Multiplex ready flow cytometric immunoassay for total insulin like growth factor 1 in serum of cattle

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The European Union has banned the use of recombinant bovine somatotropins (rbST, growth hormones) to increase milk yield in dairy cattle. As direct detection of rbST in serum is problematic, methods based on the detection of changes in multiple rbST-dependent biomarkers have high potential for monitoring rbST abuse. In this study immunoassays were developed for total insulin-like growth factor 1 (IGF-1) in cow sera. Ultimately aiming at combination with other rbST-dependent biomarker assays two multiplex formats were studied and compared critically, a multi-channel surface plasmon resonance (SPR)-based biosensor and flow cytometry combined with color encoded microbeads. Moreover, a new dedicated sample pretreatment was developed for the dissociation of complexed IGF-1 in serum, while keeping other biomarkers in solution. Compared to the SPR biosensor immunoassay, the flow cytometric immunoassay (FCIA) was more sensitive, less antibody-consuming and less vulnerable to necessary but interfering reagents from the sample treatment. In an initial in-house validation study the developed FCIA showed to be fast, specific, robust, and a high repeatability and reliability, and generated realistic IGF-1 values for bovine serum, without compromising the potential for simultaneous detection of other biomarkers. Due to the xMAP technology, in which 100 different bead sets can be measured simultaneously, the total IGF-1 assay can easily be extended with other immunoassays for candidate biomarkers. Preliminary results about a FCIA for IGF-1 multiplexing with insulin-like growth factor binding protein 2 (IGFBP2) are presented which strongly supported both the FCIA multiplex format as well as the generic nature of the developed sample pretreatment.

Introduction

Administration of recombinant bovine somatotropin (rbST) enhances growth and lactating performances of livestock,[1] however, the use is banned within the EU.[2,3] The detection of rbST abuse is complex, due to the short half-life of rbST, the similarity with the endogenous hormone (bST), the low concentrations of bST and rbST in serum and strong fluctuations therein. Therefore, research is additionally focused on the detection of changes in bST-dependent biomarkers. The following biomarkers are considered as being indicative for the (illegal) administration of (r)bST: antibodies specific for rbST, IGF-1 and its binding proteins IGFBP2 and 3 and several markers for bone and collagen turnover.[4–7] So far, none of these biomarkers has been found to be reliable for predicting rbST abuse on its own. However, a combination of biomarkers has a high potential for cost-effective screening of herds of cattle for hormone abuse.[8,9] Therefore, multiplex assays providing a detailed serum biomarker profile are promising screening tools for rbST abuse.

This study is focused on the development of immunoassays for IGF-1 detection in formats that can easily be extended with assays for other biomarkers, like IGFBPs, in a later stage. In contrast to somatotropin IGF-1 is not species-specific. bST-induced elevated levels of IGF-1 might show up in the food chain and cause adverse effects in humans. IGF-1 mediates many of the growth-promoting actions of ST.[10] In circulation, most IGF-1 is associated with acid labile subunit (ALS) and IGFBP3[11] and only a small fraction of IGF-1 circulates unbound.[5] The total concentration of IGF-1 in serum, *i.e.* free plus complexed IGF-1, is a better marker for ST abuse than the concentration of free IGF-1 as it can be detected for a prolonged period of time after ST treatment. Furthermore, breakdown of binding protein as a result of field sampling conditions could lead to an increased and therefore unrepresentative value of free IGF-1 in samples.[5] Therefore, a future multiplex assay should assess total IGF-1 concentration in addition to other biomarker concentrations. However, simultaneous immunochemical detection and quantification of IGF-1 and other biomarkers are hampered by the presence of IGF-1/IGFBP complexes. Therefore, these complexes need to be dissociated before analysis. For this, several methods exist, *e.g.* acid ethanol (AE) extraction, AE extraction followed by cryoprecipitation (AEC extraction) and sulfopropyl (SP) Sephadex extraction.[12,13] Using these methods, however, simultaneous detection of a range of markers is impossible as the IGFBPs and other proteins are (mostly) removed. Therefore, in this study, a new sample preparation procedure is developed that dissociates the complexes while keeping other proteins in solution.

