Mycobacteriology Validation Guidelines

Validation of methods relating to the *Mycobacteria tuberculosis* (MTB) laboratory include, but are not limited to, culture (MGIT, LJ), DST (phenotypic or genotypic), identification (MPT64, Hain LPA), GeneXpert (MTB ID and RIF resistance), and Interferon-Gamma Release Assays (IGRA). Validation of a laboratory test method consists of an established set of required experiments. These guidelines are intended to assist laboratories with what is expected of each type of validation/verification for TB methods. The typical positive control for a TB method is a strain of TB called H37Rv. The negative control depends on if the method is inhibitive in any way.

Each laboratory should first design a validation plan describing how they will satisfy each of these requirements. The validation plan must also detail the acceptability criteria for each element. After completing all of the validation experiments, results should be compiled and filed in an organized manner along with the package insert. All validation records should be retained for the life of the instrument. A validation summary should be prepared that contains a place for the Laboratory Director to sign, indicating the validation has been reviewed and approved.

MGIT/LJ Culture

Introduction: Culture method validation is typically detecting the presence of an organism that is intended to grow and not detecting an organism that is not intended to grow. When a lab is first validating a culture method, it is common to validate the identification method at the same time. Unless validated separately, the digestion process that is being used in the lab should be used on every specimen that is used for the validation.

Culture can be automated on liquid media using the BD Bactec MGIT or performed on solid media such as LJ or 7H9 (middlebrook) agar. A laboratory that uses the MGIT usually has a back-up method on solid culture. Culture methods require precision and accuracy but requires fewer specimens to achieve precision. The MGIT package insert also describes time to detection (TTD) and level of detection for different species. Both of these can be performed at the same time. Validations for both liquid media and solid media will look very similar as the only differences between the two methods could be that there might not be a selective agent present in the solid media and there are not multiple drawers to test.

Depending on the model of MGIT, there may be either one or three drawers. Each drawer will need to have specimens included in the validation to prove that the entire instrument works as intended. This can easily be done during the precision portion of the validation as the specimens are run in triplicate and be placed in a different drawer each day.

For a non-inhibitive method, a typical negative control is simply an uninoculated sample. If the media is inhibitive, such as the MGIT, then there can be two negative controls. In the case of the MGIT it would be an uninoculated MGIT tube with PANTA, and E. coli.

It might not be stated in the validation but every tube must have PANTA added. PANTA is lyophilized antibiotics (that have no activity against mycobacteria) reconstituted with growth supplement. Adding PANTA helps inhibit any non-mycobacteria that survives the digestion process and promotes growth of mycobacteria.

The following are the required components of validation for most TB culture methodologies. There may be acceptable alternatives to these validation methodologies. Consult the pSMILE TB specialist on deviations.

1. **Precision** is reproducibility (not applicable for solid culture but required for MGIT).
    a. Sample Criteria

- Three levels of controls (a positive control in triplicate, an uninoculated negative control with only PANTA, and a tube inoculated with E.coli and PANTA).

b. Testing and Results
- Precision will be determined by running the two negative control specimens and a positive control specimen in triplicate on three separate days and calculating the precision as indicated in the calculation below. Ensure that samples are tested on all MGIT drawers. (e.g. on day one run the five isolates in drawer A, on day two run them in drawer B, and on day three them in drawer C.
- *Precision Calculation*

  Precision % = number of repeated results in agreement/ total number of results X 100

c. Acceptability Criteria
- A culture method validation should have a 100% recovery rate when using spiked samples. Any discrepancies should be explained. With so few specimens for precision, even one not growing could be a large drop in percentage.
- Expect a level of 100%% agreement and accept no less than 90% agreement.

