Goat Milk Proteins

Immunoenzymetric Assay for the Measurement of
Goat Milk Proteins
Catalog # F240

Intended Use

This kit is intended for use in determining the presence of goat milk protein contamination in products manufactured by transgenic expression in goat milk. The kit is for **Research and Manufacturing Use Only** and is not intended for diagnostic use in humans or animals.

Summary and Explanation

Transgenic expression of therapeutic proteins in milk is a cost effective method for production of large quantities of therapeutic proteins. As therapeutic agents these proteins must be highly purified. The manufacturing and purification process of these products leaves the potential for contamination by other proteins normally found in milk. Such contamination can reduce the efficacy of the therapeutic agent and result in adverse toxic or immunological reactions and thus it is desirable to reduce the milk protein contamination to the lowest levels practical. The antibodies used in this kit were generated against clarified, non-transgenic goat milk and have been shown to react by one dimensional Western blot to approximately 30 bands also detected by SDS-PAGE with colloidal gold protein stain. However, users of this kit should confirm that its antibodies react with the majority of milk proteins found in their transgenic process. This can be determined by performing 1 or 2 dimensional western blots with the same antibody used in this kit. This antibody may be ordered separately from *Cygnus Technologies* as Catalog # 4042, or alternatively you may order the Goat Milk Protein Western Blot kit, Catalog #F245. While Western blot is a useful method it suffers from a number of limitations. Western blot is a complex and technique dependent procedure requiring a subjective interpretation of results. Furthermore, it is essentially a qualitative method which does not lend itself to obtaining quantitative answers. The sensitivity of Western blot is severely limited by the volume of sample which can be tested and by interference from the presence of high concentrations of the intended product. As such Western Blot is normally only adequate to detect milk protein contamination in upstream purification process samples. The microtiter plate immunoenzymetric assay method employed in this kit overcomes the limitations of Western blots and provides a simple to use, highly sensitive, objective, and semi-quantitative method to aid in optimal purification process development, process control, routine quality control, and product release testing.

Principle of the Procedure

The goat milk protein assay is a two-site immunoenzymetric assay. Samples which may contain goat milk proteins are reacted in microtiter strips coated with an affinity purified capture antibody. A second horse radish peroxidase (HRP) enzyme labeled anti-goat milk antibody is reacted, simultaneously resulting in the formation of a sandwich complex of solid phase antibody-milk protein-enzyme labeled antibody. The microtiter strips are washed to remove any unbound reactants. The substrate, tetramethylbenzidine (TMB) is then reacted. The amount of hydrolyzed substrate is read on a microtiter plate reader and is directly proportional to the concentration of total milk proteins present.

Reagents & Materials Provided

Component	Product #
Anti-Goat Milk:HRP Affinity purified rabbit antibody conjugated to HRP in a protein matrix with preservative. 1x12mL	**F241**
Anti-Goat Milk coated microtiter strips 12x8 well strips in a bag with desiccant	**F242***
Goat Milk Standards Clarified non-transgenic goat milk proteins in buffer, normal human IgG protein matrix with preservative. Standards at 0, 0.5, 1.5, 4, 10, and 25ng/mL. 1 mL/vial Store at –10°C to –30°C upon receipt.	**F243**
Stop Solution 0.5M sulfuric acid. 1x12mL	**F006**
TMB Substrate 3,3',5,5' Tetramethylbenzidine. 1x12mL	**F005**
Wash Concentrate (20X) Tris buffered saline with preservative. 1x50mL	**F004**

*All components can be purchased separately except # F242.

Storage & Stability

- Store Standards at –10°C to -30°C. All other reagents should be stored at 2°C to 8°C for stability until the expiration date printed on the kit.
- After prolonged storage, you may notice a salt precipitate and/or yellowing of the wash concentrate. These changes will not impact assay performance. To dissolve the precipitate, mix the wash concentrate thoroughly and dilute as directed in the 'Preparation of Reagents' section. Reconstituted wash solution is stable until the expiration date of the kit.

