# A novel assay for the detection of anti-human platelet antigen antibodies (HPA-1a) based on peptide aptamer technology

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## ABSTRACT

### Background
Neonatal alloimmune thrombocytopenia is mostly due to the presence of maternal antibodies against the fetal platelet antigen HPA-1a on the platelet integrin GPIIb-IIIa. Accurate detection of anti-HPA-1a antibodies in the mother is, therefore, critical. Current diagnostic assays rely on the availability of pools of human platelets that vary according to donors and blood centers. There is still no satisfactory standardization of these assays.

### Design and Methods
Peptide aptamer was used to detect and identify HPA-1a-specific antibodies in human serum that do not require human platelets. A peptide aptamer library was screened using an anti-HPA-1a human monoclonal antibody as a bait to isolate an aptamer that mimics the human platelet antigen HPA-1a.

### Results
This is the first report in platelet immunology of the use of a peptide aptamer for diagnostic purposes. This assay gives better results than the MAIPA currently in use, detecting around 90% of the expected alloantibodies.

### Conclusions
This assay could help define a standard for the quantitation of anti-HPA antibodies. This report also demonstrates that peptide aptamers can potentially detect a variety of biomarkers in body fluids; this is of particular interest for diagnostic purposes.

Key words: peptide aptamers, neonatal alloimmune thrombocytopenia, platelets, HPA-1a, diagnostic assay, MAIPA.

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## Introduction

Platelets are the cellular components of the blood coagulation system. Among the proteins found at the surface of platelet plasma membrane, GPIIb-IIIa integrin harbors the human platelet antigens HPA-1a/b, the most clinically important platelet antigens. These antigens result from a leukine-proline polymorphism at position 33 of the GPIIb-IIIa integrin. About 2% of Caucasian women are homozygous (HPA-1b/1b) and risk forming antibodies against the integrin of the fetus. Such antibodies may destroy fetal platelets and lead to neonatal/fetal alloimmune thrombocytopenia (NAIT).[1] Anti-platelet alloimmunization has an estimated incidence of 1 in 1,000 pregnancies and may cause *in utero* cerebral bleeds or ventriculomegaly.[2-4] Thus, screening and identification of maternal alloantibodies are critical in early detection of such alloimmunization.[5]

Up to now, all methods for detecting auto- or alloantibodies directed at platelets, such as monoclonal antibody immobilization of platelet antigen assay (MAIPA)[6] or enzyme-linked immunosorbent assay (ELISA),[7] require human platelets. These assays require the pre-collection of typed platelets carrying the HPA systems. Furthermore, these current assays use either fresh platelets or platelets conserved at low temperature. However, during their conservation, platelet glycoproteins may undergo a shedding process so that the platelet preparation might not be suitable to detect some platelet antibodies.[8,9] Although antibodies against HPA-1a antigen may still be detected after several months at low temperature, attempts to keep platelet glycoprotein expression at normal levels during long-term storage remain problematic.[10] Finally, new batches of platelets need to be collected from donors at regular intervals; this may cause results to vary between different laboratories. Thus, in the field of platelet immunology, the availability of new standardized methods to detect human platelet antibodies, including anti-HPA-1a, remains a major issue.

The technical challenge underlying this study was to provide a novel system to detect and/or identify human platelet antigen specific antibodies in human serum.

Peptide aptamers are recombinant proteins which can interact with any given protein target with high specificity. Indeed, the peptide aptamer technology allows specific peptide ligands to be isolated for any given protein or domain, including antibodies. Originally based on the two hybrid screen in yeast,[11] this has been adapted to extracellular targets in *Escherichia coli*.[12] This technology is extremely efficient in dissecting intracellular protein interaction networks at the molecular level.[13,14] It may also represent an alternative strategy to characterize new bioactive molecules of therapeutic interest for cancer and other diseases.[15-19] Peptide aptamers have a particular structure; a short variable peptide domain attached at both ends to a protein scaffold, such as thioredoxin (a scheme inspired from the structure of natural antibodies). This means they could also be valuable diagnostic tools.[20-22]

To isolate a peptide aptamer sharing structural similarities with the GPIIb-IIIa integrin, we reasoned that it should interact with anti-GPIIb-IIIa antibodies. Therefore, since the Camtran human monoclonal antibody was raised against the human platelet antigen-1a (HPA-1a) found on GPIIb-IIIa (Leu at position 33 of the β3 integrin subunit),[23] a peptide aptamer able to specifically bind this particular antibody should mimic the HPA-1a antigen. Such a peptide aptamer should, therefore, be recognized by circulating HPA-1a specific antibodies, and could potentially be used to detect these antibodies *in vitro* without requiring human platelets; a major improvement on existing assays, including MAIPA.

We characterized a peptide aptamer that mimics the HPA-1a antigen present on platelet glycoproteins. We describe how it was produced and discuss its diagnostic and clinical applications.

## Design and Methods

### FliTrx® peptide library and monoclonal antibody

The FliTrx® random peptide library, based on the system described by Lu and colleagues,[12] was obtained from Invitrogen (San Diego, CA, USA). Monoclonal antibody against GPIIb-IIIa protein specific to phenotype HPA-1a (Camtran-B2) was obtained from Cambridge laboratories.[23]

### Growth and induction of the peptide library

Growth of the bacterial cultures and general panning methods were conducted as described in the manufacturer's protocol. pFliTrx®, with the PL promoter from bacteriophage to drive expression, is propagated in *E. coli* (GI826) where the cI repressor gene is under the control of the *trp* promoter. *E. coli* cells harboring the plasmids were grown to saturation overnight at 25°C in IMC medium (1 x M9 salts, 40 mM $Na_2HPO_4$, 20 mM $KH_2PO_4$, 8.5 mM NaCl, 20 mM $NH_4Cl$, 0.2% casamino acids, 0.5% glucose, 1 mM MgCl2) containing 100 μg/mL ampicillin. Expression of the Trx-flagellin fusion proteins containing the peptide inserts was induced by 100 μg/mL tryptophan for 6 h at 25°C. A mixture of 0.1 g of non-fat dry milk, 300 μL of 5M NaCl and 500 μL 20% α-methyl mannoside was then added to 10 mL of the induced *E. coli* culture. The resulting solution was used as a peptide library ready for screening.

