D3.1

LFA feasibility study

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Lead Author:	Dr. Mark-Steven Steiner/ MICRO

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Abbreviations and Acronyms

ELISA	Enzyme linked immunosorbent assay
ELTE	Eötvös Loránd University
FH	Complement factor H
FHR	Factor H-related
FITC	Fluorescein isothiocyanate
HBT	Hycult Biotech
LFA	Lateral flow assay
LLC	Low Level Control (human deficient serum)
MICRO	Microcoat Biotechnology GmbH
POC	Point of care
SAN	Sanquin
SA-Poly	Streptavidin polymer
SciFiMed	Screening of inflammation to enable personalized medicine
UCM	Complutense University of Madrid
UMR	University Marburg
UMCG	University Medical Center Groningen
UREG	University Regensburg

Summary

This document is deliverable 3.1 (D3.1), which relates to the lateral flow assay (LFA) feasibility study for developing a highly challenging point of care (POC) device in order to quantify simultaneously seven proteins with totally different concentration ranges in human samples.
To develop a POC device for the FH protein family the first essential step of the feasibility study is to find antibody pairs against the different FH/FHR proteins, which are suitable for LFA in general. Those antibodies might not essentially be the same that are suited for ELISA application. Additionally, getting familiar with the analytical problem, the target matrix and analytes is of course also of importance during this initial phase. After finding those antibodies the detection range of the single analytes is roughly evaluated in the target matrix to early determine the boundaries of the assay. After showing that a general feasibility of the respective antibody pair for LFA is possible the antibodies can be tested for a specific multiplex LFA. Therefore, it is necessary that the antibodies have a low or no cross reactivity to the non-specific analytes of the protein famliy.
The envisioned device for SciFiMed is a 7-plex device for the different proteins of the FH family. After finding antibodies which are usable for LFA, the next step is to develop a single LFA for each antibody pair (D3.2), before a multiplex device can be developed (WP 3.3).
The work shown in this report contains preliminary work related to deliverable 3.2 (D3.2, single LFA for each FH protein) and is already needed in part in WP3.1. The proteins and antibodies for this SciFiMed feasibility study were provided by SciFiMed partners and commercial sources: UCM (recombinant FHR-1-5, human deficient serum for specific FHRs), SAN (anti-FHR-3 antibodies (clones 3.0, 3.11, 3,12, 3.13, 3.14, 3.16)) and Biotechne (anti-Factor H (FH) (clone OX23 and 556317), anti-FHR-1 (clones 4D1 and 442127), anti-FHR-2 (clone 003), anti-FHR-3 (polyclonal), anti-FHR-4 (clones AF5980 and 640212), anti-FHR-5 (clone: 3A10A5) as well as FH protein, recombinant FHR-1 and FHR-5).
The results of testing these antibodies with these antigens will be described in this report and are summarized at the end of the report (**table 2**). The testing consisted of conjugating the antibodies with biotin and FITC in order to test for the best suited pair for the LFA. The best performing antibody combinations were also tested for cross-reactivity (WP 3.2). By testing the antibody-combinations good results were provided testing the anti-FH and anti-FHR-1 antibodies in buffer. For the anti-FHR-4 the detection was possible in serum, but needs to be further improved as the detection limit is not low enough. The most improvement needs to be done for anti-FHR-3 as no suitable antibody pair for lateral flow application was identified.

Apart of testing further antibodies, the next steps need to show that more than one protein can be detected on the LFA system. Here, as a first step, it is not necessary to use antibodies against the FH/FHR family to show a proof of principle.

Additionally, it will be tested if other detection particles can be used apart of gold nanoparticles, which might further improve the detection of low concentrations. A starting point for this study were gold nanoparticles as detection particles. Another feasible way of detecting the proteins is by using liposomes as detection particle. Using those, a lower concentration of the analytes might be detectable as the liposomes might allow for a lower detection limit than gold nanoparticles. Therefore, liposomes from UREG were sent to MICRO and will be integrated into this study as soon as possible.

Contents

1. Introduction

This document is deliverable 3.1 (D3.1), which is included in task 3.1 in work package 3 (WP3): Simultaneous Quantification.

One of the goals of SciFiMed is to develop a lateral flow assay (LFA) for the Factor H (FH) protein family. **Figure 1** shows the general setup for a LFA test strip and specifically the prototyping platform used for WP3.1 with only one component. The composition of such an assay contains four pads: (1) a sample pad, where the sample is added, (2) followed by a conjugate pad, that holds the detection particle, (3) a reaction pad, where the sandwich-complex of the detection particle - a FITC-labeled antibody, the analyte and a biotin-labeled antibody - forms, and finally (4) a waste pad at the far end of the strip that absorbs not captured sample and reaction buffer. The sandwich-complex formed during passage of the reaction pad is captured at the test line via the biotinylated antibody by biotin-streptavidin interaction. Not captured detection particles can bind to the control line by an anti-species antibody directed against the antibody immobilized on the gold particles. The sandwich-complex is only formed if the analyte is present in the solution. The test line can be visually detected and its intensity can be measured reflectometrically. FHR-specific antibodies are generated in WP2, but for start of the WP3 also commercially available antibodies were sourced.

