Standard Operating Procedure

Detection of Chloramphenicol Residues in Shrimp / Fish muscle and Meat Using ELISA Technique

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1. INTRODUCTION

Chloramphenicol (CAP) is a broad spectrum antibiotic, and it causes several adverse effects in humans including fatal idiosyncratic reactions and aplastic anaemia. Therefore, CAP is banned to be used in food producing animals.

2. SCOPE

This SOP describes the procedures of an Enzyme Immuno Assay to screen CAP in shrimp / fish muscle and meat.

3. OBJECTIVE

To Screen shrimp, fish and meat samples for chloramphenicol residues

4. DEFINITIONS AND ABBREVIATIONS

4.1 TMB: Tetramethylbenzidine

4.2 CAP: Chloramphenicol

4.3 ELISA:Enzyme-Linked Immunosorbent Assay

4.4 ppb : parts per billion

5. PRINCIPLE OF THE METHOD

The microtiter based ELISA kit consists of 96 wells precoated with sheep antibodies to rabbit IgG. A specific antibody (rabbit anti-CAP), enzyme labelled CAP (enzyme conjugate) and CAP standard or sample are added to the precoated wells followed by a single incubation step. The specific antibodies are bound by the immobilised antibodies and at the same time free CAP (present in the standard solution or sample) and enzyme conjugated CAP compete for the CAP antibody binding sites

(competitive enzyme immunoassay). After an incubation time of 30 minutes, the non-bound (enzyme labelled) reagents are removed in a washing step. The amount of CAP enzyme conjugate is visualised by the addition of a chromogen substrate (tetramethylbenzidine, TMB). Bound enzyme conjugate transforms the colourless chromogen into a coloured product. The substrate reaction is stopped by the addition of sulphuric acid. The colour intensity is measured photometrically at 450 nm. The optical density is inversely proportional to the CAP concentration in the sample.

6. SAFETY CONSIDERATIONS

Wear disposable latex gloves before handling samples and while pipetting the reagents and CAP standards. Once handling is over wash hands thoroughly with soap and water. The stop solution contains 0.5 M sulphuric acid; do not allow the reagent to get into contact with the skin and eyes. Do not pipette by mouth. Do not eat, drink, smoke, store or prepare foods, or apply cosmetics within the designated work area. TMB is toxic by inhalation, in contact with skin and if swallowed; observe care when handling the substrate

7. PRECAUTIONS

All the kits should be stored at 2–8 °C in a dark place. Do not use components past expiration date and do not intermix components from different serial lots. Each well is ultimately used as an optical cuvette. Therefore, do not touch the outside or inside bottom of the wells, and prevent damage and dirt. All components should be completely dissolved before use. Take special attention to the substrate, which crystallises at 4°C. Optimal results will be obtained by strict adherence to this protocol. Careful pipetting and washing throughout this procedure are necessary to maintain precision and accuracy.

8. MATERIALS AND EQUIPMENT

8.1 Chemicals

8.1.1 Ethyl acetate ($C_4H_8O_2$)

8.1.2 Nitrogen gas (N_2)

8.1.3 Iso-octane (C_8H_{18})

8.1.4 Trichloromethane ($CHCl_3$)

8.1.5 n-hexane (C_6H_{14})

8.1.6 CAP FAST ELISA 5091CAPF kit (EuroProxima)

8.1.6.1 Sample Dilution Buffer

8.1.6.2 Rinsing Buffer

8.1.6.3 Substrate Solution

8.1.6.4 Stop Solution

8.1.6.5 Conjugate solution

8.1.6.6 Antibody solution

8.1.6.7 CAP Standards (0.04, 0.1, 0.2, 0.4, 1.0, and 4 ng/ml)

8.1.6.8 Reconstitution/ zero standard buffer

8.1.6.9 Microtiter plate

8.2 Water

8.2.1 Distilled-deionized / nanopure water

8.3 Apparatus

8.3.1 Plastic trays

8.3.2 Glass test tubes

8.3.3 Pipette tips

8.3.4 Centrifuge tubes, 15 ml, 50 ml

8.3.5 Microcentrifuge tubes, 2 ml

8.3.6 ELISA wash bottle

8.3.7 Para Film

8.3.8 Universal Bottles

8.3.9 Duran Bottle, 100 ml / 200 ml / 500 ml / 1000 ml

8.4 Equipment

8.4.1. ELISA reader (Thermo scientific)

8.4.2. Tissue homogenizer (Ultra Turrex T 25 Basic)

8.4.3. Micropipettes (Gilson / Eppendorf)

8.4.4. Laboratory Centrifuge (Centurion Scientific Ltd. / HERMLE Z 160 M)

8.4.5. .Vortex mixer (Velp)

