Development of a dog-specific Enzyme-Linked Immuno Sorbent Assay (ELISA) for detecting Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH) in plasma

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Abstract

The Hypothalamus-Pituitary-Gonadal axis is a complex system of positive and negative feedback loops. After gonadectomy the hormonal balances in the organism will shift. In dog increases in basal levels of LH and FHS have been reported after gonadectomy. In the current study we have aimed to develop a novel diagnostic Enzyme Linked Immuno Sorbent Assay (ELISA) for the detection of LH and FSH in canine plasma.

We show that with the use of cross-reactive antibodies, recognizing human FSH we were able to detect FSH plasma concentration above approximately 1,5 ng/ml. In addition a combination of a rat specific and an anti-human LH antibody LH concentrations above 10ng/ml could be detected in canine plasma, in a reproducible manner. Although the assay setup was effective and stable, additional experiments have to be performed to optimise the current test as a reliable diagnostic tool.

Introduction

The reproductive system in the bitch is under constant hormonal control by the pituitary gland, that is driven by the hypothalamus. These organs comprise the so called hypothalamic-pituitary-gonadal axis. Communication is mediated from the hypothalamus to the pituitary gland by Gonadotrophin-Releasing Hormone (GnRH). The secretory frontal lobe of the pituitary gland produces Luteinising Hormone (LH) and Follicle Stimulating Hormone (FSH) which are responsible for the growth and development of recruited follicles in the ovaries. After a proper amount of ripening, in the late pro-oestrous, ovulation occurs following a peak level of LH in the bitches plasma. Furthermore, the ovaries release hormones such as oestradiol and inhibin, which take part in regulatory mechanisms (Johnston 2001).

In the male dog, the same axis exists with the testicles responsible for the gonadal part. Pulsatile secretion of LH is responsible for paroxysmal production of testosterone. (Johnston 2001, Palmer 2017)

The hypothalamic-pituitary-gonadal axis controls itself via a system of negative and positive feedback loops. After ovariectomy, an important part of this system changes, with hormonal changes as a result. For this research, the most relevant regulatory difference is the disappearance of the long negative feedback loop which extends from the ovarian hormones to the hypothalamus. (Beijerink 2007) There, the production and secretion of GnRH are inhibited under normal circumstances, except in the pre-ovulatory stage, where there is a sudden switch to positive feedback. (Johnston 2001). The absence of the long negative feedback loop causes a lack of inhibition and therefore higher basal values of LH and FSH in the bitches plasma (Beijerink 2007).

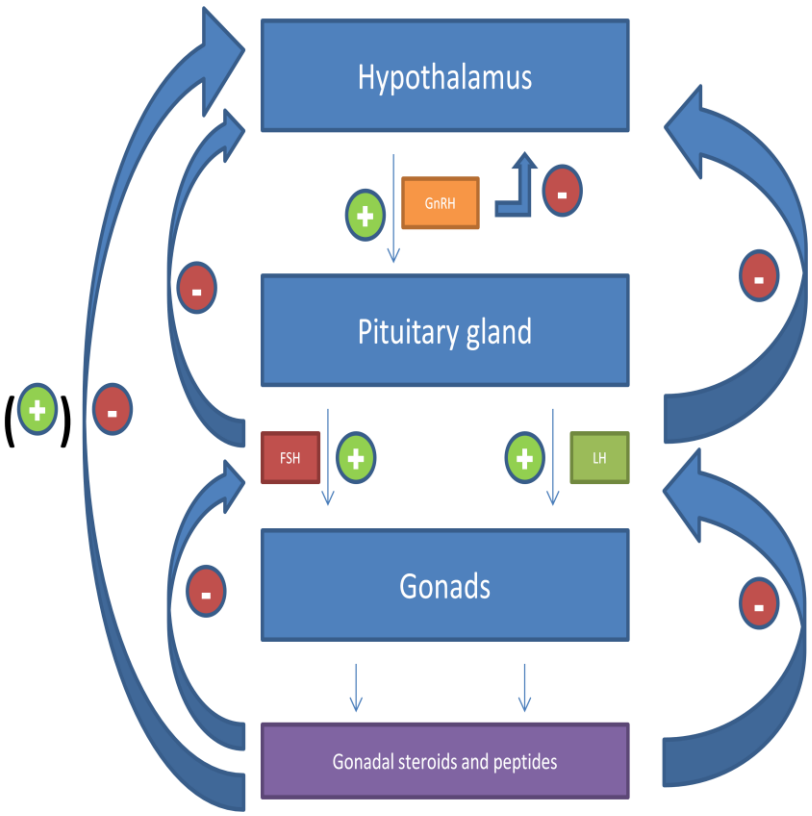

Figure 1: a schematic view of the feedback system which comprises the Hypothalamus-Pituitary-Gonadal axis. After gonadectomy, the most important hormonal change is the eradication of the long feedback loop that extends from the gonadal steroids to the hypothalamus. This eventually causes FSH and LH to constantly remain at high levels. + stands for positive feedback, - stands for negative feedback.

De Gier et al. described the ranges of LH and FSH in both intact anoestrous dogs and ovariectomised dogs. They found the range of basal LH to be 0.48–3.27 ng/ml in the intact bitch and 3.56–29.39 ng/ml in the ovariectomised bitch. Basal FSH ranges varied from 1.18–8.08 ng/ml in the intact anoestrous bitch to 13.9–82.88 ng/ml in the ovariectomised bitch. For male dogs, basal FSH levels were 0.50–6.43 ng/ml and 8.88–59.66 ng/ml in intact and gonadectomised dogs, respectively. Basal LH values were 1.01–11.25 ng/ml in the intact dog and 2.46–17.11 ng/ml in the castrated dog. (De Gier 2012). They discovered there was overlap in the ranges of LH for the male dog, which makes this parameter not useful to distinguish intact and castrated animals. FSH levels however, differ enough to be used for this purpose in both males and females. (De Gier 2012)

Determination of FSH plasma concentration can aid in analysis of the neuter, possibly after GnRH stimulation to avoid the pulsatile secretion of FSH. Neuter status can be clinically hard to determine, for example when it is unclear if a bitch is in anoestrous or when a male dog is possibly bilaterally cryptorch or castrated. Next to a clinical application a novel method to determine FH and LH would also aid in further research on these hormones and their function in the hypothalamic-pituitary-gonadal axis, for example in research on fairly recent discovered mechanism of kisspeptins, the GPR54 receptor and the KiSS1 gene. (Dedes 2012, Albers-Wolthers 2017)

The aim of the current research project was to develop a reproducible novel method to detect FSH and LH in canine plasma as a tool for future research and potential diagnostically application. In this study we compared two different diagnostic methods. The first method that we used is the Enzyme-Linked Immuno Sorbent Assay (ELISA), of which the Sandwich variety was applied. Thereby acquired results were compared to the Magpix system with magnetic beads. The Magpix methods would enable analysis of multiple parameters in a single plasma sample in the future.