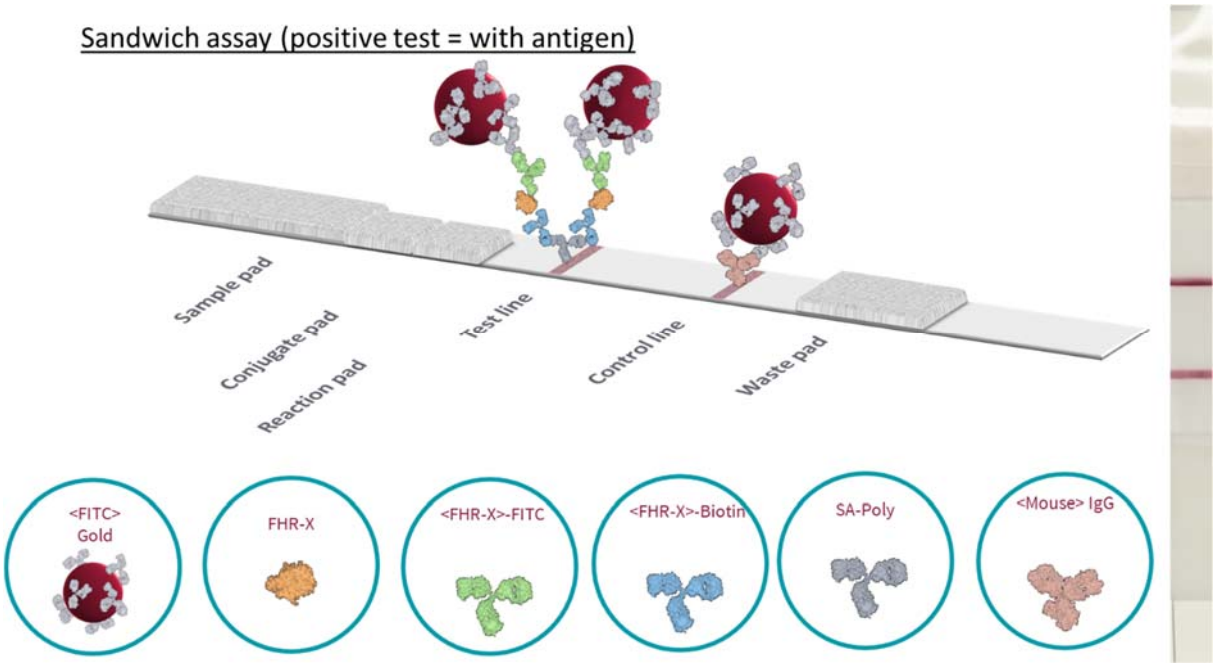

Figure 1: Setup for the feasibility testing of the different antibodies to detect the different FH proteins in single assays. The SA-poly test line is used to bind a biotinylated antibody against the test protein of the FH family (analyte). For detection of the test line a FITC-labeled antibody binds to the analyte as well, while an anti-FITC antibody conjugated to a gold particle can bind to the FITC on that antibody. At the control line an anti-mouse capture antibody will be immobilized, which interacts with the murine anti-FITC antibody on gold particles and become visible as control line.

Multiplex LFA devices can be constructed in different ways (**Figure 2**). The supposedly easiest way of construction is to add additional test lines for each additional analyte to be tested on the same test strip. This results in quite long test strips (**Figure 2a**). Another way is to test the analytes in parallel on more than one test strip, while the test strips are all contained in the same device (**Figure 2b**). Here the sample volume needs to be evenly distributed on all test strips in order to give reproducible results. This approach is well known from drugs of abuse testing devices. Both approaches can also be combined to increase the degree of multiplex. Furthermore, it is also possible to use antibodies which bind to more than one of the protein family (**Figure 2c**). The main problem here is, that the different proteins will not be distinguished (not suitable for the SciFimed goal). Finally, one can also use detection particles of different color for each specific analyte – so called spectral multiplexing (**Figure 2d**).

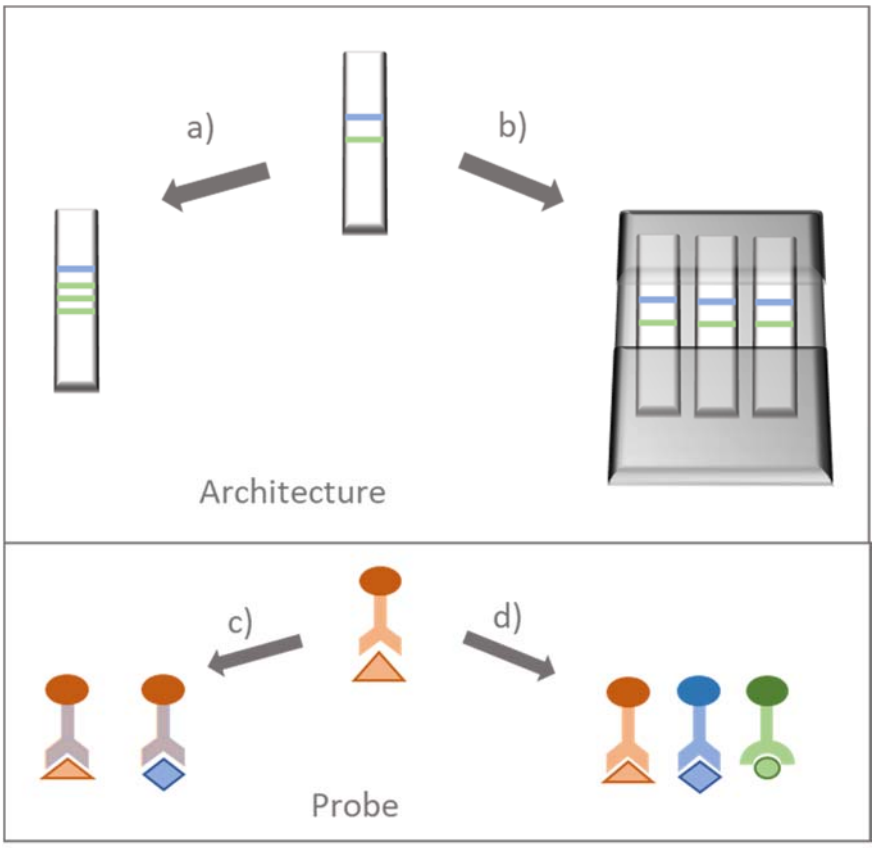

Figure 2: Four different ways of multiplex LFA: a) the number of test lines on a single test strip is increased; b) the number of test strips is improved, c) broad-selective antibodies are able to bind to different antibodies of the same class and d) the detection particle provide different signals.

In order to detect the proteins of the FH protein family in different concentrations in human samples a highly ambitious device needs to be developed. WP3 includes a study on the feasibility for a general LFA for the FH protein family and to develop a multiplex LFA in the following. Therefore, this work package is one of the key work packages of the project.

For this project it is planned to either perform the 7-plex by using strategy **a** (**Figure 2a**) or combining strategy **a** and **b** (**Figure 2a and b**). The combination of both strategies is preferred and may be supported in a future step with spectral multiplexing. A single test strip with 7 test

lines (**Figure 3**) will be quite long and might introduce additional physical issues like demand for high sample volume, longer incubation times and higher probability of unspecific binding.
A key issue that needs to be addressed from perspective of antibody generation is a possible cross-reactivity of the different target-analytes, as the proteins of the FH family have a rather high structural similarity.

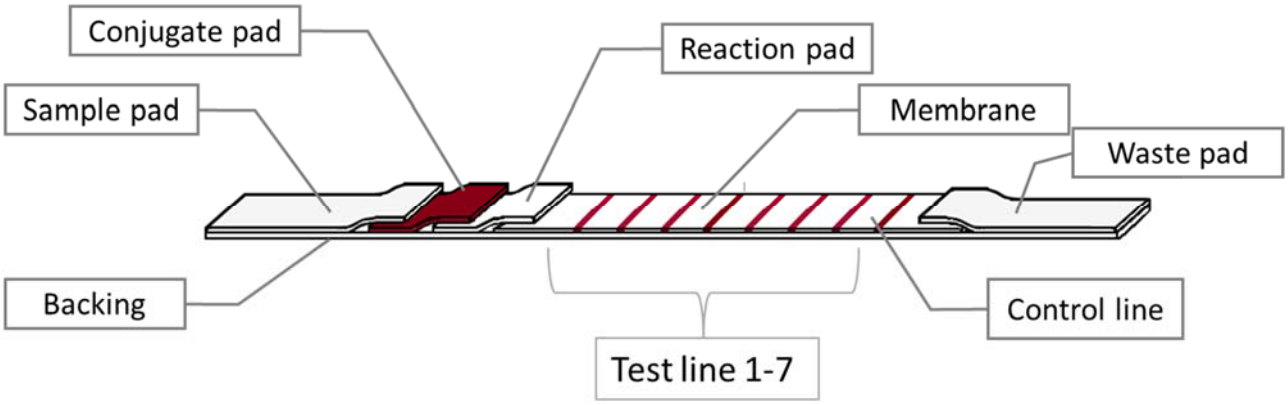

Figure 3: Construction of a 7-plex LFA. The sample, conjugate and reaction pad are overlapping each other and the reaction pad overlaps with the membrane and the waste pad. All components are hold together with a backing.

