

For laboratory use only

BioKits

Cooked Species Identification Test Kit

Sandwich enzyme immunoassay for the qualitative detection of species content in meats, meat products and feedstuffs by enzyme immunoassay

Store at 2–8°C

Information booklet for:

- Single species 48 well kits
- Four species 96 well kit (902011Q)

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1. Introduction

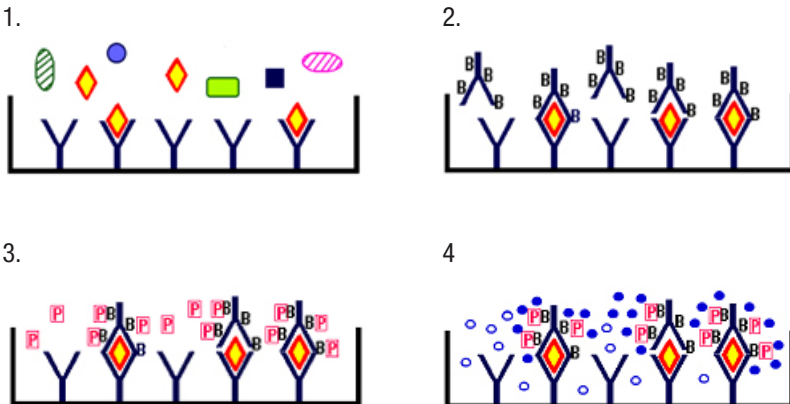
The identification of species content is performed in many countries for a variety of reasons, both economic and ethnic. Such identification may be designed e.g. to prevent the substitution of meat destined for human consumption with that of unsuitable or inferior species, or may be of importance in various religious communities where consumption of a particular species is proscribed.

Enzyme immunoassay (EIA) techniques have been implemented in the analysis of nutritive proteins, species content, additives and contaminants in food. The BioKits range of Cooked Species Identification Test Kits employs the principle of EIA based around heat stable, species-specific proteins which provide sensitive and specific tests. Routinely used around the world for control and enforcement purposes they were first to be marketed to aid in the resolution of species content in cooked meats, meat products etc. The kits have also proved successful in the detection of rendered material of animal origin in feedstuffs.

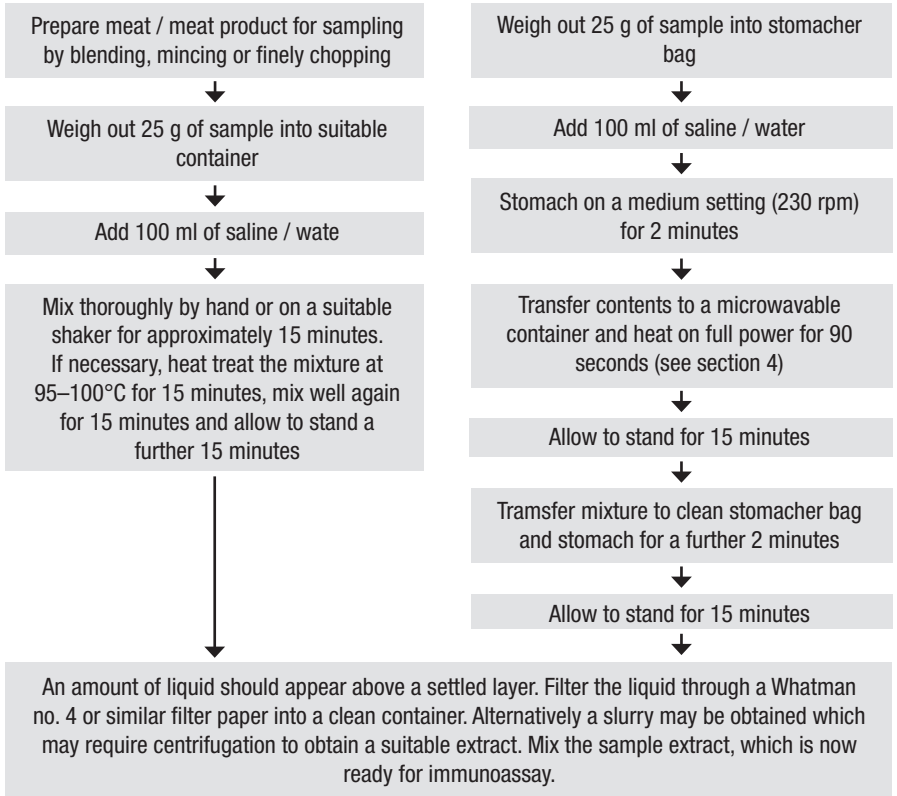
2. Principle (numbers refer to diagrams below)

