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Monoclonal antibody-based immunoassay for detection of prohibited ruminant proteins in feed

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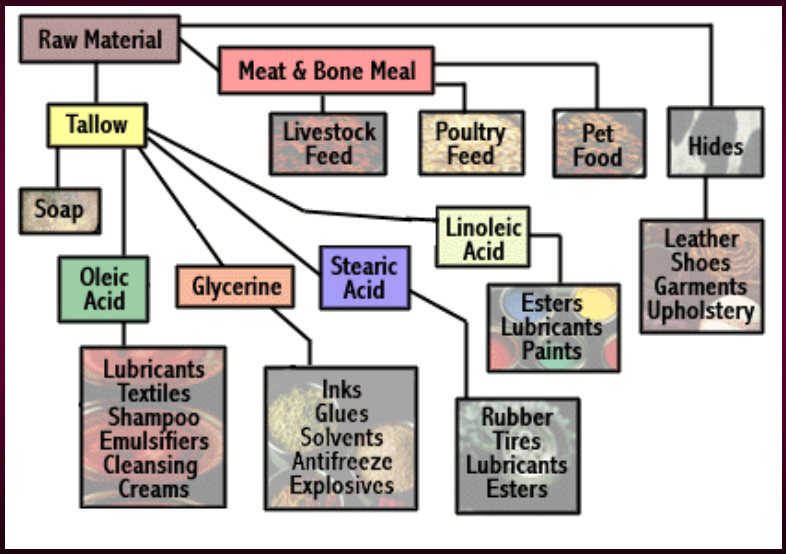
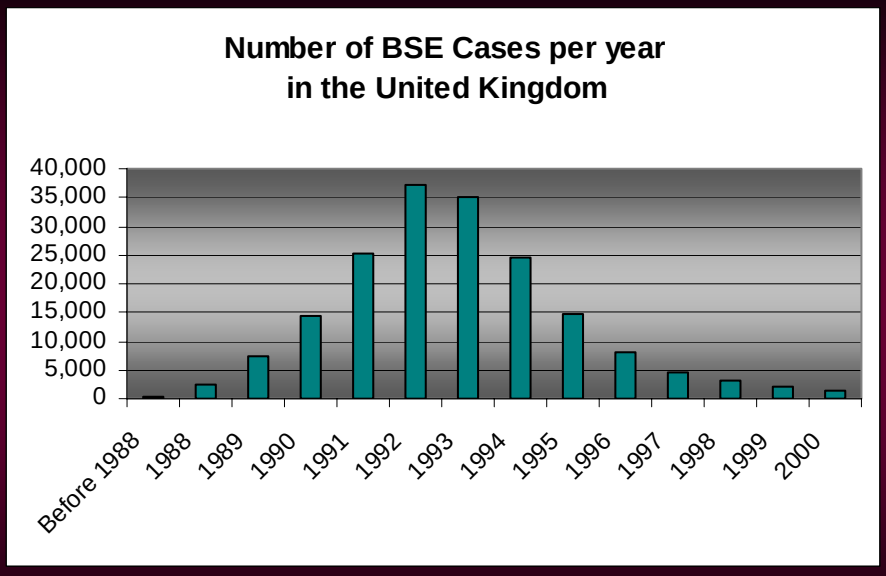
Bovine Spongiform Encephalopathy (BSE)

- Commonly known as "Mad Cow Disease"
- Originated in sheep "scrapie"
- First diagnosed in the UK in 1986
- Caused by a transmissible agent, a tiny heat-resistant protein called a prion
- Long incubation period (2-8 years) for BSE development
- New variant Creutzfeldt-Jakob Disease (nvCJD) in human

By-Products of Meat Industry





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Documented BSE Cases Worldwide

Documented Cases in Europe		Outside of Europe	
United Kingdom	181,368**	Japan	3
Ireland	811	Africa	0
Portugal	597	Australia	0
France	499	New Zealand	0
Switzerland	403	South America	0
Germany	136	United States	0
Spain	84	Canada	1
Belgium	65		
Italy	44		
Netherlands	25		
Denmark	8		
Slovakia	4		
Liechtenstein	2		
Czech Republic	2		
Luxembourg	1		
Greece	1		
Slovenia	1		
Finland	1		
Austria	1		