With this test setup the feasibility of detecting the different FH proteins is tested in WP3. For those tests it is absolutely essential to first evaluate as single parameter test which antibody clone for each of the analytes is used best as capture antibody in the test line and which antibody is used as detection conjugate. The boundaries of the detection limit of those combinations needs to be evaluated right after. This should also contain as far as possible an evaluation of cross reaction with other proteins of the FH family. After choosing a suitable antibody combination for each protein a LFA for simultaneously detection of the different proteins will be set up. Data from WP2 might not exactly be transferable from ELISA to LFA format and will at least in part be analyzed also in WP3. Therefore, a 3-plex assay will be developed first followed by a 7-plex assay. The results from WP3 are needed to develop the desired biosensor device in WP5 and for the assay validation in WP6.
To further improve the detection, with regard to sensitivity and/or multiplexing, liposomes can be used as detection conjugates. Lower concentrations of analyte might be detected by liposomes than with gold particles. Additionally, liposomes may also open up possibilities for spectral multiplexing. Therefore, liposomes from UREG will also be tested as possible detection particles.

2. Conjugation of the antibodies

Portions of all anti-FHR antibodies used in this study were first divided in halves. One half has been labeled with biotin while the other half was conjugated with FITC. The labeling protocols were optimized during that phase. After purification of the conjugates the FITC labeling ratio was measured and was in a molar ratio range between 1.24 and 3.02 (molecules fluorophores/molecules protein). After labeling, the antibodies were used to test which clone combination is best suited for the LFA. Therefore, a titration of both combinations was performed and is discussed in section 3. Those tests also showed the success of the biotin labeling.

3. Finding the best clone combination

4.1. Testing of anti-FH antibodies

Used antibodies: Anti-FH OX23 and 556317 each labeled with biotin and FITC, both were commercially obtained and are monoclonal mouse antibodies against human FH

The feasibility test for the single detection of FH was performed in standard running buffer 1 with different antibody settings (**Figure 4**). The control lines were in the expected range for all tests. The signals for the 556317-biotin with OX23-FITC combination provided a strong antigen-specific signal > 600 mV peak height starting at 0.01 µg/mL. However, the control lines became weaker as not enough detection reagent was able to reach the control line, when the antigen concentration was higher than 1 µg/mL (showing the high dose hook effect of homologous assays). This combination is clearly able to detect FH down to 0.01 µg/mL and is used for further investigation. The clone combination OX23-biotin with 556317-FITC is not able to detect FH and will not be investigated any further.

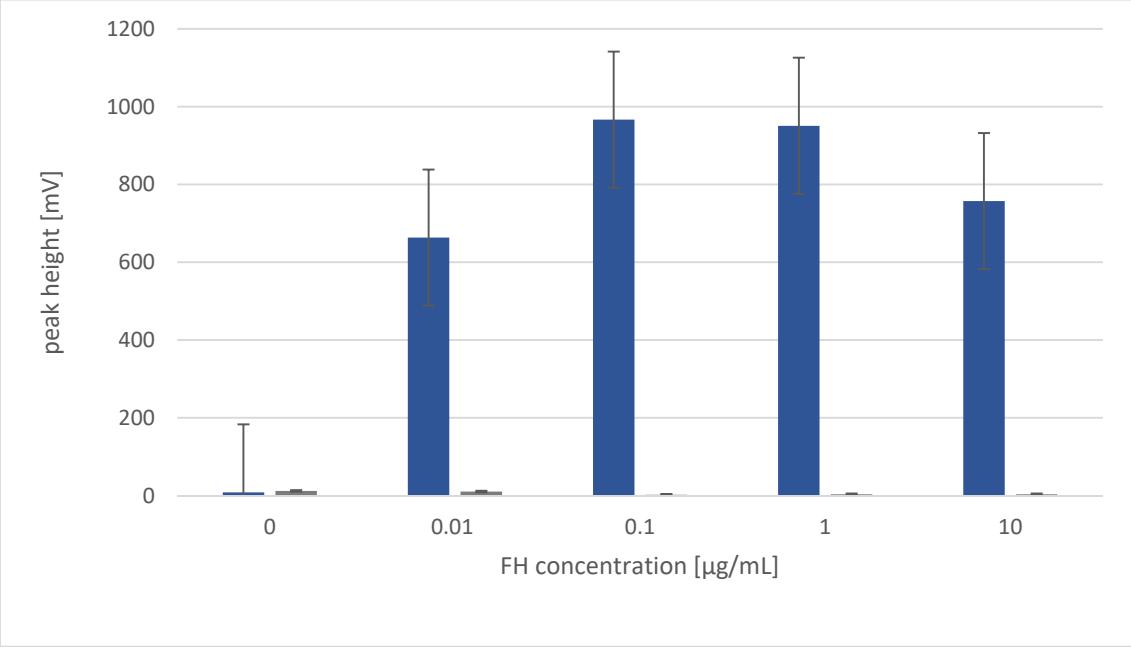

Figure 4: Titration of anti-FH 556317-biotin with anti-FH OX23-FITC (blue) and anti-FH OX23-biotin with anti-FH 556317-FITC (grey) in standard running buffer 1 spiked with FH. The antibody combination anti-FH 556317-biotin with anti-FH OX23-FITC resulted in an intense antigen-specific signal with a visible high dose hook effect between 0.1 µg/mL and 1 µg/mL. The clone combination anti-FH OX23-biotin with anti-FH 556317-FITC did not detect FH. The control lines were in the expected range (data not shown). The test was done in triplicates.

The antibody pair 556317-biotin with OX23-FITC was able to detect FH and was further validated with higher antigen concentrations. **Figure 5** shows the results with the control line. The control line decreases with a high FH concentration as not enough detection particles reach the control line. The dynamic range of this FH specific LFA was determined at 0.1 ng/mL to > 50 ng/mL with this setup.

