REPORT ON THE VALIDATION OF THE PRIOCHECK® TRICHINELLA AAD KIT OF THE PRIONICS AG COMPANY

June, 2013 – June, 2014

Index

1. Introduction

According to the Commission regulation 2075/2005 of 5 December 2005, which specifies rules on official controls for *Trichinella* in meat, each carcass must be examined for *Trichinella* in a laboratory designated by the competent authority, using one of the detection methods set out in Chapter I of Annex I or an equivalent method set out in Chapter II of Annex I. If a new method/apparatus/kit is to be used, prior to use it should be validated in accordance with the "Guidelines for the validation of apparatuses for the detection of *Trichinella* larvae in meat samples by digestion" (revision of December 21, 2012) (herein referred to as "the Guidelines") approved by the DG SANCO.

2. Evaluation round

On June 12, 2013, the Prionics AG Company (address: Wagistrasse 27a, 8952 Schlieren, Switzerland) ('Company') sent a letter to the European Union Reference Laboratory for Parasites (EURLP) with a request to validate the PrioCHECK® Trichinella AAD KIT (ANNEX 1). The Company sent the instruction manual and a description of the most important features of the Kit (ANNEX 2). On June 19, the Company informed the EURLP that the KIT should be validated for pig meat. The same day, the EURLP sent to the Company the Validation Agreement concerning the validation of the apparatus for pig meat to be performed by the EURLP and by four National Reference Laboratories (NRLs). Following the evaluation of the instruction manual, the EURLP submitted an agreement to Company and, at the same time, contacted four NRLs for *Trichinella*, which had shown a good performance at the proficiency test for *Trichinella* detection organized by the EURLP in 2013, asking their availability in validating the PrioCHECK® Trichinella AAD KIT.

The four NRLs which agreed to participate were:

- NRL for *Trichinella* of the Czech Republic
- NRL for *Trichinella* of France
- NRL for *Trichinella* of Germany
- NRL for *Trichinella* of Hungary

At the beginning of July, 2013, the Company sent the PrioCHECK® Trichinella AAD KITs to the four NRLs and to the EURLP.

The EURLP invited the NRL personnel to perform a few tests to familiarize with the kit and to check if the kit worked properly, before starting with the validation program. Once the NRL personnel acquired sufficient confidence with the kit, they contacted the EURLP and requested that the proficiency samples be forwarded.

According to the Guidelines, the proficiency samples consisted of 20 minced pork samples spiked with three encapsulated *Trichinella* larvae each for each of the five laboratories which should validate the Kit. The weight of the minced meat (free of fat and fascia) is the maximum weight (i.e., 100 g) of meat which can be digested per time according to Commission regulation 2075/2005 of 5 December 2005.

The meat samples were forwarded from EURLP to the NRLs, at the most convenient times for receiving the samples for each lab. According to the Guidelines, the EURLP first prepared the panel of 20 samples (10 + 10 samples sent by two

separate shipment at two different times, 1st and 2nd panels) for each participating lab. Since one lab did not obtain good results, a 3rd panel of 10 samples was sent to this lab (ANNEX 3, Lab 5). Afterwards, since contrasting results were obtained among several participating labs, additional 10 samples were sent to each of these laboratories (ANNEX 3, 4th panel). These last panels were tested in presence of one representative of the EURLP. Finally, due to the continuation of contrasting results among the 5 laboratories, i.e. not all participating labs correctly identified all positive samples, the EURLP carefully examined each step of the PrioCHECK® Trichinella AAD KIT protocol and identified a lack of standardization for some steps. The EURLP invited the Company to provide to the participating labs in addition to the KIT, a revision of the Instruction manual, and materials and apparatuses which has been identified as critical points in the digestion test (ANNEX 2).

The technicians performing the tests were required to fill in a form forwarded with the samples and to add comments and notes on whether or not the kit performed adequately, in terms of sensitivity, robustness, and user-friendliness required by the Guidelines.

3. Main characteristics of the PrioCHECK® Trichinella AAD KIT

The PrioCHECK® Trichinella AAD KIT consists of three components named 'digestion buffer', 'enzyme solution', and 'digestion buffer additive' which completely replace pepsin and hydrochloric acid used to digest muscle tissues according to the Commission Regulation 2075/2005. These chemical components digest muscle tissues in 20 ± 2 min at 60 °C. According to the company, up to 150 g of muscle tissues can be digested at the same time, but the KIT was tested with samples of 100 g.

