

Instruction for Use for NEO-SENSITABS™

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NEO-SENSITABS™ Antimicrobial Susceptibility Testing

Manufacturer

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Intended use

Neo-Sensitabs are tablets used for semi-quantitative *in vitro* susceptibility testing by the agar disk/tablet diffusion test procedure of common rapidly growing non-fastidious organisms, certain fastidious bacterial pathogens, and yeasts.

Principles of the procedure

Neo-Sensitabs containing a variety of antimicrobial agents are applied to the surface of a proper agar media, which has been inoculated with pure culture of clinical isolates.

Non-fastidious organism, which includes *Enterobacteriaceae*, *Staphylococcus* spp., *Pseudomonas* spp., *Acinetobacter* spp., *Enterococcus* spp. and *Vibrio cholerae*, can be tested on a media without blood or other supplement e.g. Mueller Hinton II agar (MH). *Haemophilus influenzae* requires Haemophilus test media (HTM), *Neisseria gonorrhoeae* GC agar base (GCA), *Streptococcus pneumoniae* and other streptococci require Mueller Hinton II agar + 5 % blood (MH+B). Yeast should be tested on RPMI-1640 agar or modified shadomy agar and anaerobes requires special procedures.^{1,2}

Following incubation, the plates are examined and the zone diameters of inhibition around the tablets are measured and compared with the zone interpretative tables for the individual antibiotics in order to determine the agent(s) most suitable for use in antimicrobial therapy.

Neo-Sensitabs are standardized according to the MIC breakpoints recommended by the CLSI (NCCLS).^{3,4} and EUCAST Furthermore, Neo-Sensitabs are adjusted to the MIC breakpoints recommended by "Susceptibility Testing Standardization Groups" in France and the United Kingdom). Zone diameter interpretative criteria for the different countries can be found in Neo-Sensitabs User's Guide (www.rosco.dk).¹

Reagents

Neo-Sensitabs are 9 mm tablets containing crystalline antimicrobials carefully mixed with a protective granulate. The tablets are printed on both sides with a unique 5-character code. Neo-Sensitabs are furnished in cartridges each containing 50 tablets. Cartridges can be used with a Neo-Sensitabs dispenser. The dispensers deliver 7, 9, 12 or 16 Neo-Sensitabs at a time and as the tablets are self-tamping to the media, no extra pressure on the tablets is necessary.

Storage instructions

1. On receipt check the temperature symbol on the outer container. Neo-Sensitabs with a 2 °C to 8 °C symbol should be stored in a refrigerator, and Neo-Sensitabs with a 25 °C as an upper temperature symbol on the outer container should be stored at room temperature.
2. If Neo-Sensitabs are stored in the refrigerator, allow the cartridge to reach room temperature before being opened, i.e. 30 – 60 minutes, in order to avoid water condensation on the tablets.
3. Neo-Sensitabs with the temperature symbol 2 °C to 8 °C may be left at room temperature for up to 2 months, without essential loss of activity.
4. Opened cartridges placed in a dispenser must be used within 2 months for Neo-Sensitabs with the temperature symbol 2 °C to 8 °C, and within 12 months for Neo-Sensitabs with the temperature symbol below 25 °C.

The expiry date on the cartridges applies only to cartridges with lids stored at correct temperature.

Precautions

Follow the directions for use. Neo-Sensitabs performance depends not only on the potency of the tablets, but also on use of proper inoculum and agar plates, incubation temperature, correct interpretation of the zone diameter, correct storage of Neo-Sensitabs, and on use of control cultures.⁵

Specimen

The specimen must be fully typical of the site of infection, i.e. every effort should be made to obtain a representative sample of the relevant pathogenic bacteria. See directions, which include preparation of inoculum.

PROCEDURE

Materials provided: Neo-Sensitabs as labelled.