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So far, for the detection of IGF-1 in serum, only singleplex immunoassays like Radio Immunoassays (RIA),[14,15] Enzyme-Linked ImmunoSorbent Assay (ELISA)[16] and Western Blot (WB)[13] are described. In this study, the suitability of two multiplex technologies—an SPR-based multi-channel biosensor and flow cytometry with the xMAP™ technology,[17] for the analysis of IGF-1 is being evaluated. In theory, using this particular biosensor (Biacore 3000) and flow cytometer with the xMAP™ technology (Luminex), respectively, 4 and 100 different analytes can be detected simultaneously. The feasibility of xMAP technology for multiplex screening of ovarian cancer biomarkers in human serum was demonstrated recently.[18] The sensitivity and robustness of the SPR and flow cytometry sensing formats, in combination with the newly developed sample pretreatment, are critically compared. With the best format, an inhibition immunoassay for the detection of IGF-1 in bovine serum has been developed. The performance of this first IGF-1 immunoassay format with multiplex capability has been critically compared with commercially available singleplex IGF-1 assays. Finally, preliminary results for multiplexing the IGF-1 assay with an IGFBP2 assay are presented.

Experimental

Materials and instruments

Potassium phosphate, sodium azide, sodium chloride, sodium phosphate and Tween-20 and the ultrasonic cleaner were purchased from VWR International (Amsterdam, The Netherlands) and glycine was from Duchefa (Haarlem, The Netherlands). *N*-Hydroxysulfosuccinimide sodium salt (sulfo-NHS) was supplied by Fluka (Steinheim, Switzerland) and sodium dodecyl sulfate (SDS) by Serva (Heidelberg, Germany). Bovine serum albumin (BSA), insulin-like growth factor-I (IGF-I, human recombinant), insulin like growth factor-II (IGF-II, human recombinant), insulin from bovine pancreas, MES hydrate and *N*-(3-dimethylaminopropyl)-*N*′-ethylcarbodiimide hydrochloride (EDC) were purchased from Sigma-Aldrich Chemie (Zwijndrecht, The Netherlands). Mouse anti-human IGF-1 was supplied by Spring bioscience (clone SPM406, Fremont, CA). Insulin like growth factor binding protein-2 (IGFBP2, bovine recombinant) and rabbit anti-human IGF-1 were purchased from IBT (Reutlingen, Germany). *R*-Phycoerythrin (PE)-labeled goat anti-mouse immunoglobulins (GAM-PE) and goat anti-rabbit immunoglobulins (GAR-PE) were purchased from Prozyme (San Leandro, CA) and the rabbit anti-bovine IGFBP2 was from USBiological (Swampscott, MA). Rabbit anti-human IGF-1 was from Santa Cruz Biotechnology (Santa Cruz, CA). MultiScreen HTS filter plates and a human IGF-1 singleplex Milliplex™ $_{MAP}$ Kit were purchased from Millipore (Amsterdam, The Netherlands). Protein LoBind Tubes (1.5 mL) and a table centrifuge model 5810R were supplied by Eppendorf (Hamburg, Germany). The Biacore 3000, sensor chips (CM5), HBS-EP buffer (pH 7.4, consisting of 10 mM 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid, 150 mM sodium chloride, 3 mM EDTA, 0.005% (v/v) surfactant polysorbate 20), the amine coupling kit (containing 0.1 M *N*-hydroxysuccinimide (NHS), 0.4 M *N*-ethyl-*N*′-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and 1 M ethanolamine hydrochloride–NaOH (pH 8.5)) were purchased from GE Healthcare (Uppsala, Sweden). The iDS OCTEIA IGF-1 was purchased from Lucron Bioproducts (Gennep, The Netherlands) and the Bio-tek El × 808 ultra microplate reader from Bio-tek Instruments, Inc. (Winooski, VT). The Luminex 100 IS 2.2 system consisting of a Luminex 100 analyzer and a Luminex XY Platform, which was programmed to analyze a 96-well plate, was purchased from Applied Cytometry Systems (ACS, Dinnington, Sheffield, South Yorkshire, UK). SeroMAP microspheres (bead set 025 and 078) and sheath fluid were purchased from Luminex (Austin, TX). The Snijder test tube rotator was purchased from Omnilabo International (Breda, The Netherlands). The microtiter vari-shaker was purchased from Dynatech (Guernsey, UK) and the Bio-plex Pro™II wash station was provided by Bio-Rad (Veenendaal, The Netherlands).

