

## Inventory of Methods for Mycobacterial Culture and Phenotypic Drug Susceptibility Testing (DST)

### From the culture and phenotypic DST sub-group of the STOP TB Partnership New Diagnostics Working Group

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#### Main Tests at a Glance

	Solid media				Liquid media							
	Egg-based		Agar-based		Automated				Manual			
	LJ	Ogawa	7H10	7H11 <sup>1</sup>	BACTE C 460	MBBacT ALERT	MGIT	ESP II	MGIT	CRI	MODS	NRA
<b>Culture</b>	√	√	√	√	√	√	√	√	√		√	
<b>Direct DST</b>	√ <sup>2</sup>			√ <sup>1</sup>							√	
<b>Indirect DST</b>	√ <sup>2</sup>		√	√	√	√	√	√	√	√		√

<sup>1</sup> includes Thin Layer Agar

<sup>2</sup> includes NRA

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## 1. Solid Media Culture

Culture of mycobacteria provides the definitive diagnosis of tuberculosis and is considered the gold standard for the bacteriological confirmation of the disease, whilst also allowing the opportunity to perform drug susceptibility testing. Culture on solid media is the most widely used technique

a) **Solid culture on Löwenstein-Jensen** - Among the various available media, Löwenstein-Jensen (LJ) is the most commonly used egg-based medium. The medium contains glycerol as carbon source, L-asparagine as nitrogen source, salt solutions and malachite green as an inhibitor of contaminants.

**Potential advantages:** The Löwenstein-Jensen medium is less expensive than agar-based media, easy to prepare and contamination during the preparation is limited because it is inspissated after being placed in bottles. The medium has a good buffer capacity and materials in the inoculum toxic for mycobacteria are neutralized. LJ has a long shelf life if stored in the refrigerator (several weeks). Like other solid media, LJ allows direct visual recognition of colonies characteristic of *M. tuberculosis* and growth of the contaminants.

**Considerations:** The LJ medium can be homemade or commercial but variations from batch to batch depend on the quality of the eggs used. The main disadvantage of the medium is the need for a long incubation time; it may take as long as eight weeks before cultures become positive, especially if the inoculated clinical specimen contains few bacilli.

**Requirements:** Culture on LJ require BSL-3 facilities if the tubes will be opened for speciation or further testing involving culture manipulation, and the usual equipment and consumables required to perform solid culture. An inspissator is required in order to prepare the medium. Detailed guidelines for the preparation are freely accessible on the internet (Laboratory Services in Tuberculosis Control Part III: Culture: <http://www.phppo.cdc.gov/dls/ila/documents/lstc3.pdf>)

**Product information:** potassium dihydrogen phosphate (K<sub>2</sub>HPO<sub>4</sub>), magnesium sulphate (MgSO<sub>4</sub>), magnesium citrate, L-asparagine, glycerol, malachite green dye, which can be purchased at [www.merck.com](http://www.merck.com); [www.sigmaaldrich.com](http://www.sigmaaldrich.com) and other reliable suppliers.

b) **Solid culture on agar-based medium** - The agar-based culture media are transparent, allowing better visualization of the colony as they appear on the surface of the medium. The most frequently used media are Middlebrook 7H10 and 7H11. The Middlebrook media are composed of a chemically defined base supplemented with oleic acid-albumin and designed to allow more rapid and luxuriant growth of mycobacteria. Albumin protects tubercle bacilli against the toxic effects of the reagents used in the decontamination process and increases the isolation rate in primary culture.

**Potential advantages:** The agar-based media have shown a slightly higher isolation rate than the egg-based media. As for other solid media, Middlebrook media allows visual recognition of colonies characteristic of *M. tuberculosis* and the growth of contaminants. The media is very simple to prepare and the basic ingredients are commercially available: powder base and Middlebrook OADC enrichment, and can also be purchased in packages of 10 -100

slants. Microscopic examination can be performed by turning over the Middlebrook plate and may provide earlier detection of microcolonies as soon as 10 days.

**Considerations:** The media are more expensive than the egg-based media.

**Product information:** Middlebrook 7H10 agar base, Middlebrook 7H11 agar base, OADC enrichment, prepared media in slants package can be purchased at [www.bd.com](http://www.bd.com).

c) **Ogawa-Kudoh method** – This is a culture on solid medium in which the prior decontamination of sputum does not require centrifugation. Sputum is decontaminated by an alkaline solution (NaOH at 4%) without subsequent neutralization, and inoculated on acidified modified Ogawa medium or Ogawa-Kudoh medium.

