ASPERGILLUS
Western blot
IgG

#ASP-WB24G : 24 tests
#ASP-WB12G : 12 tests
#ASP-WB96G : 96 tests

Immunoblot Assay for
*in vitro* diagnostic use

INSTRUCTIONS FOR USE

Intended use

ASPERGILLUS Western Blot (WB) IgG is a qualitative Immunoblot Assay for the confirmation of *Aspergillus* serology on human serum samples previously tested with classical IgG serology screening tests (ELISA, IF, HIA).

Introduction

*Aspergillus sp.*, from *Aspergillaceae* family and particularly from the Ascomyceta classification, is composed of about 185 species, 20 of them infecting humans. *Aspergillus fumigatus* is responsible of 80 to 90% of human infections.

Aspergillosis is an opportunistic infection which results mostly from a spores inhalation. The generated pathology depends on patients immune status: it may appear as an allergic form (allergic bronchopulmonary aspergillosis), a chronic local (aspergilloma for example) or an invasive infection. It becomes dangerous and fatal in 80% of cases when it affects immunodeficient patients.

Additionally to clinical features, specific antibodies detection is key for aspergillosis diagnosis in immunocompetent patients. ELISA and IF represent the mainly used technologies in the screening tests which are commonly completed by a confirmatory test as IPD (Immunoprecipitin detection), the current reference method.

A clinical retrospective evaluation of ASPERGILLUS WB IgG kit highlighted a good correlation with clinical features. Overall, WB proved at least as sensitive as the currently used IPD assays. This kit thus allows to confirm or contradict the presence of IgG anti-*Aspergillus* antibody in patient serum.
Principle of the test:

1/ The strips:
*Aspergillus fumigatus* antigens have been resolved by electrophoresis into bands and transferred by electro blotting onto a nitrocellulose membrane (Western Blot procedure). The antigen bearing membrane has been cut into ready to use numbered strips.

2/ The test:
Each specimen to be tested is incubated separately with a membrane strip. Specific anti-*Aspergillus* antibodies, if present in the sample, will bind to the antigens on the strip. The first wash step removes any unbound serum components. The strip is then incubated with an alkaline phosphatase-anti human IgG conjugate. Following the second wash step, the whole complex is then detected by addition of substrate (NBT/BCIP) which precipitates as a dark blue-purple colour. The colour development is stopped by rinsing the strips with distilled water. Specific IgG anti-*Aspergillus*, if present in the sample will appear on the strip as violet coloured bands. The strips are dried and their coloration remains stable for many years once stored away from direct light.

3/ Reading (see page 7 § Interpretation for details):
The presence of specific bands on the strip of is indicative of the presence of specific anti-*aspergillus* IgG in the sample. Test reading have to be done by comparing the strip with the positive control (R10).

Kit components

Nota: Numbers *in italic* correspond to volumes and quantities of the 12 tests kit #ASP-12G. Numbers *in bold* correspond to volumes and quantities of the 96 tests kit #ASP-96G.

R1 One (1) folder containing 24 (12 or 4x24) numbered TEST STRIPS
These are nitrocellulose membrane strips bearing electrophoresis separated *Aspergillus* antigens. Ready to use.

R2 One (1) vial containing 30 (30 or 125) ml of SAMPLE BUFFER:
buffer + surfactant + NaN3 (<0.1%) + red dye.
*Pink solution - Ready to use.*

R3 One (1) vial containing 30 (30 or 125) ml of ANTI IgG CONJUGATE
buffer + polyclonal goat anti-human IgG - Alkaline Phosphatase
conjugate + stabilisers + NaN3 (<0.1%) + blue dye. **Blue solution - Ready to use.**

**R5** One (1) vial containing 30 (30 or 125) ml of SUBSTRATE buffer + NBT + BCIP + stabilisers  
*Brown vial - Ready to use.*

**R6** One (1) vial containing 60 (60 or 250) ml of WASH CONCENTRATE 10X concentrated buffer + surfactant + NaN3 (<0.1%)  
*colourless solution - to be diluted 1/10 in distilled water.*

**R10** One (1) vial containing 100 (100 or 200) µl of POSITIVE CONTROL buffer + human serum positive in *aspergillus* serology + stabilisers + NaN3 (<0.1%).  
*Red/orange cap vial - Ready to use.*

- One (1) prestained Molecular Weights (MW) Standards Strip (recombinant proteins), visible on the right side of the nitrocellulose membrane (R1 folder) for estimating the MW of the revealed bands (kDa) : Blue = 250, Blue = 150, Blue = 100, Pink = 75, Blue = 50, Green = 37, Pink = 25, Blue = 20, Blue = 15.
- The kit also contains page 15 a Reference Sheet as example: the scanner copy of ASPERGILLUS WB IgG immunoblots (positive samples).