Materials required but not provided: Culture media, reagents, quality control organisms and laboratory equipment necessary to perform agar diffusion susceptibility test by standardized procedure. Prepare a McFarland 0.5 turbidity standard by adding 0.5 mL of 0.048 M BaCl₂ (1.175 % wt/vol BaCl₂·2H₂O) to 99.5 mL of 0.18 M H₂SO₄ (1% (vol/vol)) with constant stirring. Alternatively, a prepared standard could be purchased. Verify by using a spectrophotometer with a 1 cm light path and matched cuvette; absorbance at 625 nm should be 0.08-0.10.^{3,4}

I. Directions for use/Bacteria

I.1. Inoculum standardization according to CLSI (NCCLS)³ and EUCAST

Direct colony suspension method:

By suspending several morphologically similar colonies from an 18-24 h agar plate (non selective) into 4-5 mL 0.9 % NaCl solution, turbidity equivalent to the BaSO₄ standard (0.5 McFarland) can be developed. The method is equivalent to the standard CLSI (NCCLS) method and requires less time. The approach is recommended for testing the fastidious organisms like *Haemophilus* spp., *N. gonorrhoeae*, *Moraxella catarrhalis*, pneumococci/streptococci and for testing staphylococci for potential methicillin or oxacillin resistance.³

I.2. Inoculation

- a) Within 15 minutes, dip a sterile cotton swab into the adjusted suspension and remove inoculum from the swab by exerting firm pressure on the inside of the tube.
- b) Within 15 minutes swabs are used to inoculate the test plates.

- c) Inoculate the dried surface of the appropriate agar plate by streaking the swab over the entire surface. Allow the surface to dry 3-5 minutes or maximum 15 minutes before applying Neo-Sensitabs to the media.
- d) Select appropriate tablets e.g. such as recommended by CLSI (NCCLS).⁶ Use no more than nine Neo-Sensitabs per 150 mm plate or four Neo-Sensitabs per 100 mm plate when testing *H. influenzae*, *N. gonorrhoeae*, and *Streptococcus* spp.

I.3. Incubation and reading of plates

- a) Within 15 minutes, place the agar side up in a 35 °C incubator. *Haemophilus* spp., *N. gonorrhoeae*, *S. pneumoniae* and other streptococci should be incubated in an atmosphere enriched with 5 % CO₂.
- b) Examine the plates after 16-18 hours incubation (20-24 h for *N. gonorrhoeae*, *S. pneumoniae* and other streptococci). A full 24-hours incubation is recommended for the detection of Methicillin resistant *Staphylococcus aureus* (MRSA) and *Enterococcus* spp. for vancomycin resistance. Hold the plate up to transmitted light and examine the oxacillin and vancomycin zones for light growth (minute colonies) of methicillin or vancomycin resistant colonies, respectively, within apparent zones of inhibition. Any discernible growth within the zone of inhibition is indicative of methicillin or vancomycin-resistance. The edges of the zones of inhibition contain a large number of small colonies when using Trimethoprim, Sulphonamides and Trimethoprim + Sulfamethoxazole tablets. In this case zones of inhibition are measured up to colonies of normal size (disregard slight growth and measure the more obvious margin). For further details read User's Guide Neo-Sensitabs (www.rosco.dk).
- c) The diameters of the zones of complete inhibition are measured as determined by gross visual inspection. Zones are measured to the nearest whole millimeter.

II. Directions for use/Yeast

II.1. Inoculum preparation

The inoculum should result in just confluent growth. For most strains, the inoculum should contain approximately 5x10⁵CFU/ mL (McFarland 0.5 diluted 1+1 with saline). For *Candida krusei* use an inoculum equivalent to McFarland 0.5, diluted 1:10 and for *Cryptococcus* spp. use an inoculum equivalent to McFarland 1.0, undiluted.

II.2. Inoculation

- a) Plates are dried for 20-25 minutes at 35 °C before inoculation.
- b) 0.5 mL (9 cm plate) or 1.0 mL (14 cm plate) of the prepared inoculum is poured onto the agar surface (flooding) and excess liquid is removed immediately with a pipette.
- c) The open plate is dried at 35 °C for 10 minutes and the tablets are placed on the agar surface.

II.3. Incubation and reading of plates

Incubation at 35 °C overnight is suitable for most strains isolated from systemic infections. Examination and reading of the plate should be made after 18-24 hours. If growth is not yet visible with particular strains, the plates may be reincubated for up to 24 hours more. For *Cryptococcus* spp. incubate at 30 °C for 42-48 hours.

