

International Commission on Trichinellosis (ICT)

*Recommendations for Quality Assurance in Digestion Testing Programs for *Trichinella**

ICT Quality Assurance Committee (Appendix 1)

Part 2

Essential quality assurance standards for *Trichinella* digestion assays

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A. Introduction

Diagnosis and control of *Trichinella* infection in susceptible food animals and game are fundamental for ensuring consumer protection from exposure to this parasite. In this context, the effectiveness of any meat inspection system depends on the application of proper quality assurance (QA) standards (Gajadhar et al., 2009).

According to the International Commission on Trichinellosis (ICT), digestion assays for the detection of *Trichinella* larvae in meat are required to meet internationally accepted standards which include scientifically derived validation data and a design that allows routine monitoring and documentation of critical control points (Gamble et al., 2000).

For trade and food safety purposes, digestion assays are the only reliable procedures for the direct detection of *Trichinella* larvae in meat (Gamble et al., 2000; OIE, 2008). These assays

can be used on single or pooled muscle samples and rely on the enzymatic degradation of muscle fibres using acidified pepsin to release muscle larvae for subsequent isolation and identification (Nöckler and Kapel, 2007).

Of the several variations of the digestion assay, the magnetic stirrer method is the internationally accepted reference method (European Community, 2005; Webster et al., 2006; OIE, 2008) and is used as the focus of the QA standards for *Trichinella* digestion assays described in this document. A number of variations of this method currently exist, but only a few have been adequately validated (Forbes and Gajadhar, 1999). An example of a magnetic stirrer method is shown in Figure 1.

The objectives of this document are to:

1. Describe the components of the method which have the potential to influence the quality of *Trichinella* digestion testing;
2. List critical control points and minimum standards for performance of *Trichinella* digestion testing for meat inspection and surveillance;
3. Define the minimum quality assurance measures for uniform performance of *Trichinella* digestion assays with focus on the magnetic stirrer method.

B. Main components of *Trichinella* digestion testing

Artificial digestion is used for the *post-mortem* testing of carcasses for *Trichinella* infection either for inspection of meat from production animals (e.g. swine, horse, crocodile), game (e.g. wild boar, bear, walrus) or for surveillance purposes in natural populations of reservoir animals (e.g. fox, raccoon dog) (Nöckler et al., 2000; Leclair et al., 2003; Larter et al., 2011).

Since digestion assays used for the detection of *Trichinella* larvae in meat do not include internal controls to monitor the effectiveness of the detection system, other tools for quality assurance are needed. The quality and accuracy of *Trichinella* testing is dependent on the proper performance of the digestion method, the appropriate muscle collection based on the target species, adequate facilities, equipment and consumables, accurate verification of findings, and proper documentation of results (Gamble et al., 2000; Nöckler and Kapel, 2007). Thus, minimum QA standards should address the following main components:

1. Muscle sample collection and preparation for testing
2. Minimum requirements for equipment and consumables
3. Performance of the digestion assay
4. Verification of findings
5. Documentation

C. Critical control points and minimum standards

For meat inspection it is necessary to ensure a test sensitivity which allows detection of the lowest number of larvae that may cause clinical symptoms in humans (Dupouy-Camet and Bruschi, 2007; Nöckler and Kapel, 2007). Results from digestion test validation studies in pork show that a 1 g sample size reliably allows for the detection of ≥ 3 larvae per g (lpg) in muscle tissue whereas 3 and 5 g sample sizes can reliably detect ≥ 1.5 lpg and ≥ 1 lpg, respectively (Gamble, 1996; Forbes and Gajadhar, 1999).