**Additional Materials Required**

- Latex powderless gloves and tweezers for handling the strips
- Multi-channel polypropylene incubation tray for mini-bLOTS (LDBIO # WBPP-8 or equivalent)
- Rocking platform
- Filtering flask connected to vacuum system (a water aspirator is sufficient)
- Cutter or scalpel
- Flat transparent ruler
- Absorbent paper (Whatman or equivalent)
- Aluminium foil
- High quality distilled or deionised water
- Automatic pipettes, micropipettes and disposable tips for dispensing volumes of 10µl, 1.2ml and 2ml
- Graduated cylinders and flasks
- Timer
- good quality transparent adhesive tape
- Serum collection equipment

Storage and stability

All reagents are stable until the expiry date (indicated on the outer box and bottle labels) when stored between 2-8°C.
Wash buffer solution is stable for 2 months after dilution when stored at 2-8°C

Precautions

1/ Safety
- All reagents derived from human origin have been tested and found negative for antibodies to HIV, hepatitis B surface antigen and hepatitis C antibodies. However, since no test can provide complete assurance of the absence of virus, treat all controls as potentially infectious.
- For in vitro use only.
- Do not mouth pipette reagents and avoid contact with skin.
- Wear protective clothing, disposable latex gloves and eye protection while handling specimens and performing the test. Wash hands thoroughly when finished.
- Never eat, drink or smoke at the laboratory workbench.
- Most of the reagents contain sodium azide as preservative. NaN₃ may form potentially explosive metal azides with lead and copper plumbing. For disposal, reagents should be flushed away with large volumes of water to prevent azide build up.
- Both samples and kit reagents should be handled with caution, following Good Laboratory Practice. Use care to prevent aerosols and decontaminate any sample spills.
- Substrate contains NBT and BCIP which may be toxic if inhaled or after contact with skin or mucous membranes. Any substrate that comes into contact with the skin should be rinsed off with water.
- Contact a licensed professional waste disposal service to dispose of all biological material (reagents, washing buffers, tips, vials, human sera…), follow the GMP’s and observe all state and local environmental regulations.

2/ Procedural
- Use powderless gloves and tweezers when handling western blots.
- Do not use reagents after their expiry date.
- Do not mix reagents from different lot numbers.
- Do not use the kit if liquid reagents become cloudy or develop precipitate.
- Do not freeze reagents.
- Deviation from the standard protocol provided may cause erroneous results.
- Mix well the reagents before using.
- Do not use a kit which contains a bottle that presents signs of leaks.
- Avoid leaving reagents in direct sunlight and/or above 4-8°C for extended periods. Return unused components to 2-8°C.
- Unused strips should be returned into their original plastic bag and resealed.
- If specimens cannot be tested on the day of collection, they should be stored at 2-8°C for up to 3 days. If longer storage is required, samples should be frozen at -20°C ± 5. Avoid repeated freeze-thaw cycles. On thawing, mix thoroughly.
- Do not contaminate samples or components and always use fresh pipette tips for each sample and component.
- High quality distilled or deionised water is required for the wash solution. The use of poor quality or contaminated water may lead to background colour in the assay.
- Mix the wash concentrate well, before dilution.
- Always use clean, preferably disposable, containers for all reagent preparation.
- Use care to prevent inter-channel contaminations.
- Use care to prevent aerosols formation.
- Establish a distribution plan before starting the test.
- Care must be taken to ensure that specimens are dispensed correctly into their designated test channel. If a specimen is inadvertently not added to a channel, the result for that channel will be negative, regardless of the actual status of the specimen.

Specimen collection

Specimen serum should be obtained following Good Medical Practice. A minimum sample of 10µl is required for ASPERGILLUS WB IgG testing. If specimens cannot be tested on the day of collection, they should be stored at 2-8°C for up to 3 days. If longer storage is required, samples should be frozen at -20°C ± 5.

Preparation of reagents

Wash Buffer (R6) - For 4 ASPERGILLUS WB IgG assays, dilute 10 ml of Wash Concentrate 10X (R6) in 90 ml of distilled or deionised water. Swirl to mix. A squeeze bottle can be used to dispense the diluted Wash buffer during the
washing steps.
Wash buffer solution is stable for 2 months after dilution when stored at 2-8°C.

Test Procedure

1/ Establish a distribution plan for control and samples before starting the assay. Strips are in numerical order, attached by the top in the folder R1. Add a positive control to validate the test and to help in the interpretation of the result.

2/ Dispense 1.2 ml of sample buffer (R2) into the designated channels.

