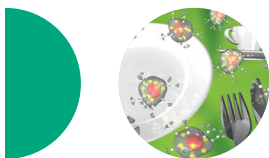




RIDASCREEN[®] FAST Milk (Art.No. R4652)

Validation Report



RIDASCREEN®FAST Milk (Art.No. R4652) Validation Report

General

Cow's milk contains 3.2 % proteins. The acidification of the milk to pH 4.6 enables two fractions to be obtained: whey (mainly β -lactoglobulin), about 20 % and curd (caseins), about 80 %. Whey contains essentially globular proteins like the β -lactoglobulin, approx. 50 % of the whey and α -lactalbumin approx. 20 % of the whey. They are synthesized in the mammary gland, while others, such as bovine serum albumin (BSA), immunoglobulin and lactoferrin, come from the blood.

β -Lactoglobulin is a strongly structured whey protein which contains 10-15 % α -helix structures, 43 % leaflet compositions and 47 % disordered areas. β -Lactoglobulin occurs naturally in the form of a 36 kDA dimer. Each subunit corresponds to a 162-residue polypeptide. The molecule possesses two disulfide bridges and one free cysteine. This structure is responsible for the main physicochemical properties and also for interaction with casein and other proteins during heat treatments. The monomeric β -lactoglobulin shows in dependence to the pH value a reversible oligomerisation: globular β -lactoglobulin forms dimers (pH 5.5 - 7.5), octamers (pH 3.5 - 5.5) or monomers (below pH 3.5, above pH 7.5). An irreversible denaturation is effected at pH > 8.6 and in conditions with a high calcium concentration. The relative resistance of β -lactoglobulin to acidic hydrolysis as well as to proteases allows some of the protein to remain intact after digestion. It is also accepted that the hydrolysis of milk proteins considerably reduces their allergenicity.

Casein (lat. Caseus = cheese) is a rough flaked curdling protein, which forms micells in the milk and precipitates under acidic conditions. The group of caseins consists of α s-caseins, β -caseins, κ -casein and γ -caseins (proteolytic protein fragment of β -casein by the milk protease plasmin). κ -Casein is cleaved into a hydrophobic (para κ -casein) and into a water soluble polar component (macropeptid) by proteolysis, e.g. by using lab ferment (in the case of cheese production).

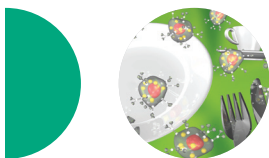
The most important allergen especially for children is β -lactoglobulin while the caseins become to be dominant later in adults. Milk consists of 10 % β -lactoglobulin (leading protein of whey) and 80 % caseins. It can be present as an ingredient or as a contaminant in raw and processed food products. According to the EU Directive 2003/89/EG from 10th November 2003, milk must be declared as an ingredient on food labels as it can induce allergic reactions. Whey (β -lactoglobulin) or caseins are often added to food products (e.g. in sausages). Therefore it is recommended to determine β -lactoglobulin or casein in food.

Test Principle

The basis of the sandwich ELISA is an antigen-antibody reaction. Detailed information is contained in the test kit insert.

Test Validation

RIDASCREEN®FAST Milk is a sandwich enzyme immunoassay for the quantitative analysis of milk in food which may contain whey, milk or milk powder as well as caseins/caseinates. Milk or milk components can be measured e.g. in sausages, ice cream, chocolate, bakery goods, cake and bread mix, soups, sauces, dressings and beverages (juice, wine, beer).



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Sample Preparation

The sample extraction is carried out using the RIDA® Extraction Solution 2 in combination with RIDASCREEN® Allergen Extraction Buffer as described in the test kit insert.

Calibration Curve

A typical standard curve for RIDASCREEN®FAST Milk is shown in appendix 1. The result is expressed as mg/kg (ppm) milk protein. To create the calibration curve the cubic spline function is recommended.

