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Specific detection and quantitation of bovine IgG in bioreactor derived mouse mAb preparations

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ABSTRACT

Monoclonal antibody and recombinant protein production benefits greatly from bovine serum as an additive. The caveat is that bovine serum IgG, co-purifies with mAbs and IgG Fc-containing fusion proteins and it presents a contaminant in the end products. In order to analytically validate the products, species specific reagents are needed that react with bovine IgG exclusively. Our attempts to find such commercially available reagents failed. Here, we report the production of species specific mAbs which recognize bovine IgG even in the presence of excess amount of mouse IgG. We present five mAbs: Bsi4028, Bsi4032, Bsi4033, Bsi4034 and Bsi4035 suitable to determine the presence of bovine IgG contamination via ELISA or immunoblotting in bioreactor derived mouse mAb preparations. To quantitate bovine IgG content we developed sensitive sandwich ELISAs capable to detect bovine IgG contaminant in the ng/ml (~10^{-11}M/l) range. Finally, we show that bovine IgG is efficiently removed from bioreactor produced mouse mAb preparation via affinity depletion columns prepared with Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035 mAbs.

1. INTRODUCTION

Monoclonal antibodies are widely used reagents in life sciences. In the past decades, monoclonal antibody (mAb) production and application as research and diagnostic tools or as therapeutic agents for various human diseases has created a great deal of interest, which is constantly on the rise (Hagemeyer et al. 2009; Siddiqui 2010; Guergova-Kuras et al. 2011; Modjtahedi et al. 2012; Elvin et al. 2013). Apart from their recombinant forms which are generated in high producer CHO or other type of cells, most of the antibodies are produced using hybridomas, either in animals *(in vivo)* or in cell culture (*in vitro*) (Schirrmann et al. 2008; Liu 2014). *In vivo* antibody manufacturing has been a widespread method of mAb production because of its reliability, high antibody yield and cost effectiveness (Jackson et al. 1996; Peterson and Peavey 1998). However, because of ethical concerns and because antibodies have to meet strict quality control requirements (purity, similarity, identity) the number of animals used for antibody production for diagnostic and scientific purposes is continuously decreasing. In parallel, in recent years, numerous bioreactors have been developed for cultivation of hybridoma cells and several types of *in vitro* antibody production methods were established (Nagel et al. 1999; Trebak et al. 1999; Ayyildiz-Tamis et al. 2014). Standard hybridoma cultures require bovine serum supplement for the effective and high yield, but also for the economically sound antibody production. Bovine serum supplemented media results in a considerable bovine protein contamination of mAb preparations. Rasmussen et al. reported that media defined as serum-free may contain up to 0.5 mg/L bovine IgG (Rasmussen et al. 2005). During the most widely used purification processes of mAbs (protein A or protein G), the contaminant bovine immunoglobulins co-purify with the specific mAbs of interest. Therefore, there is an apparent need to recognize and to reduce bovine immunoglobulin contamination of mAb preparations, additionally; monitoring mAb quality during production requires specificity, which enables detection of bovine IgG. Such requirements may be achieved by affinity reagents that react with bovine IgG exclusively. In a search for specific anti bovine antibodies (polyclonal or monoclonal) we found close to one hundred commercially available reagents, however, the majority of these were reported to react with mouse IgG as well. We also tried different vendors provided bovine IgG-specific antibodies of which the cross-reactive properties with murine IgG were not indicated, however, these antibodies also recognized murine IgG in addition to the bovine IgG. Thus, in order to fill in the need, we aimed to produce species specific mAbs that react with bovine IgG even in the presence of a huge excess of murine immunoglobulins.

2. MATERIALS AND METHODS

2.1. Purity control of the monoclonal antibodies

The purity control of mAbs produced was performed with a multi-step process involving SDS-PAGE, isoelectric focusing (IEF) and multi-Capillary Gel Electrophoresis (mCGE). For SDS-PAGE, purified mAbs (5 µg) and a protein standard (Thermo Fisher Scientific) were loaded on the 4-20% Tris-Glycine gel (Invitrogen), stained with Coomassie Brillant Blue and analyzed with densitometry (Carestream Gel Logic 2200 Pro imaging system) using Carestream MI SE software. For IEF, samples were separated on Novex pH 3-10 isoelectric focusing gel (Thermo Fisher Scientific) using Novex IEF Cathode Buffer pH 3-10 (Thermo Fisher Scientific) and Novex IEF Anode Buffer (Thermo Fisher Scientific) under conditions suggested by the manufacturer. Isoelectric focusing standards (Serva) were also run and visualized by staining with Coomassie blue R-250. Multi-Capillary Gel Electrophoresis analysis was performed as described previously (Székely et al. 2014).

2.2. Production of bovine IgG-specific mAbs

Bovine IgG (Sigma, I5506) was used as antigen for the immunization of female 7-8-week old BALB/c mice as described earlier (Guergova-Kuras et al., 2011). Hybridoma supernatants were screened for the presence of anti-bovine IgG, via a direct ELISA, using immobilized bovine-IgG as antigen. Mouse IgG cross-reactivity was also screened with mouse IgG specific ELISA. Based on the ELISA results, hybridomas producing the highest specific anti-bovine IgG titers were cloned in 96 well plates via limiting dilution. Anti-bovine IgG producer mAbs were further characterized in direct, sandwich and competitive ELISA-s. Immunoglobulin isotype of bovine IgG specific mAbs was determined by isotype specific ELISAs using goat anti-mouse IgG UNLB (Southern Biotech) antibodies as capture reagents and biotin conjugated anti-mouse IgG1, IgG2a, IgG2b and IgG3 (Southern-Biotech) antibodies and streptavidin HRP (Invitrogen) for detection of the captured antibodies. Positive controls were purified unlabeled mouse IgG1, 2a, 2b and 3 also from Southern Biotech.

2.3. Antibody production

2.3.1. *In vivo production*

Some of the anti-bovine IgG specific mAbs (Bsi4028, Bsi4029, Bsi4030, Bsi4031, Bsi4032, Bsi4033, Bsi4034 and Bsi4035) and other antibodies used in the analysis (Bsi0271, Bsi0452, Bsi0097 and Bsi0358) were produced *in vivo* in BALB/c ascites. Briefly, ascites fluid was produced by intraperitoneal injection of 1–5 × 10^6 cloned hybridoma cells into ICFA-primed BALB/c mice. All animal study protocols were approved by the Animal Care and Protection Committee at the University of Debrecen (no. 7/2011/DE MAB).

