

Development and Characterization of Novel ELISAs for the Specific Quantification of the Factor H-Related Proteins 2, 3, 4, and 5

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Keywords

Complement system · Factor H · Factor H-related proteins · Immunoassays · ELISA development

Abstract

Introduction: The complement system's alternative pathway relies on factor H (FH) for immune homeostasis. Next to FH, a group of highly similar proteins was described known as the FH-related (FHR) proteins. The FH protein family includes FH, factor H-like protein 1, and five FHR proteins (FHR-1 to -5). The exact function of the FHRs is still unknown, necessitating further research. However, the lack of highly specific assays has hindered studying their role in health and disease. This study aimed to develop novel ELISAs for reliably and specifically quantifying levels of the FHRs in human

blood. **Methods:** Novel FHR-specific antibodies were generated. Positive hybridoma clones were taken to monoclonality, verified for target specificity via ELISA and Western blot, and antibody pairs were selected for further ELISA development. During development, ELISAs were characterized and validated for specificity, stability, accuracy, and reproducibility, among others. **Results:** Monoclonal antibodies specific for FHR-2, -3, -4, or -5 were generated. Using these antibodies, four ELISAs were developed capable of quantifying FHR levels in an accurate and robust manner. Each assay showed high target specificity, good analyte

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recovery and strong reproducibility between replicates, test runs, and test laboratories. **Conclusions:** These assays enable specific and accurate quantification of FHR-2, -3, -4, and -5 in human blood. They facilitate large-scale screening of patient cohorts in a standardized manner and contribute to understanding the role of the FHRs in health and disease.

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Introduction

The complement system is a key effector mechanism of the innate immune system in the defense against pathogens. It consists of three activation pathways: the classical pathway, the lectin pathway, and the alternative pathway (AP), each leading to cleavage of the key complement component C3 and ultimately the activation of C5 and the terminal pathway [1]. Complement activation leads to the production of three main types of effectors: (1) anaphylatoxins (C3a and C5a), which are potent proinflammatory molecules; (2) opsonins (C3b, inactive C3b (iC3b), C3dg), which bind to target surfaces, thereby promoting the removal of target cells or immune complexes; and (3) the terminal membrane attack complex (C5b-9), which directly lyses targeted (opsonized) pathogens or damaged self-cells. In contrast to the classical pathway and lectin pathway, the AP plays a distinctive role in complement activation as it additionally serves as a robust amplification pathway as well as an initial activator. The AP is continuously activated by the spontaneous hydrolysis of the thioester bond in C3, forming C3(H₂O). Hydrolysis changes the structure of C3 and increases its reactivity to interact with the serine protease factor B (FB), thereby initiating the formation of the convertase C3(H₂O)Bb. Likewise, any C3b formed during complement activation can serve as a platform to generate the convertase C3bBb. These convertases initiate further downstream activation of the cascade and amplification of the immune response [2]. The spontaneous low-level C3 activation serves as a continuous immune surveillance mechanism, allowing the immune system to quickly respond to potential threats, but inadvertently also targets host cells. Therefore, the AP needs to be tightly regulated to prevent excessive activation on healthy cells and, subsequently, complement-mediated tissue damage [3].

Complement factor H (FH) is the main regulator of complement activation. It functions as a negative regulator of the AP by inhibiting its activation via several mechanisms. First, FH competes with FB for binding to

C3b. In addition, FH functions as a co-factor for factor I (FI)-mediated proteolysis of C3b, thereby forming iC3b. Both processes lead to a decrease in C3bBb convertase formation. Furthermore, FH promotes the decay of C3bBb by destabilizing the convertase complex and accelerating its dissociation to C3b and Bb [4]. As a result, circulating C3bBb levels decrease, leading to the reduction of downstream complement activation.

Next to FH, a specific group of proteins, known as the FH-related (FHR) proteins, was described [5]. The FHR proteins share structural features with FH and are believed to have arisen through duplication events during evolution [5]. Besides FH, this protein family consists of FH-like protein 1 (FHL-1) and five FHR proteins (FHR-1, -2, -3, -4, -5). FH and FHL-1 are alternative splice products of the *CFH* gene, while the FHRs are each encoded by their own gene (*CFHR1-5*) [6].

Members of the FH family share several highly conserved domains that are predominantly involved in surface recognition and play a role in host ligand recognition and complement regulation on host surfaces [7]. As also observed for FH, genetic alterations in the *CFHR* genes are associated with several diseases, such as age-related macular degeneration, atypical hemolytic uremic syndrome (aHUS), C3-glomerulopathy (C3G), systemic lupus erythematosus, and IgA nephropathy (IgAN) [8–18].

The exact function of the FHR proteins as well as their role in disease is not fully understood. However, several studies report that the FHRs share certain ligands for binding with FH, like C3b [7, 19–21]. In contrast to FH, the FHRs thereby facilitate the assembly of C3bBb, resulting in activation rather than inhibition of the AP, suggesting that FHRs may counteract the regulatory activity of FH [19, 20, 22, 23]. This is also supported by the fact that the conserved regions in the *CFHR* genes contain the major surface recognition sites of FH, but lack the sites involved in complement inhibition. This explains their ability to engage with the majority of FH ligands and, in turn, result in complement activation [24].

To further unravel the functions of the FHRs, it is of the utmost importance that circulating FHR protein levels can be assessed in large patient cohorts in a reliable and standardized way. However, accurately measuring complement protein levels has been found to be challenging [25–27]. The use of different techniques, assays, and calibrators can lead to large discrepancies in plasma levels, making it difficult to compare results between laboratories. Our consortium previously showed that assessing FH levels in a small cohort of healthy

volunteers, using several commercially available assays, yielded substantial differences in FH levels between the used assays [28]. In addition, accurately measuring levels of specific FHR family members poses a challenge due to the high degree of similarity in amino acid sequences, making it difficult to distinguish each individual FH family member [16, 28]. Various laboratories around the world have developed in-house reagents and ELISAs when studying a particular FHR protein but often with non-overlapping normal ranges for protein levels [8, 20, 29–35]. To reliably assess FHR levels, it is crucial to use specific reagents that do not cross-react with any other member of the FH protein family.

Up till now, no validated and standardized ELISAs were commercially available for measuring the FHR proteins, making it difficult to compare results between studies. In this study, we aimed to develop and validate five novel sandwich ELISAs, making use of previously described and newly generated specific antibodies, for quantifying FHR-1, -2, -3, -4, and -5 levels in human blood samples in a reliable, specific, and robust manner.

Materials and Methods

Biological Samples

Human EDTA plasma samples and CHES panels (citrate, heparin, EDTA, and serum samples from a single donor) from healthy donors were purchased from Bio-IVT (New York, USA) and stored in aliquots at -80°C . During immunoassay development, a subset of four EDTA plasma samples and two CHES panels were selected per assay and included in the measurements. A serum pool with known concentrations for FH, FHR-3, -4, and -5 was received from Sanquin Research (Sanquin Blood Supply Foundation, Amsterdam, the Netherlands) and used as a calibrator [29–31]. This serum pool was used to calibrate a pool of healthy human EDTA plasma samples purchased from BioIVT (New York, NY, USA) for each assay to be used as a standard in the ELISAs.

Murine blood samples from three different strains (CD1, C57BL/6, and Balb/C) and porcine blood samples were purchased from Innovative (Innovative Research Inc., Novi, MI, USA). Rat blood samples from two different strains (Harlan and Lewis) were purchased from Harlan (Harlan Bioproducts for Science Inc., Indianapolis, IN, USA) and Innovative (Innovative Research Inc., Novi, MI, USA), respectively. Non-human primate (cynomolgus monkey) blood samples were purchased from BioIVT (BioIVT, New York, NY, USA). All animal samples were stored at -80°C .

Recombinant and Native Purified Proteins

Recombinant human (rh) FHR-1 to FHR-5 proteins were provided by Sanquin Research (Sanquin, Amsterdam, The Netherlands) and produced with a C-terminal 6x-histidine tag by performing a transient transfection of pcDNA3.1 expression vectors in HEK293F cells as previously described by Pouw et al. [30] and based on Vink et al. [36]. Purified human FH was purchased from CompTech (Complement Technology Inc., Tyler, TX, USA).