4. Sample forwarding

Each sample of 100g consisted of pig minced meat, free of fat and fascia. *S*amples were spiked with three live larvae of *Trichinella spiralis* collected after a short period of digestion, i.e. each larva was still in the collagen capsule. Each sample was preserved in a plastic bag under vacuum, coded, and forwarded in a polystyrene box, with coolers to maintain a temperature of less than 10 °C, by an international courier. The parcels containing samples were delivered within 24-48 hours. Upon arrival, the polystyrene box was opened and the internal temperature was measured. The samples were then stored at +4 °C until testing by the Kit within 48 hours.

5. Results

5.1 First, second, and third sample panels

According to the Guidelines, the EURLP prepared a total of 100 samples (1st and 2nd panel) which were tested in the five laboratories including the EURLP in July, 2013. The results are summarised in the ANNEX 3.

Out of the 100 digested samples, the average undigested material on the sieve was 0.9 g (range 0.0 – 4.5). At least one *Trichinella* larva was detected in 90 % and 80 % of the samples of the 1st and 2nd panel, respectively. No larva was detected in 15 samples tested in 4 labs (see ANNEX 3). One larva was detected in 39 samples tested in 5 labs. Two larvae were detected in 28 samples tested in 5 labs. Three

larvae were detected in 18 samples tested in 5 labs. All larvae collected after digestion by the PrioCHECK® Trichinella AAD KIT were dead.

Due to the detection of four negative samples out of 20, lab n. 5 tested additional 10 samples (3rd panel) without improving substantially the sensitivity of the test (two negative samples, five samples with one larva, one sample with two larvae, and two samples with three larvae) (ANNEX 3).

5.2 Fourth sample panel

Due to contrasting results among the participating laboratories (see ANNEX 3) and between the two/three sample panels tested in the same laboratory, additional 10 samples were sent to each of the four labs which had had a bad performance (negative results) and 3 samples to the lab which never failed to detect the larvae. An EURLP technician skilled on the digestion technique, visited the three labs to assist the lab personnel during the digestion of the 10 samples by the PrioCHECK® Trichinella AAD KIT (see ANNEX 3).

5.3 Additional investigations

To deeply investigate during which step the larvae had been lost, the following investigations were carried out at the EURLP:

- separatory funnels of different shape were tested in parallel;
- the amount of the digestion fluid collected after the sedimentation time was increased up to 70-80 ml;
- additional 70-80 ml of digestion fluid was collected after an additional sedimentation time of 30 min
- larvae were searched in the discarded 30-50 ml supernatant.

The investigations identified the use of separatory funnels of different shape and the collection after the sedimentation step, of less than 60 ml of digestion fluid, as the two main factors imputable to the loss of larvae. The use of a separatory funnel of about 30x14 cm (HxW) and the collection of 75 ml of digestion fluid after sedimentation, allowed to correctly identify all positive samples. All larvae collected after digestion were dead.

5.4 Fifth sample panel

The Company sent to two laboratories (n. 2 and 3) new PrioCHECK® Trichinella AAD KIT with a revised Instruction manual, separatory funnels (Lenz NS 29/32) and glass test tube of 80 ml (ANNEX 5 and ANNEX 6), to perform a new round of digestions. The EURLP prepared 41 new meat samples spiked with 3 larvae which were tested in two labs by using the standardized materials and apparatuses. The two labs correctly identified the positive samples (see ANNEX 4). All larvae collected after digestion were dead.

5.5 Larva identification at the species level

Single larvae recovered after digestion by the PrioCHECK® Trichinella AAD KIT were correctly identified at the species level by molecular identification according to a validated method (multiplex PCR, EURLP method MI-02; www.iss.it/crlp/).

6. Conclusions of the evaluation rounds

In the first four evaluation rounds (ANNEX 3), the test failed to correctly identify 15% of samples as positive. Out of the 5 labs which tested the samples, only one lab (Lab 4) correctly identified all the 23 tested samples. However, it should be stressed that after sedimentation, the personnel collected 70-80 ml of the digestion fluid instead of 40-60 ml collected in the other four labs according to the Instruction manual provided with the Kit and used a separatory funnel with a HxW rate of about 30x14 cm (ANNEX 6). When the revised Instructions (ANNEX 5) were followed at the fifth round, the test allowed to correctly identified all the positive samples. Due to the digestion temperature (60 °C), all larvae collected after sedimentation were dead. However, the larvae can be identified at the species level as requested by the Commission Regulation 2075/2005.

7. Relationship between the digestion results obtained by the PrioCHECK® Trichinella AAD KIT and the Guidelines requirements

According to the Guidelines and the Commission regulation 2075/2005, the weight of the undigested material on the sieve should be lower than 5% of the weight of the muscles to be digested. The weight of the undigested material was always much lower than the 5% (average 0.9 g, range 0.0 – 4.5).