**Potential Advantages:** The method does not use equipment and can be performed in peripheral microscopy laboratories. The culture medium is an egg-based medium similar to that of Lowenstein-Jensen but less expensive because it replaces the L-asparagine by sodium glutamate. If presumptive identification of *M. tuberculosis* is done by visualization of typical *M. tuberculosis* colonies (colony morphology) it can be performed in BSL-2 laboratory conditions. The Ogawa-Kudoh method is the most simple, practical and inexpensive method to be used by National Programs to expand the use of culture in peripheral laboratories or rural settings. If the program has a well-structured network of laboratories, the tubes with positive culture in Ogawa-Kudoh medium can be sent to a reference laboratory for final identification and sensitivity test, without loss of viability of the bacillus. The culture medium is homemade or it can be prepared by the reference laboratory and sent to peripheral laboratories.

**Considerations:** The method of decontamination of sputum is considered a drastic method, using 4% NaOH for decontamination without further neutralization and should not be used for paucibacillary specimens.

**Requirements:** As with all cultures of *M. tuberculosis*, a BSL-2 laboratory is required with a bacteriological incubator. An inspissator is required in order to prepare the medium. Training of technicians who perform microscopy in biosafety precautions and recognition of the colony morphology of *M. tuberculosis* is needed.

**Product information:** potassium dihydrogen phosphate (K<sub>2</sub>HPO<sub>4</sub>), magnesium citrate, sodium glutamate, glycerol, malachite green dye can be purchased at [www.merck.com](http://www.merck.com); [www.sigmaaldrich.com](http://www.sigmaaldrich.com) or other reliable suppliers.

d) **Culture on Thin Layer Agar** – The method is based on the earlier identification of *M. tuberculosis* growth by the characteristic colony morphology, taking into account consistency, colony border and tendency towards cord formation. The thin layer method culture is performed in plastic Petri dishes with a solid media (Middlebrook 7H11) to which is added OADC and an antibiotic mixture. The *M. tuberculosis* microcolonies can be visualized with conventional microscopy as early as five days of incubation.

**Potential advantages:** The method allows both a rapid detection and presumptive identification of *M. tuberculosis* isolates by microscopy. A quadrant can also be inoculated

with PNB to permit differentiation of NTMs. It is a relatively low-cost and simple method and it does not require specialized equipment. The average time to detect the growth of mycobacteria is 8-10 days.

**Considerations:** The principal disadvantage is the requirement for manual observation of growth twice or thrice in the first two weeks which increases the work time required for TB diagnosis. Furthermore, the contamination rate reported in TLA is higher than when using Löwenstein-Jensen medium.

**Requirements:** BSL-2 facilities and the usual equipment and consumables required for mycobacterial isolation are needed. BSL-3 facilities are required for further testing such as species identification, drug susceptibility testing. Laboratory staff needs to be trained in detection of the “cording” characteristic growth of *M. tuberculosis* and biosafety precautions.

**Product information:** Middlebrook 7H11 broth base, OADC enrichment and antibiotic mixture (PANTA) can be purchased at [www.bd.com](http://www.bd.com).

## 2. Drug Susceptibility Testing on Solid Media

The conventional phenotypic methods for drug susceptibility testing are based on inoculation of cultured isolates (or for some, directly of sputum) on solid media. Three solid culture methods using egg-based or agar-based media are used around the world: the proportion method, the resistance ratio method, and the absolute concentration method. They are inexpensive and highly standardized for testing susceptibility to many drugs, but they have the major disadvantage of a long turnaround time. The most widely used is the proportion method, described further here.

**a) Proportion method** - the proportion method is the most commonly used method worldwide. The test is based in the exact determination in an inoculum of the proportion of organisms present that is resistant to a specific concentration of each drug, by comparing quantity of growth in a drug-containing and drug-free control media. When performed in egg-based media, the final reading is done after 42 days of incubation; if the test is performed on agar-based media and the medium is incubated in a 10% CO<sub>2</sub> atmosphere, results are obtained after 21 days of incubation. This delay is additional to that required for the original culture that yields the isolate for testing.

**Potential advantages:** The method does not use proprietary products or specialized equipment, is inexpensive and highly standardized for testing susceptibility to many drugs.

**Considerations:** The main disadvantage is the long time of incubation, up to 6 weeks, to report the final results. When using egg-based media, a large number of tubes are inoculated for each test and the tubes incubated up to 42 days, requiring a large incubator space. Furthermore the method is labour intensive and requires a careful quality control of all batches produced with drug susceptible and drug resistant strains for reliable results.