Previously Generated Anti-FH/FHR Monoclonal Antibodies

For developing the FHR-2, -4, and -5 ELISAs, we made use of the previously generated, monoclonal antibodies (mAbs) anti-FHR-2.11, anti-FHR-2.12, anti-FHR-4A.8, anti-FHR-4A.9, anti-FHR-5.1, and anti-FHR-5.4, described in Veuskens et al. [37] (2025), Pouw et al. [31] (2018), and van Beek et al. [29] (2017), respectively. A mAb targeting FH (clone FH.02) was previously generated [38]. This antibody binds FH and FHR-1 and was used for cross-reactivity experiments.

Mouse Immunizations and Generation of FHR Specific mAbs

All antibodies were developed and provided by Sanquin Research (Sanquin, Amsterdam, the Netherlands). To obtain specific mAbs for FHR-1, -2, and -3, new mouse immunizations and generation of hybridomas were performed as previously described [30, 37]. All experimental protocols adhered to institutional standards for animal care and use and received approval from the Central Committee for Animal Use and IVD at the Netherlands Cancer Institute (Amsterdam, the Netherlands). In short, Balb/c mice received an intraperitoneal (IP) injection of monomeric FHR-1 or full-length rhFHR-3, suspended in 50% (v/v) Montanide™ ISA V50 V2 adjuvant (Seppic, La Garenne-Colombes, France). After 4 and 6 weeks, two subsequent booster IPs were administered to each mouse. Three days after the last IP injection, the lymph nodes and spleens were isolated. Hybridomas were generated by fusing the isolated immune cells with the mouse myeloma SP2/0 cell line in a 3:1 ratio [30, 37]. The fusion process was performed in a 42% (v/v) polyethylene glycol 4,000 (Merck cat #9727, Rahway, NJ, USA) solution, and the resulting hybridomas were cultured under conditions favoring hybridoma selection. Only hybridomas demonstrating specificity for its corresponding full-length rhFHR protein in an initial ELISA screening were selected and subsequently made monoclonal through multiple

limiting dilution cultures. Next, they were cultured in IMDM supplemented with 1% (v/v) penicillin/streptomycin (Invitrogen, Waltham, MA, USA), 2.5% (v/v) fetal calf serum (Bodinco, Alkmaar, the Netherlands), 0.5 ng/mL interleukin-6 (IL-6, in-house produced), and 50 μ M β -mercaptoethanol (Merck Millipore, Darmstadt, Germany) for a period of up to 4 weeks, whereafter they were purified using a HiTrap[®] Protein A high-performance 1-mL column (GE Healthcare, Chicago, IL, USA) following manufacturer's instructions. Lastly, the isotype of the antibodies was determined using an IsoStrip[™] mouse mAb isotyping kit (Roche, Basel, Switzerland), according to the manufacturer's instructions.

Specificity and Cross-Reactivity of the mAbs

The specificity and cross-reactivity of all mAbs was validated and tested by direct ELISA and Western blotting (WB). For the direct ELISA, microtiter wells (MaxiSorp[™] 96-wells plates, Thermo Fisher Scientific, Waltham, MA, USA) were coated overnight at 4°C with either rhFHR-1, rhFHR-2, rhFHR-3, rhFHR-4A, rhFHR-4B, rhFHR-5, or native purified FH in a concentration of 500 ng/mL in PBS. After blocking (PBS + 1% BSA; pH 7.4) for 1.5 h at RT, wells were washed twice (PBS, 0.05% Tween[®]-20; pH 7.4). Subsequently, the corresponding anti-FHR mAbs were added in a concentration of 5 μ g/mL in dilution buffer (PBS; 0.1% BSA; pH 7.4) and incubated for 30 min at RT. After washes (4 $\times$), an HRP-conjugated goat-anti-mouse IgG antibody (Jackson ImmunoResearch, PA, USA) was added (0.01%, v/v, in dilution buffer) and plates were incubated for 30 min at RT. After washing (4 $\times$), TMB (K-blue aqueous tetramethylbenzidine, Neogen Europe Ltd., Ayr, UK) was added. The peroxidase reaction was stopped after 15 min, using 2% (w/v) oxalic acid. Absorbance was measured at 450 nm using a microplate reader (BioTek Synergy HT, Winooski, VT, USA).

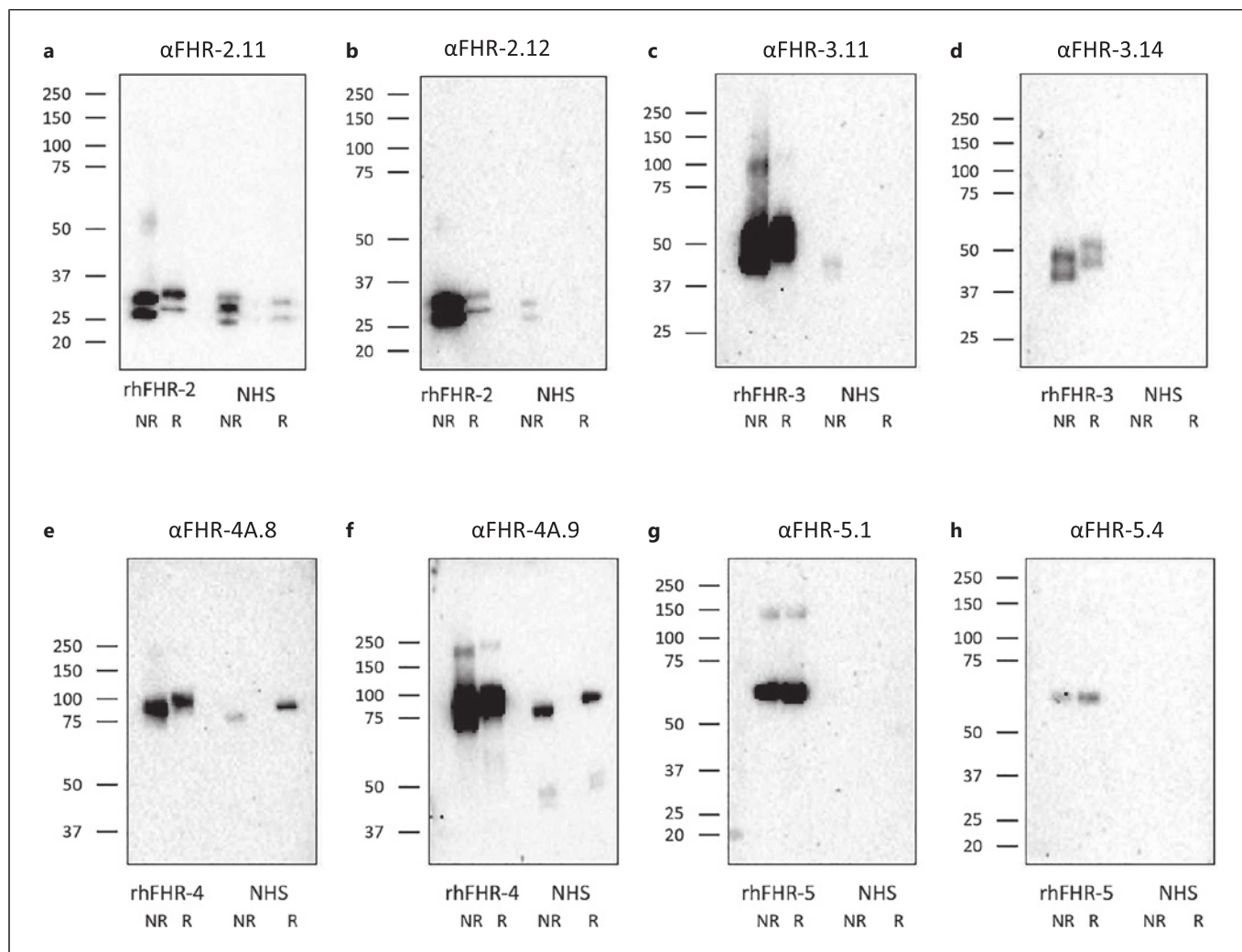
WB was performed for all mAbs that are used in the immunoassays. Normal human serum (NHS) was depleted for albumin and IgG using HPLC on a HiTrap[®] Albumin and IgG depletion column (Cytiva, Marlborough, MA, USA) following the manufacturer's instructions. The collected fractions were concentrated to 50% of the initial serum volume using Amicon[®] Ultra Centrifugal Filters (10 kDa MWCO, Millipore, Burlington, MA, USA). Depleted NHS (5%, v/v) was then prepared in NuPAGE[™] LDS Sample Buffer (Invitrogen, Carlsbad, CA, USA) with or without the addition of a reducing agent (NuPAGE[™] Sample Reducing Agent, Invitrogen). Samples for rhFHR-2, -3, -4, and FHR-5 (300