The Cooked Species Identification Test Kits are sandwich type EIA's utilising a biotin-avidin enhancement process. Proteins within the samples are extracted using water or a simple saline solution and the extract added to plastic microwells coated with a preparation of purified, species specific antibody (1). With increased concentrations of species-specific protein in the extract, more of the protein will bind to antibody attached to the well. After allowing the reaction to proceed, unbound material is removed by washing. The amount of specific protein bound to the antibody coated well is determined by reaction firstly with a biotinylated specific antibody (2) and secondly with a streptavidin peroxidase conjugate (3). Following each incubation excess reagent is removed by washing. Finally, bound peroxidase activity is determined by adding a fixed amount of TMB substrate which develops a blue colour (changing to yellow on addition of acid stop reagent) in the presence of peroxidase (4). Colour development is proportional to the original amount of specific protein in the extract and a QUALITATIVE estimate of species content may be made either by visual inspection or using a spectrophotometer or plate reader.

Diagram of Test Format:



Sample Preparation Procedure Flow Chart:



Enzyme Immunoassay Summary Flow Chart:

Procedure	Volume	Description
Addition	100 µl	Pipette sample extract or Positive Controls into appropriate assay wells
Incubate		Incubate at room temperature for 45 min
Wash		Wash wells 3 (three times) with Wash Solution
Addition	50 µl	Dispense Species Biotin into all assay wells
Incubate		Incubate at room temperature for 45 min
Wash		Wash wells 3 (three times) with Working Wash Solution
Addition	50 µl	Dispense Avidin Peroxidase Conjugate into all assay wells
Incubate		Incubate at room temperature for 15 min
Wash		Wash wells 5 (five) times with Wash Solution
Addition	100 µl	Dispense TMB Substrate solution into each assay well
Incubate		Incubate at room temperature for 45 min
Addition	50 µl	Dispense Stop Solution into all assay wells
Absorbance		Measure absorbance (450 nm) of each assay well or well contents using plate reader or spectrophotometer OR visually assess plate

3. Specifications

Limit of Detection:	<1% Beef, Pork &Poultry; <1–2% for Sheep
Number of determinations:	Single Species Kit - 48 wells Four Species Kit - 96 wells total (24 wells per species)
Sample Preparation:	Mincing, shaking or stomaching / microwave
Time required:	Sample preparation / extraction time approx 60 min (5 samples) Enzyme Immunoassay Incubation time 150 min
Specificity:	Species specific proteins
Cross Reactivity:	Each set of species specific reagents has been tested against a panel of lean meat Samples for cross reaction and been found to give a negative response to beef, pork, poultry, sheep (where appropriate) and to rabbit, horse and kangaroo (see page 11 for further detail).

4. Kit Components

Note: Exact kit contents will vary depending on the whether it is a Single Species or a Four Species Cooked Speciation Kit.

Component	Detail	Vials / bottle / pack	Ready to use
Positive Controls	Each serves as a positive control in the appropriate species test and can also be used as a negative control in any other (cooked) species test	4	✓
Species Biotin	Single Species Kit: two vials per species Four Species Kit: one vial per species	4 2	✓
Avidin Peroxidase Conjugate		1	✓
TMB Substrate		1	✓
Wash solution concentrate		1	10 fold concentrate
Stop Solution	CARE: ACID	1	✓
Beef / Pork / Poultry / Sheep Plate	Single Species Kit: 6 x 8 well strips Four Species Kit: 96 microwells (3 x 8 well strips per species) Red = Beef Yellow = Pork Blue = Poultry Green = Sheep	N/A	✓
Package insert.		N/A	N/A
Blank result form		N/A	N/A

5. Materials required but not provided

Sample Preparation:

Important Note: All samples to be tested must be COOKED. If uncooked samples are to be tested, or it is suspected that the sample is not fully cooked, it is necessary to heat treat the sample/saline mixture in a water bath at >95° for 15 min or a microwave for 90 seconds prior to mixing and filtration.

