# D3.2
# Description dynamic range LFAs

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# Disclaimer

## Abbreviations and Acronyms

| | |
|---|---|
| ELISA | Enzyme linked immunosorbent assay |
| ELTE | Eötvös Loránd University |
| FH | Complement factor H |
| FHR | Factor H-related |
| HBT | Hycult Biotech |
| LFA | Lateral flow assay |
| LOD | Lower limit of detection |
| LOQ | Lower limit of quantification |
| MICRO | Microcoat Biotechnology GmbH |
| POC | Point of care |
| SAN | Sanquin |
| SciFiMed | Screening of inflammation to enable personalized medicine |
| UCM | Complutense University of Madrid |
| UMR | University Marburg |
| UMCG | University Medical Center Groningen |

# Summary

This document is deliverable 3.2 (D3.2), which relates to the dynamic ranges of the lateral flow assay (LFA). The LFAs are developed for a highly challenging point of care (POC) device in order to quantify simultaneously seven proteins with totally different concentration ranges in human sample.

For this part of WP3 capture and detection antibodies provided from the SciFiMed partner SAN were used. These were screened for the optimal antibody pair against FHR-2, -3, -4 (still ongoing) and -5. Therefore, the antibodies needed to show a low to no cross reactivity to the non-specific analytes of the protein family.

The envisioned device for SciFiMed is a 7-plex device for the different proteins of the FH protein family. After finding antibodies which are usable for LFA, a single-plex LFA for each antibody pair was developed (D3.2). The developed single-plex LFAs are then used for the development of a multiplex LFA (Task 3.3). This has been done simultaneously for the so far developed single-plex assays. The feasibility of a first multiplex is shown, however, further development is needed. The results will be shown in D3.3.

The work shown in this report is related to deliverable 3.2 (D3.2, single LFA for each FH protein). The proteins for this part were partly provided by SciFiMed partner UCM, ELTE and partly commercially. The used depleted plasma was also provided by SciFiMed partner UCM. The testing results will be described in this report and are summarized at the end of the report. The best performing antibody combinations were tested for cross-reactivity and the detection of the analytes in plasma/whole blood. By testing the antibody combinations, good results were gained for detection of FHR-2, -3 and -5 in buffer and plasma.

# Contents

# 1. Introduction

This document is deliverable 3.2 (D3.2), which is included in task 3.2 in work package 3 (WP3). One of the goals of SciFiMed is the development of a lateral flow assay (LFA) for the Factor H (FH) protein family. **Figure 1** shows the general setup for a LFA test strip. The composition of such an assay contains four parts: (1) a sample pad, where the sample is added, (2) followed by a conjugate pad, that holds the detection particle, (3) a membrane on which the test- and control line are dispensed and a sandwich complex is formed and finally (4) a waste pad at the far end of the strip which absorbs not captured sample and reaction buffer.

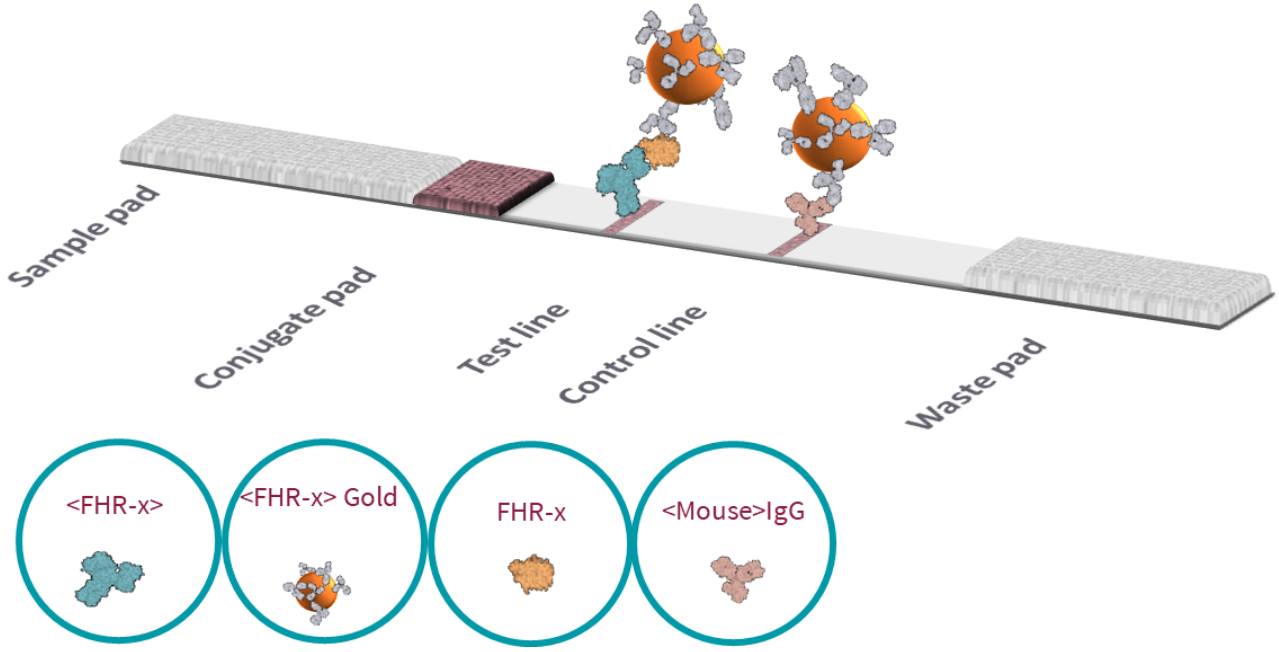


**Figure 1: Set-up of the test strips of the lateral flow assay. The test line antibody captures the analyte and by binding of the detection antibody (conjugated to gold nano particles) a sandwich complex is formed and can be measured. As control line an anti-mouse antibody was immobilized on the membrane which becomes visible after interaction with the murine anti-FITC antibody.**

The analyte first binds to the detection antibody at the conjugate pad. Next, the formed complex will be captured at the test line by the capture antibodies, forming a sandwich complex. Not captured detection particles will bind to the control line by an anti-mouse antibody directed against the antibody immobilized on the gold particles. The sandwich complex is only formed if the analyte is present in the solution. Its intensity can be measured electrometrically and is also visible by eye. FHR-specific antibodies are generated in WP2 by SAN and used for the shown work in this report. For an indirect way of detection, the test line can also be immobilized SA-poly and the gold is conjugated to FITC. To detect the Then the detection antibody has to be labeled with FITC while the capture antibody is labeled with biotin.

The main concern for the LFA is the cross-reactivity of the antibodies for other FHRs, as this would lead to incorrect results. Nevertheless, if the antibodies on one test strip exhibit cross-reactivity to the same analyte, the addition of an extra test line can be used to solve this issue.