According to the Guidelines, all muscle samples spiked with three *Trichinella* larvae should be identified as positive, i.e. at least one larva should be detected in all samples. Once all of the digestion test steps were standardized according to the EURLP suggestions, the Kit successfully satisfied the minimum requirement of the Guidelines.

8. Comments

8.1 Strongness of the PrioCHECK® Trichinella AAD KIT

- the Kit is easy to use;
- the amount of undigested tissues on the sieve is very low;
- the kit allows to perform the digestion of muscle tissues even in case of pepsin shortage on the market;
- personnel with allergic reactions to pepsin can perform the digestion of muscle tissues;
- at the end of the test all larvae are dead, then the decontamination step is not required when positive samples are detected.

8.2 Weakness of the PrioCHECK® Trichinella AAD KIT

- Since larvae obtained after digestion are not live, the larval movements cannot be of help to recognize the parasite among indigested debris in the sediment;
- the amount of undigested debris in the sediment is higher than that obtained by the pepsin-HCl digestion;
- the method shows a lower robustness compared to the pepsin-HCl digestion, requiring more accuracy in the test execution.

9. Conclusions

According to the results of the validation rounds, the PrioCHECK® Trichinella AAD KIT can be recommended to detect Trichinella sp. larvae in muscle samples of pigs when the protocol of the instruction manual (ANNEX 5) is strictly followed. The use of this kit is restricted to pork meat inspection only and the kit has been not validated for other animal species.

ANNEX 1

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Request for official listing of the PrioCHECK® Trichinella AAD in the Annex I of the EC Regulation 2075/2005

Dear Dr. Pozio,

Prionics has developed a new artificial digestion test, the PrioCHECK® Trichinella AAD, for the detection of *Trichinella spp.* larvae in meat. The assay follows a similar protocol as the traditional pepsin artificial digestion assay, but uses an alternative enzyme to the pepsin. Further it overcomes several drawbacks of the current used artificial digestion method: All components are liquid (no powder) and no acid needs to be used, secured supply, consistent quality. Therefore, the PrioCHECK® Trichinella AAD offers a good alternative to the current used artificial digestion method using pepsin.

We herewith officially request the listing of the PrioCHECK® Trichinella AAD in the Annex I of the EC Regulation 2075/2005 and ask you to initiate the necessary steps and inform us correspondingly and in due time on actions needed from our side.

Yours sincerely,
Prionics AG

Patrik Buholzer
Business Unit Manager

Dr. Markus Moser
CEO

ANNEX 2

Instruction manual of the PrioCHECK® Trichinella AAD KIT provided by the Company at the beginning of the validation process

PrioCHECK® Trichinella AAD

Artificial digestion test for *in vitro* detection of *Trichinella spp.* larvae in meat samples

Kit for 10 assays/ up to 1500 individual animals
®Prionics AG

Version 1.1_e

Package Insert

For *in-vitro* veterinary diagnostic use only
Store at 0-25°C
Product No.: 7620030

Introduction

Trichinellosis caused by the Nematode *Trichinella* is a zoonotic disease which occurs worldwide and affects a broad range of different species including mammals, birds and amphibians. Currently 11 different subspecies (8 designated as species) have been recognized in this genus. The species that are of main importance in Europe are *Trichinella spiralis*, *Trichinella britovi*, *Trichinella pseudospiralis*, and *Trichinella nativa*. *Trichinella spiralis* is found in production animals (pigs, horses) in temperate climate zones and can also be found in animals in close contact with these production animals (e.g. dogs, cats, rats). *Trichinella britovi* is mainly found in wildlife. *Trichinella pseudospiralis* is distributed worldwide and is also found in birds. *Trichinella* infections of pigs are of major concern since humans can be infected by eating raw or insufficiently cooked meat.

The artificial digestion method is the procedure currently recommended to detect Trichinella larvae in meat. According to the EC Regulation 2075/2005 each individual animal used for human consumption need to be checked for the presents of *Trichinella spp.* Larvae. Therefore meat of individual animals are pooled and checked for the presents of *Trichinella* larvae by artificial digestion.

The PrioCHECK® Trichinella AAD is a new alternative artificial digestion method. It is a reliable and fast diagnostic test for detection of *Trichinella* larvae in meat and can be used for individual carcasses testing.