2. **Accuracy** - Verification of accuracy is the process of determining that the test system is producing true, valid results.

a. Determine the Reference Method (*Selection of specimens and sample number)*
Sample Options: Samples should cover the spectrum of organisms expected to be encountered in the geographic region. DAIDS protocols only require identification of MTB but laboratories might still identify and report non-tuberculosis mycobacteria (NTM). Reference strains (such as ATCC) should be maintained in a standard manner so as not to be genetically affected by storage, passage, etc.
If non-reference strains are used, the laboratory should have a complete record of the history of the organism, including characterization, storage, and recovery from storage.
Select from the following options:
- Assaying materials with assigned values (EQA organisms and QC organisms)
- Comparing patient specimen results with a method of long standing use
  1. Verify results from inter-laboratory survey specimens
  2. Split specimens with another laboratory within the DAIDS system with successful validation and EQA history.
  3. Split patient samples with a reference laboratory
  4. Split samples between two in-house methods
- Saved specimens from previously identified cultures.
- Spiked samples using ATCC strains and organisms from previous CAP panels or patient isolates.

b. Sample Criteria (Number of Specimens)
- Testing of a minimum of 10 samples should be used. The species used should cover the range of species that the lab expects to encounter during routine testing. Multiple copies of a species can be used, such as Mtb, as long as they are not the same strain or repeated samples. If verifying the MGIT, include a non-mycobacteria species that will not grow, such as E. coli, and a MGIT tube only inoculated with phosphate buffer and PANTA. These two tubes do not count towards the 10 minimum needed for verification.

c. Acceptability Criteria
- Claims in the package insert are based on patient samples and not spiked samples.
- Due to using spiked samples for verification, expect a level of 100% agreement and accept no less than 90% agreement. Any deviations should be explained.

- The test may be considered verified if it meets the requirements initially established for performance by the users of the test and if the sensitivity and specificity are no lower than 5% below those of the reference method, those appearing in peer-reviewed journals, or those claimed by the manufacturer's marketing data used in the evaluation of test kits and reagents.
- If the sensitivity or specificity of the new or revised test does not satisfy the verification requirements, the test must be considered unverified and corrective action must be taken by the manufacturer, the user, or both. Following corrective action the new or revised test should be run again in parallel with the reference method and interpreted.

Method being Validated	Diagnostic Sensitivity and Specificity (Results from Comparison Study)		**Total**
	Positive	Negative	
Positive	***# true positive (TP)***	***# false positive (FP)***	***TP+FP***
Negative	***# false negative (FN)***	***# true negative (TN)***	***FN+TN***
Total	***TP+FN***	***FP+TN***	***N***

- Calculate the estimated Diagnostic Sensitivity

  (True positive rate) = 100 x [TP/(TP+FN)]

- Calculate the estimated Diagnostic Specificity

  (True negative rate) = 100 x [TN/(FP+TN)]

- Calculate the percent Positive Agreement

  (Positive Predictive Value) = 100 x TP/(TP+FP)

- Calculate the percent Negative Agreement

  (Negative Predictive Value) = 100 x TN/(TN+FN)

3. **Linearity, Analytical Measurement Range (AMR) and Clinical Reportable Range** are not applicable for qualitative methods.
4. **Analytical Sensitivity** is the lowest concentration of an analyte that can be measured.
   - For an FDA approved, unmodified method, the manufacturer's stated sensitivity will be used.
   - It is recommended, but not required, that a MGIT validation include a time to detection (TTD)/sensitivity portion. This is performed by preparing a 0.5 McFarland standard of M. tuberculosis ATCC strain H37Rv and making the following dilutions: 1:10, 1:20: 1:30, 1:40, 1:50. Record the TTD of each dilution.
5. **Analytical Specificity** is the determination of the effect of interfering substances.
   - For an FDA approved, unmodified method, the manufacturer's stated specificity will be used. For MGIT it is 80% based on meta-analysis publications.
6. **Reference Ranges** Not Applicable
7. **Method Approval**
   - The final decision on methodology validation and acceptance is made after a careful review of all the studies performed as part of the complete method validation process. The Laboratory Director shall make the ultimate decision on method validation. There

must be an approval with a signature from the Medical and/or Laboratory Director and preparer of validation documents with dates.

MGIT DST

Introduction: Drug susceptibility testing performed on the MGIT is considered a phenotypic susceptibility method and can be performed directly from a positive MGIT culture. Phenotypic susceptibilities for MTB look different than for other bacteria. Concentrations of drugs are done at one level for most drugs instead of multiple dilutions. This is called the critical concentration (CC). In most cases you will only see a drug and a result (resistant/susceptible) and no concentration. There are a few drugs that have a high and a low CC (most commonly Isoniazid).

