

**TECHNICAL DOCUMENT**

**Guidance and protocol for  
the serological diagnosis  
of human infection with  
*Bordetella pertussis***

**As part of the EUpert-Labnet surveillance network**

**ECDC** TECHNICAL DOCUMENT

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This report was commissioned by the European Centre for Disease Prevention and Control (ECDC), coordinated by Adoracion Navarro Torne, and produced by the members of the European Bordetella expert group 'EUpert-Labnet' as part of the project contract: Coordination of activities for laboratory surveillance of whooping cough in Member States and EEA countries (OJ/26/05/2011-PROC/2011/037)

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This protocol is intended to serve as a starting point for laboratories aiming at introducing ELISA serology for diagnosis of human *Bordetella pertussis* infections. Comments, requests or questions can be addressed to the authors.

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Disclaimer: this technical guidance is based on the latest available published data on diagnostic test performance at the time of writing. ECDC does not endorse any particular commercial product or instrument.

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## Abbreviations

CofA	Certificate of analysis
CBER	Center for Biologics Evaluation and Research
ECDC	European Centre for Disease Prevention and Control
EDTA	Ethylenediaminetetraacetic acid
EEA	European Economic Area
EMA	European Medicines Agency
EU	European Union
ELISA	Enzyme-linked immunosorbent assay
EQA	External quality assessment
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IU	International unit
LOD	Lower level of detection
LOQ	Lower level of quantitation
NIBSC	National Institute for Biological Standards and Control (NIBSC)
PCR	Polymerase chain reaction
PT	Pertussis toxin
SOP	Standard operating procedure
SPC	Statistical process control
SMI	Swedish Institute for Communicable Disease Control
WHO	World Health Organization

# 1. Introduction

Whooping cough is a bacterial respiratory infection caused by *Bordetella pertussis*. It is characterised by paroxysmal cough, whoop and posttussive vomiting. Spread takes place through air droplets produced with cough or sneezes. The most severe disease occurs in infants and young children, and it is usually milder in adolescents and adults who constitute a reservoir and are a source of spread to young children. Pertussis remains endemic worldwide and tends to be a cyclic disease, peaking every 3–5 years.

Since 2011, increases in the number of cases of pertussis have been repeatedly reported in different regions of the world, even in those with sustained high vaccination coverage. In Europe, the situation evolves similarly with many countries observing an increment in cases, mostly in infants adolescents and adults.

The laboratory diagnosis of whooping cough proves cumbersome due to variations in specificity and sensitivity of the different methods. Differences in methods and protocols between European countries have been detected.

The European Centre for Disease Prevention and Control (ECDC) addressed the harmonisation and improvement of pertussis diagnosis needed for surveillance and outbreak detection/monitoring in order to assure quality and comparability of data. Therefore, in 2011 ECDC launched the project 'Coordination of activities for laboratory surveillance of whooping cough in Member States and European Economic Area (EEA) countries'. One of the main aims of this project was to produce a consensus document on laboratory guidance for the determination of serum IgG anti-PT antibodies to pertussis by enzyme-linked immunosorbent assay (ELISA) serology for the European laboratories. The review of the scientific literature updates together with the above mentioned research work constitute the basis of the present technical report. Much of the content of the guidance in this suggested protocol has been adapted from the previous work of the EUPertstrain group of European laboratories. The reference, on which this protocol is mainly based, was published as a consensus paper of the EUPertstrain group in 2011 [1].

## 1.2 Indications for serological testing

Diagnosis of pertussis should only be attempted in patients with symptoms compatible with pertussis, such as prolonged coughing with paroxysms and/or whooping or choking. In infants, older vaccinated children, adolescents and adults the clinical course may not be typical, and prolonged coughing may be the only symptom. In these cases, diagnosis of pertussis requires laboratory methods for confirmation.

Direct and indirect diagnostic tests are available. Direct tests are real-time polymerase chain reaction (PCR) and culture whereas serological tests measure specific antibodies.

## 2. Samples

Most serological assays are validated to test serum; some may also be validated to test heparinised plasma or Ethylenediaminetetraacetic acid (EDTA)-plasma. Capillary blood samples may be used if a sufficient volume cannot otherwise be obtained. Serum or plasma must be separated as soon as possible after blood sampling (up to 24h at room temperature). If acute and convalescent serum samples taken at least three weeks apart from one individual are available, they should be tested together in one run. All serum samples may be frozen (at -20°C) after the primary assay and reanalysed later, together with a possible second sample.

