Legionnaires’ disease is caused by Legionella pneumophila and is an acute respiratory disease. It can take the course of a mild influenza-like illness (so-called Pontiac fever) or can cause pneumonia when left untreated, can show a relatively high lethality rate. (1) The mortality rate, which in the case of immunocompetent subjects can reach 20% without adequate treatment, can be reduced if appropriate antimicrobial therapy is commenced at an early stage as a result of rapid diagnosis. With regard to infections in humans, the species Legionella pneumophila is detected most frequently (80-85%) and although rarer, 18 further Legionella species occur as infectious agents of pneumonia. Strains of Legionella species isolated from patients belong mainly to the serogroup 1, however 14 other serogroups exist (2).

Since 1975, the detection of specific antigen in urine has on many occasions been described as a reliable, simple test (3,4), but until now this has been limited to the detection of serogroup 1. The test presented here detects specific Legionella antigen and recognises all L. pneumophila serogroups with a relatively wide spectrum of cross-reactivity as well as other Legionella species (5).

## Principle of test

The strips of a microplate are coated with polyclonal rabbit antibodies which react with Legionella pneumophila antigen of all serogroups as well as further Legionella species. Patient urine is added to the wells of the microplate and any Legionella antigen present will bind to the specific antibody on the solid phase. Following the first incubation, the wells are washed and a peroxidase-labeled antibody which reacts with Legionella pneumophila antigen is added which binds to the free binding sites on the antigen during a second incubation. After a further washing stage, the presence of bound peroxidase is demonstrated in a colour reaction with a substrate. The reaction is stopped by adding sulphuric acid and the optical density (OD) is measured with a spectrophotometer at 450 nm and a reference wavelength of 615-690 nm.

## Kit contains

- **MTP** 1 Microplate
  - 12 single strips with 8 wells each, coated with rabbit anti-Legionella IgG (concentration: > 0.5 µg/ml) including [MTP] frame.
- **NC** 2 x 1,5 ml
  - Negative control/Calibrator
  - Human urine, negative for Legionella antigen (ready for use, human, autoclaved)
  - Preservative: 0.095% sodium azide
- **PC** 1,5 ml
  - Positive control
  - Legionella pneumophila antigen (ready for use)
  - Preservative: 0.095% sodium azide (culture supernatant)
- **CONJ** 15 ml
  - Rabbit anti-Legionella antibodies labelled with horse-radish peroxidase (HRP) in tris buffer with protein stabiliser
  - Preservative: 0.05% Proclin-300®
- **WB** 6 ml
  - Wash buffer concentrate (500 µl), 1 M phosphate buffer pH 7.2
  - Preservative: 0.01% 2-bromo-2-nitro-1,3-propanediol
- **SUB** 2 x 13 ml
  - Substrate solution (ready for use)
  - 1.3, 5-tetramethylbenzidine (TMB) solution < 0.05 % in H₂O₂
  - See warning and precautions.
- **STOP** 15 ml
  - Stop solution (ready for use)
  - Sulfuric acid < 1N H₂SO₄
- **SB** 1
  - Storage bag with desiccant
  - Polyethylene bag for storing remaining microplate strips.
- **[WB]** 1
  - Package insert information

**Preservatives:** total concentration < 0.2%

<table>
<thead>
<tr>
<th>Materials required but not supplied with the kit</th>
</tr>
</thead>
<tbody>
<tr>
<td>Micropipettes, spectral photometer (450 nm, reference wavelength 615–690 nm), microplate washer (with bottom wash) and incubator (37°C) for [MTP]</td>
</tr>
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</table>

## Warning and precautions

Do not ingest reagents. Avoid contact with eyes and skin. All urine samples, controls and materials used for the test must be treated as being potentially infectious and appropriate safety precautions taken. The control urines have been autoclaved and sterile filtered, but should nevertheless be treated as potentially infectious. Do not pipet with mouth. According to good laboratory practice wear gloves, laboratory coat and safety glasses. Liquids and non-combustible materials should be decontaminated with sodium hypochlorite (final concentration: 3 %, activity time at least 30 minutes). Liquid waste which contains acids must be neutralised before disposal. The used [MTP] and all materials that are to be re-used must be autoclaved for 1 hour at 121°C. The [SUB] is sensitive of light and has to be protected from light. The test must be performed by well-trained and authorised laboratory technicians.

## Pipetting procedure of test performance

Allow all reagents to reach room temperature before use.

