DEPARTMENT FOR ENVIRONMENT, FOOD AND RURAL AFFAIRS

STANDARD OPERATING PROCEDURE (SOP) FA0106 v1.0

Version 1.0, July 2012

STANDARD OPERATING PROCEDURE FOR THE EXTRACTION OF A HEART MARKER PROTEIN IN RAW AND PROCESSED MEAT PRODUCTS, AND ITS SUBSEQUENT DETECTION AND QUANTIFICATION BY AN ELISA

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Approved by Authenticity Methods Working Group **Date** July 2012

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1. HISTORY/BACKGROUND

1.1 Background

The definition of meat for labelling purposes is restricted to skeletal muscle with naturally adherent fatty and connective tissue (The Food Labelling (Amendment), (England) Regulations, 2003) and parallel legislation in Scotland, Wales and Northern Ireland). These regulations state that other types of muscle, such as heart, tongue, etc., are excluded from this definition. These regulations also state that certain parts of the carcass, such as liver, kidney, lung and heart, have to be explicitly labelled as such. The generic term ‘offal’ is not permitted. Thus there is a need for robust methods for the detection and quantification of various offals in raw and processed foods. This SOP was prepared as part of Food Authenticity Programme project (FA0106) entitled ‘Transfer of offal detection assay to an ELISA platform’.

2. PURPOSE

To extract a heart marker protein from meat products followed by detection and quantification of the heart-specific protein by ELISA.

3. SCOPE

To perform ELISA assays on extracts of meat products using anti-cardiac troponin I (cTnI antibodies, in order to detect and quantify added bovine/ovine/porcine heart. The SOP can be used to analyse raw and cooked meat products.

4. DEFINITIONS AND ABBREVIATIONS

cTnI	Cardiac specific troponin I
HRP	Horseradish peroxidase
PBS	Phosphate buffered saline
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate

5. OVERVIEW OF THE PROCEDURE

The overall procedure involves the extraction of a heart marker protein from meat samples (raw or processed) in an optimal extraction buffer, prior to detection and quantification of the heart marker protein by ELISA.

6. MATERIALS AND EQUIPMENT

6.1 Chemicals

A number of these chemicals are toxic and/or carcinogenic. Use appropriate precautions and good laboratory practice (GLP) when handling (refer to Appendix 1).

Name	Formula	Molecular weight	Supplier	Product code	Grade	Special Storage	Risk phrases*
di-sodium hydrogen orthophosphate (anhydrous)	Na_2HPO_4	141.96	Fisher	S/4480/53	>99%		36/37/38
Marvel milk powder			Premier Brands UK Ltd				
Potassium chloride	KCl	74.55	Merck	101983K	99.5%		36
Potassium dihydrogen orthophosphate	KH_2PO_4	136.09	Merck	296084J	>99%		
Sodium chloride	NaCl	58.4	Melford	S0520	>99%		36/37/38
Sulphuric acid	H_2SO_4	98.07	Fisher	S/9160/PB17			23/24/25, 35, 36/37/38, 41, 48, 49
Tween®20	$C_{58}H_{114}O_{26}$	~1228	Sigma	274348	98.4%		
Urea	CH_4N_2O	60.06	Fisher	BP169-500	>99%		36/37/38

* Risk phrases: for definitions see Appendix 1

- Mouse monoclonal antibody [4C2] to cTnI supplied by Abcam, catalogue number ab10231.
- Goat anti-mouse immunoglobulins labelled with horseradish peroxidase (HRP) supplied by DakoCytomation, catalogue number P0447.
- Detergent e.g. Decon 90 supplied by SLS, catalogue number CLE1022.

6.2 Water

De-ionised water produced by a reverse osmosis system with a typical resistivity of 18.2 MΩ/cm is used.

6.3 Solutions

All the following solutions are produced in house and stored for 4 weeks at 4°C, unless otherwise stated.

- Phosphate buffered saline (PBS) [137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 (anhydrous) 1.47 mM, KH_2PO_4].

- PBS-Tween: PBS containing 0.05% (v/v) Tween-20.

- Blocking solution: PBS containing 3% (w/v) Marvel milk powder (prepare prior to use and store at 4°C for up to 24 hours).