Source: Office of International Epizootics, January 3, 2002

Food safety is the threshold issue for consumers

nvCJD in humans is believed to be associated with consumption of meat from animals infected with BSE

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Effective controls on animal feed are the key to eradication of BSE

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Current regulations on the use of rendered animal protein in feeds

- UK - prohibits the use of mammalian MBM to feed any farmed livestock (Statutory Instrument, 1996)
- EU - bans the use of processed animal protein to feed food animals (European Commission, 2001)
- US - bans the use of protein derived from ruminant tissue to feed ruminant animals (FDA, 1997)
- US – proposed expansion of feed ban (FDA, 2001)

Analytical methods to detect prohibited materials in feeds are required for successful enforcement of these regulations.

Allowed ruminant animal proteins

21 CFR 589.2000

- Blood and blood products
- Milk and milk products
- Plate waste

DNA-based techniques can not distinguish between different tissues/materials of the same species

Rendering industry non ruminant by-product

- Yields 1.5 mil tons/year Poultry meal.
- Yields 0.6 mil tons/year Feather meal.
- Yields 0.8 mil tons/year Porcine meal.
- Yields 0.1 mil tons/year Blood meal.

Source: Sparks study for NRA, June 2001

Commercial immunoassay tests for meat species identification

- Immunodiffution tests
  - Only for raw meat samples
  - Require overnight incubation
  - Variable sensitivity
  - Interference
- ELISA kits
  - Separate kits for raw and cooked meats
  - Not valid for rendered meat
  - Cost ~$ 450 per 96-well microplate
  - Qualitative result

Objectives

- **To develop rapid and reliable immunoassay for detection of ruminant protein content in feedstuffs**
  - Identify and purify a thermal-stable muscle proteins (antigens) as the species marker
  - Produce monoclonal antibodies (Mabs) specific to the thermal-stable marker protein as the recognizing reagent
  - Develop a MAb-based sandwich ELISA for detection of the target ruminant protein

A thermal-stable muscle protein, troponin I, (antigen) as the species marker

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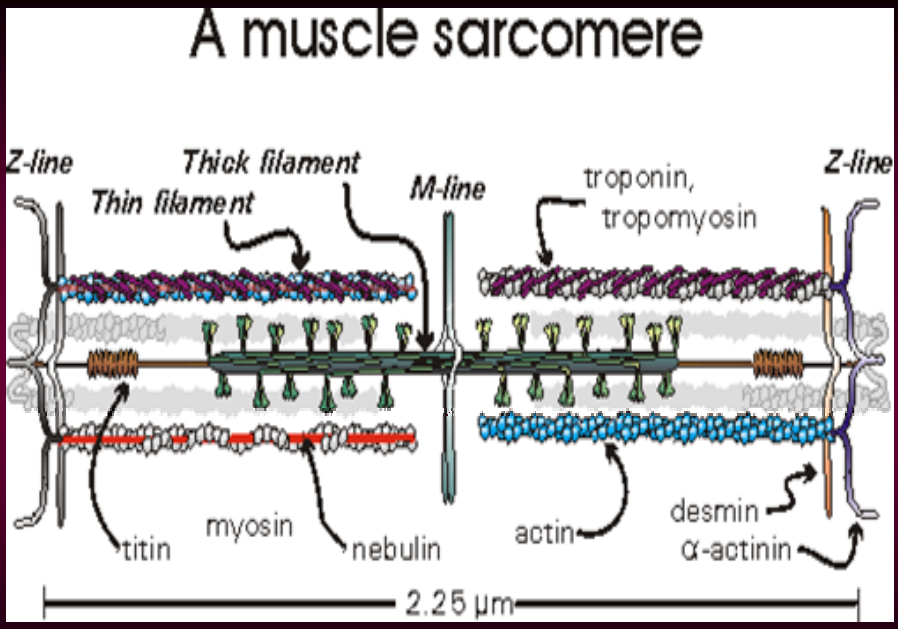