II.4. Measuring of inhibition zones

For the polyenes (Amphotericin B and Nystatin) the clear zone with no visible growth inside is measured. For these antifungals colonies inside the zone must be considered resistant mutants. For the Azoles,

Imidazoles and Terbinafine the zones must be measured up to colonies of normal size. There is often a zone of growth of partially inhibited colonies the sizes of which are smaller nearer the tablet than at the edge of the real zone. These small and medium size colonies are not resistant mutants. Imidazoles/azoles Neo-Sensitabs contain doxycycline in order to improve the quality of reading of their zones. For Fluorocytosine measure the zone up to colonies of normal size. Individual colonies inside the zone are usually *resistant* mutants (isolate and test again).

INTERNAL QUALITY CONTROL

Quality control procedure using ATCC strains should be performed at least once a week and every time a new lot of agar is introduced. The measured diameter should be within the control zone diameter limits for the specific combination of Neo-Sensitabs and control strains. Control strains limits are given in the tables and indicate the correct performance of the entire procedure.^{1,3}

RESULTS

Compare recorded zone diameter with those in the tables. Results with a specific organism may be reported as Susceptible (S), Intermediate (I) or Resistant (R)³:

Susceptible (S): The infection due to the strain tested may be expected to respond to a normal dosage of the antimicrobial agent in the tablet.

If only "S" criteria are specified: For some organism/antimicrobial combinations, the absence of resistant strains precludes defining any category other than susceptible. For strains yielding results suggestive of "non-susceptible", organism identification and antimicrobial susceptibility test results should be confirmed. Subsequently the isolates should be submitted to a Reference Laboratory for further testing.

Intermediate (I): The intermediate category implies clinical applicability in body sites where the drugs are concentrated (e.g. urine) or when high dosage of an antimicrobial can be used (e.g. betalactams). The intermediate category also comprises a "buffer zone", which should prevent small, uncontrolled technical factors from causing major discrepancies in interpretations; thus, when a zone falls within the intermediate range, the results may be considered equivocal, and if alternative drugs are not available, MIC testing may be indicated.

Resistant (R): The antimicrobial cannot be recommended for treatment in this case.

Screening and Confirmatory Tests for Extended-Spectrum Beta-Lactamases (ESBL) and Carbapenemases

Transferable plasmid-mediated beta-lactamases that produce resistance towards third generation cephalosporins and monobactams (e.g. aztreonam) have been described in strains of *Klebsiella pneumoniae*, *K. oxytoca*, *E. coli* and other Enterobacteriaceae. These enzymes are classified as extended-spectrum beta lactamases (ESBL) and Carbapenemases and they have been implicated in clinical resistance to monobactams and broad-spectrum cephalosporins. Some of these strains will show zones of inhibition below the normal susceptible strains but above the standard breakpoints broad-spectrum cephalosporins or aztreonam. These strains can be screened by using appropriate ESBL screening breakpoints. Most ESBLs are inhibited by clavulanic acid, tazobactam or sulbactam and can be readily detected by the double-disk (tablet) synergy test.⁶ Ceftazidime + Clavulanate and Cefepime + Clavulanate are very useful for confirmatory tests of ESBL. Further information may be obtained in Neo-Sensitabs User's Guide and Detection of resistance mechanisms (www.rosco.dk).¹ All ESBL strains should be reported as resistant for all penicillins, cephalosporins and aztreonam. Carbapenemase producing strains should be reported as resistant to all carbapenems.

Methicillin resistant staphylococci

Screening for MRSA and methicillin (oxacillin) resistance in coagulase negative staphylococci should be performed using Oxacillin 1 µg and Cefoxitin. Resistance indicates that all beta-lactams should be reported as resistant. Further information may be obtained in Neo-Sensitabs User's Guide (www.rosco.dk).¹

***Staphylococcus aureus* with reduced susceptibility to vancomycin (hVISA, VISA (GISA) and VRSA)**

Vancomycin-resistant *Staphylococcus aureus* (VRSA) should be detected using Vancomycin 5 µg or 30 µg. Strains with reduced susceptibility to vancomycin (hVISA and VISA (GISA)) cannot be detected by the current diffusion method. A prediffusion method should be used. Further information may be obtained in Neo-Sensitabs User's Guide and Detection of resistance mechanisms (www.rosco.dk).¹

Vancomycin resistant enterococci (VRE)

The detection of VRE by the diffusion method requires the following:

1. Use of Vancomycin 5 µg Neo-Sensitabs,
2. Incubation for full 24 hours,
3. Careful examination of the zone of inhibition.