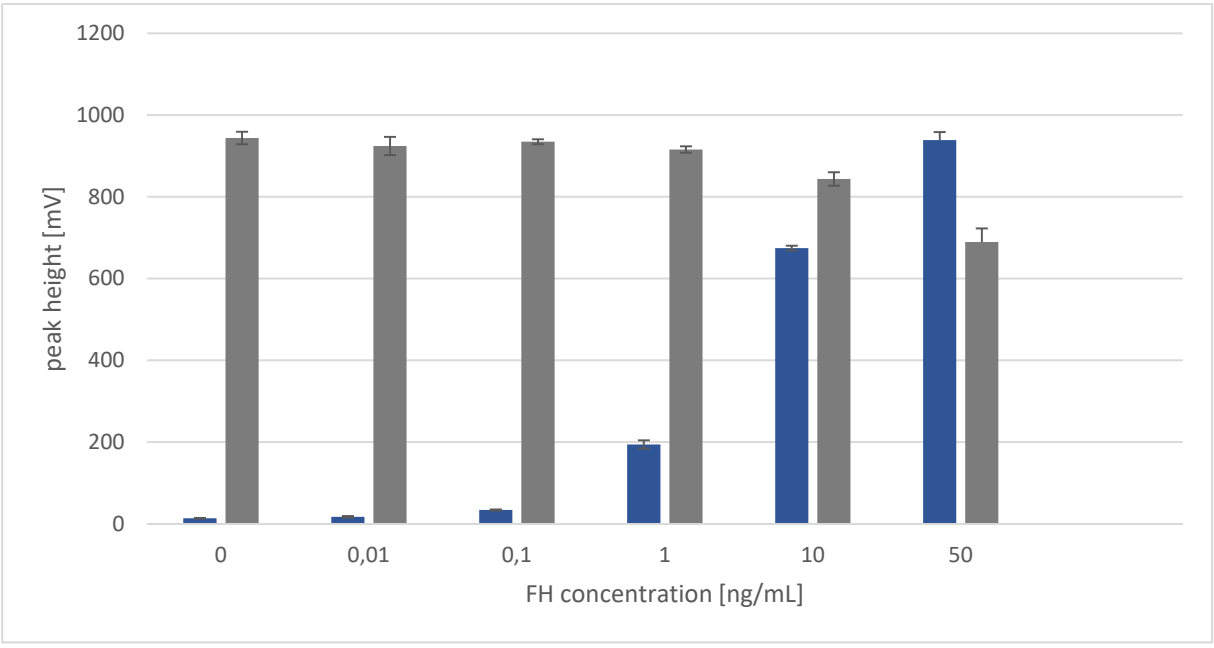

Figure 5: The combination of 556317-biotin with OX23-FITC (blue) detected FH in spiked into running buffer different in difference concentrations. Increasing FH concentrations correlated with in accumulating test line signals.

The control line is shown (grey) declined with increasing FH concentrations. The test line competed with control line in capturing detection gold particles. The test was done in triplicates. Testing of anti-FHR-1 antibodies

4.2. Testing of anti-FHR-1 antibodies

Used antibodies: anti-FHR-1 4D7 and anti-FHR-1 442127 each labeled with biotin and FITC, both were commercially obtained and are monoclonal mouse antibodies against human FHR-1

Antibody feasibility study for single LFA was performed either with recombinant FHR-1 or human serum samples diluted either in standard running buffer 1 or serum buffer, respectively. Different ratios of the antibody conjugates were investigated to determine the best assay conditions.

The first step of this analysis was to determine which antibody combination works best for the detection of the analyte. Therefore, both combinations were tested for the detection of FHR-1 in spiked standard running buffer 1. **Figure 6** shows the difference between the two combinations. Anti-FHR-1 4D7-biotin with anti-FHR-1 442127-FITC (grey) gave strong false positive results and the highest detection signal was at 10.00 µg/mL, while 4D7-FITC with 442127-biotin (blue) showed a high dose hook effect and the best detection at 0.1 µg/mL. The control lines were detected in the expected range. The higher the FHR-1 concentrations were, the lower were the control lines. Repeating the test in serum resulted in false positive signals for 4D7-biotin with anti 442127-FITC. This effect increases the more the serum is diluted. As 4D7-FITC with 442127-biotin does not show false positive results in serum this combination was investigated further.

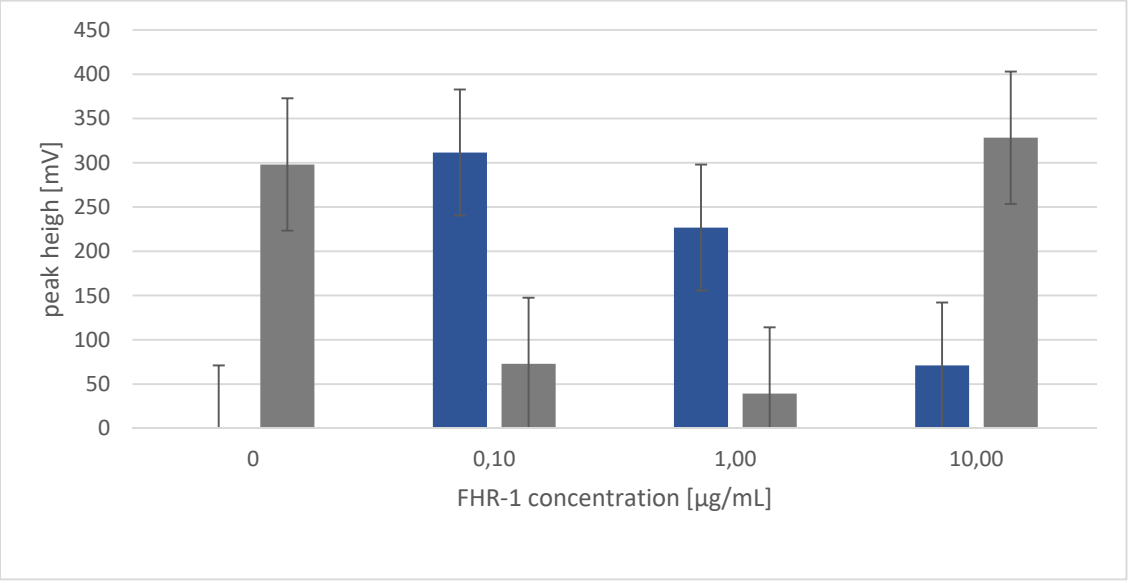

Figure 6: Comparison of the tested antibody combinations anti-FHR-1 4D7-biotin with anti-FHR-1 442127-FITC (grey) and anti-FHR-1 4D7-FITC with anti-FHR-1 442127-biotin (blue) in standard running buffer 1 spiked with FHR-1. The signals for anti-FHR-1 (clone 4D7) biotin with anti-FHR-1 442127-FITC show a false positive signal. The signals from 4D7-FITC with 442127-biotin have a high dose hook effect with the highest detection at 0.10 µg/mL while the control lines show a steady value. The test has been done in duplicates.