### Panning of the peptide library

Tissue culture plates (Nunc, 60 mm) were used for peptide library screening. Plates were coated for 1 h at 20-25°C with 20 μg of antibody diluted in 1 mL sterilized water. After the liquid was removed, the plates were washed with 10 mL sterile water and then supplemented with 10 mL of blocking solution (1% non-fat dried milk, 150 mM NaCl, 1% α-methyl mannoside and 100 μg/mL ampicillin in IMC medium) with gentle agitation for 1 h. Just before the end of the 6 h induction period of the peptide library, the blocking solution was decanted and 10 mL aliquots of the resulting solution were added to the plates. The plates were then gently agitated at 75 rpm on a shaker for 1 min and incubated for 1 h at 20-25°C. The bacterial culture was then decanted and the plates were washed by gentle agitation for 5 min with 10 mL of IMC medium containing 100 μg/mL ampicillin and 1% α-methyl mannoside. After washing an additional four times, bound bacteria were detached with 1 mL IMC by vortexing for 30 s. The remaining detached bacteria were collected from the plate and grown for the next round of biopanning. The same procedure was repeated during the 4 subsequent biopanning rounds. After 5 biopanning rounds, bacterial colonies were randomly picked from the RMG plates (1 x M9 salts, 2% casamino acids, 0.5% glucose, 1 mM $MgCl_2$, 100 μg/mL ampicillin and 1.5% agar) and grown overnight at 30°C.

### Western blotting

Identification of positive clones by Western blotting was performed essentially according to the manufacturer's protocol.

Briefly, forty clones of the RMG plate were transferred into 2 mL RM medium (1 x M9 salts, 2% casamino acids, 1% glycerol, 1 mM $MgCl_2$) containing 100 μg/mL ampicillin, and grown to saturation at 30°C with shaking. A 40 μL sample from the overnight culture was inoculated at 37°C in 2 mL IMC containing 100 μg/mL ampicillin and 100 μg/mL tryptophan until the cell density was $OD_{600}$ 0.75. Then 1.5 mL of the induced cell culture were collected by centrifugation at 10,000 g for 1 min. The pellet was re-suspended in SDS-polyacrylamide gel-loading buffer, boiled for 5 min, and electrophoresed in 8% SDS-polyacrylamide gel. Separated proteins were blotted onto a nitrocellulose membrane (PROTRAN® BA79, Schleicher and Schuell) in a liquid electrophoretic transfer cell (Bio-Rad). Membranes were then blocked with TBS (10 mM Tris pH 7.2 and 0.15 M NaCl) containing 5% dried milk overnight at 4°C, incubated with Camtran-B2 antibody, diluted 1:100 in TBS 1% dry milk, 0.05% Tween 20 for 2 h at 20-25°C. After washing three times with TBS, 0.05% Tween 20, membranes were incubated with horseradish peroxidase (HRP)-conjugated Fc specific goat anti-human IgG (Sigma A0170) at a dilution of 1:92,000 for 40 min at 20-25°C. After a further three washes with TBS 0.05% Tween 20, bound conjugate was detected using Lumi-LightPLUS Western blotting substrate (Roche). Positive samples were subsequently re-analyzed by Western blotting using human serum containing anti-HPA-1b antibodies.

### DNA sequencing

DNA of the identified clones were isolated using the Wizard® Plus SV Minipreps DNA Purification System (Promega). Nucleotide sequences were determined using the FliTrx® Forward sequencing primer (5'-ATTCACCTGACTGACGAC-3').

### Generation of Trx-HPA-1a expression plasmid

A cDNA encoding thioredoxin peptide was amplified by PCR reaction using plasmid pFliTrx® selected previously. PCR procedure was as follows: 1 min at 95°C, followed by 12 cycles at 95°C 45 s, 36°C 30 s, 72°C 45 s, then 20 cycles at 95°C 45 s, 45°C 30 s, 72°C 45 s and 72°C for 3 min to fill in the flush. At the 3'- end, the primer sequence 5'-TGTCGACCAGGTTAGCGTC-3' contained a *SalI* site, and at the 5'- end, the primer sequence 5'-TCATATGATGAGCGATAAAATTA-3' contained a *Nde*I site. The PCR product was subcloned into the pGEM®-T Easy Vector System I (Promega). The resulting plasmid construct was subsequently digested by *NdeI* and *SalI*. The digested DNA fragment was then cloned into the NdeI and SalI sites of the vector pT7-7 (based on the T7 polymerase expression system) encoding a 6 His tag as well as a stop codon downstream of the *SalI* site. This contruct was transformed into *E. coli* strain DH5α, and ampicillin-resistant colonies were isolated. All plasmids were systematically checked by DNA sequencing.

### Trx-HPA-1a expression and purification

The plasmid was transformed into the *E. coli* strain C41(DE3).[24] A freshly transformed colony was inoculated into 400 mL of 2YT medium (16% Bacto Tryptone, 10% Bacto Yeast Extract, 85.5 mM NaCl) containing 20 μg/mL ampicillin and grown at 37°C to an $OD_{600nm}$ of 0.6-0.8 before induction with 0.7 mM isopropyl β-D thiogalactopyranoside. After overnight incubation at 37°C, cells were harvested by centrifugation. The pellet was suspended and incubated for 30 min at 4°C in 10 mL of lysis buffer: 20 mM Tris-HCl pH 8.0, 20% glycerol, 500 mM NaCl, 0.1% Triton X-100, 1 mM PMSF, 5 mM DTT, 1 mg/mL lysozyme, 1/2 tablet Complete Mini EDTA-free (Roche) and 250 units/mL Benzonase (Merck). Fractions (5 mL) were disrupted by sonication for 30 s. After four times centrifugation of supernatant at 9,000 g for 30 min, the supernatant was loaded onto a 2 mL Ni-NTA-agarose column (Qiagen) equilibrated previously in lysis buffer without lysozyme and benzonase at 4°C. The column was washed with lysis buffer without lysozyme and benzonase, and then with 20 mM Tris-HCl pH 8.0, 20% glycerol, 100 mM KCl, 0.5 mM PMSF, 5 mM DTT, and 20 mM imidazole, and Trx-HPA-1a was eluted with the same buffer containing 100 mM imidazole. Fractions containing Trx-HPA-1a, as judged by SDS-PAGE, were pooled.