First line drugs are SIRE (Streptomycin, Isoniazid, Rifampicin, Ethambutol) and PZA (Pyrazinamide). Second line drugs are any drugs that are not included in the first line. Some examples are fluoroquinolones, Amikacin, and Linezolid. Drugs that do not yet have significant data collected are considered “additional drugs”. Examples of current “additional drugs” are Kanamycin, Bedaquiline, and Clofazimine. Validation of a drug requires a minimum of 20 - 30 specimens for categorical agreement (agreement with interpretation of susceptible/intermediate/resistant) for each drug being validated. The same specimen can be used for multiple drugs (i.e. resistant to isoniazid and rifampin but susceptible to other drugs). Due to limited availability of unique resistant strains, replication of the same sample also counts towards the 30 specimens needed for categorical agreement as long as it is not the control strain.

Depending on the model of MGIT, there could be either one or three drawers. Each drawer will need to have specimens to prove that the entire instrument works as intended.

The following are the required components of validation for most TB phenotypic DST methodologies:

1. **Precision** is reproducibility – the agreement of the measurements of replicate runs of the same sample. It is the process of determining the range of random error.
    a. Sample Criteria – $H37R_v$ will be
    b. Testing and Results
        - Short-term (within-run) and long-term (between-day) precision will be determined by running the $H37R_v$ control as follows:
            1. Short-term precision will be validated through repetition of isolates to reach categorical agreement for accuracy.
            2. For long-term, H37Rv will be run in triplicate over three days. Isolates will be placed in a different drawer of the MGIT each day, ensuring that all drawers will incubate isolates.
        - *Precision Calculation*

            Precision % = number of repeated results in agreement/ total number of results X 100
    c. Acceptability Criteria
        - Using spiked samples, the acceptability is expected to be 100% growth of mycobacteria samples. Any samples that fail to perform as expected will need to be explained. Below 90% agreement is considered unacceptable.
2. **Accuracy** is the true value of a substance being measured. Verification of accuracy is the process of determining that the test system is producing true, valid results.
    a. Determine the Reference Method (*Selection of specimens and sample number)*
    Sample Options: Cover the spectrum of organisms expected to be encountered. For DAIDS supported research this would only be one organism, *Mycobacterium tuberculosis*. Reference strains (such as ATCC) should be maintained in a standard manner so as not to be

genetically affected by storage, passage, etc. If non-reference strains are used, the laboratory should have a complete record of the history of the organism, including characterization, storage, and recovery from storage.
Select from the following options:

- Assaying materials with assigned values (EQA organisms and QC organisms). This is preferred method.
- Comparing patient specimen results with a method of long standing use.
    1. Verify results from inter-laboratory survey specimens
    2. Split specimens with another sufficiently accredited laboratory participating in DAIDS supported research.
    3. Split samples between two in-house methods
- Saved specimens from previously identified cultures.

b. Sample Criteria (Number of Specimens)

- A minimum of 20-30 results for categorical agreement as compared to the reference method on each antimycobacterial agent. This can be done with 3-6 different EQA/known isolates done in triplicate over 3 days for a total of 27 - 54 tests per drug. These 3-6 isolates can be used for multiple drug validations at the same time. Increasing the number of isolates tested will help cover multiple drugs being validated. Drugs being tested for 2 levels need to have more isolates tested (3 resistant at low concentration and 3 resistant at high concentration is preferred) in addition to susceptible isolates. Drugs that are not FDA cleared will need more isolates and at least 30 results for categorical agreement.

c. Acceptability Criteria

- Using spiked samples, the acceptability is expected to be 100% growth of mycobacteria samples. Any samples that fail to perform as expected will need to be explained. Below 90% agreement is considered unacceptable.
- If the sensitivity or specificity of the new or revised test does not satisfy the verification requirements, the test must be considered unverified and corrective action must be taken by the manufacturer, the user, or both. Following corrective action the new or revised test should be run again in parallel with the reference method and interpreted.

