

PROTOCOL	VERSION
DETECTION OF <i>BORDETELLA PERTUSSIS</i> VACCINE ANTIGENS (PT, PRN, FHA, FIM2, AND FIM3) BY ELISA	A

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This ELISA protocol has been optimized based on the method described by Barkoff *et al.* (<https://pubmed.ncbi.nlm.nih.gov/24925807/>). It aims to measure the expression level of *Bordetella pertussis* vaccine antigens (PT, FHA, RPRN, FIM2 and FIM3) from bacterial suspensions inactivated using commercial antibodies specific to the studied antigens.

1. MATERIALS

For information:

Designation	Brand	Reference
Immuno 96-well non-sterile transparent flat bottom plates Nunc Maxisorp (polystyrene, high bond)	Thermo Scientific	439454
Sterile SealPlate ClearLine® Film, natural colour for 96-plate	Dutscher	760201
Thermo Scientific™ Multiskan™ FC 96 Plate Microplate Photometer	Thermo Scientific	11590685
Cuvette Optical Density (OD) Reader (Thermo Scientific™ Biomate 3S Spectrophotometer)	Thermo Scientific	/
Single and multi-channel pipettes (with filter cones)	Sartorius	/
Standard sterile white polystyrene tank 50 mL	ClearLine	097802
Low Protein Binding Collection Tubes, 1,5 mL	Fisher	15342617
Thermo Scientific™ Wellwash™ Microplate Washer	Thermo Scientific	11708016

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2. SOLUTIONS AND REAGENTS

For information:

Designation	Storage	Brand	Reference
Regilait skimmed milk	Room temperature	Dutscher	731193
Physiological water	Cold room	/	/
Distilled water	Room temperature	/	/
Phosphate buffered saline solution (PBS)	Room temperature (to be used sterile)	SIGMA	P4417 (tablets)
Tween 20 - Polysorbate 20	Room temperature (to be used sterile)	Fisher BioReagents	BP337-500
TMB substrate for antibody HRP - TMB substrate set	Refrigerator	BioLegend	421101
Solution Stop TMB - Solution for TMB Substrate	Refrigerator	BioLegend	423001

Recommendations:

- 1) **The filtered PBS 1X can be kept for 1 month.**
- 2) **Attention the Tween 20 is a very viscous detergent. Keep away from light.**
- 3) **The working solution of the TMB substrate is a volume-to-volume mixture of the 2 substrates of the kit (TMB Substrate A and 110 ml TMB Substrate B). Prepare only the volume needed. Avoid exposing the solution to light.**
- 4) **All solutions (or reagents) should be handled at room temperature in order not to slow down the reaction. Remove solutions and reagents from the refrigerator 30 min before each use.**

Preparation of solutions and reagents:

1) **PBS:** Put a tablet of **PBS** in 200 mL of distilled water and filter.

Store at $+4 \pm 5^{\circ}\text{C}$ for a maximum of one month.

For the treatment of a 96-well plate, plan for 50 mL of PBS 1X.

2) **Washing buffer:** Physiological water - 0.05% Tween.

Mix 0.5 mL of Tween 20 in 1000 mL physiological water.

For the treatment of a 96-well plate, plan for 250 mL of wash buffer.

4) **Blocking/saturation buffer (TB):** 5% skim milk in PBS.

Mix 5 g of skim milk q.s.p. 100 mL of PBS 1X.

For the complete treatment (saturation, primary Ab, secondary Ab) of a 96-well plate, plan 50 mL of TB.

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3. PURIFIED ANTIBODIES, PROTEINS AND REFERENCE STRAINS

3.1. PRIMARY ANTIBODIES

Antibody (Ab)	Reference and origin	Target	Dilution in TB
Anti-PT S1	Anti-PT S3 subunit monoclonal antibody (10D6) 99/512* (mouse) - NIBSC	pertussis toxin S1 subunit	1 / 1 000
Anti-PT S3	Anti-PT S3 subunit monoclonal antibody (7E10) 99/542* (mouse) - NIBSC	pertussis toxin S3 subunit	1 / 1 000
Anti-FHA	Anti-FHA monoclonal antibody (2E9) 99/572 (mouse) or Bordetella pertussis anti FHA serum (mouse) JN1H-11- NIBSC	FHA	1 / 2 000 (or 1/ 1 000 for JN1H-11)
Anti-PRN	Pertactin monoclonal antibody, clone 69K/16 (mouse) or Pertactin monoclonal antibody, Clone 3-5 (mouse) - NIBSC	PRN	1 / 1 000
Anti-FIM2	Monoclonal antibody for Serotyping Bordetella pertussis Fimbrial Antigen 2 06-124 (mouse) - NIBSC	FIM2	1 / 10 000
Anti-FIM3	Monoclonal antibody for Serotyping Bordetella pertussis Fimbrial Antigen 3 06-128 (mouse) - NIBSC	FIM3	1 / 10 000

*A mixture of the two monoclonal antibodies 99/512 and 99/542 is used.

