, together with the availability of an effective and non-invasive preventive treatment, have stirred up the debate on implementing population-based screening for FNAIT caused by HPA-1a as well.[7] An important advantage is that only a small proportion of the pregnant population needs to be screened for alloimmunization, that is the approximately 2% HPA-1a negative women. In terms of cost-effectiveness and practical implications, the major component will be HPA-1 typing. Because currently used assays, applied in diagnostic settings, are complex and time consuming, there is need for a reliable, quick, simple and low-cost assay for high-throughput HPA-1 typing. We describe an enzyme-linked immune sorbent assay (ELISA) for HPA-1a phenotyping, modified from the assay described by Garner and colleagues.[8] The assay was extensively validated with supportive genotyping, allelic discrimination polymerase chain reaction (PCR), in exclusively pregnant women and in a high number of HPA-1a negative samples. Furthermore, the material used was plasma from three to six days-old stored tubes without handling (swirling or spinning), instead of whole blood, isolated platelets or platelet rich plasma (PRP) in order to eliminate a time-consuming and labor-intensive step and make the assay applicable to a setting for multiple testing from one tube of blood with material of suboptimal quality.

Materials and Methods

Patient samples

As part of the Dutch antenatal Screening Program for Infectious diseases and Erythrocyte immunization (PSIE), repeated red cell antibody screening and fetal *RHD* typing is offered free-of-charge at 27 weeks' gestation to all RhD and/or Rhc negative pregnant women. For this screening program, nine milliliter ethylenediamine tetra-acetic acid (EDTA) anticoagulated blood is drawn at certified, local laboratories all over the Netherlands (*n* = ± 90) and transported to our laboratory by regular surface mail or Sanquin private courier service. Upon receipt at Sanquin, blood tubes receive a numerical code with barcode, that enables automated pipetting, testing and connecting the results to clinical laboratory information system (CLIS). After regular testing for prenatal screening program, 1 – 4 days after blood drawing (depending on the day of the week and time of drawing), the tubes were stored, upright in racks without cap, for two more days at 4°C, awaiting authorization of fetal *RHD* and red cell antibody screening. After this, the samples could be used anonymously for HPA-1a testing. To ensure the applicability for high-throughput testing in a potential screening setting, the tubes were loaded into the Hamilton STARlet workstation (Hamilton Robotics, Bonaduz, Switzerland) and 20 µL of the uppermost plasma of these 3 – 6 days-old, stored tubes was automatically pipetted into a plate, without first swirling or spinning them. Platelet counts of this plasma were determined using 20 µL of the same uppermost material from the tubes that would be used in the ELISA with a Coulter T-890 counter (Coulter Electronics Ltd, Luton, United Kingdom). All subjects gave passive consent for the study. No directly linked personal or medical information was used. The study was performed in accordance with the World Medical Association Declaration of Helsinki. Confidentiality was appropriately protected according to the Dutch Medical Treatment Agreement Act, expanded in the Codes for Good Behavior and Good Use.

The collection of samples was split into two phases. During the first phase, we included 506 consecutive samples of RhD and Rhc negative pregnant women. The second phase took place as part of a current prospective observational screening study (HIP-study, HPA-screening In Pregnancy), that is aimed at gathering missing knowledge on incidence, natural history and risk assessment of FNAIT. During this second phase, 62,171 consecutive samples of the same cohort of RhD and Rhc negative pregnant women were tested.

Antibodies

A recombinant, human monoclonal antibody against HPA-1a (B2G1) was used (kindly provided by Dr. W. Ouwehand and Dr. C. Ghevaert, University of Cambridge, NHS, Blood and Transplant, Cambridge, UK)[8,9] in a concentration between 1.1 and 1.8 µg/mL in PBS with 0.2% (v/v) BSA. B2G1 was conjugated with horseradish peroxidase (HRP), using Lightning-Link HRP Conjugation

Kit (Innova Biosciences, Cambridge, United Kingdom), according manufacturers' instructions. Murine monoclonal antibody CLBthromb/1 (C17, IgG1) was used as antibody directed against glycoprotein IIIa or CD61 (Sanquin Reagents).[10]