Method being Validated	Diagnostic Sensitivity and Specificity (Results from Comparison Study)		**Total**
	Positive	Negative	
Positive	***# true positive (TP)***	***# false positive (FP)***	***TP+FP***
Negative	***# false negative (FN)***	***# true negative (TN)***	***FN+TN***
Total	***TP+FN***	***FP+TN***	***N***

- Calculate the estimated Diagnostic Sensitivity

  (True positive rate) = 100 x [TP/(TP+FN)]

- Calculate the estimated Diagnostic Specificity

  (True negative rate) = 100 x [TN/(FP+TN)]

- Calculate the percent Positive Agreement

  (Positive Predictive Value) = 100 x TP/(TP+FP)

- Calculate the percent Negative Agreement

(Negative Predictive Value) = 100 x TN/(TN+FN)

- Compare the results calculated above with the manufacturer's stated claims for Sensitivity, Specificity and Agreement found in the test kit package insert.

3. **Linearity, Analytical Measurement Range (AMR) and Clinical Reportable Range** are not applicable for qualitative methods.
4. **Analytical Sensitivity** is the lowest concentration of an analyte that can be measured. For an FDA approved, unmodified method, the manufacturer's stated sensitivity will be used.
5. **Analytical Specificity** is the determination of the effect of interfering substances. For an FDA approved, unmodified method, the manufacturer's stated specificity will be used.
6. **Reference Ranges** Not Applicable
7. **Method Approval**
   The final decision on methodology validation and acceptance is made after a careful review of all the studies performed as part of the complete method validation process. The Laboratory Director shall make the ultimate decision on method validation. There must be an approval with a signature from the Medical and/or Laboratory Director and preparer of validation documents with dates.

HAIN Line Probe Assay (LPA):

Introduction: HAIN LPA uses Polymerase Chain Reaction (PCR) to detect and amplify probes that can identify MTB in a sample and, if MTB is present, identify genotypic resistances. The instruments in this case will be a special type of incubator called a TwinCubator, a thermal cycler, and, if the lab is automating the reading portion, there will also be a GenoScan. SmartSpot has a verification panel available for each assay. Patient specimens or EQA are acceptable for validation. Validation requires a minimum of 10 samples with and without each target analyte. The target analyte is the resistance gene for each antibiotic being tested in the assay. There are two versions of the LPA that are in use in DAIDS protocols; MTBDRplus and MTBDRsl. Both versions of this assay are CE marked but **NOT** FDA cleared.

MTBDRplus: Identifies MTB and looks for genetic mutations to Rifampicin and Isoniazid only. If the organism is resistant to either of these drugs, the second line (sl) assay can be set up.

MTBDRsl: Identifies MTB and is used for detection of second line drug resistance. Requires the same equipment and is performed the same as the MTBDRplus just with different probe targets. Tests for multiple mutations that would convey resistance to fluoroquinolones (e.g. Ofloxacin or Moxifloxacin), Kanamycin, Amikacin, Capreomycin, and Viomycin

1. **Precision** is reproducibility – the agreement of the measurements of replicate runs of the same sample. It is the process of determining the range of random error.
   a. Short-term (within-run) and long-term (between-day) precision will be determined by running the negative control and positive control as follows:
      - For short-term, a negative control (such as a non-tuberculosis mycobacterium species) and a positive control, H37Rv, will be tested in triplicate in one run.
      - For long-term, the same negative control and positive control will be tested in triplicate for two additional days for a total of three consecutive days.
2. **Accuracy** is the true value of a substance being measured. Verification of accuracy is the process of determining that the test system is producing true, valid results.
   a. Determine the Reference Method (*Selection of specimens and sample number)*

For verification, a mix of samples with a variety of genotypic resistance patterns to the associated drugs and genes tested:

Kit	Drugs	Genes
MTBDRplus	Isoniazid	*katG, inhA*
	Rifampin	*rpoB*
MTBDRsl	Fluoroquinolones (Ofloxacin, moxiflocicin, levofloxacin,and gatifloxacin)	*gyrA, gyrB*
	Second Line Injectable Drugs(SLID) (kanamycin, amikacin, and capreomycin)	*rrs, eis*