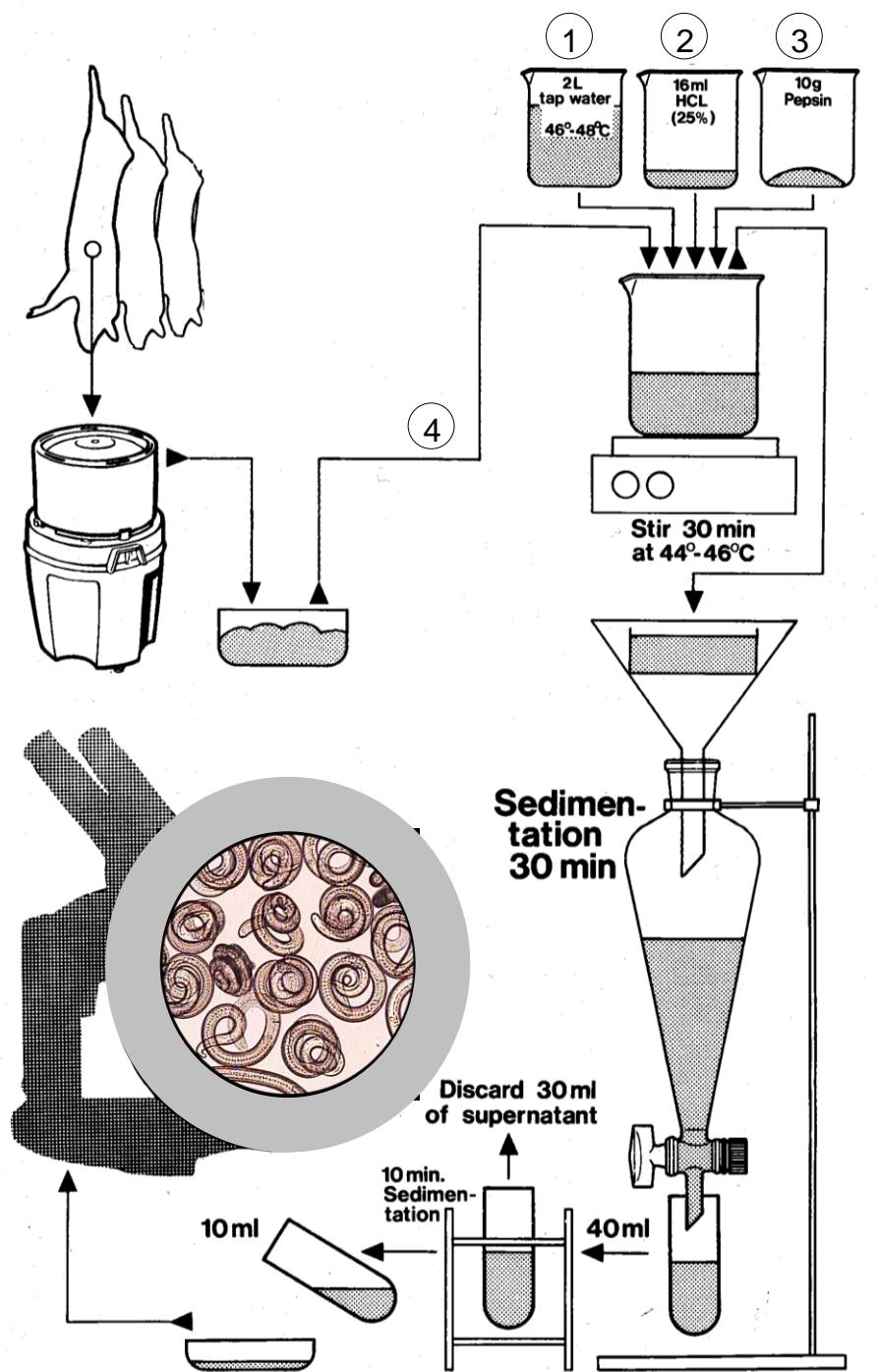


Figure 1: Example of a magnetic stirrer method for pooled sample digestion (steps labelled as 1-4 indicate the required sequential order for preparing the digest).

Digestion assays may also be used for monitoring purposes, where results are not required to assure safety for an individual carcass. The purpose of monitoring may be demonstrating freedom of infection in a herd or region, documenting a very low presence of infection, or assessing prevalence in a population. The design of sampling schemes for routine monitoring should take into account factors known to affect test performance. For example, it has been reported that the infection burden in wildlife (e.g. foxes) is low, therefore larger samples sizes are used to improve sensitivity (Malakauskas et al., 2007). Similar adjustments may also be necessary to compensate for the lower digestibility of wildlife samples relative to that of pork diaphragm, resulting in a lower relative recovery of larvae (Kapel et al., 2005).

In order to ensure reliable test performance for the required detection sensitivity, minimum standards are recommended for sample collection and preparation, equipment and consumables, assay performance, results verification, and documentation.