Further experiments showed that the variation of the ratio of the biotinylated, capture and FITC-coupled, detection antibody has a huge influence on the detection range. As shown in **Figure 7**, it turned out that the best ratio is 1:5 (4D7-FITC:442127-Biotin) as those results had the highest signal. A – undesired - hook effect is visible. For the tested ratios the signal at 0 µg/mL was nearly undetectable. The highest signal was achieved by 1:3 ratio of 4D7-FITC:442127-biotin.

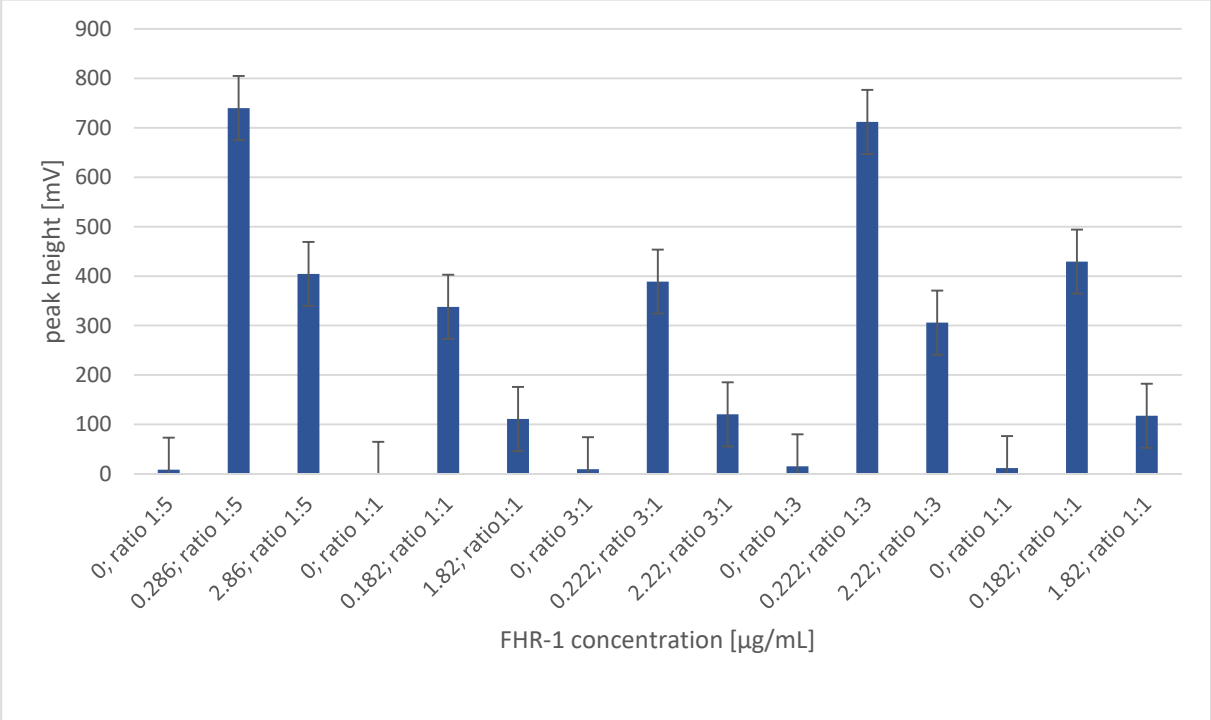

Figure 7: Test for the best antibody ratio for detection of recombinant FHR-1 in standard running buffer 1. The highest signals were detected with a ratio of 1:5 (4D7-FITC:442127 biotin). With this ratio the high dose hook effect is still visible. The test has been done in triplicates.

The detection of FHR-1 in running buffer 1 provides sufficient results for detection. By transferring the experiments to human serum with standard running buffer 1 and serum buffer, there are no detectable signals anymore. Signals are being detectable again by spiking the serum with recombinant FHR-1. Preincubation of antibodies and detection particles with the serum prior to sample application was also evaluated to improve the detection sensitivity by increasing interaction time of all test components. Neither the preincubation time, nor a higher antibody concentration improved the signal sufficiently.

4.3. Testing of anti-FHR-3 antibodies

Used antibodies: anti-FHR3.0, anti-FHR3.11, anti-FHR3.12, anti-FHR3.13, anti-FHR3.14 and anti-FHR3.16 (SAN). Poly2.2 is a polyclonal antibody from a rabbit against human and mouse FHR-3.

To find the optimal performing antibody-combination to detect FHR-3 the received antibodies from SAN and Poly2.2 (commercially bought from Biotechne) were tested. Therefore, each antibody was conjugated with biotin and FITC as described above. So far, the combinations shown in **table 1** have been tested.

Table 1: Overview about the done testing with the different antibody combinations. All tests have been done in standard running buffer 1. The used analyte was bought from biozol. The titration has been done with 0 ng/mL, 10 ng/mL and 100 ng/mL.

Biotin-conjugated antibody	FITC-conjugated antibody	Result
3.11	3.12	No sufficient signals
3.11	3.13	No sufficient signals
3.11	3.14	No sufficient signals, but highest signal so far
3.12	Poly2.2	No sufficient signals
3.12	3.0	No sufficient signals
3.12	3.11	No sufficient signals
3.12	3.13	No sufficient signals
3.12	3.14	No sufficient signals
3.12	3.16	No sufficient signals
3.13	3.11	No sufficient signals
3.13	3.12	No sufficient signals
3.13	3.14	No sufficient signals
3.14	Poly2.2	No sufficient signals
3.14	3.0	No sufficient signals
3.14	3.11	No sufficient signals
3.14	3.12	No sufficient signals
3.14	3.13	No sufficient signals
3.14	3.16	No sufficient signals
3.16	Poly2.2	Strong false positive results
3.16	3.0	No sufficient signals
3.16	3.11	No sufficient signals
3.16	3.12	No sufficient signals
3.16	3.13	No sufficient signals
3.16	3.14	No sufficient signals

All test combinations seen in **table 1** were done in standard running buffer 1. The highest received results had a signal of 570 mV and had equally strong false positive signals (3.16-

biotin with Poly2.2-FITC). The next highest signal was from 3.11-biotin with 3.14-FITC and had an intensity from 58.07 mV (**Figure 8**, yellow). As those results are not high enough to continue the testing different buffers were tested to improve the signals. The buffers were tested using 3.16-biotin with Poly2.2-FITC and 3.13-biotin with 3.14-FITC. By testing 5 different running buffers the signals did not improve, some running buffers even gave false positive signals.