Materials and methods

Antibodies

The antibodies used were murine anti-rat LH, murine anti-human LH, murine anti-human FSH and murine anti-human a-FSH. (Bio-Rad AbD Serotec GmbH, Puchheim Germany) (Table1). All antibodies we described are not dog-specific, but cross-reactive.

Capture antibody	Detection antibody
Mouse anti-human LH * Catno. MCA 5836G	Mouse anti-rat LH:biotin*
Mouse anti-rat LH* Catno. MCA 5835G	Mouse anti-human LH:biotin*
Mouse anti-human FSH (beta chain) Catno. MCA 0100-0663	Mouse anti-human FSH (alpha-biotin) Catno. MCA 0100-0662B

*Table 1: antibody pairs used in the current study. * The detection antibodies for LH were made in house, see Biotinylation below.*

Biotinylation

Both the anti-Human LH and the anti-Rat LH antibody samples were biotinylated in a 1:10 molar ratio in advance. The anti-human FSH was acquired readily biotinylated. For the anti-LH antibodies, one mg of Biotin-7-NHS (D-Biotinyl-ε-aminocaproic acid-N-hydroxysuccinamide ester was dissolved in 500 µL Dimethylsulfoxide DMSO). From this solution 5 ul (containing 3x10^-8moles) was added to 500 ul of a 1 mg/ml antibody solution (containing 3,3 x 10^-9 moles) the antibody solution and incubate for 2h at room temperature during gently stirring. Free biotin was removed by dialysis/ ultrafiltarion using a using 10.000 dalton vivaspin filter and PBS pH7.4 and stored at 4 degrees celcius in a concentration of 1mg/ml in PBS/1%BSA/0.005%thimerosal.

Plasma samples

For initial development of the assay 4 dogs were included with predetermined FSH and LH concentrations. Of these dogs 3 were client owned. One dog included was owned by the faculty of Veterinary Medicine of the university Utrecht the Netherlands. Blood collection of the client owned dogs by means of written informed consent and the blood collection of the faculty owned dogs was approved by the Central Authority for Scientific Procedures on Animals in the Netherlands.

Whole blood was collected by careful venipuncture in an EDTA collection tube. Plasma was isolated by centrifugation, separated into aliquots, frozen immediately after sampling and stored at −24 °C until further use.

#	**LH *** ***(ng/ml)***	**FSH*** ***(ng/ml)***	**Usage**	**Neuter status**
1	16,5	68,1	From 23/5/2017	Neutered
2	1,47	2,35	From 23/5/2017	Intact

3	1,15	2,19	From 5/7/2017	Intact
4	65,5	73,8	From 29/6/2017	Neutered
5	12,18	12,19	From 5/7/2017	Intact **

*Table 2: The samples used in this research *The LH and FSH values had previously been determined by IRMA and RIA, respectively ** this sample was taken from a GnRH-stimulated dog. Sample 2 and 3 were taken from the same dog, with one week in between.*

ELISA

Microtitre plates (96-wells/plate) (Corning Costar) were coated with the capture antibody at 1, 2, 4 and 8 µg/ ml in 50 µL coating buffer (Phosphate buffered Saline, PBS) varying from one hour to overnight at 4°C. Blocking was completed by incubation one hour at room temperature (RT) with 200 µL Roche Blocking Reagent, before washing the plates three times with PBS 0,1% Tween20 with the Dynex Ultrawash washing device.
Plasma samples were pre-diluted starting 1:2 with dilution buffer consisting of 1% BSA in PBS in a non-binding plate or Eppendorf tube. Fifty microlitres of pre-diluted samples were incubated for 60 min at RT in each test well of the microtiter plate. After incubation plates were washed three times with PBS 0,1% Tween20 with the Dynex Ultrawash washing device. Subsequently 50 µl of biotinylated detection antibody was incubated for 30 minutes at room temperature, followed by 3 additional was steps. For detection 50 µl of Streptavidin-Horse Radish Peroxidase 80 (SA-HRP 80) (SDT, Germany) in a 1:10.000 dilution was incubated for 15 minutes, followed by 6 wash steps with the Dynex Ultrawash. Finally 50 µl 3,3',5,5'-Tetramethylbenzidin, (Sigma Life Science,) was added to the well, and after incubation of maximum 30 minutes at room temperature in the dark, the reaction was stopped with 2N (1M) H2SO4 stop solution.
Colour development was quantified using a microplate spectrophotometer detecting the optical density at 450 nm (OD450nm) and corrected for noise by substraction of OD650.

Magpix procedure

The MagPlex® Microspheres were covalently coupled to the capture antibodies mouse anti-rat LH (35), mouse anti-human LH (36) and mouse anti-human FSH according to manufacturer's instructions.

For new tests, a plasma dilution series was made, with 50µl of diluted plasma per well. The chosen assay diluent was PBS 0.1% Tween/ 0.1% NaN_3. The Microspheres where resuspended in their stock diluent and a volume of 50µl was taken. This volume contains approximately 1000 coated Microspheres. They were added to the 50µl of diluted plasma, in order to double the plasma dilution. The biotinylated antibodies were , incubated and after washing step followed by an incubation of Streptavidin- PE. See appendix IV for more information about the Magpix procedure.

Results

In order to investigate what was the minimal concentration that could be accurately detected in the FSH sandwich ELISA, we started with a 1/50 dilution of plasma, up to 1/51200 in the Sandwich ELISA for FSH. For plasma sample 1, with a concentration of 68,10ng/ml FSH (see table 3) this resulted in a maximum corrected OD of 0,95 at the 1/50 dilution and a signal below background at higher dilutions (data not shown). These data indicate that concentration of approximately 6 ng/ml can still be detected.
To enhance the signal measured the fold dilution of the plasma was reduced and concomitantly the dilution of the detecting antibodies was increased to lower a specific binding of the antibodies and background noise (data not shown).

Ultimately, optimal signal to noise ratio for the FSH ELISA was obtained with a capture antibody 2µg/ml and detection antibody 1µg/ml. Enhancement of binding of the capture antibody by overnight coating at 4˚C resulted in a reproducible assay with a signal to noise ratio sufficient to measure FSH concentration in canine plasma. Plasma sample number 4 (neutered bitch, high amount of both FSH and LH), containing 73,8 ng/ml FSH in the undiluted sample was used to determine reproducibility and minimal concentration detected in the ELISA. With this assay setup concentrations of 1,5 ng/ml still resulted in a signal above background noise (Figure 2) The FSH Sandwich ELISA was performed at a different time point and results were compared to analyse reproducibility. The difference of corrected signal (OD- test –OD background) of the FSH ELISA performed at 2 different time points was minimal for the same sample dilutions (figure 3, 4 and 5). This observation shows that the assay can be performed in a reproducible manner within this specific laboratory setting.

In sum, a FSH sandwich ELISA that detect concentrations > 1,5 ng/ml FSH in canine plasma was developed as determined by an corrected signal of > 0,1 above background (data not shown).

