D6.1
Technical and sample validation report for ELISA

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3	06/14/2024	Added Figure 4.1	Bert Veuskens (SAN)
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Disclaimer

Summary

This report summarizes activities performed within D6.1, task 6.1-6.5: technical and sample validation for the FHR-2, 3, 4 and 5 ELISAs and follows up on the work performed within D2.2; Protocols for single ELISA detections systems. Using the newly generated antibodies (D2.1), four ELISAs were developed that are able to quantify FHR-2, FHR-3, FHR-4 and FHR-5 plasma levels in an accurate and specific manner (D2.2). In D6.1, assay development was continued and a first large prototype batch was produced according industrial standards. This batch was used to further investigate accuracy as well as reproducibility of the assays to ensure consistent and reliable results across different experiments (task 6.1). Results show that assays enable specific and accurate quantification of FHR-2, FHR-3, FHR-4, and FHR-5 levels in human plasma. In addition, an inter-laboratory variation experiment was performed to further assess assay reproducibility (task 6.2). Sample values, measured at different locations, highly correlated with each other indicating high reproducibility. Next, the utility of the assays in quantifying FHR proteins in body fluids other than plasma or serum was tested (task 6.3) and results show that these assays are also useful for quantifying the presence of FHR proteins in urine samples. As a proof-of-concept study (task 6.4), assays were used to measure FHR levels in a large cohort of 201 Dutch healthy controls. Results will be used to set a healthy control reference range. Larger clinical studies (task 6.5) are ongoing and diseases that are currently under investigation are: COVID-19, Osteoarthritis, AMD, post-operative delirium, membranous nephropathy (MN) and ANCA-vasculitis (AAV). Assay development and validation for all four FHR ELISA was successfully concluded and assays are commercially available. No deviations from DoW were reported.

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1. Sensitivity, specificity, accuracy, reproducibility (task 6.1)

For all four FHR assays, basic aspect regarding assay sensitivity (upper/lower limit of detection, range, linearity, matrix analysis), specificity (cross-reactivity FHR family members) and accuracy (recovery) were previously investigated during the feasibility phase and reported before (see periodic report 1 + 2 and deliverable report D2.2). For D6.1, assay was further developed using a first large prototype assay batch that was produced according industrial standards. This batch was used to further investigate accuracy as well as reproducibility of the assays to ensure consistent and reliable results across different experiments. Aspects that were investigated are (a.o.): upscaling, intra/inter variation, sample stability, stability assay components, batch-to-batch variation, cross reactivity different species and robustness.

1.1. Upscaling assay production and calibration of 'golden standard'.

Firstly, a large assay batch was produced to further investigate assay performance and stability. Upscaling consists of production and testing of the three main assay components: pre-coated plate batch, calibration and lyophilization of the standard and lyophilization of the detection antibody.

Plate batches passed quality control (QC) (Table 1.1). During QC, a fixed amount of FHR protein with known concentration is measured using randomly selected strips from plates selected from the plate batch. In total, 4x12 strips were selected and compared with each other regarding variation in OD signal. Variation between values within 1 strip, within 1 plate and over total number of QC plates is evaluated using the following requirements:

- Intra variation in 1 strip CV% <10
- Intra variation in 1 plate CV% <10
- Interplate variation CV% <15

CV= coefficient of variance

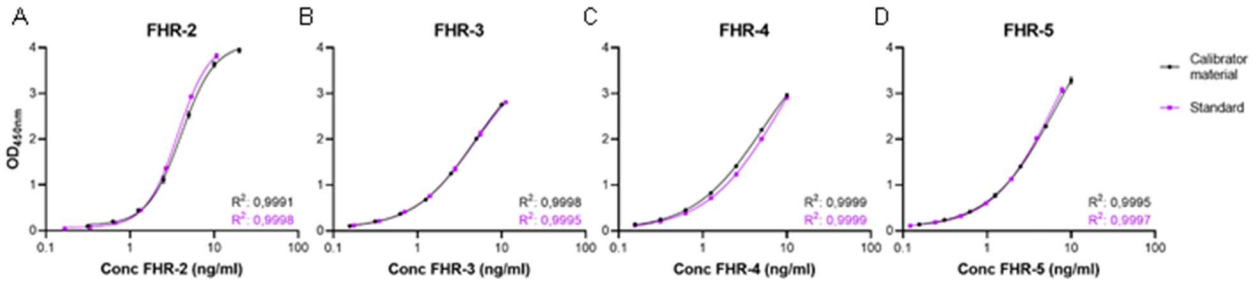
Table 1.1. Plate batch quality control.

	%CV (mean±SD)				
	Strip		Plate		Interplate*
FHR-2	4.3	1.4	5.5	0.9	8.3
FHR-3	4.3	1.5	5.6	2.2	8.9
FHR-4	2.6	0.9	3.2	1.1	4.5
FHR-5	3.4	2.2	3.9	1.6	4.4

Strip, plate and interplate (plate batch) variation (%CV) for each FHR assay. *As only 1 assay batch was produced, no mean and SD could be calculated for interplate variation.

Next, a ‘golden’ standard (calibration curve) batch was produced that will be used as internal control for further assay production in the future. The standard curve is 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. FHR-2 was calibrated against full length rhFHR-2. FHR-3, -4, and -5 were calibrated against a previously calibrated serum pool (Figure 1.1).