C1 Sample collection and preparation for testing

In order to obtain the desired sensitivity for *Trichinella* testing in domestic or wild animals, an appropriate size of muscle sample should be collected from a predilection muscle of the target animal species. An overview of predilection muscles for selected domestic and wild animals, required for collection and examination by a digestion assay is provided in Table 1. Muscle samples taken from the carcass for digestion testing should be at least twice the weight required for examination to allow for trimming of non-digestible tissues. For inspection of individual food animal carcasses for public health purposes, the sample size to be tested should be determined by a competent authority based on scientific knowledge of test sensitivity and the reason for testing.

Table 1: Predilection muscles for select animal species, which are recommended for digestion testing for *Trichinella* (Gamble et al., 2000; Leclair et al., 2003; Kapel et al., 2005, Nöckler and Kapel, 2007; Larter et al., 2011)

Animal species	Predilection muscles*
Domestic pig	Diaphragm, masseter
Horse	Masseter, diaphragm, tongue
Wild boar	Diaphragm, foreleg, tongue
Dog	Diaphragm, masseter, tongue
Bear	Diaphragm, masseter, tongue
Walrus	Tongue
Seal	Diaphragm, intercostals, tongue
Crocodile	Intercostal, masseter,
Fox	Diaphragm, foreleg, tongue
Raccoon dog	Diaphragm, foreleg tongue

*sufficient tissue of predilection muscle should be collected to allow for trimming and ensure adequate test sensitivity.

Muscle samples should be labelled upon collection and tested as soon as possible or stored under conditions (such as 2-8° C) that slow decomposition but avoid freezing. Samples which cannot be examined for some time after collection (such as wildlife surveillance) should be kept cool in labelled plastic bags until testing can be performed. Extended storage by freezing is possible, but freezing can impair digestion and result in a loss in recovery of larvae that are

not freeze-resistant; sample weight of frozen samples should be increased to compensate for the reduction in test sensitivity.

Samples tested by digestion assay should be free from non-digestible fat, tendons, fascia, etc. If tongue tissue is used, indigestible connective tissue should be removed before testing. Samples must conform to the minimum required weight after trimming. Muscle samples of insufficient weight, dehydrated muscles, or muscle samples lacking identification do not meet minimum quality requirements and should be rejected by the laboratory.

The minimum individual sample size for testing by the magnetic stirrer method following removal of non-digestible tissues depends on the required level of sensitivity. If a level of detection of at least 1 lpg of meat is required, a minimum of 5 g is required for testing, regardless of the age and origin of animal (Gamble, 1996, Forbes and Gajadhar, 1999).

The maximum sample weight in a digestion pool should not exceed 115 g for a 2 l digestion fluid. For pools with a lower total muscle weight (e.g. 50 g) the digest fluid volume and ingredients may be adjusted accordingly.

C2 Minimum requirements for equipment and consumables

All equipment used for the digestion assay must be properly cleaned prior to testing in order to avoid cross contamination. The following equipment and consumables are required for *Trichinella* testing:

- labelled collection trays or plastic bags for samples
- knives, scissors and forceps for cutting samples and removing non-digestible tissue
- calibrated scale for weighing samples and/or pepsin (accuracy $\pm 0.1\text{g}$)
- blender with a sharp chopping blade (regularly inspected and/or exchanged)
- magnetic stirrer with an adjustable heating plate
- thermometer (accurate to $0.5\text{ }^{\circ}\text{C}$, 1 to $100\text{ }^{\circ}\text{C}$)
- teflon-coated stir bar (5 cm long)
- glass beakers (minimum 3 l capacity)
- aluminium foil, parafilm or lids to cover the top of the glass beaker
- glass or plastic funnel (approx. 15 cm or larger)
- sieve made of brass or stainless steel, mesh size approx. 180-200 microns (approx. 10 cm or larger)
- conical glass separatory funnels (minimum 2.5 l capacity) preferably with teflon safety plugs
- tubes or measuring cylinders (50 or 100 ml plastic or glass)
- Petri dishes gridded with squares of 1 cm maximum dimension, or larval counting basin for trichinoscope ($180\times40\text{ mm}$) marked off into squares
- stereo-microscope with a substage transmitted adjustable light source, or trichinoscope with a horizontal table (10-20). Image capture and storage capability (camera) recommended but not required to document suspect results.
- pipettes (1, 10 and 25 ml)
- tap water heated to 46 to $48\text{ }^{\circ}\text{C}$
- hydrochloric acid (concentrated stock such as 25% or 37%)
- pepsin powder (1: 10,000 NF, 1: 12,500 BP, 2,000 FIP), granular pepsin (1:10,000 NF) or liquid pepsin (660 U/ml)
- ethanol (70-90% ethyl alcohol)
- small vials for collection of recovered larvae

Plasticware or teflonware should not be used for beakers, funnels, or separatory funnels since a rough surface and electrostatic charge may contribute to larval adherence to the inner surface of the equipment.

