

A Simple and Fast Detection Method for Bovine Milk Residues in Foods: A 2-Site Monoclonal Antibody Immunochromatography Assay

Wu Xuli, He Weiyl, Kunmei Ji, Wan Wenpu, Hu Dongsheng, Wu Hui, Luo Xinpin, and Liu Zhigang

Abstract: The ingredient declaration on food labels assumes paramount importance in the protection of food-allergic consumers. China has not implemented Food allergen labeling. A gold immunochromatography assay (GICA) was developed using 2 monoclonal antibodies (mAb) against the milk allergen β -lactoglobulin in this study. The GICA was specific for pure milk samples with a sensitivity of 0.2 ng/mL. Milk protein traces extracted from 110 food products were detected by this method. The labels of 106 were confirmed by our GICA method: 57 food samples originally labeled as containing milk were positive for β -lactoglobulin and 49 food samples labeled as not containing milk were negative for β -lactoglobulin. However, 3 food samples falsely labeled as containing milk were found to contain no β -lactoglobulin whereas 1 food sample labeled as not containing milk actually contained β -lactoglobulin. First, these negatives could be because of the addition of a casein fraction. Second, some countries demand that food manufacturers label all ingredients derived from milk as “containing milk” even though the ingredients contain no detectable milk protein by any method. Our GICA method could thus provide a fast and simple method for semiquantitation of β -lactoglobulin in foods.

Keywords: β -lactoglobulin, GICA, food allergen detection, milk allergen

Practical Application: The present method provides a fast, simple, semiquantitative method for the determination of milk allergens in foods.

Introduction

Food allergy is an abnormal immunological response because of a sensitization to a food or food component. Allergy to cow's milk proteins is a common food allergy (Mills and others 2004). Clinical manifestations of cow's milk protein allergy include gastrointestinal symptoms, urticaria, angioedema, atopic dermatitis, allergic rhinitis, asthma, chronic cough, and so on (Wal 2004). Very low dose (as low as 0.6 mg) of milk protein had been reported to elicit allergic responses (Taylor and others 2002). Because specific avoidance of cow's milk proteins is the only effective approach to prevent milk allergies, the declaration of milk ingredients even in trace amounts on food labels assumes paramount importance in the protection of milk-allergic consumers (Monaci and others 2006). In this way, the Food and Drug Administration (FDA) of United States implemented a regulation named Food Allergen Labeling and Consumer Protection Act (FALCPA), requiring food allergen labeling of all foods sold in the United States (FDA 2004.). Similar regulations have been put into execution in other developed regions, such as the European Union and Japan (Li and others 2009). Although food import and export between China and the developed countries is increasing (McKay 2007), no such regulation has been implemented in China. Milk is 1 of the 12 allergenic

ingredients whose presence has to be declared on the label of food products (European Commission 2006). Therefore, it would be essential for Chinese Customs to perform rapid detection of milk allergens.

Bovine β -lactoglobulin (β LG) is the most abundant protein (MW = 18.3 kDa) in whey, accounting for 50% of total protein in the whey fraction (Sawyer 2003). β LG has many useful functional properties and is usually added as 1 functional ingredient in food (Shimizu and others 1985; Waniska and Kinsella 1988). β LG is a major allergen of cow's milk (Koritz and others 1987) and considered to be a specific allergen marker in food products (Monaci and others 2006).

Many techniques are available for the analysis of milk protein. ELISA is the primary immunoassay methods for the detection of milk protein (Monaci and others 2006). Most of the ELISA methods developed for milk allergen analysis are time consuming and need experienced laboratory technicians. ELISA assays for determination of β LG basing on polyclonal antibodies or monoclonal antibody (mAb) have previously been reported (Mäkinen-Kiljunen and Palosuo 1992; Mariager and others 1994; Negrone and others 1998). Therefore, it is essential to develop analytical methods that can be applied in a fast and simple manner to enable legislation to detect milk allergen ingredients. Gold immunochromatography assay (GICA) is an immunochromatographic test in a mobile phase that moves the sample along a test strip. This type of assay is portable, easy to operate, and able to rapidly produce reliable results within 3 to 15 min without expensive laboratory equipment and reagents. The swiftness and convenience of GICA enables its wide application for allergen detection previously in Customs (Stephan and others 2002). Five

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rapid ELISA test kits are currently on the market and can deliver results within 30 to 45 min (Schubert-Ullrich and others 2009). The kits either detect selected milk allergens like caseins or β -lactoglobulin, or check for the presence of total milk proteins based on polyclonal antibodies. However, false positive results can be obtained with this approach because of cross-reactivity of the polyclonal antibodies (Schubert-Ullrich and others 2009). We herein developed a 2-site β LG specific, mAb-based sandwich GICA method. The high specificity of mAbs against β LG can be used to address the problem of false positives. Therefore we were able to detect β LG in food products in a fast, simple, highly selective and quantitative way.

Materials and Methods

β LG, bovine serum albumin(BSA), caseins, α -lactalbumin, ovalbumin (OVA), complete and incomplete Freund's adjuvants, goat anti-Rat IgG-HRP conjugate, 3,3'-5,5'-tetramethyl benzidine(TMB), 3-Diaminobenzidine(DAB) were purchased from Sigma (St.Louis, MO, USA). The nitrocellulose membrane, the sample pad, and the absorbent pad for GICA were from Whatman (Maidstone, Kent, U.K.). Fresh milk was obtained from the local market.

Production of anti- β LG mAbs

Two anti- β LG mAbs (clones 1H8 and 1G5) were produced and purified according to previously reported method (Chang and others 1995). The isotypes and titres were determined by standard immunoassays. The cross reactivity between BSA, caseins, α -lactalbumin and a series of food sample protein extracts (peanut, egg, fish, shrimp, cashew, wheat, soybean) was estimated by indirect ELISA assays using 1H8 and 1G5 respectively as the primary antibody. The cross reactivity between these 2 mAbs toward β LG was determined by indirect competitive ELISA (Sano and others 1998).

Epitope analysis of mAbs

As for the IgG response to β LG, ⁵¹Glu-⁶⁴Asp, ⁶⁷Arg-⁸⁸Asn, ¹²⁹Asp-¹⁴⁴Pro, ¹³⁹Arg-¹⁵⁶Thr are reported to be major epitopes (Jarvinen and others 2001). These epitopes were synthesized. Immuno dot-blotting and indirect ELISAs were performed with each purified epitope against mAbs 1H8 and 1G5, respectively.