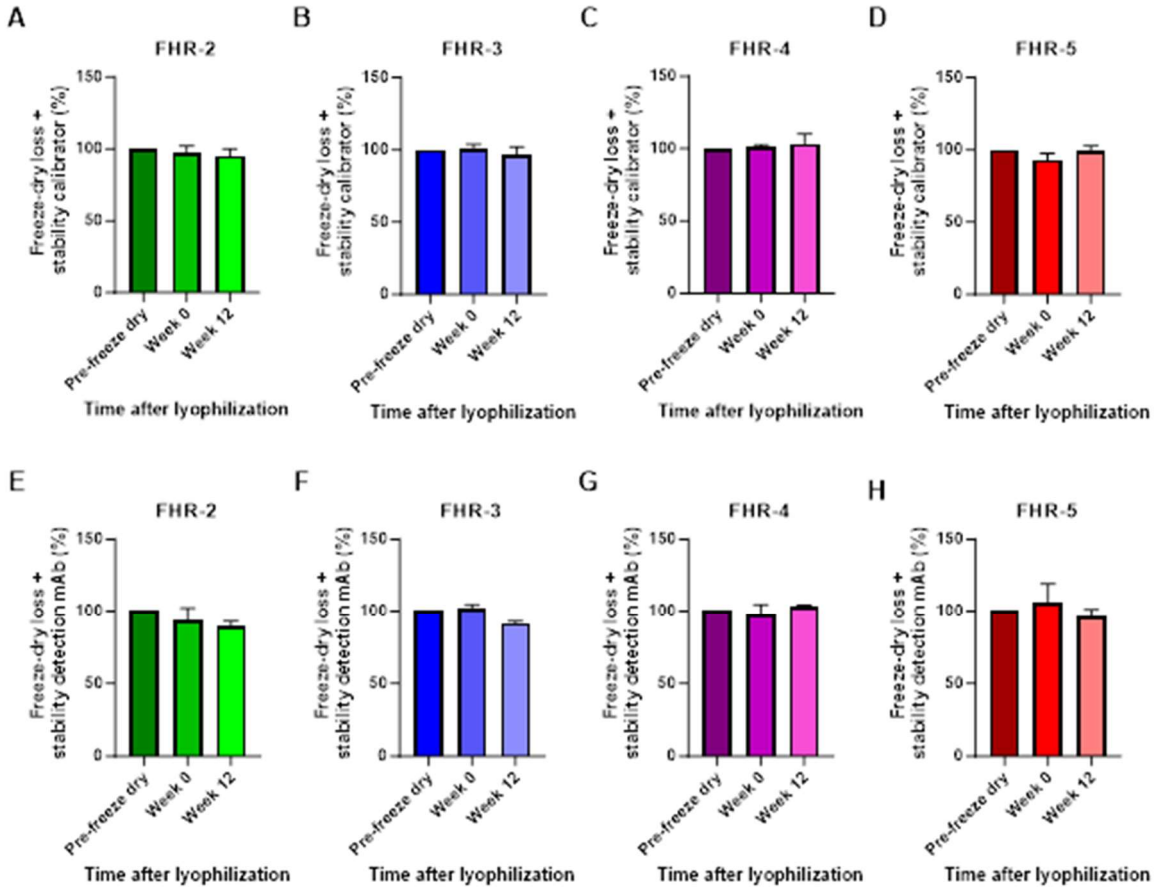
Figure 1.1 Calibration of the standard.



Calibration of the standard for (A) FHR-2, (B) FHR-3, (C) FHR-4 and (D) FHR-5. The coefficient of determination (R2) was obtained using a non-linear regression curve fit.

Freeze-dry loss and stability for both the calibrator and the detection antibody was assessed. To determine freeze-dry loss, the amount of FHR protein or labeled anti-FHR detection antibody was measured before lyophilization and this amount was set to 100%. Next, both components were lyophilized, re-measured and compared to the pre-lyophilized components. Next, real-life stability up to 12 weeks was tested. Results show minimal freeze-dry loss of both the calibrators and detection antibodies for all four FHRs assays. Next to that, components remained stable for up to 12 weeks (Figure 1.2).

Figure 1.2 Freeze-dry loss and stability of FHR calibrators and detection antibodies



Freeze-dry loss and stability of FHR calibrators (A-D) and detection antibodies (E-H) Freeze-dry loss was evaluated by comparing the components before lyophilization (pre-freeze-dry) *vs.* after lyophilization (week 0). Stability was evaluated by comparing week 0 *vs*. week 12.

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 that CV% values for inter- and intra-assay variation were <15%, indicating a good inter- and intra-assay precision (Table 1.2).

Table 1.2: Intra- and inter-variation of FHR assays

FHR-2		**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Intra-assay variation					
Operator 1	Mean aliquot 1-3 (ng/ml)	1993	2167	938	2112
	CV (%)	*2.1*	*7.8*	*14.3*	*12.2*
Operator 2	Mean aliquot 1-3 (ng/ml)	2284	2574	1060	2492
	CV (%)	*4.6*	*12.2*	*5.9*	*3.4*
Inter-assay variation					
	Mean operator 1 & 2	2139	2371	999	2302
	CV (%)	*8.2*	*13.4*	*11.5*	*11.7*

FHR-3		**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Intra-assay variation					
Operator 1	Mean aliquot 1-3 (ng/ml)	1110	675	358	802
	CV (%)	*13.8*	*4.9*	*5.2*	*2.4*
Operator 2	Mean aliquot 1-3 (ng/ml)	1072	635	353	813
	CV (%)	*10.1*	*7.2*	*9.5*	*5.9*

Inter-assay variation					
	Mean operator 1 & 2	1043	634	342	810
	CV (%)	*7.2*	*6.1*	*5.3*	*1.3*