## 3. Laboratory qualification

### 3.1. Using commercially available ELISAs

Laboratories using a commercially available IgG-anti-PT ELISA need appropriately trained staff (technicians and laboratory supervisor) knowledgeable about all relevant issues of ELISA methodology. They should have experience of performing other tests in a comparable ELISA format, and have all the technical equipment needed. The ECDC pertussis expert group recommends that all diagnostic procedures have accreditation according to EN/ISO 15189 or an equivalent standard to ensure quality data for laboratory-based surveillance.

### 3.2. Establishing an in-house ELISA

In addition to the prerequisites described above, laboratories setting up an in-house ELISA should have extensive experience with all aspects of validation requirements for the different steps of ELISA methods. They should also be familiar with their local jurisdiction's requirements for in-house ELISA, fulfilling the regulations of the in-vitro diagnostic devices regulation (EU 98/79/EC)<sup>1</sup>. Concerning the validation of bioassays, various EU guidance documents can be obtained, for example the 'Guideline on bio-analytical method validation', issued by the European Medicines Agency (EMA)<sup>2</sup>.

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<sup>1</sup> Directive 98/79/EC of the European Parliament and of the Council of 27 October 1998 on *in vitro* diagnostic medical devices. Available here <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:1998:331:0001:0037:EN:PDF>

<sup>2</sup> Available here:

[http://www.ema.europa.eu/docs/en\\_GB/document\\_library/Scientific\\_guideline/2011/08/WC500109686.pdf](http://www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2011/08/WC500109686.pdf)



## 4. Reagents for ELISA

### 4.1 Antigens

According to the recommendations stated in a recent publication [1], ELISA should be done with purified pertussis toxin (PT). Different sources of purified PT are available, and PT from some of the sources have been compared and found to give similar results [2, 3]. When other sources of PT are being used, they must be validated as recommended by Kapasi et al [2] and Xing et al [3].

### 4.2. External reference sera

WHO references are available from the National Institute for Biological Standards and Control (NIBSC) ('World Health Organization International Standard (06/140)' and 'WHO Reference Reagent (06/142)') for the measurement of human antibodies to *B. pertussis* antigens, and thus quantitative results should be reported in IU/ml [4]. Standard Operating Procedures (SOP) are recommended to be developed for defining (and documenting) handling, storage, and usage of WHO reference serum, in which the procedure for the freezing of aliquots, the vial size and type and the storage temperature should be defined. A maximum storage time of frozen aliquots should also be defined.

### 4.3. Internal working reference

Laboratories should establish their own internal working reference preparations to give results according to the WHO reference preparation. The development of an SOP for this reference is also recommended defining (and documenting): handling; storage; and usage of the internal reference serum, in which the procedure for freezing of aliquots, the vial size and type, and the storage temperature should be defined. A maximum storage time of frozen aliquots should also be defined.

### 4.4. Plates

For in-house ELISAs there seemed to be differences in antigen binding properties between plates. In general, Thermo Scientific Nunc Maxisorp (Nunc AS, Copenhagen, Denmark) plates and Greiner Microlon High Binding (Greiner Bio-One, Frickenhausen, Germany) plates seemed to work well [1, 5].

### 4.5. Conjugates

Conjugates, i.e. enzyme linked anti-human IgG or anti-human IgA, should be selected according to its quality, and the experience of other laboratories. Many laboratories using in-house anti-PT ELISAs have found that conjugates from Kieerkegaard and Perry (KP), Bethesda, MD, USA may be suitable for these tests. Both alkaline-phosphatase and horseradish-peroxidase labelling are suitable.

### 4.6. Other reagents

All other reagents should be pure reagent-grade substances; for solution of reagents, laboratories should be especially careful about the quality and purity of the water used, e.g. reagent-grade water.

## 4.7. Qualification of reagents

### Coating antigens

Review the specific information (purity, endotoxin content etc.) that is provided by the supplier in the certificate of analysis (CofA). Verify that the CofA is complete and provides all the information needed. Specify tests that can confirm the purity of the antigens and consider tests that could be performed to confirm the purity, for example using monoclonal antibodies and/or sera with known titer (highs/lows) in each assay.

### Conjugates

Review and clarify the information that is provided by supplier in CofA. Assess whether this is adequate. Laboratories may also consider testing what could be performed to verify specificity and/or sub-class reactivity/and or cross reactivity.