Sample materials

Blood serum samples were taken from 5 calves younger than 26 weeks of age and from 20 healthy, lactating cows varying in the age of two till five years old and in different stages of their lactating cycle, to reflect a normal population of diary cows. Based on the origin of these calves and cows the assumption of being untreated with rbST was justified. After blood collection, the blood samples were placed at room temperature for 4 h to coagulate. After coagulation, samples were centrifuged for 10 min at 3000*g*, and serum was collected and stored at −80 °C until further use. Serum samples were taken by a veterinarian and the experimental work was approved by the Animal Ethics Committee of Ghent University, Belgium, in accordance with local ethical requirements.

Preparation of standard solutions

A calibration curve was prepared by spiking 22.5 μL of 80 mg mL^{-1} BSA in PBS (5.4 mM sodium phosphate, 1.3 mM potassium phosphate and 150 mM sodium chloride, pH 7.4) with 2.5 μL of human recombinant IGF-1 in PBST (PBS containing 0.05% v/v Tween-20) to a final concentration of 0–1000 ng mL^{-1}.

Pretreatment of standards and serum samples

Serum samples and standard solutions were pretreated by adding 25 μL glycine solution (27.5 mM glycine pH 0.5 (pH adjusted by addition of HCl)) to 25 μL of serum sample or standard solution in a polypropylene tube under constant vortexing. Samples and standards were then incubated at room temperature for 60 min. After incubation, 50 μL glycine–SDS solution (485 mM glycine, 0.3% m/v SDS, pH 10 (pH adjusted by addition of NaOH)) was added under constant vortexing. Before analysis samples and standard solutions were further diluted to the appropriate dilution for BIA or FCIA (see Results and discussion).

SPR biosensor chip preparation

Human recombinant IGF-1 was immobilized onto the biosensor chip (CM5) surface by the use of the amine coupling kit. The biosensor surface was activated by injecting (100 μL at a flow rate of 5 μL min^{-1}) a mixture of EDC and NHS (1 : 1; v/v) into one of the four flow channels (Fcs). Then, the human recombinant IGF-1, diluted (0.05 mg mL^{-1}) in coupling buffer (10 mM sodium acetate, pH 4.5), was injected (125 μL at a flow rate of 5 μL min^{-1})

and bound to the activated carboxymethylated dextran surface *via* its primary amine groups. After coupling, remaining active groups were blocked with ethanolamine (1 M) by injecting 35 μL at a flow rate of 5 μL min^{-1}. A similar procedure was used for immobilization of insulin in the reference channel.

SPR Biosensor Immuno Assay (BIA) development

On the biosensor chip, the reference and detection Fcs were connected serially and the response of the reference Fc was subtracted from the response in the detection Fc. The Biacore 3000 operated at a temperature of 25 °C and the running buffer was HBS-EP. During analyses of standards and sera, 50 μL injections at a flow rate of 20 μL min^{-1} were applied and for regeneration, 15 μL of a 35 mM NaOH solution were injected at a flow rate of 20 μL min^{-1}. The total run time between two sample injections, including washing and regeneration steps, was 6 minutes. The relative responses measured 30 s after the sample injections ended were used for calculations. Standards and sera were pretreated as described above and further diluted with HBS-EP containing antibody to a final sample dilution of 8 times. A final antibody dilution of 150 times was used.

Bead preparation for Flow Cytometric Immuno Assay (FCIA)