Specificity

The antibodies specifically detect α -, β - and κ -caseins as well as β -lactoglobulin of cow's milk as well as sheep's, goat's and buffalo's milk.

Sensitivity

Limit of Detection (LOD)

The **Limit of Detection** or the lowest detectable concentration of milk proteins in the RIDASCREEN®FAST Milk ELISA that can be distinguished from zero matrices was determined to be 0.7 mg/kg (ppm) milk protein (see table 1).

Table 1: Determination of the Limit of Detection (LOD)

| Sample | Std 1 (OD**) | Std 2 (OD) | Mean of samples* (OD) | Standard deviation (SD) | Mean + 3 x SD (OD) | LOD (mg/kg) |
|---------------------|--------------|------------|-----------------------|-------------------------|--------------------|-------------|
| Cookie | 0.091 | 0.197 | 0.095 | 0.008 | 0.119 | 0.59 |
| Home made chocolate | 0.091 | 0.197 | 0.111 | 0.008 | 0.136 | 0.89 |
| Sausage | 0.076 | 0.173 | 0.087 | 0.005 | 0.103 | 0.70 |
| White wine | 0.076 | 0.173 | 0.081 | 0.006 | 0.098 | 0.61 |
| Mean value | | | | | | 0.7 ppm |

*mean of 10 extractions of each matrix, each measured in duplicate

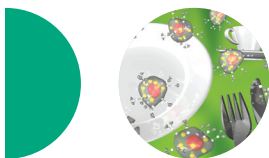
**OD = optical density

Limit of Quantification (LOQ)

The **Limit of Quantification** or the lowest concentration that can be determined in a sample with acceptable precision (repeatability) and accuracy under the stated conditions of the test was found to be 2.5 ppm milk protein (dilution factor of 100 included).

Precision (scatter of replicate readings around their mean value)

To determine the reproducibility and repeatability of the RIDASCREEN®FAST Milk ELISA intra-assay and inter-assay coefficients were calculated.



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Intra-Assay Variation (repeatability)

Within run variation was calculated by measuring the standards in one assay run (n=6). The within assay variation over the standard curve is 9.1 % (data not shown). The average intra-assay variation of samples has been found at 6.1 % (see table 2) with a recovery between 84 - 99 % (see table 2).

Table 2: Determination of the Intra-Assay Variation (repeatability) of spiked samples (n=6)

| Sample | Target milk protein concentration (mg/kg) | Concentration measured (mg/kg) | Standard deviation | CV* (%) | Recovery (%) |
|---|---|--------------------------------|--------------------|---------|--------------|
| Milk-free cookie (heated) | 0 | < LOQ | | | |
| milk-free bread mixture spiked with milk powder (heated) | 380 | 375.9 | 26.4 | 6.4 | 98.9 |
| milk-free infant formula spiked with milk powder (non heated) | 13 | 10.9 | 10.9 | 5.7 | 83.5 |
| Mean value | | | | 6.1 | 91.2 |

*CV = Coefficient of variation

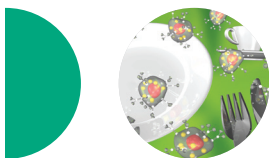
Inter-Assay Variation (reproducibility)

Between run variation was determined by repeated measurements of the standard curves from different test kits of one production batch performed by three technicians (n=3 runs). In each run duplicates of samples and standards have been measured. The mean between assay variation for the RIDASCREEN®FAST Milk is 8.6 % over the standard curve (data not shown). The inter-assay variance of one negative and two spiked food samples is 5.9 % (see table 3). The results are related to the milk protein content and expressed as mg/kg milk protein.