- Primary antibody [diluted in blocking solution, 1:1000] (prepare prior to use).

- Horseradish peroxidase conjugated secondary antibody [diluted in blocking solution, 1:1000] (prepare prior to use).

- Phosphate buffered saline containing 8 M urea [137 mM NaCl, 2.68 mM KCl, 8.1 mM Na_2HPO_4 (anhydrous) 1.47 mM, KH_2PO_4, 8 M urea].

6.3.1 Meat and offals

Appropriate offals and minced meat purchased from butcher for use in the production of standards.

6.4 Commercial kits

- Bio-Rad Protein Assay (Bio-Rad code 500-0006)

- 1-Step™ Ultra TMB-ELISA (Thermo Scientific 34028)

6.5 Plasticware

Pipette tips (0.1 – 10 µl)	Fisher code FB34521
Pipette tips (yellow 200 µl)	Sarstedt code 70.760.002
Pipette tips (blue 1000 µl)	Sarstedt code 70.762
Eppendorf tubes (500 µl)	Sarstedt code 72.699
Eppendorf tubes (1.5 ml)	Sarstedt code 72.690
50 ml tubes (red topped)	Sarstedt code 62.547.004
25 ml tubes (yellow topped)	Sarstedt code 63.9922.254
8 ml flat base tube	Sarstedt code 63.542.709
25 ml graduated pipettes	Sarstedt code 86.1685.001
10 ml graduated pipettes	Sarstedt code 86.1254.001
5 ml graduated pipettes	Sarstedt code 86.1253.001
Eppendorf CombiTips® Plus 1ml	SLS code E0030069234
NUNC Maxisorp clear flat bottom plates	SLS code 442404

6.6 Equipment

Liquidiser (1 l) with coffee mill attachment	- Kenwood BL300T
Heating oven	- LTE Scientific Ltd. OP150-MF
MP FastPrep®-24	- MP Biomedicals

SiLi Type ZY beads (1.4 – 1.6 mm)	-Sigmund Lindner UK product code 9615-41
End-over-end tumbler	- e.g. Stuart rotator SB2
Centrifuge	- Eppendorf 5810R
Centrifuge for Eppendorf tubes	- Hettich Mikro 22R
ELISA plate washer	- Biochrom ASYS Atlantis 4 plate washer (product number G021102002)
ELISA plate reader	- Bio-Rad model 680 microplate reader
Orbital shaker	- e.g. Stuart SSL1
Magnetic stirrer and stirring bar	
Vortex mixer	

Gilson Micropipettes
Multichannel pipette
Eppendorf Multipipette® Plus
Disposable weighing boats (44 x 44 mm, 80 x 80 mm and 140 x 140 mm)
Eppendorf tube rack
Scissors
Disposable nitrile gloves
Sterile cork board
Scalpels
Spatula
Aluminium foil
Cling film
Absorbent paper
Timer

7. PROCEDURE

The procedure is divided into 3 sections:

7.1 Preparation of skeletal muscle and heart mixtures for use as standards
7.2 Extraction of standards and unknown meat products
7.3 Detection and quantification of heart by ELISA

7.1 Preparation of skeletal muscle and heart mixtures for use as standards

Suitable standards need to be prepared to enable the analysis of unknown meat products/samples with cTnI (heart). This is achieved using skeletal muscle samples spiked with known amounts of heart.

7.1.1 Obtain skeletal muscle (minced beef, lamb and pork) and heart (beef, lamb and pork) from a butcher. Homogenise sufficient heart for the preparation of a range of standards in the coffee mill attachment of the Kenwood blender for 30 seconds**.**

7.1.2 Prepare "meatballs" containing known amounts of heart (bovine/ovine/porcine). Generally the meat used is of the same species as the offal. See table for example of beef heart.

Weight beef meat (g)	**Weight beef heart (g)**	**% heart in standard**
42.5	7.5	15
45.0	5.0	10
47.5	2.5	5
49.0	1.0	2
50.0	0.0	0

7.1.3 The standard mixtures of minced meat and homogenised heart are prepared according to the table. Using the coffee mill attachment of the Kenwood blender, homogenise again in three bursts of 10 seconds to ensure complete mixing and to produce a homogeneous sample.