Reagents:

- Sodium Chloride
- Purified water

Equipment:

- Domestic blender or mincer.
- Miscellaneous laboratory plastic and/or glassware, including measuring cylinders, pipettes and containers suitable for meat extracts.
- Whatman no. 4 (or similar) filter papers.
- Boiling water bath (if samples require heat treatment - see above).

Microwave Extraction Method:

- Microwave (850 W or equivalent*)
- Stomacher

Enzyme Immunoassay:

- Precision micropipette and disposable tips capable of delivering 50 & 100 microlitres.
- Reagent wash bottle (for plate washing).
- Enzyme Immunoassay (Optional):
 - Precision repeating dispenser capable of delivering 50 & 100 microlitres.
 - Microwell washer, (eg. NUNC Immuno Wash 8).
 - Spectrophotometer (set at 450 nm) and semi micro (1 ml) cuvettes.
 - Microwell plate reader, fitted with 450 nm interference filter.

*For microwaves of different power output cooking times can be adjusted as follows:

$$\frac{\text{Cooking time in seconds} \times \text{Wattage of Microwave used for validation}}{\text{Wattage of Microwave to be used}}$$

$$\text{e.g. } \frac{90 \times 850}{750} = 102 \text{ seconds}$$

6. Safety / COSHH Note:

“Good laboratory practice” techniques should be employed when using this kit; if such practices are used the reagents constitutes a very low potential risk to health. Safety clothing (lab coat, glasses and gloves if necessary) should be worn and skin contact with reagents avoided; do not ingest. Any contact with skin/eyes should be treated by washing/irrigation. It is also important to be aware of the allergic, toxic or infectious potential of analytical samples. Further information is available in the MSDS (Material Safety Data Sheet).

7. Preparation and extraction of samples

7.1. Preparation of sample extraction buffer

Prepare a saline solution (0.9% [0.15M] Sodium Chloride, preferably in purified water) for use in the extraction of meat samples. Approximately 100ml saline solution required per sample to be extracted. Alternatively water may be used for the extraction.

7.2. Preparation & extraction of samples

Prepare meat/meat product for sampling by blending, mincing or finely chopping (care must be taken to properly clean equipment between samples to avoid cross contamination); the more finely divided and homogeneous the sample the better the analytical result.

In view of the sensitivity of the method, care must be taken at this stage not to cross contaminate samples; any equipment/utensils used must be either disposable or thoroughly washed between extractions.

Shaking Extraction Method:

1. Weigh out 25 g of the prepared meat sample into a clean container
2. Add 100 ml of saline solution or water, stopper and mix thoroughly by hand or on a suitable shaker for 15 minutes. Allow to stand for 10–15 minutes.
3. Mix well again for approximately 15 minutes and allow to stand for a further 10–15 minutes.

Alternatively the samples can be extracted using a stomacher and microwave as follows,

Stomacher / Microwave extraction method:

1. Weigh out 25 g of finely chopped meat into a stomacher bag
2. Add 100 ml of saline and stomach on a medium setting (230 rpm) for 2 minutes.
3. Transfer contents to a suitable microwavable container and heat on full power for 90 seconds (take care that mixture does not boil over). Allow to stand for 10-15 min.
4. Transfer mixture to a clean stomacher bag and stomach for a further 2 minutes on a medium setting.
5. Transfer mixture back to a suitable microwavable container and allow to stand for a further 10–15 min.