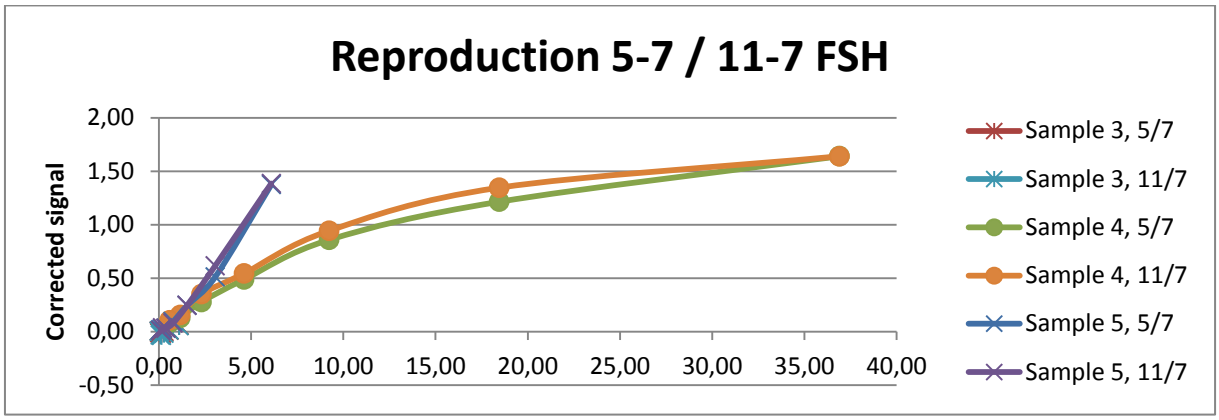

Figure 2: The signals as found in two consecutive tests under similar conditions. Signal is measured by OD450-OD650. The FSH content is calculated by multiplication of the predetermined FSH value of the plasma by the dilution factor (e.g. dog 1 plasma contains 73,8ng/ml, ½ dilution equals 36,9 ng/ml etc.). The sample data for the two consecutive tests are plotted together and are visually comparable

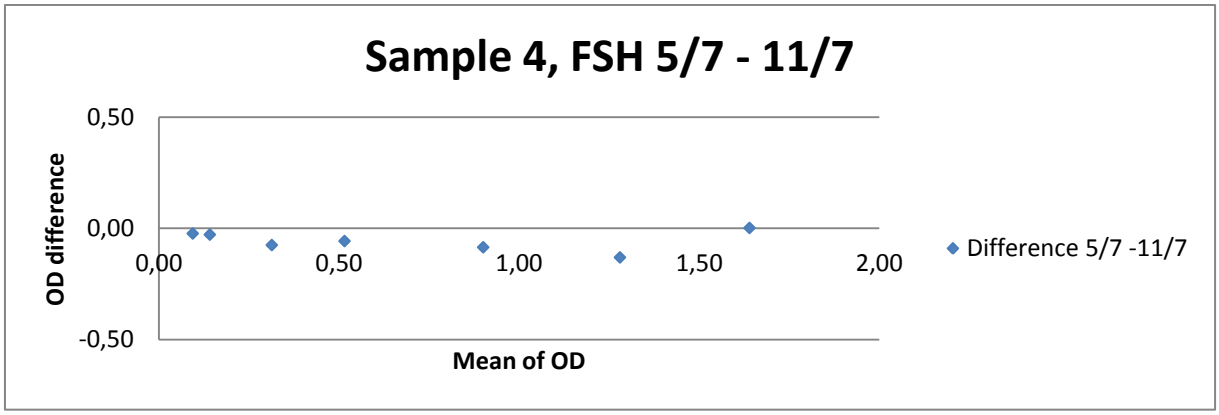

Figure 3: In this graph the difference between the two consecutive tests is plotted on the vertical axis and the mean of the OD is plotted on the horizontal axis, to assess if the magnitude of the difference depends on the height of the signal

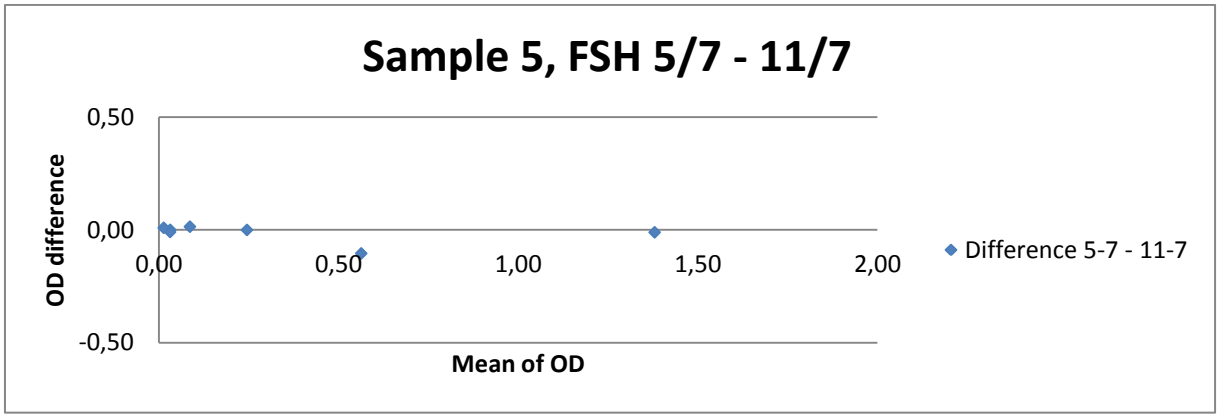

Figure 4: In this graph the difference between the two consecutive tests is plotted on the vertical axis and the mean of the OD is plotted on the horizontal axis, to assess if the magnitude of the difference depends on the height of the signal

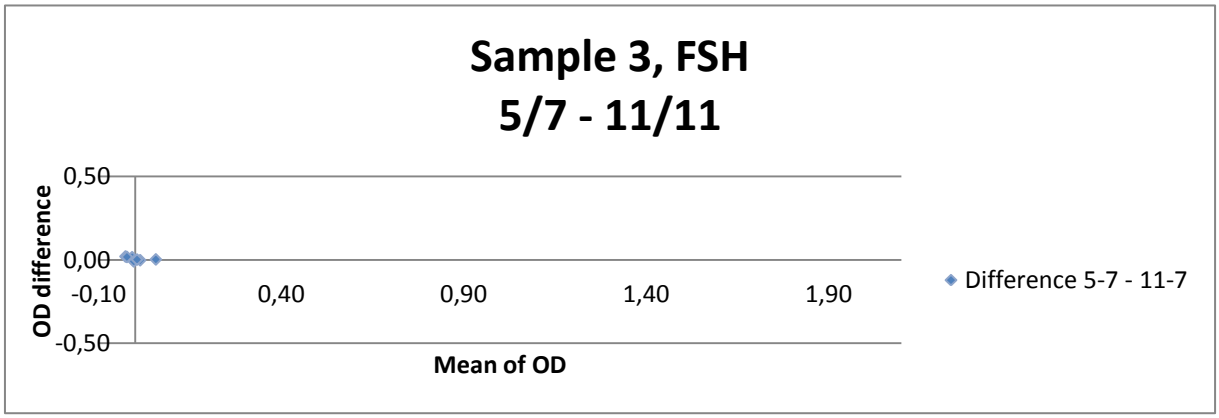

Figure 5: In this graph the difference between the two consecutive tests is plotted on the vertical axis and the mean of the OD is plotted on the horizontal axis, to assess if the magnitude of the difference depends on the height of the signal