## 4.8. Qualification of analysts

Internal SOP's should define how analysts are being trained in the ELISA procedure, who should train them, and who may give permission that an individual analyst can perform the assays themselves. Repeated trainings in ELISA methodology and pitfalls should be regularly performed and documented.

## 5. ELISA methodology

### 5.1. IgG-anti-PT ELISA SOP

Several principally similar SOP for IgG-anti-PT have been developed and published [6–8]. If laboratories want to implement the ELISA as an in-house method, they should decide which SOP to follow. Any of the authors can be contacted for advice and/or training.

### 5.2. Supporting SOPs

Define all necessary supporting SOPs as outlined above and below to keep the procedure well controlled.

### 5.3. Calculation of results

Develop SOPs to define the data reduction process that is used for calculating the results, i.e. parallel line assay, four-parameter logistic (4PL) calculation, or similar calculation. In the SOP, identify those approved to perform calculations, and those approved to check and verify calculations. Provide clearly defined procedure for dropping data points, and specify how many points can be excluded. Evaluate whether all data should be used, or only those data points in range between 10–90% (or 5–95%) of highest standard. Define criteria for assessing the validity of the standard curve on each plate. The procedure may include an assessment for parallelism between test and reference sera [9].

### 5.4. Statistical analysis

#### Lower level of quantitation

For in-house assays the lower level of quantitation (LOQ) and the lower limit of detection (LOD) should be defined. In the ELISA SOP, define how data between LOQ and LOD are being handled and procedures for reporting of values below the lower limit of quantitation should be described.

#### Linearity

A dilutional linearity assessment should be done to define range of assay, in which positive sera should be diluted in dilution buffer and assessment of whether samples and controls have the same slope.

#### Intra-assay and inter-assay variability

Precision should be evaluated by repeat analysis of samples that are comparable to the samples that will ultimately be tested in the assay.

#### Ruggedness

Data should be produced to verify the acceptability of approved ranges for time and temperature, which should be narrow but practical, as this tends to minimise the assays needed to verify acceptability.

## 6. Quality control

### 6.1. Internal quality control

Define the laboratory's own material for internal quality control and validate the material according to a separate SOP. A statistical review of available data that can assess assay stability over time is recommended, as well as approaches that could be used to monitor stability, such as using the relevant control sera in statistical process control (SPC) procedures. Using a serum proficiency panel to monitor stability is also recommended which may be used for training and qualification of technicians in addition to stability monitoring.

### 6.2. External quality assessment

Perform regular external quality assessment (EQA) schemes as these are now offered by many European quality assessment organisations.

### 6.3. Accreditation and CE marking

As all other diagnostic tests IgG- and IgA-anti-PT ELISAs should preferably be accredited according to EN 15189 or EN 17025 or equivalent standard, consider your own jurisdiction's requirements as for using in-house ELISA for quantifying antibodies for diagnostic procedures.

## 7. Commercially available IgG-anti-PT assays

Many commercial assays for measuring IgG anti-PT are available in the EU, and some of these have been compared and found to be suitable as compared to an in-house ELISA [10].

### 7.1. CE marking

All commercial assays that are distributed in the EU have to be CE marked. It must be noted, however, that CE marking does not mean that these assays have been validated to serve the clinical purpose to diagnose pertussis, and that assays with unsuitable antigen composition can also be CE marked according to the current legislation. The lack of technical specifications or stringent performance criteria may leave performance less than desirable, as the intended performance level is left to the manufacturer. Therefore, pertinent validation of commercial assays is recommended.

### 7.2. Validation procedure

The consortium of the new ECDC project EUpert-labnet recently decided that one group from EU reference laboratories (SMI, Stockholm, Sweden) may validate commercial assays as compared to its own in-house ELISA procedures. In addition to the published comparison [10], this is expected to provide additional guidance on the use of commercially available IgG anti-PT ELISA assays.

## 8. Reporting of serological results

Concentrations of antibodies to *B. pertussis* antigens should be quantitatively expressed in international units (IU/ml), as reference preparations are available. The numerical values of IU/ml are equivalent to the previously used ELISA units/ml (EU/ml) derived from the human reference preparations lot 3, lot 4, and lot 5 from the Center for Biologics Evaluation and Research (CBER/US Food and Drug Administration, Bethesda, MD, USA) [4].