For the treatment of a complete 96-well samples plate, provide 2 mL of primary Ab diluted in TB for each of the antibodies.

For the treatment of a 96-well standard range plate, provide 2.5 mL of primary Ab diluted in TB for each of the antibodies.

Recommendation:

Keep the Ab in the ice. Gently mix them by hand then quickly centrifuge with the minispin.

3.2. SECONDARY ANTI-MOUSE ANTIBODY

Antibody	Reference	Dilution in TB
Anti-Mouse IgG (γ-chain specific) - Peroxidase antibody produced in goat, affinity isolated antibody, buffered aqueous solution	A3673 - SIGMA	1/1 000

For the treatment of a 96-well plate, allow 10 mL of the 1/1 000 dilution of secondary Ab (by mixing 10 µL of Ab in 9 990 µL of TB).

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3.3. POSITIVE CONTROLS

3.3.1. Preparation of purified protein standards

Purified antigens (Ag)	Reference and origin	Initial concentration (µg/mL)
PT	10/158 or 15/126 - NIBSC	50 µg/mL or 20 µg/mL
FHA	10/160 or 90/520 - NIBSC	50 µg/mL or 20 µg/mL
PRN	10/156 or 18/154 - NIBSC	50 µg/mL or 20 µg/mL

Dilute each antigen at 1.0 µg/mL in PBS 1X (1/50 dilution).

For each antigen (Ag), make the series dilutions to 1:2 directly in plate 96 wells.

1. Add 100 µL of 1X PBS to each well in columns 2 to 12.
2. Add 200 µL of each Ag, previously diluted to 1.0 µg/mL, to the wells in column 1 (in duplicate).
3. Transfer 100 µL from column 1 to column 2 to perform the first dilution, and continue with the serial 1:2 dilutions across the following columns. After mixing in column 12, discard 100 µL from the last wells.

Attention, the final volume of each well must be 100µL.

Well	Calibration range of concentration in µg/mL
1	1.0µg/mL
2	0.5µg/mL
3	0.25µg/mL
4	0.125µg/mL
5	0.0625µg/mL
6	0.03125µg/mL
7	0.015625µg/mL
8	0.0078125µg/mL
9	0.00390625µg/mL
10	0.00195313µg/mL
11	0.00097656µg/mL
12	0.00048828µg/mL

Recommendation:

It is preferable to aliquot the mother solutions. Mix them very gently, then quickly centrifuge them with the Minispin. To avoid denaturing the Ag, keep them in ice for the time of handling.

3.3.2. Reference strain: *Bordetella pertussis* Tohama (OD_{650nm} = 0.2)

A volume V = 1 000 µL of the reference strain *B. pertussis* Tohama (CIP 81.32) suspension is first prepared in a calibrated manner (OD_{650nm} = 1).

This suspension is then diluted 1/5 to obtain a suspension at OD_{650nm} = 0.2.

Plan for 1000 µL for a 96-well plate.

Add the positive control as a duplicate for each virulence factor tested/plate.

List of *B. pertussis* strains recommended by the NRC as positive controls for the study of vaccine antigen production using ELISA or Western blot techniques:

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- *B. pertussis* CIP 81.32: PT positive, PRN positive, FHA positive, FIM2
- *B. pertussis* CIP 112613: PT negative, PRN positive, FHA positive, FIM3
- *B. pertussis* CIP 112615: PT positive, PRN negative, FHA positive, FIM2 (MRBP)
- *B. pertussis* CIP 112614: PT positive, PRN negative, FHA negative, FIM3

4. PLATE PLAN

An example of a plate plan is given below.

	1	2	3	4	5	6	7	8	9	10	11	12			
A	FR8171	FR8171	FR8171	FR8171	FR8171	FR8171	FR8171	FR8171	FR8171	FR8171	FR8171	FR8171			
B	FR8558	FR8558	FR8558	FR8558	FR8558	FR8558	FR8558	FR8558	FR8558	FR8558	FR8558	FR8558			
C	FR8665	FR8665	FR8665	FR8665	FR8665	FR8665	FR8665	FR8665	FR8665	FR8665	FR8665	FR8665			
D	FR8668	FR8668	FR8668	FR8668	FR8668	FR8668	FR8668	FR8668	FR8668	FR8668	FR8668	FR8668			
E	FR8670	FR8670	FR8670	FR8670	FR8670	FR8670	FR8670	FR8670	FR8670	FR8670	FR8670	FR8670			
F	FR8673	FR8673	FR8673	FR8673	FR8673	FR8673	FR8673	FR8673	FR8673	FR8673	FR8673	FR8673			
G	Tohama	Tohama	Tohama	Tohama	Tohama	Tohama	Tohama	Tohama	Tohama	Tohama	Tohama	Tohama			
H												BLK			
	PT - 99/512 + 99/542 1/1000			FHA - 99/572 1/2000			PRN - 69K/16 1/1000			FIM2 - 06-124 1/10000			FIM3 - 06-124 1/10000		
	1	2	3	4	5	6	7	8	9	10	11	12			
A	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.00195313	0.00097656	0.00048828			
B	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.00195313	0.00097656	0.00048828			
C	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.00195313	0.00097656	0.00048828			
D	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.00195313	0.00097656	0.00048828			
E	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.00195313	0.00097656	0.00048828			
F	1	0.5	0.25	0.125	0.0625	0.03125	0.015625	0.0078125	0.00390625	0.00195313	0.00097656	0.00048828			
G															
H															