Enzyme-linked immunosorbent assay (ELISA) based HPA-1a typing

Flat-bottom Maxisorp 96-well plates (Nunc) were coated with C17 at a concentration of 3 µg/mL in 0.1M sodium carbonate buffer (Merck) and then incubated for a minimum of 12 hours to a maximum of five days at 4°C. Before use, the plates were washed with wash buffer (phosphate-buffered saline (PBS), 0.05% Tween-20) and blocked for one hour with NaCl 0.2% bovine serum albumin (BSA), to prevent non-specific binding. After proving the ability of using supernatant, from tubes stored in racks at 4°C for 3 – 6 days, the assay was optimized for optimal concentrations for type and dilution of monoclonal antibody used for coating of the plate (C17, 3 µg/mL), amount of plasma (20 µL), HRP-incubation time (45 minutes), washing method (3 washing steps with phosphate-buffered saline (PBS), 0.05% Tween-20), substrate incubation time (30 minutes) and optimally HRP-conjugated (1:2 ratio) B2G1 (supplemental table S7.1). A 6-step protocol for the HPA-1a ELISA was applied: 20 µL of plasma sample was automatically pipetted using a Hamilton STARlet workstation (Hamilton Robotics, Bonaduz, Switzerland) into each well. Subsequently, 20 µL HRP-conjugated B2G1 was added to each well and plates were centrifuged for 5 minutes at 550 g and incubated for a total of 45 minutes, including spinning time, in the dark at room temperature. Thereafter, plates were washed three times with the washing buffer and 50 µL of HRP-substrate solution (TMB/DMSO 10 mg/mL) was added and incubated for 15 minutes in the dark at room temperature. The reaction was stopped with 50 µL of sulfuric acid (H_2SO_4) and quantified using an ELISA reader (Biochrom Anthos, Cambridge, United Kingdom). An overview of this assay is depicted in supplemental figure S7.1. Single samples of HPA-1bb and HPA-1aa platelets isolated from healthy donors, stored at 4°C, were diluted to a concentration of 7.0×10^7/mL and used as positive and negative control in each plate.

HPA-1 Genotyping using allelic discrimination polymerase chain reaction (PCR)

A Xiril robotic workstation (Xiril) was used for automatic pipetting of the samples into the plate. Automated DNA extraction was performed with M-PVA magnetic beads (PerkinElmer Chemagen) from 200 µL EDTA anticoagulated blood. After normalizing DNA level to 5 ng/µL, an allelic discrimination PCR assay using FAM-labeled HPA-1a and VIC-labeled HPA-1b MGB-probe. Primer and probe sequences were as follow: forward primer, 5' – CTG ATT GCT GGA CTT CTC TTT GG – 3'; reverse primer, 5' – AGC AGA TTC TCC TTC AGG TCA CA – 3'; HPA-1a MGB probe, 5' – CTG CCT CTG GGC TC – 3' (5' FAM labelled); HPA-1b probe, 5' – CTG CCT CCG GGC TC – 3' (5' VIC labelled). We used Xiril robotic workstation for pipetting of 25 µL, using 12.5 µL Sensifast High ROX mastermix (Bioline), 5 µL extracted DNA, and primers and probes at final concentrations of 10 and 5 pmol/µL, respectively Cycling conditions were 30 seconds at 63°C and 5 seconds at

95°C, followed by 45 cycles of denaturation for 10 seconds at 95°C and annealing and elongation for 20 seconds at 63°C, completed by 30 seconds of 63°C using StepOne-Plus Real-Time PCR System (Applied Biosystems). Interpretation of results was performed with StepOne™ software (version 2.3).

Statistical analyses

Validation and standardization were performed by testing the intra-assay and the inter-assay variation and calculation of coefficients of variation (CV). Inter-assay was assessed twice, by ten-fold repeated measurement of 30 samples in the same assay by the same technician under the same conditions. Intra-assay variation was calculated in three ways. First, by measuring 30 samples on the same day by three different technicians (operator-to-operator variation). Second, by measuring the same 30 samples on three different days by the same technician (day-to-day-variation), this was performed twice. Third, by measuring the same 30 samples by both the pipetting robot and technicians in different rooms. Mean CVs were calculated based on variation in ELISA OD values. Diagnostic accuracy was evaluated using contingency tables, calculating sensitivity, specificity, predictive values, and cases falsely detected as HPA-1a positive or negative. The diagnostic value for different cut-off values was assessed by plotting sensitivity vs. specificity, and analyze the area under these curves (AUC). Statistical analyses were performed using SPSS (version 23.1) and GraphPad Prism (version 8.0) was used for producing plots and graphs.