## 9. Interpretation of serological results<sup>3</sup>

Pertussis toxin is contained in substantial amounts in all acellular vaccines licensed in Europe. Thus, the immune response against infection or vaccination cannot be distinguished and pertussis vaccination may interfere with the interpretation of serological results. Due to a continuous circulation of *Bordetellae* in the population, IgG-anti-PT are detectable in the majority of all adolescent and adult populations tested so far.

Measurement of IgG-anti-PT is not meaningful in neonates and young infants. It is of diagnostic value in older children, adolescents and adults. For adolescents and adults with coughing less than three weeks, PCR and measurement of IgG-anti-PT are recommended [1]. If coughing lasted at least 2–3 weeks, measurement of IgG-anti-PT is regarded to be sufficient. In outbreak situations, PCR should be used and IgG-anti-PT should be measured irrespective of duration of symptoms.

### 9.1. Dual-sample serology

Dual sample serology based on  $\geq 100\%$  increase in antibody concentration or on  $\geq 100\%$  decrease in antibody concentration is a sensitive and specific way for serological diagnosis [1, 7]. However, even in paired sera no antibody increase may be seen after infection due to the secondary immune response, and the diagnosis may also be based on a decrease of antibodies, which may be too slow to reach 50% between the acute and convalescent sample. Even if there is no  $\geq 100\%$  increase or decrease, if one of the samples shows antibody-levels above the cut-off for single sample serology (see section 9.2 below), the result can be interpreted as evidence of recent infection with *B. pertussis*.

### 9.2. Single sample serology

In clinical practice, diagnosis is mostly based on single sample serology using a single or a more continuous cut-off. For single-sample serology, various cut-off values for IgG-anti-PT have been proposed [1]. Comparison of ROC curve analyses with data from Denmark, the Netherlands, and the United Kingdom showed for all three countries that the single cut-off with optimal sensitivity and specificity may be in the range between 60 IU/ml and 75 IU/ml.

It may be sensible to use a dual cut-off between 62 and 125 IU/ml to define a recent infection for patients who were not vaccinated during the last twelve months. If diagnosis cannot be confirmed with certainty from a single serum, but is deemed to be necessary according to the clinical symptoms, antibodies should be measured in a second (convalescent) serum sample at two to four weeks interval. In case of non-availability of a second serum sample, measurement of IgA anti-PT antibodies can be an alternative, but no broadly accepted cut-off is available for this antibody subclass. Considering its relatively high specificity and low sensitivity, a cut-off near the minimal level of quantification, which may be between 10 IU/ml and 20 IU/ml, may seem reasonable.

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<sup>3</sup> Text adapted from 1. Guiso N, Berbers G, Fry NK, He Q, Riffelmann M, Wirsing von König CH. What to do and what not to do in serological diagnosis of *pertussis*: recommendations from EU reference laboratories. Eur J Clin Microbiol Infect Dis. 2011;30:307-12.

# 10. Protocol

## IgG-anti-pertussis toxin ELISA for serological diagnosis of pertussis

### Intended use

The test measures IgG -antibodies to pertussis toxin (PT) of *Bordetella pertussis* in human serum samples. The results are expressed in International Units/ml (IU/ml) related to a reference preparation. Suggestions for interpretation are given in a recent publication [1].

Due to the specificity of IgG antibodies to PT for diagnosing pertussis, these antibodies are measured primarily. In cases where the IgG anti-PT level does not allow a diagnostic interpretation, IgA antibodies against PT can be measured.

Diagnostic pertussis serology has to be interpreted with great caution within one year after vaccination with pertussis vaccines due to cross-reactions to vaccine-induced antibodies.

### Summary of the tests

The test is a 'sandwich-ELISA' in which a solid phase (Microtiter plate) is coated with the PT antigen. If the sample contains antibodies, they will bind to PT while unbound serum is washed away. A secondary antibody to human IgG is added that reacts with the antibodies bound to PT. These antibodies are conjugated to an enzyme. Unbound secondary antibody is washed away, and the activity of the antibody-bound enzyme is measured by addition of an enzyme substrate. The content of antibodies is expressed in relation to a standard curve constructed from a reference preparation. Results are estimated by a four-parameter logistic calculation and expressed as IU/ml.

### Material

Serum (human) or also plasma (EDTA or heparin), minimal volume 50 µl. Diagnostic samples and controls must be the same type of serum or plasma.