Two-site mAb ELISA for β LG

We set up a 2-site mAb ELISA. All experiments were performed according to Ji, and others (Ji and others 2011). In brief, biotin-conjugated mAb 1G5 was used as the capture antibody and mAb 1H8 as the coating antibody. A standard curve was profiled with serially diluted β LG protein and the detection limit was determined.

Two-site mAb-based GICA for β LG

GICA was established according to the method of Lee and others (Lee and others 2006). First, 100 mL boiled chloroauric acid (0.01%) was blended with 1.5 mL 1.5% trisodium citrate and was boiled for another 15 min. The pH of the cooled down solution was adjusted to 8.2 by 0.2 M K₂CO₃. Purified 1H8 was then added to the buffer under stirring for 15 min. OVA was added to the solution and 10% NaCl was added to bring NaCl to a final concentration of 1%. The resulting solution was centrifuged at 614 g for 10 min and the supernatant was centrifuged at 15,344 g for 60 min. The resulting colloidal gold- β LG-1H8 complex was stored in 0.02 mol/L, pH 7.4 Tris-HCl containing

40 mg/100 mL polyethylene glycol (M.W. 20 kDa), 50% glycerol and 0.02 % NaN₃. The GICA test strips were composed of water absorbing cellulose, nitrocellulose membrane and water absorbing paper. The colloidal gold- β LG-1H8 complex was applied to the water-absorbing cellulose and capture antibody 1G5 was absorbed onto nitrocellulose membrane as the detection line. An anti-mouse sheep IgG was dotted above the capture antibody as the control line. The dried membrane was blocked by 10% fresh OVA and stick to a white plastic sheet and cut into strips of 0.2 cm×8 cm for ready use. The specificity of GICA strip was tested with BSA, caseins, α -lactalbumin and a series of food sample protein extracts (peanut, egg, fish, shrimp, cashew, wheat, soybean).

Detection of β LG in foods using GICA at China customs

We obtained 110 exported and imported food product samples from the Shenzhen Entry-Exit Inspection and Quarantine Bureau of China. The food samples were ground into a fine powder and dissolved in extraction buffer (1 g of powder or 1mL liquid in 20 mL extraction buffer, 20 mmol/L Tris HCl, 1 M NaCl, 2% Tween, pH 8.0) at 50°C for 20 min. After rigorously shaking every 10 min, the solutions were centrifuged at 22,095 g for 10 min. The supernatant was then tested by GICA.

Results

Anti- β LG mAbs

The mAbs were identified by Western blotting. Both mAbs bound β LG (18 kDa) (Figure 1). Isotyping assays verified that mAb 1H8 and 1G5 were both IgG1 subtype. The titers of both mAbs exceeded 1:4000000 in titration assay. BSA, caseins, α -lactalbumin, and a series of food sample protein extracts (peanut, egg, fish, shrimp, cashew, wheat, soybean) did not show any reactivity with mAb 1H8 and 1G5 (Figure 1). Indirect ELISA inhibition assays showed that mutual inhibition of mAb 1H8 and 1G5 was less than 4% (Table 1). This indicated that these 2 mAbs

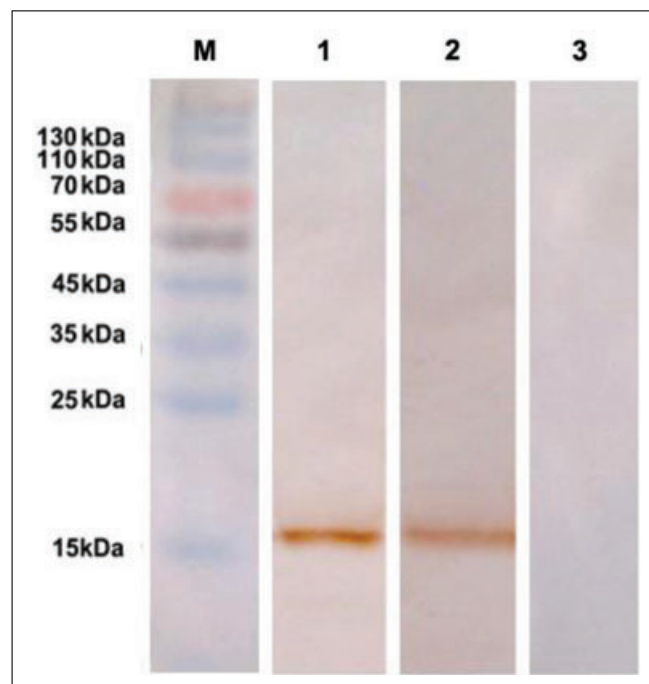


Figure 1—Immunoblot analysis of mAb 1G5 and 1H8 specificity. M, protein marker; 1, mAb 1G5 + β LG; 2, mAb 1H8 + β LG. 3, negative control.

Table 1—Cross reactivity of mAb 1H8 and 1G5 as determined by indirect competitive ELISA.

Competitive biotinylated mAb dilutions	1: 8000	1: 4000	1: 2000
Inhibition ratio, mAb 1H8: mAb 1G5	2.13%	3.05%	3.88%
Inhibition ratio, mAb 1G5: mAb 1H8	2.86%	3.11%	3.50%

recognized distinct epitopes and specificity without cross-reactivity which could potentially be used to develop a 2-site mAb sandwich immunoassay.

Epitope analysis

The reactivity of the 4 synthesized β LG fragments toward the 2 mAbs was tested through immuno dot-blotting and indirect ELISA assays. As shown in Figure 2, mAb 1H8 showed ELISA reactivity against polypeptide ⁶⁷Arg-⁸⁸Asn, and mAb 1G5 showed ELISA reactivity against polypeptide ⁵¹Glu-⁶⁴Asp (Figure 3). Reactivities were confirmed by immuno dot-blotting analysis (Figure 3).

Two-site mAb ELISA for β LG

We used mAb biotin-conjugated mAb 1G5 as capture antibody and 1H8 as coating antibody and developed a 2-site mAb ELISA. The optimum dilution fold for the capture antibody (biotin-conjugated mAb 1G5) was 1:2000 and that for the coating antibody (mAb 1H8) were 1:5000. The ELISA detection limit of β LG was 0.8 ng/mL, and a linear curve was plotted between 1.6 and 50 ng/mL ($R^2 = 0.9903$; Figure 4).