ng) were similarly prepared. Samples were heated for 10 min at 70°C. A 10 μ L sample was loaded onto Novex NuPAGE[™] 4–12% Bis-Tris gels (Invitrogen, Carlsbad, CA, USA) for separation, followed by WB onto nitrocellulose membranes using the Novex iBlot Gel Transfer Kit (Invitrogen, Carlsbad, CA, USA). Membranes were blocked overnight at 4°C with 1% (v/v) Western blocking reagent (Roche, Basel, Switzerland) in PBS. Next, membranes were incubated for 1 h at RT with 2 μ g/mL of the following mAbs: anti-FHR-2.11, -2.12, -3.11, -3.14, -4A.8, -4A.9, -5.1, and FHR-5.4 [29, 31, 37]. Membranes were washed three times for 5 min with PBS containing 0.1% Tween[®]-20 and incubated for 1 h with HRP-conjugated RM-19 (rat, anti-mouse, anti-Kappa, 0.5 μ g/mL in 0.5% (v/v) Western blocking reagent in PBS, Sanquin Research, The Netherlands). After three additional washes with PBS 0.1% Tween[®]-20 and PBS (5 min each), blots were developed using ECL (Thermo Fisher Scientific, Waltham, MA, USA) and imaged with a Chemidoc[™] MP System (BioRad, Hercules, CA, USA). Antibody specificity for native FHR-3 was further determined by using immunoprecipitation prior to WB as previously described using NHS and a mix of biotinylated antibodies (polyclonal anti-FH, polyclonal anti-FHR-3, anti-FHR-5.4, anti-FHR-2.1) to recognize the whole FH family [30].

Development of FHR-2, -3, -4, and -5 Sandwich ELISAs

ELISAs for the in vitro quantification of FHR-2, -3, -4, and -5 in human blood samples were developed at Hycult Biotech (Uden, the Netherlands). Each assay consists of a 96-wells plate coated with a mAb specific to the target FHR protein. Plasma (EDTA, citrate and heparin) and serum samples were diluted at least 100 times in assay-specific dilution buffer and, next to a calibrator, incubated in the precoated wells for 30 min at RT. Subsequently, plates were washed using washing buffer and incubated with an HRP-conjugated monoclonal anti-FHR antibody. After washing, TMB substrate was added, and the reaction was stopped with 2% (w/v) oxalic acid. The optical density (OD) was measured at 450 nm using a microplate reader (BioTek Synergy HT). Unless otherwise specified, this protocol was used to perform sample measurements and evaluate several assay characteristics such as specificity, sensitivity, recovery, sample stability, intra- and inter-assay variability. During development, variation between measurements or conditions was assessed by calculating the coefficient of variation (CV) over multiple determinations of a single sample.

Assay performance was evaluated using the following general requirements. The OD_{450nm} of the highest



(Figure continued on next page.)

concentration of the standard curve (S1) should be $1.7 \leq OD_{450nm} S1 \leq 3.5$, while the blank must be $OD_{450nm} \leq 0.2$. Additionally, the signal-to-noise ratio must be >10 ($OD_{450nm} S1/OD_{450nm} blank >10$). Lastly, when calculating sample concentrations over multiple dilutions/determinations, the CV (over at least three dilutions/determinations) must be below 15% to report results as "reliable."

Specificity and Cross-Reactivity of the Immunoassays

Specificity and cross-reactivity of the immunoassays were investigated for FH and all FHR proteins using native purified FH and rhFHRs. For all proteins, the signal was measured in each FHR assay as described

above. In addition, it was also investigated whether the assays cross-react with FHR proteins from species other than humans. The animal plasma samples (murine, rat, porcine, and monkey) were tested up to a ten-fold higher concentration than the recommended concentration for human plasma samples.

Recovery of FHR Proteins in EDTA Plasma Samples

The recovery of the target analyte in each respective FHR assay was determined in EDTA plasma samples. EDTA plasma samples from different donors with different concentrations were mixed in the ratios 100:0, 75:25, 50:50, 25:75, and 0:100 and incubated for 30 min at RT. Following the above-mentioned protocol, sample

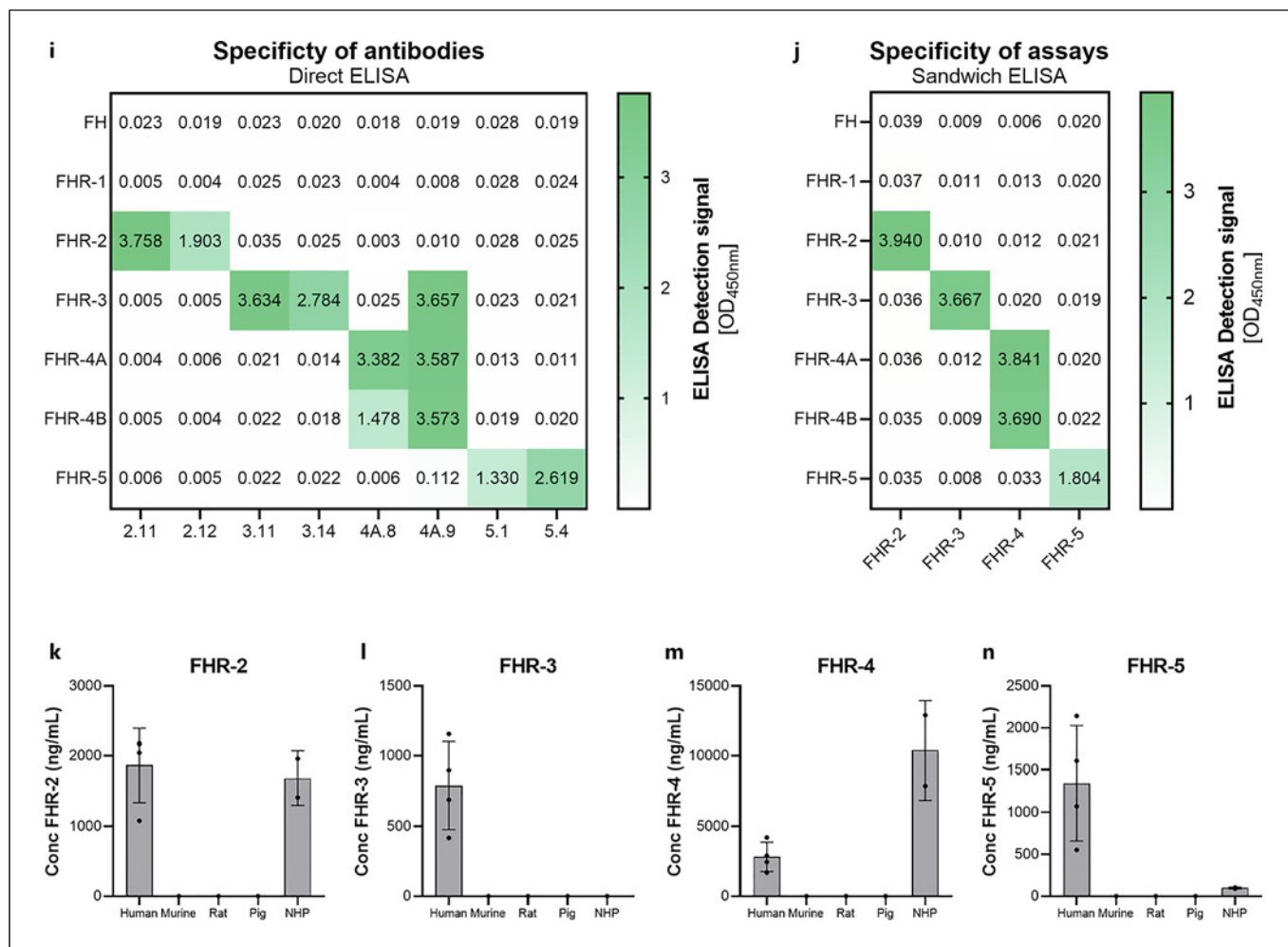


Fig. 1. Specificity and cross-reactivity of monoclonal antibodies (mAbs) and sandwich ELISAs. **a–h** Western blotting (WB) with mAbs against the factor H-related (FHR) proteins on recombinant human FHR (rhFHR) proteins (rhFHR-2, -3, -4A, -5) and normal human serum (NHS) in reduced (R) and non-reduced (NR) conditions. **a, b** The FHR-2 mAbs (clone 2.11 and 2.12) recognize the FHR-2 monomer (24–29 kDa) and the FHR-2/2 dimer (48–58 kDa). **c, d** The FHR-3 mAbs (clone 3.11 and 3.14) recognize FHR-3 (50 kDa). **e, f** The FHR-4 mAbs (clones 4A.8 and 4A.9) both recognize FHR-4A (86 kDa), and clone 4A.9 additionally recognizes