Sample Options:

- Assaying materials with assigned values (EQA organisms and QC organisms). This is preferred method.
- Comparing patient specimen results with a method of long standing use.
    1. Verify results from inter-laboratory survey specimens
    2. Split specimens with another sufficiently accredited laboratory participating in DAIDS supported research.
    3. Split samples between two in-house methods
    4. Saved specimens from previously identified cultures.

b. Sample Criteria (Number of Specimens)

- A minimum of 10 samples for each expected result will be used (for example, 10 samples resistant for each drug and 10 susceptible samples for each drug).

c. Acceptability Criteria

- Claims in the package insert are based on patient samples and not spiked samples.
- Due to using spiked samples for verification, expect a level of 100% agreement and accept no less than 90% agreement. Any deviations should be explained.
- The test may be considered verified if it meets the requirements initially established for performance by the users of the test and if the sensitivity and specificity are no lower than 5% below those of the reference method, those appearing in peer-reviewed journals, or those claimed by the manufacturer's marketing data used in the evaluation of test kits and reagents.
- If the sensitivity or specificity of the new or revised test does not satisfy the verification requirements, the test must be considered unverified and corrective action must be taken by the manufacturer, the user, or both. Following corrective action the new or revised test should be run again in parallel with the reference method and interpreted.

Method being Validated	Diagnostic Sensitivity and Specificity (Results from Comparison Study)		**Total**
	Positive	Negative	
Positive	***# true positive (TP)***	***# false positive (FP)***	***TP+FP***
Negative	***# false negative (FN)***	***# true negative (TN)***	***FN+TN***
Total	***TP+FN***	***FP+TN***	***N***

- Calculate the estimated Diagnostic Sensitivity

  (True positive rate) = 100 x [TP/(TP+FN)]
- Calculate the estimated Diagnostic Specificity

  (True negative rate) = 100 x [TN/(FP+TN)]
- Calculate the percent Positive Agreement

  (Positive Predictive Value) = 100 x TP/(TP+FP)
- Calculate the percent Negative Agreement

  (Negative Predictive Value) = 100 x TN/(TN+FN)

- If the Hain MTBDRplus assay demonstrates increased sensitivity as compared to the comparative methods, please refer to the method notes below for consideration in method approval and conclusion.

3. **Linearity, Analytical Measurement Range (AMR) and Clinical Reportable Range** are not applicable for qualitative methods.
4. **Analytical Sensitivity** is the lowest concentration of an analyte that can be measured.
   For an FDA approved (as well as WHO endorsed), unmodified method, the manufacturer's stated sensitivity will be used.
5. **Analytical Specificity** is the determination of the effect of interfering substances.
   For an FDA approved (as well as WHO endorsed), unmodified method, the manufacturer's stated specificity will be used.
6. **Reference Ranges** are not Applicable
7. **Method Approval**
   The final decision on methodology validation and acceptance is made after a careful review of all the studies performed as part of the complete method validation process. The Laboratory Director shall make the ultimate decision on method validation. There must be an approval with a signature from the Medical and/or Laboratory Director and preparer of validation documents with dates.
   Method Notes:

   The GenoType MTBDRplus only detects those resistances that have their origins in the rpoB, katG, and inhA regions examined here. Resistances originating from mutations of other genes or gene regions as well as other RMP and INH resistance mechanisms will not be detected by this test.

   Theoretically, a resistance can exist in spite of a wild type pattern. If the sample contains a strain that has developed a heteroresistance and the resistance is caused by a mutation not covered by the mutation probes, the wild type pattern will appear. Similarly, if the sample contains more than one M. tuberculosis complex strain (due to mixed culture or contamination) and one of these harbors a mutation not covered by the mutation probes, the wild type pattern will also appear.

The test only works within the limits of the genomic regions the primers and probes were chosen from. As with any detection system based on hybridization, the test system on hand bears the possibility that sequence variations in the genomic regions the primers and probes were chosen from but the detection of which the test was not designed for may lead to false results. Due to the high variability of bacterial genomes, it is possible that certain subtypes might not be detected.

