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Q-detect™

QDXS-0036 (3 x 12 tests)

1 Intended use

Q-detect™, an *in vitro* whole blood interferon gamma (IFNγ)-release assay (IGRA) for Q fever, measures the response of white blood cells to *Coxiella burnetii*. Q-detect™ is an indirect assay to detect both active and latent *Coxiella* infections. Q-detect™ does not measure the presence of *Coxiella* directly.

The test results must be used to determine whether a subject has been exposed to Q fever. A positive result supports the diagnosis of Q fever or Q fever-related chronic fatigue syndrome (CFS) and a decision to perform or not to perform additional medical and diagnostic tests, to vaccinate or to prescribe immune suppressants.

Other medical and diagnostic tests are needed to confirm active infection. It is not known which cross-reactions with other bacteria occur.

2 Summary and explanation of the test

2.1 Q fever

Q fever is caused by an infection with *C. burnetii*, a bacterium that grows intracellularly and which is passed from animals to humans through the air. The incubation period is 2-3 weeks.

The disease leads to atypical pneumonia and liver abnormalities but is also often asymptomatic. Chronic Q fever, without treatment often lethal, sporadically occurs after infection. After an epidemic in the Netherlands, it mostly concerned people without any history of acute Q fever. In approximately 20% of people with acute Q fever, CFS develops, that seriously affects the quality of life of patients.

The available methods to establish a *Coxiella* infection consist of the measurement of antibodies and, in the first two weeks of the disease, molecular diagnostics using PCR. A skin test for the purpose of pre-vaccination screening is registered in Australia.

2.2 Basic principles of the test

Q-detect™ is an *in vitro* test in which the response of blood cells to stimulation with *Coxiella* is measured (Schoffelen et al., 2013). After exposure, cells in the blood recognize the bacterium and, as a result, produce IFNγ. The heat-killed bacterium used in the assay originates from a Dutch *Coxiella* strain and has been inactivated by heating at 99 °C for 30 min. IFNγ production is measured using an ELISA (Enzyme-Linked Immunosorbent Assay).

2.3 Implementation in brief

For Q-detect™, blood is collected in standard tubes coated with lithium heparin. The blood is divided over tubes or a 96-wells plate. Wells contain heat-killed bacteria, a positive control or medium as a negative control. Samples are placed at 37 °C within 12 hours. After 24 hours, IFNγ is measured. There are two versions of the test: a small-scale version and the version for screening large numbers. Only plasma collection and the stimulation step differ.

2.4 Review

The test result is considered to be positive if, after stimulation with *Coxiella*, at least 16 pg/mL IFNγ is formed and the relative value with respect to the positive control is larger than 0.40. Phytohemagglutinin (PHA) serves as positive control. A sample with a poor response to PHA (<40 pg/mL) cannot be assessed when the sample did not respond to *Coxiella* as well. The negative control is used to detect non-specific IFNγ formation.

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2.5 Time needed to perform the essay

Table 1. Time needed to perform the essay

Step	Action	Time	Working hours
1	Preparation solutions	60 min	60 min
	Stimulation	30 min per 12 samples	30 min
	Incubation samples	24 hours	0
2	Coating	16 hours	60 min
	IFNγ measurements	4 hours in case of 1-5 ELISA-plates (1-60 samples)	90 min

3 Components and storage

3.1 Required, supplied materials

The supplied reagents are sufficient for 3 plates of 12 samples each. An excess of stimulation reagents and ELISA plates are supplied to account for partially-filled plates. The reagents have been validated in accordance with the mentioned combination of lot numbers in the Certificate of Analysis. Innatoss will not guarantee correct results if components of the kit are replaced.

Table 2. Required, supplied materials and reagents

	Quantity	Storage conditions
Stimulation plate	5	17-25 ˚C
ELISA plate	5	17-25 ˚C
Seals	10	17-25 ˚C
Q-detect™ Antigen Cb2009-02629	300 µL	-20 °C
Positive control (PHA)	400 µL	-20 °C
Block solution	2 x 2 mL	-20 °C
Assay buffer (5x)	2 x 20 mL	-20 °C
Anti-IFNγ coating antibody	375 µL	-20 °C
Anti-IFNγ biotin conjugate	375 µL	-20 °C
IFNγ standard	1 vial	-20 °C
Streptavidin-HRP conjugate	10 µL	-20 °C
TMB ELISA substrate	50 mL	2-8 °C
PBS (10x)	2 x 250 mL	17-25 ˚C
Wash buffer detergent	2 x 2 mL	17-25 ˚C