2.3.2. *In vitro production*

Monoclonal antibodies used for the evaluation (Bsi0271, Bsi0452, Bsi0097 and Bsi0358) were produced *in vitro* using the CELLine™. Two-Compartment Bioreactor Technology (BD Biosciences). Briefly, monoclonal hybridomas were seeded into the "cell" compartment, and were maintained in DMEM (PAA Laboratories) supplemented with 16% fetal calf serum (FCS Gold, PAA Laboratories), 2 mM L-glutamine (PAA Laboratories), and antibiotics (PAA Laboratories), while the nutrient compartment medium was identical with that of the cell compartment except it was serum-free.

2.4 Antibody purification from ascites and from bioreactor preparations.

Immunoglobulin was purified from ascites fluid, using a two-step affinity chromatography procedure consisting of a thiophilic adsorbent column (Thermo Fisher Scientific) and a Protein G-Sepharose 4 FF (GE Healthcare) column purifications followed by protein concentration measurement with the BCA protein assay kit (Thermo Fisher Scientific). Bovine and mouse IgG were purified from sera using this method as well. Purification of cell culture supernatants from BD CELLine System was achieved by the protein G method.

2.5. Antibody labeling

Purified mAbs were biotinylated with EZ-Link® Sulfo-NHS-Biotin (Thermo Fisher Scientific) following the manufacturer's instructions. Bound biotin quantitation was determined with the HABA method and calculated with the HABA calculator found conveniently on the Pierce Biotechnology web site (http://www.piercenet.com/haba/habacalcmp.cfm).

2.6. ELISA assays

2.6.1. ELISA 1. : Screen ELISA

The assay was performed as follows: flat-well, half-area ELISA plates, (Costar3690) were coated with 30 µl, 1 µg/ml of bovine IgG (Sigma) or 1 µg/ml of IgG from murine serum (Sigma) in carbonate coating buffer (15 mM Na_2CO_3, 35 mM $NaHCO_3$, pH 9.6), hereinafter CCB, for 60 minutes, without shaking at 37 °C. Plates were washed 2 times with phosphate-buffered saline containing 0.05% Tween (PBS-T). Remaining free binding sites were blocked with blocking buffer (PBS-B) containing 5 mg/ml polyvinylpyrrolidone (PVP, Sigma) in PBS-T for 30 minutes at 37 °C without shaking, then the plates were washed two times with PBS-T. Serum samples from the immunized BALB/c mice and hybridoma culture supernatant were diluted in PBS-B and incubated for 1 h at 37 °C without shaking. Plates were then washed three times with PBS-T and incubated for 30 minutes at 37 °C, without shaking, with HRP labelled goat anti-mouse IgG-HRP (Southern-Biotech) diluted to 1:5000 in PBS-B. After washing the plates four times with PBS-T, enzyme reaction was developed using (0.01%) hydrogen-peroxide (diluted from 30 % stock solution, VWR) as substrate and TMB as chromogen (0,1 mg/ml Sigma) at 37 °C, without shaking for 2-3 minutes. Reactions were stopped with 30 µl/well of 4N H_2SO_4. The progression of the colorimetric reactions were measured with reading at 450 nm using the Multiskan Ascent (Model number: 354) microplate reader (Thermo Fisher Scientific) at room temperature. Raw measurements were background (incubated with PBS-B instead of plasma samples) corrected.

2.6.2. ELISA 2. : Sandwich ELISA detection of "bovine" IgG (polyclonal commercial Ab, Vendor#1)

Assays were performed as described in 2.6.1, with the exception of the following details: For the quantification of bovine IgG, quantitative sandwich-ELISAs, using species specific anti-IgG antibody pairs were carried out. Purified, unlabeled anti-bovine IgG antibodies were used as capture reagents, and the bound antigen (bovine IgG) was detected with anti-bovine IgG-HRP conjugate**.** For the determination of bovine IgG levels, we used HRP labeled and unlabeled anti-bovine IgGs from different vendors. The assays were carried out as follows: ELISA plates were coated with 1 µg/ml of capture antibody (obtained from vendor 1 or vendor 2) for 60 minutes at 37^{o}C, diluted in CCB. After incubation the plates were blocked using PBS-B, then incubated with *in vitro* and *in vivo* produced antibodies, bovine or mouse IgG from bovine and mouse sera. Captured antibodies were detected with the commercially

available HRP-conjugated polyclonal anti-bovine IgG-s. IgG from murine serum (Sigma) and bovine IgG (Sigma) were used as standards and controls.

2.6.3. ELISA 3. : Sandwich ELISA detection of "mouse" IgG (polyclonal commercial Ab)

Assays were developed as described in 2.6.1, with the exception of the following details: Plates were coated with 30 µl/well of 1 µg/ml goat anti-mouse IgG UNLB (Southern Biotech) at 37 °C for 60 minutes in CCB, without shaking. After washing the plates for 2 times with PBS-T wells were blocked at 37 °C for 30 minutes in 60 µl/well of PBS-B. Plates were washed then 3 times, and subsequently incubated with *in vitro* or *in vivo* produced antibodies, bovine or mouse IgG from bovine or mouse sera, at 37 °C for 60 minutes as antigens without shaking. Antigens were detected with HRP labelled goat anti-mouse IgG (Southern-Biotech) secondary antibodies diluted to 1:5000.

2.6.4. ELISA 4. : Direct ELISA for characterization of anti-bovine mABs

Measurements were done as described in 2.6.1, with the exception of the following details: Eight anti-bovine mAbs produced by hybridoma monoclones were first analyzed with direct ELISA using bovine IgG as immobilized antigen. Plates were coated with 1 µg/ml of bovine IgG (Sigma) in CCB for 60 minutes at 37 °C, without shaking. Non-specific binding was blocked with PBS-B. Biotinylated bovine IgG specific mAbs were diluted in PBS-B and incubated for 1 h at 37 °C without shaking and antigens were detected by streptavidin HRP (Invitrogen) diluted to 1:4000.

2.6.5. ELISA 5. : A representative sandwich ELISA

Tests were performed as described in 2.6.1, with the exception of the following details: Five mAbs showing higher relative affinity to bovine IgG were further characterized using a sandwich ELISA protocol as follows, ELISA plates were coated with 1 µg/ml of capture antibody (Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035) diluted in CCB. Plates were washed then three times and incubated with the ten-fold dilution series (starting at 1 µg/ml) of bovine IgG (Sigma) at 37 °C for 60 minutes as antigen without shaking. Captured bovine IgG was detected with biotinylated mouse anti-bovine mAbs and streptavidin HRP (Invitrogen). All possible capture / detection mAb combinations were tested.

2.6.6. ELISA 6: Competitive Inhibition ELISA

Assays were done as described in 2.6.1, with the exception of the following details: In order to determine the relative affinity of anti-bovine mAbs, all the studied mAbs were tested with competitive ELISA on immobilized antigen (bovine IgG). Briefly, the wells were coated with 1 µg/ml bovine IgG, washed with PBS-T and blocked with PBS-B, then incubated with biotinylated mAbs in fixed concentration (1 µg/ml) pre-mixed with a dilution series of individual unlabeled competitor mAbs starting from 10 µg/ml and developed with streptavidin-HRP. All possible capture / inhibitor combinations were tested.