FHR-3. **g, h** The FHR-5 mAbs (clone 5.1 and 5.4) recognize FHR-5 monomer (65 kDa) and dimer (130 kDa). **i** Specificity of the mAbs used in the immunoassays was further confirmed in a direct ELISA using rhFHR and native plasma-derived FH (pdFH). **j** Target specificity of the sandwich ELISAs was confirmed using rhFHR and pdFH. **k–n** Cross-reactivity of the immunoassays was investigated against other species. Animal samples were 10x less diluted than human samples and tested in the immunoassays using the human samples as a reference. Murine ($n = 3$), rat ($n = 2$), porcine ($n = 1$), and non-human primate ($n = 2$) samples were tested.

concentrations of all mixed samples were measured and the recovery in each sample was determined by comparing the obtained vs the expected value, based on the following equation:

$$\text{Recovery (\%)} = \frac{\text{Measured concentration of mixed sample}}{\text{Expected concentration } [(\% \times \text{sample A}) + (\% \times \text{sample B})]} \times 100$$

Requirements were met if the calculated recovery was between 80 and 120%.

Stability Testing

Benchtop and freeze-thaw stability of FHR-2, -3, -4, and -5 was evaluated by incubating undiluted samples for 1-, 2-, and 16-h intervals at RT and on ice prior to measurements. Stability was assessed by comparing the obtained sample values to a baseline sample (sample thawed for 10 min and directly measured).

Freeze-thaw stability was investigated by repeatedly freeze-thawing (0–4 cycles) different aliquots of the same

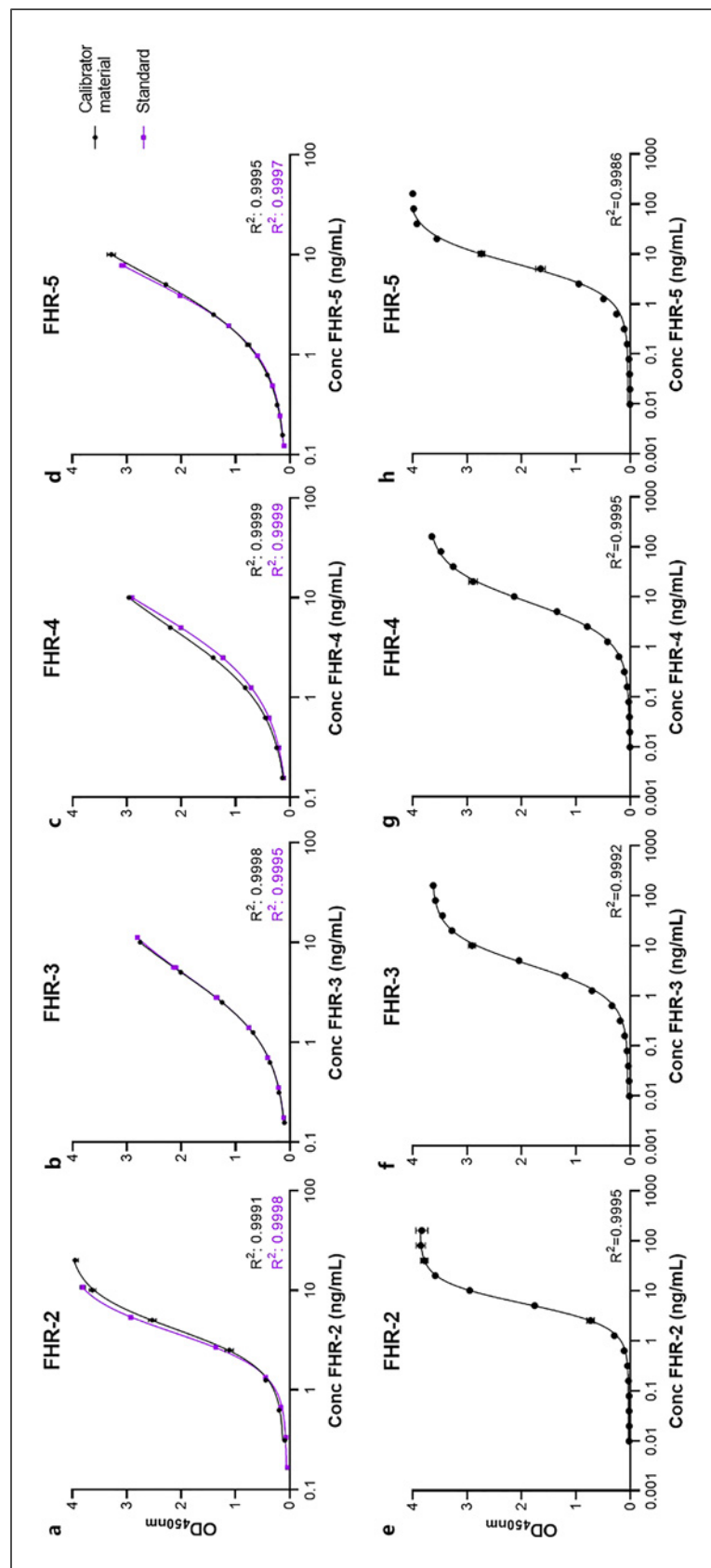


Fig. 2. Calibration and sigmoidal shape of the standard curve. **a–d** The immunoassays consist of a standard curve (calibration curve; depicted in purple) based on EDTA plasma. The standards were calibrated against the calibrator material that was either a recombinant human factor H-related (rhFHR) protein or a previously calibrated serum pool (depicted in black). The FHR-2 assay was calibrated using rhFHR-2. Assays for FHR-3, -4, and -5 were calibrated against a previously calibrated serum pool. **e–h** Standard curves for all immunoassays ranged from 0.156 to 10 ng/mL. For each assay, elongated curves are shown to visualize the sigmoidal dose-response curves. The coefficient of determination (R^2) was obtained using a non-linear regression curve fit.