Table 3: Determination of the Inter-Assay Variation (reproducibility) of spiked samples

| Sample | Target milk protein concentration (mg/kg) | Concentration measured (mg/kg) | Standard deviation | CV* (%) | Recovery (%) |
|---|---|--------------------------------|--------------------|---------|--------------|
| Milk-free cookie (heated) | 0 | < LOQ | | | |
| milk-free bread mixture spiked with milk powder (heated) | 380 | 445.6 | 22.9 | 5.1 | 117.3 |
| milk-free infant formula spiked with milk powder (non heated) | 13 | 13.6 | 0.9 | 6.7 | 104.9 |
| Mean value | | | | 5.9 | 111.1 |

*CV = Coefficient of variation



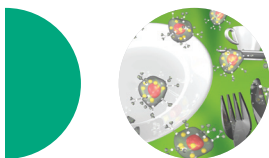
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Samples from the market

More than 40 various food samples have been collected from the supermarket, looking for declaration with or without milk. No false positive or false negative samples have been found (see table 4).

Table 4: Measurement of samples from the market

| Sample | Milk declared | Result (mg/kg) |
|--|-----------------------------|----------------|
| sausage | milk-free | < LOQ |
| boiled sausage 1 | milk-free | < LOQ |
| boiled sausage 2 | milk-free | < LOQ |
| sausage with pepper | milk-free | < LOQ |
| strawberry cream powder | n.s. | < LOQ |
| baking mixture for brownies (non heated) | n.s. | < LOQ |
| baking mixture for brownies (heated) | n.s. | < LOQ |
| baking mixture for chocolate cake (non heated) | n.s. | 896 |
| convenience blend for spaghetti Bolognese | n.s. | < LOQ |
| hot salsa sauce | n.s. | < LOQ |
| asia sauce | n.s. | 17 |
| cereals | n.s. | < LOQ |
| chocolate cake | n.s. | 6 |
| lemon muffin | n.s. | < LOQ |
| cake | n.s. | 16 |
| cookies with almond | n.s. | < LOQ |
| surimi prawns | n.s. | < LOQ |
| butter | butter milk; 0.1 % protein | 248 |
| Asia soup | traces of milk | 4 |
| convenience blend for Bolognese au gratin | traces of milk | 40 |
| sweet cream butter | 0.7 % protein | 5247 |
| convenience blend for roasted chicken | whey product | 239 |
| semi-bold margarine | buttermilk; < 0.5 % protein | 4591 |
| dark chocolate | traces of milk | 493 |
| white chocolate | whey powder/milk powder | 105014 |
| chocolate | whey powder | 108716 |
| buttermilk | 3.4 % protein | 105930 |
| zwieback | condensed milk/whey powder | 431 |
| cream cheese | n.s. | 91817 |
| mix of ewe's and goat's milk cheese (feta) | n.s. | 41566 |
| ewe's milk cheese (feta) | n.s. | 17559 |
| buffalos mozzarella | n.s. | 34800 |
| ewe's milk cheese | n.s. | 2249 |
| cheese 1 | cow's milk | 25090 |
| Emmentaler cheese | cow's milk | 23595 |
| alpine cheese (cow milk based) | cow's milk | 30674 |
| cheese with wild garlic | cow's milk | 30471 |
| Appenzeller cheese | cow's milk | 33245 |
| Parmesan | cow's milk | 43880 |
| Grana Padano | cow's milk | 38685 |
| Peccorino | cow's milk | 27381 |
| cheese 2 | cow's milk | 41498 |



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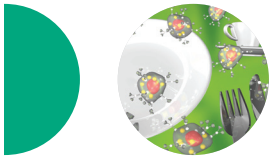
Cross reactivity

More than 70 compounds have been evaluated. The RIDASCREEN®FAST Milk test kit determines β -lactoglobulin and caseins from cow's, sheep's, goat's and buffalo's milk. Some of the results are listed in table 5.

It is also shown in table 5 that the RIDASCREEN®FAST Milk test kit has no cross reaction to other milk proteins, neither to α -lactalbumin nor to lactoferrin.