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5. EXPERIMENTAL PROTOCOL

5.1. PREPARATION OF THE BACTERIAL SUSPENSIONS FOR COATING

- From bacterial strains grown on **BGS medium**, prepare a suspension with an OD at 650 nm of 1.0 in physiological water.
- **Aliquot 400 µL of each suspension** into a low-binding eppendorf tube, then centrifuge for 10 min at 10°C and 8,000 rpm. Label the eppendorf tubes properly.
We will call these tubes the OD4.
- Remove all supernatant from the eppendorf tube (using a micropipette ensure that no liquid remains inside).
- While waiting to perform the ELISA, store the pellet at -20°C, or proceed directly to the next step (inactivation and dilution)

5.2. THERMAL INACTIVATION OF SUSPENSIONS AND COATING OF ELISA PLATES

- Resuspend each OD4 tube in **400 µL of physiological water**, then **thermally inactivate the suspensions at 56°C for 1 hour in a dry bath.**
- Dilute the suspensions 1:5 to obtain an **OD_{650nm} of 0.2.**

Prepare a final volume of 1500 µL per sample: mix 300 µL of the inactivated OD4 suspension with 1200 µL of 1X PBS to obtain an OD_{650nm} of 0.2.

1. Coating (2 wells/sample)

Samples plate (see page 5):

For each sample, **add 100 µL of the suspension at OD_{650nm} = 0.2** to each well in columns 1 to 10, with the same sample loaded across one row.

For each row, 5 virulence factors (**PT**, **FHA**, **PRN**, **FIM2**, and **FIM3**) are tested in duplicate (2 wells per virulence factor, i.e., 10 wells in total).

Each sample therefore corresponds to one row. A maximum of 6 samples can be processed per plate, using rows A to F.

Do not forget to add (see page 5):

- A line of **positive controls** (line G): 2 Tohama wells OD_{650nm} = 0.2 per virulence factor (i.e. 10 wells in total).
- One **negative control** line (line H): 2 wells of PBS 1X per virulence factor (10 wells in total).

Standard range plate (see page 5):

On a separate plate, carry out the standard ranges for the 3 virulence factors **PT**, **FHA** and **PRN** (see section 3.3.1) using the dedicated antigens. **No standard range is made for FIM2 and FIM3 (no purified proteins available).**

Film the plates and leave **the coating step overnight at room temperature.**

5.3. ELISA

2. **Wash 3x with the washing buffer** - 200 µL/well.
3. **Blocking** with the **TB** - 150 µL/well.

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4. **Incubate 1h at 37°C.**
5. **Wash 3x with the washing buffer** - 200 µL/well.
6. Distribute **primary antibodies** in accordance with the plate plane - **100 µL/well.**
 - Anti-PT antibodies diluted in the TB (1/1 000).
 - Anti-FHA antibodies diluted in the TB (1/2 000).
 - Anti-PRN antibodies diluted in the TB (1/1 000).
 - Anti-FIM2 antibodies diluted in the TB (1/10 000).
 - Anti-FIM3 antibodies diluted in the TB (1/10 000).
7. Apply a new film and close the plate tightly.
8. **Incubate for 2h at 37°C.**
9. **Wash 3x with the washing buffer** - 200 µL/well.
10. Distribute the **secondary antibody** - **100 µL/well.**
 - Secondary antibody diluted in TB (1/ 1 000)
11. **Incubate 2h + 37 °C.**
12. **Wash 3x with the washing buffer** - 200 µL/well.
13. Add the **TMB Substrate** - **100 µL/well.**
14. Incubate **1h at room temperature.**
15. Add the **STOP TMB Solution** - **50 µL/well.**
16. Measure the absorbance at wavelengths **450 and 570 nm** in the Multiskan plate reader.

Analyses of the results: Compare sample values with negative control and Tohama to identify positive and negative isolates (to be confirmed with sequencing).

Procedure:

- The absorbance at 450 nm corresponds to the reading wavelength, whereas the absorbance at 570 nm corresponds to background signal. **For each well, the absorbance measured at 450 nm should therefore be corrected by subtracting the value measured at 570 nm.**
- Calculate the **mean and standard deviation** of the duplicates.
- Construct a graph with the samples and controls on the x-axis, and the mean absorbance ± standard deviation on the y-axis. In our experience, we consider mean absorbance values above 0.2 for PT, FHA, and PRN, and above 0.3 for FIM-2 and FIM-3, to be positive.
- For quantification, use the standard curve. Calculations can be performed using the online software MyAssays (<https://www.myassays.com>).