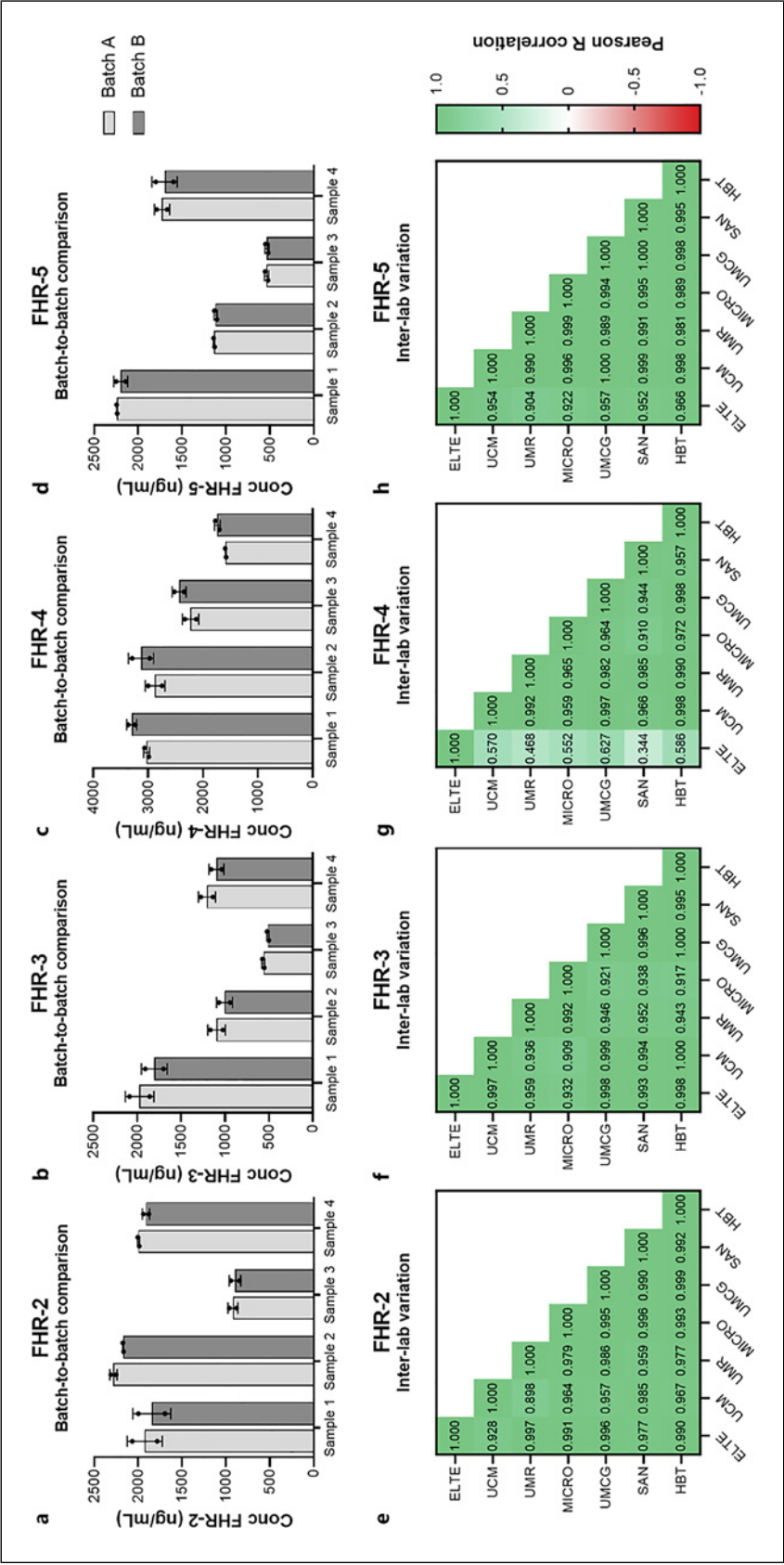


Fig. 3. Reproducibility of assay development and assay performance. **a–d** Batch-to-batch comparison. For each ELISA two independently produced batches were compared for their ability to quantify the FHR protein level in EDTA plasma samples ($n = 4$). **e–h** Inter-laboratory variation (variation in multiple determinations of a sample in several assay runs performed at different laboratories) was determined by comparison of the obtained data from seven different laboratories. Pearson's R correlation was used to determine whether the results from different laboratories correlate to each other. ELTE, Eötvös Loránd University (Hungary); UCM, Complutense University of Madrid (Spain); UMR, Philipps University Marburg (Germany); MICRO, Microcoat Biotechnology GmbH (Germany); UMC, University Medical Center Groningen (the Netherlands); SAN, Sanquin (the Netherlands); HBT, Hycult Biotech (the Netherlands).

Table 1. Intra- and inter-variation of FHR assays

FHR-2	Sample 1	Sample 2	Sample 3	Sample 4
<i>Intra-assay variation</i>				
Operator 1				
Mean aliquot 1–3, ng/mL	1,993	2,167	938	2,112
CV, %	2.1	7.8	14.3	12.2
Operator 2				
Mean aliquot 1–3, ng/mL	2,284	2,574	1,060	2,492
CV, %	4.6	12.2	5.9	3.4
<i>Inter-assay variation</i>				
Mean operator 1 and 2	2,139	2,371	999	2,302
CV, %	8.2	13.4	11.5	11.7
FHR-3	Sample 1	Sample 2	Sample 3	Sample 4
<i>Intra-assay variation</i>				
Operator 1				
Mean aliquot 1–3, ng/mL	1,110	675	358	802
CV, %	13.8	4.9	5.2	2.4
Operator 2				
Mean aliquot 1–3, ng/mL	1,072	635	353	813
CV, %	10.1	7.2	9.5	5.9
<i>Inter-assay variation</i>				
Mean operator 1 and 2	1,043	634	342	810
CV, %	7.2	6.1	5.3	1.3
FHR-4	Sample 1	Sample 2	Sample 3	Sample 4
<i>Intra-assay variation</i>				
Operator 1				
Mean aliquot 1–3, ng/mL	3,013	2,822	2,252	1,739
CV, %	3.8	4.5	5.2	3.0
Operator 2				
Mean aliquot 1–3, ng/mL	3,164	2,839	2,262	1,778
CV, %	1.6	2.0	5.8	2.9
<i>Inter-assay variation</i>				
Mean operator 1 and 2	3,088	2,857	2,256	1,754
CV, %	3.7	3.4	4.7	2.9
FHR-5	Sample 1	Sample 2	Sample 3	Sample 4
<i>Intra-assay variation</i>				
Operator 1				
Mean aliquot 1–3, ng/mL	2,346	1,191	584	1,792
CV, %	0.7	3.3	3.4	0.3
Operator 2				
Mean aliquot 1–3, ng/mL	2,222	1,166	569	1,739
CV, %	3.6	3.5	3.7	3.1
<i>Inter-assay variation</i>				
Mean operator 1 and 2	2,284	1,178	577	1,766
CV, %	3.7	3.3	3.5	2.5

Intra-assay variation (variation in multiple determinations of a sample within 1 assay run) and inter-assay variation (variation in multiple determinations of a sample in several assay runs) were determined for each factor H-related (FHR) assay by calculating the mean concentration and CV using three aliquots of four samples in two assay runs (performed independently by two operators). A CV below 15% indicates low variation for the intra- and inter-assay variation. CV, coefficient of variance.

Table 2. Summary of assay characteristics of the novel FHR assays

Assay	FHR-2	FHR-3	FHR-4	FHR-5
Standard range, ng/mL	10–0.156	10–0.156	10–0.156	10–0.156
Quantification limit (ULoQ–LLOQ), ng/mL	20–0.156	80–0.01	40–0.156	20–0.04
Recovery, %	91–101	88–116	81–110	89–97
Intra-assay variation, %	3.4–12.2	5.9–10.1	1.6–5.8	3.1–3.7
Inter-assay variation, %	8.2–13.4	1.3–7.2	2.9–4.7	2.5–3.7
Cross-reactivity against FH family	None	None	None	None
Cross-reactivity in other species (mouse, rat, pig, NHP)	Non-human primate	None	Non-human primate	None
Matrices to be used (^a = recommended)	EDTA plasma ^a Citrate plasma Heparin plasma Serum	EDTA plasma ^a Citrate plasma Heparin plasma Serum	EDTA plasma ^a Citrate plasma Heparin plasma Serum	EDTA plasma ^a Citrate plasma Heparin plasma Serum

Assay characteristics of the FHR-2, -3, -4, -5 immunoassays are summarized in an overview regarding assay range, quantification limits, recovery, inter-/intra-assay variation, cross-reactivity, and matrix information. ULoQ, upper limit of quantification; LLOQ, lower limit of quantification.

sample on ice. All samples were then measured and compared to the sample that was not subjected to any additional freeze-thaw cycles. For both the benchtop and freeze-thaw stability, a concentration varying between 80 and 120% of the reference/baseline sample was considered acceptable.

Intra- and Inter-Assay Variation

For the intra-assay variation, a comparison was made between multiple determinations of the same samples within a single ELISA plate. For the inter-assay and inter-lab variation, a comparison was made between multiple determinations of the same samples when independently tested by different operators/laboratories in a blinded fashion. A CV% <15% indicates low intra- and inter-assay variation and a low inter-laboratory variation.

Statistical Analysis

For statistical analysis and data visualization Graph-Pad Prism 9.5 was used. Non-linear regression (sigmoidal four-parameter-logistic (4 PL) curve fit) was used to determine the dose-response curves and subsequently to determine the coefficient of determination (R^2) of those curves. Pearson R correlation was used to determine the inter-laboratory variation.

One-way ANOVA (Dunnett's multiple comparisons test) was used to compare the effect of different incubation times on the benchtop stability of

EDTA plasma samples ($n = 4$). A $p < 0.05$ was considered statistically significant ($*p < 0.05$, non-significant [ns]).

Results

Specificity of FHR mAbs

Specificity of the mAbs was assessed using WB and direct ELISA. Fifteen mAbs were produced for FHR-1 (online suppl. Table 1; for all online suppl. material, see <https://doi.org/10.1159/000545139>). However, none of these antibodies could be used to develop a specific FHR-1 immunoassay as fourteen of these mAbs were cross-reactive to FH and/or several of the other FHR proteins. The mAb that showed no cross-reactivity to any of the FH family members was only reactive against the recombinant protein and did not recognize native human FHR-1 in serum (data not shown). As no specific mAbs could be generated, the development of a FHR-1 specific immunoassay was terminated.