The controls and the blank should be pipetted last. After pipetting the controls and samples immediately begin with incubation of the plate.

<table>
<thead>
<tr>
<th>step 1</th>
<th>well [µl]</th>
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<tbody>
<tr>
<td>A1</td>
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<td>B1</td>
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<td>C1</td>
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<td>D1</td>
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<tr>
<td>E1</td>
<td></td>
</tr>
<tr>
<td>Blank</td>
<td></td>
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</tbody>
</table>

**Blank** -- -- -- 100

**A1** B1 C1 D1 E1

**NC** double test -- 100 **[NC]** -- --

**PC** -- -- 100 **[PC]** --

**Sample** -- -- -- 100

**[WB]**

<table>
<thead>
<tr>
<th>step 2</th>
<th>well [µl]</th>
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</table>

**WB** 550 550 550 550 550

**[CONJ]** 100 100 100 100 100

**[MTP]**

<table>
<thead>
<tr>
<th>step 3</th>
<th>well [µl]</th>
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</table>

**WB** 550 550 550 550 550

|        |           |
|        |           |
|        |           |
|        |           |
|        |           |

**[CONJ]** 100 100 100 100

## Testing is performed under aseptic and microbiologically controlled conditions.

## Storage

The reagents are stable up to the stated expiry dates on the individual labels when stored at 2-8°C. After opening reagents have to be used within 30 days.

For repeatedly testing store the reagents immediately after usage at 2-8°C. The [MTP] stripes are sealed separately in an aluminium bag and must be at room temperature before opening. Return unused strips into the storage bag with the desiccant and store in this way at 2-8°C. Do not touch the upper rim or the bottom of the wells with fingers.

## Washing procedure

**The wash procedure is critical.** Insufficient washing will result in poor precision and unspecific reactions.

**W1:** wash 5 times with wash buffer. For that remove the liquid in the well and dispense with 300 µl washing buffer. Fill the well with at least 250 µl washing buffer (total volume 550 µl). This washing procedure is repeated 5 times. Tap out the plate briefly after washing. Perform next steps immediately afterwards. Do not allow the plate to dry out.

## Reagent preparation

Dilute the wash buffer concentrate using deionised or destilled water (1:100, e.g. 1 ml plus 500 µl). The prepared wash buffer is stable for 2 weeks when stored at 2-8°C. Should crystallisation occur after storage at 2-8°C, the wash buffer concentrate can be brought back into solution by warming to 37°C. Mix the buffer well before diluting. All other test components of the test kit are ready for use.

## Sample preparation

Urine samples should be collected in standard sterile containers, stored at room temperature and tested within 24 hours. Alternatively, the samples may be stored at 2-8°C for 14 days or frozen at –20°C.

In the case of urine samples containing large particulate matter, the clear supernatant should be used. Frozen urine samples containing high concentrations of salts (phosphates, urates) can produce precipitation of large quantities of salts on thawing. After mixing and sedimentation the clear supernatant can be used or the crystals redissolved by warming to 37°C.

## Test performance

**The protocol (see pipetting procedure) has to be followed strictly.** Use 100 µl of positive control, negative control and sample in each well. The strips are not sealed with a self-adhesive film.

Measure the extinction immediately or after stop at 450 nm using a spectral photometer (reference wavelength: 615-690 nm).

## Quality control

The positive and negative control must be included in every test run for evaluation of the patient samples and monitoring of test performance. Known positive or negative urine samples may be included as additional controls. The mean value of the positive controls (blank subtracted) should be less than 0.100. The OD value of the positive control must be greater than 0.600. The OD value of the blank must be less than 0.150. If these conditions are not met, the test results are invalid and the test must be repeated.
Calculation of the cut-off value

The blank value is subtracted from all extinction values of the controls and samples.

The cut-off value is calculated from the mean OD value of the negative controls (NCx) + 0.200. Cut-off value = (NCx) + 0.200.

Example: NCx = 0.023, cut-off = 0.023 + 0.200 = 0.223

The grey zone is localised: OD > mean OD of NC + 0.100 and OD < of mean OD of NC + 0.200.

Example: NCx = 0.023, grey zone = 0.123 – 0.223.