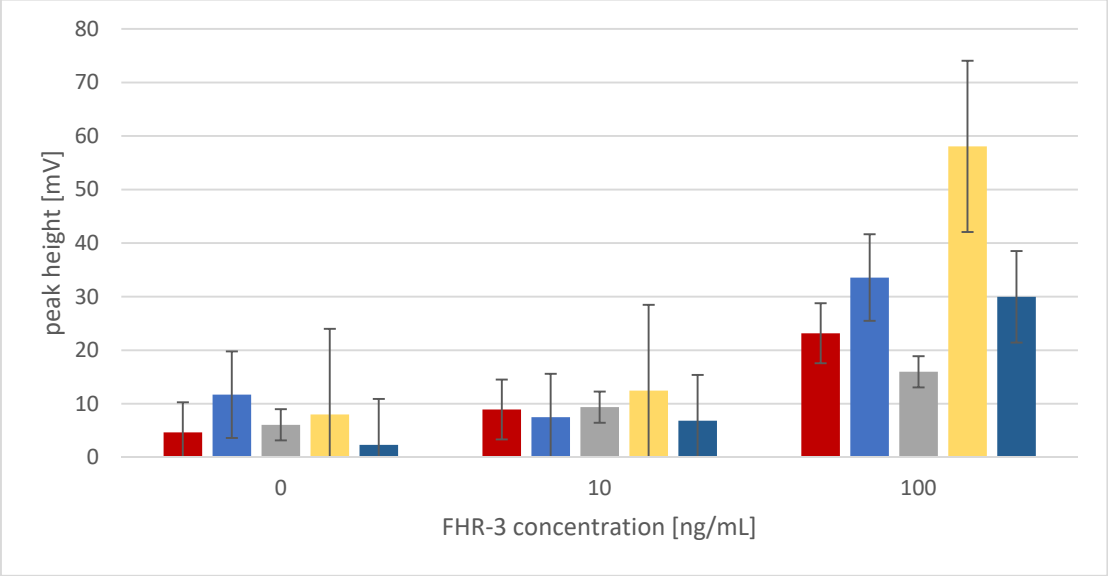

Figure 8: Titration of anti-FHR-3 3.12-biotin with anti-FHR-3 Poly2.2-FITC (red), anti-FHR-3 3.0-FITC (bright blue), anti-FHR-3 3.11-FITC (grey), anti-FHR-3 3.14 (dark blue) and anti-FHR-3 3.11-biotin with anti-FHR-3 3.14-FITC (yellow) in standard running buffer 1. All combination had really low signals, even at a high concentration of the FHR-3 (100 ng/mL). Low false positive signals were detectable as well. The highest signals were detected for 3.11-biotin with 3.14-FITC (yellow). In order to make the figure well arranged the control lines are not shown were however in the expected range. The tests have been done in standard running buffer 1.

To find out whether the used analyte is the issue, FHR-3 received from UCM was used as analyte for the testing. Using the different analyte improved the results slightly, by reaching a signal of 70 mV. This showed that the used commercial analyte is functional and that so far, no well-working antibody combination has been found.

4.4. Testing of anti-FHR-4 antibodies

Used antibodies: Anti-FHR-4 AF5980 (polyclonal sheep IgG against human FHR-4) and anti-FHR-4 640212 (monoclonal mouse antibody against human FHR-4) each labeled with biotin and FITC

In order to find out which antibody combination yields better results both combinations have been tested in standard running buffer 1. So far, no satisfying results have been found. For both combinations the control line signal decreases with a higher analyte concentration and a false positive signal was detectable as well (**Figure 9**). For AF5980-biotin with 640212-FITC

the false positive signal is weaker than for the other antibody combination. Both combinations have their peak at 100 ng/mL, with a higher concentration the signal decreases.

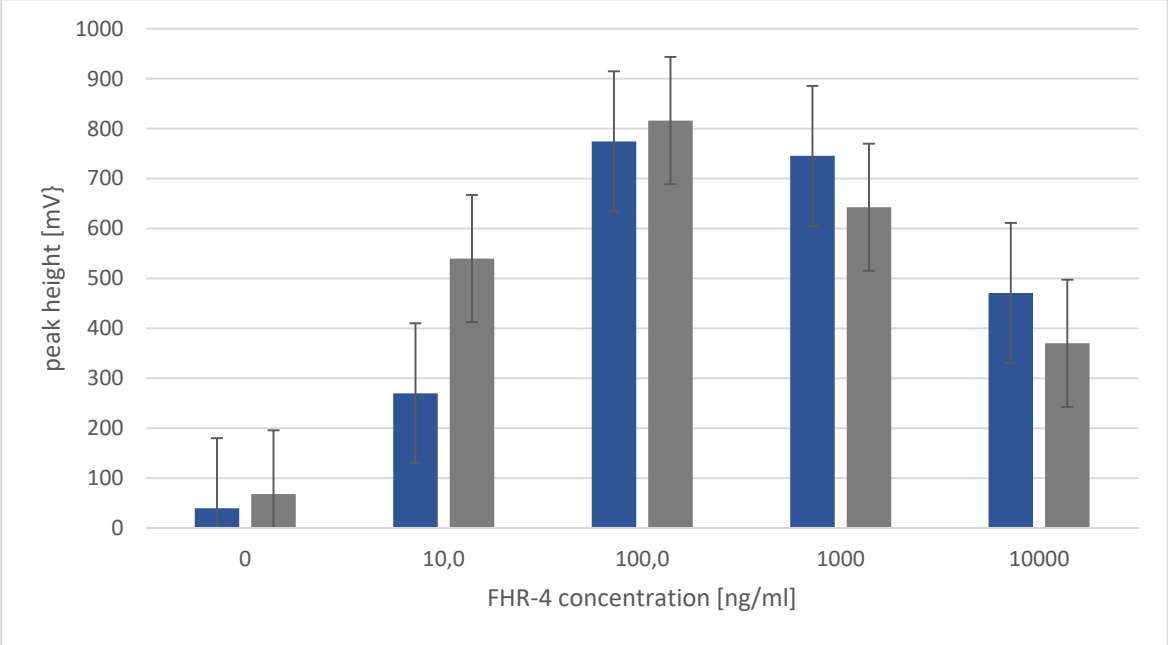

Figure 9: Titration of both antibody combinations anti FHR-4 AF5980-biotin with anti FHR-4 640212-FITC (blue) and anti FHR-4 640212-biotin with anti FHR-4 AF5980-FITC (grey) in standard running buffer 1 spiked with FHR-4. The testing has been done in triplicates. Both combinations show a false positive signal and have the highest signals at a concentration of 100 ng/mL. For a better overview are the control lines not shown in the diagram. They were, however, in the expected range.

Different ratios of the antibody combinations were tested in order to find out whether the results can be improved. By using a higher amount of the antibody AF5980-biotin strong false positive signals were detectable. By changing the ratio to a higher rate of AF5980-FITC false positive signals did not occur, but the signal strength decreased. Therefore, the best ration of this clone combination is 1:1.