By continued experiments in different set-ups, the minimally detectable concentration of LH was determined. Optimisation of the capture and detecting antibody concentrations showed that the highest optical density was found with a capture antibody concentration of 4µg/ml. Other concentrations used were 8, 2 and 1µg/ml. An interesting observation from this test

was the fact that the results of the two antibody couples (LH 35 C/ LH 36:Bio D and LH 36 C/LH 35:Bio D), seemed to be interchangeable. This was concluded as the signals were approximately equally high in both ways (0,296 vs. 0,246, respectively). Arbitrarily, we chose to resume our further testing with anti-LH 35 as capture and anti-LH 36:Bio as detection antibody. To investigate if the signal could be optimised by increasing the concentration capture antibody, 8µg/ml and 4µg/ml were used for coating. New plasma was added to the test, sample 4 (65,5ng/ml LH). As expected, this sample rendered the highest signal, up to 0,91 above background (figure 6). In addition a concentration of approximately 10ng/ml could still be detected with a signal of at least 0,1 above background.

Sample dilution / Combinations	1/50	1/100	1/200	1/400	1/800	1/1600	1/3200	1/6400	1/12800	1/25600	1/51200
35 - 1 - 36 Bio	0,30	0,25	0,26	0,40	0,35	0,39	-0,04	-0,03	-0,03	-0,02	0,01
35 - 2 - 36 Bio	0,29	0,24	0,23	0,39	0,38	0,36	-0,02	-0,02	-0,03	-0,02	-0,02
36 - 1 - 35 Bio	0,14	0,08	0,20	0,19	0,20	0,19	-0,02	-0,02	-0,02	-0,02	-0,02
36 - 2 - 35 Bio	0,22	0,16	0,17	0,19	0,25	0,23	-0,04	-0,05	-0,05	-0,04	-0,05
FSH - 1 - FSH Bio	0,31	0,26	0,23	1,01	0,93	0,95	-0,02	-0,02	-0,02	-0,01	0,00
FSH - 2 - FSH Bio	0,21	0,14	0,15	-0,02	-0,02	-0,02	-0,03	-0,03	-0,03	-0,02	0,00

Table 3: the optical density values, corrected for background, as found in the initial setup of the LH specific Sandwich ELISA. The 'combinations' in the first column are capture antibody, sample number, detection antibody. For example, the first row: anti-LH 35, plasma sample 1 and biotinylated anti-LH 36

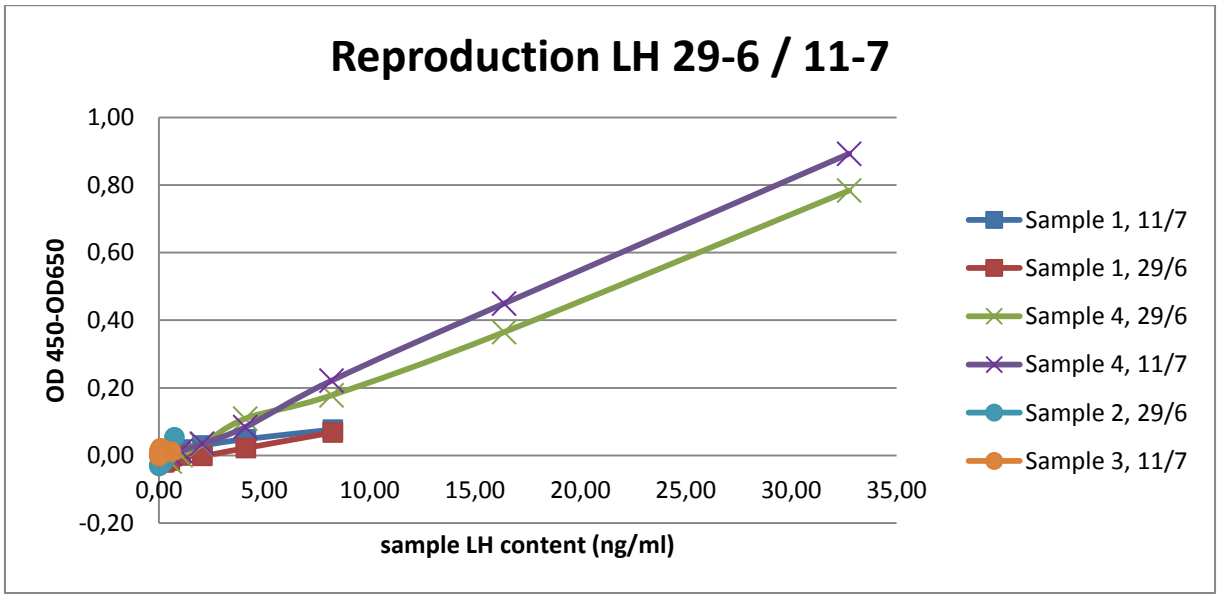

Figure 6: Corrected signal of LH concentration of two consecutive tests. Plasma samples were diluted on 2 different occasions. for LH and corrected signal was determined as

described in Materials and Methods. Data points represent average of duplicate analysis and data of separate test are plotted in the same graph to determine comparability of corrected signal over time.

To assess reproducibility of the result the assay was performed on multiple days with multiple plasma samples and corrected OD values were compared (figure 6). Corrected OD measures for sample 4 on different days were almost identical as also depicted in figure 7. For sample 1 a similar analysis was performed (figure 8).
As the plotted differences show, the differences of the two tests are small, with no visible relationship between altitude of the signal and the magnitude of differences.