Materials & Equipment Required But Not Provided

Microtiter plate reader spectrophotometer with dual wavelength capability at 450 & 650nm. *(If your plate reader does not provide dual wavelength analysis you may read at just the 450nm wavelength.)*
Pipettors - 25µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (400 - 600 rpm)
Distilled water
1 liter wash bottle for diluted wash solution

Precautions

- For Research or Manufacturing use only.
- Stop reagent is 0.5M H_2SO_4. Avoid contact with eyes, skin, and clothing.
- The hIgG used in preparation of the kit standards has been tested by FDA approved methods and has been found negative for antibody to human immunodeficiency virus (HIV-I & HIV-II), antibody to Hepatitis C virus, and for Hepatitis B surface antigen. No known test method can offer total assurance that HIV, Hepatitis B and C, or other infectious agents are absent. *Handle these reagents as if they were potentially infectious.* At the concentrations used in this kit, none of the other reagents are believed to be harmful.
- This kit should only be used by qualified technicians.

Preparation of Reagents

- Bring all reagents to room temperature.
- Dilute wash concentrate to 1 liter in distilled water, label with kit lot and expiration date, and store at 4°C.

Procedural Notes

1. Complete washing of the plates to remove excess unreacted reagents is essential to good assay reproducibility and sensitivity. If duplicate CVs are poor or if the absorbance of the 0 standard is greater than 0.200, evaluate plate washing system for proper performance.
2. High Dose Hook Effect may be observed in samples with very high concentrations of milk proteins. Samples greater than 200µg/mL may give absorbances less than the 25ng/mL standard. High Dose Hook samples are most likely to be encountered from samples early in the purification process. If a hook effect is possible samples should also be assayed diluted. If the absorbance of the undiluted sample is less than the diluted samples this may be indicative of the hook effect. Such samples should be diluted until the dilution adjusted value remains constant. The diluent used should be compatible with accurate recovery. Prospective diluents can be tested for recovery by using them to dilute the 25ng/mL standard, as described in the "Limitations" section below. Alternatively, the sample diluent used to prepare the kit standards, Catalog # F243A can be ordered from *Cygnus Technologies.*

Limitations

- The standards used in this assay are comprised of clarified non-transgenic goat milk. Western blot analysis of the antibodies used in this kit demonstrates that they recognize 30 distinct protein bands from goat milk also detectable by one-dimensional SDS electrophoresis under reducing conditions. However there can be no guarantee that this assay will detect all non-transgenic proteins or protein fragments from transgenic goat milk. Hence, the suggested arbitrary dose units for reporting unknown samples is in "ng/mL of total immuno-reactive goat milk protein equivalents."
- Before exclusively relying on this assay to detect goat milk proteins it is advisable to validate it relative to other methods of detection such as PAGE silver stain, HPLC, and Western blot to ensure that this assay does not fail to react to any important contaminants. The assay may not be strictly quantitative for a given milk protein contaminant due to the potential for variable reactivity of different proteins. If absolute quantitation of a particular known contaminant is necessary and this contaminant does not give essentially 100% recovery relative to the standards supplied with this kit it may be necessary for the user to provide their own standards. Consult *Cygnus Technologies* Technical Services department for issues regarding quantitation.
- Certain sample matrices may interfere in this assay. High or low pH, detergents, urea, high salt concentrations, and organic solvents are some of the known interference factors. Sample matrix interference can be determined by a 'spike and recovery' experiment in which 1 part of the

25ng/mL standard is diluted into 4 parts of the matrix containing no or very low milk protein contaminants. This diluted standard when assayed as an unknown should give a value of ~ 4 to 6 ng/mL above the base level of the undiluted sample. Consult *Cygnus Technologies* Technical Service Department for advice on how to quantitate the assay in problematic matrices.