3/ Wear powderless gloves. Don't touch the strips with fingers, handle strips with tweezers. Using a flat transparent ruler (well cleaned and dried) and a cutter (or a scalpel), cut out as many strips R1 as necessary: hold the strips in place by pressing the ruler firmly on the strips (numbers can be seen through the ruler) and cut out the strips along the bookbinding. Ensure that you keep the numbers on the strips.

4/ Using tweezers, place one membrane strip, face up, into each channel of the tray following the distribution plan. Return the remaining strips to their bag and reseal. Shake the tray gently to ensure that the strips are well covered with the buffer. Incubate for 1 min to thoroughly wet the strips.

5/ Dispense 10 µl of each specimen and 10µl positive control into the channels following the distribution plan. Shake the tray gently (avoid cross contamination) after each dispense for mixing. Incubate on the rocking platform (about 10 cycles/mn) for 90 min ± 5mn at 18-25°C.

6/ Wash step :
   6.1 - Aspirate the liquid from each channel with a disposable tip connected to a vacuum system or by flipping over the incubation tray. Dispense about 2 - 3 ml of diluted Wash Buffer into each channel. Shake the tray gently and aspirate the liquid from the channels or flip over the tray.
   6.2 - Dispense another 2 - 3 ml of diluted Wash Buffer into each channel. Incubate on the rocking platform for 3-5 mn. Aspirate the liquid from the channels flip over the tray.
   6.3 - Repeat step 6.2 one more time.

7/ Put 1.2 ml of anti IgG conjugate (R3) into each channel of the tray.
   Shake the tray gently to ensure that the strips are well covered with the buffer, face up. Incubate on the rocking platform for 60 min ± 5mn at 18-25°C.
8/ Wash step: Repeat step No 6.

9/ Dispense 1.2 ml of NBT/BCIP substrate (R5) into the channels. Shake the tray gently to ensure that the strips are well covered with the substrate, face up. Incubate on the rocking platform for 30 to 60 min at 18-25°C. Cover the tray with a piece of aluminium foil. Stop the colour development by aspirating the liquid from the channel or by flipping over the incubation tray and dispensing 2 ml of distilled water. Repeat this step one more time.

10/ Take the strips at the top, using the tweezers and transfer the strips face up onto a sheet of filter paper and let air dry. Leave them to dry about 15mn: the colour of the strips becomes much lighter after drying.

11/ Keeping them in place with a dry and clean flat ruler, stick the top of the strips to the paper with a transparent adhesive tape. Warning: strips are more fragile when dried.

**Interpretation**

**Molecular Weight (MW) identification tools**

- The MW of unknown bands can be estimated by comparing their positions with those of bands on the prestained MW Standards Strip (R1 folder). Before using, cut out the Standards Strip from the folder R1 with the help of a flat ruler and a cutter.
- The strip obtained from the R10 positive control, when tested with an adjoining membrane strip at the same time as the patient’s sample, is the most precise positive standard of the kit. It will supply an exact model for the definite identification of the specific 16, 18-20, 22 and 30 kDa bands. These bands are so called:

  **P16, P18-20, P22, P30**

- Other non specific bands can also appear above 30 kDa on the positive control.
- The kit also contains one (1) Reference Sheet: a scanner copy of *Aspergillus* immunoblot testing (negative and positive results, see page 15). It can only be used for example.
Interpretation

Remarks:
- Some sera show very weak bands (shadows) which can be seen or not depending on the reader. A strip showing two or more such very pale bands among the four specific bands is considered as a positive WB result.

- Other specific bands with a MW of 10 and 15 kDa may be present on the strip. Because their very low frequency they are not used for the diagnostic.

Limitations of use:

1/ Results of ASPERGILLUS WB IgG should be considered in the context of all available clinical, epidemiological and laboratory data before diagnosing an Aspergillus infection.

2/ Bands may have variable appearances: thin, thick, more or less coloured... It is recommended therefore to start using ASPERGILLUS WB IgG assay by running some known sera for training.

3/ A negative ASPERGILLUS WB IgG result does not exclude the possibility of an infection.

Trouble Shooting

"Bands are pale, without contrast": The concentration of specific antibodies in the serum could be very low (cf. Interpretation p.8).

"Bands become rapidly large and dark so that they can't be clearly interpreted". The concentration of specific antibodies in the serum can be very high. Monitor
colour development (last incubation) to avoid over-development

"The strip seems partly coloured with fuzzy bands, wave-like stains":
The strip was not totally covered with reagent during one of the incubation steps. Artifact stains may be present on the strip at the dispensing place when the sample has not been mixed after dispensing.

"The strip presents a high background staining":
Washing steps were insufficient or the last incubation step was too long. Check the technical procedure, especially the washing steps and the quality of the deionised water. Monitor colour development to avoid over-development. Exceptionally, the sample can be the cause of the phenomenon (non-specific binding). The WB is not interpretable. Non-specific binding may concern only one part of the strip. The WB is then not interpretable on this part only.