Testing those combinations in human serum with serum buffer yields stronger signals for 640212-biotin with AF5980-FITC (**Figure 10**, grey). The strongest signal for this antibody combination is in a 1:20 dilution, with a higher dilution the signal decreases, showing a high dose hook effect. For AF5980-biotin with 640212-FITC (blue) the strongest signal was detected with an undiluted serum. Spiking serum with a 1:200.000 dilution with recombinant FHR-4 yields high signal again. Those results show that both antibody combinations are able to detect FHR-4 in human serum. The spiking of the diluted serum shows that measured results for the undiluted serum has a similar value, thus either the used serum contains a similar amount of FHR-4 or cross reactions is detected.

To define the optimal antibody combination a cross-reactivity test has been done against FHR-1. The results of this test can be found in section five.

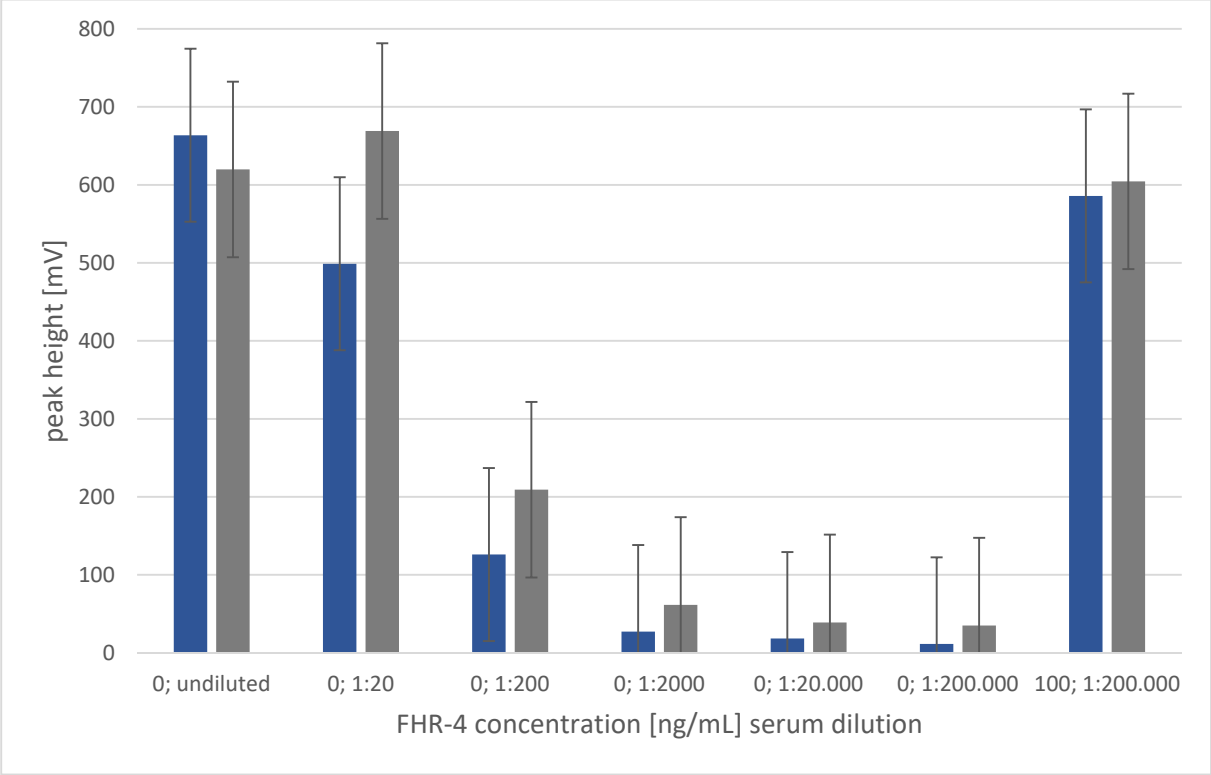

Figure 10: Titration of the tested clone combinations anti FHR-4-AF5980 biotin with anti FHR-4-640212 FITC (blue) and anti FHR-4- AF5980 FITC with anti FHR-4 640212 biotin (grey) in serum. The test has been done in triplicates. The control lines were detectible in the expected range, while the test lines were decreasing with a higher dilution. For a better overview are the control lines not shown in the diagram. Only the “100 ng/mL 1:200.000” serum had been spiked with FHR-4 and therefore, has a high signal.

5. Cross reaction between of the clone combinations and different analytes

Apart of finding suitable antibody combinations of the different antibodies to detect the proteins of the FH family, it is important to also test those antibodies for their cross-reactivity. To know which antibody combinations have a cross reactivity to other proteins is extremely important as those would falsify the results. In order to be able to eliminate the cross reactivity, this cross-reactivity first needs to be known. As all antibodies have a high similarity to each other, it is very likely that cross-reactivity takes place and each antibody needs to be tested against all proteins of the FH family.

5.1. Cross reactivity of anti-FHR-1 antibodies to recombinant FHR-4

The cross-reactivity has currently only been tested for the anti FHR-1 442127-biotin with anti FHR-1 4D7-FITC combination, as this combination has reached better results in the previous tests. As **Figure 11** shows, very low cross reactivity was detectable in standard running buffer 1. This cross-reactivity was as strong as the false positive signal at about 10 mV which is a rather low signal and is therefore neglectable. The antibody pair still needs to be tested for cross-reactivity with the other analytes as well as it still needs to be tested with all native analytes.

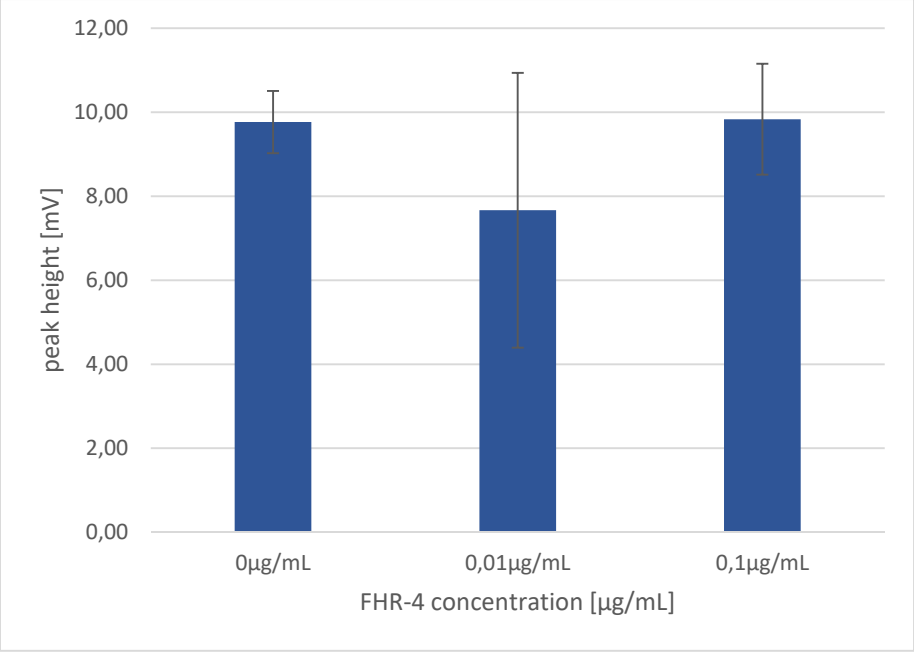

Figure 11: Test for cross reactions with the antibody combination anti-CFHR-1-442127-biotin with 4D7-FITC with FHR-4. Low signals were detectable. For a better overview are the control lines not shown in the diagram. They were, however, in the expected range. The test has been done in triplicates. The test has been done in standard running buffer 1.