The development of single-parameter tests within this work package, including the determination of cross-reactivity, is essential for the advancement of a multiplex device. In this work package, suitable antibody combinations for each protein are evaluated and tested for their cross-reactivity. Subsequently, the detection process will be tested using a human matrix (plasma). It is important to note that the data obtained from WP2 cannot be directly transferred from ELISA to LFA format. Therefore, antibody combinations used for the specific FHR ELISAs will be partially re-analyzed in WP3 for the LFA format. The results from WP3 are crucial for the development of the desired biosensor device in WP5 and for the validation of the assay in WP6.

# 2. General concept of the test setup

The antibodies developed by SAN were always tested as detection and as capture antibodies. The capture antibodies were conjugated to gold nanoparticles, while the detection antibodies were dispensed on nitrocellulose membranes. For these initial tests, only small amounts of antibodies were needed. The optimal antibody combination was identified by testing different concentrations of the desired analyte (i.e., specific FHRs) for each antibody pair. The best antibody combination was then utilized to optimize the test setup. Following the optimization phase, the lower limit of detection was established, and a cross-reactivity test was conducted. In certain assays, the development process commenced using an indirect approach similar to D3.1, where a biotin-FITC system was employed. If the final antibody combination for a specific FHR showed a good performance, antibody combinations were screened for the next analyte (i.e., another FHR). After completing individual LFA assays for two separate analytes, they were subsequently tested in a multiplex format. The challenge here is that the same running buffer has to be used, the analytes should have similarly intense signals and more conjugate pads are needed, which influences the flow through the test strip.

# 3. Finding the best clone combination

## 3.1. Testing for anti FHR-2

Used antibodies: Anti-FHR-2.11, Anti-FHR-2.12, Anti-FHR-2.13, Anti-FHR-2.14 and Anti-FHR-2.15 each conjugated to gold nanoparticles and dispensed on a nitrocellulose membrane. All antibodies were provided by the SciFiMed partner SAN.

While testing the antibody combinations in buffer spiked with FHR-2, the results varied highly. Some combination hardly showed any test line signal at all and some caused an aggregation of the gold nanoparticles. The best combination for FHR-2 was with the anti-FHR-2.13 conjugated to the gold nanoparticles, while the anti-FHR-2.11 or the anti-FHR-2.12 was immobilized as the capture antibody onto the membrane. To gain good results different running buffers were tested. The gold conjugations showed aggregation in running buffer 1 to 3, therefore, only running buffer 4 can be used for the detection of FHR-2. The results of the best antibody combinations, while using running buffer 4, to detect FHR-2 in plasma are shown in **Figure 2**. It can be seen that both combinations are able to detect FHR-2 in plasma. As the combination anti-FHR-2.11 with anti-FHR-2.13 gained higher signals than the combination anti-FHR-2.11 with aFHR-2.12, it was chosen for optimization.

After finding the optimal conditions for the conjugation to gold nanoparticles the limit of detection (LOD) and limit of quantification (LOQ) were identified. The LOD is 0.67 ng/mL and the LOQ is 1.95 ng/mL.

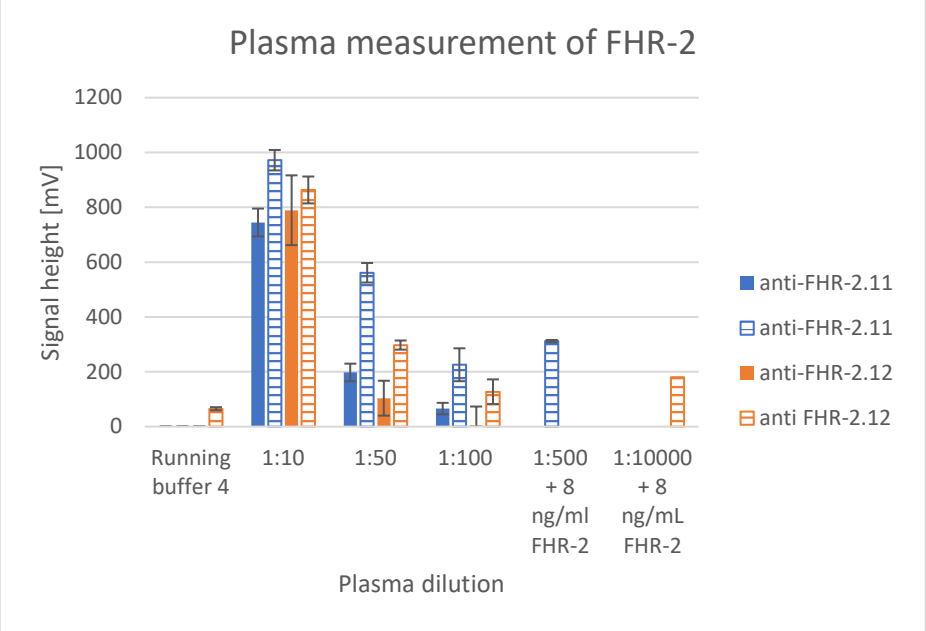


**Figure 2: Results of plasma testing of the two best antibody combinations in plasma. Blue: anti-FHR-2.11 immobilized on the membrane, anti-FHR-2.13 conjugated to gold nanoparticles; orange: anti-FHR-2.12 immobilized on the membrane, anti-FHR-2.13 conjugated to gold nanoparticles; filled columns: unoptimized gold conjugations, striped columns: optimized anti-FHR-2.13 gold conjugation was used. Using anti-FHR-2.12 as capture antibody the visual detection by eye is possible up to a plasma dilution of 1:50, for anti-FHR-2.11 until 1:100. The optimized conjugation condition shows a more sensitive detection of FHR-2. Only the optimized conjugation conditions were spiked with 8 ng/mL FHR-2 at a dilution of 1:500 or 1:10000. n=3.**

As **Figure 2** shows, the optimized gold conjugations (stripped columns) gain higher signals than the unoptimized conjugations (filled columns) and, therefore, allow the detection of higher diluted samples. By spiking FHR-2 in a high diluted plasma sample, the detection of FHR-2 is possible again. The chosen concentration was 8 ng/mL. This concentration of FHR-2 equates the current knowledge of a plasma concentration of a 1:250 dilution[1], however, the measured signal is higher than the signal of the 1:100 dilution. This is most likely caused by less matrix effects of the plasma in a higher dilution. To confirm the possibility of detecting FHR-2 in a more complex matrix, the detection was also conducted using whole blood. Clear test lines were observed and measured, demonstrating the feasibility.

[1] A. E. van Beek *et al.,* „Factor H-Related (FHR)-1 and FHR-2 Form Homo- and Heterodimers, while FHR-5 Circulates Only As Homodimer in Human Plasma“ (eng), *Frontiers in immunology*, Jg. 8, S. 1328, 2017.