Results

ELISA optimization

The CV of the intra-assay variation was between 2.9% and 4.7%. The mean CV in the inter-assay variation test, 30 samples tested by three different technicians on three (sequential) days, was 5.2% (range: 1.4% – 10.2%). The HPA-1a positive control platelets had a mean OD value of 1.720 (range 1.577 – 1.849) and were clearly discriminated from HPA-1a negative control platelets, which had OD values ranging from 0.021 to 0.042 with a mean OD of 0.031.

Effect of platelet counts

Platelet counts of 162 samples were determined. The mean platelet count in the supernatant, that was pipetted from the stored tubes, was 7.1×10^7/mL (range 1.5 – 34.8 10^7/mL; IQR 43 – 140 x 10^7/mL). Of 70 samples (HPA-1a positive, $n = 59$; HPA-1a negative, $n = 11$) the platelet counts are plotted against the corresponding ELISA OD (Figure 7.1). An ELISA OD below 1.000 in samples with HPA-1a positive platelets only occurred in samples with a platelet count below 8.0×10^7/mL. Regardless of platelet count (range 5.5×10^7/mL – 36.2×10^7/mL), all samples with HPA-1a negative platelets, all genotyped as HPA-1bb, tested in this series had an ELISA OD below 0.110 (range 0.021 – 0.104).

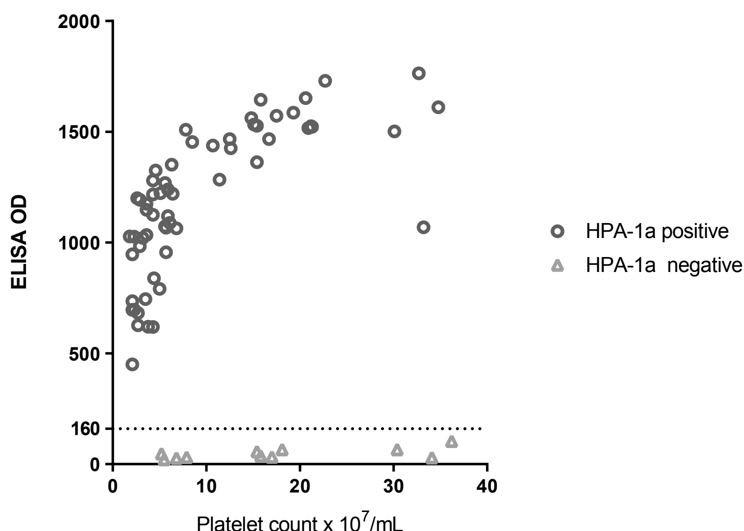

Figure 7.1 – Effect of platelet count on ELISA OD

ELISA, enzyme-linked immunosorbent assay; HPA, human platelet antigen; OD, optic density.

Samples and study population

Phase I. 506 consecutive samples of pregnant women were phenotyped by HPA-1a ELISA and genotyped by an HPA-1 allelic discrimination assay (Figure 7.2A). Of these, 13 women were genotyped as HPA-1bb (2.6%) and all had an OD < 0.100 (range: 0.028 – 0.058). All but two HPA-1a positive samples had an OD > 0.300. These two were both genotyped as HPA-1ab and had OD values of 0.072 and 0.149.

Phase II. During the second phase, 62,171 consecutive samples of pregnant women were tested (Figure 7.2B). To minimize the risk of missing HPA-1a negative pregnant women, the cut-off value for supportive genotyping of the HPA-1a ELISA was first set at 0.500 and later lowered to 0.300. A total of 1,902 samples underwent genotyping, 77 with OD < 0.500 but above 0.300 and 1,825 with OD < 0.300. The median OD of the 1,572 HPA-1bb samples was 0.035 (range 0.014 – 0.160). A total of 156 women (*n* = 32 < 0.500 and *n* = 124 < 0.300, respectively) were HPA-1ab and 174 (*n* = 45 and *n* = 129, respectively) were HPA-1aa. The mean OD positive controls was 1.591 (SD 0.084) and the mean OD of negative controls was 0.062 (SD 0.005).