## Apparatus, reagents and consumables

### Apparatus

Immunoplate I Microtiter plates (MaxiSorp, Nunc, Wiesbaden)

Microplate Washer

Refrigerator (+4°C)

Incubator (+37°C)

Microplate Reader

### Reagents

#### Primary reagents

Pertussis toxin (native, not detoxified), aliquotted. Purified PT is available from many sources, e.g. Statens Serum Institut (SSI DIAGNOSTICA), List Biological Laboratories Inc, Protein Express Inc, GlaxoSmithKline Biologicals and Sanofi Pasteur. The toxin must be stored according to the manufacturer's instructions.

- bovine serum albumin (BSA) (reagent grade)
- skim milk powder (reagent grade)
- TWEEN 20
- glycerol (reagent grade)
- antibodies (goat) to human IgG, affinity chromatography purified, alkaline phosphatase (Kirkegaard and Perry, Order # Nr. 075-1002, Medac, Wedel, Germany)
- Alkaline phosphatase DGKC (DaiSys, Diagnostic Systems GmbH, Holzheim, Germany) + substrate



### Secondary reagents

- Carbonate buffer 50 mM, pH 9,6
  - 1,59 g Na<sub>2</sub>CO<sub>3</sub>
  - 2.9 g NaHCO<sub>3</sub>
  - in 1.0 l Aqua dest.
- Phosphate buffered NaCl (PBS), 0.85%, pH 7.2 (ready for use):
  - 4.25 g NaCl
  - 1.12 g KH<sub>2</sub>PO<sub>4</sub>
  - 4.47 g Na<sub>2</sub>HPO<sub>4</sub> x 2 H<sub>2</sub>O
  - in total 400 ml Aqua dest.
- Bovine serum albumin (BSA)-Stock 5 %:
  - 2.5 g is dissolved in 50 ml PBS
- Buffer for incubation and dilution [PBS with 0.2 % BSA (w/v) and 0.05 % TWEEN 20 (v/v)]:
  - 480 ml (PBS) ready to use
  - 20 ml Bovine serum albumin (BSA)-Stock dilution 5 %
  - 250 µl Tween 20
- Washing buffer [PBS with 0.05 % TWEEN 20 (v/v)]:
  - 500 ml PBS
  - 250 µl Tween 20
- 1 M NaOH (reagent grade)
- Conjugate: One bottle of lyophilized conjugate is dissolved in 1ml glycerol solution (1 ml Glycerol plus 1ml distilled water.).
  - conjugate dilutions ready for use: in incubation buffer, must be checkerboard titrated for the actual lot of conjugate antibodies.

### Standards and controls

#### Standards:

In-house secondary standard related to WHO International Standard (06/140) (NIBSC, Potters Bar, UK) [4].

Reference serum is dissolved in 1 ml distilled water and frozen in 100 µl aliquots. Before use it is 1:100 diluted.

The related WHO reference contains:

- PT-IgG 335 IU/ml
- PT-IgA 65 IU/ml

#### Control:

NIBSC Working-Reference serum (is used as control).

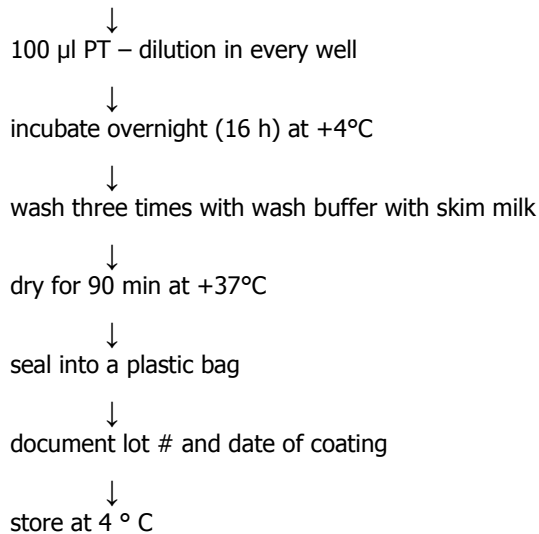
The reference serum is dissolved in 1 ml distilled water and frozen in 100 µl aliquots. This working reference contains:

- PT-IgG: 106 IU/ml (94–118)
- PT-IgA: 18 IU/ml (13–31)

## Procedure

### Coating of plates

PT is diluted in carbonate buffer to give 1 µg/ml



(maximal storage time: PT-plates one month)