Assessment of the 2-site mAb-based GICA for its sensitivity and specificity

The GICA was able to detect as low as be 0.2 ng/mL milk protein in crude extracts (Figure 5). GICA strips did not react with BSA, caseins, α -lactalbumin and food sample protein extracts from peanut, egg, fish, shrimp, cashew, wheat, soybean (data not

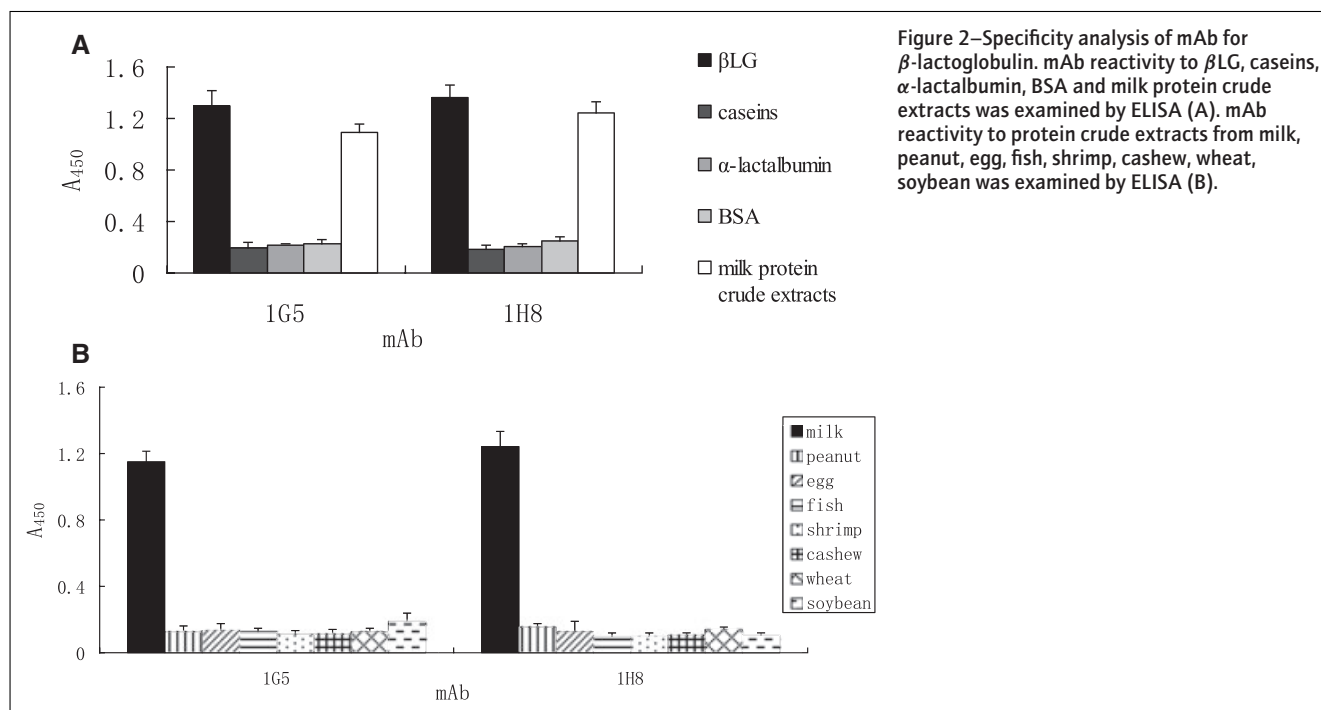
shown). These results showed that our GICA strips demonstrated good sensitivity and specificity.

Detection of β LG in foods with GICA by China customs

GICA was used to detect β LG in 110 exported and imported food product samples from the Shenzhen Entry-Exit Inspection and Quarantine Bureau of China (Table 2). As β LG is an abundant protein in bovine milk, we determined the accuracy of food labeling by checking the presence or absence of β LG. GICA tests of β LG revealed that 106 of these samples showed consistent results with their original labels (57 food samples labeled as containing milk allergen and 49 samples labeled as not containing milk allergen). However, 3 food samples labeled as containing milk tested negative for β LG in GICA test whereas 1 food sample labeled as not containing milk got positive GICA result. These results show that GICA could be effective for possible detection of milk residues in food products.

Discussion

Milk is the one of the major allergen sources. In recent years a few enzyme immunoassays which are highly specific and sensitive in detecting even trace amounts of milk allergens have been published and reviewed by Schubert-Ullrich and others (Schubert-Ullrich and others 2009). However, most of the methods developed for milk allergen analysis in food are labor intensive, time consuming, requiring expensive laboratory equipment, and well-trained technicians, which adds to the inconvenience of the tests. In this study, the sensitivity of ELISA was determined as 0.8 ng/mL β LG, and GICA was determined as 0.2 ng/mL total milk extract. Its sensitivity was lower than that of the ELISA system (0.03 ng/mL) developed by Negroni and others for β LG detection (Negroni and others 1998). However, the GICA method described here is much simpler and faster than an ELISA assay. The strips could be easily used by consumers without any intensive training thereby making it more accessible to large volume food sample testing in the Customs environment.