"A dark purple precipitate appears in the buffer during the last incubation (colour development step)" :
The substrate can partially precipitate in the buffer during the last incubation. This will not influence the normal colour development of the bands on the strips. Solid particles of precipitate will be washed out during the last washing step. Don't mind and continue the incubation following the test procedure.

Quality control

A positive control is included in the kit. It is useful for:
- the validation of the technical procedure (the bands must appear clearly on the strip),
- the precise identification of the significant bands.
Performance characteristics

The evaluation of the kit was made in four references laboratories specialized in aspergillosis diagnostic.

1/Sensitivity

A panel of 308 sera was retrospectively collected in four University Hospitals from 158 patients according clinical and serological data. IPD has been performed in each laboratory as part of the patients’ routine diagnostic work-up. Sera were divided into two categories: chronic aspergillosis (n=267) and colonization (n=41).

<table>
<thead>
<tr>
<th>Group</th>
<th>Sera (s)</th>
<th>WB (%)</th>
<th>IEP (%)</th>
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<tr>
<td><strong>Chronic Aspergillus diseases</strong></td>
<td></td>
<td></td>
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<tr>
<td>Chronic Pulmonary Aspergillosis</td>
<td>197</td>
<td>93,9%</td>
<td>87,3%</td>
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<tr>
<td>Uncomplicated Aspergilloma</td>
<td>13</td>
<td>100,0%</td>
<td>100,0%</td>
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<tr>
<td>ABPA</td>
<td>57</td>
<td>98,2%</td>
<td>86,0%</td>
</tr>
<tr>
<td><strong>Aspergillus colonization</strong></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Cystic fibrosis +</td>
<td>18</td>
<td>100,0%</td>
<td>72,2%</td>
</tr>
<tr>
<td>Cystic fibrosis -</td>
<td>23</td>
<td>52,2%</td>
<td>39,1%</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td>308</td>
<td>92,2%</td>
<td>83,1%</td>
</tr>
</tbody>
</table>

Remarks:
- Among the positive results, 84% show at least a three specific bands WB pattern. This underlines the easiness to read and to make the interpretation of the WB results.
2/ Specificity

Specificity of ASPERGILLUS WB IgG was performed on sera from 213 Blood Donors (n=212)

<table>
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<th>WB(%)</th>
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<tr>
<td>Specificity</td>
<td>94%</td>
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Remarks:
- This low percentage of positive results among Blood Donor’s sera could be explained by the ubiquity of Aspergillus and the presence of an immunological response due to a contact in some people.

3/ Reproducibility:
- Inter-lots Reproducibility:
  8 positive serums were twice tested in the same serie with reagents resulting from 2 different lots (antigens from different production lots): the correlation between both tests for each serum is satisfactory.
- Intra-series Reproducibility:
  8 positive sera were twice tested in the same series with reagents resulting from the same lot: the correlation between both tests for each serum is total.

4/ Interferences:
Although no particular interference was found with hemolyzed, icterical or lipidic sera, it is recommended to interpret results of such samples with caution.
Personal notes
References:


SUMMARY OF ASSAY PROCEDURE:

This summary is for quick reference only.
Read the full instructions for use before starting an assay.

1/ Establish a distribution plan

2/ Dispense 1.2ml R2 (sample buffer - pink) into each channel.

3/ Place strips R1, face up, into each channel following distribution plan. Shake gently.

4/ Dispense 10µl samples and control R10 following distribution plan.
   Shake gently the tray after each dispense.
   Incubate 90 mn on rocking platform.

5/ Wash 3 times with 1/10 diluted R6.

6/ Dispense 1.2ml R3 (IgG conjugate - blue) following distribution plan.
   Shake gently the tray.
   Incubate 60 mn on rocking platform.

7/ Wash 3 times with 1/10 diluted R6.

8/ Dispense 1.2ml R5 (substrate), shake gently.
   Incubate 60 mn on rocking platform (check the colour development regularly).

9/ Stop the reaction by rinsing 2 times with distilled water.

10/ Transfer strips on a filter paper – Let air dry 15mn.

11/ Compare IgG pattern to the positive control R10
ASPERGILLUS WB IgG
Immunoblot Examples

A minimum of two well defined bands among P16, P18-20, P22, P30 is indicative of the presence of specific anti-Aspergillus IgG in the patient’s sample.

The diagnosis of Aspergillosis must be done taking clinical and epidemiological features, imaging and all biological data into account.

Remark: Some sera show very weak bands (shadows), more or less present depending on the readers. The presence on the strip of two or more such very pale bands among the four specific bands is considered as a positive WB result.
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