For FHR-3, six new mAbs were generated (online suppl. Table 2). No cross-reactivity of anti-FHR-3.11 and anti-FHR-3.14 was observed using WB analysis and direct ELISA (Fig. 1c, d, i). Additionally, immunoprecipitation of native FHR-3 from NHS was performed and visualized on WB for each of the new mAbs, which showed the expected signal of 37–50 kDa for FHR-3

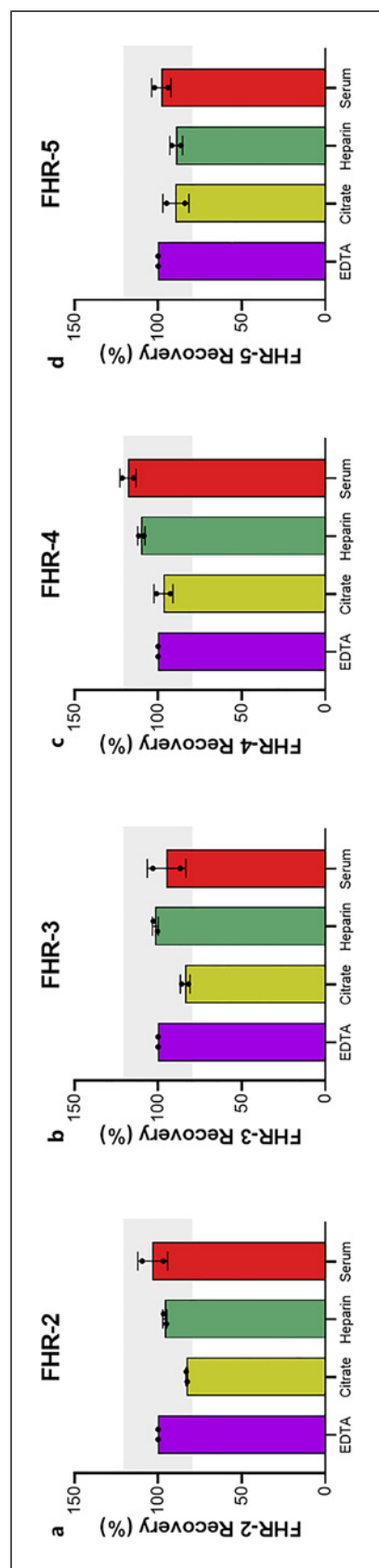


Fig. 4. a–d Matrix analysis A matrix analysis was performed by comparison of different sample types. The EDTA plasma sample (recommended sample type) was used as reference to determine the recovery in citrate plasma, heparin plasma and serum ($n = 2$). A recovery between 80 and 120% (depicted in the gray area) is considered a good recovery, indicating minimal differences between sample types.

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ine suppl. Fig. S1). Although no cross-reactivity was observed for anti-FHR-3.14 in multiple techniques, some cross-reactivity was observed when capturing FH using the FH.02 antibody in combination with anti-FHR-3.14 as detection antibody (online suppl. Fig. S2).

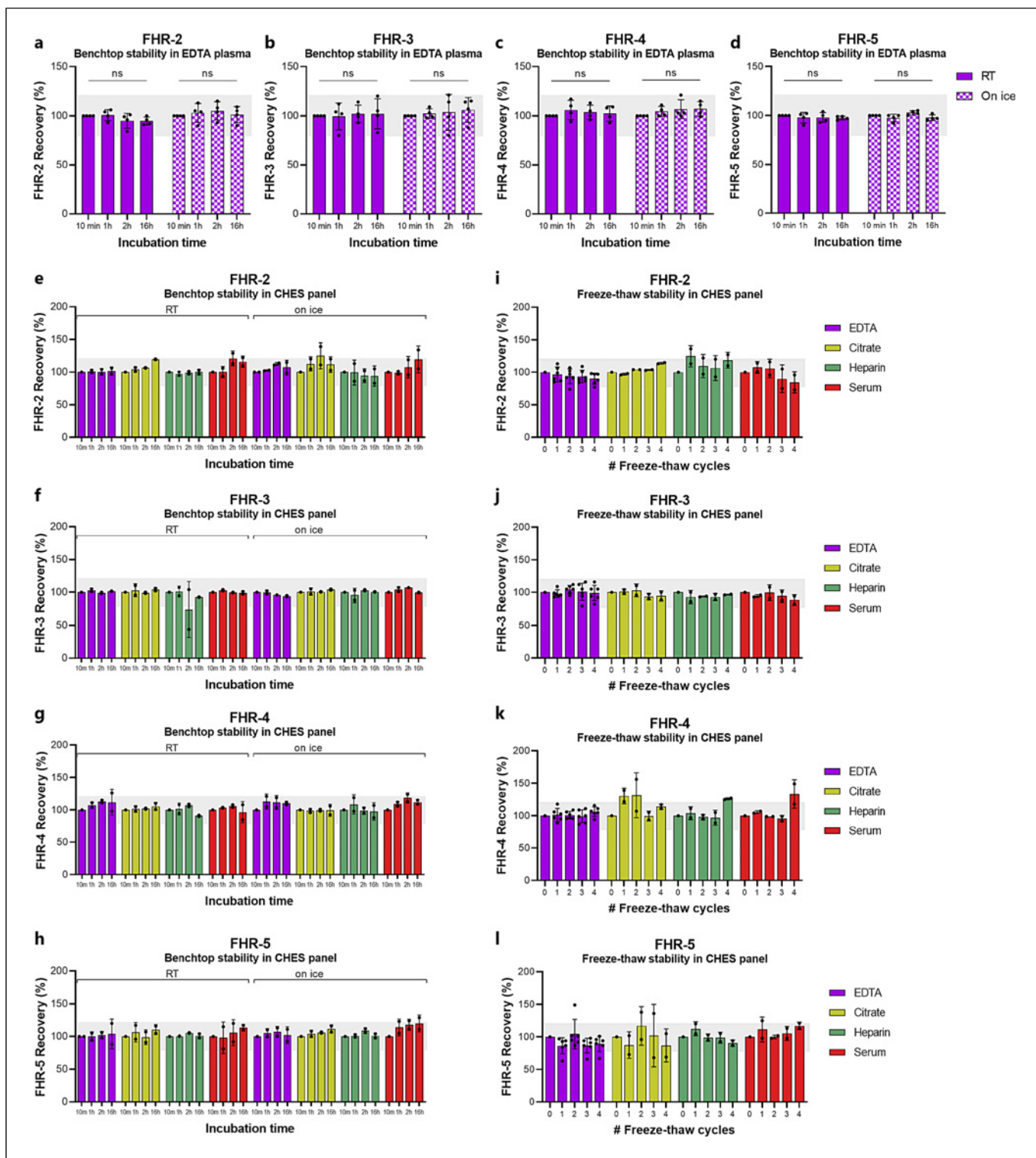
Previously generated mAbs were used for the recognition of FHR-2 [37], FHR-4 [31], and FHR-5 [29]. Specificity of these mAbs was reevaluated by WB analysis and direct ELISA as shown in Figure 1. Our data verify results on FHR-2 specificity of anti-FHR-2.11 and anti-FHR-2.12 (Fig. 1a, b, i) [37]. Next, we also confirmed the specificity of anti-FHR-4A.8 for FHR-4, while cross-reactivity of anti-FHR-4A.9 with both FHR-4 and FHR-3 was observed (Fig. 1e, f, i) [31]. Lastly, corresponding with earlier data, we showed specificity of anti-FHR-5.1 and anti-FHR-5.4 targeting FHR-5 (Fig. 1g, h, i) [29].

Development and Characterization of FHR-2, -3, -4, and -5 Sandwich ELISAs

To determine suitable antibody pairs, the antibodies were tested in each possible sandwich combination using cross-testing experiments (data not shown). The antibody pairs that were identified as most promising were anti-FHR-2.11 (capture) and anti-FHR-2.12 (detection) for measuring FHR-2, anti-FHR-3.11 (capture), and anti-FHR-3.14 (detection) for measuring FHR-3, anti-FHR-4A.8 (capture), and anti-FHR-4A.9 (detection) for measuring FHR-4, and anti-FHR-5.4 (capture), and anti-FHR-5.1 (detection) for measuring FHR-5. All further experiments were performed using the aforementioned antibody pairs. Note that the selected FHR-4 antibody pair differs from the previously published FHR-4A specific ELISA by Pouw et al. [31] (2018), whereas the selected anti-FHR-5 antibody pair is identical as the pair used by van Beek et al. [29] (2017) but reversed in respective use (coat vs. detection).