2.6.7. ELISA 7: Sandwich ELISA detection of "bovine" IgG (BSI monoclonal Ab) in the presence of murine IgG

ELISAs were performed as described in 2.6.1, with the exception of the following details: Plates were coated with 1 µg/ml of anti-bovine mAb (Bsi4028) CCB for 60 minutes at 37 °C temperature, then blocked with PBS-B, washed three times and incubated with *in vitro* and *in vivo* produced antibodies, including bovine or mouse IgG from sera or fixed, low amount of bovine IgG (10 ng/ml) in the presence of varying amounts of murine IgG. Incubation was carried out at 37 °C for 60 minutes without shaking. Biotinylated bovine IgG specific mAbs were diluted in PBS-B and incubated, finally binding was detected via streptavidin HRP (Invitrogen) diluted to 1:4000.

2.6. Western Blot Analysis

For immunoblot analysis, 1 µg of bovine IgG purified from sera, or 2 µg serum samples from different species (human, gorilla, simian, tiger, lion, dog, horse, camel, alpaca, pig, goat, sheep, rabbit, BALB/c mouse, SCID mouse, rat, crow, swan, eagle, turkey, chicken, painted turtle, Moorish tortoise) were loaded for SDS-PAGE onto 5–12% Tris-glycine gels. After separation the samples were transferred to a nitrocellulose membrane (Whatman). The membranes were blocked with 5 mg/ml PVP in PBST, and incubated either with goat anti-mouse IgG HRP (Southern Biotech) or polyclonal anti-bovine IgG Peroxidase produced in rabbit (Vendor#1), or the Bsi4028 mAb conjugated to biotin first then with Streptavidin HRP (Invitrogen) for 1 hour. Immunoblots were developed by Pierce™ ECL Western Blotting Substrate (Thermo Fisher Scientific) according to the manufacturer's guide and detected with the Gel Logic 2200 Pro Imaging System (Carestram).

2.7. Affinity removal of bovine IgG from artificial mixes and from bioreactor produced mouse mAb preparations

2.7.1. Anti-bovine IgG affinity column preparation: we used 1.5 ml sepharose slurry of Protein G Sepharose 4 Fast Flow (GE Healthcare) (about 1 ml settled sepharose). In total, 10 mg of anti-bovine IgG specific mAbs (2-2-mg of each, Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035) were added to the Protein G Sepharose, supplemented with cross linking agent DMP (Thermo Scientific) in binding buffer (0.1M Na_2PO_4, 0.01% Tween-20, pH 8.2) in 10 ml final volume. Subsequently the sepharose slurry was washed once with 2 ml PBS, twice with 2 ml 0.2 M Triethanolamine buffer (pH 8.35).

2.7.2. Removal of bovine IgG from mixes of mouse mAb plus bovine IgG and from mouse mAb preparations.

Anti-bovine affinity column was washed two times with 5 ml PBS, samples were added then in a 15 ml centrifuge tube, incubated overnight at 4˚C on a rotator (Stuart Tube Rotator SB3, Bibby Scientific). During the next day, the Sepharose slurry was loaded to a glass column equipped with a filter, and the flow through and wash 5× (1 ml PBS) samples were collected. Columns were re-used after elution of bound material with 0.1 M glycine-HCl (pH 2.7) elution buffer followed by washing with wash buffer. Mouse IgG and bovine IgG concentrations were detected by ELISA 3 and 7 respectively.

2.8. Calculations, statistics.

IC_{50} concentrations of the different monoclonal antibodies in the competitive inhibition assays were calculated using GraphPad Prizm software. Statistical analysis of the specific and background ELISA signals was performed with *t-test* with equal variances using Microsoft Excel.

3. RESULTS AND DISCUSSION

3.1. Routine purity control procedures fail to detect non-clonal (bovine) IgG contamination in mAb prepations

Purity controls of murine mAbs are performed routinely using either CGE and/or SDS-PAGE (Szekely et. al 2014). An example showed on figure 1 (panels A, B, D and E) indicates that the purity of the mAbs produced both *in vivo* and *in vitro* in our laboratories is high, as determined either by CGE or SDS-PAGE. No detectable contamination was observed using these methods. However, analytical iso-electrofocusing reveals the presence of some non-uniformity (Fig 1 panels C and F), most likely emerged from the altered processing of the IgG molecules which may include glycosylation variants (e.g. sialylation), deamidation or C-terminal lysine processing variants, introducing negative (sialic acid) or positive (unprocessed lysine variant) charges to the molecules (He et al. 2009). None of the deployed analytical procedures can detect however, the presence of non-clonal antibodies (i.e. bovine IgG) in the "production" culture fluid, which co-purifies with the product. We conclude that although the first results of the analytical checking of our antibodies seem to be favorable, we still do not know how much non-clonal antibody (bovine IgG) is present in our preparations.

3.2. Commercial anti-bovine IgG reagents cross-react with mouse IgGs.

In order to specifically detect the presence of bovine IgG in bioreactor produced mouse mAbs, we ran sandwich ELISA experiments first, where commercially available polyclonal antibodies were used. The sandwich ELISA was sensitive (approx 3 ng/ml) and recognized bovine IgG (purified IgG or serum IgG) dose-dependently (Figure 2 panel A). In addition, the results indicated that our bioreactor produced monoclonal mAb preparations contain bovine immunoglobulin (Figure 2 panel A). However, when we extended the analysis and used mouse ascites (*in vivo*) produced mAbs or mouse sera derived pure mouse IgG, these were all positive in the sandwich ELISA (ELISA 2.), indicating that our assay, that is supposedly bovine IgG specific, in fact, cross reacts with mouse IgG (Figure 2 panel C). We have tested another set of antibodies from a different vendor and the components of the sandwich ELISA-s were exchanged between the two vendors' products, but neither these actions nor optimization procedures including concentration titration of components resulted in more specific results (results not shown). Western blot analysis showed that indeed the commercial antibodies declared to be bovine Ig specific recognize both bovine and murine IgG (Figure 2

panel C). On the contrary, the reagents declared to be mouse Ig specific (ELISA 3.), clearly do not cross react with bovine IgG (Figure 2 panel D).