During the whole procedure, beads were pelleted by centrifugation (3 min, 10 000*g*) and after removal of the supernatant, pelleted beads were resuspended by vortexing (1 min) and sonication (1 min), unless mentioned otherwise. IGF-1 and IGFBP2 were coupled to seroMAP beads using a two-step carbodiimide reaction. The coupling was carried out using 2.5×10^6 beads present in 200 μL of the stock bead suspension. Beads were removed from their storage buffer after acclimatization to room temperature (5 min), resuspension by constant vortexing (5 min) and sonication (1 min) and transferred to a LoBind tube. Then, the beads were pelleted and resuspended in 100 μL of water. This bead suspension was pelleted and resuspended in 80 μL of activation buffer (NaH_2PO_4, pH 6.2). Sulfo-NHS (10 μL of 50 mg mL^{-1} in water) was added to the beads and mixed gently by vortex. Immediately after that 10 μL of EDC (50 mg mL^{-1} in water) were added to the beads and mixed gently by vortex. The beads were incubated for 18 min at room temperature in the dark under continuous mixing (head over head). After the incubation, the activated beads were pelleted and resuspended in 250 μL of 50 mM MES (pH 5.0). This wash procedure with 50 mM MES was repeated twice. After the wash procedure, the pelleted beads were resuspended in 500 μL of a 100 μg mL^{-1} IGF-1 or IGFBP2 protein solution in MES buffer. This bead suspension was incubated for 2 h at room temperature in the dark under continuous mixing (head over head). Afterwards, the coupled beads were pelleted, resuspended in 500 μL of blocking buffer (PBS, 0.1% BSA, 0.02% Tween-20 and 0.05% NaN_3) and incubated for 30 min in the dark at room temperature under continuous mixing (head over head). After this blocking step, the beads were pelleted and resuspended in 500 μL of blocking buffer. This wash procedure was repeated twice with 500 μL of blocking buffer and, finally, the beads were stored in blocking buffer at 2–8 °C in the dark until use. Under these storing conditions, beads were stable for over a year. Human IGF-1 was coupled to seroMAP bead set 025 and IGFBP2 to bead set 078, the numbers referring to different color codes of the beads.

Inhibition FCIA development

Standards and sera were pretreated as described above and further diluted with PBST containing 0.1% BSA (standards) or PBST (sera) to an 80 times final dilution. Of the pretreated and diluted serum samples or standard solutions, 100 μL were added to a filter bottom microtiter plate. Hereafter 10 μL of a 1500 times diluted anti-IGF-1 antibody were added and the mixture was incubated for 15 min on a microtiter plate shaker. Then, beads (10 μL diluted suspension containing about 1250 beads) were added to each well and incubated for 1 h on a microtiter plate shaker. After incubation, the plate was centrifuged (1 min, 130*g*) and the beads were washed with 200 μL PBST. After washing, 125 μL of a 625 times diluted GAM-PE solution were added and incubated for 30 min on a microtiter plate shaker. After this incubation step, the plate was centrifuged and 125 μL of PBST were added per well. Then, the beads were detected in the flow cytometer (50 μL $well^{-1}$ were measured in 50 s until 100 events per bead set were reached). A similar procedure was followed for the IGFBP2 assay using a 120 000 times dilution of the anti-IGFBP2 antibody.

Results and discussion

IGF-1 BIA *versus* FCIA for IGF-1

Inhibition immunoassay development in buffer. Antibodies against bovine IGF-1 were not commercially available. However, antibodies raised against human IGF-1 could also be applied for the detection of bovine IGF-1, as both IGF-1s are identical.[19] One anti-human IGF-1 monoclonal antibody (Mab) and two polyclonal antibodies (Pabs) were tested for their sensitivity, affinity and specificity in the BIA and the as best selected Mab was applied in the FCIA. Typical dose–response curves in buffer obtained with the assays in both formats and with the Mab are shown in Fig. 1. In the BIA, this MAb was used at a final dilution of 150 times and the curve displayed a 50% inhibition (IC_{50}) at a concentration of *ca.* 40 ng mL^{-1}. In the FCIA, a much higher final dilution of the antibody (18 000 times) could be used and this resulted in a much more sensitive assay with an IC_{50} at 1 ng mL^{-1}. When using this Mab in an 18 000 times dilution

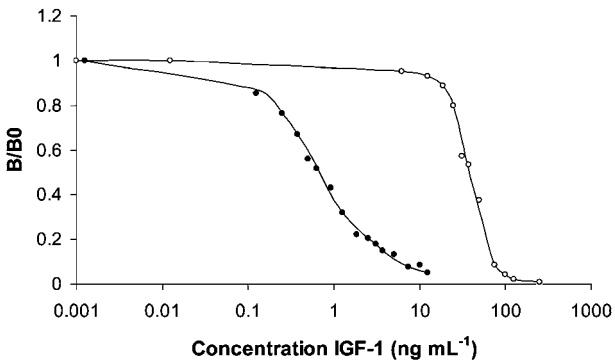

Fig. 1 Typical dose–response curves for IGF-1 in buffer obtained in the BIA (○) and the FCIA (●).

combined with an 80 times sample dilution, mean fluorescence intensities (MFIs) were found to be 1000 and 50 for a blank standard solution and a standard solution containing 1000 ng mL^{-1} IGF-1, respectively. Hardly any inhibition was observed with diluted serum, when compared with the maximum response in buffer. This indicates that most of the expected IGF-1, around 80 ng mL^{-1},[14,20] was in the complex and that the applied antibody could not bind to complexed IGF-1. This demonstrated the need for a sample pretreatment that could dissociate the IGF-1/IGFBP/ALS complexes for the detection of total IGF-1 concentration.