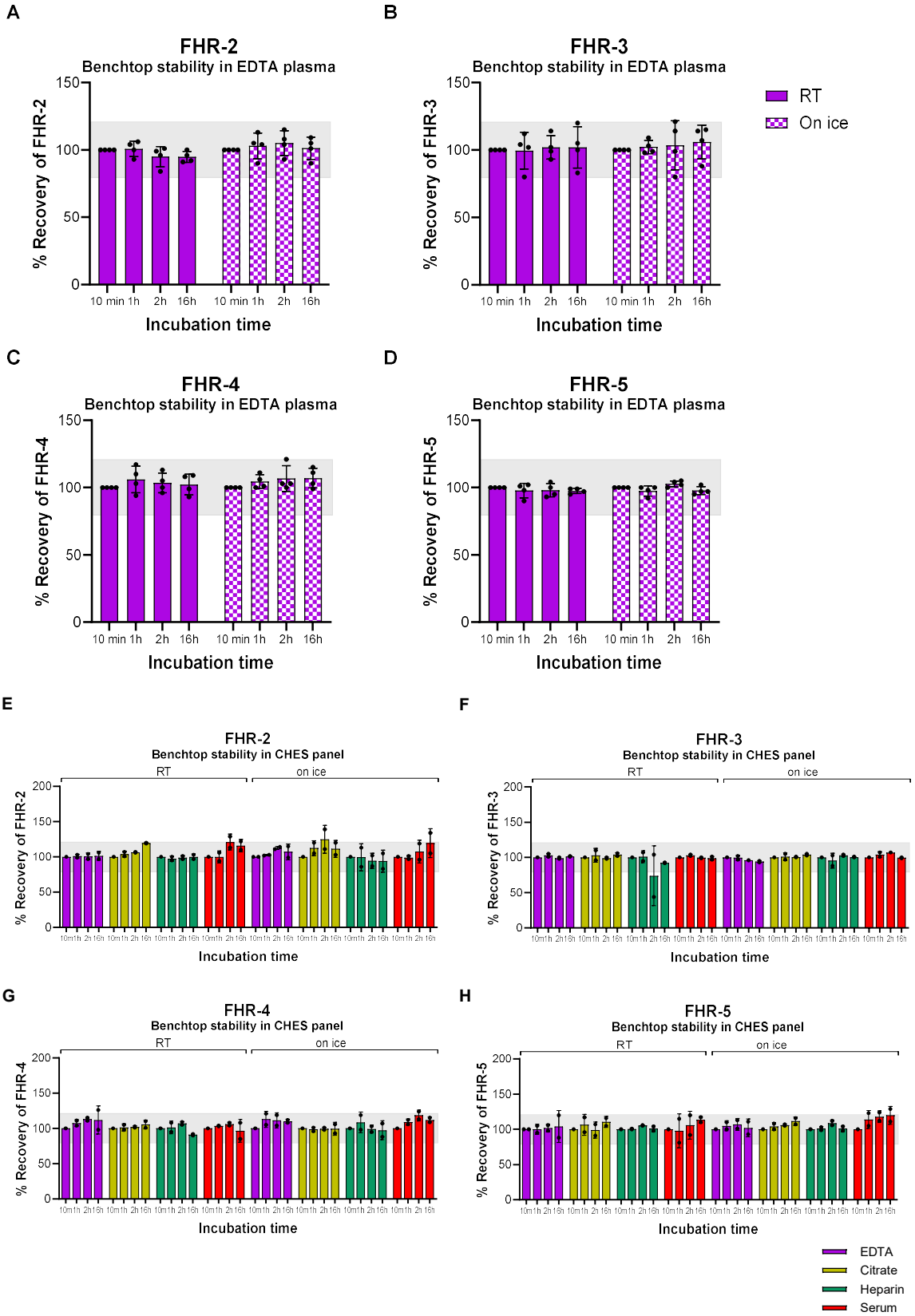
FHR-4		**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Intra-assay variation					
Operator 1	Mean aliquot 1-3 (ng/ml)	3013	2822	2252	1739
	CV (%)	*3.8*	*4.5*	*5.2*	*3.0*
Operator 2	Mean aliquot 1-3 (ng/ml)	3164	2839	2262	1778
	CV (%)	*1.6*	*2.0*	*5.8*	*2.9*
Inter-assay variation					
	Mean operator 1 & 2	3088	2857	2256	1754
	CV (%)	*3.7*	*3.4*	*4.7*	*2.9*

