B. INTERPRETATION OF RESULTS

For the interpretation of results, an **IRPC** (Relative Index x100) value of each sample is required. The following formula is applied to obtain the IRPC value (using mean OD_{405} values obtained with the repetitions of Positive Control):

$$IRPC = \left[\frac{((OD_{405}\ Sample\ (+)) - (OD_{405}\ Sample\ (-))}{((Mean\ OD_{405}\ Control\ +\ (+)) - (Mean\ OD_{405}\ Control+\ (-))} \right] x\ 100$$

Where (+) Indicates odd well and (-) indicates even well.

IRPC VALUE	RESULT
LESS THAN 30	**Negative**
EQUAL OR GREATER THAN 30 AND LESS THAN OR EQUAL TO 35	**Uncertain**
GREATER THAN 35	**Positive**

TEST DEVELOPMENT

1.		1/40 - 50 µl/well (x2: odd well(+) and even well (-))
2.		+36 ºC - +38 ºC / 60 minutes
3.		3 times
4.		Conjugate Solution - 50 µl/well
5.		+36 ºC - +38 ºC / 60 minutes
6.		3 times
7.		Substrate Solution - 50 µl/well
8.		+20 ºC - +25 ºC (Room Temperature) 15 minutes
9.		Stop Solution - 50 µl/well
10.		405 nm

CIVTEST®

LABORATORIOS HIPRA, S.A. Avda. la Selva, 135 . 17170 Amer (Girona) Spain
Tel. (34) 972 43 06 60. Fax (34) 972 43 06 61. hipra@hipra.com

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CIVTEST® SUIS MHYO

Detection and quantification of porcine antibodies against *M. hyopneumoniae*, by indirect ELISA

PRINCIPLE OF TEST

The **CIVTEST® SUIS MHYO** is a test based on a two-well indirect enzyme immunoassay (ELISA). The columns of the kit are coated alternately with an antigen enriched in *M. hyopneumoniae* antigen-determinats (odd columns, marked "+") and a generic *Mycoplasma* antigen (even columns; marked "-"). Thus, each sample should be assayed in two adjacent wells, one from an odd column (+) and another from an even column (-). During the incubation of a positive sample in the well, *M. hyopneumoniae* specific antibodies bind to the antigen adsorbed to the odd-well (+) and also generic antibodies could bind to the antigen present in the even-well (-). These antibodies remain in the well after washing off the unbound material, then a conjugated is added that binds any attached swine antibody. After that, unbound conjugate is washed away and a peroxidase-specific chromogenic substrate is added to both wells. To read the test result the absorbance of the even well (-) must be subtracted from the absorbance of the odd well (+). The resulting absorbance value is proportional to the amount of *M. hyopneumoniae* specific antibodies present in each sample.

KIT COMPOSITION (ENOUGH FOR 225 TEST)

PRODUCT	QUANTITY
96-well microplates (divided into 2x8 well-strips) alternately coated with specific (odd columns; +) and generic (even columns, -) *M. hyopneumoniae* antigen.	**5**
Vial N°0: Washing Solution (10x).	**2x100 ml**
Vial N°1: Sample Diluent Solution (3x) containing green dye.	**100 ml**
Vial N°2: Conjugate Solution: MAb anti-porcine IgGs/HRPO conjugate solution ready to use containing red dye.	**30 ml**
Vial N°3: Substrate solution: ABTS solution. Ready to use.	**30 ml**
Vial N°4: STOP Solution: Oxalic acid solution. Ready to use.	**30 ml**
Vial N°5: Positive Control: Positive Control Serum.	**0.5 ml**
Microplate adhesive cover.	**5**
Kit insert.	**1**
Certificate of analysis.	**1**

The preservative used in the liquid reagents is ProClin 300.

Materials required but not provided.
+36 °C - +38 °C incubator, precision single and /or multichannel pipettes with disposable pipette tips, tubes or dilution plate for diluting samples, 96-well plate reader, distilled or deionised water and plate-washing device.

PRECAUTIONS FOR USERS

Carefully read this kit insert. Store all reagents between +2 and +8 °C (do not freeze). Unused strips must be stored between +2 and +8 °C sealed inside the plastic bag with silica gel as moisture can damage the plates. Even though the antigen has been inactivated during the manufacturing process, the antigen-coated plates should be treated as a potential source of *M. hyopneumoniae*. Do not expose substrate solution to strong light or any oxidising agents. ***ABTS substrates are very sensitive even to trace levels of contamination and should not therefore be returned to the bottle once removed. Approximately 25% overage is supplied to allow the removal of a little over the exact requirements.*** Do not use components past expiry date and do not intermix components from kits with different lot numbers. Careful pipetting and washing throughout the procedure are necessary to maintain precision and accuracy. Do not pipette by mouth. Use gloves during the process. The stop solution is an organic acid, which is toxic, and may be corrosive, handle with care. All waste materials must be properly decontaminated prior to disposal. For veterinary use only.

Unopened reagents, correctly stored, are stable until the expiry date printed in the external label.

TEST PROCEDURE

A. REAGENT PREPARATION

All reagents must be allowed to come to room temperature before use.