Test Principle

Meat, e.g. diaphragm, is pooled in pieces up to 150 g. Digestion Buffer and Enzyme Solution are added to the meat pieces and digested at 60°C. After subsequent sedimentation and washing steps, the solution is checked for the presents of larvae under a trichinoscope or stereo-microscope (15 - 20 times magnification).
The PrioCHECK® Trichinella AAD uses a serin-endopetidase of the enzyme group subtilisine and no hydrochloric acid is used making the method more confinement than the traditional artificial digestion method.

Kit Components

Store kit at 0-25°C until expiry date. See kit label for actual expiry date. The shelf life of diluted, opened or reconstituted components is noted below, where appropriate. Chemical hazard data are available in section "Safety Regulations and R&S Statements" (Appendix IV).

Component 1
Digestion Buffer (20x)
Two bottles containing 500 ml Digestion Buffer. Shake slightly before use.

Component 2
Enzyme Solution (Ready-to-use)
1 bottle containing 500 ml Enzyme Solution.

Component 3
Digestion Buffer Additive
One bottle containing 15 ml Digestion Buffer Additive

Additional Kit Contents:

Package Insert

Additional Material Required

General:
Laboratory equipment according to national safety regulations.

- Water
- Graduated cylinder
- Beaker, 3 liter
- Pipette
- Thermometer accurate to 0,5°C within the range of 1°C to 100°C
- Funnel
- Separation funnel, 2 liter
- Stainless steel mesh, mesh size 180 microns,

Sample preparation:
- Blender

Analysis of Results:
- stereo-microscope at a 15 to 20 times magnification or trichinoscope
- Petri dish with grid

Test Procedure

Precautions
National guidelines for working with animal samples must be strictly followed. The PrioCHECK® Trichinella AAD must be performed in laboratories suited for this purpose.
Samples should be considered as potentially infectious and all items which contact the samples as potentially contaminated.

Chemical hazard data are available in section "Safety Regulations and P&L Statements" (Appendix IV).

Notes

To achieve optimal results with the PrioCHECK® Trichinella AAD, the following aspects must be considered:

- **The Test Procedure protocol must be strictly followed.**
- Kit components of different kit lot numbers must not be used together.
- The digestion solution must have 60°C ± 2°C

SAMPLE PREPARATION

- Using a knife or scissors and tweezers for cutting specimens

SAMPLE PREPARATION:

Preparatory Steps
- Cutting specimens using a knife or scissors.
- Pooling samples up to 150 g.
  E.g. for pork: diaphragm up to 150 pieces of 1 g is pooled. Higher amounts for wild animals are recommended. Please review to official Regulation EC 2075/2005 for details of individual species.

Note: Recommended is the use of 100 g to150 g tissue for one pool. If less than 100 g tissue is used, the volumes of the Digestion Buffer working solution, Enzyme Solution and the rinsing volumes are adapted accordingly. Do not use less than 10 g of tissue.

ARTIFICIAL DIGESTION

- Provide 2 l of Digestion Buffer 1x (100ml ± 5 ml Digestion Buffer 20 x + 1900 ml water) in in the digestion vessel (e.g. a beaker)
- Add 0.5-1ml of Digestion Buffer Additive
- Add 50 ml ± 2 ml Enzyme Solution
-
- Heat up to a temperature of 60°C ± 2°C.
- Add the chopped meat to the digestion solution and wash out all the chopped meat with Digestion Buffer working solution.
- Incubate for 20 min ± 2 min at 60°C under strong stirring conditions (≥750 rpm). Insulate the digestion vessel to prevent heat loss e.g. by using aluminum foil.

  Note: During stirring, the digestion fluid must rotate at a sufficiently high speed to create a deep whirl without splashing. Make sure that stirring is sufficient to prevent any accumulation of meat. This will lead to an incomplete digestion.

- The digestion fluid is poured through the stainless steel mesh into a separation funnel. For 100 g - 150 g of meat a 2 liter separation funnel is required.
- Rinse the beaker properly with water (max. volume of 200 ml). Pour the water through the stainless steel mesh into the separation funnel.

  Note: The Digestion is considered satisfactory if not more than 5 % of the starting sample weight remains on the sieve (for pork only).

- Let the digestion fluid stand for 30 min ± 2 min in the separation funnel for sedimentation.
- After sedimentation a sample of 40 ml - 60 ml digestion fluid is quickly run off into a measuring cylinder or centrifugation tube.
- Allow the 40 ml - 60 ml suspension to stand for 10 min ± 1 min for sedimentation.
- Discard 30 ml - 50 ml of the supernatant by carefully sucking off the upper layer. Leave a volume of not more than 10 ml the cylinder or centrifugation tube.