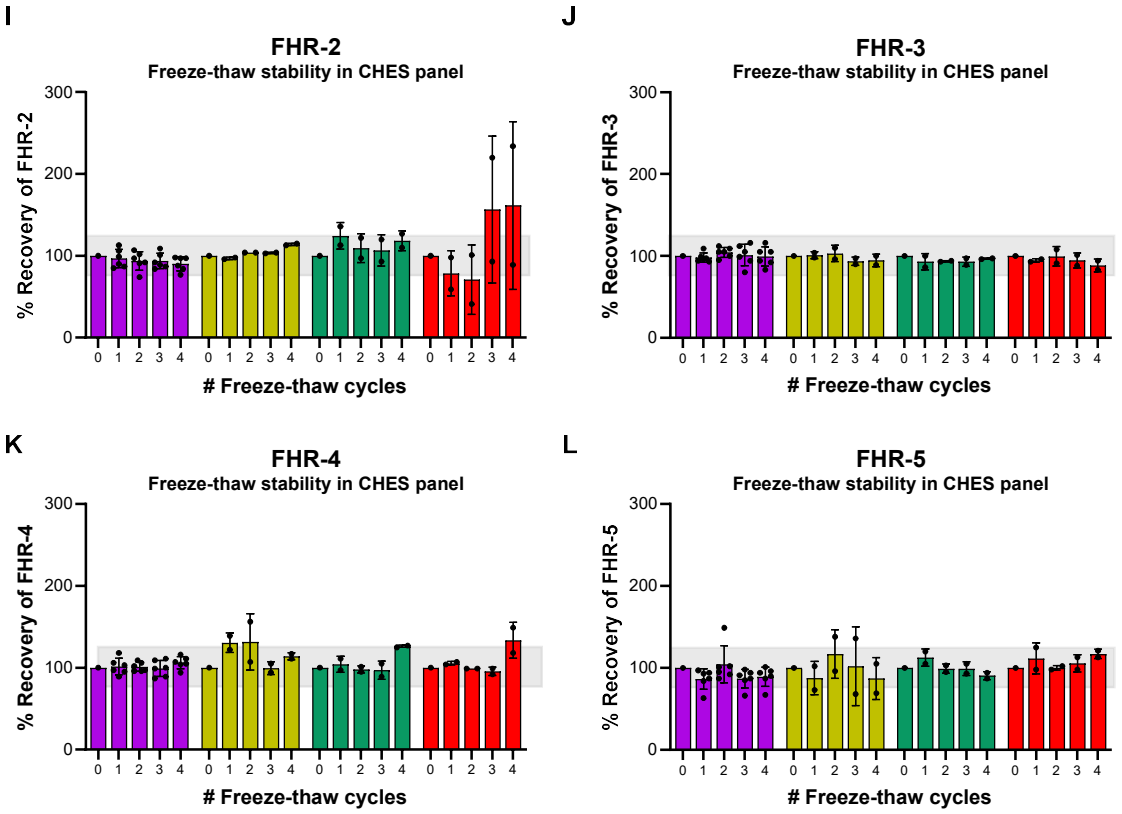
FHR-5		**Sample 1**	**Sample 2**	**Sample 3**	**Sample 4**
Intra-assay variation					
Operator 1	Mean aliquot 1-3 (ng/ml)	2346	1191	584	1792
	CV (%)	*0.7*	*3.3*	*3.4*	*0.3*
Operator 2	Mean aliquot 1-3 (ng/ml)	2222	1166	569	1739
	CV (%)	*3.6*	*3.5*	*3.7*	*3.1*
Inter-assay variation					
	Mean operator 1 & 2	2284	1178	577	1766
	CV (%)	*3.7*	*3.3*	*3.5*	*2.5*

1.3. Benchtop and freeze-thaw stability

Next, the impact of sample handling is evaluated by the benchtop stability (the time a sample is stable at RT or on ice) and the freeze-thaw stability (the number of freeze-thaw cycli a sample can tolerate before protein levels are affected). In addition, benchtop stability of the standard was also tested. Requirements for evaluation of these aspects was set to 80-120% recovery when compared to a reference sample (10 min. RT or on ice). Benchtop stability was tested for several individual EDTA samples (n=4) next to two CHES (citrate, heparin, EDTA and serum) panels. For each FHR assay it was observed that EDTA samples are stable for at least 16 hours at RT or on ice (Figure 1.3). Although the variation in observed FHR levels increases over time, no significant differences were observed in EDTA samples when compared to the reference sample (10 minute sample incubation at RT or on ice). For the CHES panels, a similar pattern was observed as for the individual EDTA plasma samples. Although variation in observed FHR levels increased over time, in general they did not differ significantly from the reference sample (Figure 1.3). Subsequently, freeze-thaw stability was evaluated for the aforementioned sample types/matrices. Results show that for each FHR protein, the levels remained within range and did not significantly change with the number of freeze-thaw cycli (Figure 1.3). However, as expected, variation in levels and inconsistency of measurements increased with the number of freeze-thaw cycli. This was most evident for serum samples 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 quite robust and are minimally affected by sample type and/or sample handling.

Figure 1.3 Benchtop and freeze-thaw stability of samples



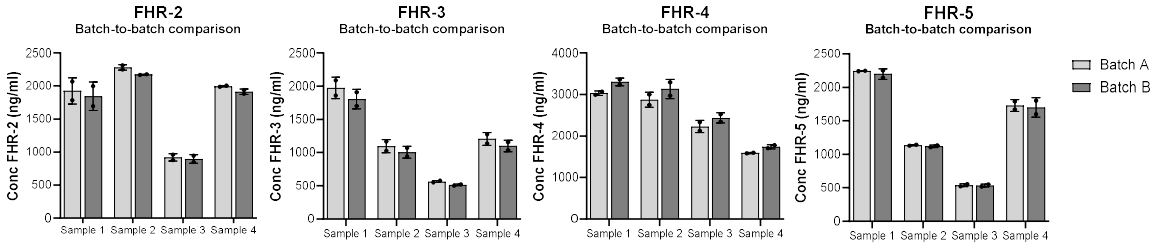


Evaluation of benchtop (A-H) and freeze-thaw (I-L) stability of EDTA plasma and CHES samples

1.4. Batch-to-batch variation

Assay performance of two different ELISAs batches were compared to evaluate (possible) batch-to-batch differences. Batch A and batch B were produced independently of each other, using antibodies from two independent culture batches. A small set of EDTA plasma samples (n=4) were measured in both ELISA batches and concentrations were compared to determine whether performance between batches was similar. Overall, differences in sample values between batch A and B were low (CV% values ranging from 2.6-4.8% for FHR-2; 8.3-9.0% for FHR-3; 9.0-9.4% for FHR-4 and 0.6-2.0% for FHR-5) and did not differ significantly from each other. All observed CV% values were below <15%, indicating low batch-to-batch variation (Figure 1.4).