**Requirements:** similar to those required for culture on LJ medium.

**Product information:** antibiotic powders can be purchased at [www.merck.com](http://www.merck.com) [www.sigmaaldrich.com](http://www.sigmaaldrich.com) - and other reliable suppliers.

**b) Nitrate reduction assay (NRA)** – The nitrate reduction assay is a very simple and rapid technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding potassium nitrate to the culture medium.

**Potential advantages:** The NRA is an inexpensive novel phenotypic method. It is straightforward, does not require specialized equipment and uses slightly modified but otherwise conventional LJ media (it can also be done in liquid media – see below) with the same methodology and the same concentration of antimicrobial drugs that is used for the proportion method. However, instead of awaiting the appearance of MTB colonies visible to the naked eye on the surface of the media, the readout is a colour change revealed in the media itself (after addition of a specific reagent to the culture tube) due to reduction of the nitrate. This reduces the time to detection from inoculation of the cultured isolate from 42 days by the proportion method to 10 days. The sensitivity and specificity for the detection of INH and RIF resistance are comparable to the conventional methods. Biosafety problems are limited as the test is performed in a solid medium, though the addition of a reagent to an open culture has the potential to generate aerosols and therefore should be performed in a biological safety cabinet.

**Considerations:** NRA does not work for the (rare) nitrate reductase negative *M. tuberculosis* strains, neither for *M. bovis*. However this assay has been endorsed by the WHO (2010).

**Requirements:** BSL-3 facilities and the usual equipment and consumables required to perform solid culture. Laboratory staffs need to be well trained in biosafety precautions, mycobacterial techniques and quality control issues.

**Product information:** The NRA-specific reagents sulfanilamide, N-1-naphthylethylenediamine Dihydrochloride and antibiotic powders can be purchased at [www.merck.com](http://www.merck.com) [www.sigmaaldrich.com](http://www.sigmaaldrich.com) or other reliable suppliers.

**c) Thin Layer Agar DST** – The method relies upon the rapid detection of *M. tuberculosis* microcolonies in agar plates by conventional microscopy. The thin layer agar method for drug susceptibility testing is performed in plastic Petri dishes with a solid media (Middlebrook 7H11) supplemented with OADC.

**Potential advantages:** The method can be applied for the simultaneous diagnosis of TB and resistance to RMP and INH, directly from sputum samples. It is a relatively low-cost and simple method and does not require specialized equipment. In 2010, the WHO determined that TLA showed promise for direct DST but that there was as yet insufficient evidence to endorse the method. Further operational field data are awaited, particularly for direct DST.

**Considerations:** The principal disadvantage is the requirement for manual observation of the plates on alternate days in the first two weeks. Further studies are still needed to confirm the accuracy of the method.

**Requirements:** BSL-3 facilities. Laboratory staff well trained in detection of the “cording” characteristic growth of *M. tuberculosis* and biosafety precautions.

**Product information:** antibiotic powders can be purchased at [www.merck.com](http://www.merck.com) [www.sigmaaldrich.com](http://www.sigmaaldrich.com) or other reliable suppliers. Middlebrook 7H11 broth base, OADC enrichment can be purchased at [www.bd.com](http://www.bd.com).

### 3. Automated Liquid Culture Systems

Liquid systems are more sensitive for detecting mycobacteria, and may therefore increase the case yield by 10% over solid media, as well as reducing the time to result. Positive cultures require some form of confirmatory identification to confirm the presence of *Mycobacterium tuberculosis* complex – this might be microscopy of an aliquot of media for acid-fast bacilli, molecular or biochemical testing, or recognition of characteristic phenotypic growth in media. Cultures containing both *M. tuberculosis* and non-tuberculous mycobacteria (NTM) may occur more frequently with liquid than solid media, and these can cause confusion on subsequent DST as NTM growth may be mistaken for highly resistant *M. tuberculosis* strains.

Although culture on liquid media reduces turnaround time compared with solid culture, two to four weeks are still needed to obtain results with liquid cultures.

Both automated and manual liquid culture systems exist. The high cost of automated systems is an issue for resource-limited countries.

**a) Culture on BACTEC460TB radiometric system** – BACTEC460TB was the first semi-automated system to come on the market and to reach the goal of detecting mycobacterial growth within 14 days. The system uses the medium BACTEC12B, which consists of Middlebrook 7H9 broth enriched and added <sup>14</sup>C-labeled palmitic acid as carbon source. The palmitic acid is metabolized by microorganisms releasing <sup>14</sup>CO<sub>2</sub> which is monitored by specialized equipment. The amount of <sup>14</sup>CO<sub>2</sub> produced is proportional to bacterial growth.