An amount of liquid should appear above a settled layer; filter the liquid through Whatman no. 4 or similar (preferably fluted) filter paper into a clean container; alternatively a slurry may be obtained which may require centrifugation to obtain a suitable extract. Mix the sample extract, which is now ready for the immunoassay (see page 7).

Note: When analysing dry animal feeds, the method of von Holst (2000) is recommended. In order to compensate for the effect of concentration during the rendering and drying stages 20 ml of water is added to 6 g of dry feed and left for 15 minutes to swell. 100 ml of saline is added to this mixture, which is mixed and heated at 95–100°C for 15 minutes. The mixture is then filtered (fluted filter paper) and, if appropriate, adjusted to pH 6.5–7.5 with small amounts (a few microlitres) of 2 M NaOH or HCl.

8. Enzyme Immunoassay

8.1. Preparation

1. Prepare diluted sample extracts (section 7.) and kit materials (see below).
2. Remove all reagents from the kit box and allow to reach ROOMTEMPERATURE (18–22°C) before starting the test.
3. Positive Species Control, Species Biotins, TMB Substrate & Stop Solution are supplied ready to use, NO PREPARATION is necessary, simply mix by repeated inversion (do not shake).
4. **Wash solution concentrate:** Supplied as a 10 fold concentrate. Dilute 1:9 in purified water to prepare Working Wash Solution. For example add 100ml (\pm 1 ml) to a volumetric flask /cylinder and make up to 1.0 litre (\pm 10 mls) with purified water.
5. **Beef / Pork / Poultry / Sheep Plate:** Open the foil pouch. Take out the plate, remove the strips not required and return them to the pouch taking care that the desiccant capsule is present; reseal the pouch carefully.

Note: With a pencil, number the columns in sequence on the upper frosted edge of the strips in use; this preserves the identity of the strips should they become detached from the frame.

8.2. Test Procedure

The Cooked Species Identification Test Kits can be divided into a variety of layouts depending on the number of samples to be analysed. It is recommended when first familiarising oneself with the kit that smaller test runs are performed. All reaction wells are run singly and the results may be recorded on the form provided. One well is used for the **POSITIVE CONTROL**, two for Negative Controls and the remainder for test samples (Example test layouts are given on the enclosed assay worksheet).

Note: When testing has been started, all steps should be completed without interruption.

For anti-beef and anti-sheep coated wells, Sheep and Beef positive controls respectively must be included as a Negative control, to compensate for any possible cross-reactivity of these closely related species. **A minimum of TWO different negative controls should be run in each species test.**

Step by Step Procedure

Step	Procedure	Volume	Description
1	Addition	100 µl	Pipette Sample Extract or Positive controls into appropriate assay wells
Note: Use a separate disposable tip for each pipetting step to avoid cross-contamination.			
2	Mix/Incubate		Mix by hand, cover and Incubate at room temperature for 45 min without shaking
3	Wash		Wash 3 times with Wash Solution*
4	Addition	50 µl	Dispense Species Biotin into each relevant assay well
5	Mix/Incubate		Mix by hand, cover and Incubate at room temperature for 45 min without shaking
6	Wash		Wash 3 times with Wash Solution*
7	Addition	50 µl	Dispense Avidin Peroxidase Conjugate into all test wells
8	Mix/Incubate		Mix by hand, cover and Incubate at room temperature for 15 min without shaking
9	Wash		Wash 5 times with Wash Solution*
10	Addition	100 µl	Dispense TMB Substrate into each assay well
11	Mix/Incubate		Mix by hand, cover and Incubate at room temperature for 45 min without shaking
12	Addition	50 µl	Dispense Stop Solution into each assay well
13	Mix		Mix for 10 seconds (± 1 sec) gently by hand to stop colour development and uniformly distribute the Stop Solution. Colour changes from blue to yellow.