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Thin Filament

Actin

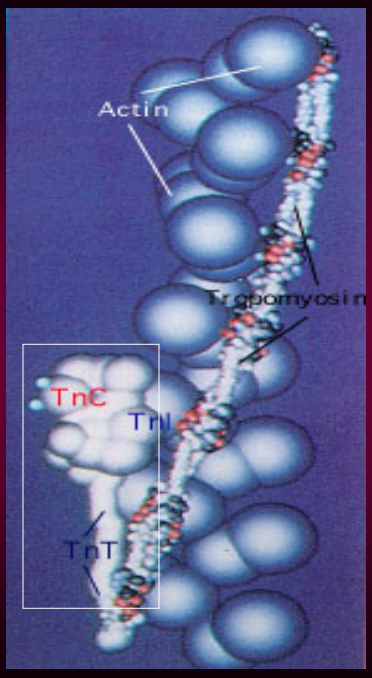
Tropomyosin

Troponin

Tn C

Tn I

Tn T



Purification of Troponin I

Skeletal Muscle

0.4 M LiCl ↓

Troponin Complex

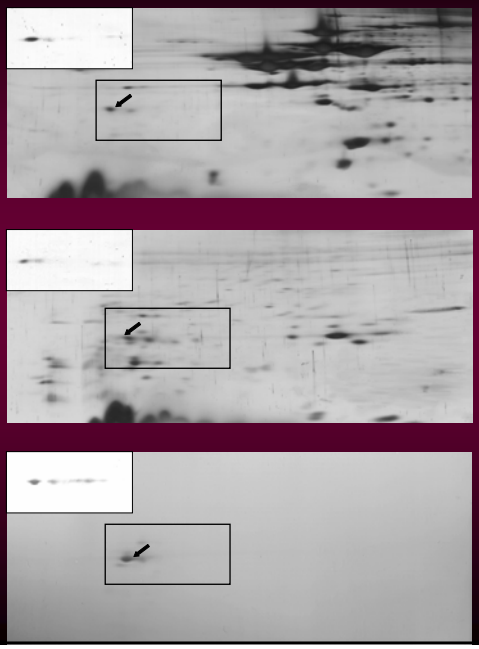
DEAE-Cellulose Chromatography ↓ ↓

Troponin I & T | Troponin C

CM-Cellulose Chromatography ↓ ↓

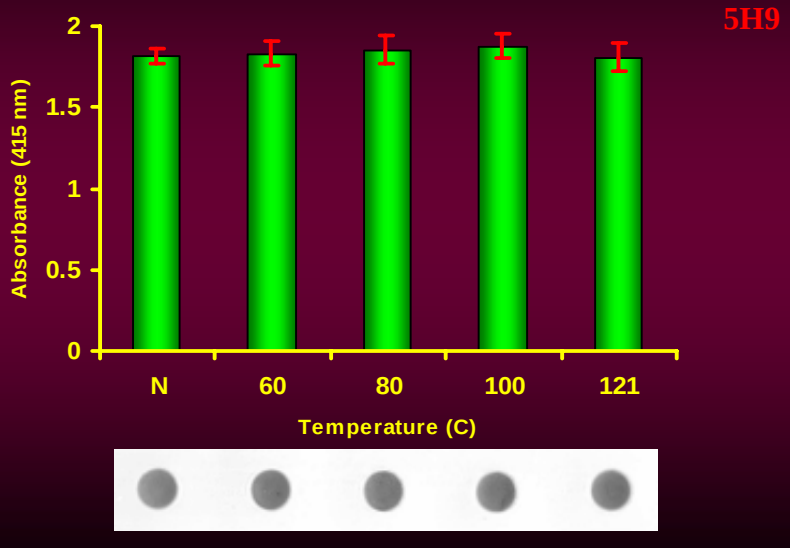
Troponin I | Troponin T

Specificity of MAbs to TnI by 2-D gel electrophoresis



Thermal stability of TnI

ELISA and dot blot of heat-treated TnI



Development of monoclonal antibodies against TnI

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Why monoclonal antibodies?