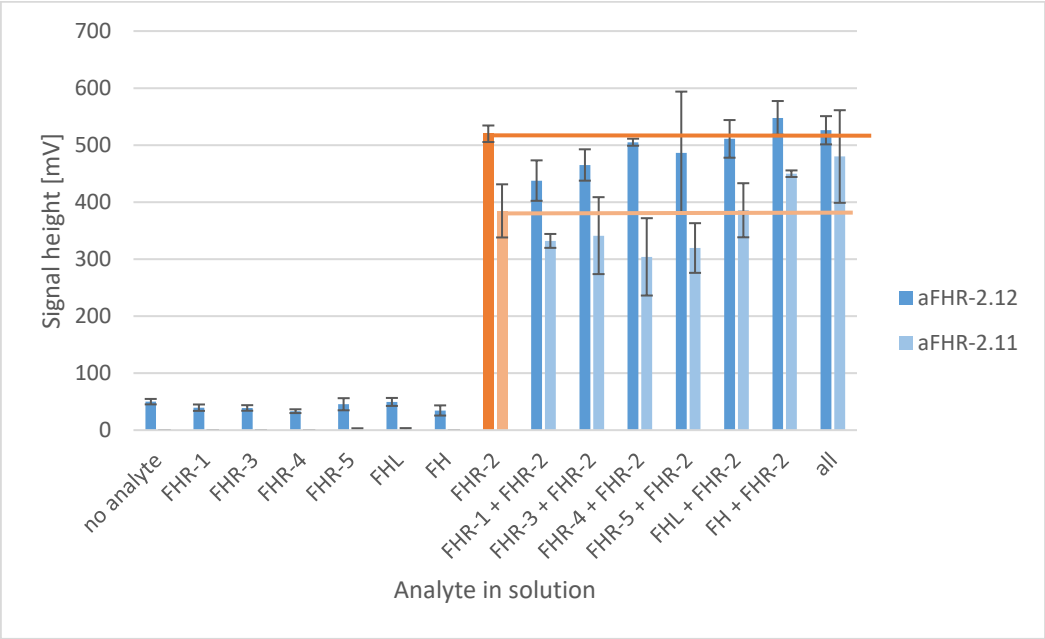


**Figure 3: Test of cross reactivity using anti-FHR-2.12 or anti-FHR-2.11 as capture and anti-FHR-2.13 as detection antibody. The orange column indicates the signal when only FHR-2 is present in the solution with a concentration of 8 ng/mL. The horizontal orange line indicates the signal height to visualize the changed height cause by cross reactivity. All members of the factor H protein family had a signal of ~ 30-50 mV with anti-FHR-2.12 as detection antibody. Those signals were not visible by eye. FHR-1 and FHR-3 in the same solution as FHR-2 decrease the signal intensity. FH causes a higher signal. When all FH-proteins are present in the same solution the signal does not change. The concentrations of the FHR proteins correlate to the FHR concentrations in plasma when 8 ng/mL FHR-2 is present. Using anti-FHR-2.11 as capture antibody causes lower signals in general as well as a stronger cross reactivity. n=3.**

Next, cross reactivity for other members of the FH protein family was assessed. The results are shown in **Figure 3**. With anti-FHR-2.11, no cross-reactivity was observed when using samples lacking FHR-2 but containing a single recombinant or purified protein of the other FH family. However, when either FHR-1, -3, -4 or -5 was added to a sample containing FHR-2 the signal intensity of FHR-2 decreased. In contrast, FH caused an increase of the signal when this protein was added to a sample containing FHR-2. Because of these unforeseen problems, FHR-2.12 was used to replace FHR-2.11 as capture antibody and the assay's cross-reactivity was reassessed. With FHR-2.12 as a capture antibody, weak false positive results were measured with each cross reagent. However, since the test lines for those signals were not visually observable, this does not present a significant problem. As 8 ng/mL FHR-2 caused a clearly visible test line, this concentration was used for the cross reactivity. The concentration of the other proteins correlated with the FHR-2 concentration: for each protein the “normal”

plasma concentration was calculated under the assumption that the plasma concentration for FHR-2 is 8 ng/mL. [2345678]The used concentrations are listed in **Table 1**.

**Table 1: Used concentration of FHR-1, -3, -4, -5, FHL and FH for measurement of the cross reactivity of the FHR-2 test strip. The concentrations were calculated under the assumption that the plasma is diluted to a FHR-2 concentration of 8 ng/mL.**

| Protein | Concentration |
|---|---|
| **FHR-1** | 162 ng/mL |
| **FHR-3** | 1,5 ng/mL |
| **FHR-4** | 67 ng/mL |
| **FHR-5** | 15 ng/mL |
| **FHL-1** | 133 ng/mL |
| **FH** | 800 ng/mL |

By changing the capture antibody from anti-FHR-2.11 to anti-FHR-2.12, less cross reactivity was seen when the other FH family members were added to a sample containing FHR-2. Only FHR-1 still resulted in change in signal (-15.8%), demonstrating cross-reactivity. However, importantly, the signal for the sample containing all FH family members was in the same range as the signal for the sample containing only FHR-2. The strongest difference in cross reactivity for the two antibody combinations was observed when FHR-4 was added to a sample containing FHR-2. Using anti-FHR-2.12 as a capture antibody the signal decreased by 2.9% when FHR-4 was added to a sample containing 8 ng/mL FHR-2, while it decreased by 21,0 % when the aFHR-2.11 was used as capture antibody. A signal change of 15% is considered as cross reactivity. Therefore, anti-FHR-2.12 does not have a cross reactivity whereas anti-FHR-2.11 has a cross-reactivity against FHR-4. The calculated change of the signal intensity for the other proteins are shown in **Table 2**.

[2] N. R. Medjeral-Thomas *et al.,* „Circulating complement factor H-related proteins 1 and 5 correlate with disease activity in IgA nephropathy“ (eng), *Kidney international*, Jg. 92, Nr. 4, S. 942–952, 2017.

[3] A. E. van Beek *et al.,* „Factor H-Related (FHR)-1 and FHR-2 Form Homo- and Heterodimers, while FHR-5 Circulates Only As Homodimer in Human Plasma“ (eng), *Frontiers in immunology*, Jg. 8, S. 1328, 2017.

[4] M. A. Friese *et al.,* „Different regulation of factor H and FHL-1/reconectin by inflammatory mediators and expression of the two proteins in rheumatoid arthritis (RA)“ (eng), *Clinical and experimental immunology*, Jg. 121, Nr. 2, S. 406–415, 2000.

[5] R. Sofat *et al.,* „Distribution and determinants of circulating complement factor H concentration determined by a high-throughput immunonephelometric assay“ (eng), *JOURNAL OF IMMUNOLOGICAL METHODS*, Jg. 390, 1-2, S. 63–73, 2013.