FOR ABSORBANCE MEASUREMENT USING A PLATE READER:

14	Read absorbances using a microplate reader fitted with a 450 nm filter, blank the reader on air. Record results on the worksheet provided.
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FOR ABSORBANCE MEASUREMENT USING A SPECTROPHOTOMETER:

14	Transfer 100 µl from each well to a cuvette; add 900 µl distilled water to each cuvette. Read absorbances at 450 nm against a water blank. Record results on the worksheet provided.
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FOR VISUAL INSPECTION:

14	<p>Place the MICROWELL MODULE on a white background and assess the wells for the presence or absence of a definite yellow colouration.</p> <p>The POSITIVE CONTROLS [e.g. Beef Control in the Beef strip(s); Pork Control in the Pork strip(s) etc] should be a medium yellow while Negative Controls (e.g. Pork & Sheep in BEEF strip; Sheep & Poultry in PORK strip etc) should be virtually colourless. Test samples are classified as positive if they are visibly similar in colour to, or deeper yellow than the respective POSITIVE CONTROL and negative if they are either visibly lighter in colour than the POSITIVE CONTROL or virtually colourless like the negative CONTROLS.</p>
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NOTE: Plate reader/Spectrophotometer data only: The use of a cut-off value, based on the absorbance values generated by the Negative Control wells, gives greater sensitivity than visual assessment and enables the QUALITATIVE classification of low level contamination below the level of the POSITIVE CONTROLS.

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To calculate a cut-off value, take the mean absorbance of the Negative Control wells and multiply by a factor, F (in our hands F=2.5 for beef, pork, poultry and sheep; F=5.0 for horse). Test samples with an absorbance value greater than the cut-off value are significantly higher than the Negative Control and can be classified as Positive. If a test sample gives a result very close to the cut-off value, its extraction and/or analysis should be repeated. It is important to note that because the colour of the Negative Control, and therefore the cut-off value, is related e.g. to reagent quality, sample type and individual technique the value of the factor, F, may vary slightly from day to day, person to person or laboratory to laboratory.

Setting the Cut-off value yourself:

If absorbance values obtained from X% samples are consistently higher than the suggested cut-off value, recalculate the factor, F as follows:

$$F = \frac{X\% \text{ lean meat Absorbance}}{\text{Mean Absorbance of Negative Control wells}}$$

The recalculated factor is used in Section 2 of the Results Form to determine the new cut-off value. If absorbance values obtained from 1–2% samples are consistently lower than the suggested cut-off value, it is suggested that you test samples equivalent to e.g. 2 or 3% meat to ensure that they are detected using the suggested cut-off; you should also increase your quoted limit of quantitation.

***Plate Washing Procedure:** Empty the wells by inverting over a sink and, in one rapid movement, flicking out the contents. Using a 500 ml wash bottle containing Wash Solution fill each well to the top, allow to stand for 20–30 seconds then empty the plate as described above: repeat this washing sequence for a total of three washes. Remove residual liquid and bubbles by tapping upside down on several layers of absorbent tissue.

When inverting the plate be sure to squeeze the plastic frame at the centre of the long edges to prevent the strips from falling out of the frame.

Alternatively, at the end of the incubation period aspirate the contents of all wells in column 1 using a Microwell washer then fill wells with Working Wash Solution. Complete this sequence for each column of wells (all wells are now filled with Wash Solution). Return the Microwell washer to column 1 and repeat the aspiration/fill sequence four times (each column will have been washed five times). Finally, use the Microwell washer to aspirate all the wells

then tap the plate upside down on several layers of absorbent tissue to remove residual droplets of Wash Solution and bubbles.

9. Interpretation of Results

Each Positive Control is equivalent to a sample containing significant levels of the relevant meat; 100% lean meat samples will give significantly more colour in the test. In our laboratories, using the cut-off value described below, diluted lean meat extracts and “spiked” samples give consistent responses with the following limits of detection for each specific kit species:

Species	Limit of Detection
Beef	<1%
Pork	<1%
Poultry	<1%
Sheep	<1-2%

However, each laboratory should ideally verify its own detection limits for the test, preferably using fresh, lean meat samples.