8.4.6. .Multidispenser

8.4.7. Analytical balance (Range 0.0000 g – 0.1000 g, Last digit 0.0001g, Sartorius)

8.4.8. Analytical balance (Range 0.0000 g- 15.0000 g, Last digit 0.0001g, OHAUS)

8.4.9. Refrigerator

8.4.10. Water bath

8.4.11. Test tube rotator (GFL)

8.4.12. Dri-block heater (Techne, DB.3A)

8.4.13. Glass pipettes

9. REAGENTS AND SOLUTIONS

9.1 Rinsing Buffer

Dilute 2 ml of concentrated rinsing buffer (8.1.6.2) with 38ml of water (8.2.1).

Prepare buffer freshly before used.

9.2 Sample Dilution Buffer

Dilute 3 ml of sample dilution buffer (8.1.6.1) in 9 ml of water (8.2.1). This is adequate for about 10 samples.

9.3 Conjugate Solution

Reconstitute the vial of lyophilised conjugate (CAP-HRPO) (8.1.6.5) with 4 ml of reconstitution/zero standard buffer (8.1.6.8). Mix thoroughly and keep in the dark until use. Stable for 2 months at 2 ^{o}C to 8 ^{o}C in dark place.

9.4 Antibody Solution

Reconstitute the vial of lyophilised antibodies (8.1.6.6) with 4 ml of reconstitution/ zero standard buffer (8.1.6.8).Mix thoroughly and keep in the dark until use. Stable for 6 months at 2 ^{o}C to 8 ^{o}C in dark place.

10. PROCEDURE

10.1 Preparation of Samples

Remove the samples (shrimp, fish or meat) from the freezer and thaw the samples by keeping in the room temperature or over night in refrigerator. Once the samples are thawed: a) for shrimp samples, randomly pick three shrimps and remove the carapace and sliced into small pieces using a knife and a cutting board. b) For fish and meat a chunk of muscle tissue about 25-30 g will be cut and sliced into small pieces. Then, homogenize (8.4.2) and weigh 3 ± 0.05g of tissue into a clean glass tube and record the sample weight.

10.2 Preparation of Quality Control Samples

10.2.1 Negative Control/ Blank sample

Take a known blank sample of the same matrix as the test samples.

10.2.2 Positive Control

Take a known blank sample of the same matrix as the test samples, and weigh one test portion of 3 ± 0.05g. Fortify the blank sample as below. Dilute the standard solution 100 ng/ml ten times to yield 10 ng/ml standard solution by mixing 25 µl of 100 ng/ml standard solution with 225 µl of water. Let the sample stand 15 minutes before continue with step 10.3

Concentration of the sample	Standard Concentration	Volume to add
0.3 µg/ kg	10 ng/ml	90 µl

10.3 Sample Extraction

Add 6 ml of ethyl acetate (8.1.1) to the test portion, and mix for 10 minutes using the test tube rotator (8.4.11). Centrifuge (8.4.4) the sample at 2000 g for 10 minutes. After centrifugation, pipette out 4 ml of the ethyl acetate into a glass tube. Evaporate the ethyl acetate at 50 °C (8.4.12) under a mild stream of nitrogen (8.1.2). Dissolve the fatty residue in 1 ml of either iso-octane (8.1.3) / trichloromethane (8.1.4) (2:3, v/v) or n-hexane (8.1.5). Add 1.0 ml of sample dilution buffer (9.2) and vortex for 1 min, and then centrifuge at 2000 g for 10 minutes. Pipette out 50 µl portions of the upper layer (if iso-octane / trichloromethane was added at the previous step) or 50 µl portions of the bottom layer (if n-hexane was added at the previous step) for the test.

10.4 Procedure for ELISA

- Pipette 100 µl of reconstitution/ zero standard buffer (8.1.6.8) in duplicate (well A1, A2).
- Pipette 50 µl of reconstitution/ zero standard buffer (8.1.6.8) in duplicate (well B1, B2)
- Pipette 50 µl of each standard solution (8.1.6.7) in duplicate wells (well C1, C2 to F1, F2 i.e., 0.04, 0.1, 0.2, and 0.4ng/ml). Alternatively, a single well for the standards may be used instead duplicating.
- Pipette 50 µl of each sample solution in duplicate into remaining wells of the microtiter plate (8.1.6.9).
- Add 25 µl of conjugate (CAP-HRPO) (9.3) into all wells, except wells A1 and A2.
- Add 25 µl of antibody solution (9.4) into all wells, except wells A1 and A2.
- Seal the microtiter plate with parafilm (8.3.7) and shake the plate for 1 min.
- Incubate for 30 min in the dark at room temperature (20–25°C).
- Discard the solution from the microtiter plate and wash 3 times with rinsing buffer (9.1). Each cycle of rising include pipetting of 300µl of the rinsing buffer to the wells and empty the wells. After the 3rd washing cycle tap the plate onto a paper towel to make sure the wells do not contain any rinsing buffer.
- Pipette 100 µl of substrate solution (8.1.6.3) into each well, and incubate for 15 minutes at room temperature (20–25°C).
- Add 100 µl of stop solution (8.1.6.4) into each well.
- Read the absorbance values immediately at 450 nm using the ELISA reader (8.4.1).