**Potential advantages:** Comparing with the conventional culture on solid medium, BACTEC460TB significantly improves the rate of recovery of mycobacteria from clinical samples. The detection time of *M. tuberculosis* is variable depending on the bacterial load in the clinical specimens, but on average is about 10 to 15 days shorter than solid culture. The addition of NAP (p-nitro-α-acetylamino-β-hydroxy-propiofenone) to the culture medium allows the distinction between *M. tuberculosis* and other non-tuberculous mycobacteria (NTM, also known as mycobacteria other than tuberculosis, MOTTs), since NAP inhibits the growth of *M. tuberculosis*.

**Considerations:** Beyond the limitations of culture in liquid medium, for BACTEC460TB the main issues driving the phasing out of this platform relate to cost, the need for radioisotope disposal, extensive use of syringes with potential hazard of needle stick injury and lack of computerized data management. The system requires specialized equipment.

**Requirements:** BACTEC460TB requires the decontamination of clinical specimens, BSL-2 facilities and the usual equipment and consumables required for mycobacterial isolation are

also needed. Laboratory staff should be well trained in biosafety precautions and procedures for isolation of mycobacteria.

**b) Culture on automated, continuously monitoring systems** – Various fully automated continuously monitoring **non-radiometric** systems are commercially available:

The **BACTEC MGIT960** uses the same fluorescence quenching-based oxygen sensor as manual MGIT (see below) to detect microbial growth.

The **MB/BacT ALERT 3D** employs a colorimetric carbon dioxide sensor.

The technology used in **ESP culture System II** is based on the detection of pressure changes in the headspace above the broth medium in a sealed bottle.

All systems use a similar medium, which is a Middlebrook 7H9 broth supplemented with growth factors and antibiotic mixture.

**Potential advantages:** All have similar performance and operational characteristics. Recovery rate and time to detection of mycobacteria are similar to those of BACTEC460TB. All share the advantages over the BACTEC460TB system of having continuous monitoring, being less labor-intensive, having electronic data management. But importantly, they do not use radioisotopes.

**Considerations:** These systems are continuously monitoring, and the bottles are incubated in the instruments for all the incubation time. These systems are both instruments and space intensive. Both the ESP II and BACTEC MGIT 960 systems have limitations with regard to specimen type. Direct inoculation of blood for mycobacterial culture is not an intended use of either system, though blood bottles are available for MB/BacT ALERT. Since all methods use liquid culture, a rapid identification *M. tuberculosis* complex test is required. The main limitation of these systems is the high cost. Training is relatively straightforward.

**Requirements:** BSL-2 facilities are required for processing specimens and culture inoculation. Culture manipulation (conventional identification, sub culturing and DST activities) must be performed in BSL-3 facilities. A rapid method to differentiate *M. tuberculosis* complex from NTM is essential. A stable source of power and maintenance of equipment by qualified manufacturers are required.

**Product information:** MB/BacT ALERT 3D system MB/BacT ALERT 3D 60 system ([www.biomerieux.com](http://www.biomerieux.com)), ESP Culture System II ([www.trekds.com](http://www.trekds.com)) and BACTECMGIT960 ([www.bd.com](http://www.bd.com)).

## 4. Drug Susceptibility Testing on Automated Liquid Culture Platforms

Indirect detection of drug resistance using cultured isolates can be accomplished in days rather than weeks as in the traditional tests. Experience with direct DST (using a sputum sample rather than an isolate) is limited and has not been validated to date.

**a) DST on BACTEC460TB system** – This is a semi-automated and qualitative method using the same medium and equipment used for culture in BACTEC 460.

**Potential advantages:** The median time for obtaining susceptibility patterns using a cultured isolate is less than 10 days, which is as rapid as that of the fully automated systems. The system can be used to test all first and important second line drugs including pyrazinamide, quinolones and rifamycins.

**Considerations:** The major drawback of the method is again the need for radioisotope disposal, the extensive use of syringes (with potential hazard of needle stick injury), and the lack of computerized data management. Others limitations are the possibility of cross contamination between cultures during reading, the cost and the requirement for specialized equipment. Finally, preliminary isolation of strains involves additional cost and delay.

**Requirements:** BSL-3 facilities. Laboratory staff trained in biosafety precautions and mycobacterial techniques.

**Product information:** antibiotics as lyophilized powders, BACTEC12B vials and BACTEC machine can be purchased at [www.bd.com](http://www.bd.com).