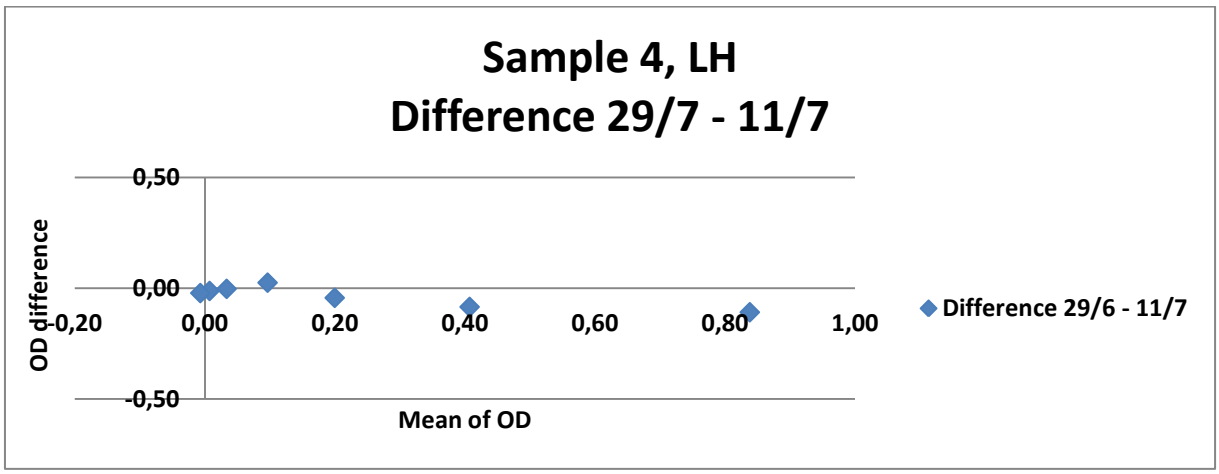

Figure 7: In this graph the difference between the two consecutive tests is plotted on the vertical axis and the mean of the OD is plotted on the horizontal axis, to assess if the magnitude of the difference depends on the height of the signal.

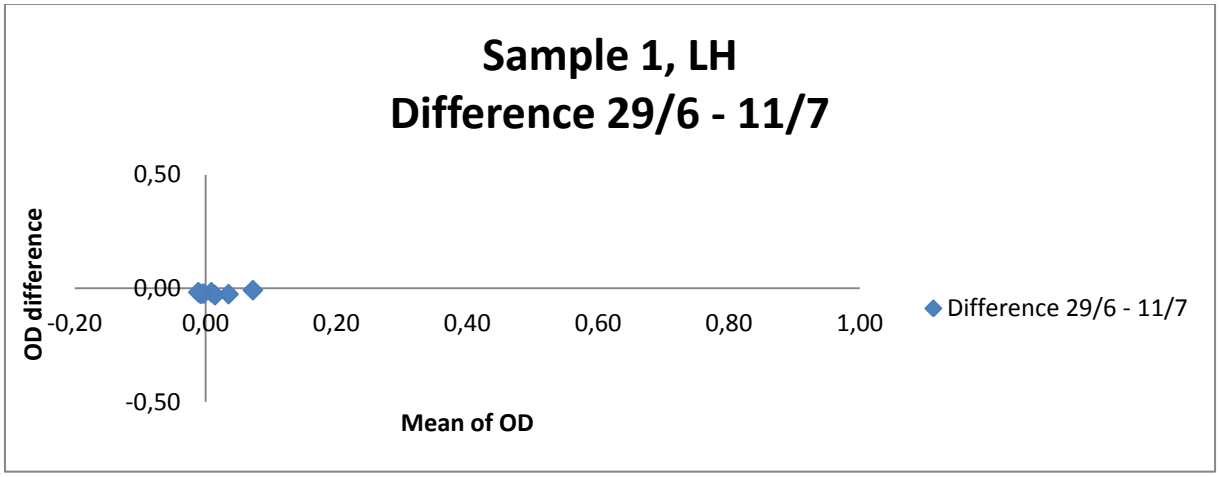

Figure 8: In this graph the difference between the two consecutive tests is plotted on the vertical axis and the mean of the OD is plotted on the horizontal axis, to assess if the magnitude of the difference depends on the height of the signal

In addition to a sandwich ELISA for FSH and LH a Magpix approach for detecting LH and FSH was assessed. The Magpix has the advantage that multiple analytes can be addressed in the same biological sample, thereby enabling multiple analysis with small blood samples.

For the Magpix system the following test results were found (table 4).

		Sample 1		**Sample 2**		**Sample 4**	
	Dilution	FSH	LH	FSH	LH	FSH	LH
A	1/2	42	23	41	23	36	21
B	1/4	40	22	48	22	36	20
C	1/8	39	22	44	21	32	20
D	1/16	38	21	38	21	30	20
E	1/32	33	20	37	20	32	19
F	1/64	35	20	32	20	34	21
G	1/128	36	20	34	20	25	19
H	0	41	21	39	21	37	21

Table 4: the test results of the Magpix multiplex testing device. MFI stands for mean fluorescence intensity. Both the MFI for FSH and LH were determined in all three samples, and in each dilution.

Unfortunately the results were very low, and were similar to background as depicted by in row H in table 4.Therefore these results can be considered irrelevant.
In an attempt to optimise this assay for the analysis of FSH and LH in canine plasma a positive control with Goat anti-Mouse was included. In this assay the positive control resulted in a significant signal with an MFI of 11762, however analysis of both FSH and LH concentration present in the canine plasma analysed (73,8ng/ml and 65,5ng/ml respectively) did not result in a signal above background (data not shown).

Discussion

In both male and female dogs the hypothalamus-pituitary-gonadal axis is an important feedback mechanism involved in their reproduction. Both FSH and LH play a key role in this mechanism by stimulating the ovaries and testes. As the plasma FSH levels differ between animals before and after gonadectomy, this hormone can be used to evaluate neuter status. Furthermore, both hormones are an interesting subject for further research regarding their respective roles and secretion, for example.

Therefore, it is essential that there be a reproducible assay to determine the plasma levels of these hormones. In this research, we have attempted to use two different techniques – Sandwich ELISA and Magpix – to develop said assays. For both hormones a separate Sandwich ELISA is used, but the assay can be combined in one Magpix test. The initial test setup was a standard protocol Sandwich ELISA, with a titration of FSH an LH.

The initial experiments developing the sandwich ELISA for both LH and FSH yielded insufficient results, and no concentration dependent signal could be detected (data not shown). Fortunately after subsequent optimisation of the ELISA protocol a reproducible assay was obtained.

There are multiple ways to further optimise a sandwich ELISA. A more secure analysis of the optimal concentrations of enzyme and biotinylated detection antibodies can be explored, by means of changing the used amounts of both substances. On the one hand this could increase the signal, but it is likely that it will increase the background as well, as we have also observed higher backgrounds for the FSH tests, when using more detection antibody.

Plasma incubation time was eventually increased to an overnight incubation step in an attempt to increase signals. This resulted in increased signal for plasma sample 4, a sample with a LH concentration of 65.5ng/ml. At a concentration of 32.75ng/ml (at 1:2 dilution) a signal of 0,89 above background was detected. This is a sufficient positive signal as an OD of 0.1 above background is considered positive (personal communication Peter van Kooten).