Calibration or certification as appropriate should be performed at least once a year for all instruments used for measurements, i.e. scale, thermometers and pipettes and all equipment should be routinely maintained. This is also a requirement for ISO/IEC 17025 accredited labs.

C3 Performance of the digestion assay

C3.1 Blending of muscle samples

Single or pooled meat samples should be chopped by a blender, grinder, or similar device (one which is easy to thoroughly clean) to increase the surface of the sample for enzymatic degradation. The blending procedure should be adjusted to maximize digestion efficiency (Gajadhar et al., 2009). The intensity of blending is dependent on both speed and time, and on the construction and maintenance of the blending device. Blending should be continued until no visible pieces of meat remain (usually 5-10 seconds at maximum speed). Too little blending may result in incomplete digestion, while too much blending could possibly damage any muscle larvae present in the samples (Gamble et al. 2000).

After blending, the chopped meat should be transferred to a 3 l glass beaker which contains the digest fluid (see Section 3.2 of this document). A small amount of digest fluid may be added to the meat in the blender (max. 100 ml per 100 g meat) to facilitate tissue homogenization and transfer of homogenate from the blender into the glass beaker (OIE, 2008). To avoid larval loss due to adhering muscle tissue, the chopping blade and the blender bowl should be rinsed with a small quantity of digest fluid which should then be poured into the glass beaker.

The blender bowl should be made of acid resistant material (glass or stainless steel) to avoid etching which could result in adherence of larvae.

C3.2 Preparation of the digest fluid

The most critical step is the sequence of mixing of the constituents of the digestive fluid. This sequence should be in the following order: 1) water, 2) hydrochloric acid (HCl) and 3) pepsin. This sequence will prevent degradation of pepsin which could occur if it is exposed to concentrated HCl. It is recommended that mixing of digest fluid take place under a fume hood to prevent exposure of analysts to acid fumes and/or pepsin dust. Initially, 16 ml of 25% HCl acid or an equivalent volume of 37% (see Section 2 of this document) is added to a glass beaker containing 2 l of tap water which should be preheated to 46-48°C. Care should be taken not to exceed this temperature, as higher temperatures could destroy any larvae present in the samples, or reduce the activity of the pepsin. Stock solutions of HCl are available in formulations other than 25% and the volume used must therefore be adjusted accordingly to achieve a final concentration of 0.2% (pH=1-2) (max. pepsin activity pH=1.5-1.6). The pH of the digest fluid can easily be checked using pH (litmus) paper. Following addition of the HCl, a magnetic stir bar is placed in the beaker, the beaker is placed on a preheated stir plate and the equipment is set to commence stirring.

Ten grams of pepsin should then be added to the digest fluid. The pepsin used for the preparation of digest fluid must have the appropriate activity required for digestion. The activity is expressed either in “NF” (US National Formulary), “BP” (British Pharmacopoeia) or “FIP” (Fédération Internationale de Pharmacie) units (see Section 2 of this document).

Storage conditions and shelf life of the pepsin should be displayed on the label of the storage container. The use of granular or liquid pepsin is recommended to reduce the risk of aerosolisation and possible allergic reaction to analysts. The final concentrations of pepsin in the digest fluid should be 0.5%. Up to twofold higher concentrations of pepsin may be used without harm to *Trichinella* larvae, but should only be considered when muscle samples do not digest adequately using standard conditions.

The chopped meat is the final ingredient to be added to the digest (Figure 1).

C3.3 Digestion of chopped meat in the glass beaker

A maximum of 1:20 ratio of meat to digest fluid in the glass beaker and a constant temperature of 44-46°C should be used throughout the process to facilitate an efficient and rapid digestion. Beakers and fluid may be pre-heated (e.g. to 46-48°C) to maintain an initial temperature within the required range. To maintain a constant temperature and decrease evaporation during digestion, the glass beaker should be covered with aluminium foil or similar material and the temperature should be regularly monitored with a thermometer. An electronic thermostat is recommended. During stirring, the digest fluid must be stirred at a sufficiently high speed to create a deep vortex without splashing.