3.2 Required, but not supplied materials

- Biosafety cabinet class 2.
- 37 °C incubator. No CO_2 is required.
- Calibrated pH meter.
- Orbital shaker and plate washer for microtiter plates.
- Calibrated pipettes to pipette volumes of 1 µL to 1000 µL and calibrated multichannel pipettes of 20 µL to 300 µL, with disposable tips.
- Microplate spectrophotometer with 450 nm filter.
- When using tubes for stimulation: polypropylene microtubes with cap (e.g. micro Micronic tubes or Eppendorf Safe Lock).
- Ultrapure water.
- Na_2CO_3 (≥ 99.8%) and $NaHCO_3$ (≥ 99.5%) or ready-for-use carbonate/bicarbonate buffer.
- RPMI medium, without phenol red and glutamine (Dutch modification).
- Gentamicin 10 mg/mL.
- Sodium pyruvate 100 mM.
- Glutamine 200 mM or GlutaMAX.
- Sulfuric acid 2 M (stop solution).

3.3 Storage and processing

3.3.1 Kit storage

Kit reagents must be stored in accordance with the instruction on the label. Please note: different components must be stored at different temperatures.

3.3.2 Storage of working solutions

Instructions for preparing and storing the reagents are shown in Table 3.

4 Warnings and precautions

4.1 Warnings

In the EU this kit is for *in vitro* diagnostic use only. Outside the EU for research use only. A negative result does not exclude the possibility of a *Coxiella* infection; false-negative results may be caused by the infection phase, abnormalities in the immune function, or incorrect assay execution. A false-positive result may be the result of incorrect execution. A positive result is not sufficient to diagnose a *Coxiella* infection, but should be followed by further medical and diagnostic research. Only in this way, Q fever or CFS can be diagnosed.

4.2 Precautions

4.2.1 Biosafety

- CAUTION: human blood is potentially infectious material. Treat it as if it might be infected. Work in a biosafety cabinet class 2 where possible. Blood and plasma samples may only be processed with a pipette. Preferably use pipette tips with filter.

- The following risk and safety phrases apply to the stop solution: contains sulfuric acid; irritating; P260, P264, P280, and P363[1].

- The following risk and safety phrases apply to the TMB ELISA substrate solution: H302, H314, H315, H319, H332, and H335[1].

4.2.2 General Safety

- The Q-detect™ kit may only be used by trained personnel.
- When working with Q-detect™, wear a lab coat, safety glasses and disposable gloves.
- Check the Material Data Safety Sheets (MSDS) of the kit and individual components. These can be obtained via support@innatoss.com.
- Dispose of unused reagents and diagnostic samples according to local regulations.
- Add 1 chlorine tablet per liter (0.15 ppm) to the waste bottle of the plate washer to disinfect washing liquid. Avoid aerosol formation when emptying the waste.
- Disinfect surfaces and possibly contaminated equipment with 70% ethanol.
- In case of emergencies if chemicals are involved: call your on-site safety services.

4.2.3 Use of reagents

- Read the entire instructions before use. Deviations from the Q-detect ™ manual can lead to incorrect results.
- Provide calibrated and validated laboratory equipment.
- Do not use the kit if unused vials are damaged or the expiry date has passed.

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[1] P260: Do not breathe dust/fume/gas/mist/vapors/spray, P264: Wash skin thoroughly after handling, P280: Wear protective gloves/protective clothing/eye protection/face protection, P363: Wash contaminated clothing before reuse.
H302: Harmful if swallowed, H314: Causes severe skin burns and eye damage, H315: Causes skin irritation, H319: Causes serious eye irritation, H332: Harmful if inhaled, H335: May cause respiratory irritation.

- Q-detect™ antigen, PHA, anti-IFNγ coating antibody, anti-IFNγ biotin conjugate, assay buffer and IFNγ standard with different Q-detect™ batch numbers should not be mixed or used interchangeably. Other reagents (Streptavidin-HRP conjugate, wash buffer and TMB ELISA substrate solution) can be interchanged, provided that the expiry date has not passed and that the batch numbers are recorded.

5 Blood collection, transport and storage

Blood for Q-detect™ is collected in a lithium heparin tube. Other anti-coagulants interfere with the test. The validation was performed with BD vacutainer tube cat. number 368884.

Collect 3.5 to 4 mL of blood by means of venipuncture. This procedure must be performed by a trained and qualified health professional. Mix the tube by inversion. Blood may be stored for up to a maximum of 12 hours **at room temperature** (RT) until step 1 is performed: the stimulation step. Do not place the tubes in the refrigerator, nor in the freezer. Note the ambient temperature during transport.