Buffer exchange and sample concentrations were carried out by centrifugation with Vivaspin Concentrator membranes (Vivascience; cut off 5 kDa) and 20 mM Tris pH 8.0, 10 mM NaCl, 5 mM DTT. Samples were applied onto 1 mL Q-Sepharose columns (Mono Q HR 5/5, Amersham Pharmacia Biotech). The column was washed with 20 mM Tris pH 8.0, 10 mM NaCl, 5 mM DTT and Trx-HPA-1a was eluted with a linear gradient from 10 mM to 1 M NaCl. Trx-HPA-1a rich fractions were pooled, concentrated to 1-15 mg/mL, equilibrated in 4% glycerol, 20 mM Tris pH 8, 100 mM KCl, 5 mM DTT on Vivaspin Concentrators and stored at -20°C until used. In these storage conditions, the final Trx-HPA-1a product was stable for at least six months. Protein concentrations were determined using Bradford reagent.

### Analysis of purified Trx-HPA-1a

A protein aliquot was equilibrated in sterile water. Mass spectra were acquired using an electrospray API 165 (Applied Biosystems) instrument. The protein was identified by peptide mass fingerprinting and MALDI-TOF (Voyager-DE™ PRO, Applied Biosystems).

### Human sera

The sera used in this study are from voluntary blood donors. The donations were collected according to French blood donation regulations and the laws in force, and in accordance with the Public Health Code (art. L1221-1).

### Purification of total IgG from human sera

Human serum equilibrated with 10% 1 M Tris-HCl pH 8 was loaded onto a 250 μL protein A-sepharose bead column (P3391, Sigma-Aldrich) equilibrated previously in 100 mM Tris-HCl pH 8. The column was washed with 10 volumes of 100 mM Tris-HCl pH 8 and 10 volumes of 10 mM Tris-HCl pH 8. Total IgG was eluted with 100 mM glycine pH 3. Eluted fractions were neutralized with 10% 1M Tris-HCl pH 8. Eluted fractions containing IgG were concentrated and the buffer exchanged using Vivaspin Concentrators (membrane cut off 5 kDa) and PBS. Samples were analyzed by SDS-PAGE; IgG concentrations were determined using Bradford reagent.

### Immunoprecipitations

For immunoprecipitation, 1 μg Trx-HPA-1a protein was incubated with 30 μg IgG extract from human sera in buffer A (PBS, 0.5 M trehalose) or buffer A with 0.01% Tween 20 or with 0.1% NP40 or with 0.05% Triton X-100 for 2 h at 20-25°C. Ten percent 1M Tris-HCl was added to the mixture and incubated with 25 μL of protein A-sepharose beads previously equilibrated in 100 mM Tris-HCl pH 8 at 4°C 45 min. The beads were then washed three times with 100 mM Tris-HCl pH 8, 0.2% Tween 20 at 4°C for 10 min and then three times with 10 mM Tris-HCl pH8, 0.2% Tween 20. To collect the protein A-sepharose-IgG-Trx-HPA-1a complex, the mixture was centrifuged for 2 min at 10,000 g. Proteins were eluted by boiling in SDS sample buffer and the eluate was resolved on a 15% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane (0.1 μm PROTRAN® BA79, Schleicher and Schuell) in a liquid electrophoretic transfer cell (25 mM Tris, 250 mM Glycine, 3.5 mM SDS). The membranes were then blocked with TBS 5% dried milk at 4°C overnight, then incubated with anti-His antibody

(His-probe, Santa Cruz Biotechnology), diluted 1:200 in TBS 1% dried milk, 0.05% Tween 20 for 1 h at 20-25°C. After three washes with TBS, 0.05% Tween 20, membranes were incubated with HRP-conjugated goat anti-rabbit IgG (Dakocytomation) at a dilution of 1:3,000 for 50 min at 20-25°C. After a further three washes with TBS 0.05% Tween 20, bound conjugate was detected using Lumi-LightPLUS Western Blotting Substrate.

### Immunocapture assay

Interactions between Trx-HPA-1a and human serum containing antibodies against HPA-1a were tested by immunocapture. A solid phase assay for the detection of IgG antibodies to platelets assay was used (Capture P®, Immucor). The pool of platelets was replaced by Trx-HPA-1a protein. Microtitration plates with conic wells were coated overnight at 4°C with 5, 10, 50 or 100 ng of Trx-HPA-1a per well in carbonate buffer (15 mM $Na_2CO_3$, 35 mM NaHCO3 in deionized water). After washing with PBS, 50 µL serum were added to each well and incubated at 37°C for 45 min. After six washes, 50 µL of Capture-P indicating erythrocytes (red blood cells carrying anti-human IgG antibody) were added to each well. Microplates were centrifuged at 1,500 rpm for 1 min 30 s before reading.

### ELISA assay

The detection of HPA-1a specific antibodies in human serum was carried out by ELISA using microtitration plates (Nunc) coated overnight at 4°C with 100 ng of Trx-HPA-1a per well in carbonate buffer. The plates were rinsed twice with 250 µL PBS. Non-specific sites were blocked by a 1 h 30 min incubation at 20-25°C with 250 µL PBS containing 1% bovine serum albumin (BSA; A7030, Sigma). The plates were rinsed once with 250 µL PBS and subsequently incubated for 45 min at 20-25°C on a shaking incubator with 30 µL of human serum diluted with 70 µL PBS. After three washes with PBS containing 0.05% Tween 20 and one wash with PBS, the plates were incubated for 40 min at 20-25°C on a shaking incubator with HRP-conjugated Fc fragment specific goat anti-human IgG (Jackson ImmunoResearch). After three washes with PBS, 0.05% Tween 20, and two washes with PBS, the binding of anti-HPA-1 antibody was detected by the addition of OPD peroxidase substrate (1,2-phenylenediamine dihydrochloride) according to the manufacturer's instructions (DakoCytomation). Absorbance was measured at 492 nm and followed during 20 min with an ELISA plate reader. Reactions were stopped by adding 100 µL 0.5 M $H_2SO_4$. Samples showing optical densities higher than 0.1 were considered to be positive.