Diagnostic test evaluation

Predictive values as well as diagnostic accuracy of the HPA-1a ELISA can only be optimally deduced from a tested population that reflects the prevalence of the tested condition in the total population, which is phase I of this study. With cut-off values of 0.075 – 0.200, sensitivity

remains 100% and diagnostic accuracy is 99.6 – 99.8% (Table 7.1, Figure 7.3A). HPA-1a positive predictive value is 100%, with HPA-1a negative predictive values varying from 87 – 93%, depending on different cut-offs.

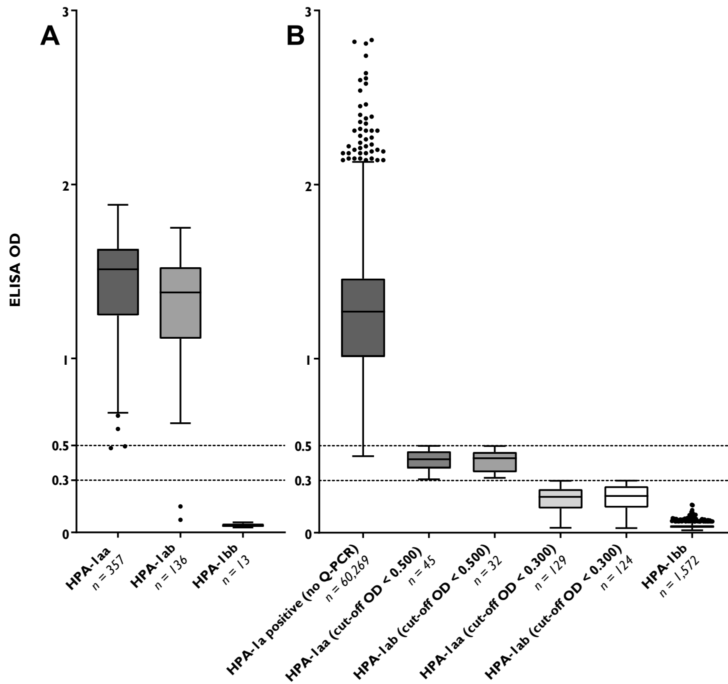

Figure 7.2 – Distribution of ELISA OD for different genotypes

A. Phase I, 506 consecutive samples. OD < 0.300 in 15 (HPA-1bb, *n* = 13; HPA-1ab *n* = 2; HPA-1aa, *n* = 0), OD > 0.300 in 491 (HPA-1bb, *n* = 0; HPA-1ab, n = 135; HPA-1aa, n = 357). **B.** Phase II, 62,171 consecutive samples. PCR in 1,902, because of OD from 0.300 - 0.500 (*n* = 77) or OD < 0.300 (*n* = 1,825).

Boxes represent 25th and 75th percentiles; lines in boxes represent median values; horizontal lines outside boxes represent 1.5 times IQR; dots represent outliers.ELISA, enzyme-linked immunosorbent assay; HPA, human platelet antigen; IQR, inter-quartile range; OD, optic density; PCR, polymerase chain reaction.

Extrapolation the diagnostic test evaluation towards phase II would imply considering that samples that did not undergo PCR (OD values: median 1.231, range 0.440 – 2.830) would have been genotyped as HPA-1a positive. Based on all genotyping data, phase I and II combined, threshold values above 0.160 would mean identifying all HPA-1a negative cases and resembles a sensitivity of 100%. This would correspond with a specificity of 99.9% (Figure 7.3B).