MPT64 Antigen:

Introduction: A rapid antigen test using a lateral flow cartridge that is very similar to a rapid strep test. Tests for the presence of a protein found in MTB but not BCG. There are MTB strains that are MPT64 negative but they are rare and tend to be regional (most commonly found in China). The most popular version from SD Bioline/ BD is CE marked and FDA cleared. Precision specifications are not covered in the package insert and is not necessary for validation. Validation can follow the qualitative guidelines.

This identification method is FDA cleared for direct sputum specimens but most labs use it on positive MGIT tubes. The validation would need to use specimens from how the lab intends to use the test. If directly from sputum, the validation could use spiked sputum. If the lab is using the test for positive MGIT tubes, the tubes would need to be spiked and placed on the MGIT to incubate and be flagged as positive to have the correct concentration of Mtb for detection.

The following are the required components of validation for MPT64 antigen methodology:

1. **Precision** is not applicable
2. **Accuracy** is the true value of a substance being measured. Verification of accuracy is the process of determining that the test system is producing true, valid results.
    a. Determine the Reference Method (*Selection of specimens and sample number)*
    Sample Options: Cover the spectrum of organisms expected to be encountered. Reference strains (such as ATCC) should be maintained in a standard manner so as not to be genetically affected by storage, passage, etc.
    If non-reference strains are used, the laboratory should have a complete record of the history of the organism, including characterization, storage, and recovery from storage.
    Select from the following options:
        - Assaying materials with assigned values (EQA organisms and QC organisms)
        - Comparing patient specimen results with a previously validated method
            1. Verify results from inter-laboratory survey specimens
            2. Split specimens with another sufficiently accredited laboratory.
            3. Split patient samples with a reference laboratory
            4. Split samples between two in-house methods
        - Saved specimens from previously identified cultures.
        - Outside source of organisms for identification and/or susceptibility testing
        - Spiked samples using ATCC strains and organisms from previous CAP panels or patient isolates.
3. **Linearity, Analytical Measurement Range (AMR) and Clinical Reportable Range** are not applicable for qualitative methods.
4. **Analytical Sensitivity** is the lowest concentration of an analyte that can be measured.
    For an FDA approved, unmodified method, the manufacturer's stated sensitivity will be used.
5. **Analytical Specificity** is the determination of the effect of interfering substances.
    For an FDA approved, unmodified method, the manufacturer's stated specificity will be used.
6. **Reference Range** is not applicable

7. **Method Approval -** The final decision on methodology validation and acceptance is made after a careful review of all the studies performed as part of the complete method validation process. The Laboratory Director shall make the ultimate decision on method validation. There must be an approval with a signature from the Medical and/or Laboratory Director and preparer of validation documents with dates.

GeneXpert

Introduction: The Cepheid GeneXpert uses a cartridge system that utilize RT-PCR for identification of organisms and most of these cartridges can also perform genotypic DST, as is the case with the MTB/RIF cartridge. It is considered semi-quantitative and has several different cartridges that can detect organisms. During validation, the bays used should be rotated unless performing precision (not required). If a lab needs to replace a faulty bay, they do not need to re-validate that bay/instrument. The calibration and verification that is performed during installation is adequate due to each cartridge having its own internal QC.

MTB/RIF: Detects the presence of MTB in direct sputum samples. If a specimen is positive for MTB, the cartridge can also detect Rifampicin resistance if there is a sufficient number of organisms in the sample. SmartSpot offers a verification panel for this cartridge. This cartridge is FDA cleared.

MTB/RIF Ultra: Performs the same function as the MTB/RIF cartridge but has a much lower level of detection. Can potentially give a false positive Rifampicin resistance if there is very little organism present in the specimen. Uses the same verification panel as the MTB/RIF. This cartridge is **not** FDA cleared but it is recommended by WHO so DAIDS accepts the Ultra cartridge.