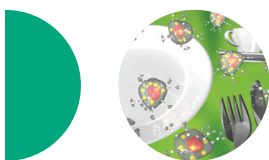
Table 5: Overview of different foods tested for cross-reactivity

| Matrix | OD | (mg/kg) milk protein |
|------------------------|-------|----------------------|
| α -lactalbumin | 0.140 | < LOQ |
| lactoferrin | 0.125 | < LOQ |
| wheat flour | 0.090 | < LOQ |
| oat flour | 0.093 | < LOQ |
| rice flour | 0.092 | < LOQ |
| maize flour | 0.080 | < LOQ |
| millet | 0.077 | < LOQ |
| buckwheat flour | 0.078 | < LOQ |
| soybean flour | 0.067 | < LOQ |
| lupine flour | 0.175 | < LOQ |
| lupine protein isolate | 0.065 | < LOQ |
| teff flour | 0.071 | < LOQ |
| white beans | 0.085 | < LOQ |
| kidney beans | 0.077 | < LOQ |
| pinto beans | 0.071 | < LOQ |
| green pea | 0.072 | < LOQ |
| chickpea | 0.132 | < LOQ |
| lentils | 0.073 | < LOQ |
| soy milk | 0.067 | < LOQ |
| soy protein | 0.089 | < LOQ |
| tofu | 0.095 | < LOQ |
| sunflower kernel | 0.093 | < LOQ |
| pumpkin seed | 0.180 | < LOQ |
| apricot kernel | 0.089 | < LOQ |
| pine nut | 0.090 | < LOQ |
| poppy seed | 0.082 | < LOQ |
| chestnut | 0.114 | < LOQ |
| linseed | 0.097 | < LOQ |
| sesame | 0.092 | < LOQ |
| sesame (roasted) | 0.092 | < LOQ |
| cashew (raw) | 0.068 | < LOQ |
| walnut (raw) | 0.067 | < LOQ |
| almond (raw) | 0.072 | < LOQ |
| macadamia | 0.097 | < LOQ |
| pecan nut | 0.099 | < LOQ |
| coconut | 0.092 | < LOQ |
| pistachio | 0.088 | < LOQ |
| brazil Nut | 0.091 | < LOQ |
| hazelnut (raw) | 0.106 | < LOQ |
| peanut (raw) | 0.116 | < LOQ |



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| Matrix | OD | (mg/kg) milk protein |
|----------------------------|-------|----------------------|
| bovine gelatin | 0.098 | < LOQ |
| porcine gelatin | 0.096 | < LOQ |
| fish gelatin | 0.092 | < LOQ |
| lecithin | 0.084 | < LOQ |
| bovine serum albumin (BSA) | 0.095 | < LOQ |
| ovalbumin | 0.099 | < LOQ |
| fetal calf serum (FCS) | 0.089 | < LOQ |
| whole egg powder | 0.088 | < LOQ |
| egg white powder | 0.093 | < LOQ |
| apricot | 0.093 | < LOQ |
| orange juice | 0.095 | < LOQ |
| red wine | 0.092 | < LOQ |
| white wine | 0.092 | < LOQ |
| kiwi | 0.094 | < LOQ |
| coffee | 0.087 | < LOQ |
| cocoa | 0.081 | < LOQ |
| cocoa butter | 0.153 | < LOQ |
| margarine | 0.083 | < LOQ |
| ketchup | 0.150 | < LOQ |
| soy sauce | 0.095 | < LOQ |
| mustard seed | 0.092 | < LOQ |
| celery seed | 0.089 | < LOQ |
| celery powder | 0.145 | < LOQ |
| fennel seed | 0.089 | < LOQ |
| anis | 0.087 | < LOQ |
| caraway | 0.107 | < LOQ |
| black cumin | 0.181 | < LOQ |
| cumin | 0.081 | < LOQ |
| curcuma | 0.138 | < LOQ |
| onion, granulated | 0.081 | < LOQ |
| onion, freeze-dried | 0.087 | < LOQ |
| paprika powder, sweet | 0.090 | < LOQ |
| paprika powder, hot | 0.079 | < LOQ |
| jalapeno pepper | 0.088 | < LOQ |
| pepper, white | 0.082 | < LOQ |
| pepper, black | 0.078 | < LOQ |