DETECTION

- Pour the remaining 10 ml into a larval counting basin or Petri dish. Rinse the cylinder or centrifugation tube with not more than 10 ml of water which has to be added to the sample in the larval counting basin or Petri dish.
- Subsequently the sample is examined by trichinoscope or stereo-microscope at a 15 to 20 times magnification.

  Note: In case of a suspect area or parasite like shape, a higher magnification of 60 to 100 times must be used.

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PrioCHECK® Trichinella AAD

RESULT INTERPRETATION

If **larvae are found** during examination under trichinoscope or stereo-microscope the sample is considered positive.
Further investigations are required: Identfication of the species of the found larvae by PCR technique.

Note: Found larvae might be send to the National Reference Labor for Trichinella for further investigations.

If no larvae are found during examination under trichinoscope or stereo-microscope the sample is considered negative.

Appendix I

Notice
This manual is believed to be complete and accurate at the time of publication. In no event shall Prionics AG be liable for incidental or consequential damage in connection with or arising from the use of this manual.

Liability
Prionics AG warrants its products will meet their applicable published specification when used in accordance with their applicable instructions and within the declared products life time. Prionics AG makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose. The warranty provided herein and the data, specifications and descriptions of Prionics AG products appearing in Prionics AG published catalogues and product literature may not be altered except by express written agreement signed by an officer of Prionics AG. Representation, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.
In the event of a breach of the foregoing warranty, Prionics AG's sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Prionics AG promptly of any such breach. If after exercising reasonable efforts, Prionics AG is unable to repair or replace the product or part, then Prionics AG shall refund to the customer all monies paid for such applicable product or part.

Prionics AG shall not be liable for consequential, incidental, special or any other indirect damages resulting from economic loss or property damage sustained by any customer from the use of its products.

Prionics AG is an ISO 9001:2000 certified company.

Appendix II

Safety Regulations and P&L Statements

National Safety Regulations must be strictly followed.

P&L Statements

Component 1
Digestion Buffer (20x)
 Xi; Sensitising
Hazard Code: R43: May cause sensitisation by skin contact.

Component 2
Enzyme Solution (Ready-to-use)
 Xi: Irritant
Xn: Sensitising
Hazard Code: R37/38-41: Irritating to respiratory system and skin. Risk of serious damage to eyes.
R42: May cause sensitisation by inhalation.

Component 3
Digestion Buffer Additive (Ready-to-use)
The product does not have to be labeled due to the calculation procedure of the "General Classification guideline for preparations of the EU" in the latest valid version.

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Critical Points – PrioCHECK® Trichinella AAD

- **Temperature of digestion solution**
  - Do not let temperature fall below 58°C. Important for a proper digestion.

- **Adding of meat**
  - Keep temperature sensor in the solution to keep temperature.

- **Stirring of digestion fluid**
  - During stirring, the digestion fluid must rotate at a sufficiently high speed to create a deep whirl without splashing. Important for a proper digestion.

- **Pouring digestion solution trough sieve**
  - Control sieve for clogging before use. Check that no holes are blocked.
  - Using a wash bottle for washing the sieve after pouring the digestion solution trough the sieve. Increase of sensitivity due to washing down of larvae. Especially for dead larvae (C-shaped larvae).

- **Separation Funnel**
  - Check if the valve fits properly.
  - Open valve fully during separation step after sedimentation. It is not critical if more than 40 ml (max. 60ml) are poured out during separation step.

- **1st sedimentation step**
  - Slightly tapping of the separation funnel during the 1st sedimentation step after about 5 to 10 minutes can increase the sensitivity. Especially for dead larvae (C-shaped larvae). Repeat tapping three times. Let the digestion solution rest in the separation funnel (without tapping) at least 10 minutes before the sedimentation step is finished.

- **2nd sedimentation step**
  - Remove supernatant carefully from the top (using a pipette) after 2nd sedimentation step.

- **Proficiency samples**
  - Rinse the bag, in which the proficiency samples were delivered, carefully with water. Larvae might stick to the plastic.
  - Add the meat ball in one part. Do not chop up or mash the meat in the plastic bag. The meat shall be chopped in the baker with the digestion solution after added as whole meat ball.