[6] R. B. Pouw *et al.,* „Complement Factor H-Related Protein 3 Serum Levels Are Low Compared to Factor H and Mainly Determined by Gene Copy Number Variation in CFHR3“ (eng), *PloS one*, Jg. 11, Nr. 3, e0152164, 2016.

[7] M. Hebecker und M. Józsi, „Factor H-related protein 4 activates complement by serving as a platform for the assembly of alternative pathway C3 convertase via its interaction with C3b protein“ (eng), *The Journal of biological chemistry*, Jg. 287, Nr. 23, S. 19528–19536, 2012.

[8] K. A. Vernon *et al.,* „Acute presentation and persistent glomerulonephritis following streptococcal infection in a patient with heterozygous complement factor H-related protein 5 deficiency“ (eng), *American journal of kidney diseases : the official journal of the National Kidney Foundation*, Jg. 60, Nr. 1, S. 121–125, 2012.

**Table 2: Change of the signal intensity of the FHR-2 test line with 8 ng/mL FHR-2 with calculated concentrations of the other FHR proteins. The change of the signals is calculated to the signal intensity of FHR-2 when no other proteins were in the solution. A signal change of > 15% is considered cross reactivity.**

| Analyte | Capture antibody | |
|---|---|---|
| | anti-FHR-2.12 | anti-FHR-2.11 |
| **FHR-1** | -15.8 % | -13.7 % |
| **FHR-3** | -10.5 % | -11.1 % |
| **FHR-4** | -2.7 % | -21.0 % |
| **FHR-5** | -6.4 % | -16.9 % |
| **FHL-1** | -1.7 % | +0.3 % |
| **FH** | +5.35 % | +17 % |

The calculated LOD for the FHR-2 test strip with anti-FHR-2.12 as capture and anti-FHR-2.13 as detection antibody is 0.68 ng/mL and is therefore in the same range as using anti-FHR-2.11 as capture antibody. The calculated LOQ is 1.8 ng/mL. For the complete dynamic range, the highest measurable concentration still needs to be determined. This will be done during the measurement of the standard curve.

### 3.2. Testing for anti FHR-3

Used antibodies: Anti-FHR-3.1, Anti-FHR-3.4, Anti-FHR-3.11 and Anti-FHR-3.12, each conjugated to gold nanoparticles and dispensed on a nitrocellulose membrane. All antibodies were provided by the SciFiMed partner SAN.

In D3.1 it was described that the antibody combinations to detect FHR-3 only gain weak signals. Since then, it was observed that the signals increased using a fresh FHR-3 lot and it was decided to use anti-FHR-3.1 as capture and anti-FHR-3.4 as detection antibody. The LFA study was performed in running buffer 1 as well as in human plasma and whole blood. The change to running buffer 4 was only necessary for the development of the multiplex assays as the development of the FHR-2 assay showed that running buffer 4 has to be used for the FHR-2 detection. It was not used from the beginning as the development of the FHR-3 assay was done prior the development of the FHR-2 assay.

In initial testing of D3.1, the detection antibody was labeled with FITC and a murine anti-FITC antibody was labeled to gold nanoparticles. To simplify the procedure, a direct conjugation of the antibody to the gold nanoparticles was performed. Next, the best conditions of the gold conjugation were tested. Subsequently, the capture system was simplified as well by a direct dispensing of anti-FHR-3.1. So far, the assays were always performed in running buffer 1. By using direct dispensed anti-FHR-3.1 false positive results were gained. Different running buffers were tested to gain results without false positives. The best results were gained using running buffer 3. The calculated LOD for the test system is 0.12 ng/mL. The LOQ is 0.46 ng/mL. The antibody combination was also tested in human plasma, using different

dilutions. A visible test line developed when plasma was undiluted or diluted up to 1:500, However, when the plasma sample was diluted at 1:500 the test line was hardly visible anymore. Therefore, a recovery testing was performed by adding 1 ng/mL FHR-3 to the 1:500 dilution, which caused a 239% higher signal, showing that the recovery experiment was successful.

Afterwards the combination was tested for cross reactivity. **Figure 4** shows the results of the cross-reactivity when FHR-3 was combined with another FH family member in the same sample. Significant cross-reactivity of the FHR-3 assay was observed for FH and FHR-4, as a signal reduction of 57% occurred by the addition of FH and 21% by the addition of FHR-4. This problem was addressed by changing the capture antibody to anti-FHR-3.11. The same observation was done by SciFiMed Partner SAN as anti-FHR-3.4 is cross reactive against FH, while anti-FHR-3.1 is cross reactive against FHR-4.

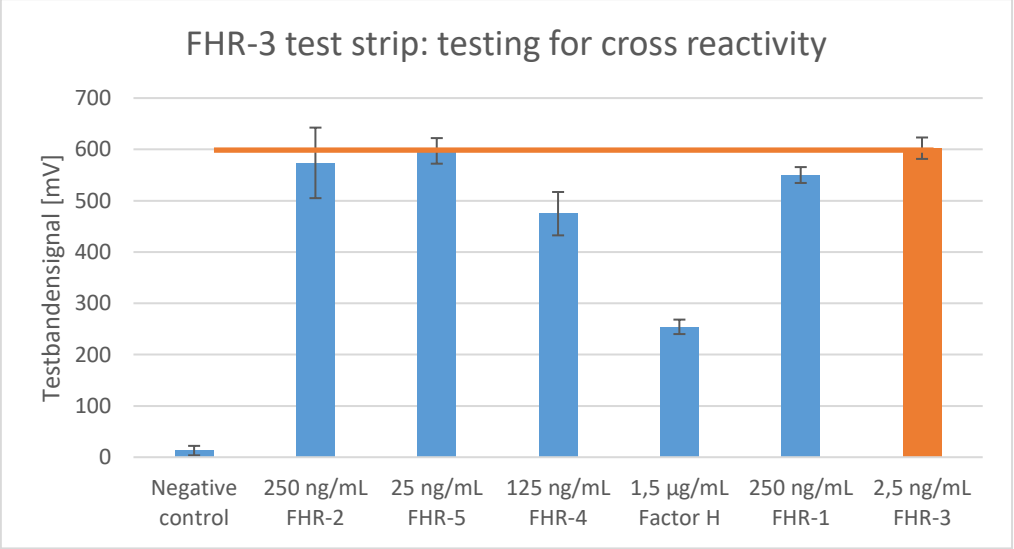


**Figure 4: Test of cross reactivity using anti-FHR-3.1 as capture and anti-FHR-3.4 as detection antibody. For each test 2.5 ng/mL FHR-3 was in the solution. A test with 2.5 ng/mL FHR-3 was done as reference signal (orange). Testing the FHR-proteins without FHR-3 in the solution did not cause significant signals and the results are not shown. The highest cross reactivity was caused by FH followed by FHR-4 and FHR-1. The concentrations of the FHR proteins correlate to the FHR concentrations in plasma when 2.5 ng/mL FHR-3 is present. As negative control only running buffer was applied. n=3.**

In collaboration with SciFiMed partner SAN, it was decided to test anti-FHR-3.11 and -3.12 as capture antibodies for the FHR-3 LFA assay. Both were able to detect FHR-3 in combination with the anti-FHR-3.4 as a detection antibody in running buffer 4. As depicted in **Figure 5**, the use of anti-FHR-3.11 and -3.12 as capture antibodies resulted in a lower signal for FHR-3 compared to the use of anti-FHR-3.1 as capture antibody. The loss of the sensitivity can be addressed by the plasma dilution. Whether this is necessary, will be addressed in the development of the multiplex and the final set up of the FHR-3 test strip.