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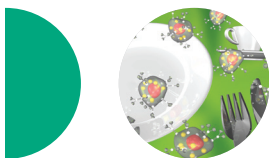
Recovery of spiked samples

Food matrices measured below the LOQ have been spiked with NIST SRM 1549 skim milk powder (36.7 % protein) and two other skim milk powder preparations as well as with a combination of β -lactoglobulin and casein equal to a certain amount of mg/kg milk protein to measure the recovery (see table 6a). Processed and non processed samples have been used. The spike samples were extracted according to the test kit insert. A mean recovery over all was found at 97.5 %.

Table 6a: Recovery (%) of milk protein in spiked samples

| Sample matrix | Spike material | Calculated as milk protein (mg/kg) | Milk protein concentration measured (mg/kg) | Recovery (%) |
|--------------------------------|-----------------------------------|------------------------------------|---|--------------|
| Spikes with milk powder | | | | |
| heated samples | | | | |
| bread | NIST SRM 1549 SMP | 381.0 | 429.2 | 112.7 |
| mixed ground meat | NIST SRM 1549 SMP | 463.0 | 482.3 | 104.2 |
| white bread | NIST SRM 1549 SMP | 734.0 | 588.5 | 80.2 |
| Non heated samples | | | | |
| milk free infant formula | NIST SRM 1549 SMP | 13 | 12.0 | 92.3 |
| milk free infant formula | NIST SRM 1549 SMP | 367.5 | 517.3 | 140.8 |
| pancake | NIST SRM 8435 SMP | 120.0 | 108.1 | 90.1 |
| soy formula | NIST SRM 1549 SMP | 122.0 | 89.9 | 73.7 |
| Spikes of liquids | | | | |
| spike control | SMP 1 | 55.0 | 47.9 | 87.1 |
| wheat flour | SMP 1 | 55.0 | 41.3 | 75.1 |
| red wine | SMP 1 | 55.0 | 40.3 | 73.3 |
| spike control | SMP 2 | 55.0 | 58.7 | 106.7 |
| wheat flour | SMP 2 | 55.0 | 53.7 | 97.6 |
| red wine | SMP 2 | 55.0 | 48.1 | 87.5 |
| wheat flour | NIST SRM 1549 SMP | 5.0 | 5.4 | 108.0 |
| apple juice | NIST SRM 1549 SMP | 15.0 | 16.9 | 112.7 |
| infant formula | NIST SRM 1549 SMP | 45.0 | 39.3 | 87.3 |
| spike control | NIST SRM 1549 SMP | 65.0 | 57.8 | 88.9 |
| apple juice | NIST SRM 1549 SMP | 65.0 | 70.7 | 108.8 |
| apple juice | NIST SRM 1549 SMP | 10.0 | 12.2 | 122.0 |
| apple juice | NIST SRM 1549 SMP | 50.0 | 50.4 | 100.8 |
| Casein + BLG spikes | | | | |
| wheat flour | 4 mg/kg casein and 0.5 mg/kg BLG | 5.0 | 5.2 | 104.0 |
| infant formula | 12 mg/kg casein and 1.5 mg/kg BLG | 15.0 | 14.3 | 95.3 |
| apple juice | 36 mg/kg casein and 4.5 mg/kg BLG | 45.0 | 42.4 | 94.2 |
| Mean value | | | | 97.5 |

SMP = skim milk powder
BLG = β -lactoglobulin



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Food matrices measured at LOQ and multiples of LOQ have been spiked with NIST SRM 1549 skim milk powder (36.7 % protein) to measure the recovery (see table 6b). One spike control and two samples have been used. The spike samples were extracted according to the test kit insert. A mean recovery over all was found at 120.1 %.