7.1.4 Transfer the "meatball" to a square of aluminium foil, wrap it securely and cook in a pre-heated oven at 180°C for 30 minutes.

7.1.5 If the unknown meat product is raw, homogenise in three bursts of 10 seconds to ensure complete mixing and to produce a homogeneous sample using the coffee mill attachment of the Kenwood blender. Take a 50 g representative sample, form into a "meatball" and transfer to a square of aluminium foil, wrap it securely and cook in a pre-heated oven at 180°C for 30 minutes.

7.1.6 If the unknown meat product is already cooked, homogenise (3 x 10 seconds) the whole product if it weighs less than 50 g (e.g. a whole burger) or homogenise a 50 g representative sample.

7.2 Extraction of standards and unknown meat products

7.2.1 Allow all cooked standards and unknown meat products to cool for 1 hour at 20°C.

7.2.2 Take three 5 g representative samples from the unknown cooked meatballs/processed meat samples and place each in a 50 ml tube with 20 g of SiLi Type ZY beads and 30 ml PBS 8 M urea and extracted (sections 7.2.3 to 7.2.5) using the MP FastPrep® 50 ml rotor.

7.2.3 The MP FastPrep® is used according to the manufacturer's instructions. The homogeniser is set to 4 m/s for 30 seconds.

7.2.4 Tumble the tubes end-over-end for 16 hours at 20°C.

7.2.5 Centrifuge tubes at 1,800 x g for 15 minutes. Harvest the supernatants and store in aliquots at -20°C.

7.3 Detection and quantification of heart by ELISA

7.3.1 Coat wells of the ELISA plates with antigen diluted 1:40 in PBS (50 µl/well). Cover with plate film and incubate at 37°C for 1 hour.

7.3.2 Remove the plate film and using the Biochrom ASYS Altantis 4 plate washer aspirate the antigen and wash the wells twice with PBS followed by 2 washes with distilled water.

7.3.3 Dispense 200 µl blocking solution into each well using a multichannel pipette, cover with film and incubate for 2 hours at 20°C on an orbital shaker set at 40 rpm to block excess protein binding sites.

7.3.4 Remove the plate film and using the Biochrom ASYS Altantis 4 plate washer aspirate the antigen and wash the wells twice with PBS followed by 2 washes with distilled water.

7.3.5 Add 50 µl primary antibody solution to each well, cover the plate and incubate overnight at 4°C.

7.3.6 Remove the plate film and using the Biochrom ASYS Altantis 4 plate washer aspirate the antigen and wash the wells twice with PBS-Tween followed by 2 washes with distilled water.

7.3.7 Add 50 µl secondary antibody solution to each well, cover the plate and incubate for 2 hours at 20°C whilst shaking on an orbital shaker set at 40 rpm.

7.3.8 Remove the plate film and using the Biochrom ASYS Altantis 4 plate washer aspirate the antigen and wash the wells twice with PBS-Tween followed by 2 washes with distilled water.

7.3.9 Add 100 µl of 1-Step™ Ultra-TMB substrate into each well and start timing the reaction when the solution is added to the first well. A blue colour will develop.

7.3.10 When the blue colour is dark enough, stop the reaction with 50 µl /well 2.5 M H_2SO_4.

7.3.11 Read the absorbance values at 450 nm.

7.3.12 Using a data handling software (e.g. Excel), arrange the results for each plate so that the standards and samples are labelled. Prepare a standard plot by subtracting the 0% beef heart signal from the other standards and unknowns.

7.3.13 For non-linear data use a polynomial fit; produce a standard plot using GraphPad Prism software or similar curve fitting software, and calculate the amount of cTnI in the unknown samples. Use values for the unknown sample that fall within the range of the standards.

7.3.14 Calculate the percentage heart in the unknown sample and take the mean value for the 3 replicates.

8. APPENDIX

Risk phrases are a system of codes and phrases for labeling hazardous chemicals and compounds.

R23/24/25	Toxic by inhalation, in contact with skin and if swallowed
R35	Causes severe burns
R36	Irritating to eyes
R37	Irritating to respiratory system
R36/37/38	Irritation to eyes, respiratory system and skin
R41	Risk of serious damage to eyes
R48	Danger of serious damage to health by prolonged exposure
R49	May cause cancer by inhalation