Figure 1.4 Reproducibility of assay development and assay performance

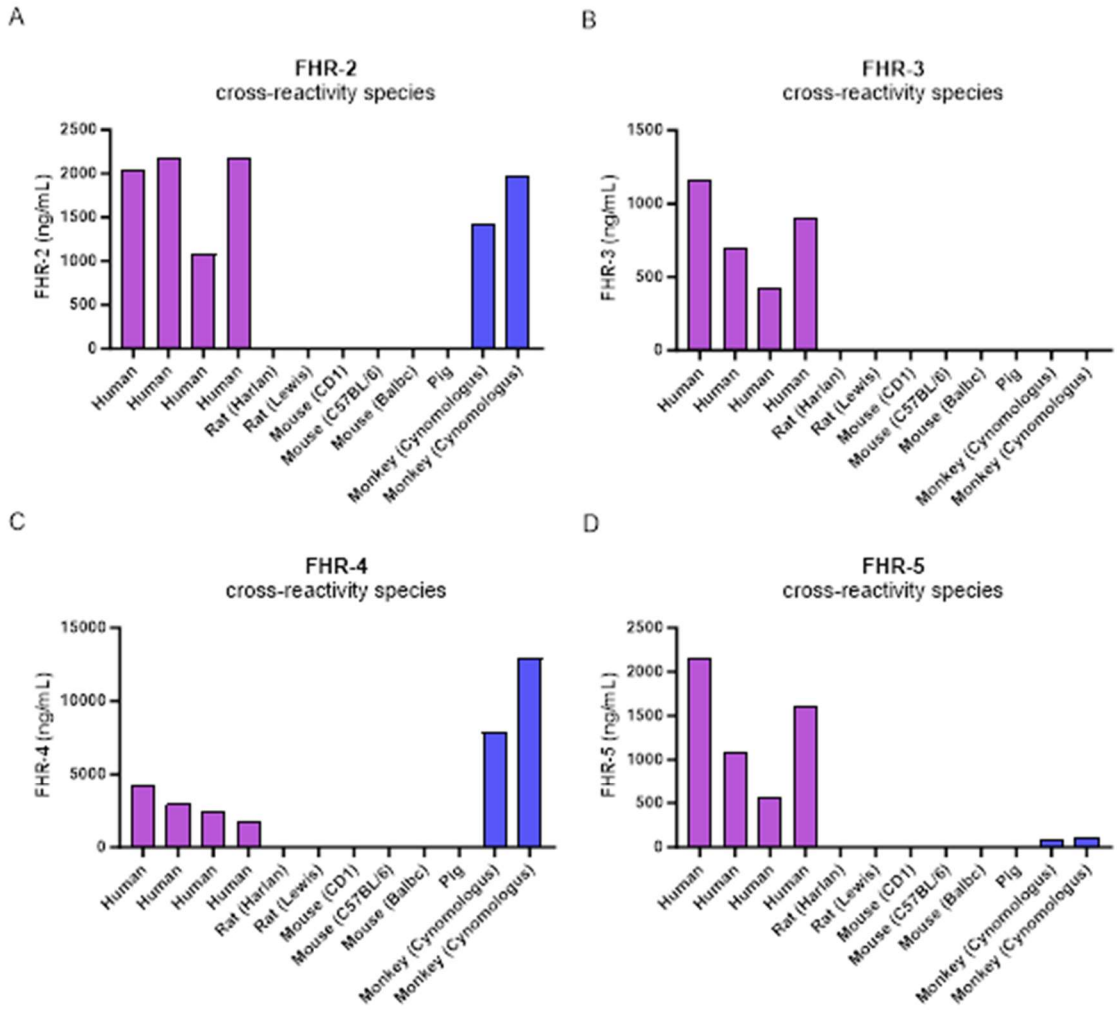


Evaluation of batch-to-batch variation for (A) FHR-2, (B) FHR-3, (C) FHR-4 and (D) FHR-5.

1.5. Cross-reactivity species other than human

To determine whether the FHR ELISAs could also be used for measuring FHR-2, FHR-3, FHR-4 and FHR-5 levels in species other than humans, cross-reactivity was investigated in samples obtained from mouse, rat, pig and non-human primates (NHP). The FHR-3 and FHR-5 assays show no cross-reactivity against other species. For FHR-2 and FHR-4, cross-reactivity was observed for non-human primates (Cynomologus) (Figure 1.5).

Figure 1.5 Cross-reactivity different species



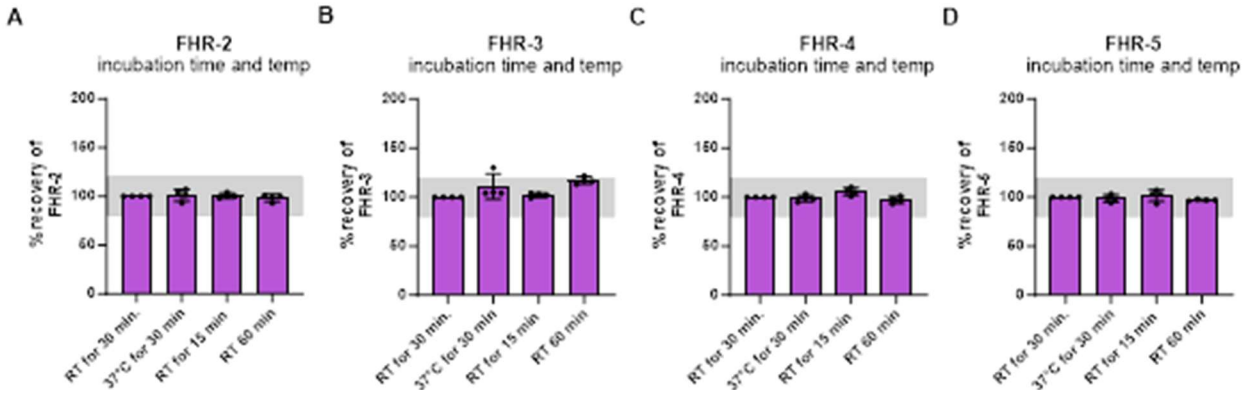
Evaluation of assay cross-reactivity with species other than human for (A) FHR-2, (B) FHR-3, (C) FHR-4 and (D) FHR-5.