**b) Fully automated nonradiometric DST methods** - The fully automated continuously monitoring nonradiometric systems for drug susceptibility testing (DST) are broth-based methods. They are qualitative methods that do not estimate the percentage of resistant bacilli. All are designed to test most first line drugs, and some, notably MGIT, are used for pyrazinamide and second line agents as well.

**Potential advantages:** The fully automated DST systems are rapid methods; the mean average time for delivery of a final result from starting with an isolated strain is 7-10 days. All offer standardized media, drugs and supplements. When compared to conventional solid media DST, agreement is high and these are thus currently considered the gold standard for DST in industrialized countries.

**Considerations:** The main disadvantages of these methods are their high cost and the requirement for specialized equipment. Finally, preliminary isolation of strains involves additional cost and delay.

**Requirements:** BSL-3 facilities, equipment and consumables required to perform sub-culture, specialized instruments and a stable continuous source of power.

**Product information:** MB/BacT ALERT 3D system ([www.biomerieux.com](http://www.biomerieux.com)), VersaTREK ([www.trekds.com](http://www.trekds.com)) and BACTECMGIT960 ([www.bd.com](http://www.bd.com)).

## 5. Manual Liquid Culture

Middlebrook 7H9 and Dubos broth are the most widely used liquid media for manual liquid culture.

**Potential advantages:** Apart from the advantages described above, Middlebrook 7H9 and Dubos can be easily prepared from commercially available basic ingredients. Tween 80, a

surfactant, can be added to the media allowing a more homogeneous growth of *M. tuberculosis*. Middlebrook 7H9 broth is used as the basal medium for several *in vitro* tests and for preparing the inoculum for indirect drug susceptibility tests.

**Considerations:** There is debate about whether the characteristic cording of colonies of *M. tuberculosis* visualized under the microscope is sufficient for speciation and particularly whether such identification can reliably identify mixed cultures containing *M. tuberculosis* and NTM. The use of liquid media should be carefully planned and implemented only in laboratories which have enough background on solid culture and with biosafety facilities that allow safe working.

**Requirements:** BSL-2 facilities, relevant equipment and consumables are required to perform mycobacterial isolation and direct drug susceptibility testing (which does not require culture manipulation). For indirect drug susceptibility testing or speciation that requires manipulation of positive liquid cultures, BSL-3 facilities are required. Laboratory staff should be trained in biosafety and procedures for isolation of mycobacteria.

**Product information:** Middlebrook 7H9 broth base, Dubos broth base, OADC enrichment, prepared media in slants package can be purchased at [www.bd.com](http://www.bd.com).

**a) MODS** – The Microscopic Observation Drug Susceptibility (MODS) assay is a low-cost, manual liquid mycobacterial culture method utilizing microscopic observation for detection and direct drug susceptibility testing. It is based on the observation that *Mycobacterium tuberculosis* grows faster in liquid than on solid media, and can be differentiated from NTM by its characteristic serpentine “cording” growth which can be observed under the microscope long before the naked eye could visualize colonies on solid medium. MODS is a tissue-culture plate assay which uses OADC-enriched Middlebrook 7H9 liquid medium supplemented with the antibiotic mixture PANTA.

**Potential advantages:** Despite being a liquid culture method, MODS allows differentiation between *M. tuberculosis* and NTM. So far, the multi-center studies have demonstrated that MODS is sensitive and slightly faster than the automated commercial broth-based systems. MODS is a low-cost, rapid and simple liquid culture method which has no need for special instrumentation other than an inverted light microscope.

**Considerations:** Since MODS is a tissue-culture plate assay there is a potential biohazard related to *M. tuberculosis* growing in liquid culture in plates requiring transport from incubator to microscope; however plates are never opened and are kept within a sealed transparent plastic bag from inoculation of sample to discard. The method is more labor intensive than automated systems since it requires visual observations at least twice a week.

**Requirements:** MODS requires the decontamination of clinical specimens, BSL-2 facilities and conventional equipments plus an inverted microscope, and consumables required for mycobacterial isolation. Laboratory staffs need to be well trained in detection of the “cording” characteristic growth of *M. tuberculosis* in liquid media, and in biosafety precautions.

**Product information:** Middlebrook 7H9 broth base, OADC enrichment and antibiotic mixture (PANTA) can be purchased at [www.bd.com](http://www.bd.com).

**b) Culture on MB redox system** - MB Redox is a modified serum-supplemented Kirchner medium with a colorless tetrazolium salt which is reduced to a pink-, red-, or violet-colored formazan during the growth of mycobacteria. In this way the mycobacterial colonies acquire a coloring which can be seen by visual observation. Both the recovery rates and time to detection of *M. tuberculosis* is similar to those of the automated broth-based culture systems.