A high background signal was observed when the ELISA plates were blocked with PBS/BSA and even higher with Roche BB as block/ assay diluent. This signal can be at least partly due to a-specific binding of the FSH-bio antibody. In the biotinylation confirmation the detection of the FSH:Bio with goat-anti-mouse antibody resulted in a signal that did not decrease with increasing dilution. Suggestive of a-specific binding to the ELISA plate. (see appendix III). A possible explanation is a protein-protein interaction between the detection antibody and capture antibody or blocking agent, or a difference in biotinylation ratio compared to our home made anti-LH biotinylated antibodies. Optimisation of the capture antibody and detecting antibody ration in the assay with a higher dilution of the detecting antibody decreased the background signal (data not shown) suggesting that indeed the protein-protein interaction between the capture and the detecting antibody was responsible for the high background signal.

Another measure in attempt to lower the background was the increase in blocking buffer from 150µl to 200µl. We have not repeated tests with similar conditions apart from the increase in block buffer, so the significance of this change cannot be quantified. Nevertheless, it can be expected this will not alter results in a negative way as the decanting/washing step leaves the

wells in similar state except for a probably higher reaching block of the wells, and direct binding of anything but capture antibody and block buffer to the wells should be avoided

To further optimise the signal to noise ratio and potentially enhance the detection in the LH ELISA overnight sample incubation was examined. However, corrected OD values measured in this test were lower compared to a similar test without overnight sample incubation (data not shown). Stability of the LH and FSH in plasma are not likely responsible for this observation, as Livesey et al. have found that both FSH and LH are stable up to 8 days in 4°C and can be frozen and thawed for 5 times without degradation of the hormones. (Livesey 1980).
However, no protease inhibiting factors were used in the incubation buffers, which can be used to prevent micro-organisms from breaking down peptides. The overnight incubation has been done under cooled circumstances, which we expected to be enough to keep the material stable, but S. Aydin refers to the lack of protease inhibiting factors as the most common pre-analytical error for ELISA, but stresses this is most relevant for peptide measuring, while FSH and LH are bigger proteins (Aydin 2015, Pierce 1981).
Importantly this alternative protocol was not further explored and it cannot be excluded that the assay can be optimised in the future.

The fact that patient plasma in limited quantities was used to perform these tests complicated the process. For example reproducibility was only addressed with a duplicate analysis due to low plasma quantities available.

Although the data show that FSH and LH could be accurately detected in the Sandwich ELISA, further research is essential to confirm the reliability of the assay. To accurately quantitate the FSH and LH concentration the use of a standard curve with recombinant LH/FSH would be essential. Especially since we found differences in the OD measured between different samples with a putative identical concentration. Because of this discrepancy of signals we can at least conclude the ELISA does not show identical results to the IRMA and RIA, but this does not prove that either test is accurate and more research is required.

We used a signal of 0.1 more than the background as a relevant signal. As sometimes double the background signal is chosen as a significant signal, this could be disputed. Even though the values of the dogs that should be in the high range (such as sample 1) sometimes differed, both sample 2 and 3 never had a corrected signal of 0,1 or higher. This can give us an indication that future follow up test will probably have low false positives, though more data are clearly warranted to back up this assumption.

In the current research project the development of the Magpix assay for detection of FSH and LH was unsuccessful. The coating of beads was sufficient and was not likely to be responsible for the lack of detectable signal as the positive control resulted in high fluorescence as expected. Furthermore, the same antibodies did work for the Sandwich ELISA. Currently no satisfactory explanation for the failure of this particular assay was found. One improbable theory could be impaired binding capacity of hormone of the capture antibodies, due the strong binding on the Magplex beads. These beads bind covalently to the capture antibody, whereas the capture antibodies bind with Van der Waals-forces to the

ELISA plate (Esser 1988). It could be possible that the more potent binding of the Magplex beads causes the capture antibodies to change structure and lose their binding capacity.

Based on the corrected OD values obtained in the experiments described the lower detection limits for the Sandwich ELISA that follow are 10ng/ml LH and 1,5ng/ml FSH. However, these values should be interpreted with caution, as the detection limit is based on the concentration of LH and FSH previously determined in the plasma. As mentioned before, these numbers were found using a different test method, and it is necessary to assess values in the Sandwich ELISA itself to validate these numbers. This should be done in comparison to a standard curve. The upper detection limit for this test has not been achieved, but hardly seems relevant, as this is expected to be much higher than the range of variation in both FSH and LH concentrations.

As means of qualitative testing to distinguish intact and spayed bitches, this test has shown to be useful, after all the anoestrous intact animals yield no relevant signals while the castrated animals do indeed yield signals. This can only be said with a strong caution at the moment, as the signals differ greatly between animals. It cannot be excluded by these tests that an animal that should test 'positive' (i.e., castrated and therefore in the high range of LH/FSH) might be tested false negative. Vice versa, false positives are also not unlikely, but have not yet occurred in our research. The strongest test results have been found for FSH, which makes this test the most reliable, knowing that ranges of FSH also differ more than ranges of LH in the intact vs neutered dog. Given our aim of the research already had determined that a qualitative assay for FSH would be the most useful, due to the reference values as determined by De Gier et al., this goal was achieved. This implicates the Sandwich ELISA for FSH could be used for future neuter status check-ups.

Quantitative analysis with the Sandwich ELISA is not yet possible for neither LH nor FSH. In order to analyse quantitatively, a proper calibration line would preferably be made with recombinant LH/FSH, instead of plasma. Sensitivity in the lower ranges of this test, i.e. 10ng/ml LH and 1,5ng/ml FSH and less, appears to be too low to even represent a distinct value of OD. The results in our testing were not consistent enough, hence it seems not sensible to state any Limits of Detection and Limits of Quantitivity yet.

Multiplex testing with the Magpix device does not work in the current setting. If the theory we described in the discussion is true – about the conformation change due to binding – it will certainly be a challenge to overcome this problem. We have not found more techniques that could be applied within our time-schedule, and it is not sure if the technique could be fixed.

In the near future this research will be continued. The most relevant and therefore chosen step is the acquirement of a standard curve. Therefore, canine recombinant FSH and LH are being developed to include in future test set-ups.

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K. Albers-Wolthers et al., Identification of a novel kisspeptin with high gonadotropin stimulatoy activity in the dog, 2014, Neuroendocrinology 99, Pp. 178-189

Further sources

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Appendix I

Concept of Sandwich ELISA

IgG is an immunoglobulin consisting of two disulphide bridge bound gamma heavy chains accompanied by two light chains that can be either of the kappa or lambda type. Both the heavy chain and the light chain possess variable domains and therein hypervariable domains which are responsible for the interaction with antigens.

The biotinylation of Detection antibody is based on a covalent binding of biotin on the amino-groups situated on the antibody. We used D-Biotinoyl-ε-Aminocaproic Acid N-Hydroxysuccinamide Ester. The aminocaproic acid functions as a spacer, that facilitates Avidin-Biotin Complex formation (Addendum Biotin, Roche 2004).

Avidin-biotin Complex (ABC). Avidin has four binding sites for biotin, but usually binds less than four molecules. The Avidin type used in this research was Streptavidin, bound to 80 molecules of Horseradish Peroxidase. ABC's were not readily added to the ELISA, but were formed by the addition of SAV:HRP80 (enzyme-labelled avidin, LAB) to the biotinylated capture antibodies.