6 Instructions for use

6.1 Step 1 – Stimulation step

6.1.1 Included and not included materials

See chapter 3.

6.1.2 Procedure

Perform the following actions under aseptic conditions. Working in a biosafety cabinet class 2 is preferable, to protect the sample as well as the user.

When using 96-wells plates

1. Label a stimulation plate (U-bottom polypropylene plate) with patient numbers and stimulus.
2. Dilute the antigen suspension with full medium according to the Certificate of Analysis. Make 40 µL antigen solution per sample with an excess of at least 20 µL.
3. Dilute the PHA solution with full medium according to the Certificate of Analysis. Make 40 µL PHA solution per sample with an excess of at least 20 µL.
4. Add 20 µL full medium to rows C and D, 20 µL antigen solution in rows E and F, and 20 µL PHA solution to rows G and H. Rows A and B remain empty.
5. Before distributing the blood, mix the tube by inverting the tube (5x). Add 180 µL blood to each well, in such a way that for each patient 2 wells are filled with negative control, 2 with antigen and 2 with positive control. Cover the plate with a seal. This step should be performed within 12 hours of blood drawing.
6. Place the plate for 24 ± 1 hour in an incubator at 37 ± 1 °C.
7. After 24 hours, place the plate in the refrigerator at 2-8 °C or, alternatively, determine the concentration of IFNγ immediately.
8. Store the plate with the whole blood for up to a max. of 3 days at 2-8 °C. If the sample is to be stored for longer periods, the plasma can be pipetted off and transferred to a clean 96-wells plate. This may be stored for up to a maximum of 14 days at 2-8 °C. Longer storage is possible at -20 °C.

When using incubation tubes

When preferred, plastic tubes can be used in step 1 instead of a 96-wells plate. In that case, more antigen is required.

1. Label tubes with patient number and stimulus.
2. Dilute the antigen suspension with full medium according to the Certificate of Analysis. Make 80 µL antigen solution per sample with an excess of at least 20 µL.
3. Dilute the PHA solution with full medium according to the Certificate of Analysis. Make 80 µL PHA solution per sample with an excess of at least 20 µL.
4. Add 40 µL full medium to 2 tubes, 40 µL antigen solution to 2 tubes and 40 µL PHA solution to 2 tubes. Ensure that the stimulus is at the bottom of the tube.

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5. Before distributing the blood, mix it by inverting the tube (10x). Add 360 µL blood to each tube. Close the vials with a matching cap. This step should be performed within 12 hours of blood drawing.
6. Place the plate in an incubator at 37 ± 1 °C. The blood cells sediment during incubation.
7. After 24 hours, remove the samples from the incubator and pipette 150 µL of the supernatant. Centrifugation is not necessary. Transfer the plasma into a clean tube and keep it for up to 14 days at 4 °C. Longer storage is possible at -20 °C.

6.2 Step 2: determination of IFNγ production

6.2.1 Included and not included materials

See chapter 3.

6.2.2 Procedure

Make the required solutions with the reagents in the kit as defined in Table 3.

1. Coat well A1 up to and including B11 of the ELISA plate and a sufficient amount of wells for the samples with 100 µL coating buffer. Use the same plate setup as used for stimulation.
2. Cover with a seal and incubate overnight at RT. The seal can be used throughout the ELISA procedure.
3. Wash 5x with 300 µL wash buffer.
4. Add 200 µL block buffer and incubate 60-75 min at RT.
5. Make a 1:1 dilution series of the IFNγ standard in assay buffer. The standards contain:
   500 – 250 – 125 – 62.5 – 31.3 – 15.6 – 7.8 – 3.9 – 1.95 – 0.977 - 0 pg/mL IFNγ.
6. Wash the plate 5x with 300 µL wash buffer and label the plate.
7. Add 100 µL of each of the IFNγ standards to well A1 up to and including B11.
8. Mix plasma samples or whole blood samples well before adding. Freezing sometimes causes aggregates. Removal is possible by centrifugation at 12.000 ***g*** during10 min.
9. Negative control and *Coxiella* stimulated samples: Add 50 µL assay buffer to C1 up to and including F12. Add 50 µL plasma or whole blood to the appropriate wells (see stimulation plate setup).
10. Positive control: add 80 µL assay buffer to G1 up to and including H12. Add 20 µL plasma or whole blood to the appropriate wells (see stimulation plate setup).
11. Cover each plate with a seal and incubate for 60-75 min at RT on an orbital shaker at about 600 rpm, depending on the shaker. Shaking should be gentle with motion, but without splashing.
12. Wash 5x with 300 µL wash buffer.
13. Add 100 µL freshly prepared biotin conjugate to all wells.
14. Cover with a seal and incubate for 60-75 min at RT.
15. Wash 5x with 300 µL wash buffer.
16. Add 100 µL freshly prepared streptavidin-HRP conjugate solution to the appropriate wells.
17. Cover with a seal and incubate for 30-40 min at RT.
18. Wash 5x with 300 µL wash buffer.
19. Add 100 µL TMB ELISA substrate solution and incubate for some minutes, according to the Certificate of Analysis. Avoid direct sunlight.
20. Then add 100 µL diluted stop solution. Add the diluted stop solution in the same order and at approximately the same speed as the substrate.
21. Measure the optical density (OD) of each well with a microplate spectrophotometer at 450 nm within 30 min after stopping the reaction.