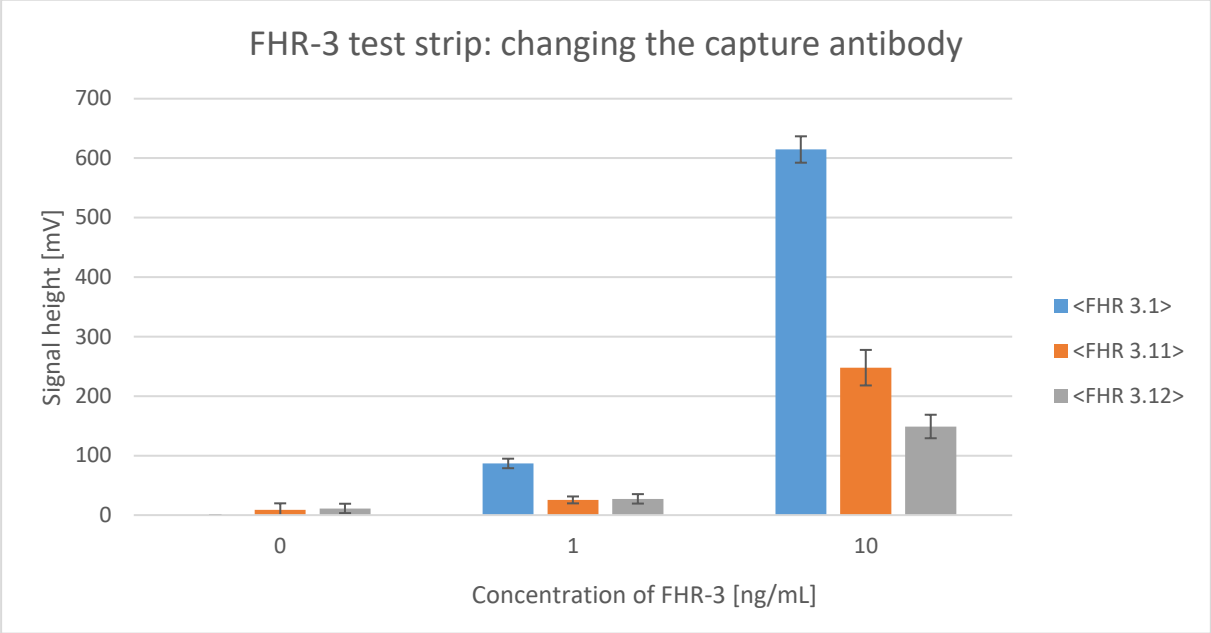


**Figure 5: Comparison of anti-FHR-3.1, -3.11 and -3.12 as capture antibody for the FHR-3 assay. Anti-FHR-3.1 gains the strongest signals. By changing the detection antibody to anti-FHR-3.11 the signal decreases by 60 % and by changing the capture antibody to anti-FHR-3.12 the signal decreases by 76%. n=3.**

Subsequently, the cross-reactivity for these new capture antibodies in the FHR-3 LFA assay was re-analyzed as shown in **Figure 6**. For the anti-FHR-3.11, a significant reduction of 60-70 % was observed in the FHR-3 signal when FH was added. The reduction is caused by the anti-FHR-3.4 detection antibody. Furthermore, the addition of FHR-4 also caused a reduction in the FHR-3 signal. The FHR-3 signal did not change with the anti-FHR-3.11 as a capture antibody with the addition of FHR-1, FHR-2, or FHR-5. For the anti-FHR-3.12, a significant reduction was observed in the FHR-3 signal when FH was added as well, which is again caused by the anti-FHR-3.4 detection antibody. In contrast, using the anti-FHR-3.12 as capture antibody, no major changes were observed in the FHR-3 signal when FHR-4 was added. The FHR-3 signal did not change either with the anti-FHR-3.12 as a capture antibody with the addition of FHR-1, FHR-2, or FHR-5. For both assays, the FHR-3 signal increases by addition of FHL-1.

Although anti-FHR-3.11 is cross reactive to FHR-4 it was chosen as capture antibody for the developed multiplex test strip (a test strip against FHR-2, FHR-3, and FHR-5). Crucial for the decision was the signal height. Using anti-FHR-3.11 as capture antibody, 210 % higher signals were measured than for FHR-3.12 (only FHR-3 present). As FHR-3 has the lowest concentration of all FHR-proteins in human plasma a higher sensitivity is needed. As a future direction, the detection antibody will be replaced to reduce the currently observed cross reactivity with FHR-4 and FH. Here the same antibody as SciFiMed partner HBT uses will be evaluated.