3.3. Production and characterization of anti-bovine-IgG, species specific monoclonal antibodies

Monoclonal antibodies were generated against bovine IgG. Eight hybridomas were selected for cloning via limiting dilution, resulting in thirty-five IgG producing monoclonal hybridomas of which eight (one from each hybridomas) were further characterized in direct, sandwich, and competitive ELISA-s. Based on isotype ELISA tests (not shown), all the eight monoclonal hybridomas produced IgG1 isotype. Five of the eight hybridomas produced anti-bovine IgG with high affinity (Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035), giving appreciable signal already at 100 ng/ml bovine IgG coating concentration (ELISA 4.), while the remaining three (Bsi4029, Bsi4030, Bsi4031) showed reduced relative affinities providing relatively low signal even at 1 µg/ml bovine IgG concentration used for coating (Figure 3 panel A). Subsequently, the five better performing mAbs were further characterized with sandwich ELISA in combinations. In these experiments all five mAbs were used as capture mAbs, reacted with a series of concentrations of purified bovine IgG; the captured antigen was then detected with all five biotinylated mAbs via streptavidin-HRP complexes in separate experiments, in a total of twenty-five different combinations (ELISA 5.). The sandwich ELISAs demonstrated that each of the mAb couples produces very similar results and shows comparable characteristics. The results are illustrated with one representative data set, where biotinylated Bsi4035 was used as the detection antibody (Figure 3 panel B). In all cases 10 ng/ml bovine-IgG produced reproducible high signal, the limit of detection was ≥ 1 ng/ml, from which point specific signals were significantly higher (p<0.05) than the background signals.

In order to be able to find optimal pairs for sandwich ELISA-s, we further characterized the five selected mAbs in an inhibition test to query whether the relative affinities differed significantly from each other. In these experiments, fixed amount of biotinylated monoclonal antibodies were mixed with a series of different concentrations of all the five bovine IgG specific unlabeled antibodies individually and were added to the wells of the ELISA plates containing fixed amount of the antigen, bovine-IgG. ELISA-s were developed with HRP-Streptavidine as usual (ELISA 6.). The IC50s (50% inhibitory doses) were calculated and were normalized to the "self" sandwich, which was the setup where the biotinylated mAb was the same as the unlabeled inhibitor. Important to note here, that because of the dimeric

structure of IgG sandwich ELISAs can be built with a single epitope specific mAb. Using the table (Figure 3 panel D) we attempted to rank the mAbs, however we noted only negligible differences, thus we concluded that any of the mAbs (Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035) would be fit for the development of a species specific sandwich ELISA alone or in combination with any other.

Finally, in order to test species specificity, from lower vertebrate to hominid and human, we performed immunoblotting and sandwich ELISA experiments where for the western blotting sera from a wide variety of vertebrate species were separated by SDS-PAGE under reducing conditions, and after blotting, were probed with a polyclonal antibody declared to be bovine IgG specific (Figure 4 panel A) and with Bsi4028 (Figure 4 panel C) or Bsi4032 (not shown). Sandwich ELISA results obtained with polyclonal antiserum declared to be bovine specific is shown on Figure 4, panel B, while reactivity of Bsi4028 (results obtained with Bsi4032 are not shown) is presented on Figure 4 panel D. Overall, the results indicate, that mAb Bsi4028 and Bsi4032 react with a limited species-specific (species specificity with limited cross-species recognition) epitope (both native and denatured), present on the heavy chain of the ruminant mammal species (bovine, goat and sheep) and possibly in a related but evolutionary more distant and therefore weaker version in lion, tiger, simian monkey, gorilla and human. In addition to the IgG heavy chain reactivity of bovine IgG, a weak reaction of the bovine light chain is also observed. This reactivity may be due to the presence of a related weakly reactive epitope on the bovine light chain. Most importantly, the mAb Bsi4028 does not react with rodent IgG and IgG from lower vertebrate like swan, eagle, turkey, chicken, painted turtle, moorish tortoise. While the rabbit anti-bovine IgG polyclonal antibody detects epitopes present on both light and heavy chains of bovine IgG and all other species from rodents up to a hominid (gorilla) and human. Species from reptiles to birds, at lover level of the phylogenetic tree fail to react with the polyclonal sera. The reaction pattern indicates that Bsi4028 and Bsi4032 react in a limited species-specific manner. Results shown on Figure 3 panel C strongly suggest that each of the mAb-s (Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035) react with the same epitope of bovine IgG. As Bsi4028 and Bsi4032 provided identical results in these experiments taken together with the results in western blot experiments we suggest that mAbs Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035, all possess identical limited species specificity. Further, sandwich ELISA experiments indicate that limited species specificity at least for BSI4028 and BSI4032 are valid with respect to natural (see ELISA results) and denatured (SDS-PAGE, denaturing conditions in western blot experiments) epitopes (Figure 4).

3.4. Sandwich ELISA development for the detection of bovine IgG contamination.

In order to generate a quantitative assay to determine bovine IgG contamination in bioreactor produced mouse mAb, we developed a sandwich ELISA, using Bsi4028 mAb as capture and the Bsi4032-biot as detection mAbs (ELISA 6.) to test whether the low 10 ng/ml concentration of bovine IgG would be detectable in the presence of excess mouse IgG. As shown on Figure 5 A, 10 ng bovine IgG can be adequately quantified even in the presence of a wide range, (from 5 ng/ml to 0.5 mg/ml, up to $5x10^4$-fold excess) of mouse IgG. Figure 5 B shows that the sandwich ELISA between the Bsi4028 capture and the Bsi4032-biot detection mAbs detects bovine IgG contamination in an *in vitro* produced mouse mAb preparation. Finally, we used this sandwich ELISA to quantify the bovine IgG contamination of different monoclonal antibody preparations in parallel with two other sandwich ELISA-s, which we constructed using commercially available antibodies declared to be bovine IgG specific. The samples were diluted to set the mAb concentrations of the preparations between 21 and 35 µg/ml, and their bovine IgG content was determined. The antibody pair Bsi4028 and Bsi4032-biotin bovine IgG specific mAb pairs did not detect any bovine IgG in preparations derived from mouse ascites, while both ELISA-s constructed from commercial antibodies reported the presence of variable amount of bovine IgG. Bovine IgG contamination was detectable in the *in vitro* generated mAb preparations with all three sandwich ELISA pairs (Table 1.), but the detected amount of bovine IgG was considerable lower in case of the Bsi4028 and Bsi4032-biotin pair. While with the Bsi4028 and Bsi4032-biotin based sandwich ELISA we detected between 0.3 and 8.3 % as "contamination", the two commercial preparations detected between 7.1 and 32.7 % or between 32.5 and 69.2 % bovine IgG contamination respectively.