- Defined biological properties
- Consistent specificity
- Continuous supply of uniform reagent
- Reduce the cost of the assay

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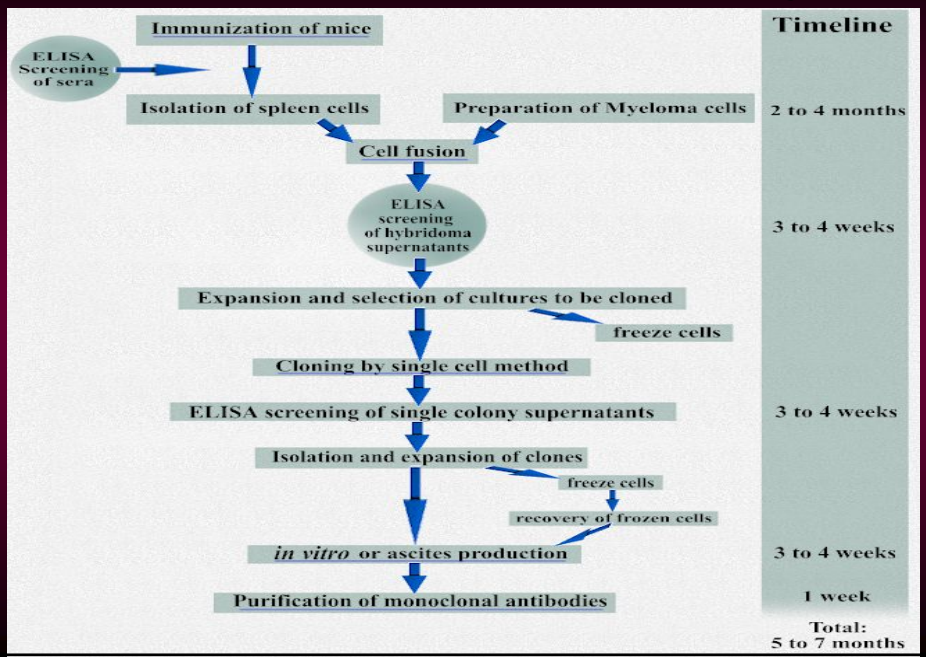
Production of monoclonal antibody



Mab	Isotype	Specificity
2G3	IgG1	All Animal Species
2F8	IgG2b	Mammalian
3E12	IgG1	Poultry
2A8	IgG1	Ruminant
5D2	IgG1	Chicken &Turkey
9C6	IgG1	Chicken
5H9	IgG1	Pork
3E3	IgG1	Horse

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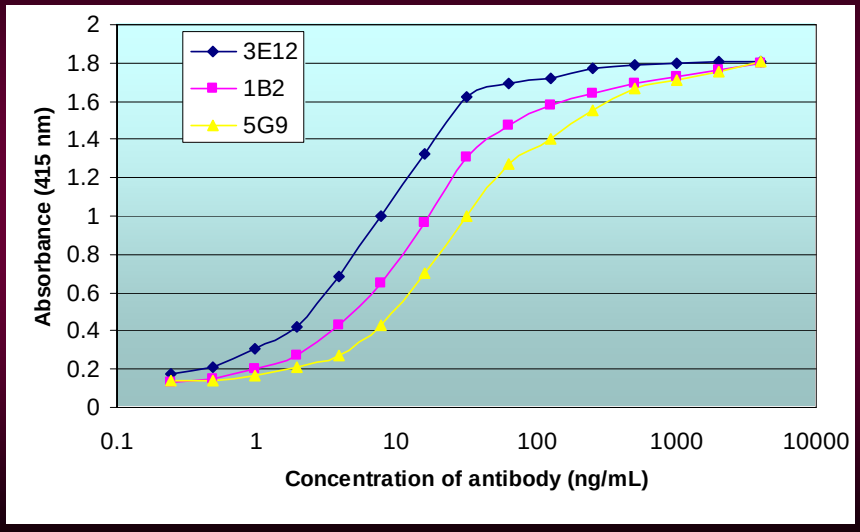
Characteristics of anti-TnI MAbs

Monoclonal Antibody	Class	Species Specificity
3E12	IgG	Beef, Sheep, Deer
1B2	IgG	Beef, Sheep
5G9	IgG	Beef, Sheep