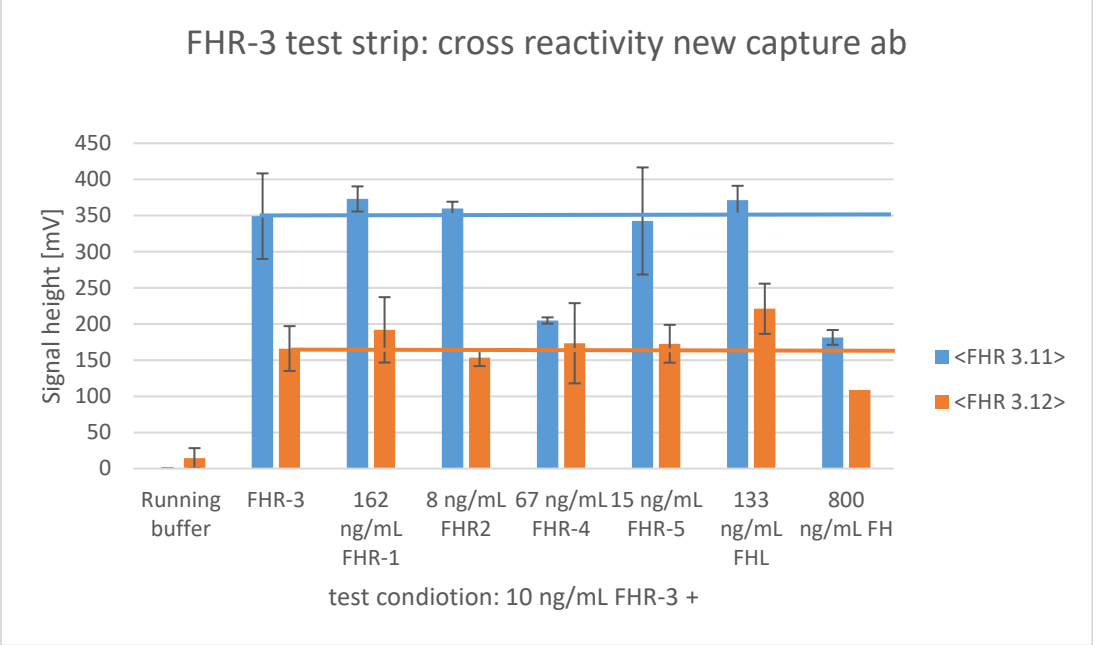


**Figure 6: Comparison of anti-FHR-3.1, -3.11 and -3.12 as capture antibody for the FHR-3 assay and their performance by the cross-reactivity testing. The horizontal orange/blue line indicates the signal height of only FHR-3 in the solution to visualize the changed height cause by cross-reactivity. Both capture antibodies show a lower signal when FH is present. Anti-FHR-3.11 also shows a cross reactivity against FHR-4, while FH increases the signal of FHL for anti-FHR-3.12. As negative control only running buffer was applied to the test strips. For all other measurements 10 ng/mL FHR-3 was in the solution as well as the shown amount of analyte under the column. n=3.**

### 3.3. Testing for anti FHR-4

Used antibodies: Anti-FHR-4A.04, Anti-FHR-4A.07, Anti-FHR-4A.08, Anti-FHR-4A.09 and Anti-FHR-4A.11 each conjugated to gold nanoparticles and dispensed on a nitrocellulose membrane. All antibodies were provided by the SciFiMed partner SAN.

Anti-FHR-4A.08 as capture and anti-FHR-4A.09 as detection antibody gained the best results. Those antibodies are also used by SciFiMed partner HBT for the ELISA production. As the multiplex against FHR-2, FHR-3 and FHR-5 was in development before the development of the FHR-4 LFA, running buffer 4 was used from the beginning making it easier to add the FHR-4 assay to a 4 or 6-multiplex later on. As a next step the cross-reactivity was tested.

**Figure 7** shows the results of the cross-reactivity testing. No cross-reactivity of the antibodies against the other proteins of the FH-family was measured. Even when all analytes are in the same solution the signal does not increase. Future experiments will include further cross-reactivity tests with human plasma which is depleted of specific proteins of the FH family. These depleted human plasmas are not commercially available, and are currently being produced by other SciFiMed partners.

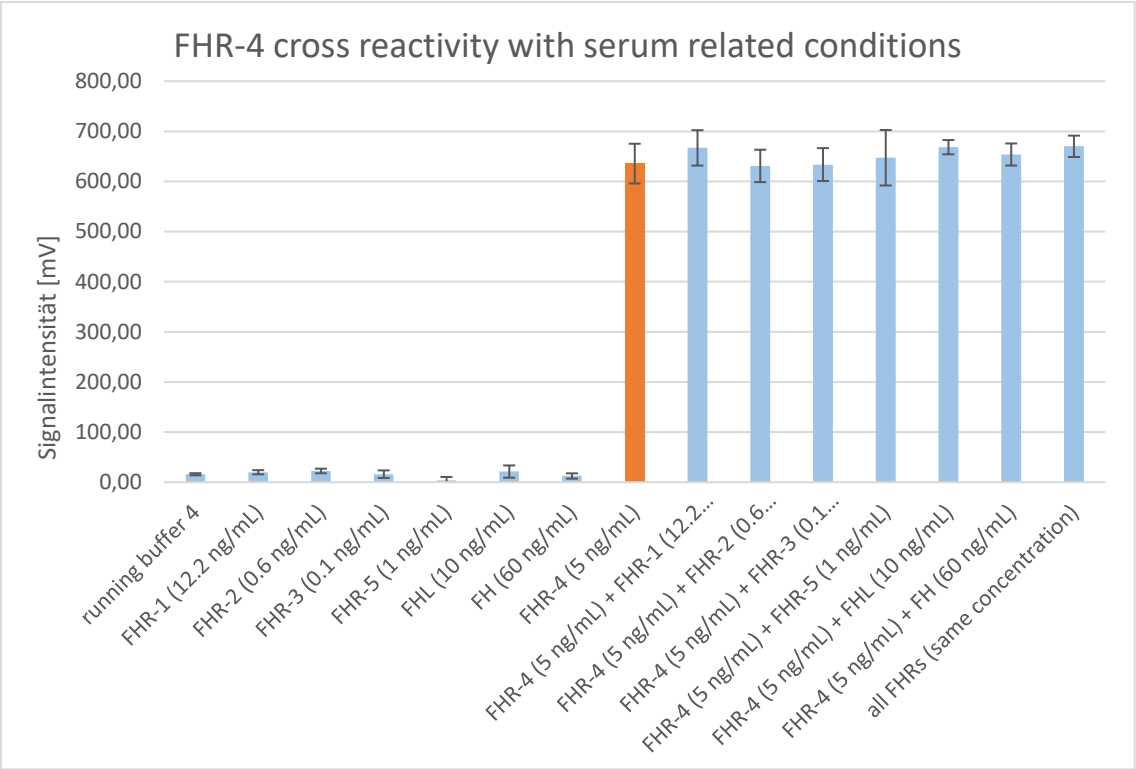


**Figure 7: Test of cross reactivity using anti-FHR-4A.08 as capture and anti-FHR-4A.09 as detection antibody. As negative control running buffer 4 was used. On the left part of the figure the FHR proteins were alone in the running solution and tested for cross reactivity. The orange bar shows the signal for 5 ng/mL FHR-4. On the right sight of the orange bar the FH-proteins are tested for cross reactivity when FHR-4 is present. The used concentration of the analytes were the calculated concentrations the analytes would have, if 5 ng/mL FHR-4 would be present in human plasma. No cross reactivity was detected. n=3.**

Furthermore, the conditions of the conjugation of anti-FHR-4A.09 to gold nanoparticles will be optimized. Once these conditions are established, the LOD as well as the detection in plasma need to be measured. After finalizing the optimization of the single LFA for FHR-4, the assay will be tested in the multiplex format.