### MAIPA assay

The MAIPA assay was performed as described by Kiefel and colleagues[6] except multiwell dishes were used.

### Serum depletion assay

Increasing concentrations of Trx-HPA-1a (0.1-1,000 ng) diluted in 30 µL PBS were added to 50 µL of human serum containing or not polyclonal anti-HPA-1a or 15 ng of Camtran-B2 antibody diluted in 50 µL human serum devoid of anti-HPA-1a antibodies. After incubation at 20-25°C for 25 min, the mixture was pre-incubated with 3 µL of Ni-NTA agarose beads and the complex alloantibodies or Camtran/Trx-HPA-1a was pulled down by centrifugation at 13,000 rpm for 2 min.

## Results

Here, we used the FliTrx® screening system to isolate peptide aptamers interacting with an anti-HPA-1a human monoclonal antibody (Camtran-B2[23]). In this particular system, the aptamers are exposed on the surface of the flagellum of *E. coli* allowing the extracellular interactions to be seen, in contrast to the two hybrid screen which is devoted to intracellular interactions.[12,13]

### Screening of the peptide aptamer library

To set up a new detection assay for antiHPA-1a antibodies, we screened a peptide aptamer library using the FliTrx® strategy and the Camtran-B2 antibody as bait. After the last screening round, 48 bacterial colonies were picked up and tested for expression of peptide aptamer able to bind to the Camtran antiHPA-1a antibody. Western blotting analysis showed that 25 of them exhibited a positive signal at 63 kDa, the expected molecular weight of recombinant thioredoxin (Trx). Results obtained with a subset of positive and negative clones are shown in Figure 1. Nine positive clones were re-tested by Western blotting using an anti-HPA-1b serum, instead of the Camtran anti-HPA-1a antibody. No signal could be detected under these conditions (*data not shown*), suggesting that the peptide aptamer did not cross react with anti-HPA-1b antibodies.

Plasmids from these nine positive clones were re-isolated and the Trx open reading frame (ORF) was subcloned in frame with a 6HIS tail at the C-terminus, into the pT7-7 expression vector. The plasmids were resequenced after the subcloning steps. Interestingly, all were identical, with the same dodecapeptide-encoding sequence within the Trx ORF (Figure 2A) suggesting that one single aptamer had been selected. This probably reflects the high affinity of this particular aptamer for the bait. Further rounds of screening at lower stringencies may allow additional aptamers to be isolated that are able to interact with the Camtran antibody. It should be noted that the dodecapeptide sequence did not show any similarity to GPIIb-IIIa, including the HPA-1a encompassing region, or to any peptide sequence in available databases

### Characterization of peptide aptamer interaction with HPA-1a-specific antibodies

The specificity of the interaction between the peptide aptamer (hereafter named Trx-HPA-1a) and antiHPA-1a antibodies was confirmed by immunoprecipitation. To this end, the Trx-HPA-1a recombinant protein was purified to homogeneity by affinity chromatography using Ni-NTA agarose beads. The purity of the protein was checked by gel electrophoresis and mass spectrometry (Figure 2B). Immunoprecipitations were performed using

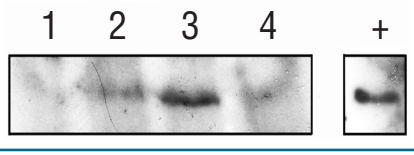


**Figure 1. Isolation of a peptide aptamer that binds specifically to the anti-HPA-1a monoclonal antibody. Western blotting analysis of whole protein extracts from bacterial clones (lanes 1-4) selected following FliTrx® screening steps. Membranes were probed with the Camtran-B2 anti-HPA-1a monoclonal antibody. Positive clones are characterized by 63 kD band (lanes 2 and 3). A sample from HPA-1a platelets was used as a positive control (right lane, +).**