MTB XDR: Is not used to detect the presence of MTB but instead is used as a reflex test in the case that either the MTB/RIF or Ultra cartridge detects Rifampicin resistance. Detects resistance to Isoniazid, fluoroquinolones, Amikacin, Kanamycin, Capreomycin, and Ethionamide. This cartridge needs a newer version of the GeneXpert instrument and which requires a new instrument validation. This newer instrument can also run all of the previous cartridges but has the ability to perform 10 PCR cycles at once instead of the previous version of the instrument's 4 cycles. This allows the new cartridges to detect more analytes at once. There is a verification panel and a validation panel for the XDR cartridge available from SmartSpot.

1. **Precision** is not applicable.
2. **Accuracy** is the true value of a substance being measured. Verification of accuracy is the process of determining that the test system is producing true, valid results.
    a. Determine the Reference Method (*Selection of specimens and sample number)*
    Sample Options: Cover the spectrum of organisms expected to be encountered. Reference strains (such as ATCC) should be maintained in a standard manner so as not to be genetically affected by storage, passage, etc.
    If non-reference strains are used, the laboratory should have a complete record of the history of the organism, including characterization, storage, and recovery from storage.
    Select from the following options:
        - Assaying materials with assigned values (EQA organisms and QC organisms)
        - Comparing patient specimen results with a previously validated method.
            1. Verify results from inter-laboratory survey specimens
            2. Split specimens with another sufficiently accredited laboratory.
            3. Split patient samples with a reference laboratory
            4. Split samples between two in-house methods

- Saved specimens from previously identified cultures.
- Outside source of organisms for identification and/or susceptibility testing (commercial validation panel is available from SmartSpot)
- Spiked samples using ATCC strains and organisms from previous CAP panels or patient isolates.

b. Sample Criteria (Number of Specimens)
   - Testing on a minimum of 10 specimens for each expected result will be used (for example, 10 samples resistant for each drug and 10 susceptible samples for each drug). Analytes include the resistance genes that each cartridge can detect. The selection of specimens needs to include resistant and susceptible isolates for each drug that the cartridge can detect.

c. Acceptability Criteria
   - Claims in the package insert are based on patient samples and not spiked samples.
   - Due to using spiked samples for verification, expect a level of 100% agreement and accept no less than 90% agreement. Any deviations should be explained.
   - The test may be considered verified if it meets the requirements initially established for performance by the users of the test and if the sensitivity and specificity are no lower than 5% below those of the reference method, those appearing in peer-reviewed journals, or those claimed by the manufacturer's marketing data used in the evaluation of test kits and reagents.
   - If the sensitivity or specificity of the new or revised test does not satisfy the verification requirements, the test must be considered unverified and corrective action must be taken by the manufacturer, the user, or both. Following corrective action the new or revised test should be run again in parallel with the reference method and interpreted.

Method being Validated	Diagnostic Sensitivity and Specificity (Results from Comparison Study)		**Total**
	Positive	Negative	
Positive	***# true positive (TP)***	***# false positive (FP)***	***TP+FP***
Negative	***# false negative (FN)***	***# true negative (TN)***	***FN+TN***
Total	***TP+FN***	***FP+TN***	***N***

   - Calculate the estimated Diagnostic Sensitivity

     (True positive rate) = 100 x [TP/(TP+FN)]

   - Calculate the estimated Diagnostic Specificity

     (True negative rate) = 100 x [TN/(FP+TN)]

   - Calculate the percent Positive Agreement

     (Positive Predictive Value) = 100 x TP/(TP+FP)

   - Calculate the percent Negative Agreement

     (Negative Predictive Value) = 100 x TN/(TN+FN)

3. **Linearity, Analytical Measurement Range (AMR) and Clinical Reportable Range** are not applicable for qualitative methods.
4. **Analytical Sensitivity** is the lowest concentration of an analyte that can be measured.
   For an FDA approved (as well as WHO endorsed), unmodified method, the manufacturer's stated sensitivity will be used.

5. **Analytical Specificity** is the determination of the effect of interfering substances.
   For an FDA approved (as well as WHO endorsed), unmodified method, the manufacturer's stated specificity will be used.
6. **Reference Ranges** Not Applicable
7. **Method Approval**
   The final decision on methodology validation and acceptance is made after a careful review of all the studies performed as part of the complete method validation process. The Laboratory Director shall make the ultimate decision on method validation. There must be an approval with a signature from the Medical and/or Laboratory Director and preparer of validation documents with dates.