1.6. Assay robustness

To test assay robustness, several condition were testing thereby deviating from the recommended protocol (sample incubation at RT for 30 min). When samples are incubated at 37 °C or for a shorter/longer time than 30 min, variation in sample values tented to increase but remained within 80-120% deviation from baseline (value measured at RT, 30 min. sample incubation) (Figure 1.6). Also different plate washing procedures were tested and compared to the default washing procedure (manual wash). Also here, variation in sample values tented

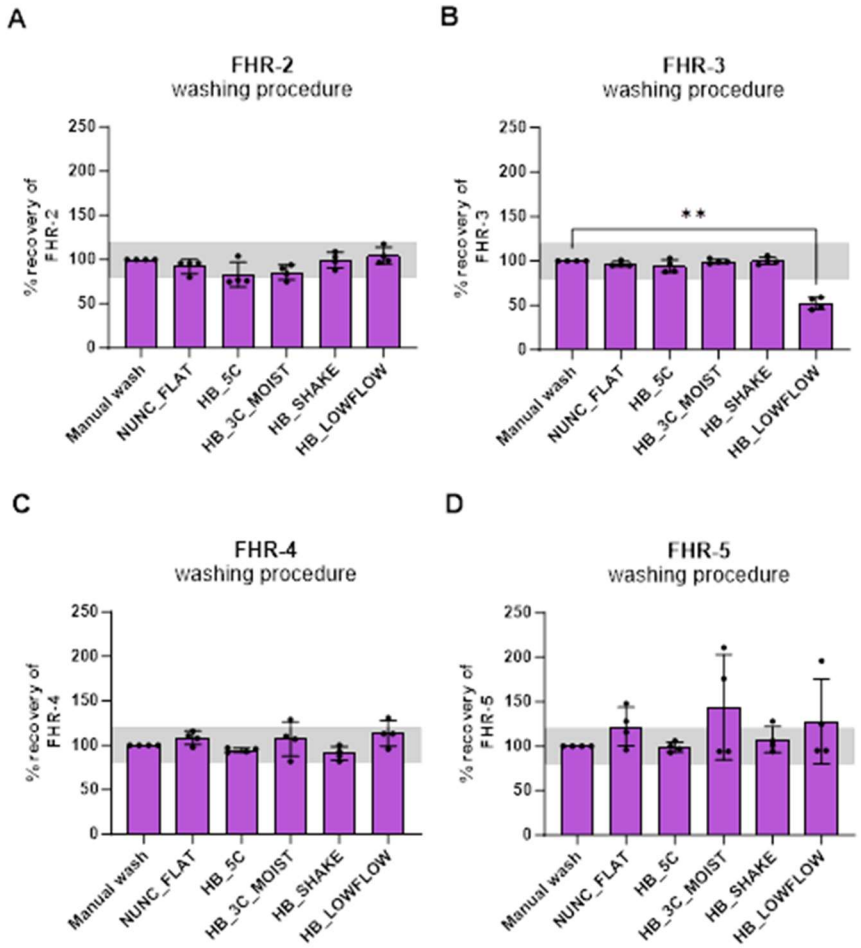
to increase when more rigorous washing programs were used, and reached statistical significance for FHR-3 for one washing program Figure 1.7). Overall, these data show that all FHR assay are robust, as deviating from the recommended protocol introduces only a modest increase in sample variation. However, it is advised not to deviate from the recommended protocol.

Figure 1.6 Robustness – assay performance using different incubation temp. and timepoints



(A-D) Assessing the effect of different incubation temperatures and times on assay performance for each FHR assay.