Washing Solution (10x) (Vial N°0): To reconstitute add 1 volume of Washing Solution concentrate to 9 volumes of **distilled or deionised water** (e.g. to prepare 200 ml of reconstituted Washing Solution, mix 20 ml of the concentrate solution with 180 ml of **distilled or deionised water**). We recommend that this should be used within 7 days.

Sample Diluent Solution (3x) (Vial N°1): To reconstitute add 1 volume of Sample Diluent Solution concentrate to 2 volumes of **distilled or deionised water** (e.g. to prepare 60 ml of reconstituted Sample Diluent Solution, mix 20 ml of the concentrate solution with 40 ml of **distilled or deionised water**). We recommend that this should be used within 2 days.

NOTE: Crystals may form in the washing and sample diluent solutions due to the high concentration of salts. If reconstituting the whole volume, the bottle should simply be repeatedly inverted prior to the reconstitution step. If you need to reconstitute only a portion of the volume (to be used for testing) is necessary to ensure that the crystals have been completely redissolved before preparing dilution. To speed up the process can submerge the bottle in a bath at +28 - +37 °C for 10-15 min. To avoid crystal formation in the concentrated 10x wash solution It can be stored at room temperature after the first precipitated resuspension.

B. SAMPLE PREPARATION

Both the positive control and the rest of the samples should be diluted 1/40 in Sample Diluent Solution (1x). In all cases should be dispensed 50 µl of this 1/40 dilution in two adjacent wells of the plate, one corresponding to an odd column (+) and another corresponding to an even column (-).

Two alternative procedures are suggested for dilution:

(1) If you have 96-well U-bottomed serology plates and multichannel pipette capable of delivering precise volumes of 5.0 µl you can perform dilution 1/40 directly on the serology plate. To do this, mix 5.0 µl of sample and 195.0 µl of Sample Diluent Solution (1x) in the serology plate and then transfer 50.0 µl to each of the two selected wells on the ELISA plate. **The Positive Control should be treated the same way but will be tested in duplicate.**

(2) If you have 96-well U-bottomed serology plates and a multichannel pipette capable of delivering precise volumes of 10.0 µl **is recommended to perform the 1/40 dilution in two steps as follows: (a)** For each serum dispense 70.0 µl of Sample Diluent Solution (1x) in a well of the serology plate and 40 µl of Sample Diluent Solution (1x) to two adjacent wells of the ELISA plate (one (+) and one (-)). **(b)** Transfer 10.0 µl of sample to the 70.0 µl of Sample Diluent Solution in the serology plate (dilution 1/8; shake pipetting up and down 3 or 4 times). **(c)** With the same tip, then transfer 10 µl of this 1/8 dilution to the odd well (+) of the ELISA plate that already contains 40 µl of Sample Diluent Solution (1x). **(d)** Then, do the same with the even well (-). **Following this protocol you will consume a single pipette tip for each sample. The Positive Control should be treated the same way but will be tested in duplicate.**

C. TEST DEVELOPMENT

A. Allow the reagents to come to room temperature and ensure adequate mixing by swirling or inversion.
B. Record sample and control locations on a 12x8 template sheet. **Each plate or test must include two positions for the Positive Control and one position for the Control Diluent (+ and – wells filled only with Sample Diluent Solution (1x)).**
1. Remove the adhesive cover from the plate and add: **(a) 50 µl of the 1/40 diluted Positive Control, (b) 50 µl of Sample Diluent Solution (1x) for the Control Diluent wells and (c) 50 µl of the 1/40 diluted samples to the appropriate wells. Remember that for every sample or Control should dispense two adjacent wells (+ and -).**(See Section B. Sample Preparation).
2. Cover the plate with the adhesive cover and **incubate 60 minutes at +36 °C - +38 °C.**
3. Remove the adhesive cover and **wash the plate 3 times** with reconstituted Washing Solution (300 µl per well). Invert and firmly tap dry on absorbent paper.
4. **Add 50 µl of Conjugate Solution (Vial N°2)** to each well.
5. Cover the plate with the adhesive cover and **incubate 60 minutes at +36 °C - +38 °C.**
6. Remove the adhesive cover and wash the plate 3 times with reconstituted Washing Solution (300 µl per well). Tap dry as above.
7. **Add 50 µl of Substrate Solution (Vial N°3)** to each well. Shake gently the plate for 2 seconds.
8. Develop the chromogenic reaction for **15 minutes at room temperature (+20 - +25 °C)** in the dark.
9. **Add 50 µl of Stop Solution (Vial N°4)** to each well. Mix by gently tapping the side of the plate.
10. Wipe the under-surface of the plate free of dust etc. with a soft tissue. **Read** the plate using a Microtiter Plate Reader **at 405 nm** having first blanked on air. Record the results.

READING RESULTS

A. TEST VALIDATION

The test is valid if:

OD_{405}*	VALUE
MEAN CTR+(+)	**>= 0.6**
(MEAN CTR+(+)) – (MEAN CTR+(-))	**>= 0.5**
(CONTROL DILUENT (+) – CONTROL DILUENT (-))	**< 0.1**

** (+) Indicates odd well and (-) indicates even well.*