Table 6b: Recovery (%) of milk protein in spiked samples at LOQ, 2x LOQ and 5x LOQ

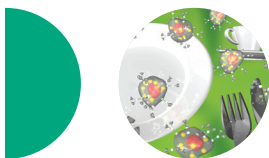
| Sample matrix | Spike material | Spiked milk protein (mg/kg) | Milk protein concentration measured (mg/kg) | Recovery (%) |
|-------------------|-------------------|-----------------------------|---|--------------|
| LOQ | | | | |
| spike control | NIST SRM 1549 SMP | 2.5 | 4.0 | 160.0 |
| apple juice | NIST SRM 1549 SMP | 2.5 | 3.0 | 120.0 |
| wheat flour | NIST SRM 1549 SMP | 2.5 | 2.4 | 96.0 |
| 2 x LOQ | | | | |
| spike control | NIST SRM 1549 SMP | 5.0 | 7.4 | 148.0 |
| apple juice | NIST SRM 1549 SMP | 5.0 | 5.8 | 116.0 |
| wheat flour | NIST SRM 1549 SMP | 5.0 | 5.5 | 110.0 |
| 5 x LOQ | | | | |
| spike control | NIST SRM 1549 SMP | 12.5 | 14.2 | 113.6 |
| apple juice | NIST SRM 1549 SMP | 12.5 | 14.8 | 118.4 |
| wheat flour | NIST SRM 1549 SMP | 12.5 | 12.4 | 99.2 |
| Mean value | | | | 120.1 |

SMP = skim milk powder

The RIDASCREEN®FAST Milk ELISA detects caseins and β -lactoglobulin in equal parts with approx. 66 % casein and approx. 55 % β -lactoglobulin recovery (see table 6c). Cow's milk contains approx. 80 % casein and 10 % β -lactoglobulin. The spiked casein and β -lactoglobulin amount was converted to the amount of total milk protein (spiked casein x 100 / 80 and spiked β -lactoglobulin x 100 / 10). The measured milk protein content was compared to the theoretically calculated value and the recovery was determined.

Table 6c: Recovery (%) of casein and β -lactoglobulin spikes recalculated to the amount of milk protein

| Spike material | Spiked casein (mg/kg) | Spiked BLC (mg/kg) | Calculated milk protein (mg/kg) | Concentration measured of milk protein (mg/kg) | Recovery (%) |
|--------------------------|-----------------------|--------------------|---------------------------------|--|--------------|
| casein 1 | 54.0 | | 67.5 | 39.3 | 58.2 |
| casein 2 | 54.0 | | 67.5 | 48.6 | 72.0 |
| casein 3 | 54.0 | | 67.5 | 46.0 | 68.1 |
| Mean value | | | | | 66.1 |
| β -lactoglobulin 1 | | 6.75 | 67.5 | 45.3 | 67.1 |
| β -lactoglobulin 2 | | 6.75 | 67.5 | 22.3 | 33.0 |
| β -lactoglobulin 3 | | 6.75 | 67.5 | 42.8 | 63.4 |
| Mean value | | | | | 54.5 |



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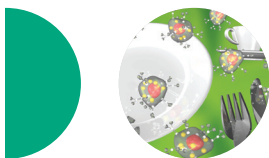
Stability of the Test

The stability of the test is routinely checked by R-Biopharm's quality assurance laboratory after defined storage intervals. Test kits are stored in a cold room at temperatures of 2 - 8 °C (35 - 46 °F). Before testing the kit components are equilibrated to room temperature (20 - 25 °C / 68 - 77 °F). Real time stability of the test will regularly be controlled according to the total quality management schedule of the company.