Interpretation of the results

Urine samples with an extinction below the cut-off are considered as being negative. Note: A negative result does not exclude the possibility of a Legionella infection. Urine samples with an extinction equal to or greater than the cut-off are considered to be positive for the detection of Legionella antigen. Positive samples can be repeated for confirmation. Weak positive samples in the region of OD > 0.200 must be repeated for confirmation. If the repeated sample is above the cut-off again the sample is to be considered as positive. Urine samples with an extinction within the grey zone should be repeated in testing. If the OD-value is again in the grey zone than the sample is considered as positive. If the OD value of the sample is below the grey zone than the sample is negative.

Diagnostic interpretation

Result above the cut-off or repeatedly within the grey zone = positive; presumed positive for Legionella antigens in urine. This gives rise to the suspicion of an existing or past Legionella infection (weak positive samples must be repeated for confirmation, see above).

Result < NCx + 0.200 = negative; presumed negative for Legionella antigens in urine. The possibility of a Legionella infection cannot be excluded, as the sensitivity of the test is not sufficient to recognise absolutely all cases of all Legionella species. Variations can arise in antigen secretion. Follow-up samples should be tested.

Limitations of the method

This test was validated using urine samples. Other materials (e.g. plasma, serum or body fluids) which can contain Legionella antigen have not been tested. In spite of its broad reactivity with various Legionella species, the Bio-Rad Legionella Urine Antigen EIA is not able to detect all 39 Legionella species with equal sensitivity (6,7). The diagnosis of legionellosis cannot be made solely on the basis of clinical or radiological evidence. Antigen detection, the results of cultures and serological tests provide further aids for the diagnosis alongside the clinical findings.

The secretion of Legionella antigen in urine can vary, depending on the patient, any accompanying diseases and the treatment. Furthermore, secretion can be variable in one and the same patient over time, thus requiring testing of a sequence of several urine samples. Early treatment with appropriate antibiotics can reduce the secretion of antigen in some patients. Antigen secretion can persist in some patients over a longer period. Appropriate antibiotics can reduce the secretion of antigen in some patients. Variations can arise in antigen secretion. Follow-up samples should be tested.

Performance characteristic

350 urine samples from healthy subjects and 176 urine samples from subjects with a suspected urinary tract infection were tested. 475 samples were negative in the Legionella urine antigen test. One of the subjects with a suspected urinary tract infection was weakly positive. (specificity = 99.78%).

46 urine samples from patients with Legionella confirmed by culture were tested with the Bio-Rad Legionella Urine Antigen EIA test. All 46 urine samples produced a positive result. The table below presents the distribution of the absorbance values of all 46 samples.

<table>
<thead>
<tr>
<th>Distribution of extinction values of negative samples</th>
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</thead>
<tbody>
<tr>
<td>Absorbance</td>
</tr>
<tr>
<td>Number of samples (total 476)</td>
</tr>
</tbody>
</table>

Specificity of Legionella Urine Antigen EIA: 99.8%

Sensitivity of Legionella Urine Antigen EIA: 100.0%

Note: It is to be expected that the absolute sensitivity of the Legionella Urine Antigen EIA in patients with definite legionellosis is 50-70% (CDC Publication 8) shows 55.9% with comparable tests. The European Multicenter Study of the European Working Group on Legionella Infections EWGLI (5) showed a sensitivity of 94.6% for Legionella pneumophila serogroup 1 and a sensitivity of 86% including samples of serogroups 2, 3, 4, 6, 10.

Literature


Trouble Shooting Guide

1) NC value higher than criteria of validity > 0.100 OD:
   a) SUB turned blue due to oxidation or contamination.
   b) Washing fault: Perform 5x wash cycles/washing step. Use Bio-Rad WB as contained in the kit.
   c) Incubation fault: Temperature too high, incubation time was exceeded or plate was not incubated directly after finishing of pipetting.
   d) Wavelength fault: Measurement without reference filter will increase OD values approximately + 0.120 OD.
   e) Contamination with the lid of the Bio-Rad EIA kit.

2) Yellow coloration in all wells:
   a) WB contamination; Prepare new washing buffer.
   b) CONTamination; Repeat test with reagents from unopened vials. Use reagents under less microbial conditions.

3) Mean value of Bio-Rad EIA below < 0.600 OD:
   a) Exceed of expire date.
   b) Temperature too low or fall below incubation time.
   c) Washing fault: Too intensive washing or mechanic contact of manifold and solid phase of the well.
   d) Contamination of Bio-Rad EIA kit.

Key: Underline = Addition or significant change ► Deletion of text