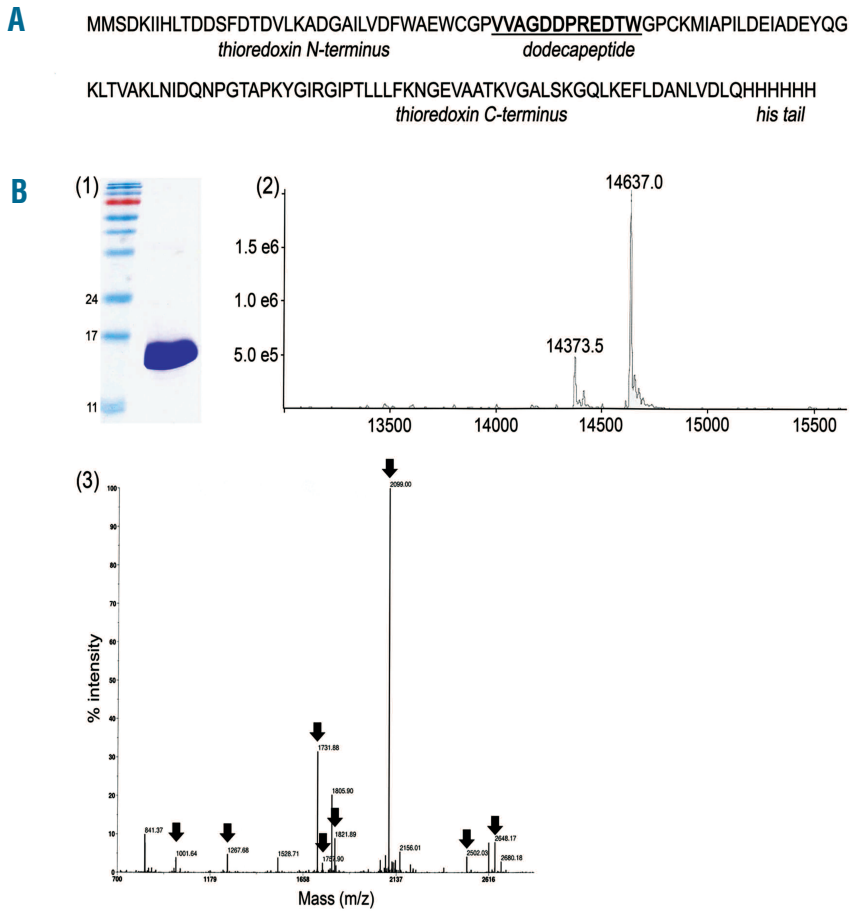


**Figure 2.** Characterization and verification of peptide aptamer. (A) Sequence of the Trx-HPA-1a protein. The protein contains a twelve aminoacid-long sequence cloned in frame into the active site of thioredoxin, as well as a poly-histidine tail in C-terminus. All sequenced positive clones exhibited the same twelve aminoacids. (B) Analysis of the purified Trx-HPA-1a protein. (1) Detection of the recombinant protein by SDS-PAGE (Coomassie blue staining). Protein (20 μg) purified by anion exchange chromatography was run on a 20% polyacrylamide gel. Molecular weight markers are shown on the left. (2) Electrospray mass spectrum of the Trx-HPA-1a protein. A major peak at the expected size (14637 Da) is detected. The minor peak (14373.5 Da) corresponds to Trx-HPA-1a lacking two methionine residues at the N-terminus. (3) MALDI-TOF spectrum obtained after tryptic digestion of Trx-HPA-1a. Black arrow (➡) corresponds to peaks at the expected size. Together these peaks match 80% of the Trx-HPA-1a sequence.

two serum samples containing or not anti-HPA-1a antibodies, as previously determined by MAIPA assay; these samples were from a mother with a thrombopenic baby and an anonymous donor, respectively. The serum samples were incubated with the purified Trx-HPA-1a protein and subsequently immunoprecipitated using protein A-coated beads. The presence of Trx-HPA-1a in the immune complexes was then checked by Western blotting using an anti-His antibody. Immunoprecipitations were carried out with or without detergents (Tween 20, NP40 or Triton X-100). Figure 3 shows that, in the presence of detergents, the Trx-HPA-1a protein was preferentially immunoprecipitated when incubated with the anti-HPA-1a positive serum sample, as compared to the anti-HPA-1a negative sample. This shows that the Trx-HPA-1a protein specifically interacted with the anti-HPA-1a antibodies present in the serum. To further assess the specificity of the interaction between Trx-HPA-1a and the anti-HPA-1a antibodies, we checked that Trx-HPA-1a did not interact with antibodies directed against the main alternate epitope found on GPIIb-IIIa, namely HPA-1b (proline instead of leukine at position 33 on the GPIIIa subunit). Serum samples with or without anti-HPA-1a or anti-HPA-1b antibodies were analyzed by immunocapture assay in which the purified Trx-HPA-1a protein was used to coat microwells. The immunocapture assay confirmed the interaction of Trx-HPA-1a with anti-HPA-1a antibodies, but not with anti-HPA-1b antibodies (Figure 4). As expected, the double negative sample (*e.g.* devoid of both anti-HPA-1a and -1b) gave no signal.

### *A new ELISA-based detection assay for anti-HPA-1a antibodies using the recombinat Trx-HPA-1a protein*

On the basis of the above observations, indicating that the Trx-HPA-1a protein did specifically interact with anti-HPA-1a antibodies, we set up a new ELISA assay to detect anti-HPA-1a antibodies using the recombinant Trx-HPA-1a protein coated onto microwells. First, the experimental conditions of the assay were optimized using serum from a woman with a NAIT newborn. This sample was previously shown to be anti-HPA-1a positive by MAIPA assay and genotyped as homozygous for the HPA-1b allele (1b/1b); it is routinely used as a standard positive control at the "Etablissement Français du Sang" (EFS-Lyon).

Optical density (OD) increased linearly with time at least during the first 20 min of incubation (Figure 5A) showing that the enzyme-catalyzed colorimetric reaction is in apparent steady state conditions. Also, the OD value closely depended on the amount of coated Trx-HPA-1a protein, increasing linearly together with coated Trx-HPA-1a; saturation was reached for amounts higher than 200 ng (igure 5B). Finally, as expected, OD was correlated to the concentration of serum sample used in the assay (Figure 5A and B). The standard conditions of the ELISA assay used in the subsequent studies described here were determined according to this first series of data (incubation time 20 min; sample dilution 1:3; 100 ng coated Trx-HPA-1a).

The assay was then carried out on additional serum samples provided by the EFS, previously classified as HPA-1a negative using MAIPA. Eight samples were tested with the new ELISA; for all samples OD was close to negative

control value ($OD_{20min}$ < 0.03). Figure 5C shows the kinetics of one representative sample (negative control); the standard positive control routinely used at the EFS-Lyon is shown for comparison ($OD_{20min}$=0.4).

To confirm that this new ELISA protocol accurately detected circulating anti-HPA-1a antibodies, we performed this analysis on a significantly larger number of blood samples and compared the results obtained with the canonical MAIPA assay.[6] For a number of samples, MAIPA was carried out both on whole serum and protein A-purified immunoglobulins (IG-MAIPA) (Table 1). A total of 118 samples from anonymous donors were found to be negative using both MAIPA and ELISA (Table 1, lines 1 and 2). Among these, 5 were chosen at random for genotyping; all were found homozygous (HPA-1a/1a) (Table 1, line 2). As expected, all these samples, which tested negative with MAIPA (or IG-MAIPA), also tested negative with ELISA.

We also analyzed a series of samples from 68 patients suffering from thrombopenic diseases (Table 1, lines 3-24). (Samples were from the EFS-Lyon, the INTS Paris and the University of Tromsø, Norway.) Among them, 23 suffered from platelet refractory disease (PR) (Table 1), 45 were women whose infants suffered from NAIT (Table 1). Twenty-nine NAIT or PR samples tested negative for anti-HPA-1a antibodies with either MAIPA, IG-MAIPA or ELISA (Table 1, lines 3-10). In some of them, the MAIPA assay showed the presence of anti-HPA-1b, anti-HPA-3a, anti-HPA-5a or anti-HPA-5b antibodies (Table 1, line 4-9). In particular, 4 NAIT or PR samples, that were genotyped as 1a/1a on the HPA locus, tested positive for anti-HPA-1b antibodies both with MAIPA and IG-MAIPA, whereas, as expected, they tested negative for anti-HPA-1a with the ELISA assay (Table 1, lines 4 and 5).