11. INTERPRETATION OF RESULTS

Positive samples

Absorbance values corresponding tissue concentration above 0.3 ppb.

Negative samples

Absorbance values corresponding tissue concentration below 0.3 ppb.

12. SCHEMATIC REPRESENTATION OF THE ELISA PROCEDURE

Test Sample

1. Cut thawed 25-30 g of shrimp, fish or meat it into smaller pieces
2. Homogenize the tissue, weigh 3 ± 0.05 g and transfer into a glass tube

↓

Fortification

1. Fortify the blank sample by adding 90 µl of 10 ng/ml CAP

↓

Extraction

1. Add 6 ml of ethyl acetate to the test portion
2. Mix 10 minutes in the test tube rotator.
3. Centrifuge at 2000 g for 10 minutes
4. Pipette out 4 ml of ethyl acetate layer into a new glass tube
5. Evaporate the ethyl acetate in the new glass tube at 50°C under mild N_2 gas flow
6. Dissolve the fatty residue in 1 ml of either iso-octane / trichloromethane or n-hexane.
7. Add 1 ml of sample dilution buffer and vortex for 1 min.
8. Centrifuge at 2000 g for 10 mins.
9. Pipette out 50 µl portions from bottom or top layer depending on the extraction solution.

↓

ELISA

1. Pipette 100 µl of reconstitution/ zero standard buffer in duplicate (well A1, A2).
2. Pipette 50 µl of reconstitution/ zero standard buffer in duplicate (well B1, B2)
3. Pipette 50 µl of each standard solution in duplicate well (well C1, C2 to F1, F2 i.e., 0.04, 0.1, 0.2, and 0.4ng/ml).
4. Pipette 50 µl of each sample solution in duplicate into remaining wells of the microtiter plate.
5. Add 25 µl of conjugate (CAP-HRPO) into all wells, except wells A1 and A2.
6. Add 25 µl of antibody solution into all wells, except wells A1 and A2.
7. Seal the microtiter plate with parafilm and shake the plate for 1 min.
8. Incubate for 30 min in the dark at room temperature (20–25°C)
9. Discard the solution from the microtiter plate and wash 3 times by pipetting with 300µl of rinsing buffer to each well. After the final rinse tap the plate onto a paper towel.
10. Pipette 100 µl of substrate solution into each well, and incubate for 15 minutes at room temperature (20–25°C).
11. Add 100 µl of stop solution () into each well.

Schematic Representation of Pipetting the ELISA Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2										
B	B1	B2										
C	C1	C2										
D	D1	D2										
E	E1	E2										
F	F1	F2										
G	G1	G2										
H	H1	H2										

A1, A2: Blank wells.

B1, B2: Zero Standards

C1, C2 to F1, F2: CAP Standards 0.04, 0.1, 0.2, 0.4 ng/ml

G1, G2: Negative Control

H1, H2: Positive Control

13. REPEATABILITY, SELECTIVITY AND UNCERTAINTY CALCULATION

Repeatability

Precision of the ELISA method for each matrix was assessed using repeatability data (intra-assay), which were expressed as %RSD values.

Matrix	**Repeatability (%RSD)**
Shrimp	20.3
Chicken	16.4
Fish	11.6

Selectivity

This ELISA kit uses a specific antibody raised in rabbits against protein conjugated CAP. The reactivity pattern of this antibody is:

Cross reactions:
- Chloramphenicol : 100%
- Chloramphenicol-glucuronide : 100%
- Thiamphenicol : < 1%
- Florphenicol : < 1%

Uncertainty calculation at 0.3 ppb

Average recovery (at 0.3 ppb)	75.31%
LOD	0.02 ppb
Uncertainty calculation at 0.3 ppb	
Mean	0.226 ppb
Sample Standard Deviation	0.039
Sample Size	9
Standard Uncertainty	0.013
Degrees of Freedom	8
95% t-Value	2.306
95% Confidence Deviation	0.030 ppb
95% Confidence Interval (Min)	0.196 ppb
95% Confidence Interval (Max)	0.422 ppb