ANNEX 3

Results of 1-4 sample panel digestion by the PrioCHECK® Trichinella AAD KIT (three *Trichinella* larvae per sample)

Panel of samples	**Lab 1 F/P[a]**	**Lab 2 F/P[a]**	**Lab 3 F/P[a]**	**Lab 4 F/P[a]**	**Lab 5 F/P[a]**	**Total F/P[a]**	**% of positive results**
1st panel	0/10	1/9	1/9	0/10[d]	3/7	5/45	90
2nd panel	6/4	1/9	2/8	0/10[d]	1/9	10/40	80
3rd panel	n.d.[c]	n.d.	n.d.	n.d.	2/8	2/8	80
4th panel	0/10	0/10	0/10	0/3[d]	5/5	5/38	88
Total	6/24	2/28	3/27	0/23	11/29	22/131	86
% of positive results	80	93	90	100	72.5	85	-
undigested material ≥ 5 g[b]	0/30	0/30	0/30	0/23	0/40	0/153	-

[a] F/P = fail/pass according to the Guidelines, i.e. 1-3 undetected larvae/at least one larva is detected;
[b] according to the Guidelines and the Commission Regulation 2075/2005, the undigested material on the sieve should be <5 g;
[c] n.d. = not done;
[d] after sedimentation, a sample of 70-80 ml (instead of 40-60 ml as suggested in the instruction manual) has been collected.

ANNEX 4

Results of the fifth sample panel digestion by the PrioCHECK® Trichinella AAD KIT (three *Trichinella* larvae per sample)

Panel of samples	**Lab 1 F/P[a]**	**Lab 2 F/P[a]**	**Lab 3 F/P[a]**	**Lab 4 F/P[a]**	**Lab 5 F/P[a]**	**Total F/P[a]**
5th panel	n.d.[c]	0/30	0/11	n.d.[e]	n.d.	0/41
% of positive results	-	100	100	-	-	-
undigested material ≥ 5 g[b]	-	0	0	-	-	-

[a] F/P = fail/pass according to the Guidelines, i.e. 1-3 undetected larvae/at least one larva is detected;
[b] according to the Guidelines and the Commission Regulation 2075/2005, the undigested material on the sieve should be <5 g;
[c] n.d. = not done;
[d] after sedimentation, a sample of 70-80 ml (instead of 40-60 ml as suggested in the instruction manual) has been collected;
[e] The fifth panel has not been sent to Lab 4, since this lab had already obtained 100% of positive results for the previous 3 sample panels tested. The fifth panel has not been sent to Lab 1 and Lab 5, since three (Labs 2, 3 and 4) had obtained positive results.

ANNEX 5

Revised Instruction manual of the PrioCHECK® Trichinella AAD KIT

PrioCHECK® Trichinella AAD

Artificial digestion test for *in vitro* detection of *Trichinella spp.* larvae in meat samples

Kit for 10 assays/ up to 1500 individual animals
©Prionics AG

Version 1.1_e

Package Insert

For *in-vitro* veterinary diagnostic use only
Store at 0-25°C
Product No.: 7620030

Introduction

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The artificial digestion method is the procedure currently recommended to detect *Trichinella* larvae in meat. According to the EC Regulation 2075/2005 each individual animal used for human consumption need to be checked for the presents of *Trichinella* spp. Larvae. Therefore, meat of individual animals are pooled and checked for the presence of *Trichinella* larvae by artificial digestion.

The PrioCHECK® Trichinella AAD is a new alternative artificial digestion method. It is a reliable and fast diagnostic test for detection of *Trichinella* larvae in meat and can be used for individual carcasses testing.

Test Principle

Meat, e.g. diaphragm, is pooled in pieces up to 115 g. Digestion Buffer and Enzyme Solution are added to the meat pieces and digested at 60°C. After subsequent sedimentation and washing steps, the solution is checked for the presents of larvae under a trichinoscope or stereo-microscope (15 - 20 times magnification).
The PrioCHECK® Trichinella AAD uses a serin-endopetidase of the enzyme group subtilisine and no hydrochloric acid is used making the method more confinement than the traditional artificial digestion method.

Kit Components

Store kit at 0-25°C until expiry date. See kit label for actual expiry date. The shelf life of diluted, opened or reconstituted components is noted below, where appropriate. Chemical hazard data are available in section "Safety Regulations and R&S Statements" (Appendix IV).

Component 1
Digestion Buffer (20x)
Two bottles containing 500 ml Digestion Buffer.
Shake slightly before use.

Component 2
Enzyme Solution (Ready-to-use)
1 bottle containing 500 ml Enzyme Solution.

Component 3
Digestion Buffer Additive
One bottle containing 15 ml Digestion Buffer Additive

Additional Kit Contents:

Additional Material Required

General:
Laboratory equipment according to national safety regulations.