New sample pretreatment. The newly developed sample pretreatment to detect total IGF-1 consists of an incubation in acid and the addition of glycine–SDS. To test the suitability of the new sample preparation in combination with the BIA and the FCIA, IGF-1 standard solutions (for constructing a calibration curve) and 5 calf sera were treated according to this procedure and to an acid ethanol precipitation, widely used for routine measurements of total IGF-1 in mammalian sera.[12,16] IGF-1 concentrations in the sera were determined in both the BIA and the FCIA, see Fig. 2. Acid ethanol prepared samples resulted in a high ethanol concentration and a low pH and therefore, the extracts were adjusted to neutral pH and diluted 50 times prior to the BIA. At lower dilutions, antibody binding to the chip was diminished and the chip was unstable. However, in the 50 times diluted sera, IGF-1 concentrations were too low for detection (Fig. 2, asterisk). Applying the new sample preparation procedure, an 8 times dilution of sera and standard solutions could be used. However, also in this case, the IGF-1-coated chip was not very stable as a result of the high SDS concentrations. Due to the high sensitivity of the FCIA, the samples could be diluted 80 times, herewith reducing matrix problems, and, as the beads were only used for a single analysis, the problem of surface capacity reduction was avoided. The total IGF-1 concentrations in sera assessed in the FCIA, using acid ethanol and the new sample preparation correlated well, see Fig. 2. This indicates that the newly developed sample preparation generated realistic total IGF-1 concentrations in sera. The higher IGF-1 concentrations found in serum with the BIA in comparison to the FCIA using the new sample pretreatment were caused by a decrease of antibody binding capacity of the chip as a result of the high SDS concentration. This loss of antibody binding capacity resulted in an erroneously high IGF-1 concentration due to the inhibition format of the assay. The main advantage of the new sample preparation over the acid ethanol precipitation, *i.e.* dissociating the IGF-1/IGFBP complexes while keeping the proteins in solution for multiplexing, will be demonstrated below.

From the dose–response curves of IGF-1 (Fig. 1), it is clear that much more sensitive assays, a factor of *ca.* 40, can be developed with the flow cytometry approach in comparison to the SPR approach. Such a difference in sensitivity between the two formats was also observed for detection of plant proteins in milk.[17] In addition, in the FCIA the applied antibodies can be diluted to a factor of *ca.* 150 times extra, which is an explanation for the improved sensitivity and obviously is more (cost) efficient. The extra sensitivity was essential for the analysis of sera to circumvent matrix problems and problems with interfering substances used for sample preparation. Furthermore, FCIA's are more suitable for high throughput screening as the analysis time is about 30 minutes for a whole 96 well plate whereas over 10 hours are needed for analyzing a complete 96 well plate using the BIA. In conclusion, for multiplex detection of biomarkers for rbST abuse, the FCIA format was more suitable than the BIA format and we focused our follow-up research on this assay format.

FCIA potential for applicability to real samples

To cope with the problem that serum samples will always contain endogenous IGF-1 levels (blank serum does not exist) calibration curves were constructed using BSA solutions spiked with known concentrations of IGF-1 and they were treated according to the newly developed sample pretreatment protocol. This resulted in typical average calibration curves with an IC_{50} of 83 ng mL^{-1} (sample dilution factor taken into account) and a measurement range up to 400 ng mL^{-1} as shown in Fig. 3. Naturally occurring IGF-1 concentrations in cows of around 80 ng mL^{-1} have been described in literature.[14,20] Using our new assay, an average

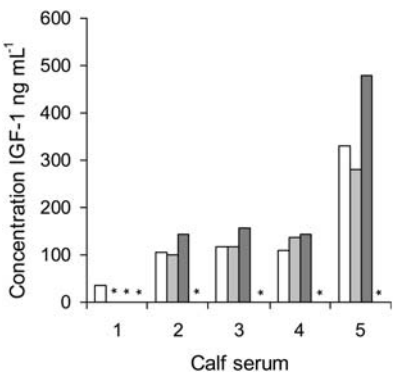

Fig. 2 IGF-1 concentrations in sera determined by different methods and sample preparations: FCIA with new sample preparation (white) and with acid ethanol treatment (light grey), BIA with new sample preparation (dark grey) and with acid ethanol treatment (*, IGF-1 was not detectable).