Titration curves of purified MAbs against bovine TnI by indirect ELISA



Mab-based Enzyme-linked ImmunoSornt Assay

Requires a pair of Mabs that bind to the different sites on the target molecule

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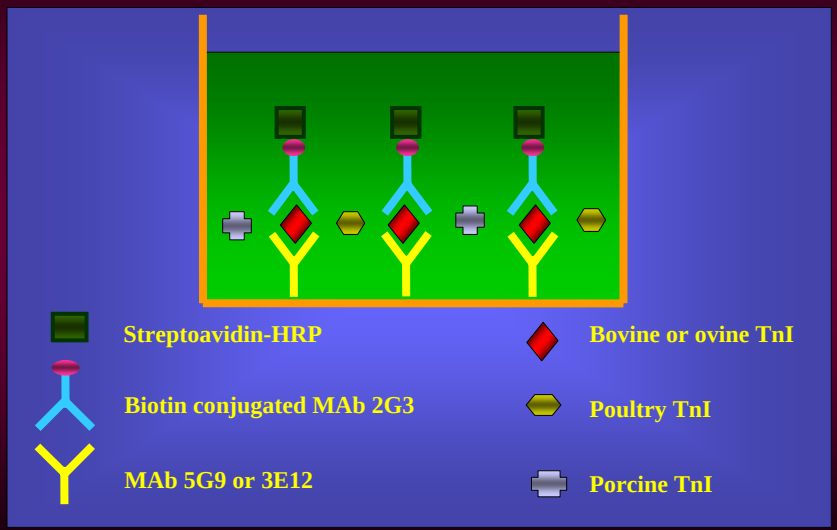
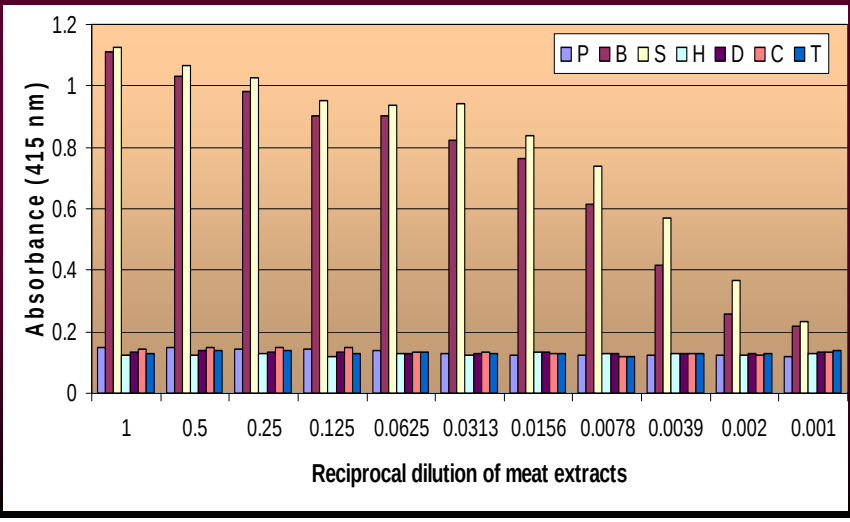
Illustration of monoclonal antibody sandwich ELISA



Specific reactivity of Sandwich ELISA to meat meals produced from heated muscles (132 °C/ 2 bar, 2 hr). MAb 5G9: capture antibody, Biotin labeled MAb 2G3: detecting antibody.
P=pork; B=beef, S=sheep; H=horse, D=deer, C=chicken; T=turkey