- Avoid the assay of samples containing sodium azide (NaN_3) which will destroy the HRP activity of the conjugate and could result in the under-estimation of milk protein levels.

Assay Protocol

- The assay protocol is a simultaneous incubation of sample with HRP conjugated antibody. This yields a sensitivity (limit of detection) of ~0.1ng/mL and requires 2.5 hours to complete.
- The protocol specifies the use of an approved microtiter plate shaker or rotator for the immunological step. These can be purchased from most laboratory supply companies. If you do not have such a device it is possible to incubate the plate without shaking however it will be necessary to extend the first immunological incubation step by one hour in order to achieve comparable results to the 2 hour shaking protocol. **Do not shake during the 30 minute substrate incubation step as this may result in higher backgrounds and worse precision.**
- Bring all reagents to room temperature. Set-up plate spectrophotometer to read dual wavelength at 450nm for the test wavelength and ~650nm for the reference.
- Thorough washing is essential to proper performance of this assay. Automated plate washing systems or other vacuum aspiration devices are not recommended. The manual method described in the assay protocol is preferred for best precision, sensitivity and accuracy. A more detailed discussion of this procedure can be obtained from our Technical Services Department or on our web site. In addition, a video demonstration of proper plate washing technique is available in the 'Technical Help' section of our web site.
- All standards, controls, and samples should be assayed in duplicate.
- Maintain a repetitive timing sequence from well to well for all assay steps to insure that all incubation times are the same for each well.
- Make a work list for each assay to identify the location of each standard, control, and sample.
- If the substrate has a distinct blue color prior to the assay it may have been contaminated. If the absorbance of 100µL of substrate plus 100µL of stop against a water blank is greater than 0.1 it may be necessary to obtain new substrate or the sensitivity of the assay may be compromised.
- Strips should be read within 10 minutes after adding stop solution since color will fade over time.

Assay Protocol

1. Pipette 25µL of standards, controls and samples into wells indicated on work list.

2. Pipette 100µL of anti-Goat Milk:HRP (#F241) into each well.

3. Cover & incubate on rotator at 400-600rpm for 2 hours at room temperature, 24°C ± 4°C.

4. Dump contents of wells into waste. Blot and gently but firmly tap over absorbent paper to remove most of the residual liquid. Overly aggressive banging of the plate or use of vacuum aspiration devices in an attempt to remove all residual liquid is not necessary and may cause variable dissociation of antibody bound material resulting in lower ODs and worse precision. Fill wells generously to overflowing with diluted wash solution using a squirt bottle or by pipetting in ~350µL. Dump and tap again. Repeat for a total of 4 washes. Wipe off any liquid from the bottom outside of the microtiter wells as any residue can interfere in the reading step. Do not allow wash solution to remain in wells for longer than a few seconds. Do not allow wells to dry before adding substrate.

5. Pipette 100µL of TMB substrate (#F005).

6. Incubate at room temperature for 30 minutes. DO NOT SHAKE.

7. Pipette 100µL of Stop Solution (#F006).

8. Read absorbance at 450/650nm

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL milk protein equivs.
1A	0ng/mL	0.049	0.051	
1B	0ng/mL	0.052		
1C	0.5ng/mL	0.086	0.089	
1D	0.5ng/mL	0.092		
1E	1.5ng/mL	0.175	0.178	
1F	1.5ng/mL	0.180		
1G	4ng/mL	0.334	0.332	
1H	4ng/mL	0.329		
2A	10ng/mL	0.826	0.804	
2B	10ng/mL	0.781		
2C	25ng/mL	2.168	2.099	
2D	25ng/mL	2.030		
2E	Sample A	2.304	2.330	>25
2F	Sample A	2.356		
2G	Sample B	0.089	0.092	0.53
2H	Sample B	0.095		

Quality Control

- Precision on duplicate samples should yield average % coefficients of variation of less than 10% for samples greater than 2 ng/mL and < 25 ng/mL. CVs for samples < 2 ng/mL may be greater than 10%.
- It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL "total immuno-reactive milk protein equivalents" (See Limitations section above). This data reduction may be performed through computer methods using curve fitting routines such as point-to-point, spline, or polynomial methods. Data may also be manually reduced by plotting the absorbance values of the standard on the y-axis versus concentration on the x-axis and drawing a smooth point-to-point line. Absorbances of samples are then interpolated from this standard curve.