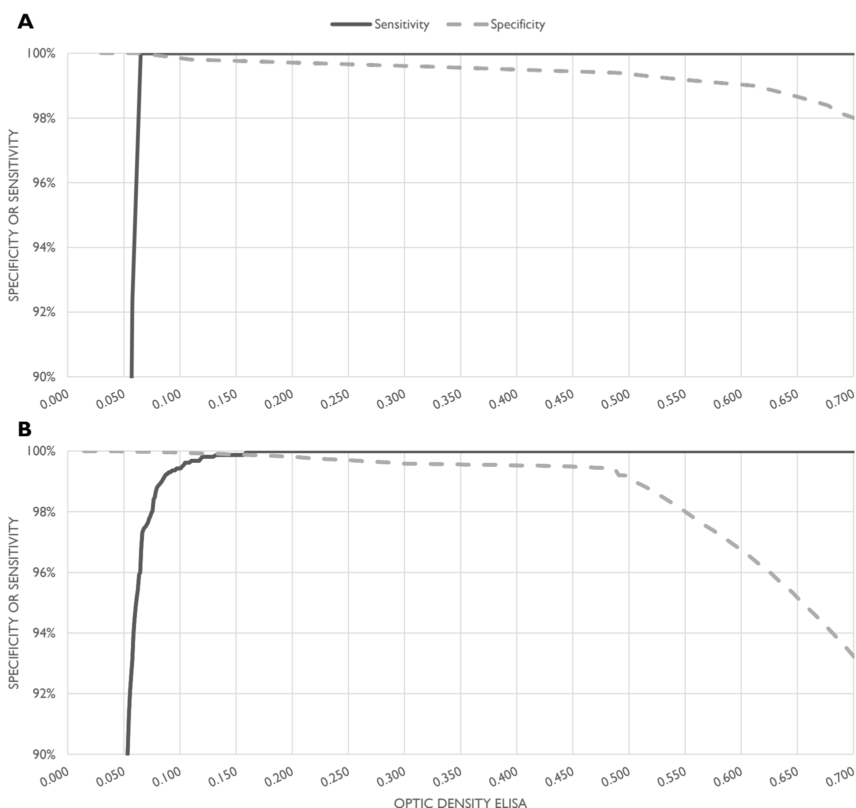

Figure 7.3 – Diagnostic evaluation of HPA-1a ELISA

A. Phase I, 506 consecutive samples. **B.** Phase I+II, 62,677 samples. During phase II all samples with an OD > 0.500 were marked as HPA-1a positive without genotyping.

ELISA, enzyme-linked immunosorbent assay; HPA, human platelet antigen; OD, optic density.

Costs

A potential screening setting for all pregnant women in the Netherlands would mean a total of 750 samples a day. By using in-house available MoAbs, equipment, reagents and disposables, costs are kept at a minimum. The total material costs per sample in a potential future screening setting would be €0,25 per sample, of these the biggest contributor is the amortization cost of a pipetting robot (€0.22 per sample). Another great part of costs for serological HPA-1a typing are labor costs. The hands-on time for this quick ELISA, testing three 96-well plates, is three hours for a single junior technician. Lastly, costs for follow-up testing, for example genotyping and antibody screening, need to be taken into account as well. These would mainly depend on the setting and design of the program, as well as on cut-off values used after validation with specific material sent in for testing in such programs.

Table 7.1 – Diagnostic evaluation of HPA-1a ELISA – Phase I (*n* = 506)

Cut-off OD value	Accuracy	Sensitivity	Specificity	AUC	HPA-1a negative predictive value	HPA-1a positive predictive value
<0.075	99.8%	100.0%	99.8%	99.9%	0.93	1.0
<0.100	99.8%	100.0%	99.8%	99.9%	0.93	1.0
<0.150	99.6%	100.0%	99.6%	99.8%	0.87	1.0
<0.200	99.6%	100.0%	99.6%	99.8%	0.87	1.0

AUC, area under the curve; ELISA, enzyme-linked immunosorbent assay; HPA, human platelet antigen; OD, optic density.

Discussion

Fetal and neonatal alloimmune thrombocytopenia is one of the most important causes of severe thrombocytopenia in term born neonates and a potentially life-threatening condition. Prevention of this disease by timely detection in potential antenatal routine HPA-screening has been debated for over decades. One of the unsolved complicating quests is the cost-effectiveness of such a screening program. Whereas just 2% of pregnant women is HPA-1a negative and will need follow-up testing, the major proportion of costs will be determined by HPA-1a typing of the complete pregnant population. Therefore, a reliable, quick, simple and low-cost ELISA assay, suitable for high-throughput serological HPA-1a typing was designed.

A couple of prospective HPA-1a screening studies that use an ELISA for HPA-1a typing have been performed thus far.[11-14] However, this study is the first to describe extensive validation with samples solely from pregnant women, to report in detail on (low) costs and to use moderate quality material, making it highly applicable for potential screening settings. Despite using these moderate quality samples the assay reaches a high diagnostic accuracy.