The time usually required for complete digestion of muscle is 30 min. In the case of muscle samples which are less digestible such as wildlife tissues (see Section 1 of this document), the digestion time can be increased but should not exceed 60 min in total (Kapel et al., 2005).

Time-temperature conditions below the recommended values could result in incomplete digestion of the muscle tissue. Conversely, overheating (> 50°C) or prolonged digestion times could result in the inactivation of pepsin or destruction and loss of larvae.

C3.4 Filtration of the digest fluid

Following digestion, the fluid should be poured carefully (to avoid overflow) through a sieve (see Section 2 of this document) into a separatory funnel. Sieves do not need to be calibrated since they are used to retain undigested debris and are not intended for measurement; however, sieves should be properly cleaned after use (without scratching) to avoid altering the mesh size. Sieves must be free of debris prior to use to allow digest fluid to pass through.

After pouring the digest fluid into the separatory funnel, the glass beaker and the sieve should be rinsed with an additional volume of tap water (minimum of 100 ml) into the separatory funnel to avoid larval loss due to larvae adhering to the surfaces of the beaker or to residual tissue on the sieve.

The weight of any appreciable amount of undigested tissue remaining on the sieve should be determined by weighing the sieve on a scale that has been tared (zeroed) using a clean identical sieve. The digestion process is considered compliant if residual debris remaining on the sieve consists primarily of undigestible non-muscle tissue (typically consisting of fascia and connective tissue). If visible muscle tissue remains on the sieve, the whole procedure must be repeated (i.e., start procedure again from Section 1) using fresh muscle samples. If no more muscle tissue can be obtained from the same carcass or pool of carcasses, the undigested portion (if consisting of muscle tissue) from the sieve should be digested again using freshly prepared digest solution and examined in addition to the initial digest.

C3.5 Sedimentation of the digest fluid in the separatory funnel

The sedimentation step of the completed digest in the separatory funnel allows sufficient time for larvae to settle at the bottom of the fluid column for subsequent recovery and enumeration. Although not necessary, gentle tapping of the funnel wall (e.g. every 10 min) may facilitate the larvae settling to the bottom of the funnel.

Coiled live larvae sediment at a rate of about 1 cm per min. The digest fluid should remain undisturbed in the funnel for a minimum of 30 min. If the sedimentation time is less than 30 minutes not all larvae may have settled and may not be recovered in the collected sediment.

If muscle samples have been frozen prior to digestion, larvae of freeze-susceptible *Trichinella* species are likely to be dead. As dead larvae un-coil upon release from the muscle tissue, their sedimentation speed decreases. Therefore, sedimentation time for frozen muscle samples where dead larvae are expected should be extended for up to 60 min. DNA degradation occurs quickly in dead larvae, precluding the molecular identification of larvae at the species or genotype level.

C3.6 Collection of the primary and secondary sediment

After sedimentation in the separatory funnel, approximately 40 ml (or as prescribed by the particular validated method used) of the digest fluid (primary sediment) should be quickly dispensed (free-flowed) into a 50 ml tube. The stopcock of the separatory funnel must be fully opened to ensure that no larvae are trapped on the edge of the opening or fail to be flushed out due to low flow velocity. The primary sediment should be allowed to stand for 10 min so that larvae can again settle to the bottom. If the volume of the primary sediment is too small, larvae may remain in the digest fluid in the separatory funnel and will be lost. Conversely, there may be more debris if the volume of the primary sediment is too high.

After sedimentation for 10 min, 40 ml of the supernatant should be carefully withdrawn from the 50 ml tube by aspiration from the top, without disturbing the sediment, leaving a volume of 10 ml. This secondary sediment should be poured into a gridded Petri dish or larval counting basin. To remove larvae which may stick on the surface of the inner wall, the tube should be rinsed with 10 ml of water which is then added to the Petri dish or larval counting basin.

The collection of the primary and secondary sediment may be modified by using a double separatory funnel technique (Forbes and Gajadhar, 1999; Gajadhar and Forbes, 2002). In this method, approximately 125 ml of the digest fluid (primary sediment) from the first separatory funnel should be poured into a 500 ml separatory funnel and the volume is adjusted to 500 ml with tapwater at room temperature. This mixture should be allowed to settle for an additional 10 min, after which a sample of 20 ml (secondary sediment) should be recovered directly into a gridded Petri dish for the identification of the larvae.