### 3.4. Testing for anti FHR-5

Used antibodies: Anti-FHR-5.1 and Anti-FHR-5.4 each conjugated to gold nanoparticles and dispensed on a nitrocellulose membrane. All antibodies were provided by the SciFiMed partner SAN.

The first tests for the FHR-5 assay was performed using a FITC-biotin system, which was always used in D3.1. Here both antibodies were conjugated once to FITC and once to biotin. Using a streptavidin test line and anti-FITC antibody conjugated to gold nanoparticles, anti-FHR-5.4 performed well as detection while anti-FHR-5.1 performed well as capture antibody.

After establishing these findings in D3.1, we proceeded with this deliverable by testing the optimal conjugation conditions for the detection of FHR-5. By changing the pH during the conjugation, the signal could be increased by 830%.

The cross-reactivity as well as the LOD were determined with a directly immobilized capture antibody on the nitrocellulose membrane, as well as for an indirect detection where anti-FHR-5.1 was conjugated to biotin. **Figure 8** shows the results of cross-reactivity with direct dispensed anti-FHR-5.1 (final format of the test strip). No cross-reactivity was observed when using samples lacking FHR-5 but containing single recombinant or purified proteins of the other FH family members (data not shown). The results show that the antibody combination has no significant cross reactivity to the other members of the FH-family (FHL-1 was not tested). For this test, the FH family proteins were added in the calculated concentration they would have if 2 ng/mL FHR-5 would be in plasma. The cross-reactivity analysis was repeated with 150 % of the concentration of the FH family proteins (data not shown). This did not cause significant cross reactivity for FHR-1, FHR-2, FHR-3 or FH. However, the addition of FHR-4 in150 % of the plasma concentration did cause a reduction in FHR-5 signal of 13.4 %.

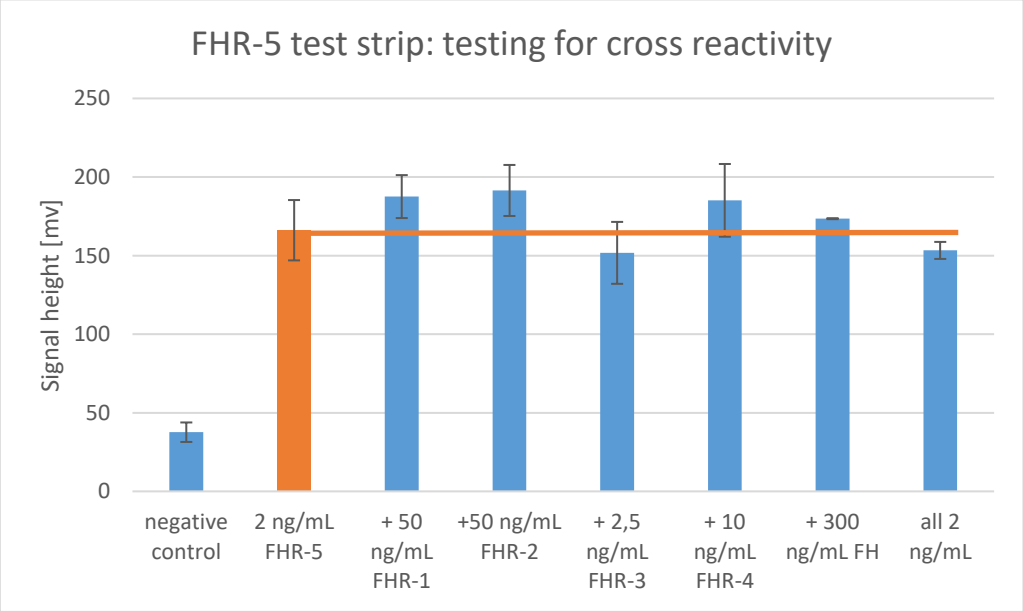


**Figure 8: Test for cross reactivity with anti-FHR-5.1 immobilized on the nitrocellulose membrane and anti-FHR-5.4 conjugated to gold nanoparticles. For each test 2 ng/mL FHR-5 was in the solution. A test with only 2 ng/mL FHR-5 was done as reference signal (orange) the horizontal orange line indicates the signal height for easier visualization. Testing the FHR-proteins without FHR-5 in the solution did not cause signals significantly different from the negative control and the results are not shown. No significant cross-reactivity was detected. The concentrations of the FHR proteins correlate to the FHR concentrations in plasma when 2.5 ng/mL FHR-5 is present. The test was also done with 150 % of the proteins in comparison to the plasma concentration of FHR-5. Here the cross reactivity was comparable, only FHR-4 caused a lower signal (87 % of the FHR-5 signal). n=3.**

The final set up for the assay was also tested in running buffer 3 as well as human plasma and whole blood. FHR-5 was also detected in human plasma as shown in **Figure 9**. A signal for FHR-5 was detected up to a dilution of 1:1000, whereas a dilution of 1:5000 in running buffer 3 gave an equal signal to our negative control. Of note, for a reliable detection of an analyte in an LFA assay, the signal height should be around 400 mV. By doing a recovery test it was

shown that by spiking 2 ng/mL FHR-5 to 1:50000 dilution the detection of FHR-5 was possible again, even though the signal was below 200 mV.