**Potential advantages:** MB Redox is rapid, sensitive, easy to handle, and does not require additional costly instrumentation. MB Redox is a ready-to-use medium that contains both OADC and the antibiotics mixture (PACT).

**Considerations:** As in all others liquid culture system, the major disadvantage of MB Redox is that the system is only able to detect growth and does not differentiate the *M. tuberculosis* complex from other species. An external method must be applied. MB Redox uses proprietary products and long-term agreements with the suppliers must be addressed before the implementation of the method.

**Requirements:** MB Redox requires the decontamination of clinical specimens, as well as BSL-2 facilities and the usual equipments and consumables required for mycobacterial isolation. BSL-3 facilities are required for further culture manipulation. Laboratory staffs need to be trained in biosafety precautions and procedures for isolation of mycobacteria.

**Product information:** MB Redox 4 mL can be purchased at [www.heipha.de](http://www.heipha.de).

**c) Culture on Bio FM broth** – The Bio FM medium is the Middlebrook 7H9 medium with OADC and VCA (Vancomycin - Colistin - Amphotericin B) supplements. The system contains a chromogenic indicator that changes from dark blue to violet in response to mycobacterial growth. It is reported that the aspect of the culture gives a first indication of the species identification - the presence of flakes indicate *M. tuberculosis* and cloudiness non tuberculous mycobacteria - however biochemical identification remains obligatory.

**Potential advantages:** The method allows both a rapid detection and presumptive identification of *M. tuberculosis* isolates. It is a simple method and does not require specialized equipment. Since the method uses dye to detect the growth it has the potential to be applied to sensitivity tests.

**Considerations:** The disadvantages are the requirement for manual observation of growth (twice or thrice in the first two weeks, which increases the worktime required for TB diagnosis) and the relatively high cost of the consumables and the use of proprietary products. The method has been evaluated only at reference laboratory level. Therefore, large studies in peripheral laboratories are urgently needed before being placed in routine use.

**Requirements:** BSL-2 facilities and the usual equipments and consumables required for mycobacterial isolation are needed. BSL-3 facilities are required for further testing such as species identification, drug susceptibility tests. Laboratory staffs need to be well trained in biosafety precautions.

**Product information:** Middlebrook 7H9 broth base and OADC enrichment can be purchased at [www.bd.com](http://www.bd.com) , antibiotic can be purchased at [www.sigma.com](http://www.sigma.com) . The complete Bio FM broth is a commercial medium manufactured by BIO-RAD.

**d) Culture on MGIT system** – In addition to the automated platform described above, MGIT tubes may be used manually without the MGIT machine. The Mycobacteria Growth Indicator Tube was developed by BD to circumvent some limitations of the BACTEC460TB system, such as the use of radioisotopes and cross contamination of cultures during reading. MGIT contains a modified Middlebrook 7H9 broth in conjunction with a fluorescence quenching-based oxygen sensor (silicon rubber impregnated with a ruthenium pentahydrate) to detect mycobacterial growth. The growth of mycobacteria depletes the oxygen present in the medium and the indicator fluoresces brightly when the tubes are illuminated with UV light. This is observed with the naked eye.

**Potential advantages:** Compared with the BACTEC460TB system MGIT offers several advantages - it does not use radioisotopes, has little chance of culture cross contamination, does not use needles for inoculation, and the manual version does not require specialized equipment (a Wood's lamp or a transilluminator can be used as the UV light source).

**Considerations:** According to the manufacturer, MGIT is not intended for mycobacterial culture of urine, blood or bone marrow samples. As in all other liquid culture systems a rapid method to differentiate *M. tuberculosis* complex from NTM is essential. The major drawback is the high cost and the use of proprietary products.

**Requirements:** MGIT requires the decontamination of clinical specimens naturally infected as sputum, BSL-2 facilities and the usual equipments and consumables required for mycobacterial isolation. BSL-3 facilities are required for further testing such as species identification, drug susceptibility tests. Laboratory staffs need to be well trained in biosafety precautions and procedures for isolation of mycobacteria.

**Product information:** MGIT Mycobacteria Growth Indicator Tube 7 mL, MGIT Growth Supplement and PANTA (Antibiotic Mixture) can be purchased at [www.bd.com](http://www.bd.com)

## 6. Drug Susceptibility Testing with Manual Liquid Culture

### 1. Indirect Methods

**a) MGIT system** – the same tubes as for detection are used, only supplemented with the antibiotic to be tested. Tubes are inoculated with a defined volume and concentration of mycobacterial suspension from an earlier culture.