For monoclonal antibody production in bioreactor systems (*in vitro*), the use of serum-free media are recommended, however, for various reasons a significant amount of mAbs are produced in culture media containing bovine protein supplement. The use of bovine serum in hybridoma culture medium will introduce bovine immunoglobulins to the culture fluids which may considerably influence the further application of antibodies produced in bovine serum supplemented media (Darby et al. 1993). As a solution, specifically defined serum-free media have been formulated to support hybridoma growth (Tarleton and Beyer 1991; Federspiel et al. 1991). However, several authors reported the decline and loss of mAb productivity during long-term culture of hybridomas adapted to serum-free media (Frame and Hu, 1990; Ozturc and Palsson, 1990). Moreover, Rasmussen et al. reported that media defined as serum-free may contain even 0.5 mg/l bovine IgG (Rasmussen et al., 2005). These alarming observations indicate that mAbs produced even in serum-free defined media may contain bovine IgG impurities. Thus, there is a definitive need to precisely determine bovine IgG contamination is

mAb preparations. To detect and quantify the presence of bovine IgG in mAbs preparations produced in bioreactors where bovine serum supplemented culture medium is used, we attempted to measure bovine IgG content with commercial anti-bovine antibodies, however, our results were inconsistent. Here we show that the commercially available anti-bovine antibodies cross react with mouse IgG and therefore falsely "recognize" mouse IgGs not only if produced in bioreactor but also in IgG preparations derived from mouse ascites which does not contain bovine proteins at all. Our data show that cross-reactivity of commercially available anti bovine Ig inhibits or at least severely limits their use for analytical quantitation of bovine IgG contamination. The apparently limited species specific anti-bovine IgG reacting mAbs (Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035) reported here, did not show any detectable cross-reactivity with mouse IgGs, neither in ELISA nor in Western-blot applications. These results qualify Bsi4028, Bsi4032, Bsi4033, Bsi4034, Bsi4035 mAbs for analytical quantitation of bovine IgG contamination in *in vitro* produced mouse mAbs and mouse IgG Fc-containing fusion proteins as shown by our western blot and sandwich ELISA assays.

3.5. Removal of bovine-IgG from mixes that contain mouse IgG.

In order to validate the apparently bovine IgG specific mAbs for the use of specific removal of bovine IgG from biological mixes that contain excess amount of mouse IgG, first we tested artificial mixes. We show that from mixes containing 10% and 20% excess of bovine IgG as compared to mouse IgG, we were able to remove most of the bovine IgG as after removal we observed either no detectable bovine IgG or ≤ 0.03%. (Table 2.). Moreover, we show that a bioreactor derived mouse mAb preparation can also be efficiently depleted of bovine IgG (Table 2.).

Conclusion.

Our results indicate the existence of a sufficiently antigenic IgG epitope of bovine IgG, which allows generation of mABs with (limited) species-specificity. The limited species-specific mAbs are readily applicable for sensitive detection of bovine IgG in the presence of excess amount of mouse IgG, moreover the mAbs are useful for specific removal of bovine IgG from mixes that contain excess amount of mouse IgG.

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Figure legends:

Fig. 1. Purity control of monoclonal antibodies. *In vitro* (upper panels; using bioreactors), or *in vivo* (lower panels; mouse ascites) produced monoclonal antibodies (Bsi0452-4 and Bsi0452-5 respectively) were subjected to Multi Capillary Gel Electrophoresis (mCGE) (A and D); SDS-PAGE (B and E) or Isoelectric focusing (IEF-PAGE) (C and F).

Fig. 2. Reactivity of commercially available anti bovine IgG and anti-mouse IgG specific reagents with different mouse and bovine IgG preparations. Sandwich ELISAs for quantitative determination of bovine (A) or mouse IgG-s (B) were set using commercially available reagents as described in materials and methods (ELISA 2.; ELISA 3.). Serial dilutions of serum purified mouse and bovine polyclonal IgG-s and mouse monoclonal antibodies produced eiter *in vitro* in bioreactors or *in vivo* in mice were tested in the ELISA-s. Murine and Bovine IgGs isolated from sera of the animals were separated at various amounts (1 or 0.5 □g) using SDS-PAGE, were blotted to NC membrane and detected with anti-bovine IgG HRP (C), or with goat anti-mouse IgG HRP (D).

Fig. 3. Portrayal of selected anti-bovine monoclonal antibodies. Eight anti-bovine IgG mAbs were first analyzed with direct ELISA (ELISA 4.) using bovine IgG as immobilized antigen (A). Five mAbs showing higher relative affinity to bovine IgG were further characterized using a sandwich ELISA (ELISA 5. see materials and methods) in all possible combinations. The results are illustrated with one representative data set, where biotinylated Bsi4035 mAb was used as the detection antibody (B). To further analyse the antibodies an inhibiton ELISA was set (ELISA 6. see materials and methods) and used to examine possible interactions between the mAb-s (C). Results with one representative antibody (Bsi4035) is plotted (C). 50% inhibitory doses were calculated and were normalized to the "self" sandwich, which was the setup where the biotinylated mAb was the same as the unlabeled inhibitor (D).

Fig. 4. Immunoblot analysis of species specificity of anti-bovine antibodies. Sera samples from different species [human (*Homo sapiens sapiens*), gorilla *(Gorilla gorilla)*, Simian *(Erythrocebus patas)*, tiger *(Panthera tigris)*, lion *(Panthera leo)*, dog *(Canis familiaris)*, horse *(Equus caballus)*, camel *(Camelus bactrianus)*, alpaca *(Vicugna pacos)*, pig *(Sus scrofa domestica)*, goat *(Capra aegagrus hircus)*, sheep *(Ovis aries aries)*, rabbit *(Oryctolagus*

cuniculus domestica), BALB/c mouse *(Mus musculus)*, SCID mouse *(Mus musculus, scid/scid)*, rat *(Rattus rattus)*, crow *(Corvus cornix)*, swan *(Cygnus olor)*, eagle *(Aquila heliaca)*, turkey *(Meleagris gallopavo domesticus)*, chicken *(Gallus gallus domesticus)*, painted turtle *(Trachemys scripta)*, Moorish tortoise *(Testudo graeca)* were separated using reducing SDS-PAGE and after immobilization were probed with a commercial anti-bovine IgG (Panel A) and, Bsi4028 mAb (Panel C). Sera from different species (as on panels A and B) were tested in sandwich ELISA experiments using commercial anti-bovine IgG (Panel B) and, Bsi4028 mAb (Panel C) as detection reagents (ELISA 2 and ELISA 7, respectively in Materials and Methods section).

Fig. 5. Demonstration of specific recognition of bovine IgG of anti bovine mAb-s in the presence of varying amount of murine IgG. Cross reactivity with bovine IgG was tested in a sandwich ELISA (ELISA 7. Bsi4028 and Bsi4032-Biot) using fixed, low amount of bovine IgG (10 ng/ml) in the presence of varying amount (covering 6 order of magnitude) of murine IgG (panel A). Measurement of bovine IgG content of different IgG preparations using the sandwich ELISA (panel B).