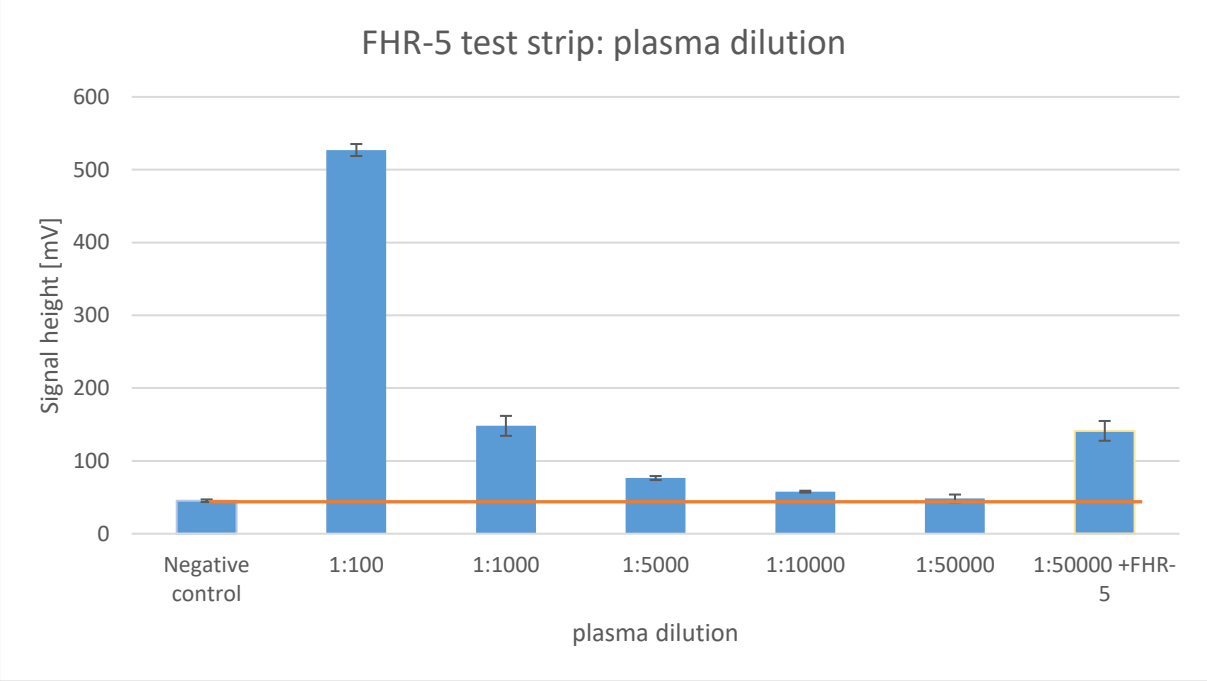


**Figure 9: Measurements of different plasma dilutions to detect FHR-5 using anti-FHR-5.1 as capture and anti-FHR-5.4 as detection antibody. The signal height of the buffer control is indicated by an orange line at 45 mV. FHR-5 can be detected up to a dilution of 1:1000 in running buffer 3. The recovery experiment shows that by diluting the plasma to a not detectable level and spiking it with 2 ng/mL FHR-5 the detection of FHR-5 is possible. n=3.**

Since the LFA against FHR-2 only yielded good results with running buffer 4, the running buffer for the LFA against FHR-5 had to be changed in order to conduct a multiplex test. As depicted in **Figure 10**, the use of running buffer 4, instead of running buffer 3, the false positive signal in the negative control decreased. Furthermore, the FHR-5 signal was lower overall compared to running buffer 3. In conclusion, a change of the running buffer is possible, even though the LOD changed from 0.009 ng/mL (running buffer 3) to 0.875 ng/mL (running buffer 4), as this concentration is under the plasma concentration of FHR-5 the change of the LOD is not considered as a problem. To confirm these results, a plasma dilution curve was re-tested comparing both running buffers. In accordance, these experiments also showed a lower FHR-5 signal using running buffer 4. For a dilution of 1:100 the signals with running buffer 4 were 51.6% lower than using running buffer 3. The feasibility of using buffer 4 for the FHR-5 LFA, despite the lower sensitivity, makes it possible to develop a multiplex assay for the combined detection of FHR-2 and FHR-5.

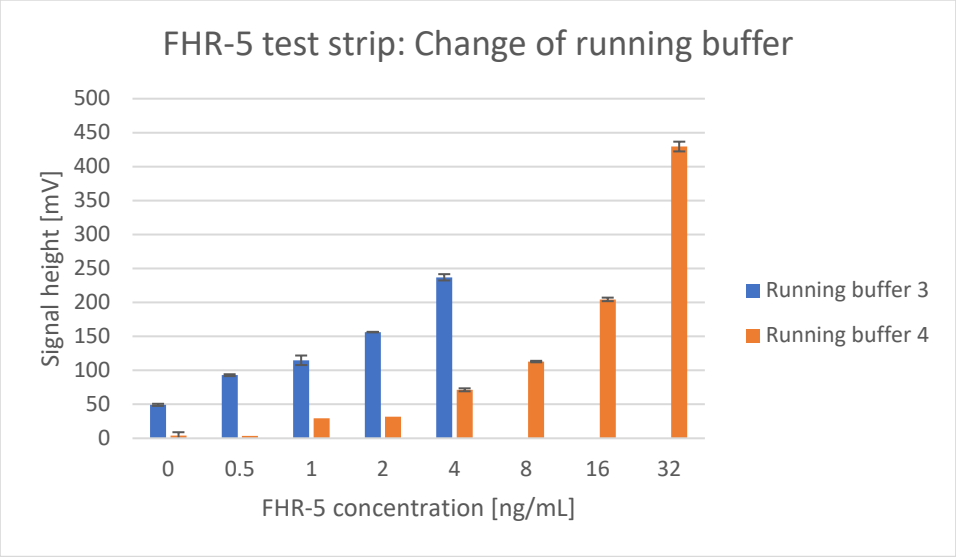


**Figure 10: Measurements of the LFA against FHR-5 in different running buffers using anti-FHR-5.1 as capture and anti-FHR-5.4 as detection antibody. The concentrations 8, 16 and 32 ng/mL were not measured in running buffer 3. By changing the running buffer, the false positive signal was minimized, but the signals were less intense. n=3.**

# Conclusion

The D3.2 report gives an overview of the development status of the single plex test strips against the proteins of the FH protein family with antibodies provided by SciFiMed partner SAN. The feasibility is shown for test strips against FHR-2, -3 and -5, while the testing against FHR-4 is still ongoing. The current state of the development is shown in **Table 3**. The developed FHR-3 assay will undergo some optimization by changing the detection antibody. For all developed assays a standard curve will be measured and the assays will be used for the development of a multiplex assay. The development of an LFA against FHR-1 has not been started so far.

**Table 3: Overview of the testing state of the different antibody combinations with the next planned steps for further improvement.**

| LFA specific for | Antibody combination | | Tested in buffer | Tested in plasma | Cross reactivity | Next steps |
|---|---|---|---|---|---|---|
| | Capture | Detection | | | | |
| FHR-1 | tbd | tbd | tbd | tbd | tbd | tbd |
| FHR-2 | aFHR-2.12 | aFHR-2.13 | LOD: 0.67 ng/mL; LOQ: 1.95 ng/mL | 1:100 | FHR-1 | Measurement of a standard curve and cross reactivity in depleted serum |
| FHR-3 | aFHR-3.1 | aFHR-3.4 | LOD: 0.12 ng/mL; LOQ: 0.46 ng/mL | 1:500 | FH and FHR-4 | - |
| FHR-3 | aFHR-3.11 | aFHR-3.4 | tbd | 1:100 | FH and FHR-4 | Change of detection antibody; Standard curve for new detection + capture |

| | | | | | | |
|---|---|---|---|---|---|---|
| | | | | | | antibody; determine new LOD and LOQ |
| **FHR-4** | aFHR-4A.08 | aFHR-4A.09 | tbd | tbd | none | Optimization of gold conjugation; Determination of LOD and LOQ, plasma testing |
| **FHR-5** | aFHR-5.1 | aFHR-5.4 | LOD: 0.875 ng/mL | 1:5000 | none | Measurement of a standard curve and cross reactivity in depleted serum |