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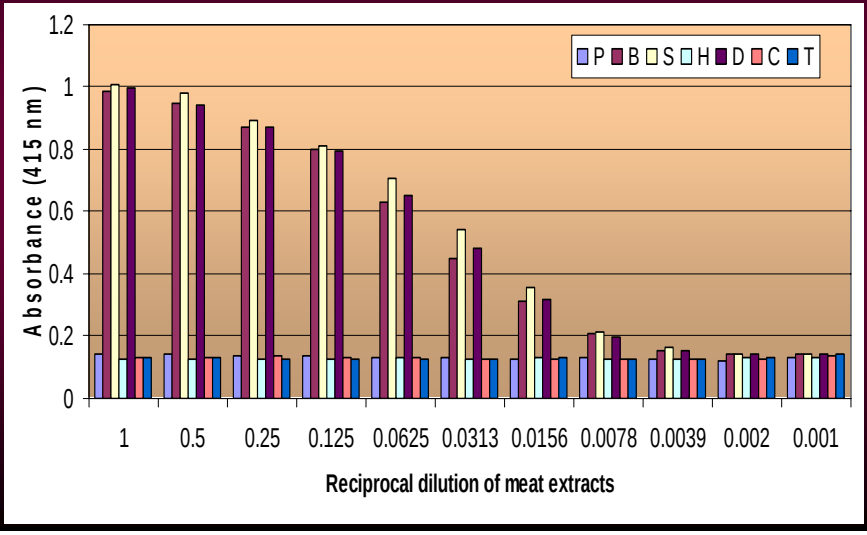
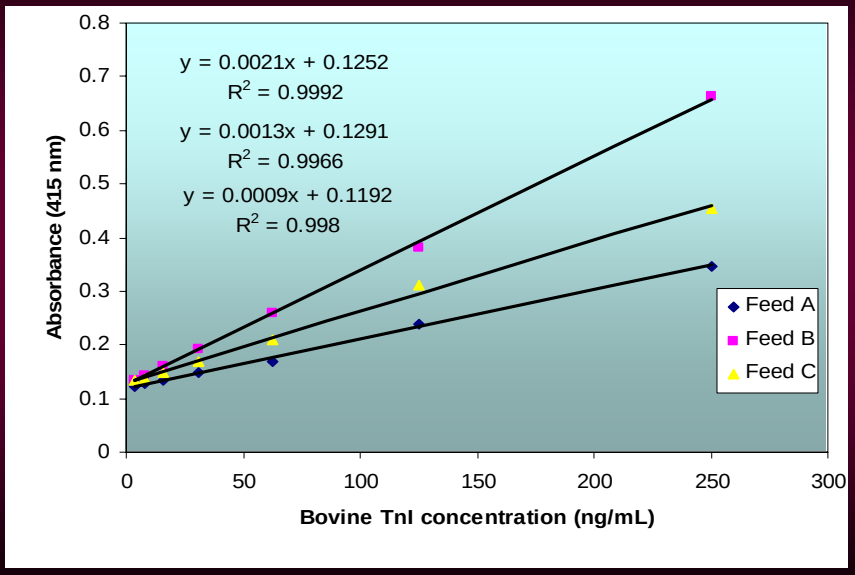
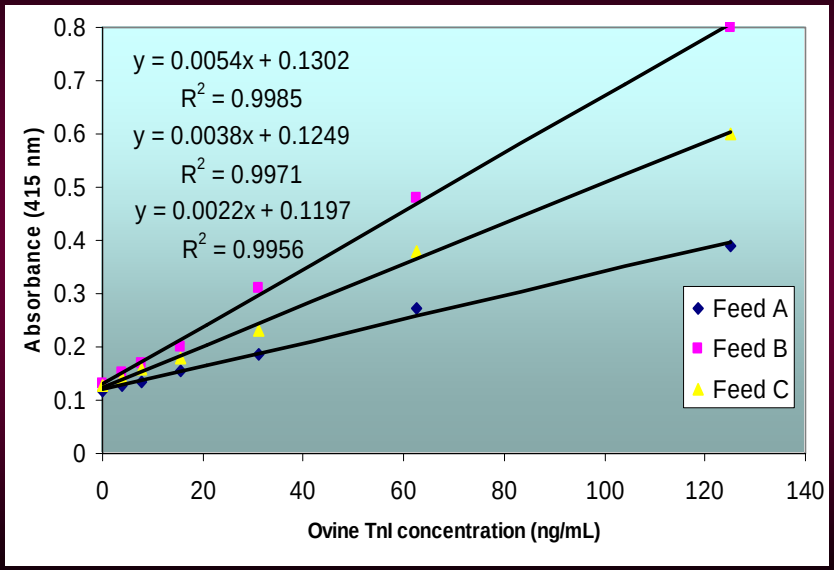


Calibration curves of bovine TnI in different feed matrices



Calibration curves of ovine TnI in different feed matrices



Quantitative detection of 5, 0.5, and 0.05% (w/w) of heat-treated (132°C, 2 h) beef and sheep meat meals in three different feeds with coexistence of 10% pork, chicken, or turkey meat meal.