Performance Characteristics

Cygnus Technologies has validated this assay by conventional criteria as indicated below. This validation is generic in nature and is intended to supplement but not replace certain user and product specific qualification and validation that should be performed by each laboratory. At a minimum each laboratory is urged to perform spike and recovery and dilutional linearity studies in their sample types. Each laboratory and technician should also demonstrate competency in the assay by performing a precision study similar to that described below. A more detailed discussion of recommended user validation protocols can be obtained by contacting our Technical Services Department or at our web site.

Sensitivity

The lower limit of detection (**LOD**) is defined as that concentration corresponding to a signal two standard deviations above the mean of the zero standard. LOD is ~0.1 ng/mL.

The lower limit of quantitation (**LOQ**) is defined as the lowest concentration, where concentration coefficients of variation (CVs) are <20%. The LOQ is ~0.42 ng/mL.

Precision

Intra (n=20 replicates) precision was determined on 3 pools with low (~1.3ng/mL), medium (~3.1ng/mL), and high concentrations (~20ng/mL). Inter assay (n=6) CV's were determined on 2 control samples at 4 and 25ng/mL. The % CV is the standard deviation divided by the mean and multiplied by 100.

Pool	Intra-assay %CV	Inter-assay %CV
Low	12.1%	-
Medium	6.4%	7.2%
High	2.4%	5.4%

Specificity/Cross-Reactivity

In sandwich ELISA, cross reactivity can manifest itself either as a false increase in analyte levels (positive cross reactivity) or as a false decrease in analyte (negative cross reactivity) when analyte present in the sample competes with the cross reactant for the kit antibodies. Other materials that may not cross react in the true immunological sense may simulate cross reactivity due to non-specific interactions that can result in either a false increase or false decrease in the apparent analyte concentration. Because of the very defined nature of the samples proposed for testing in this assay, an extensive study into cross reactivity was not attempted. However, it is recommended that each user evaluate known materials in their sample matrices for cross reactivity or non-specific interferences by testing those materials with and without milk proteins spiked into them. "Purified" serum proteins and diluted sera from various species were evaluated for positive cross reactivity as indicated in the Table below. Only goat and sheep serum proteins showed significant cross reactivity. The very low cross reactivity for BSA, human serum albumin and bIgG may not be due to those proteins but rather to some contaminating impurity.

Cross-Reactivity		
Analyte	**Analyte concentration used for calculating cross-reactivity**	**% Cross-Reactivity (wt/wt)**
Bovine serum albumin	8µg/mL	0.026
Bovine IgG	16µg/mL	0.046
Bovine serum	700ng/mL	1.37
Goat IgG	100ng/mL	15.9
Goat serum	65ng/mL	22.5
Human serum albumin	1mg/mL	0.000019
Human IgG	1mg/mL	0
Mouse serum	60µg/mL	0
Sheep serum	125ng/mL	24.0

Hook Capacity

Increasing concentrations of milk proteins > 25 ng/mL were assayed as unknowns. The hook capacity, defined as that concentration which will give an absorbance reading less than the 25 ng/mL was ~200 µg/mL.

Ordering Information/ Customer Service

Cygnus Technologies also offers kits for the extraction of Host Cell DNA. The following kits are available:

- Residual Host Cell DNA extraction:

  Cat # D100W, DNA Extraction Kit in 96 deep well plate
  Cat # D100T, DNA Extraction Kit in microfuge tubes

To place an order or to obtain additional product information contact *Cygnus Technologies*:

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