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Table 3. Preparation, storage and storage life of the required reagents

	Reagents	Solvent	Storage life	Implementation
Full medium	RPMI, Gentamicin, Sodium pyruvate and GlutaMAX		1 month at 2-8 °C	Add to 5 mL RPMI 1640 without phenol red and glutamine: 25 µL Gentamicin 10 mg/mL, 50 µL sodium pyruvate 100 mM and 50 µL GlutaMAX 200 mM.
Carbonate buffer	Na_2CO_3 $NaHCO_3$	water[2]	2 weeks at 2-8 °C	Dissolve 2.11 g Na_2CO_3 in 200 mL water; dissolve 3.36 g $NaHCO_3$ in 400 mL water; mix half of each solution and, if necessary, add one of both until the pH is 9.6 ± 0.1. As an alternative, ready-for-use carbonate/bicarbonate buffer can be used.
Coating buffer	Anti-IFNγ coating antibody	carbonate buffer	Prepare fresh	Add 100 µL antibody per plate to 10 mL carbonate buffer. In case of fewer samples: prepare 100 µL per well.
Wash buffer	PBS 10x and wash buffer detergent	water[2]	2 weeks at RT	Mix 100 mL PBS (10x) and 900 mL water. Add 500 µL wash buffer detergent and mix well. 750 mL is needed per plate.
Block buffer	Block solution	PBS (1x)	24 h at RT 48 h at 2-8 °C	Thaw the block solution rapidly to prevent aggregation. Homogenize and mix 400 µL with 20 mL PBS (1x) per plate.
Assay buffer	Assay buffer 5x	water[2]	1 day	Dilute the concentrated assay buffer 5x.
IFNγ stock		water[2]	Store at -20 °C. Three freeze-thaw cycles are acceptable.	Reconstitute the freeze-dried IFNγ standard in 500 µL water[2]. Mix gently to avoid foaming and ensure that all substances are fully dissolved. Aliquot if necessary. This stock contains 4500 pg/mL IFNγ.
IFNγ standard		assay buffer	Prepare fresh	Dilute the IFNγ stock in assay buffer to a concentration of 500 pg/mL. Use this to prepare other standard solutions.
Biotin conjugate	Anti-IFNγ biotin conjugate	assay buffer	Prepare fresh	Add 100 µL anti-IFNγ biotin conjugate to 10 mL assay buffer. In case of fewer samples: make 100 µL per well.
Streptavidin-HRP conjugate solution	streptavidin-HRP conjugate	assay buffer	4 hours	Mix 1 µL streptavidin poly-HRP with 10 mL assay buffer per plate. The tube contains a small volume. Spin down before use.
Diluted stop solution	H_2SO_4 2 M	water[2]		Mix 9 mL 2 M sulfuric acid with 1 mL water per plate.

[2] Ultra pure water

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7 Calculations and interpretation of the test

7.1 Generating the standard curve

- Extensive data analysis instructions can be requested via support@innatoss.com.
- Make a standard curve by plotting the log of the OD (y-axis) versus the log of the IFNγ concentration (increased by an *offset* of 0.01) of the standards in pg/mL (x-axis). Calculate the best fitting line for the standard curve using non-linear regression.
- The calculations can be performed using software supplied with microplate readers, and standard spreadsheet or statistical software to determine the four parameters of the standard curve (OD_{min}, OD_{max}, EC_{50} and slope). Innatoss can provide help here.
- Calculate the IFNγ concentration of the samples, using the OD values and the standard curve.
- Measured values outside the range of the standard curve are cut off at the detection limit (0.6 pg/mL), or the highest standard * 1.05.

7.2 Quality Control

The accuracy of the test results depends on generating a good standard curve.

