

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 powerful 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 and in routine quality control of final product.

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, tetramethyl benzidine (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

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

Storage & Stability

- * All reagents should be stored at 2°C to 8°C for stability until the expiration date printed.
- * The substrate reagent should not be used if its stopped absorbance at 450nm is greater than 0.1.
- * 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.)
Microtiter plate washing system
Pipettors - 25µL and 100µL
Repeating or multichannel pipettor - 100µL
Microtiter plate rotator (150 - 200 rpm)
Distilled water
1 liter container for wash solution storage

Precautions

For research or manufacturing use only.

Stop reagent is 0.5N H₂SO₄. Avoid contact with eyes, skin, and clothing. At the concentrations used in this kit none of the other reagents are believed to be harmful.

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.* 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, and 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 minus a

substrate blank 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. 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 (NaN₃) 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. 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.

* 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.

Calculation of Results

The standards may be used to construct a standard curve with values reported in ng/mL “total immunoreactive 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.

Assay Protocol

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

2. Pipette 100µL of anti-goat milk:HRP into each well.

3. Cover, transfer to rotator, and incubate for 2 hours at 180rpm, at room temperature, 24°C ± 4°.

4. Aspirate, wash with 300µL of diluted wash solution and aspirate. Wash a total of 4 times.

5. Pipette 100µL of substrate.

6. Incubate for 30 minutes. Do Not Shake on rotator for this step.

7. Pipette 100µL of stop solution.

8. Read absorbance at 450/650nm blanking on the Zero standard.

Example Data

Well #	Contents	Abs. at 450nm	Mean Abs.	ng/mL milk protein equivs.
1A	Zero Std	0.049		
1B	Zero Std	0.052	0.051	
1C	0.5ng/mL	0.086		
1D	0.5ng/mL	0.092	0.089	
1E	1.5ng/mL	0.175		
1F	1.5ng/mL	0.180	0.178	
1G	4ng/mL	0.334		
1H	4ng/mL	0.329	0.331	
2A	10ng/mL	0.826		
2B	10ng/mL	0.781	0.804	
2C	25ng/mL	2.168		
2D	25ng/mL	2.030	2.099	
2E	sample A	2.304		
2F	sample A	2.356	2.330	>25
2G	sample B	0.089		
2H	sample B	0.095	0.092	0.53

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%.

- For optimal performance the absorbance of the substrate when blanked against water should be < 0.1.

- It is recommended that each laboratory assay appropriate quality control samples in each run to insure that all reagents and procedures are correct.

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 validation which should be performed by each laboratory.

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	8 µg/mL	0.026
Bovine IgG	16 µg/mL	0.046
Bovine serum	700 ng/mL	1.37
Goat IgG	100ng/mL	15.9
Goat serum	65 ng/mL	22.5
Human serum albumin	1 mg/mL	0.000019
Human IgG	1 mg/mL	0
Mouse serum	60 µg/mL	0
Sheep serum	125 ng/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 standard was ~200 µg/mL.

Ordering Information/ Customer Service

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

Tel: 910-454-9442 Fax: 910-454-9443

Email: cygnustec@aol.com

Website: www.cygnustechnologies.com

4701 Southport Supply Rd. SE, Suite 7
Southport, NC 28461 USA