Of the other NAIT or PR samples, 18 samples that tested positive for anti-HPA-1a both with MAIPA and IG-MAIPA also tested positive with ELISA (Table 1, lines 11-13); interestingly, most of them were genotyped 1b/1b (Table 1, lines 11 and 12).

Therefore, on the basis of the above analyses carried out on a set of 165 samples (Table 1, lines 1-13), the results from the ELISA assay appear to be strictly correlated with those obtained from MAIPA (147 samples testing anti-HPA-1a negative, 18 samples testing anti-HPA-1a positive).

However, 2 additional NAIT samples, which were found to be anti-HPA-1a positive according to both MAIPA and IG-MAIPA, and being genotyped 1b/1b, did test negative with ELISA (Table 1, line 14) indicating that the Trx-HPA-1a protein did not interact with circulating anti-HPA-1a antibodies present in these particular samples.

Finally, among the 19 samples (15 NAIT, 4 PR) that were either weakly positive (Table 1, lines 15-18) or negative (Table 1, lines 19-24) according to MAIPA, 18 samples were found anti-HPA-1a positive with the ELISA assay (Table 1, lines 15-19 and 21-24). In addition, most of them (14 of 19 samples) also tested positive with IG-MAIPA (Table 1, lines 15 and 17-21). Seventeen samples were genotyped as 1b/1b on the HPA locus (Table 1, lines 15, 16, 18-20, 22-24) indicating that anti-HPA-1a antibodies may be present in these samples. Thus, taken together, these observations suggest that the new ELISA assay described here could detect anti-HPA-1a antibodies in serum samples from patients who were originally classified as anti-HPA-1a negative, according to the MAIPA assay currently in use.

### *Use of the Trx-HPA-1a peptide aptamer in a depletion assay for therapeutic purposes*

On the basis of the specific interaction of the Trx-HPA-1a recombinant protein with anti-HPA-1a antibodies, we eval-

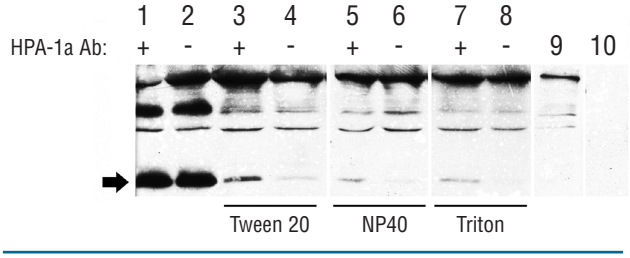


**Figure 3.** Characterization of the interaction between Trx-HPA-1a protein and anti-HPA-1a antibodies. Two human serum samples, with (1, 3, 5, 7) or without (2, 4, 6, 8) HPA-1a-specific alloantibodies were incubated with purified Trx-HPA-1a protein and immunoprecipitated with protein A-sepharose. Immunoprecipitates were analyzed by Western blotting using anti-His antibody to detect Trx-HPA-1a. Buffers used for immunoprecipitation were PBS (lanes 1, 2, 9, 10), PBS-Tween 20 (0.01%) (lanes 3, 4), PBS-NP40 (0.1%) (lanes 5, 6), PBS-Triton X-100 (0.5%) (lanes 7, 8). Negative controls without Trx-HPA-1a (9) or without protein A-sepharose (10) are shown. A signal corresponding to the apparent molecular weight of Trx-HPA-1a (16,500 Da) is clearly detected in lanes 1-3, 5 and 7; Black arrow (➡). In the absence of detergent (lanes 1, 2) the Trx-HPA-1a aptamer is immunoprecipitated, irrespective of the presence of anti-HPA-1a antibodies, while in the presence of detergent (lanes 3-8) the aptamer is preferentially immunoprecipitated when incubated with the serum that contains HPA-1a-specific antibodies. Additional bands in the upper part of the blot correspond to immunoglobulins that are detected in a non-specific manner.

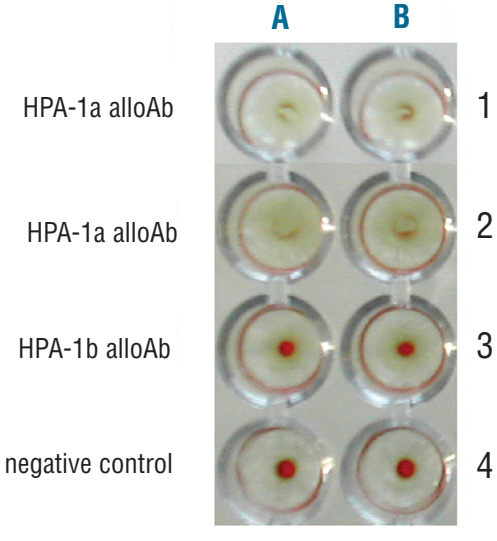


**Figure 4.** Detection of anti-HPA-1a antibodies in human serum using the Trx-HPA-1a protein (Immunocapture assay). The following human serums were used: 2 different samples from patients with NAIT babies, containing anti-platelet HPA-1a alloantibodies (rows 1, 2), one sample containing anti-platelet HPA-1b alloantibody (row 3), one negative control (row 4). Wells were coated with 50 ng (column A) or 100 ng (column B) of Trx-HPA-1a protein. Interaction between Trx-HPA-1a and alloantibodies is only detected with sera containing anti-HPA-1a immunoglobulins (row 1, 2).