Figure 1.7 Robustness – assay performance using different washing procedures

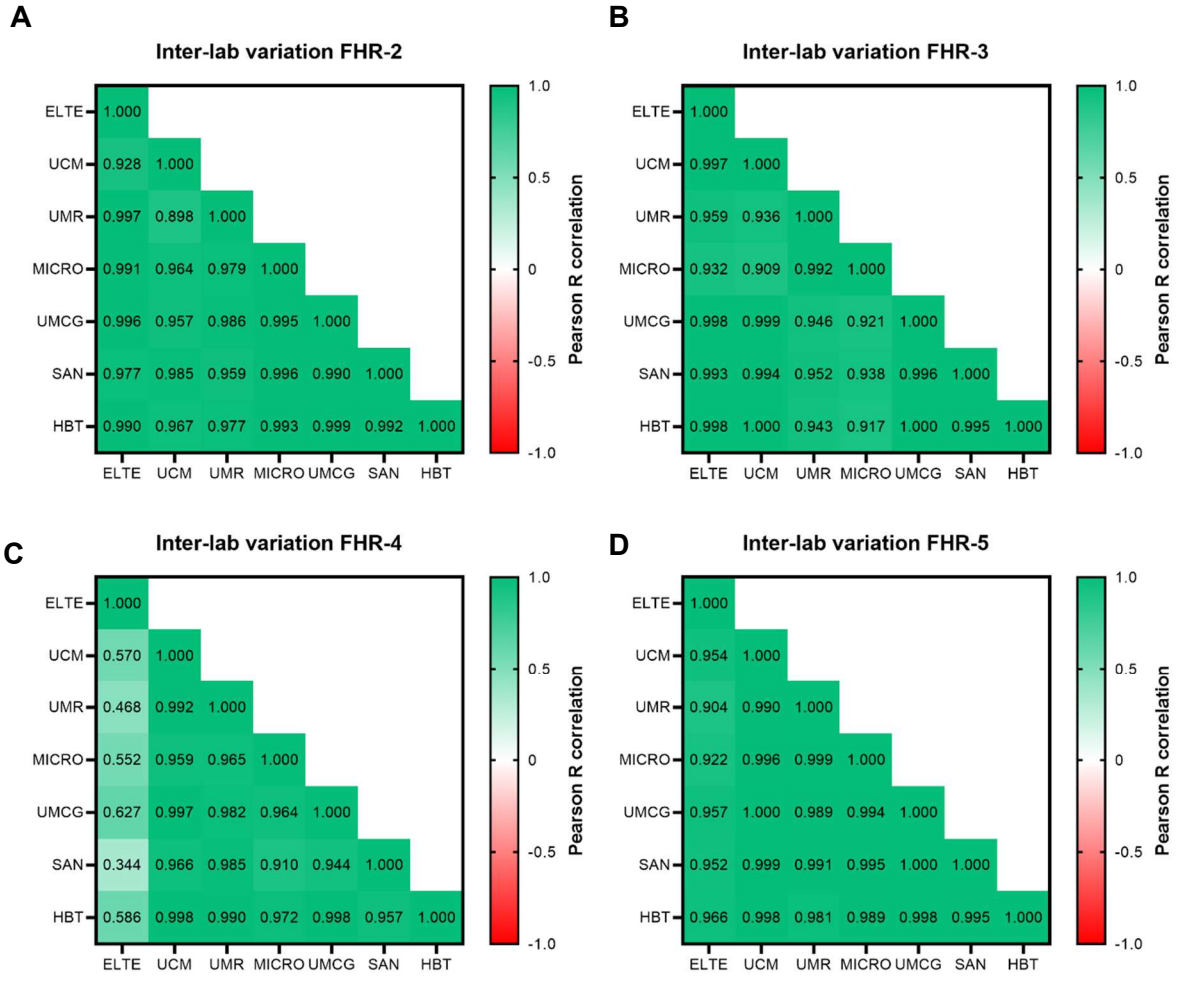


(A-D) Assessing the effect of different washing programs on assay performance for each FHR assay.

2. Inter-lab variability (task 6.2)

Reproducibility of the assays was also assessed by measuring and comparing the same set of samples at seven different laboratories, spread across Europe. Pearsons R correlation was used to determine whether the results from different laboratories correlate to each other. For the FHR-2, FHR-3 and FHR-5 assays, sample concentrations measured at different locations highly correlated with each other (R ≥0.898, ≥0.909, ≥0.904, respectively) (Figure 2.1), indicating good reproducibility. Results obtained for FHR-4 showed high correlations between sample values measured at six different locations (R ≥0.910), whilst the correlations for one location were low to average (R = 0.344-0.627).

Figure 2.1 Inter-laboratory variation

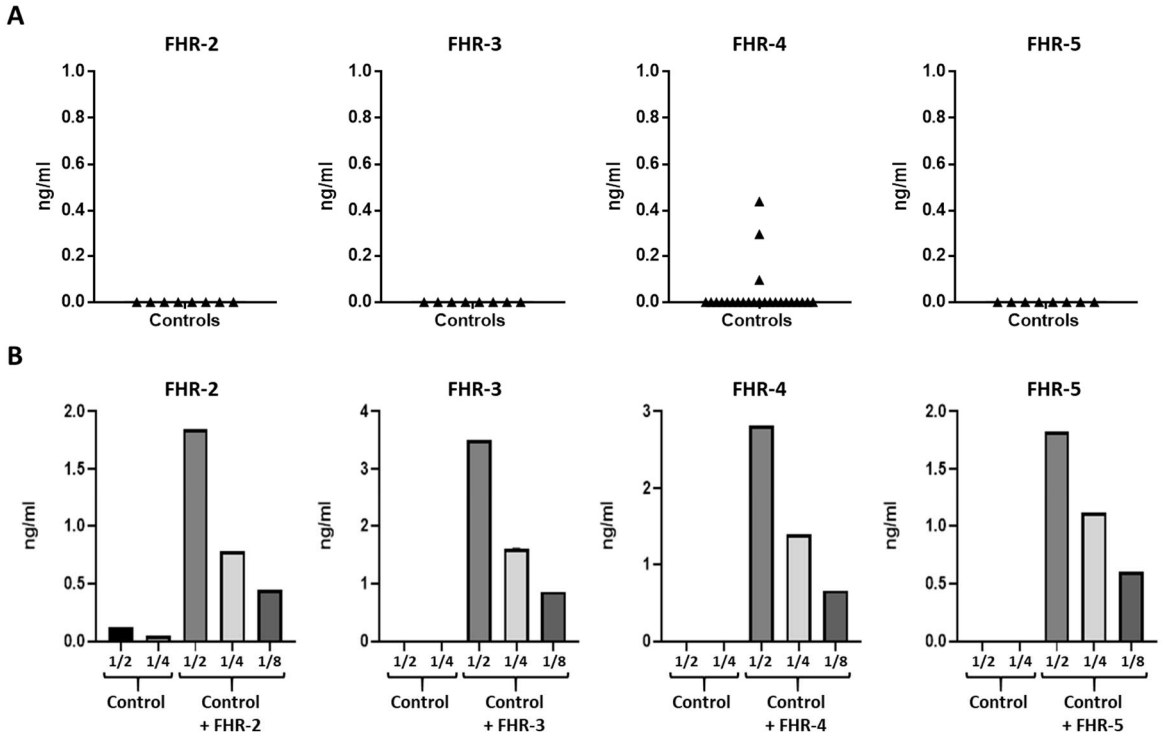


Inter-laboratory for (A) FHR-2, (B) FHR-3, (C) FHR-4 and (D) FHR-5. Inter-laboratory variation was determined by comparison of data from seven different laboratories. Pearsons R correlation was used to determine whether the results from different laboratories correlate to each other