### Preparation of plates and samples

- Plates are brought to room temperature one hour before use or washed immediately and one hour with 50 µl of incubation buffer.
- Plates are washed three times with washing buffer.
- Produce primary dilution from samples, controls and standards with incubation buffer:
  - standard
  - patient sera
  - positive control
    - IgG-antibodies: 10 µl sample + 1000 µl incubation buffer
  - negative control can be also included
- Sequential dilutions are done in the microtiter plate:
  - patient samples and control:
    - first well (A / E) 200 µl primary dilution, next three wells (B-D / F-H) 100 µl buffer
    - transfer 100 µl sequentially from first well to next well and mix by pipetting
    - discard 100 µl from last well
  - standard:
    - first well (A) 200 µl primary dilution, next seven wells (B-H) 100 µl dilution buffer
    - transfer 100 µl sequentially to next well and mix by pipetting
    - discard 100 µl from last well

**Running the test**

Plates are filled with 100 µl material according to the following scheme:

Row 1 Well A, B: nothing (Blank)

Row 1 Well C, D: buffer, and antiserum (Ab-control)

	<b>IgG</b>
Row 1 Well E: positive control	1:100
Row 1 Well F: positive control	1:200
Row 1 Well G: positive control	1:400
Row 1 Well H: positive control	1:800

	<b>IgG</b>
Row 2 Well A: Standard	1:100
Row 2 Well B: Standard	1:200
Row 2 Well C: Standard	1:400
Row 2 Well D: Standard	1:800
Row 2 Well E: Standard	1:1600
Row 2 Well F: Standard	1:3200
Row 2 Well G: Standard	1:6400
Row 2 Well H: Standard	1:12800

<b>Patient Nr.</b>	<b>IgG</b>
Row 3 Well A: #1	1:100
Row 3 Well B: #1	1:200
Row 3 Well C: #1	1:400
Row 3 Well D: #1	1:800

Rows 4–12 as Row 3 with four wells in different dilutions for each patient sample

- ↓
- Plates are sealed and are incubated for 60 min at 37° C
- ↓
- Plates are washed five times with washing buffer
- ↓
- 100 µl of enzyme marked conjugate (anti-IgG) is filled into all wells except A1 and B1.
- ↓
- Plates are sealed and are incubated for 60 min at 37°C
- ↓
- Plates are washed five times with washing buffer
- ↓
- Add 100 µl freshly prepared AP-reagent (p-nitrophenylphosphate) to all wells
- ↓
- Plates are incubated at room temperature for 30 min in the dark.
- ↓
- Enzyme reaction is stopped by adding 100 µl of 1M NaOH
- ↓
- Plates are measured in a micro plate reader with the respective program at 405 nm–650 nm. Apply a four-parameter logistic to do a standard curve.

## Results

### Run validation

All runs are technically validated according to the following criteria:

- Reagent control (Mean) (Blank) (A1, B1) must be <0,200
- Antibody control (Mean) (C1, D1) must be <0.150
- Standard curve must have a fit of  $r=1.00$  or  $0.99$
- A curve fit of  $r = <0,99$  may be corrected by omitting one or two outliers by the laboratory supervisor
- Results of control must be :
  - PT-IgG: 106 IU/ml (range: 94–118)

### Results

- According to the standard curve, the amount of antibodies is estimated for every sample as anti PT in IU/ml
- No antibodies detected; < 2 IU/ml
  - sera with an OD of less than the antibody control are regarded as negative
  - sera with a calculated concentration of <2 IU/ml are regarded as negative.
- Values that are not used for calculation:
  - OD beyond the highest or lowest OD of the standard curve.
- A value in IU/ml is calculated as a mean from the remaining values. This mean is reported in the protocol and transferred to the patient report.

### Reference ranges

Reference ranges are age-dependent.

Reference ranges are dependent on vaccination status. Pertussis serology cannot or can only be very carefully interpreted until one year after a vaccination with acellular pertussis vaccine.

### Interpretation

When interpretation is not possible, a second serum sample should be obtained two to four weeks later and analysed in one run with the first sample.

If no second sample can be obtained [11], IgA-anti-PT may additionally be measured.

Guidance regarding cut-off values are presented in the introductory text.

Results are clinically validated by the laboratory supervisor.

### Limits of the procedure

#### Sensitivity

Analytical sensitivity (lower limit of detection) was estimated to be around 2 IU/ml for the antibodies tested. The lower limit of detection may also depend on the lot of antigen used.

Diagnostic sensitivity was estimated between 85% and 93% according to age groups.

#### Specificity

Diagnostic specificity was found to be ~ 95% in unvaccinated children.

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