Conclusion

With the new RIDASCREEN®FAST Milk a sensitive ELISA is available which allows a quantitative and fast determination of milk or milk ingredients in food like bakery goods, chocolate, sausages, ice cream, wine, beer and juice. The results are expressed as mg/kg milk protein and are able to be calculated to milk content. The factor to recalculate milk protein to milk is 31.25 because milk contains 3.2 % protein (example: 1 ppm milk protein x 31.25 = 31 ppm milk).

**For further question or information please contact R-Biopharm AG directly,
phone: +49 (0) 61 51 - 81 02-92 or sales@r-biopharm.de.**



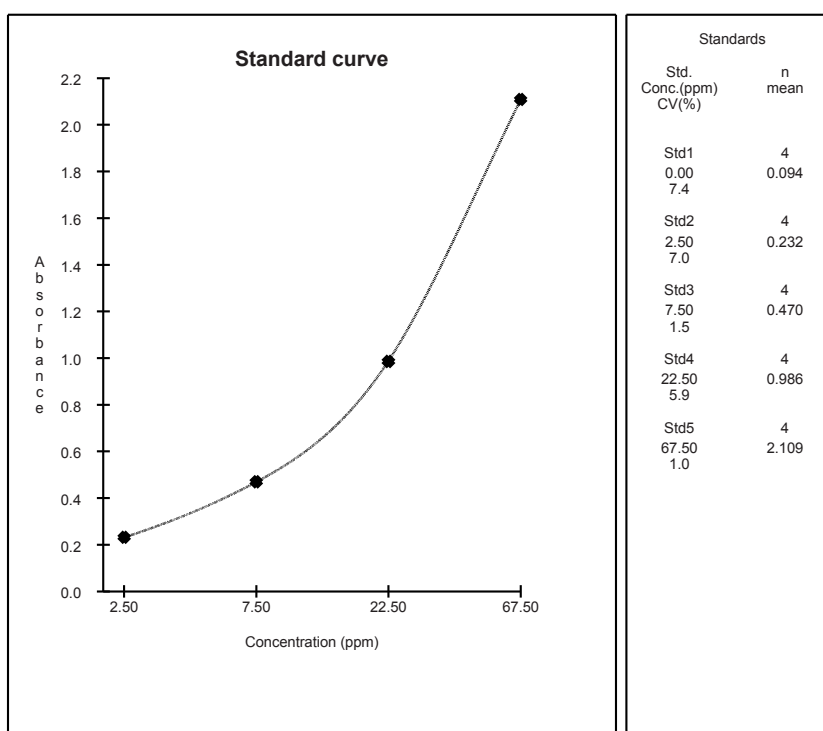
RIDASCREEN®FAST Milk (Art.No. R4652) Validation Report - Appendix 1

QUALITY ASSURANCE CERTIFICATE

RIDASCREEN®FAST Milk

Art. No.: R4652 Lot: 13490 Expiry: 2011-08

R-Biopharm AG, Darmstadt, Germany certifies that this batch has been approved by the Quality Assurance Department and conforms with specifications



| | Lot No. | Expiry |
|-----------------------------|---------|---------|
| Microwell plate | 15410 | 2012-09 |
| Standards | 15460 | 2012-10 |
| Conjugate | 13480 | 2011-08 |
| Buffer1 | 13480 | 2011-11 |
| Additive 1 | 15420 | 2012-04 |
| Red Chromogen Pro | 15380 | 2013-03 |
| Stop solution | 14330 | 2015-07 |
| Washing buffer | 03489 | 2012-04 |
| Extr. buffer | 14420 | 2013-03 |
| RIDA® Extraction Solution 2 | 15410 | 2012-09 |

Please note:

The absorbance for the standards may decrease during the shelf life of the kit. The general shape of the curve will remain similar, while the slope might change slightly. Furthermore refer to product leaflet 8. Indication of instability or deterioration of reagents.

sign.: Edda Rohm
Quality Assurance Representative

Date: 2010-12-08

Remark:

This document has been created electronically and is therefore valid without a signature.