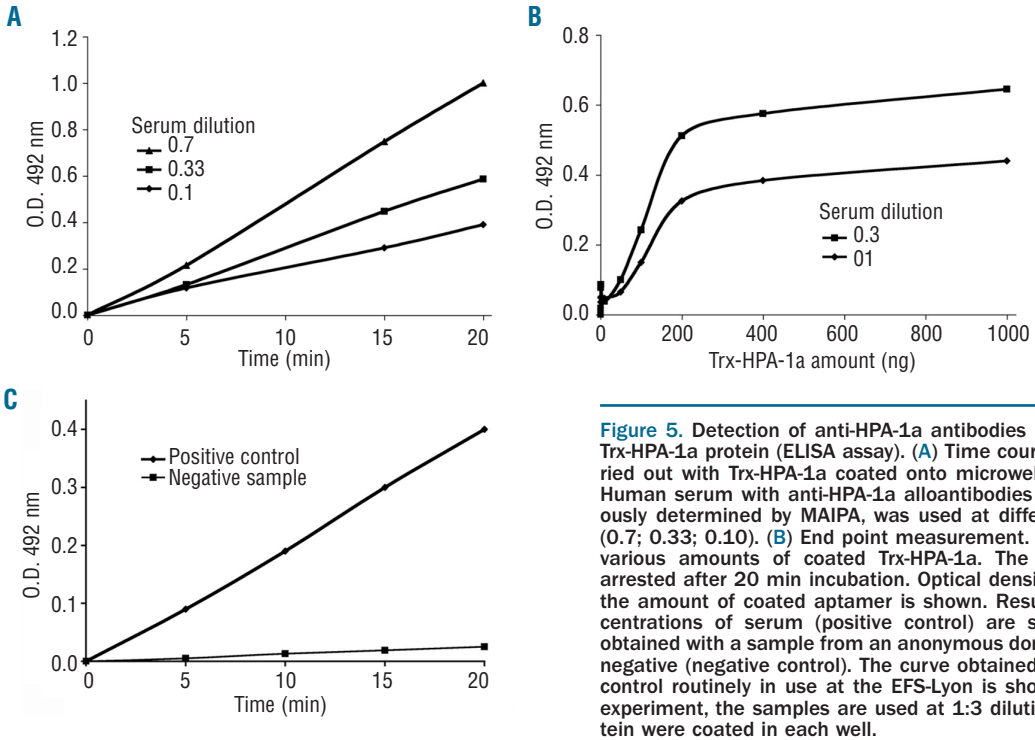


**Figure 5. Detection of anti-HPA-1a antibodies in human serum using the Trx-HPA-1a protein (ELISA assay). (A) Time course analysis. ELISA was carried out with Trx-HPA-1a coated onto microwell plates (100 ng per well). Human serum with anti-HPA-1a alloantibodies (positive control), as previously determined by MAIPA, was used at different dilutions as indicated (0.7; 0.33; 0.10). (B) End point measurement. ELISA was carried out with various amounts of coated Trx-HPA-1a. The colorimetric reaction was arrested after 20 min incubation. Optical density ($OD_{20min}$) as a function of the amount of coated aptamer is shown. Results obtained with two concentrations of serum (positive control) are shown. (C) Typical kinetics obtained with a sample from an anonymous donor previously tested MAIPA negative (negative control). The curve obtained with the standard positive control routinely in use at the EFS-Lyon is shown for comparison. In this experiment, the samples are used at 1:3 dilution, 100ng Trx-HPA-1a protein were coated in each well.**

uated its ability to neutralize anti-HPA-1a positive serum samples. The ability of Trx-HPA-1a to neutralize alloantibodies from 2 women with NAIT infants was investigated; the monoclonal Camtran antibody was used for calibration. The Trx-HPA-1a protein could neutralize anti-HPA-1a reactivity in a dose-dependent manner, 1 μg Trx-HPA-1a protein neutralizing 50-60% of the anti-HPA-1a antibodies present in 50 μL of serum (Figure 6). Importantly, this depletion test confirmed the specificity of the peptide aptamer, showing the absence of cross reactivity with anti-platelet HLA class 1 antibodies (*Online Supplementary Appendix*). This shows that the Trx-HPA-1a protein can efficiently lower anti-HPA-1a reactivity in the blood.

## Disussion

Peptide aptamers offer a versatile way to isolate ligands with high specificity for a given protein. They have been successfully used to characterize inhibitors of a number of targets involved in cell signaling,[25,26] cell cycle[11] and cell death.[15,18] Therefore, peptide aptamers and their derivatives may be particularly useful to dissect intracellular protein interaction networks. Peptide aptamers are also of potential therapeutic interest in a number of pathologies, including cancer.[19,27]

In addition, due to their high specificity, peptide aptamers are interesting tools to detect proteins, antigens or antibodies in body fluids, including the blood. However, so far they have rarely been used for diagnostic purposes.

### *A peptide aptamer specific for human anti-HPA-1a antibodies*

We describe the isolation and characterization of a peptide aptamer (Trx-HPA-1a ) that specifically recognizes anti-HPA-1a antibodies in the blood. Together our data indicate that, although it shares no sequence homology with GPIIb/IIIa, the variable region of the Trx-HPA-1a aptamer interacts specifically with anti-HPA-1a antibodies. Indeed this aptamer interacts with the Camtran monoclonal antibody and a number of circulating anti-HPA-1a antibodies (Table 1) but not with anti-HPA-1b, anti-HPA-3a, anti-HPA-5a and anti-HPA-5b antibodies (Figures 2 and 3, Table 1). This suggests that the 3D structure of this twelve aminoacid-long region of the Trx-HPA-1a aptamer closely resembles the HPA-1a antigen. However, the fact that 2 samples were found to be negative using the ELISA assay described here, even though these serum samples contained anti-HPA-1a antibodies, as shown both by MAIPA and IG-MAIPA, and confirmed by genotyping (Table 1, line 14), indicates that Trx-HPA-1a may not exhibit all of the antigenic determinants of the HPA-1a antigen. This suggests that the Trx-HPA-1a protein probably interacts with cross-reacting idiotypes in the antigen binding site that are shared by some but not all anti-HPA-1a antibodies. Additional screenings of the library may lead to the characterization of new peptide aptamers interacting with those idiotypes that are not recognized by Trx-HPA-1a. Given this, experimental determination of the 3D structure of Trx-HPA-1a would be of major interest.