The ELISA is approved if the following conditions are met:

- The average OD-value of the zero standard is <0.120. If the zero-standard > 0.120, the washing procedure must be checked and exposure of the substrate to sunlight.
- The average OD-value of the 1.95 pg/mL standard is above that of the standard with 0.977 pg/mL. This is a measure of sensitivity.
- The average OD-value for the standard with 500 pg/mL IFNγ is between 1.500 and 3.000.

7.3 Interpretation of the results

The IFNγ concentration is calculated for each sample, where the average is calculated on log-basis. Important for the interpretation:

- *Coxiella*-induced IFNγ production Prod = cox – neg
- The Relative *Coxiella* Response in relation to PHA (CoxRR). This is calculated with the formula:

$$CoxRR = (\log(cox)-\log(neg))/(\log(pos) - \log(neg))$$

Q-detect™-results are interpreted on the basis of the following criteria:

IFNγ	**Conclusion**
Negative control (neg) > 40 pg/mL	Inconclusive
Positive control (pos) < 40 pg/mL	Inconclusive, but positive if (cox-pos) > 64
Pos/Neg ratio < 10	Inconclusive, but positive if (cox-pos) > 64
Prod < 16 pg/mL **or** CoxRR < 0.40	Negative
Prod between 16 and 64 pg/mL **and** CoxRR between 0.40 and 0.60	Borderline
Prod > 64 pg/mL **and** CoxRR between 0.40 and 0.60	Slightly positive
Prod > 16 pg/mL **and** CoxRR between 0.60 and 0.80	Moderately positive
Prod > 16 pg/mL **and** CoxRR > 0.80	Strongly positive

7.3.1 Inconclusive results

Inconclusive results rarely occur. Inconclusive results due to a low positive or high negative value usually will not change by repeating the test. In case of abnormalities in blood collection or transport to the laboratory, the Q-detect™ test must be repeated with a new blood sample. Repetition of the ELISA with stimulated samples is sufficient if an incorrect execution is the cause of the anomalous results.

8 Quality aspects

8.1 Clinical research

There is no gold standard for Q fever diagnostics. Therefore, the sensitivity and specificity of the Q-detect™ test is detected on the basis of several studies in which more than 3000 people participated in total. The sensitivity is determined during a research study in the Dutch village Herpen in which IFA and Q-detect™ measurements were carried out. The specificity of Q-detect™ is determined by evaluating positive results in individuals with a low risk of Q fever in the city of Enschede.

8.2 Sensitivity

8.2.1 Vaccination studies

Schoffelen et al. investigated the performance of the *Coxiella* IGRA in pre-vaccination screening (2013). Out of 1278 participants with an interpretable IGRA, 144 were positive in an immunofluorescence assay. In 163 seronegative participants, a response to *Coxiella* was demonstrated by means of a skin test. This shows that a T cell such as the skin test and IGRA is more sensitive than serology.

8.2.2 QHORT

As part of the QHORT study (Wielders et al. 2014), a comparison was made between IFA and Q-detect™. In 78% of participants (n = 394) a positive Q-detect ™ response was found. The relatively low sensitivity was the reason for the optimization of protocol and logistics.

8.2.3 Herpen

The Q-Herpen II study combined patients and healthy participants. From 1517 people in a high-incidence area, blood was collected for IFA and Q-detect™. Both the previously used Nine Mile antigen and the Innatoss antigen Cb02629 were used. The results were comparable. Of the Cb02629 group (N = 822), 18 were undecided, 482 people were positive in Q-detect™, 518 were positive in IFA and/or Q-detect™. Consequently, the sensitivity of the test is 93%, in which 10% of the IFA positive samples cannot be confirmed by Q-detect™. This is in agreement with the observation that a large number of seronegative people have a T cell-dependent positive skin test (Schoffelen, 2013).

8.3 Specificity

Q-detect ™ was conducted with 109 volunteers from an area with a presumed lower risk of Q fever. 19 people tested positive, of which 9 had formed detectable antibodies against Coxiella. Therefore, the specificity was at least 90%.

9 Literature

- Schoffelen T, Joosten LA, Herremans T, et al. Specific interferon γ detection for the diagnosis of previous Q fever. Clin Infect Dis. 2013; 56:1742-51.
- Wielders, CC. Long term follow-up of acute Q fever patients after a large epidemic. Utrecht University, 1st July 2014.

10 Technical service

For technical service and questions, contact support@innatoss.com.

11 Important changes

Changes with respect to the June 2016 version of this manual: the Certificate of Analysis is referred to for the dilution of the antigen and PHA, and for the TMB incubation time. Also, the result interpretation has been slightly changed based on post marketing experience.

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