After phase I, setting the threshold to discriminate between HPA-1a positive and negative samples would lead to a cut-off value of 0.061 (mean + 2SD, 0.041 + 2*0.010). With cut-off values above 0.075 a 100% sensitivity is reached, which corresponds with a zero false-negative rate, so that no HPA-1a negative samples would be missed. In phase II the number of HPA-1a negative samples tested increased drastically, up to 1,585 in total. The cut-off value for discrimination between positive and negative would be 0.065 (mean + 2SD, 0.037 + 2*0.014). However, to achieve a 100% sensitivity, threshold for supportive genotyping or phenotyping should be increased to 0.160. Nonetheless, eliminating the possibility of missing HPA-1a negative samples, comes at the cost of falsely identifying some HPA-1a positive women as possible HPA-1a negative and therefore theoretically avoidable additional testing.

In the current prospective study HPA-1a negative cases are genotyped with allelic discrimination, which is a rather expensive step. One might argue that in a potential screening setting it is more cost-efficient to skip this step and either perform direct antibody screening in all women with ELISA results suggesting HPA-1a negativity or test maternal platelets by flow cytometry for the presence of the HPA-1a epitope with a monoclonal antibody before performing testing for the presence of anti-HPA-1a antibodies. For the tested cohort, a threshold of 0.160 resulted in 1,629 samples identified for additional testing, of which 57 would not have required this testing (false-HPA-1a negative rate 0.03). Also, in potential future screening setting, after identifying alloimmunized pregnancies, additional testing might to be performed to identify pregnancies at high risk of bleeding complications. For this, HLA-DRB3*0101 type, anti-HPA-1a antibody titer, Fc-glycosylation pattern or endothelial binding properties might be assessed.[15-20]

The strength of using moderate quality material in our assay is a limiting factor for not achieving an even higher accuracy and specificity as well. Also, the currently used volume is relatively small, 20 µL plasma. The amount of left-over material was carefully managed, because it was not only used in this study for the HPA-1a ELISA, but also for follow-up testing in the prospective HIP-study. Obviously, with increasing volume and quality of material in a potential screening setting, the amount of platelets added to the assay can be doubled or tripled. This will lead to higher OD values of the HPA-1a positive cases, but not the HPA-1a negative samples. Therefore, the line between HPA-1a positivity and negativity will be more discriminative, leading to even less falsely identified HPA-1a negative cases that need further follow-up testing and would undergo the above-mentioned, unnecessary antibody screening or repeated HPA-1a ELISA. Additionally, plasma platelet counts can be lowered by unnoticed thrombocytopenia in pregnant women as well. Although we did not specifically test our assay for this, this would not complicate the performance of the assay. No HPA-1a negative women will be missed, because these samples will still have low ELISA OD values. There is only a chance that samples of HPA-1a positive women have an ELISA OD below threshold and undergo avoidable follow-up testing.

By using a specific recombinant HPA-1a antibody that is directly labeled, there will be no cross-reactivity with specific or non-specific antibodies in maternal plasma.

This highly optimized assay minimizes the costs of serological HPA-1a typing to €0.25 per sample, excluding labor costs. Previously reported costs by Turner and colleagues were £3.01 per sample for ELISA and £16.19 for HPA-1 genotyping, without any further details or specification.[12] Reported costs from the largest prospective screening study, based on typing over 100,000 women, were €1.72 per sample when using flow-cytometry and €21.28 per sample when using HPA-1 genotyping by PCR, again without any further details or specification.[21]