**Potential advantages:** MGIT drug susceptibility testing is available as a manual or fully automated system. Media, supplements and lyophilized stock solutions of antibiotics are provided by the manufacturer. It can be used for DST to INH, RIF, EMB, PZA and SM. Good performance is also reported for the second-line drugs kanamycin and ofloxacin.

**Considerations:** The systems use patented products

**Requirements:** BSL-3 facilities. Laboratory staff trained in biosafety precautions and procedures for isolation of mycobacteria.

**Product information:** MGIT Mycobacteria Growth Indicator Tube 7 mL, MGIT Growth Supplement and lyophilized stock solutions of antibiotics can be purchased at [www.bd.com](http://www.bd.com).

**b) Colorimetric methods** – Colorimetric methods are based on the use of a redox indicator added to the culture medium after *M. tuberculosis* has been exposed in vitro to different antibiotics. The growth indicators used are the tetrazolium salts: XTT [2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide], MTT [3(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide] and the redox indicators Alamar blue and resazurin. These assays are performed in microtiter plates or in culture tubes, with minimum inhibitory concentrations (MICs) of the anti-tuberculosis drugs available in 8 days.

**Potential advantages:** The assay shows a good agreement with standard susceptibility tests especially for RIF and INH. The method does not require specific equipment and shows the same time to results as the MGIT system. The microdilution is a rapid, low-cost assay and provides quantitative results in the form of MIC (though the utility of such detail is debated). In addition the test can be used for second-line drugs.

**Considerations:** The method can be used only for indirect testing. The major drawback is a concern for biosafety, especially in the laboratory without extensive experience in liquid culture.

**Requirements:** BSL-3 facilities and laboratory trained staff.

**Product information:** antibiotic powders can be purchased at [www.merck.com](http://www.merck.com) [www.sigmaaldrich.com](http://www.sigmaaldrich.com) and other reliable suppliers. Middlebrook 7H9 broth base, Dubos broth base, ADC enrichment can be purchased at [www.bd.com](http://www.bd.com).

**c) Nitrate reductase assay in liquid medium (NRA)** – The nitrate reductase assay is a simple and rapid technique based on the capacity of *M. tuberculosis* to reduce nitrate to nitrite, which is detected by adding sodium nitrate to the culture medium. It can be done on both solid (see above) and liquid media.

**Potential advantages:** The NRA in liquid medium is a simple method to test drug susceptibility and requires neither specialized equipment nor patented supplies. The main advantage of the method is an average time to positivity (TTP) of 6.5 days compared with 7–12 days for those that performed the NRA in the solid medium. The available studies had been performed on Middlebrook 7H9, but can be accomplished with any suitable liquid medium for *M. tuberculosis* growth.

**Considerations:** NRA does not work for nitrate reductase negative *M. tuberculosis* strains, nor for *M. bovis*. There are very few studies in the literature evaluating the accuracy of the method, but they show good agreement with standard methods, especially for INH and RIF.

Evaluations in low resource setting are needed to assess the accuracy and reproducibility of the method. The procedure on liquid medium poses higher biosafety concerns.

**Requirements:** BSL-3 facilities and usual equipments and consumables required to perform liquid culture. Laboratory staff well trained in biosafety precautions, mycobacterial techniques as well in quality control issues.

**Product information:** The reagents sulfanilamide, N-1-naphthylethylenediamine dihydrochlorid and antibiotic powders can be purchased at [www.merck.com](http://www.merck.com), [www.sigmaaldrich.com](http://www.sigmaaldrich.com) or other reliable suppliers. Middlebrook 7H9 broth base, ADC enrichment and lyophilized stock solutions of antibiotics can be purchased at [www.bd.com](http://www.bd.com).

## 2. Direct Methods

**a) MODS** – The microscopic observation drug susceptibility assay (MODS) is a rapid, low-cost, low-tech direct DST method which reliably detects resistance to INH and RIF directly from the sputa in relative short time.

**Potential advantages:** MODS is a relatively low-cost and simple liquid culture method. It is rapid compared with solid agar culture methods, and does not require special instrumentation other than an inverted light microscope.

**Considerations:** Requires meticulous technique during inoculation and plate handling to prevent cross-contamination. Biosafety concerns relate to the handling of liquid cultures in 24-well plates, which must be contained in a sealed transparent plastic bag, and not opened. The method is more labor intensive than automated systems since it requires visual observations at least twice a week.

**Requirements:** BSL-2 facilities. Laboratory staffs need to be well trained in detection of the “cording” characteristic growth of *M. tuberculosis* in liquid media and biosafety precautions.