3. Different body fluids (task 6.3)

Next, we tested the utility of the assays in quantifying FHR proteins in body fluids other than plasma or serum. First, we investigated whether these proteins could be detected in urine samples. To do this, we incubated a series of urine samples from healthy individuals at 1/4 dilution. As shown in Figure 3.1A, the FHR proteins tested were not detected in the majority of the control samples analysed. Exceptionally, in the case of the FHR-4 assay, where more samples were analysed, low protein levels were detected in 3 out of 24 samples. Next, we wanted to verify whether this result was due to the fact that FHRs were not present in the urine samples, or whether, on the contrary, they could not be detected due to technical problems caused by the nature of a urine sample. For this purpose, we added to a urine sample from a healthy individual (control) the amount of each of the FHRs proteins to leave them at the theoretical concentration of 2.5 ng/ml in the urine sample. We then tested both the urine sample without added FHRs (control) and the samples with added FHRs in the assays. In this case, the samples were incubated at 1/2, 1/4, or 1/8 dilutions. As shown in Figure 3.1B, the FHRs proteins were detected in the urine samples to which the FHRs were added in a dose-dependent manner. Therefore, we can conclude that these kits are useful for quantifying the presence of FHR proteins in urine samples and that samples from healthy individuals are not expected to contain FHR-2, FHR-3, FHR-4 and FHR-5 proteins.

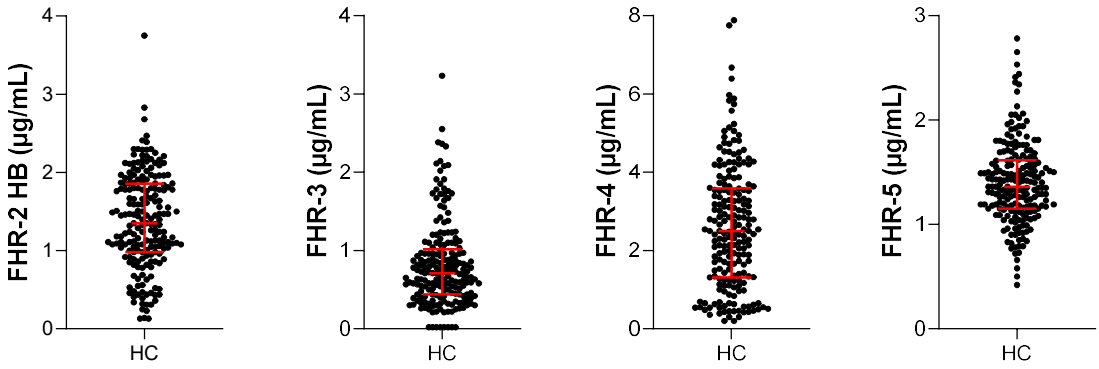
Figure 3.1 Quantification of FHR proteins in urine samples.



4. Proof-of-concept for cohorts (task 6.4)

Using the newly developed ELISA kits, levels for FHR-2, -3, -4 and -5 were determined in serum of 201 Dutch healthy controls (HC) (Figure 4.1). FHR-2 and -5 were found to have similar levels with a median concentration of 1.35 µg/mL (IQR:0.98-1.86) and 1.36 µg/mL (IQR: 1.15-1.62) respectively. FHR-3 was found to be the lowest in serum with a median of 0.71 µg/mL (IQR: 0.44-1.02). Additionally, eight donors were found to carry a homozygous deletion for *CFHR3/CFHR1* as confirmed with MLPA, resulting in no detectable FHR-3 in the ELISA. Lastly, FHR-4 had the overall highest levels of the measured FHRs with a median of 2.50 µg/mL (IQR:1.32-3.59). These results are part of a larger study on normal ranges and genetic influence in healthy controls performed by SAN and HBT, planned for publication in 2024.

Figure 4.1 Normal range in Dutch health controls.



5. Initiation of larger clinical studies (task 6.5)

Larger clinical studies are in preparation. For instance, a large study is planned in which the association between FHR genetic variants, FHR protein levels, disease onset and severity will be investigated in patients suffering from kidney disease. Other diseases that are currently under investigation and for which SciFiMed has provided assays are: COVID-19, Osteoarthritis, AMD, post-operative delirium, membranous nephropathy (MN) and ANCA-vasculitis (AAV). Results are expected in Q3 and Q4 2024.

6. Overview and conclusions

Assay development for all four FHR ELISA was succesfully concluded (Table 6.1) and assays are commercially available. Task 6.1 to 6.5 are finalized. Assays are distributed within SciFiMed but also outside the consortium. Clinical studies are ongoing and will be concluded in Q3 and Q4 2024.

Table 6.1 Evaluation criteria of developmental phase

Criteria	FHR-2 ELISA	FHR-3 ELISA	FHR-4 ELISA	FHR-5 ELISA
Upscaling	👍	👍	👍	👍
Inter-intra variation	👍	👍	👍	👍
Freeze-thaw stability of standard and samples	👍	👍	👍	👍
Benchtop stability standard and samples	👍	👍	👍	👍
Freeze-dry loss standard and detection antibody	👍	👍	👍	👍
Batch-to-batch variation	👍	👍	👍	👍
Stability	👍	👍	👍	👍
Robustness	👍	👍	👍	👍
Measuring patient cohorts (externally WP6)	Ongoing	Ongoing	Ongoing	Ongoing

6.1 Deviation from Discription of Action (DoA), mitigation actions and risk assessment.

No deviations from DoA. All tasks for D6.1 are completed within time (due date 30-06-2024).

No mitigation actions needed.