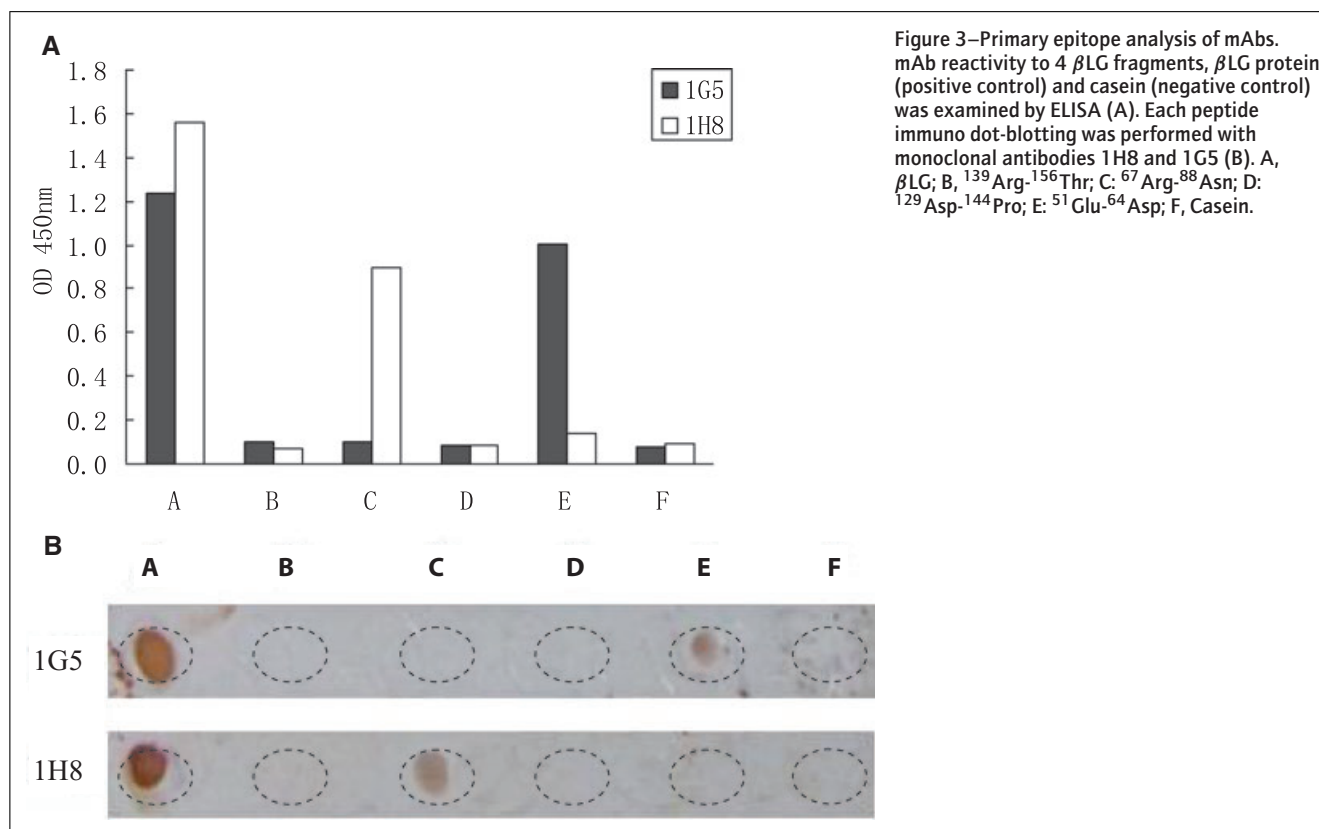


Figure 3—Primary epitope analysis of mAbs. mAb reactivity to 4 β LG fragments, β LG protein (positive control) and casein (negative control) was examined by ELISA (A). Each peptide immuno dot-blotting was performed with monoclonal antibodies 1H8 and 1G5 (B). A, β LG; B, $^{139}\text{Arg}-^{156}\text{Thr}$; C, $^{67}\text{Arg}-^{88}\text{Asn}$; D, $^{129}\text{Asp}-^{144}\text{Pro}$; E, $^{51}\text{Glu}-^{64}\text{Asp}$; F, Casein.

Threshold doses for allergic reactions can be as low as 0.6 mg of milk proteins (Taylor and others 2002). Our GICA strips could detect as low as 0.2 ng/mL total milk extract, which could detect most milk products and therefore benefit consumers with milk allergies.

The key to a successful 2-site mAb immunoassay is to develop 2 mAbs with high titers, high specificity and essentially no cross reactivity (Sander and others 1997). Fortunately, our 2 mAbs

recognized 2 contiguous, separate epitopes in a small region ($^{51}\text{Glu}-^{88}\text{Asn}$ of β LG) without cross reactivity. This situation is very preferable for a 2-site immunoassay of milk allergens. As foods have to undergo harsh processing practice such as high temperature treatment thereby degrading the β LG protein into smaller peptides (Barreto and others 2003). Two epitopes far apart on the protein molecule may fail to be detected because of fragmentation from the food processing procedures. Thus a 2-site mAb immunoassay based on 2 noncross reactive mAbs which recognize distinct epitopes in an adjacent region of the antigen could stand a bigger chance of reacting with the peptide fragments. Another point to keep in mind is that the milk protein may be degraded into small peptides with lower allergenicity in food processing. Our system may cause overestimation of the allergenicity of the samples tested. β LG is notorious for its propensity to aggregate and become insoluble following heat processing. Downs and others evaluated quantitative β LG kits for detection of milk residues in a simple

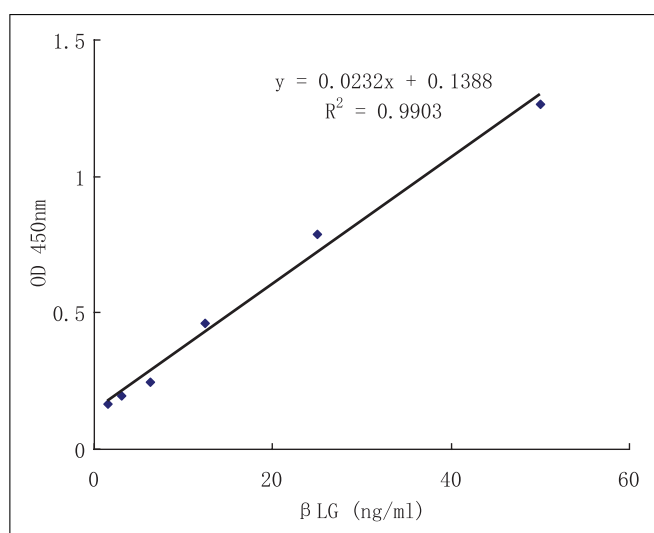


Figure 4—Sandwich ELISA with biotinylated mAb 1G5 as the capture antibody and mAb 1H8 as the coating antibody. The standard curve is linear for β LG from 1.6 to 50 ng/mL and the detection limit was 0.8 ng/mL ($R^2 = 0.9903$).

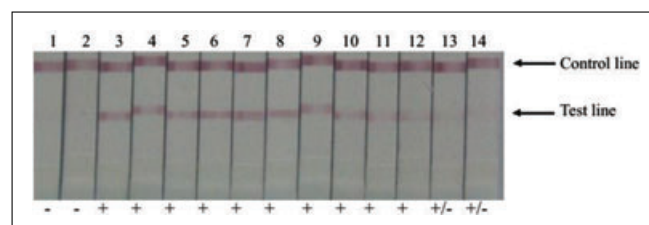


Figure 5—Detection of milk crude protein extracts with GICA strips; 1, Extract buffer; 2, PBS; 3, 100 ng/mL; 4, 50 ng/mL; 5, 25 ng/mL; 6, 12.6 ng/mL; 7, 6.3 ng/mL; 8, 3.1 ng/mL; 9, 1.6 ng/mL; 10, 0.8 ng/mL; 11, 0.4 ng/mL; 12, 0.2 ng/mL; 13, 0.1 ng/mL; 14, 0.05 ng/mL. The concentrations indicated are the quantity of total protein per mL of crude milk extract. +: positive; -: negative; +/ -: not definitive.