C3.7 Microscopic examination

The Petri dish or larval counting basin containing the secondary sediment should stand undisturbed on the examination stage of the stereomicroscope for at least 1 min to allow larvae to settle before microscopic examination. Following this initial settling, microscopic examination should be done as soon as possible. If the secondary sediment is kept for longer than 30 min before reading, it should be properly labelled and kept secure.

Accurate focussing of the stereomicroscope on the lower layer of the sediment is crucial for the identification of larvae and should be established prior to microscopic examination of the test sample. This can be done by focussing the microscope to ensure that gridlines of the Petri

dish or larval counting basin are easily visualised through the digest fluid. Because the gridlines are on the outer surface of the bottom of the plate, the focus must be adjusted slightly upwards to the inner surface of the plate to bring larvae into sharp focus.

The secondary sediment must be transparent enough to enable easy identification of any larvae. If not, the secondary sediment must be washed again as follows: 1) transfer the secondary sediment and a tap water rinse of the Petri dish or larval counting basin into the tube, adding additional tap water to a total volume of approximately 40 ml; 2) allow to sediment for 10 min; 3) carefully withdraw the supernatant (see Section 3.6 of this document), leaving a volume of 10 ml. This sediment and a 10 ml tap water rinse of the tube should then be poured into the original gridded Petri dish or larval counting basin.

The final 20 ml of recovered sediment and rinse fluid is systematically examined grid by grid with a stereomicroscope or trichinoscope at a 10-20 X magnification. Any suspect *Trichinella* or other nematode findings should be verified by examination at 60-100 X magnification.

Suspect or positive *Trichinella* results from pooled samples must be traced back from the pool to the carcass of origin via digestion of progressively smaller numbers of pooled samples of increased sample size from the implicated carcasses. Pooled samples with negative results should be ruled out and those yielding positive results continue to be sampled and tested until digestion of tissue from an individual carcass demonstrates the source of the positive result (link to lab certification document).

Following performance of the digestion method, specific decontamination and cleaning procedures must be employed for all equipment (e.g. blender/grinder compartment, glassware and counting chambers) and lab table surfaces. *Trichinella* larvae are inactivated after contact with hot water ($\geq 70^{\circ}\text{C}$ for at least 1-2 min). However, it is important to note that larvae requiring subsequent confirmation by microscopic or molecular assays should not be inactivated using hot water in the Petri dish or larval counting basin (see Section 3.4 below).

C4 Verification of findings

Knowledge of the basic morphological characteristics of *Trichinella* larvae, including dimensions, shape, and colour is required to verify findings. First stage larvae are approximately 0.7 to 1.1 mm in length and 0.03 mm in width. The most distinguishing feature of *Trichinella* larvae, not recognized by stereomicroscopy but by compound microscopy, is the stichosome, which consists of a series of discoid cells lining the oesophagus and occupying the anterior half of the body. Live *Trichinella* larvae may appear coiled (when cold) or motile (when warm), or C-shaped when dead (Gamble et al., 2000).

If positive or doubtful findings occur the larvae should be transferred with a minimal volume of fluid (such as 5-10 ul) into a small vial (1-2 ml) filled with 70-90% ethyl alcohol (final concentration) for preservation as soon as possible and subsequent molecular identification (PCR) should be performed at a qualified reference laboratory. After attempting to transfer a single larva, the pipette tip used in the transfer should be examined under a stereomicroscope to ensure that the larva did not remain on the tip.

C5 Documentation

Each laboratory must have a system of adequate documentation which demonstrates that *Trichinella* testing was correctly performed according to appropriate QA standards, and facilitates adequate traceback (see Part 5 of these recommendations).

A laboratory worksheet (Table 2) should be used by analysts to record data for test reports and is therefore a critical document for quality audits and traceback investigations. Key components of the laboratory worksheet include sample tracking information, documentation that the method has been performed correctly by qualified personnel, documentation of problems and irregularities, and a written record of results (Gajadhar et al., 2009). Laboratory worksheets should be stored according to the requirements of a competent authority.

Table 2: Example of a laboratory worksheet for recording data when testing pooled samples by digestion assay

SOP				Pepsin: Lot N.	Packet N.				
Sample code	Pork (g)	Horse (g)	Wild boar (g)	Other animal species _____	Digestion time (min.)	Residual tissue on sieve (g)	Negative	Positive	No. of larvae/g

NOTES

Date	Analyst	Signature	Date	Supervisor signature
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APPENDIX 1 ICT Quality Assurance Committee Members

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