- Water
- Graduated cylinder
- Glass beaker, 3 liter
- Pipette
- Thermometer accurate to 0,5°C within the range of 1°C to 100°C
- Stainless steel mesh, mesh size 180 microns

Special Material
- Separation funnel according to Squibb; Pyrex; Capacity: 2000mL; Plug stopcock No.: 6; Standard taper Stopper No.: 38; With polyethylene stopper and PTFE stopcock
  e.g. Fisher Scientific , Catalog No.: 10-437-5F
- Glass cylinder or glass test tube, capacity 80 ml, diameter x length: 40 x 115 mm

Sample preparation:
- Blender

Analysis of Results:
- stereo-microscope at a 15 to 20 times magnification or -trichinoscope
- Petri dish with grid

Test Procedure

Precautions
National guidelines for working with animal samples must be strictly followed. The PrioCHECK® Trichinella AAD must be performed in laboratories suited for this purpose.
Samples should be considered as potentially infectious and all items which contact the samples as potentially contaminated.

Chemical hazard data are available in section "Safety Regulations and P&L Statements" (Appendix IV).

Notes

To achieve optimal results with the PrioCHECK® Trichinella AAD, the following aspects must be considered:

- **The Test Procedure protocol must be strictly followed.**
- Kit components of different kit lot numbers must not be used together.
- The digestion solution must have 60°C ± 2°C

SAMPLE PREPARATION

- Using a knife or scissors and tweezers for cutting specimens

SAMPLE PREPARATION:

Preparatory Steps
- Cutting specimens using a knife or scissors.
- Pooling samples up to 115 g.
  E.g. for pork: diaphragm up to 115 pieces of 1 g is pooled. Please review to official Regulation EC 2075/2005 for details of individual species.

Note: Recommended is the use of 100 g to115 g tissue for one pool. If less than 100 g tissue is used, the volumes of the Digestion Buffer working solution, Enzyme Solution and the rinsing volumes are adapted accordingly. Do not use less than 10 g of tissue.

ARTIFICIAL DIGESTION

- Provide 2 l of Digestion Buffer 1x (100ml ± 5 ml Digestion Buffer 20 x + 1900 ml water) in in the digestion vessel (e.g. a beaker)
- Add 0.5-1ml of Digestion Buffer Additive
- Add 50 ml ± 2 ml Enzyme Solution
- Heat up to a temperature of 60°C ± 2°C.
- Add the chopped meat to the digestion solution and wash out all the chopped meat with Digestion Buffer working solution.
- Incubate for 20 min ± 2 min at 60°C under strong stirring conditions (≥750 rpm). Insulate the digestion vessel to prevent heat loss e.g. by using aluminum foil.

  Note: During stirring, the digestion fluid must be rotated at a sufficiently high speed to create a deep whirl without splashing. Make sure that stirring is sufficient to prevent any accumulation of meat. This will lead to an incomplete digestion.

- The digestion fluid is poured through the stainless steel mesh into the separation funnel (see specification under section 'Additional Material Required').
  Rinse the beaker properly with water (max. volume of 300 ml). Pour the water through the stainless steel mesh into the separation funnel.
- Accurately rinse the stainless steel with water using a squeeze bottle.

  Note: The Digestion is considered satisfactory if not more than 5 % of the starting sample weight remains on the sieve.

- Check that the separation funnel is leveled vertically
- Let the digestion fluid stand for 30 min ± 2 min in the separation funnel for sedimentation.
- After sedimentation a sample of 75 ml digestion fluid is quickly run off into the cylinder (see specification under section 'Additional Material Required').
- Allow the suspension to stand for 10 min ± 1 min for sedimentation.
- Discard 65 ml of the supernatant by carefully sucking off the upper layer. Leave a volume of not more than 10 ml the glass cylinder or glass test tube .

DETECTION

- Pour the remaining 10 ml into a larval counting basin or Petri dish. Rinse the glass cylinder or glass test tube with not more than 10 ml of water which has to be added to the sample in the larval counting basin or Petri dish.

- Subsequently the sample is examined by trichinoscope or stereo-microscope at a 15 to 20 times magnification.

  Note: In case of a suspect area or parasite like shape, a higher magnification of 60 to 100 times must be used.

  Remark: The recovered larvae are killed during the procedure. No life larvae are recovered! The DNA of the isolated larvae is intact and the determination of the species can be done by using standard techniques.
  After parasite collection, positive fluids (digestive juice, supernatant fluid, washings, etc.) do not need to be decontaminated by heating since larvae are dead because the digestion test is performed at 60 °C± 2 °C.