Interferon- Gamma Release Assay (IGRA)

Introduction: A whole blood test that measures the white cell response to the presence of MTB antigens. If a person has been exposed to MTB, their T-cells will release IFN-γ when exposed to MTB specific antigens. There are two IGRA assays; the T-SPOT TB and QuantiFERON-TB Gold. Both are FDA approved for diagnosis of latent TB or TB exposure. IGRA relies on an ELISA and is considered a semi-quantitative method but the validation is treated as qualitative.
To perform the test, a whole blood specimen can be collected in a single green-top lithium-heparin tube. That blood is then transferred into 4 tubes (Nil, TB1, TB2 and Mitogen tubes) and incubated before performing an ELISA. The ELISA portion can be performed manually on micro-titer plates and then placed into a plate reader with specific software or completely automated on an immunology instrument such as the Diasorin Liaison.
Nil: Negative control
TB1: Low level reaction
TB2: High level reaction
Mitogen: Tests for non-specific reaction. Works as a negative control for the patient's immune system to show that the blood is capable of reacting to the TB1 and TB2 tubes. Reduces indeterminate reactions.

1. **Precision** is not applicable.
2. **Accuracy** is the true value of a substance being measured. Verification of accuracy is the process of determining that the test system is producing true, valid results.
   a. Determine the Reference Method (*Selection of specimens and sample number)*
      Sample Options: Due to the nature of the test, previously tested specimens are not an option. If possible, whole blood specimens should be used for validation. Chose one of the options below
      - Comparison testing with another lab that is performing QuantiFERON-TB Gold In-Tube (QGIT) or QuantiFERON-TB Gold Plus (QFT-Plus) testing. These specimens should be whole blood specimens. The lab would coordinate with another lab with which they already have a relationship. Samples taken at the first lab would be drawn in duplicate and one set would be sent to the second lab for parallel testing.
      - In house comparison of patients previously tested with QuantiFERON-TB Gold In-Tube (QGIT) or QuantiFERON-TB Gold Plus (QFT-Plus). If possible, new whole blood specimens should be obtained for these patients. As time goes on, this option is less likely to occur since QGIT is not very common anymore.
      - Validation over time using samples from CAP, UKNEQAS or other approved External Quality Assurance (EQA) Program. This is the most likely option for our labs starting the QFT-Plus. Samples would most likely come from UKNEQAS. EQA events usually have 2 specimens for testing so multiple back panels would be needed (if they are available). Could take several months to get 20 total specimens and they might not

be divided evenly between positive and negative. Exceptions for total numbers can be made if it takes too long to gather 10 positives and 10 negatives.

- In house comparison to culture positive patients. The most time-consuming option as positive MTB cultures take weeks to be confirmed positive. Timing of testing could also be difficult as the whole blood collected needs to be transferred to testing tubes within 53 hours of collection. **Avoid this option if possible** as some culture positive patients could test negative for QFT due to prolonged active infection reducing a patient's immune response over time.
- Combination of the previous options. **Avoid this if possible** as it would make for a convoluted summary.

3. **Linearity, Analytical Measurement Range (AMR) and Clinical Reportable Range** are not applicable for qualitative methods.
4. **Analytical Sensitivity** is the lowest concentration of an analyte that can be measured. For an FDA approved, unmodified method, the manufacturer's stated sensitivity will be used.
5. **Analytical Specificity** is the determination of the effect of interfering substances. For an FDA approved, unmodified method, the manufacturer's stated specificity will be used.
6. **Reference Ranges** Not Applicable
7. **Method Approval -** The final decision on methodology validation and acceptance is made after a careful review of all the studies performed as part of the complete method validation process. The Laboratory Director shall make the ultimate decision on method validation. There must be an approval with a signature from the Medical and/or Laboratory Director and preparer of validation documents with dates.

Validation of Stains

Refer to qualitative guidelines for specimen numbers and acceptability criteria.

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