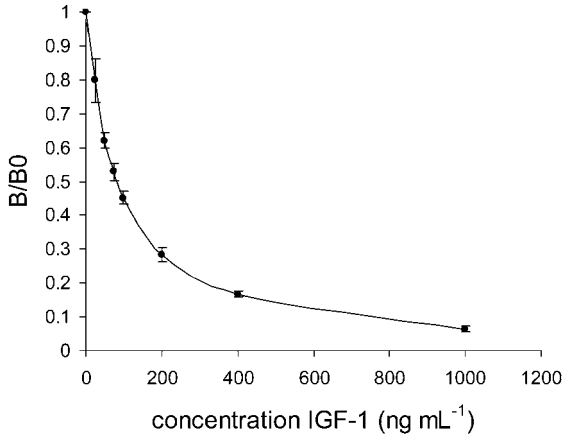

Fig. 3 A typical IGF-1 calibration curve obtained in the FCIA constructed by analyzing a BSA solution spiked with known concentrations (sample dilution factor taken into account). The average characteristics are derived from three different curves. Error bars indicate the SD.

Table 1 Intra- and inter-assay precision data for the IGF-1 FCIA

Serum sample	Concentration IGF-1/ng mL^{-1}	Intra-assay CV (%) ($n = 3$)	Inter-assay CV (%) ($n = 4$)
Cow 1	51	5.1	4.3
Cow 2	74	7.7	3.5
Cow 3	92	4.2	6.4
Cow 4	86	3.4	11.3
Cow 5	49	3.3	4.8
Calf 1	130	5.1	7.7
Calf 2	218	2.9	8.0
Calf 3	320	3.2	15.1

IGF-1 concentration of 82 ± 20 ng mL^{-1} was found in the serum samples from 20 cows. After rbST application, the concentration of IGF-1 increases two to three fold,[14,21] which can easily be distinguished from the basal IGF-1 concentrations with the developed assay.

Initial in-house validation study of the developed FCIA

As an initial in-house validation the intra- and inter-assay precision, recovery, and specificity were assessed.

To estimate the intra-assay variance, IGF-1 concentrations in sera of 5 cows and 3 calves were determined in triplicate. For the inter-assay variance, results of 4 different days were combined, see Table 1. The assay showed a high repeatability, with coefficients of variation of <7.7% in intra-assay variance and <11.3% in inter-assay variance. Only for very high IGF-1 concentrations, and thus in the less sensitive part of the calibration curve, the inter-assay variance was found to be 15.1%. The relatively high IGF-1 concentration in calf sera compared to the concentration in cow sera reflects the fast growth of calves as previously observed.[21]

The recovery could only be evaluated by analysing fortified sample material, unfortunately no certified reference material is available. The recovery was determined by adding recombinant IGF-1 at two concentrations, 25 and 50 ng mL^{-1}, to three cow sera. The average recovery found was 98% with a standard deviation of 10%.

The specificity was assessed by studying the cross-reactivity of IGF-2 and insulin, two proteins very closely related to IGF-1: no cross-reactivity was observed, clearly showing that the newly developed method is suitable for specific detection of naturally occurring IGF-1 concentrations and elevated IGF-1 concentrations after bST treatment in serum of cows.

Critical comparison of the developed FCIA with alternative IGF-1 immunoassays

As the amino acid sequence of bovine and human IGF-1 is identical,[18] in theory, commercially available test kits for IGF-1 in human serum should also be applicable to bovine serum. To further evaluate the applicability of the developed FCIA, the performance was compared with two commercially available immunoassays, a human IGF-1 singleplex FCIA and a human IGF-1 ELISA, see Table 2. Both assays are aimed at total IGF-1 concentrations and therefore include a sample treatment for dissociating the IGF-1/IGFBP/ALS complexes. The assays are performed according to the manufacturers protocol.