RESULT INTERPRETATION

If larvae are found during examination under trichinoscope or stereo-microscope the sample is considered positive.
Further investigations are required: Identification of the larva species by PCR technique.

Note: Detected larvae might be sent to the National Reference Laboratory for Trichinella for their identification at the species level.

If no larvae are found during examination under trichinoscope or stereo-microscope the sample is considered negative.

Appendix I

Notice
This manual is believed to be complete and accurate at the time of publication. In no event shall Prionics AG be liable for incidental or consequential damage in connection with or arising from the use of this manual.

Liability
Prionics AG warrants its products will meet their applicable published specification when used in accordance with their applicable instructions and within the declared products life time. Prionics AG makes no other warranty, expressed or implied. There is no warranty of merchantability or fitness for a particular purpose. The warranty provided herein and the data, specifications and descriptions of Prionics AG products appearing in Prionics AG published catalogues and product literature may not be altered except by express written agreement signed by an officer of Prionics AG. Representation, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.
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Prionics AG is an ISO 9001:2000 certified company.

Appendix II

Safety Regulations and P&L Statements

National Safety Regulations must be strictly followed.

P&L Statements

Component 1
Digestion Buffer (20x)
Xi; Sensitising
Hazard Code: R43: May cause sensitisation by skin contact.

Component 2
Enzyme Solution (Ready-to-use)
Xi: Irritant
Xn; Sensitising
Hazard Code: R37/38-41: Irritating to respiratory system and skin. Risk of serious damage to eyes.
R42: May cause sensitisation by inhalation.

Component 3
Digestion Buffer Additive (Ready-to-use)
The product does not have to be labeled due to the calculation procedure of the "General Classification guideline for preparations of the EU" in the latest valid version.

Contact

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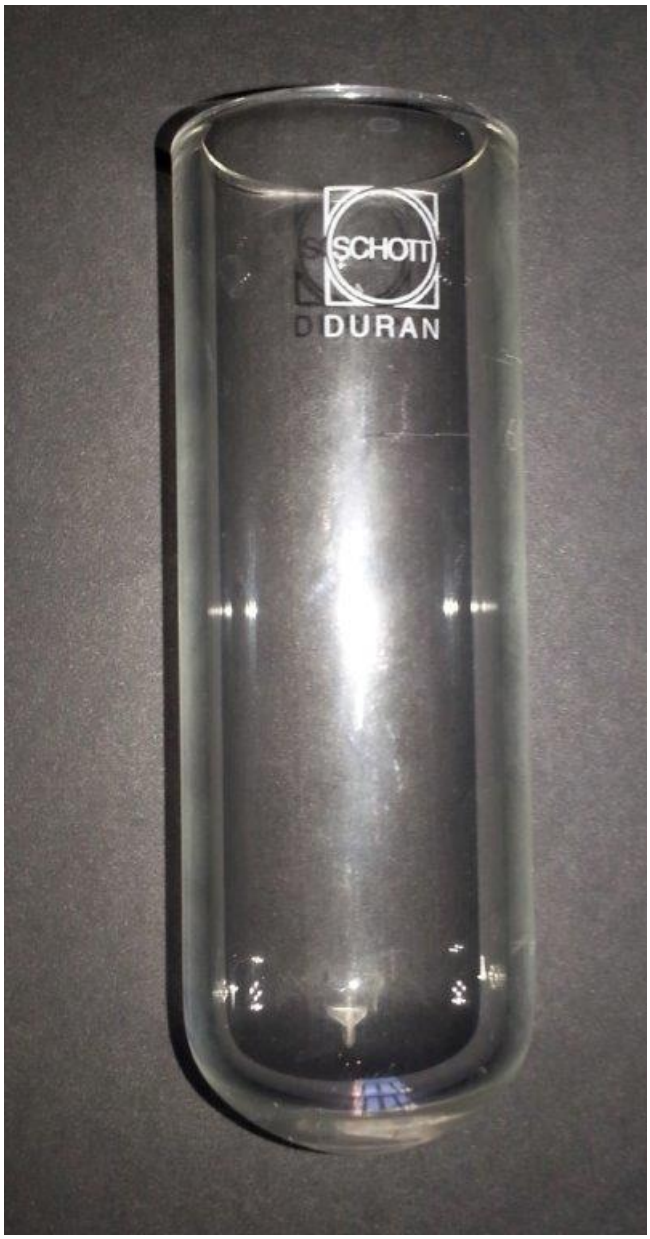
ANNEX 6

Separatory funnel and test tube supplied by Prionics with the PrioCHECK® Trichinella AAD KIT

Separatory funnel



Glass test tube 80 ml