Table 1. Determination of bovine IgG contamination of different monoclonal antibody preparations (*in vivo* and *in vitro* produced mAb) Bovine IgG content was determined using a sandwich assay derived from BSI's monoclonal antibodies in parallel with two sandwich ELISA-s constructed from commercial reagents declared to be bovine IgG specific.

Table 2. Removal of bovine-IgG from mixes that contain mouse IgG. The efficiency of depletion of bovine IgG contamination via the bovine IgG removal column was tested using the mixture of mouse and bovine IgG (Experiment 1 and 3) 10% and 20% respectively, or *in vivo* produced Bsi mAb (Bsi0452) with added bovine IgG contamination (Experiment 4) or *in vitro* produced Bsi mAb Bsi1328, (Experiment 2). Bovine IgG was quantified via sandwich ELISA between the Bsi4028 capture and the Bsi4032-Biot. No bovine IgG contamination was detected in Experiment 2-4, while the vast majority of the bovine IgG was removed with a single column chromatography step resulting in merely traces amount (≤0.03%) of bovine IgG in experiment 1.

TABLES:

mAb	Production	Mouse IgG specific SW ELISA	Bovine IgG specific SW ELISAs		
			BSI	vendor 1.	vendor 2.
Bsi0271-2	*in vivo*	32µg/ml	< 1ng/ml	0.50µg/ml	4.80µg/ml
Bsi0271-3	*in vitro*	21µg/ml	0.30µg/ml	5.60µg/ml	12.00µg/ml
Bsi0452-4	*in vivo*	25µg/ml	< 1ng/ml	0.50µg/ml	6.40µg/ml
Bsi0452-5	*in vitro*	24µg/ml	0.08µg/ml	1.70µg/ml	7.80µg/ml
Bsi0097-5	*in vivo*	35µg/ml	< 1ng/ml	2.40µg/ml	12.00µg/ml
Bsi0097-6	*in vitro*	26µg/ml	2.30µg/ml	8.50µg/ml	18.00µg/ml
Bsi0358-4	*in vivo*	24µg/ml	< 1ng/ml	0.05µg/ml	7.70µg/ml
Bsi0358-5	*in vitro*	24µg/ml	2.00µg/ml	4.80µg/ml	10.00µg/ml

Table 1.

Ratio (%) of bovine IgG vs. mouse IgG	**Loaded**	**Recovered**
Experiment 1.	10%	≤ 0.03%
Experiment 2.	≤ 0.03%	n.d.
Experiment 3.	20%	n.d.
Experiment 4.	10%	n.d.

n.d.: No detectable (< 0.01%) bovine IgG after depletion.

Table 2.

FIGURES :

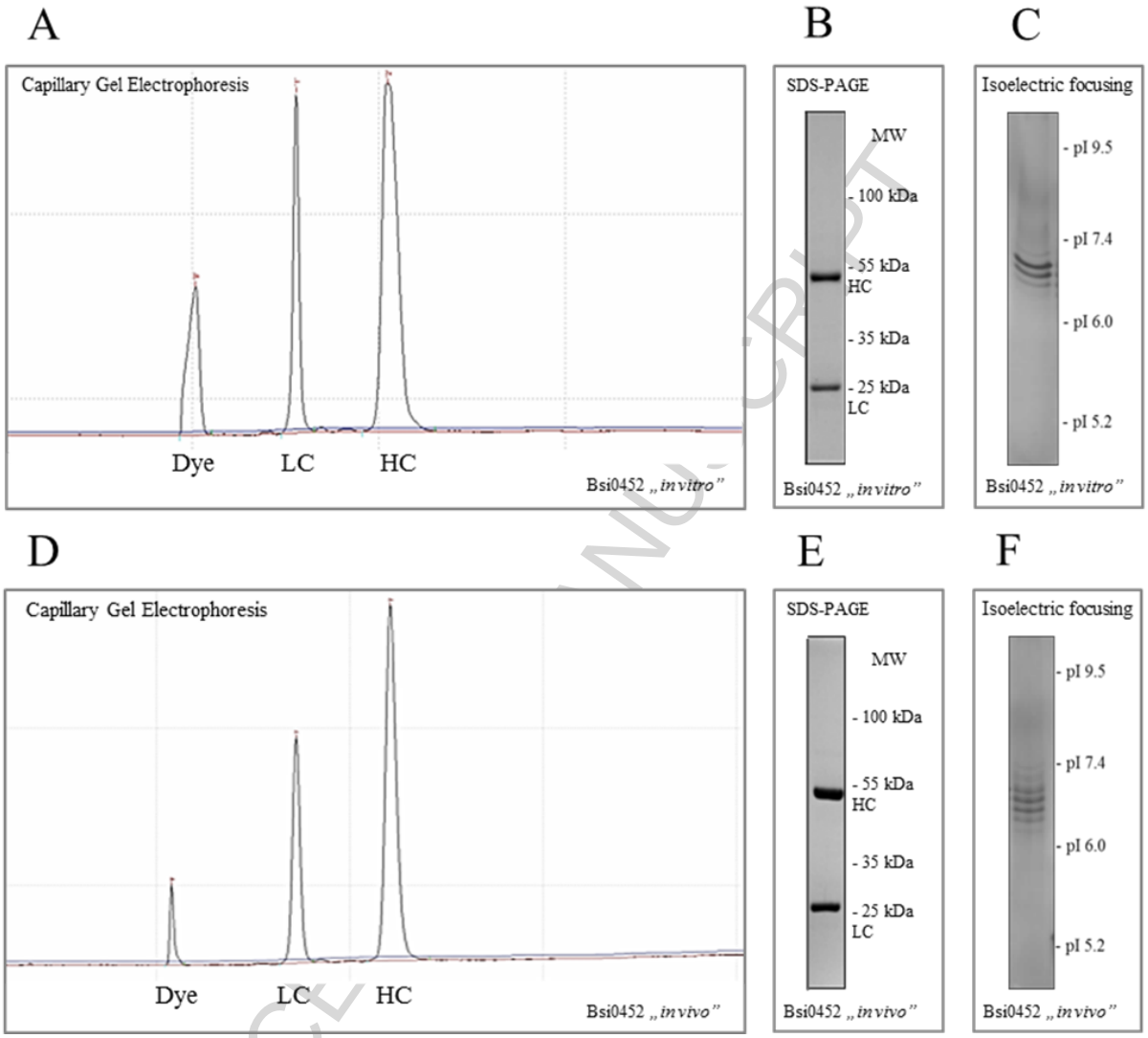


Figure 1.