	5% Beef	0.5% Beef	0.05% Beef
Pork/Feed A	652 (18)	58.3 (2.3)	5.4(3.5)
Pork/Feed B	638 (24)	62.8 (1.3)	5.0 (1.0)
Pork/Feed C	621 (21)	65.8 (4.6)	5.7 (3.2)
Chicken/Feed A	686 (19)	64.6 (1.7)	5.3 (2.3)
Chicken/Feed B	714 (43)	67.8 (1.4)	6.0 (0.3)
Chicken/Feed C	635 (10)	68.6 (1.2)	7.5 (1.9)
Turkey/Feed A	718 (33)	71.4 (2.4)	5.6 (2.9)
Turkey/Feed B	681 (15)	71.6 (0.3)	7.6 (2.7)
Turkey/Feed C	707 (19)	69.2 (2.2)	7.3 (2.2)

Results are expressed as ng/mL of TnI in sample extract. Values represent the mean (standard deviation) of three repeat measurements

Quantitative detection of 5, 0.5, and 0.05% (w/w) of heat-treated (132°C, 2 h) beef and sheep meat meals in three different feeds with coexistence of 10% pork, chicken, or turkey meat

	5% Sheep	0.5% Sheep	0.05% Sheep
Pork/Feed A	534 (23)	57.6 (2.5)	6.3 (0.7)
Pork/Feed B	565 (19)	52.9 (4.0)	5.2 (0.6)
Pork/Feed C	592 (22)	59.5 (3.6)	7.0 (0.9)
Chicken/Feed A	587 (43)	57.9 (3.2)	5.5 (0.8)
Chicken/Feed B	608 (37)	56.8 (3.1)	5.1 (0.2)
Chicken/Feed C	559 (30)	61.5 (4.6)	6.9 (0.3)
Turkey/Feed A	598 (47)	61.0 (1.3)	7.7 (1.3)
Turkey/Feed B	607 (24)	59.1 (3.3)	6.9 (0.8)
Turkey/Feed C	613 (26)	61.5 (2.0)	7.5 (0.4)

Results are expressed as ng/mL of TnI in sample extract. Values represent the mean (standard deviation) of three repeat measurements

Unique features of the Mabs-based sandwich ELISA

- Monoclonal antibodies
  - Ensuring a continuous supply of a homogeneous and biologically defined immunoreagent
- Simple sample extraction
- Simple qualitative assay procedure
- Specific to ruminant muscle protein (not blood or milk)
- Quantitative assay can be achieved by using a calibration curve.
- Low detection limit (<0.05% w/w or 4 ng TnI/ml)
- Same assay for raw, cooked and severely heat-treated samples

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MELISA-TEK™

Microwells Ruminant Kit

ELISA Technologies Co.

AgriScreen Ruminant MBM Statistics

Test for MBM in Animal Meals

- Sensitivity 97.6%
- Specificity 99.5%
- 170 Positive, 187 Negative
- n = 357

AgriScreen Ruminant FEED Statistics

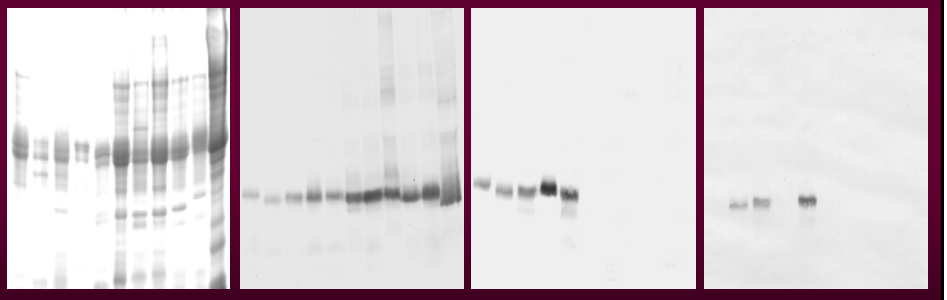
Test for MBM in Animal Feeds

- Sensitivity 98.7%
- Specificity 99.5%
- 308 Positive, 218 Negative
- n = 526
- Submitted for AOAC Validation

Formats of immunoassay

Detection of heat-treated muscles from different animals by MAbs to TnI

SDS-PAGE MAb 7F7 MAb 7A12 MAb 2A8



P B S H D C T K G O F P B S H D C T K G O F P B S H D C T K G O F P B S H D C T K G O F

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Development of mammalian-specific and animal-specific assays in our laboratory are in progress

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