Sensitive strains show a sharp edge of the zone, while resistant strains show a hazy edge. A prediffusion method with Vancomycin 30 µg Neo-Sensitabs produces excellent results. Further information may be obtained in Neo-Sensitabs User's Guide (www.rosco.dk).¹

High-level aminoglycoside resistance (HLR)

High-level resistance to aminoglycosides is an indication that an enterococcal isolate will not be affected synergistically by a combination of a penicillin or glycopeptide plus an aminoglycoside. Screening for high-level gentamicin and streptomycin resistance should be performed on enterococcal isolates from blood or CSF. High-content Neo-Sensitabs like Gentamicin 250 µg, Kanamycin 500 µg and Streptomycin 500 µg are used to screen for this type of resistance.

LIMITATIONS OF DIFFUSION METHODS

The purpose of using Neo-Sensitabs is to provide fast and accurate antimicrobial susceptibility testing. Acceptable results derived from quality controls strains do not guarantee accurate results with all patient isolates. Where atypical or inconsistent results are encountered, repeat testing and/or repeat identification procedure should be performed to ensure accurate results. Unexpected results should be considered for reporting and isolates could be sent to reference laboratories for further testing.

Dangerously misleading results can occur when certain antimicrobials are tested against specific microorganisms.^{3,6} Resistant mechanisms in some species are more difficult to detect than others, and some results may appear active in vitro, even though the antimicrobial agents are not effective clinically. These combinations include the following:

- All beta lactam antibiotics (except oxacillin, methicillin) against methicillin-resistant staphylococci
- Cephalosporins, aminoglycosides (except testing for high level resistance), clindamycin, and trimethoprim + sulfamethoxazole against enterococci
- First and second-generation cephalosporins and aminoglycosides against *Salmonella* spp. and *Shigella* spp.
- Cephalosporins against *Listeria* spp.
- Glycopeptides against *S. aureus* with reduced susceptibility to vancomycin.
- Cephalosporins and aztreonam against ESBL-producing *E. coli*, *K. pneumoniae* and *P. mirabilis*

Routine reporting of results from strains isolated from the CSF could be dangerously misleading for patient care in the following cases:

- Agents administered orally only
- 1st and 2nd generation cephalosporins (except Cefuroxime sodium)
- Clindamycin
- Macrolides
- Tetracyclines
- Fluoroquinolones

Some antimicrobials are associated with the emergence of resistance during prolonged therapy. As a consequence, isolates initially susceptible may become resistant within a few days after initiation of treatment. This occurs most frequently in:

- *Enterobacter*, *Citrobacter*, and *Serratia* spp. with third generation cephalosporins
- *Pseudomonas aeruginosa* with most antimicrobials
- Staphylococci with quinolones

Essentially all isolates of *Enterobacter aerogenes*, *E. cloacae*, *Citrobacter freundii*, *Providencia* spp., *Proteus* spp. (except *P. mirabilis*), *Serratia marcescens*, *P. aeruginosa*, possess the genes for Group I beta-lactamase production. Therefore, there is no useful information from demonstrating in vitro induction of the enzyme. Laboratories should focus on repeat testing (every 3-4 days) of isolates repeatedly recovered from infected patients during therapy to detect selection of clones that constitutively produce Group I beta-lactamase.

REFERENCES

- 1) Neo-Sensitabs User's Guide. 2013. www.rosco.dk.
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- 3) National Committee for Clinical Laboratory Standards. 2009. Performance Standards for Antimicrobial Disk Susceptibility Tests. Approved Standard M2-S10. NCCLS, Wayne, Pa., USA.
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- 5) Ericsson H.M. and J.C. Sherris. 1971. Antibiotic susceptibility testing. Report of an international collaborative study. Acta Path. Microbiol. Scand. Sec. B, Suppl. 217: 1-90.
- 6) Clinical and Laboratory Standards Institute/NCCLS. 2013. Performance Standards for Antimicrobial Susceptibility Testing. M100-S23. 23rd Informational Suppl. CLSI, Wayne, Pa., USA.