To obtain a solution corresponding to 1% lean meat, a further 1:99 dilution of the 100% sample extract must be made (e.g. take 0.1 ml extract and add 9.9 ml of saline solution or, preferably, another negative meat extract, in a clean tube). This solution approximates to a sample extract from a product adulterated with 1% of the relevant meat. Using this approach or performing spike/recovery experiments, 1–2% solutions or 1–2% spiked meat/meat products run in the appropriate test (Beef, Pork, Poultry and Sheep) should consistently give a positive result. In the case of horse speciation 5% solutions or 5% spiked meat/meat products should consistently give a positive result.

The kits are designed to assist in the QUALITATIVE assessment of species type in COOKED meats/meat products etc. Although the antigenic protein used in the preparation of the test reagents is prepared from lean muscle, the tests are NOT muscle-specific. A wide variety of other body tissues, including bone and also some gelatins, blood, meat exudates/drip, egg white/yolk and cows milk/milk products, may give positive results in the relevant test(s). Concentrated meat extracts can also be potent sources of species contamination. It should be noted that, because of the

- variations in content of these widely differing test matrices
- treatment of products prior to testing (e.g. during cooking/heating processes)
- presence of interfering factors such as salinity, acidity/alkalinity etc
- different responses of individual species proteins, tissues etc to heat treatment
- technical limitations of the methodology
- batch to batch differences in kits

the amount of residual protein in the extract, and hence reactivity in the test, may vary considerably.

Such factors must be taken into consideration when interpreting results. The tests are QUALITATIVE and CANNOT be regarded as QUANTITATIVE unless appropriate validation is performed on each batch received.

10. Performance Characteristics

The kit is tested at room temperatures of 18–22°C. Under these conditions Negative Control wells in each species test should appear virtually colourless to the naked eye, while the Positive Controls will give a medium yellow colouration. Performance of the test above or below these temperatures may necessitate either the reduction or extension (respectively) of incubation times.

Significant visible colour in any of the Negative Control wells may indicate contamination of the Substrate solution, splashing of Peroxidase Conjugate during addition to other wells or poor washing technique. Colouration of the Negative Control wells or poor agreement between replicate samples/wells is an indication of a problem during the performance of the test and any results from that test should be interpreted with caution.

11. Specificity

Each set of species specific reagents has been tested against a panel of lean meat samples for cross reaction and been found to give a negative response to beef, pork, poultry, sheep, horse (where appropriate) and to rabbit and kangaroo when tested according to the limits of detection of each species test.

Species Tested	Test				
	BEEF	PORK	POULTRY	SHEEP	HORSE
BEEF	+++++	-	-	-	-
PORK	-	+++++	-	-	-
CHICKEN	-	-	+++++	-	-
TURKEY	-	-	++++	-	-
SHEEP	-	-	-	+++++	-
BUFFALO	+	-	-	-	NT
GOAT	-	-	-	++++	NT
RED DEER	+	-	-	-	+++
HORSE	-	-	-	-	+++++
BISON	+++	-	-	-	NT
DUCK	-	-	+++	-	-
QUAIL	-	-	++++	-	NT
KANGAROO	-	-	-	-	-
RABBIT	-	-	-	-	-

- Negative + v.weak positive +++++ strong positive NT Not tested

ANALYSIS OF GELATINS

Unlike cooked/processed meats, for which the kit is intended or rendered meat & bone meal for which the kit has proved successful, gelatin presents special difficulties. Gelatin cannot be classed as meat; commercial gelatin is a highly processed, refined product which has undergone a number of chemical and physical treatments during its manufacture. Consequently, false negative results can be seen due to destruction of antigens. False positive results are also possible, due to the gelling properties of gelatin causing non-specific binding of kit reagents, even when antigens known to react in the test may have been destroyed. Studies on gelatins have demonstrated inconsistencies and lower reactions when using the test (Hofmann et al., 1999). It is our opinion that the kit may not reliably speciate commercial gelatin in isolation or products where gelatin is the sole animal protein source since, at present, there is no means of validating results.