Source: Handbook immunochemical staining methods, Chapter 1: Antibodies Pp. 1-6 and Chapter 4: Staining methods paragraph Avidin-Biotin Methods, p.16, T. Boenisch, DAKO Corp. 1989, Carpinteria California

Appendix II

Exemplary protocol Sandwich ELISA for canine plasma FSH

Materials

- Dilution fluid for coating: Dulbecco's Phosphate Buffered Saline 1x (from now on: PBS)
- Blockbuffer & assay diluent: PBS with 1%Bovine Serum Albumin (from now on: PBS/BSA)
- Corning Costar 96 wells plate type 9018.
- Biotinylated mouse anti-Human FSH antibodies, which express a cross-reaction to canine FSH. Stored in the refrigerator. The Anti-FSH was purchased at Bio-Rad AbD Serotec GmbH, Puchheim Germany. Product code 0100-0662B.
- Enzyme: Streptavidin Horse Radish Peroxidase (bound in the order of 1:80, from now on: SAV-HRP80) stored in the freezer, kept in fluid state by dilution in glycerol. Lot #446571
- Substrate: 3,3',5,5'-Tetramethylbenzidin from Sigma Life Science PCode 1002288380, stored in the refrigerator (From now on: TMB)
- H2SO4 (Sulfuric Acid) 2N
- Non-binding polypropylene dilution plates and cups
- Dynex Ultrawash ELISA washer, using PBS/Tween 20 (0,10%) and distilled H_2O. Washing steps were done three times unless mentioned differently.

Procedure of dilution series

1. Fill the first row of wells with 100µl PBS/BSA
2. Fill the other rows with 50µl PBS/BSA
3. Add plasma in the requested concentration to the first well. Aspirate and eject at least thrice for proper mixing
4. Aspirate 50µl of the wells in the first row, and add this to the next in line
5. Again aspirate and eject at least thrice for mixing
6. Follow this procedure up until the last well, which is used as negative control.

Assay procedure

1. Coat 96 wells plate with 50 uL **capture antibody**, diluted in a requested concentration in PBS. Incubate at least 1 hour on mixing plate
2. Remove fluid
3. Block with 150 uL PBS/BSA per well, incubate for at least 15 minutes
4. Remove fluid
5. Add the plasma in the requested order as made in the dilution series. Incubate for at least 1 hour
6. Washing step
7. Add 50 uL **biotinylated detection antibody** to all wells, incubate for at least 30

minutes

8. Washing step
9. Add 50 uL SAV-HRP80 in a 1:10,000 dilution with PBS/BSA to all wells and incubate for at least 15 minutes
10. Double washing step (so 6 times total)
11. Add 50µL substrate (TMB) and incubate for 30 minutes ór stop when the negative control starts to change colour
12. 50 uL stop solution → 2N H_2SO_4
13. ELISA reading: new reading → endpoint, 3sec mixing, dual, 450 with 650 as reference (the data on 650 nm are noise and are filtered out)

Appendix III

Biotinylation of detection antibodies, absorbance values (OD450-OD650)

sample dilution	1:100	1:200	1:400	1:800	1:1600	1:3200	1:6400	1:12800	1:25600	1:51200	1:102400	1:204800
mIgG 35-biotin	1,427	1,339	1,298	1,32	1,357	1,351	1,342	1,249	0,986	0,682	0,426	0,243
mIgG 36-biotin	1,03	1,122	1,074	1,056	1,056	1,038	0,953	0,869	0,65	0,411	0,232	0,131
mIgG fsh-biotin	1,717	1,742	1,743	1,732	1,742	1,705	1,585	1,608	1,624	1,615	1,606	1,604

Table 1: the absorbance values corresponding to the dilution of detection antibodies in a direct ELISA, without plasma sample.

Capture antibody: anti-mouse Dako Z0420 2ug/ml in PBS
Sample dilution in PBS-1%BSA
SA-HRP80 dilution in PBS-1%BSA
Substrate: TMB
Stop solution: 2N H_2SO_4

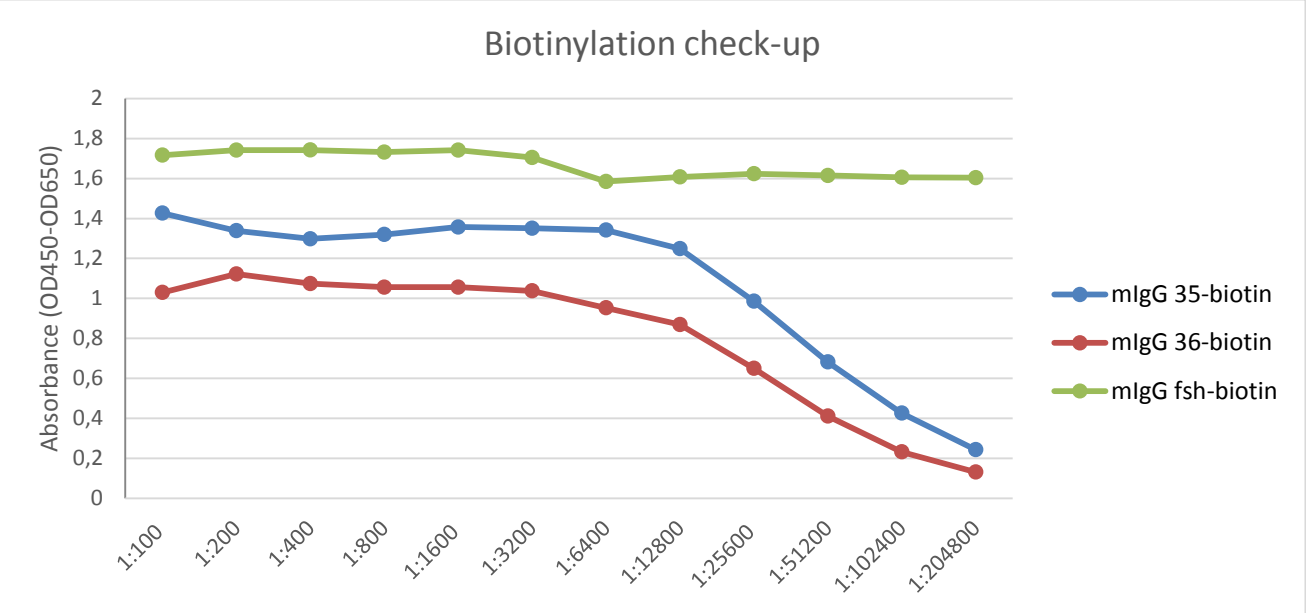
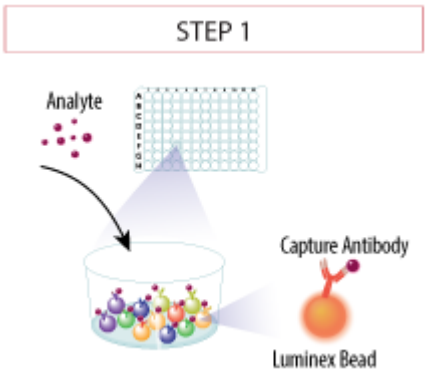