**Product information:** rifampicin and isoniazid powder can be purchased at [www.merck.com](http://www.merck.com), [www.sigmaaldrich.com](http://www.sigmaaldrich.com) or other reliable suppliers. Middlebrook 7H9 broth base, OADC enrichment and lyophilized stock solutions of antibiotics can be purchased at [www.bd.com](http://www.bd.com).

**b) Slide DST** – It is the oldest method of testing sensitivity to drugs, introduced in 1941. A smear is prepared on a glass microscope slide from the sputum sample. The slide is decontaminated and immersed in a liquid medium with and without drugs and incubated at 37°C. After 7 to 10 days of incubation, the bacteria on the slide are killed by heat, and the slide is stained and observed at 100× magnification under optical microscope for visualization of the presence of microcolonies of *M. tuberculosis*.

**Potential advantages:** The slide DST is a direct method. The assay shows a good agreement with standard indirect susceptibility testing, especially for RIF and INH. The method does not require specific equipment and shows the same time to results as the MGIT system. The slide DST is a simple and low-cost assay. It is designed for peripheral laboratories.

**Considerations:** the slide DST was widely used before 1960, but until now there are very few evaluations of the method; before considering its use, further studies under routine conditions are needed. The method can only be used for smear positive sputum samples. It uses liquid culture and manipulation of slides with heat-treated microcolonies. Before its implementation, proper training for both the visualization of microcolonies and biosafety issues is necessary.

**Requirements:** BSL-2 facilities and laboratory trained staff.

**Product information:** antibiotic powders can be purchased at [www.merck.com](http://www.merck.com) [www.sigmaaldrich.com](http://www.sigmaaldrich.com) either reliable supplier. The liquid medium can be prepared in the laboratory or can use a Middlebrook 7H9 broth base, Dubos broth base, ADC enrichment, which can be purchased at [www.bd.com](http://www.bd.com).

## 7. Miscellaneous

### Mycobacteriophage plaque-based assays (commercial version is FASTPlaque-Response)

These are phage amplification-based tests which depend upon the replication of MTB-specific phages in the presence of *M tuberculosis* in clinical specimens. Phage replication is thus the indicator (or *readout*) for TB detection. Phage replication is identified by the lysis of a rapidly growing indicator organism, usually *Mycobacterium smegmatis*, which leaves plaques on an agar plate lawn. Phage assays have been developed for direct use with sputum specimens and have been adapted to give direct drug susceptibility testing through the incorporation of rifampicin into the assay – viable (rifampicin resistant) organisms support phage replication whilst dead (rifampicin susceptible) organisms do not. The assay is available as a commercial kit or as an in-house method.

**Potential advantages:** The assay is rapid. Both commercial and in-house assays can be performed directly on sputa without the need for primary isolation and may provide results in 1–4 days. The phage assay does not require sophisticated equipment.

**Considerations:** The promise of early laboratory diagnostic accuracy studies was not maintained once the commercial assay was transferred into an operational setting, and the commercial plaque-based assay was dropped from the FIND portfolio. Training of laboratory staff is needed (as with all assays) though the techniques are new and not generalizable. Specific media, reagents and supplies of phage, sensor cells, are needed. Further refinement and evaluation work is required if this platform is to fulfill the requirements of WHO STAG TB endorsement.

**Requirements:** BSL-3 facilities. Laboratory staff well trained in mycobacterial techniques and biosafety precautions.

**Product information:** A commercial kit can be obtained at [www.biotec.com](http://www.biotec.com).

### E-test

The Etest is based on the diffusion of antibiotic on the surface of Petri dish agar media inoculated with *M. tuberculosis*, and consequent formation of a zone of inhibition in

sensitive strains. The antibiotic strip has a concentration gradient of the antibiotic, for establishing the MIC. It is an in-house method.

**Potential advantages:** The E-test performed in a large Petri dish permits testing for the MIC of more than one antibiotic. The assay does not require sophisticated equipment other than that required for culture of MTB, and it is a relatively low-cost assay.

**Considerations:** Although not a complicated test, the preparation of the inoculum is critical for reliable results. **The few studies on the test do not show good agreement with the gold standard.** The turnaround time is much longer than for the other rapid phenotypic tests. Further investigation must be undertaken to determine the efficacy and its feasibility. It can only be used as an indirect method and is not widely used.

**Requirements:** BSL-3 facilities. Laboratory staff well trained in mycobacterial techniques and biosafety precautions.

**Product information:** The antibiotics strips can be obtained at [www.abbdisk.com](http://www.abbdisk.com).