ANALYSIS OF RENDERED ANIMAL “MEAT & BONE MEAL”

It has been reported (see references, page 13) that the kits can successfully determine species content in feeds containing rendered animal meat and bone meal (MBM) and that the Cooked Pork and Beef speciation tests can be used to determine the “adequacy” of rendering processes by monitoring the reduction in activity after heating. Although Neogen has performed an amount of animal feed testing it has not been able to obtain sufficient quantities of MBM samples to adequately validate this aspect of the test’s performance.

In order to provide Quality Control data, MBM samples available to Neogen are tested with each batch of reagent (pork data on C of A). It is recommended that customers confirm for themselves, e.g. with known adequately and inadequately heated samples, that **each lot of kits** they receive meet their requirements. Since several kit lot numbers may contain the same combinations of specific Pork reagents, customers should check with their distributor or Neogen to determine whether they have previously tested the kit lot they propose to use.

11. Example Data

1. Record visual assessment or absorbance of Controls.

	SPECIES			
	Beef STRIP	Pork STRIP	Poultry STRIP	Sheep STRIP
	Visual A450	Visual A450	Visual A450	Visual A450
Positive Control (+)	+++ 1.051	+++ 0.836	+++ 0.709	+++ 1.769
Negative Control (-)	- 0.112	- 0.073	- 0.076	- 0.136
Negative Control (-)	- 0.072	- 0.070	- 0.073	- 0.093
Mean Absorbance of the Negative Control wells (C)	0.088			
F*	2.5			
Cut Off** = C x F	0.220			

2. Record visual assessment or absorbance of SAMPLES

Sample ID	Poultry STRIP		Sample ID	Sheep STRIP	
	Abs Value	Cut-off / Visual +/-		Abs Value	Cut-off / Visual +/-
Beef T1	0.074	-	Beef T1	0.199	-
Pork T2	0.066	-	Pork T2	0.090	-
Chicken T3	0.883	+++	Chicken T3	0.068	-
Horse T4	0.079	-	Horse T4	0.077	-
Kangaroo T5	0.127	-	Kangaroo T5	0.078	-
Sheep T6	0.078	-	Sheep T6	1.832	+++

12. Shelf Life

The Cooked Species Identification Test Kits should be stored at 2–8°C. The shelf life of unopened kit components is indicated by the expiry date on the respective labels. Once the kit reagents have been opened, exposure to elevated (i.e. room) temperatures should be minimised.

Diluted wash buffer: Once diluted 1:9 the wash buffer is stable at room temperature in a sealed clean container for at least 1 week.

Diluted assay diluent: Fresh assay diluent should be prepared for each assay.

Extraction buffer: Fresh extraction buffer should be prepared daily.

Extracted Samples: The sample extracts may be stored at 2–8°C for up to 36 hours. If prolonged storage is required the extracts must be kept frozen (< 20°C) where they are stable for several months.

Kit Reagents: The kit should be stored at 2–8°C. The shelf life of unopened kit components is indicated by the expiry date on the respective labels. Once the kit reagents have been opened, exposure to elevated (i.e. room) temperatures should be minimised.

Beef / Pork / Poultry / Sheep Plate: must be kept dry; if necessary (desiccant turns off-white/cream) the desiccant capsule can be re dried by placing in a 100°C oven (changes to yellow-orange in colour).

Providing these instructions are complied with the opened kit reagents should be stable for many weeks or months at 2–8°C.

13. References

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Neogen Corporation (“Neogen”) warrants the product supplied (“the product”) against defects in materials and workmanship when used in accordance with the applicable instructions for a period of one year from the date of shipment of the product or if shorter, for a period not to extend beyond a product’s printed expiration date. If the customer establishes that the product does not conform to this limited warranty, Neogen shall, at its option, replace such product with a similar product or allow the Customer credit for their invoice value, but Neogen will have no further liability to the Customer. Neogen makes no other warranties expressed or implied, including but not limited to any implied warranties or merchantability or fitness for a particular purpose. Neogen does not warrant against damages or defects arising in shipping or handling, or out of accident, or improper or abnormal use of the product. Neogen shall not be liable for any damages (including special or consequential damages) or expenses arising directly or indirectly from the use of its product.



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