### *A sensitive assay for the detection of anti-HPA-1a antibodies*

Although a thorough statistical analysis would require an even larger number of samples from thrombopenic patients and/or mothers with NAIT newborns, our data show that the protein A-purification step increases the sensitivity of the MAIPA, a number of serum samples found to be anti-HPA-1a negative using MAIPA, testing

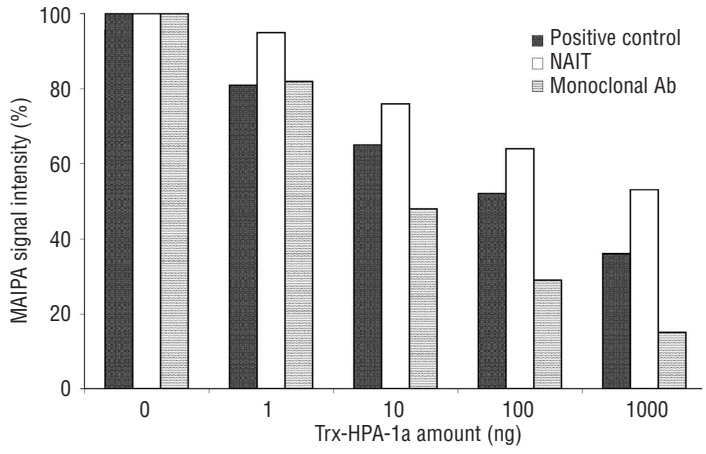


**Figure 6.** Depletion assay. Analysis of the MAIPA signal intensity as a function of the amount of Trx-HPA-1a protein. Depletion was carried out on the serum of 2 mothers whose infants were diagnosed with NAIT ("Positive control" used in EFS and "NAIT"), as well as the monoclonal anti-HPA-1a antibody ("monoclonal Ab"). The signal decreases as a function of the amount of Trx-HPA-1a, showing the neutralization of anti-HPA-1a antibodies.

positive by IG-MAIPA. Thus, since these samples also tested positive using the new ELISA assay described here, our data support the idea that this new ELISA allows the detection of anti-HPA-1a antibodies in samples that were first classified as HPA-1a negative according to existing assays. Among the 39 samples from patients harboring the 1b/1b genotype, in which the presence of anti-HPA-1a antibodies could be thus suspected, 19 clearly tested anti-HPA-1a positive by MAIPA, 30 tested positive by IG-MAIPA, and 33 tested positive by ELISA (Table 1). As a result, taken together, the results from these 3 different assays classified 92.3% of these samples to be anti-HPA-1a positive. Therefore, the combined use of MAIPA and ELISA seems to significantly improve the detection of anti-platelet antibodies in the blood.

In addition, this new ELISA is rapid and easy to perform routinely. Furthermore, this method could be used to set up a standard assay for the quantitation of anti-HPA antibodies. MAIPA and other assays currently used to detect platelet-associated antibodies depend on the availability of human platelets;[6,28,29] since pools of platelets used in these assays vary from one blood center to another, accurate comparison of the results obtained by the different laboratories is still a challenge.[3,30,31] Although an international standard for the quantitation of anti-HPA-1a antibodies has been proposed,[32] the development of new strategies that do not rely on the availability of human platelets is still required.[33-36] In this respect, it should be noted that beads coated with recombinant β3-integrin fragments were recently used to successfully detect anti-HPA-1a antibodies.[37] It would be interesting to compare these two novel detection assays. Such *in vitro* assays, including the new ELISA assay described in the present report, may be of interest for diagnostic purposes. However, more clinical samples need to be analyzed before these novel assays can be recommended for routine use.

In addition to diagnostic applications, the Trx-HPA-1a protein may also be an interesting therapeutic tool for immune thrombocytopenia, including NAIT; indeed, our results suggest that immobilized aptamer columns could be used to remove anti-HPA-1a from the blood.

In conclusion, this study demonstrates that peptide aptamers can be used to detect specific antibodies in human serum. Our data also suggest that peptide aptamers can detect a variety of biomarkers in body fluids and are, therefore, of potential interest for diagnostic purposes.

**Table 1.** Detection of anti-HPA1a antibodies using MAIPA and ELISA. Serum samples were from anonymous donors, thrombopenic patients or mothers with NAIT babies. Healthy: donors without refractory platelets; NAIT: women with thrombopenic infants. Results from the 118 samples from anonymous donors are shown. The MAIPA assay was performed using GPIIbIIIa (or GPIa, lines 7-9) as antigen on either crude serum (MAIPA) or protein A-purified immunoglobulins (IG-MAIPA). ELISA was carried out by measuring optical density at a given time point ($OD\ 20_{min}$). Genotyping for the HPA-1 locus is shown when available.

| Line | People tested | Clinical context | HPA-1 genotype | MAIPA | IG-MAIPA | ELISA HPA-1a |
|---|---|---|---|---|---|---|
| 1 | 113 | Healthy | nd | – | – | – |
| 2 | 5 | Healthy | 1a/1a | – | – | – |
| 3 | 13 | PR | nd | – | – | – |
| 4 | 2 | NAIT | 1a/1a | α -1b | α -1b | – |
| 5 | 2 | PR | 1a/1a | α -1b | α -1b | – |
| 6 | 1 | PR | nd | α -3a | α -3a | – |
| 7 | 3 | NAIT | nd | α -5a | α -5a | – |
| 8 | 2 | PR | nd | α -5a | α -5a | – |
| 9 | 3 | NAIT | nd | α -5b | α -5b | – |
| 10 | 3 | NAIT | 1b/1b | – | – | – |
| 11 | 1 | PR | 1b/1b | α -1a | nd | + |
| 12 | 16 | NAIT | 1b/1b | α -1a | α -1a | + |
| 13 | 1 | NAIT | nd | α -1a | α -1a | + |
| 14 | 2 | NAIT | 1b/1b | α -1a | α -1a | – |
| 15 | 1 | NAIT | 1b/1b | +/– α -1a | α -1a | + |
| 16 | 1 | NAIT | 1b/1b | +/– α -1a | nd | + |
| 17 | 1 | NAIT | nd | +/– α -1a | α -1a | + |
| 18 | 3 | PR | 1b/1b | +/– α -1a | α -1a | + |
| 19 | 7 | NAIT | 1b/1b | – | α -1a | + |
| 20 | 1 | NAIT | 1b/1b | – | α -1a | nd |
| 21 | 1 | NAIT | nd | – | α -1a | + |
| 22 | 1 | NAIT | 1b/1b | – | nd | + |
| 23 | 2 | NAIT | 1b/1b | – | – | + |
| 24 | 1 | PR | 1b/1b | – | – | + |

*-: anti-HPA-1a negative; +: anti-HPA-1a positive; nd: not determined; +/-: samples testing negative or weakly positive at MAIPA EFS -Lyon and INTS – Paris, depending on the date of blood sampling. (α–"nx"): presence of anti-HPA-nx-specific antibodies.*

## Authorship and Disclosures

*The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.*

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