Table 2—Representative (*n* = 20) GICA analysis of milk allergen β LG in food samples from Chinese customs.

Food description	Imported (I)/ Exported (E)	Labeling for milk presence	GICA results (<i>n</i> = 3)
Cookies, almond	E	+	Positive
Cookies, walnut	E	+	Positive
Cookies, peanut	E	+	Positive
Sugar, milk	E	+	Positive
Sugar, strawberry	I	—	Negative
Sugar, orange	E	—	Negative
Sugar, chocolate	I	—	Negative
Cake, nuts	E	—	Negative
Cake, lotus seed	E	—	Negative
Cake, milk	E	+	Positive
Chocolate I	I	—	Negative
Chocolate II	I	—	Negative
Chocolate III	I	+	Positive
Chocolate, Milk	I	+	Positive
Chocolate, strawberry	E	+	Positive
Wafer, peanut	I	—	Negative
Chocolate, liqueur	I	—	Negative
Hazelnuts, milk	I	+	Positive
Corn bar	I	—	Negative
Rusk, wheat	I	—	Negative

Note: Theoretically, in our experiments, GICA positive results indicated that the concentrations of the foods were greater than 200 μ g milk total protein/kg food sample whereas negative results were less than 200 μ g milk total protein/kg food sample.

cereal matrix and found lack of detection because of insolubility and extraction issues (Downs and Taylor 2010). The detection fluctuation in processed samples is likely because of protein modifications which affect the immunoreactivity of the antigens detected by the ELISA methods. Therefore, caution should be exercised in equating the amount of β LG to allergenicity.

In our previous studies, GICA was developed to detect the peanut allergen Ara h1 (Ji and others 2011). We used DNASTar software to select 5 potential antigenic peptide fragments of Ara h1. Their corresponding coding cDNA sequences were cloned and expressed. And this experiment was time consuming and labour intensive. In this study, we directly synthesized reported IgG epitopes of β LG. Immuno dot-blotting instead of West-blotting was performed with each purified epitope. Therefore, epitope analysis of mAbs for target protein in this study was more simple and accurate than our previous work.

Caseins are often used instead of total milk proteins or whey proteins in food processing. Although our method is specific and sensitive for detecting β LG, it would give false negative results for milk samples with only caseins. First, the false negatives could be because of the addition of a casein fraction instead of total milk proteins or whey proteins. Second, some countries including the USA demand that food manufacturers label all ingredients derived from milk as “containing milk” even though the ingredients contain no detectable milk protein by any method. Thus these companies may only adhere to the regulatory requirements of the country of origin. For these samples, Customs may have to turn to other alternatives especially caseins-specific tests. Currently, there are at least 3 commercial lateral flow strip tests on the market detect total milk which could be a real advantage over this method (Schubert-Ullrich and others 2009). However, these kits based on polyclonal antibodies demonstrated lower sensitivity and specificity compared with our method. Our method is perhaps more useful for possible detection of milk residues in products that do not declare the presence of milk.

Some food labeling regulations to protect allergic consumers from accidental consumption of food allergens have been issued (Food and Drug Administration 2004.; Vierk and others 2007;

Ji and others 2009). Although China in the biggest world food trader, it does not have such regulations. Applying GICA as part of the process to examine food imported or exported will assist Chinese Customs Authorities to detect any food allergens present. However, further work is needed to continue to improve this process.

Acknowledgments

This study was supported in part by research funding from the Natural Science Funding of Guangdong Province (Nr. S2012010008514) to Xuli Wu, the funding from the High-tech Industrialization Funding of Guangdong Province (Nr. 2009B011300010) to Ji Kunmei, and the Key Laboratory Project of Shenzhen (Nr. SW201110010) to Zhigang Liu.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Chromatogram of $^{51}\text{Glu-}^{64}\text{Asp}$.

Figure S2. Ms Spectrum of $^{51}\text{Glu-}^{64}\text{Asp}$.

Figure S3. Chromatogram of $^{67}\text{Arg-}^{88}\text{Asn}$.

Figure S4. Ms Spectrum of $^{67}\text{Arg-}^{88}\text{Asn}$.

Figure S5. Chromatogram of $^{129}\text{Asp-}^{144}\text{Pro}$.

Figure S6. Ms Spectrum of $^{129}\text{Asp-}^{144}\text{Pro}$.

Figure S7. Chromatogram of $^{139}\text{Arg-}^{156}\text{Thr}$.

Figure S8. Ms Spectrum of $^{139}\text{Arg-}^{156}\text{Thr}$.

Figure S9. Certificate of Analysis to the Synthetic of $^{51}\text{Glu-}^{64}\text{Asp}$.

Figure S10. Certificate of Analysis to the Synthetic of $^{67}\text{Arg-}^{88}\text{Asn}$.

Figure S11. Certificate of Analysis to the Synthetic of $^{129}\text{Asp-}^{144}\text{Pro}$.

Figure S12. Certificate of Analysis to the Synthetic of $^{139}\text{Arg-}^{156}\text{Thr}$.

1 **Supplementary Data**

2 **Supplementary Material**

3 **MATERIALS AND METHODS**

4 **Material:** Epitopes were synthesized by GenScript Co., USA.

5 **Apparatus:** Protein electrophoresis and blotting equipments were from Bio Rad
6 (Hercules. CA. USA) . Microplate Readers MK3 were from Thermo (Franklin. MA.
7 USA) .Equipment for making GICA test strip was purchased from Bio-Dot (Irvine.
8 CA. USA) .

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Supplementary Results

1. Production of mAbs against β LG

β LG specific mAbs were produced according to a standard protocol (Chang JH, Sutherland WM, Parsons SJ. Monoclonal antibodies to oncoproteins. *Methods Enzymol* 1995; 254:430-45.). Five Balb/c mice were immunized with subcutaneous and intraperitoneal injections of 100 μ g β LG in complete Freund's adjuvant (Sigma) at 4-week intervals. Approximately 2 months later, the selected mouse received intrasplenic and intraperitoneal injections of 10 μ g β LG, and 4 days later the spleen cells were fused with NS-1 (Sp2/0) myeloma cells. Supernatants were screened for specific antibody production by indirect ELISA. Antibody positive hybrids were cloned twice by limiting dilution, and frozen cell stocks were stored in liquid nitrogen. Two anti- β LG mAbs from a total of 60 were selected: named clones 1H8 and 1G5. Antibody subtype was analyzed by ELISA with an isotyping kit for mAbs according to the manufacturer's instructions (Sigma, St. Louis, MO, USA). The mAbs were produced as ascites and purified by affinity chromatography over recombinant Protein G (Gamma Bind Plus; GE, USA). Ascites titres of these mAbs were assayed by indirect ELISA. The identification of these mAbs was assayed by immunoblot analysis.