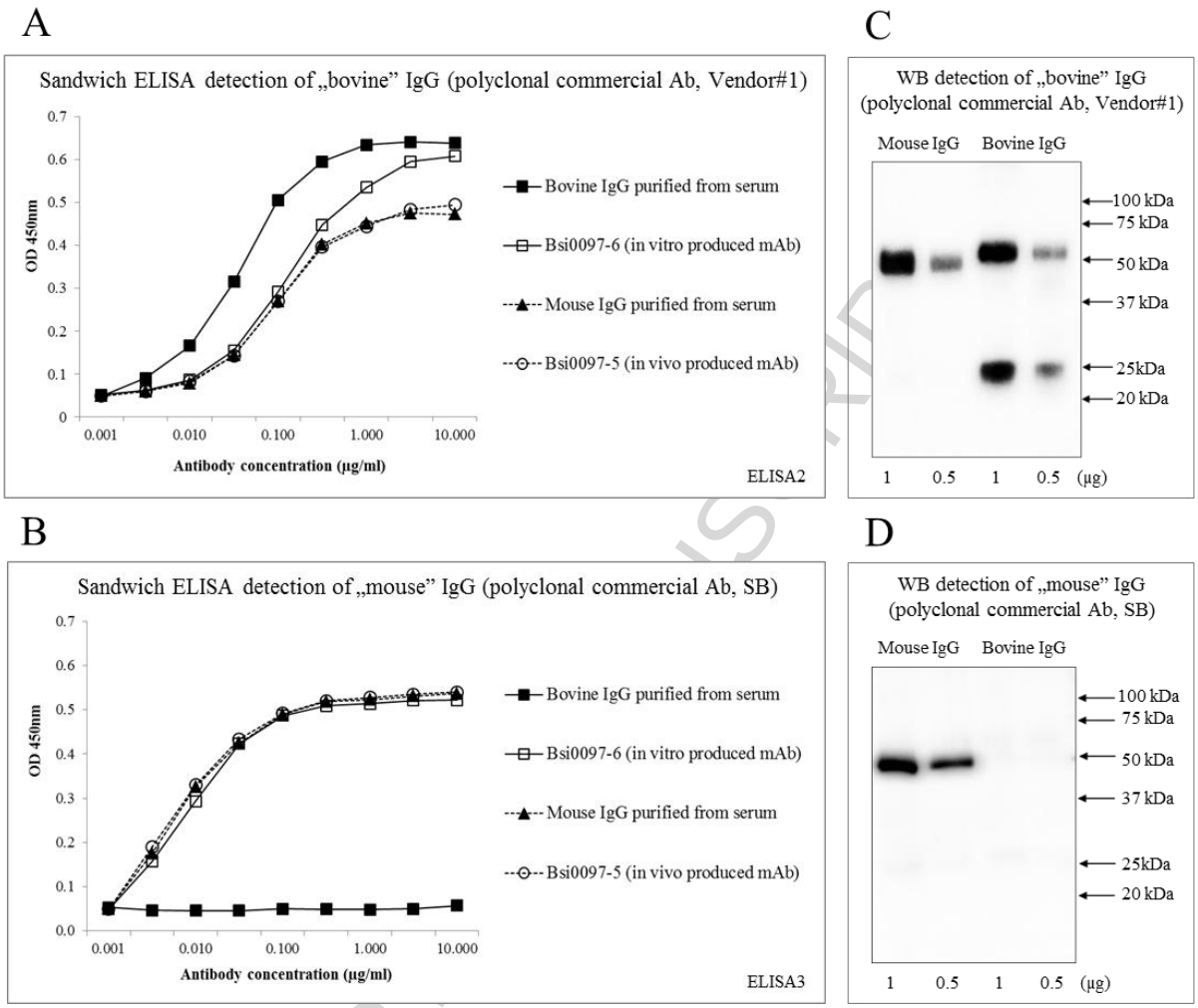
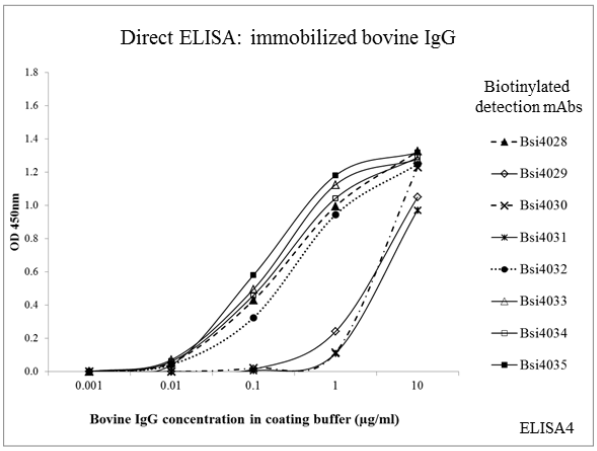
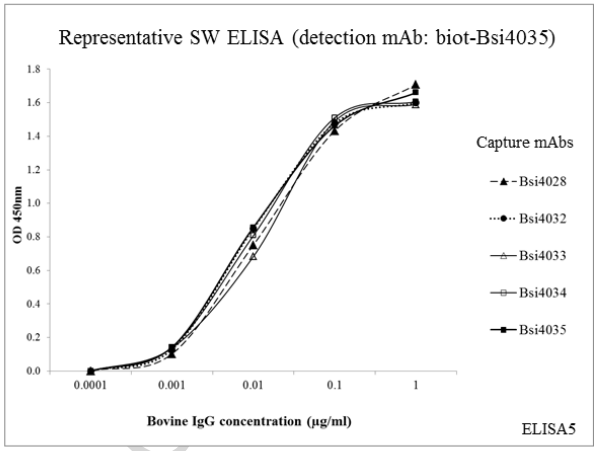


Figure 2.

A



B



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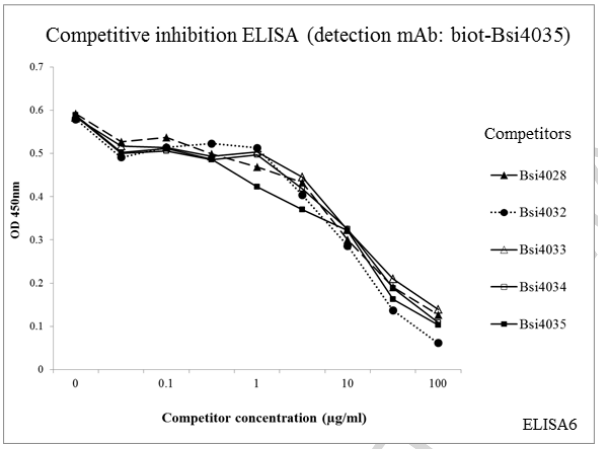


D

Summary Table of relative IC_{50} -s of Competitive inhibition ELISA

Competitor antibody	Biotinylated antibody: Bsi4028	Bsi4032	Bsi4033	Bsi4034	Bsi4035
Bsi4028	**1.00**	0.83	1.20	1.36	0.91
Bsi4032	0.87	**1.00**	0.92	0.77	1.12
Bsi4033	0.76	0.94	**1.00**	1.21	1.22
Bsi4034	0.87	0.83	0.81	**1.00**	1.37
Bsi4035	1.51	0.95	1.30	1.14	**1.00**

Figure 3.