Besides costs, prospective studies have reported on the use of serological HPA-1a typing in a screening setting as well. The first study screened a cohort 24,417 pregnant women, and besides notice of 20 cases that turned out to be HPA-1a positive after PIFT, no information on diagnostic accuracy or costs of the assay were provided.[11] A whole-blood ELISA kit for simultaneous HPA-1a typing and anti-HPA-1a detection, was described in two prospective studies in Ireland (4,090 women) and Scotland (26,206 women).[12,13] Supportive genotyping was performed in only 67 pregnant women, of which 54 turned out to be HPA-1bb. Again, no further information on diagnostic accuracy or performance of the ELISA was provided.[13] The largest prospective study screened 100,448 pregnant women in Norway.[14] Screening was performed by ELISA, flow-cytometry and PCR, with unknown distribution. Their ELISA assay used was previously described by Garner and colleagues, and used whole-blood samples.[8] The performance analysis of the assay was done in samples of 1,947 (random) donors and no pregnant women. Genotyping and PIFT were performed in samples that were considered HPA-1a negative and an equivalent of randomly selected HPA-1a positives. Overall both ELISA and supportive testing (genotyping and PIFT) were performed in 91 samples, of which 44 were HPA-1a negative. The flow-cytometry assay was previously described by Killie and colleagues and used a commercially available FITC-labeled mouse IgG1 anti-CD61 (clone SZ21) specific for HPA-1a and is a very quick assay with only a 10-minute incubation without washing.[22] They tested 45,960 samples and genotyped the 1,121 samples that were typed HPA-1a negative after flow-cytometry. Of these, 1,112 were typed HPA-1bb and 9 HPA-1ab. These samples tested were either pregnant women or blood donors, the exact distribution was not described.

Compared to the previously described assays in screening settings this study adds an extensive validation in a high number of pregnant women instead of healthy blood donors. Furthermore, since we have genotyped the 330 samples with the lowest OD values above the threshold, of which none were found to be HPA-1bb, we were able to demonstrate the excellent sensitivity of the assay. Further, the use of low-moderate quality material that was not spinned or swirled, like in the other studies that used blood drawn only for the purpose of HPA-1a typing, is important and reduces labor costs. When potentially adding a HPA-1a screening to an already existing serological prenatal screening it is highly preferable that the sample can be used from the same tube or same blood drawing moment. Also, without having to twist or spin for example 750 tubes a day (in case of a Dutch national screening program) the assay becomes more fit for high-throughput usage. Lastly, because the validation and confirmation with genotyping was also part of our national screening study, we were able to include a large amount of HPA-1a negative pregnant women.

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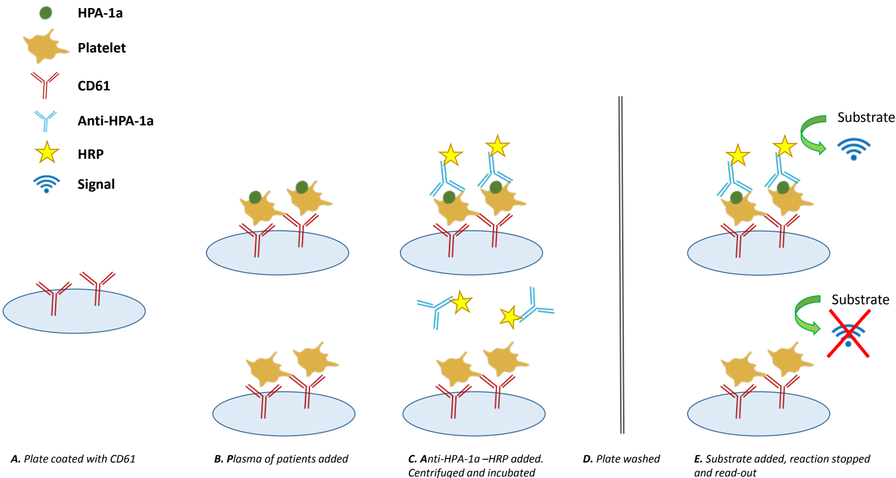
Supplemental material

Supplemental table S7.1 – Optimization of ELISA

Variable	Conditions tested
Coating plate	Y2. RFGP56, **C17** (1ug/mL and **3ug/mL**)
Amount of plasma	10ul, **20ul,** 30ul, 40ul
HRP-incubation time	30 minutes, **45 minutes** and 60 minutes
Washing method	**3** or 5 washing steps
Substrate incubation	10 minutes, 15 minutes and **30 minutes**
Concentration B2G1	1.8mg/ml: 1:500, **1:1000**, 1:5000
HRP conjugation B2G1	1:1, **1:2** and 1:4

ELISA, enzyme-linked immunosorbent assay; HRP, horse-radish peroxidase.



Supplemental figure S7.1 – HPA-1a ELISA