2. Epitopes were synthesized for primary epitope analysis of mAbs

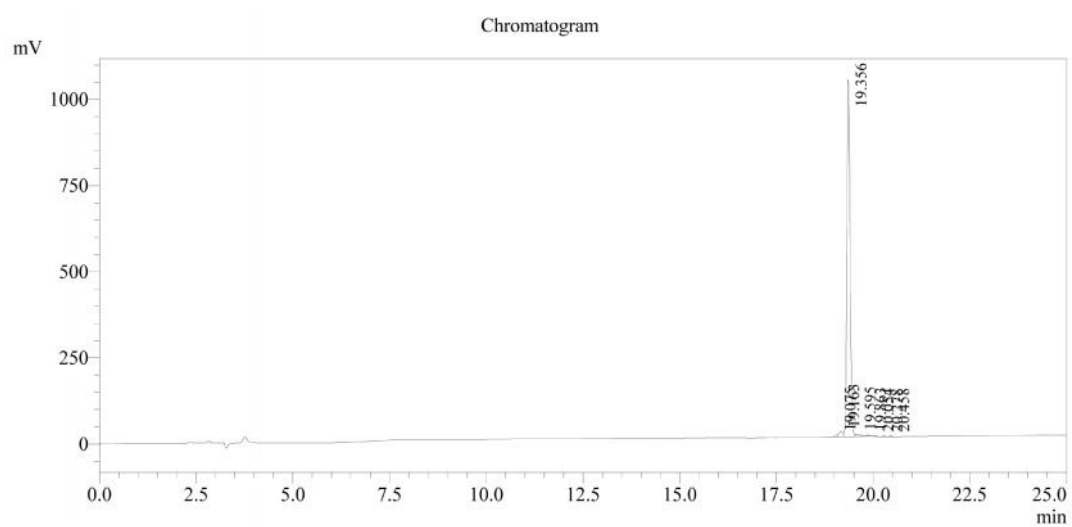


Fig.1 Chromatogram of $^{51}\text{Glu}-^{64}\text{Asp}$

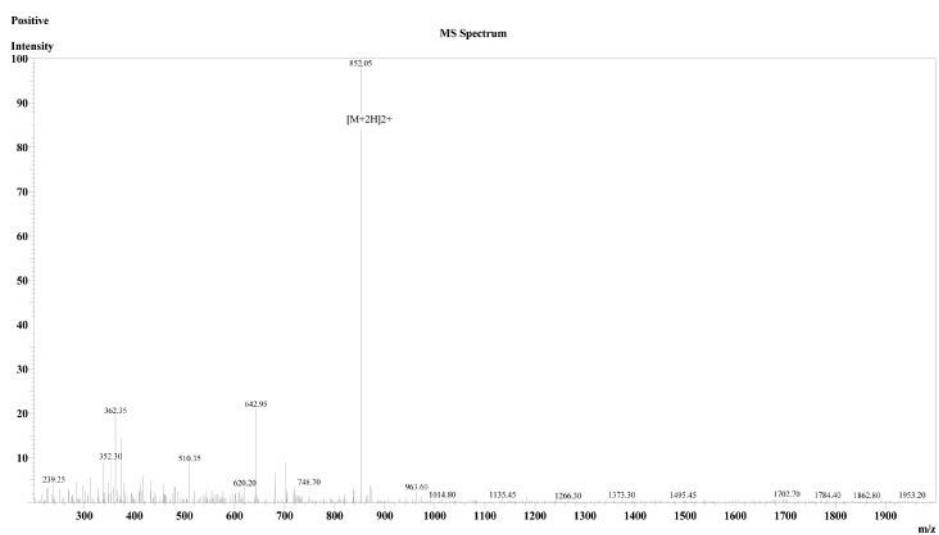


Fig.2 Ms Spectrum of $^{51}\text{Glu}-^{64}\text{Asp}$

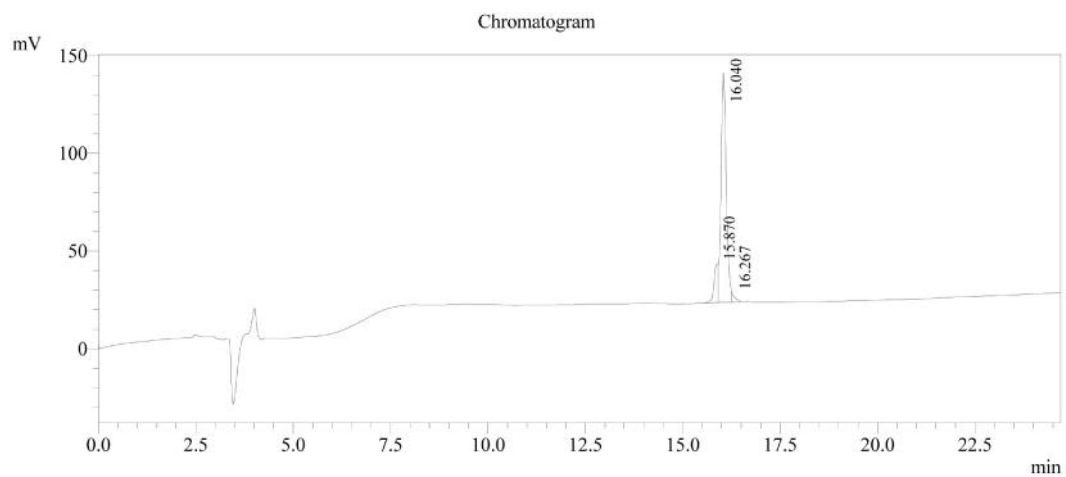


Fig.3 Chromatogram of $^{67}\text{Arg}-^{88}\text{Asn}$

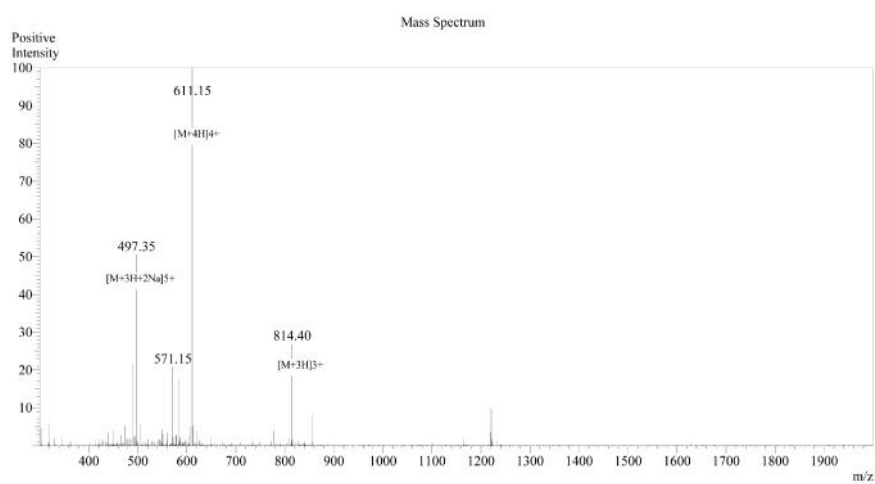


Fig.4 Ms Spectrum of $^{67}\text{Arg}-^{88}\text{Asn}$

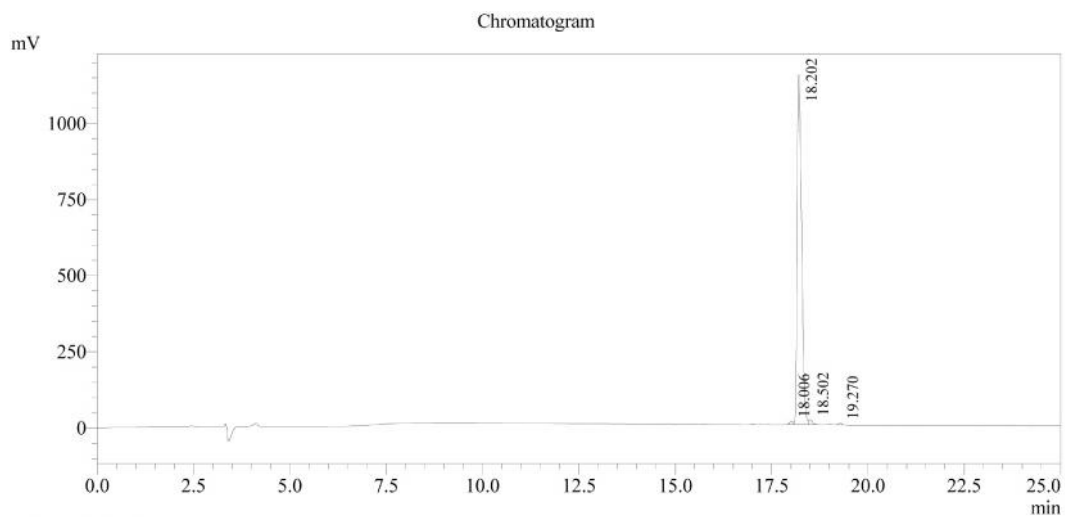


Fig.5 Chromatogram of $^{129}\text{Asp}-^{144}\text{Pro}$

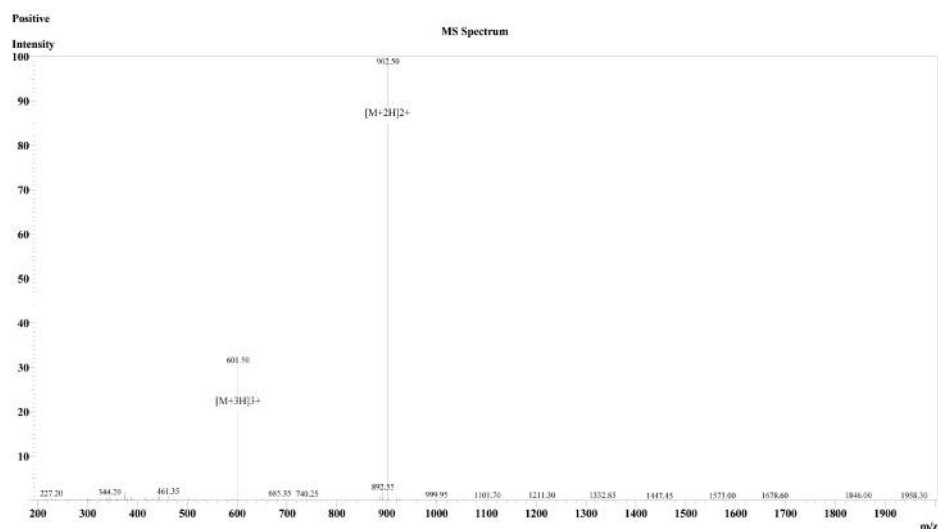


Fig.6 Ms Spectrum of $^{129}\text{Asp}-^{144}\text{Pro}$

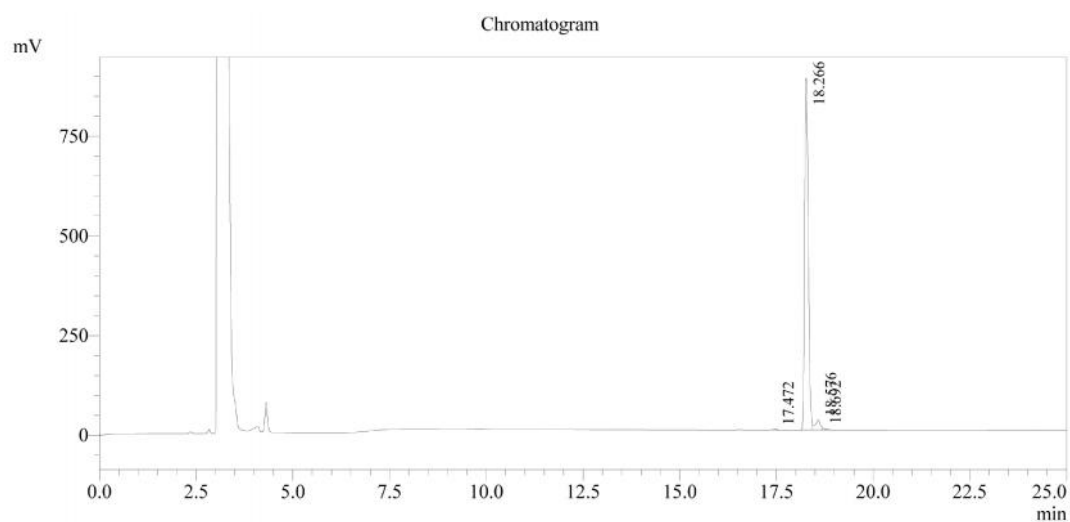


Fig.7 Chromatogram of $^{139}\text{Arg}-^{156}\text{Thr}$

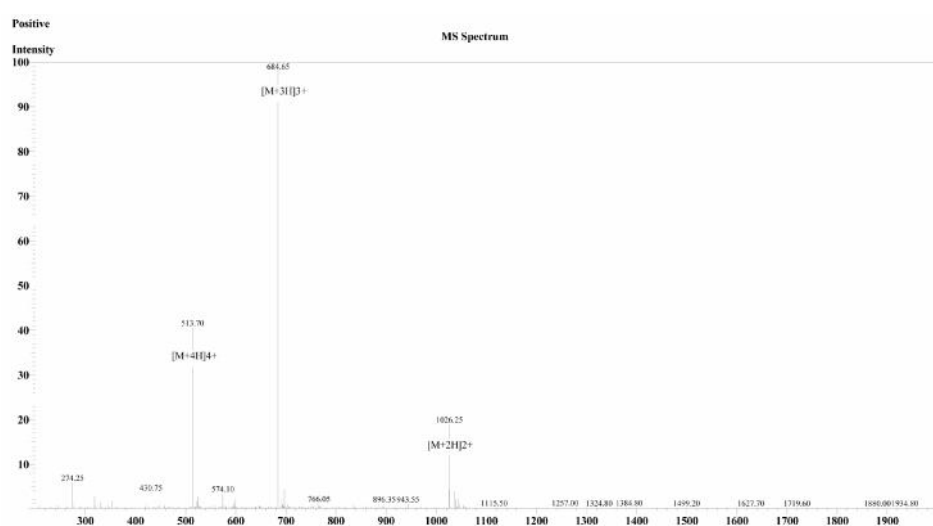


Fig.8 Ms Spectrum of $^{139}\text{Arg}-^{156}\text{Thr}$

CERTIFICATE OF ANALYSIS

Product Name	51-64
Order ID	110478_1
Lot No.	110478001092710ZW
Sequence	EGDLEILLQKWEND
Modification	N/A
Length	14AA
Storage	-20°C

Test Items	Specifications	Results
Molecular Weight	Theoretical MW:1701.84	Consistent
HPLC purity	>70%	96.1%
Appearance	White lyophilized powder	Conforms
Quantity	1-4 mg	4mg

Caution:

For laboratory or further manufacturing use only. Not intended for household use. If you have any questions about the Certificate of Analysis, please contact our customer service representative at 1-877-436-7274 (Toll-Free), or 1-732-885-9188.

Certified by:  Date: 2010-10-11

Quality Assurance Department

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Fig.9 Certificate of Analysis to the Synthetic of ⁵¹Glu-⁶⁴Asp

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CERTIFICATE OF ANALYSIS

Product Name	67-88
Order ID	110478_2