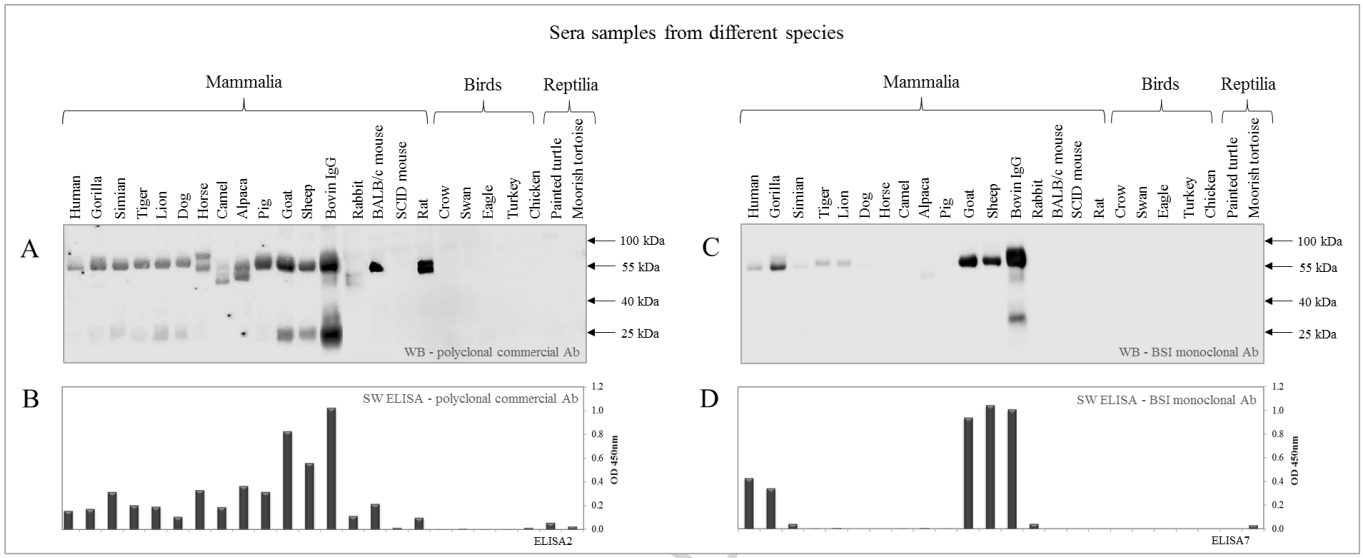
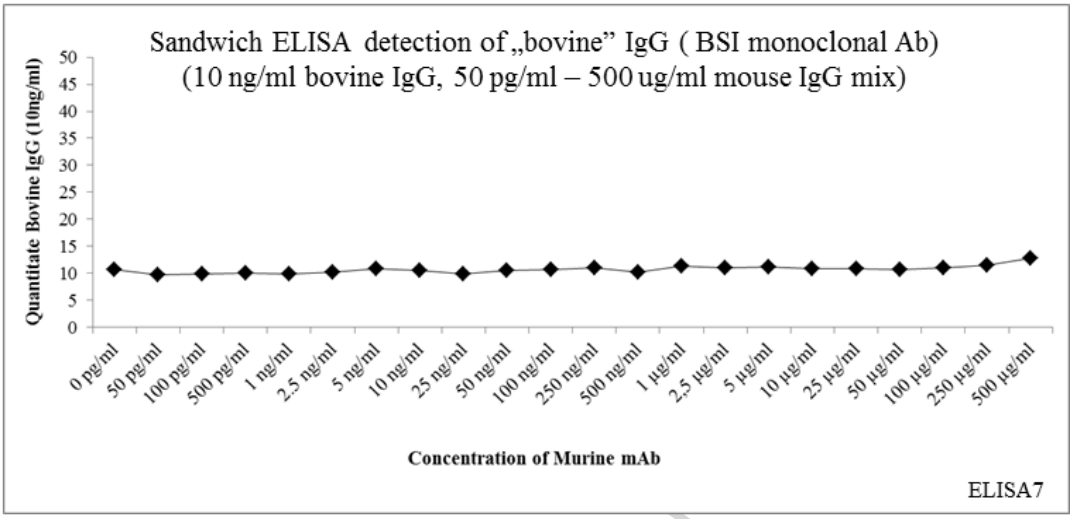


Figure 4.

A



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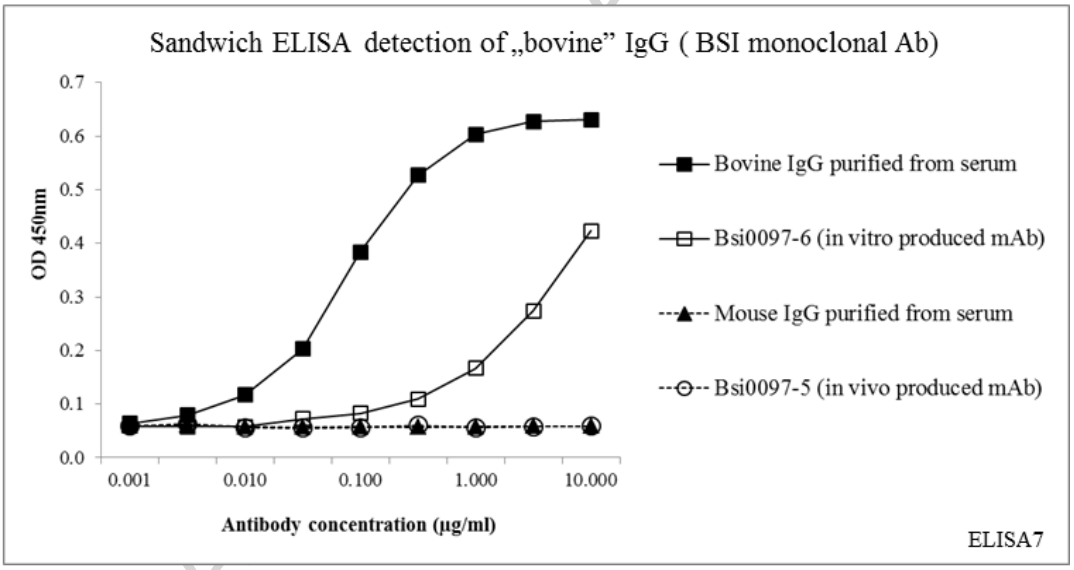

Figure 5.