Using the identified mAb pairs, four novel sandwich ELISAs were developed for quantification of FHR-2, -3, -4, and -5 (Fig. 1j). While clone anti-FHR-4A.9 is cross-reactive with FHR-3, combining it in a sandwich ELISA with the specific anti-FHR-4A.8 antibody as capturing antibody ensures assay specificity for FHR-4 as shown in Figure 1f.

To determine whether the ELISAs could also be used for measuring FHR-2, -3, -4 and -5 levels in species other than humans, cross-reactivity was investigated in samples obtained from mouse, rat, pig, and non-human primates. The assays for FHR-3 and -5 showed no cross-reactivity against other species. In the ELISA for FHR-2 and -4,



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(For legend see next page.)

cross-reactivity was observed for non-human primates (*Macaca fascicularis*) (Fig. 1k–n).

For enabling quantitative measurements of the target protein, a standard curve was created by calibrating a pool of normal human EDTA plasma against a previously calibrated serum pool with a known concentration for the corresponding FHR protein (FHR-3, -4, and -5) [29–31], or against the recombinant protein when no calibrated plasma or serum pool was available (FHR-2). For each of the developed assays, the new standard curves, based on the plasma pool, were similar to the curves obtained using the initial calibrator material (Fig. 2a–d).

For each of the ELISAs, an elongated standard curve was assessed to determine the upper and lower limit of quantification (ULoQ and LLoQ) and to visualize the sigmoidal shape of the dose-response curves. For the FHR-2 assay, the curve progression was linear between 1.25 and 20 ng/mL (ULoQ: 20 ng/mL, LLoQ: 0.156 ng/mL) and resulted in a coefficient of determination of $R^2 = 0.9995$ (Fig. 2e). For the FHR-3 assay, the curve was linear between 0.625 and 10 ng/mL (ULoQ: 80 ng/mL, LLoQ: 0.01 ng/mL) with $R^2 = 0.9992$ (Fig. 2f). The FHR-4 assay showed a linear progression between 1.25 and 20 ng/mL (ULoQ: 40 ng/mL, LLoQ: 0.156 ng/mL) with $R^2 = 0.9995$ (Fig. 2g) and the FHR-5 assay showed linearity between 1.25 and 20 ng/mL (ULoQ: 20 ng/mL, LLoQ: 0.04 ng/mL) with $R^2 = 0.9986$ (Fig. 2h). For each ELISA, sample linearity and parallelism with the standard curve was evaluated. Linearity was assessed by calculating the CV across at least three consecutive sample dilutions. A CV below 15% was considered indicative of good linearity. This requirement was met for each of the ELISAs (data not shown).

Accuracy and Reproducibility of the Novel FHR ELISAs

To assess assay accuracy and reproducibility, recovery of the target analyte and both intra- and inter-assay variation were evaluated. First, recovery of the FHR proteins in each subsequent assay was analyzed in EDTA

plasma samples with a known FHR concentration by mixing them in different ratios: 100:0, 75:25, 50:50, 25:75 and 0:100. Results showed an adequate recovery for each assay, with results between 80 and 120% (online suppl. Table 3).

Next, the performances of two ELISA batches were compared to evaluate for possible batch-to-batch differences. Both batches were produced independently of each other, using antibodies from two independent hybridoma cultures. A small set of EDTA plasma samples ($n = 4$) were measured in both ELISA batches and concentrations were compared to determine whether assay performance was similar. Overall, differences in sample values between the two batches were within set criteria (CV <15%, FHR-2: CV = 2.6–4.8%, FHR-3: CV = 8.3–9.0%, FHR-4: CV = 9.0–9.4%, FHR-5 = 0.6–2.0%), indicating minimal batch-to-batch variation (Fig. 3a–d).

Furthermore, reproducibility of the assays was evaluated by assessing the intra-assay variation (variation of multiple measurements of the same sample in a single test run) and the inter-assay variation (variation of multiple measurements of the same sample in several test runs performed by different operators). Each of the assays showed an adequate inter- and intra-assay variation with CV values below 15% (Table 1).

In addition, assay reproducibility was further assessed by measuring and comparing the same set of samples at seven different laboratories, spread across Europe. For the FHR-2, -3, and -5 assay, sample concentrations measured at different locations strongly correlated ($R^2 \geq 0.898$, ≥ 0.909 , ≥ 0.904 , respectively, Fig. 3e, f, h), indicating good assay reproducibility. Results obtained for the FHR-4 assay showed strong correlations between sample values measured at six different locations ($R^2 \geq 0.910$), while the correlations for one location were low to average ($R^2 = 0.344$ – 0.627 , Fig. 3g). All single determinations from each laboratory were also compared to all other laboratories and summarized in online supplementary Table 4.

Fig. 5. The effect of sample type and pre-analytical sample handling on observed FHR protein levels. The effect of sample handling on the assessment of factor H-related protein (FHR) levels is evaluated by testing the sample benchtop stability and the freeze-thaw stability. For benchtop stability testing, samples were stored at room temperature (RT) or on ice on indicated time intervals ranging from 10 min (min) to 16 h (h). The concentrations of each FHR was measured for each incubation time and sample concentrations were compared to the 10-min reference sample using a one-way ANOVA. A recovery between 80 and 120% is considered a good recovery (depicted in gray area).

a–d The sample benchtop stability is performed on EDTA plasma samples ($n = 4$). Comparison of the samples to the 10-min reference sample showed no significant differences. **e–h** A CHES panel ($n = 2$) was used to determine the effect of sample type on benchtop stability. For each sample type the sample recovery was compared to the 10-min reference samples. **i–l** To determine the freeze-thaw stability, sample aliquots were exposed to 0–4 freeze-thaw cycles prior to assessing the FHR protein concentrations. A CHES panel ($n = 2$) and several additional EDTA plasma samples ($n = 4$) were evaluated. All samples were compared to a reference sample (no freeze-thaw cycles).

Overall, each ELISA showed to be able to measure the FHR proteins in a specific, accurate, and reproducible manner. The assay characteristics of each of the novel ELISAs are summarized in Table 2.

Evaluation of Sample Matrix, Sample Benchtop Stability, and Sample Freeze-Thaw Stability

As mentioned earlier, accurately measuring complement protein levels in general can be challenging. One reason might be the spontaneous *ex vivo* activation of complement during sample processing. Hence, appropriate pre-analytical sample handling is a necessity to avoid erroneous results [25, 39]. Factors that might influence (the degree of) spontaneous complement activation are sample type (matrix influence), benchtop stability (the duration samples remain stable at RT or on ice), and freeze-thaw stability (the number of freeze-thaw cycles a sample can endure without affecting protein level). Therefore, these aspects were also investigated for the newly developed FHR ELISAs. To evaluate matrix influence, EDTA plasma, the preferred sample type for measuring individual components of complement activation, was compared to citrate plasma, heparin plasma, and serum (CHES panel). For this, CHES panels of two donors were used. The mean level observed in EDTA plasma for each FHR protein was used as a reference value (100%) and compared to the other sample types. For all tested matrices, each of the assays resulted in a target recovery between 80 and 120%, indicating a minimal impact of sample type (Fig. 4).

Next, benchtop stability and the freeze-thaw stability were assessed. Also here, the criterium was set to 80–120% recovery when compared to a reference sample (10-min sample incubation at RT or on ice). Benchtop stability was tested for several individual EDTA samples ($n = 4$) next to two CHES panels. For each FHR assay, EDTA samples were stable for at least 16 h at RT or on ice (Fig. 5a–d). Although the variation in observed FHR levels increased over time, no significant differences were observed in EDTA samples when compared to the reference sample. For the CHES panels, a similar pattern was observed (Fig. 5e–h). In a few individual cases, levels were found to be slightly below 80% or above 120% recovery. However, in the majority of the cases, the mean recovery of the two measurements was within range.

Subsequently, freeze-thaw stability was evaluated for the aforementioned matrices. Results show that for each FHR protein, the levels largely remained within range and did not change with the number of freeze-thaw cycles (Fig. 5i–l). A limited number of exceptions were found in which the recovery was slightly above the accepted 120%.

As expected, variation in levels and inconsistency of measurements increased with the number of freeze-thaw cycles. This was most evident for serum and citrate plasma samples. Based on these results, EDTA and heparin plasma samples seem to be less sensitive for freeze-thawing. Overall, these results show that the FHR concentrations, obtained using these newly developed ELISAs, are robust and are minimally affected by sample type and/or pre-analytical sample handling.