Table 2 IGF-1 concentrations in sera determined with the developed FCIA and two commercially available assays

	Measured concentration IGF-1/ng mL^{-1}		
Serum sample	FCIA newly developed	FCIA commercially obtained	ELISA commercially obtained
Cow 1	51	Low	52
Cow 2	74	Low	73
Cow 3	92	Low	95
Cow 4	86	Low	58
Cow 5	49	Low	22
Calf 1	130	11	153
Calf 2	218	37	224
Calf 3	320	43	330

Concentrations measured with the newly developed FCIA and the ELISA were comparable and in good agreement with concentrations found in literature.[14,20] On the other hand, using the commercially available FCIA, only very low IGF-1 concentrations were detected, even lower than the lowest standard. This indicates that this assay was not suitable for the detection of IGF-1 in bovine serum. Total analysis time of our FCIA and the ELISA was similar, *ca.* 4 hours. With the commercially available FCIA, it took over 20 hours to get the results. In general, for IGF-1 detection methods described in literature, *i.e.* Western blot,[13] RIA[14,15] and ELISA,[16] the analysis times were much longer than for our FCIA. Moreover, the disadvantages of working with radio-active materials and laborious gel separations are obvious. The main advantage of the developed FCIA is that it is suitable for multiplex detection without adding extra analysis time. In particular when a more extended and detailed serum profile is desired, the FCIA, combined with the xMAP technology, is a very promising multiplex format indeed.[18]

Multiplexing potential for IGF-1 and IGFBP2 FCIA's

To demonstrate the FCIA multiplex capability for additional rbST dependent biomarker immunoassays in cow serum, preliminary results were obtained with a FCIA developed for the detection of IGFBP2. A commercially available anti-bovine IGFBP2 antibody displayed an IC_{50} value of 3 ng mL^{-1} using a 120 000 times dilution (see Fig. 4). Other bovine BPs are not commercially available, thus requiring isolation and purification from serum prior to evaluation. The new developed sample

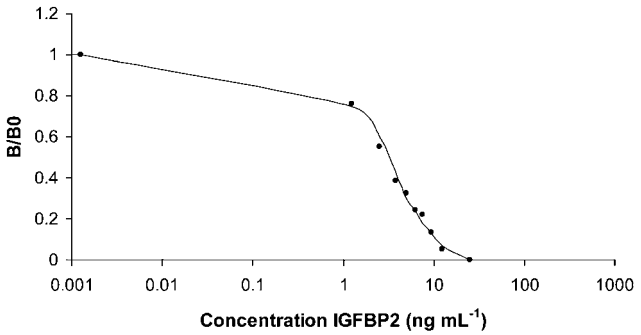

Fig. 4 Typical IGFBP2 calibration curve obtained in the FCIA in buffer.

preparation procedure was successfully tested with standard solutions containing known concentrations of IGFBP2 and found not to discriminate against protein biomarkers such as IGFBP2. Preliminary results obtained in cow sera were in good agreement with literature values,[4] IGFBP2 levels being roughly 0.6 μg mL^{-1}. These results underline that the newly developed sample pretreatment keeps IGFBP2 in solution, as most likely also other relevant protein biomarkers. Note that in contrast to conventional acid ethanol pretreatment no precipitate was observed after the treatment of the serum samples thereby supporting our hypothesis of the multiplexing capability for IGF-1 with other relevant candidate biomarkers. A fully validated FCIA for IGFBP2 will be developed shortly and described elsewhere.

Conclusions

Compared to the BIA, the FCIA developed in this study was found to be more sensitive, less antibody-consuming and less susceptible to interfering substances necessary for sample treatment. A new sample preparation procedure for obtaining total IGF-1 concentrations in serum, while keeping other proteins in solution, was developed. The total IGF-1 concentrations in cow sera using acid ethanol and the new procedure correlated well and generated realistic total IGF-1 concentrations. The high recovery found in spiking experiments also showed the applicability of the new sample treatment for total IGF-1 concentration measurements in serum. In addition and in contrast to conventional acid ethanol sample preparation, IGFBP2 could be detected adequately in solutions and sera treated with the new sample preparation procedure, therefore this method seems to be promising for developing multiplex assays.

The developed FCIA is fast, specific, robust, has high repeatability and reliability and generated realistic IGF-1 concentrations in bovine serum samples, without losing the ability for simultaneous detection of other biomarkers. It is envisaged that multiplex FCIA's, featuring many color-encoded microbead immunoassays and combined with the generic sample preparation developed, can provide a rapid screening for detailed biomarker profiles in serum, ultimately pinpointing to rbST abuse in cattle.

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