Lot No.	110478002092710LQ
Sequence	AQKKIIAEKTKIPAVFKIDALN
Modification	N/A
Length	22AA
Storage	-20°C

Test Items	Specifications	Results
Molecular Weight	Theoretical MW:2439.95	Consistent
HPLC purity	>70%	86.9%
Appearance	White lyophilized powder	Conforms
Quantity	1-4 mg	4mg

Caution:

For laboratory or further manufacturing use only. Not intended for household use. If you have any questions about the Certificate of Analysis, please contact our customer service representative at 1-877-436-7274 (Toll-Free), or 1-732-885-9188.

Certified by:  Date: 2010-10-11

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Fig.10 Certificate of Analysis to the Synthetic of $^{67}\text{Arg-}^{88}\text{Asn}$

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CERTIFICATE OF ANALYSIS

Product Name	129-144
Order ID	110478_3
Lot No.	110478003092710BF
Sequence	DDEALEKFDKALKALP
Modification	N/A
Length	16AA
Storage	-20°C

Test Items	Specifications	Results
Molecular Weight	Theoretical MW:1803.03	Consistent
HPLC purity	>70%	97.9%
Appearance	White lyophilized powder	Conforms
Quantity	1-4 mg	4mg

Caution:

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Certified by:  Date: 2010-10-06

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Fig.11 Certificate of Analysis to the Synthetic of $^{129}\text{Asp-}^{144}\text{Pro}$

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CERTIFICATE OF ANALYSIS

Product Name	139-156
Order ID	110478_4
Lot No.	110478004092710BF
Sequence	ALKALPMHIRLSFNPTQL
Modification	N/A
Length	18AA
Storage	-20°C

Test Items	Specifications	Results
Molecular Weight	Theoretical MW:2050.48	Consistent
HPLC purity	>70%	95.3%
Appearance	White lyophilized powder	Conforms
Quantity	1-4 mg	4mg

Caution:

For laboratory or further manufacturing use only. Not intended for household use. If you have any questions about the Certificate of Analysis, please contact our customer service representative at 1-877-436-7274 (Toll-Free), or 1-732-885-9188.

Certified by:  Date: 2010-10-10

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Fig.12 Certificate of Analysis to the Synthetic of ¹³⁹Arg-¹⁵⁶Thr

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3. Two-site mAb ELISA for β LG

Purified mAb 1G5 (1:1000) was dissolved in PBS buffer and reacted with NHS-activated biotin (NSH-Biotin) in a ratio according to the manual of the manufacturer (Pierce Inc.). The reaction was performed in the dark and the unconjugated NSH-biotin was removed by a filter column centrifugation with a molecular cut-off value of 50 kDa. 100ng of mAb 1H8 was coated onto ELISA plate as coating antibody at 4 °C overnight. The wells were washed and blocked by 200 μ l blocking buffer (2% OVA-PBS-T) at 37 °C for 2 h. 100 μ l serially diluted native β LG was blotted onto each well and reacted at 37 °C for 1 h and was washed three times. The biotin-conjugated mAb 1G5 was diluted at the ratio of 1:2000 by 0.1% OVA-PBS-T as the capture antibody. It was then added onto each well and incubated at 37 °C for 1 h before 3 washes by PBST. TMB and H₂O₂ substrate was added to the wells at 37 °C for 10 min and the reaction was stopped by 2M H₂SO₄ and the optical density was determined by a ELISA microplate reader. A standard curve was established by profiling the concentration of n β LG to the optical density and the detection limit was determined. A positive reaction was defined when the optical density of the working well is over 2.1 fold of that of the negative control well.