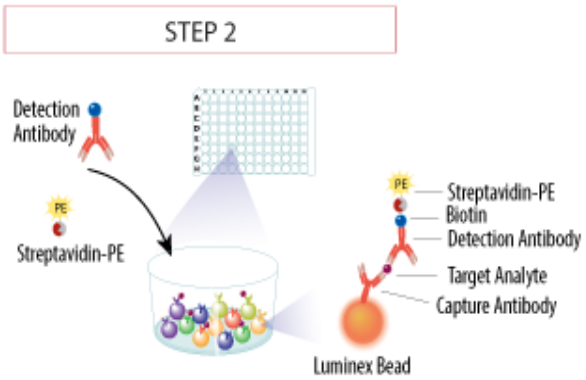
Figure 1: the biotinylation check-up. On the horizontal axis the dilution of detection antibodies is depicted. The vertical axis shows the yielded optical density values, for a wave-length of 450nm corrected for 650nm. The detection antibodies were captured directly by goat-anti-mouse antibodies which were bound on the plates.

Appendix IV

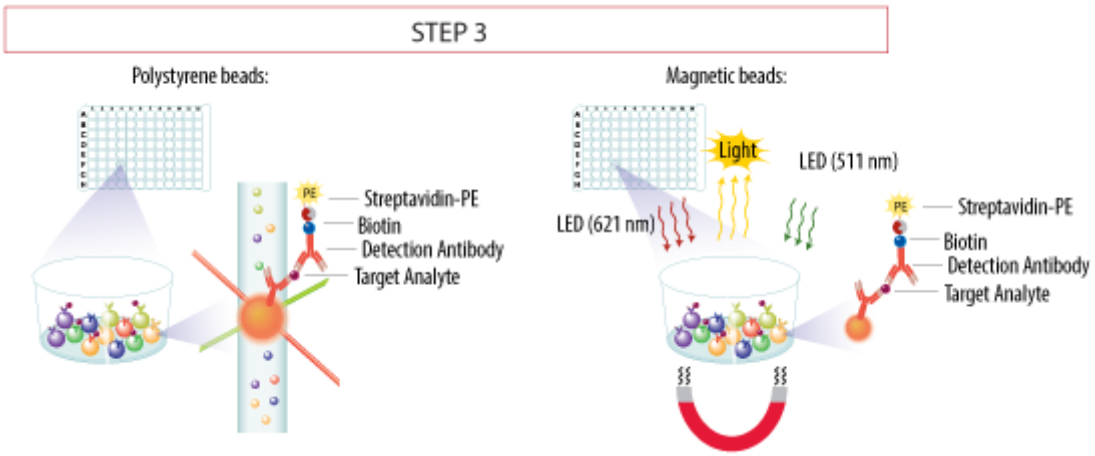
Principle of Luminex Magpix.



The beads are all distinguishable by colour. They are bound to specific capture antibody. This is added to the sample.



Corresponding detection antibodies, which are biotinylated, are added. Subsequently, Steptavidin Phycoerythrin (SAV-PE) is added to bind the biotin. The PE is a fluorochrome which can be detected.



Two lasers are used to measure the sample. One is able to identify the coloured beads, the other scans for possible fluorochrome intensity, which is a direct measure for the amount of antigen. Together they identify and quantify antigen presence.

Images found on R&D systems website, https://www.rndsystems.com/resources/technical/luminex-assay-principle

Appendix V

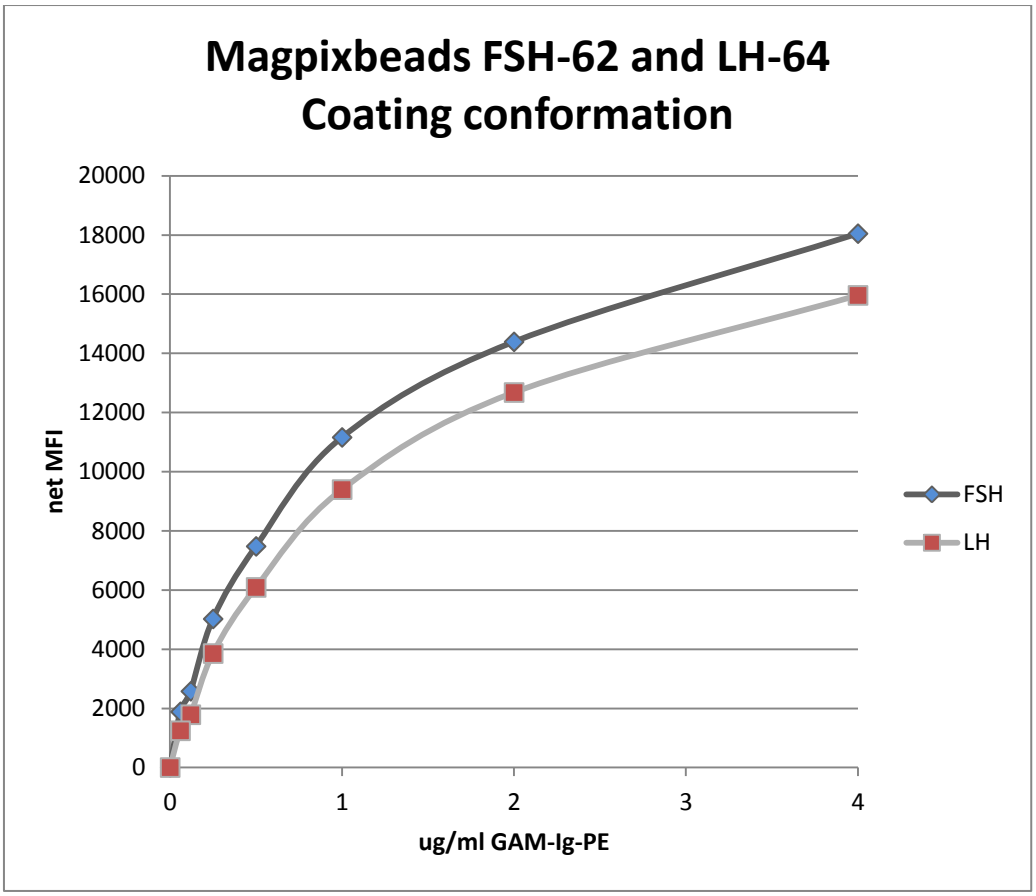


Figure 1: the coating confirmation of the Magplex beads used in the Magpix test. The horizontal axis shows the dilution of goat-anti-mouse antibodies, coupled to a fluorochrome. The vertical axis shows the net Mean Fluorescence Intensity as found by reading the samples with a Luminex Magpix device.