5.2. Cross reactivity of anti-FHR-4 antibodies to recombinant FHR-1

The used antibody combinations anti FHR-4 AF5980-biotin with 640212-FITC (blue) and 640212-biotin with AF598-FITC (grey) were tested regarding their cross reactivity against recombinant FHR-1 (**Figure 12**). The test was done in standard running buffer 1. Both antibody combinations show false positive signals. The combination with AF5980-biotin shows a higher cross reactivity, which increases with a higher concentration of FHR-1. Reaching a concentration of 0.1 µg/mL the cross-reactivity has a signal at about 70 mV, while the cross-reactivity of 640212-biotin has a signal of about 20 mV. The antibody combination 640212-biotin with AF598-FITC has a less distinct cross reactivity, which stays on the same level as the false positive results (about 20 mV)). Therefore, a clear statement of the cross reactivity cannot be given. In order to give a clear statement, the false positive signals need to be minimized. Testing the cross reactivity in a depleted serum will give more information. Afterwards the cross-reactivity with the other proteins of the Factor H family need to be tested.

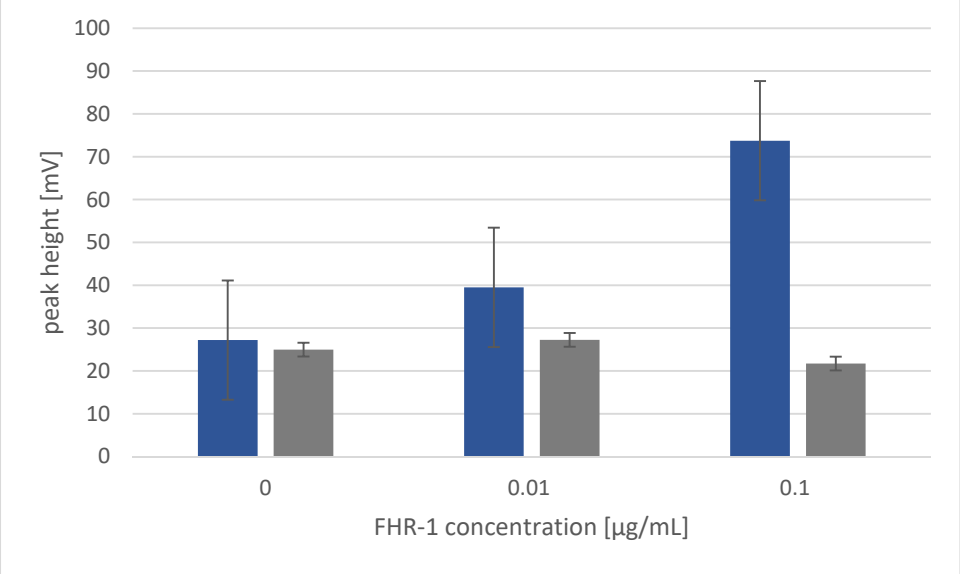

Figure 12: Test for cross reactions with the antibody combination anti FHR-4 AF5980-biotin with 640212-FITC (blue) and anti FHR-4 640212-biotin with AF598-FITC (grey) with FHR-1 in standard running buffer 1. Both combinations perform cross reactions. The cross-reactivity form anti FHR-4 AF598-biotin with 640212-FITC is stronger than the cross-reactivity of the other antibody combination. For a better overview are the control lines not shown in the diagram. They were, however, in the expected range. The test has been done in triplicates.

6. Conclusion

The D3.1 report gives an overview of the testing for suitable clone combinations to detect the proteins of the FH-family in an LFA format and to prepare the way for multiplex detection of those proteins. So far anti-FHR-1, anti-FHR-3 and anti-FHR-4 antibodies as well as anti-FH antibodies were tested. **Table 2** gives an overview of the testing state. To further investigate the cross reactivity and the detection in serum it might be necessary to use depleted serum.

So far, the tested antibody combinations (apart of anti-FHR-3 antibodies) give results in the desired analyte concentration range. For FHR-3 it will be necessary to screen for further

antibody combinations provided by SAN. All antibodies tested so far still need more evaluation, in order to find optimal ratio of antibodies and to give good results for serum test. Other matrices should also be included as far as applicable. Antibody combinations against FHR-2, FHR-5 and Factor H like-protein still need to be tested.

The concept for multiplexing was worked out and will be evaluated as soon as at least three FHR-x antibody pairs with stabile performance are found or be basically evaluated with other biologically relevant molecules.

Table 2: Overview of the testing state of the different antibody combinations with the next planned steps for further improvement. The results of the remaining tests of anti-FHR-3 are stated in table 1 and not shown here again. By changing the test composition (e.g. different ratio of antibodies, pre-incubation) the results of anti-FHR-3 might be improved. If an improvement is not possible further clone combinations need to be screened.

LFA specific for	Antibody combination		Tested in Puffer	Tested in Serum	Limit of detection	Cross reactivity	Next steps
	Biotin capture	FITC detector					
FH	556317 -biotin	OX23 -FITC	Good results	Not done	below 0.01 ng/mL	Not tested yet	Serum test
	OX23 -biotin	556317 -FITC	No detection of the analyte	Not done	-	-	none
FHR-1	4D7 -biotin	442127-FITC	False positive	False positive	-	-	none
	442127 -biotin	4D7-FITC	High dose hook effect; (5:1 as best ration)	No signals	<286 ng/mL	FHR-4 (neglect-able)	Improve-ment serum assay
FHR-3	3.16-biotin	Poly2.2-FITC	False positive	-	-	-	Test of other ratios and pre-incubation
	3.11-biotin	3.14-FITC	Low signals	-	-	-	Test of other ratios and pre-incubation
FHR-4	640121 -biotin	AF5980-FITC	weak false positive signals; highest signal at 100 ng/mL	High dose hook effect	<10 ng/mL		Testing for cross reactivity
	AF5980 -biotin	640121 -FITC		good signals without hook effect	<10 ng/mL	FHR-1 (high)	Reduction of cross reactivity