Discussion

Unlike FH, the functions and physiological roles of the FHR proteins remain largely elusive. They share structural similarity with FH, but lack corresponding regulatory domains, suggesting differences in their complement-modulating activities. However, studying their role in health and disease has been hampered by limited availability of specific antibodies and assays. Next to that, existing tools often lack the specificity and sensitivity required to accurately detect and quantify FHRs in biological samples in a standardized way [27, 28]. While use of specific reagents is crucial for reliable testing, the similarity in amino acid sequence among the FH protein family complicates efforts to develop specific antibodies tailored to each protein. To overcome this challenge, we successfully developed four ELISAs enabling us to quantify total protein levels of FHR-2, -3, -4, and -5 in plasma and serum samples in a highly specific, accurate, and reproducible manner. The antibodies used in the ELISA development are highly specific as no cross-reactivity was observed for FH nor for FHR proteins other than their targets, in both a direct and sandwich ELISA setup. This was further confirmed by WB analysis. The only exception was the previously reported anti-FHR-4A.9 antibody, which did not only recognize FHR-4 but was also cross-reactive with FHR-3 [31]. By using this mAb with the highly specific clone anti-FHR-4A.8 as capturing antibody, no cross-reactivity was observed in the FHR-4 sandwich ELISA. For the anti-FHR-3.14 antibody, the specificity for FHR-3 was debatable as no cross-reactivity was shown in direct ELISA, WB, or immunoprecipitation, but cross-reactivity was shown when using the anti-FHR-3.14 mAb as detection antibody in a sandwich ELISA in which FH was captured using an anti-FH antibody (anti-FH.02). No further tests were performed as it was shown that anti-FHR-3.11 shows no cross-reactivity and thereby ensures specificity of the antibody pair. Unfortunately, we were not able to develop an immunoassay for FHR-1 as we were unsuccessful in

generating FHR-1 specific antibodies. Generating specific reagents for FHR-1 is challenging as the first two N-terminal domains share a 98–100% sequence similarity to FHR-2 and the remaining three domains share a 95–100% sequence similarity to FH [7].

The newly developed immunoassays showed low intra- and inter-assay variation as well as high recovery of the target protein. Furthermore, no batch-to-batch variation between two independently produced ELISA batches was observed. Next, an inter-laboratory comparison test was conducted assessing the performance of the assays over seven different laboratories. Sample concentrations measured at different laboratories highly correlated with each other. For FHR-4, high correlations between sample values were observed for six different laboratories, while the correlations for one laboratory were low to average. This was likely due to performance and/or pipetting errors, and the FHR-4 results from this location were considered outliers. Overall, these results demonstrate that FHR levels can now be quantified in an accurate and reproducible manner.

Robust immunoassays are particularly important within the complement field as it has been shown that measuring complement components in a reliable way is challenging [25, 27]. Large variations can be induced by differences in sample type and pre-analytical sample handling like sample collection and storage conditions among others [25, 27, 40]. In general, it is strongly recommended to use EDTA plasma for analyzing complement activation components [40]. In addition, it is advised to thaw samples on ice, assess them within 1 h, and prevent multiple freeze-thaw cycles [25, 41]. Our overall results regarding sample type and pre-analytical sample handling showed that FHR concentrations in individual samples remained stable over time in most cases. Concentrations for the majority of samples that were left on the bench for 16 h at RT were still within set criteria. Regarding sample type, protein levels seem to fluctuate more in citrate plasma and serum samples that are incubated for a longer time (>2 h) versus samples that are used within 1 h after thawing. These data illustrate that, over time, the FHR proteins become less stable. EDTA plasma and heparin plasma samples seemed to be more stable over time. However, studies showed binding of FH, FHR-1, and FHR-5 to heparin [42, 43]. Therefore, the use of heparin as anticoagulant could interfere with reliable detection of these proteins. Also, the assessment of the freeze-thaw stability showed that FHR concentrations remained stable after multiple freeze-thaw cycles. However, as expected, it is observed that after an increasing number of freeze-thaw cycles, the variation

between measurements can increase. This was mostly the case in citrate plasma and serum samples. Similar as with the benchtop stability, EDTA plasma and heparin plasma show to be less affected by sample handling. These data support earlier research showing that EDTA plasma is less sensitive to repeated freeze-thawing than other types of plasma samples or serum [41, 44]. Although the assessment of the benchtop stability and freeze-thaw stability showed only minimal differences in measuring FHR levels, it is still deemed to follow the recommendations of using EDTA plasma within 1 h after thawing the sample on ice [25, 41].

This study has several limitations. When studying cross-reactivity of the newly developed antibodies, only recombinant FHR proteins were used as no plasma-derived native protein was available. Recombinant proteins can have structural differences to native proteins due to post-translational modifications. These structural differences may affect protein folding and, therefore, epitope binding/accessibility, resulting in an altered affinity of the antibody for the target analyte. To minimize the differences between native and recombinant proteins, the rhFHRs were produced in human cells. Furthermore, our WBs show similar results to earlier published immunoprecipitations performed with these mAbs, suggesting that the mAbs have a similar recognition to plasma-derived FHRs and the rhFHRs [29, 31, 37].

Next to that, the sample set evaluated in this study is limited in size and only derived from healthy volunteers. Except for gender, no additional information was available for these samples. The goal of this study was to develop novel ELISAs and in that light, these samples are only used for technical evaluation of assay properties rather than determining reference ranges in healthy individuals. Determining these reference ranges is important for the interpretation of results and can aid to further unravel functions of the FHRs and their role in disease [45]. Furthermore, in future studies, it would also be of added value to explore other matrices beyond blood as there are several complement-associated diseases known to target specific organs like e.g., the kidneys or eyes among others [8, 10].

Lastly, FHR-1, -2, and -5 circulate as homodimers (FHR-1/1, -2/2, and -5/5) and as FHR-1/2 heterodimers [29]. The newly developed ELISA kits for FHR-2 and FHR-5 quantify total protein levels, and thus, all types of species are quantified. Although it is known that specific protein characteristics, such as affinity for ligand-binding, are influenced by dimer formation [20], the clinical significance of quantifying specific dimers has yet to be determined.

In summary, we have developed four novel ELISAs for the quantification of FHR-2, -3, -4, and -5 protein levels in plasma and serum samples. The newly developed ELISAs show a high target recovery, no cross-reactivity against other FH family members and good intra- and inter-assay precision, indicating that the quantification of the FHR proteins in these ELISAs is accurate and robust. These novel ELISAs will add in our further understanding of the function of the FHR proteins and their role in disease pathology.

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Statement of Ethics

All experimental protocols for mouse immunizations adhered to institutional standards for animal care and use and received approval from the Central Committee for Animal Use and IVD at the Netherlands Cancer Institute (Amsterdam, the Netherlands). Human samples were used for the technical evaluation of the assay properties. These samples were all ordered from BioIVT (New York, NY, USA). The following statement concerning informed consent is published by BioIVT [46]: “The Department of Health and Human Services regulations for the protection of human subjects (45 CFR §46.116 and §46.117) and Good Clinical Practice (GLP), (ICH E6 R2) require that informed consent will be sought from each prospective subject or the subject’s legally authorized representative and will be appropriately documented in writing.”

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Conflict of Interest Statement

E.J.M.T. and M.R. are employees of Hycult Biotech. R.B.P. and M.C.B. are co-inventors of patents and patent applications describing the therapeutic use of anti-FH antibodies. All other authors have no conflicts of interest to declare.

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Author Contributions

M.R., B.R.J.V., E.J.M.T., and R.B.P. designed and supervised the study. M.R., B.R.J.V., M.C.B., and G.M. performed the experiments and analyzed the data. E.J.M.T., M.R., R.B.P., B.R.J.V., L.L.C., E.G.J., B.U., A.M.M.A., M.J., G.M., A.M.M.A., F.P., and D.P. contributed to the inter-laboratory testing. M.R. and E.J.M.T. wrote the first draft of the manuscript. B.R.J.V. and R.B.P. critically evaluated the manuscript. All authors contributed to the article and approved the submitted version.

Data Availability Statement

The raw data supporting the findings of this study alongside the assay development trajectory are intellectual property of Hycult Biotech and are, therefore, regarded as confidential and not publicly available. This raw data will be made available upon reasonable request by the corresponding author (E.J.M.T.); Erik J.M. Toonen, R&D department Hycult Biotech b.v., Frontstraat 2A, 5405 PB, Uden, the Netherlands, e.toonen@hycultbiotech.com.

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