SUBJECT: **Interfering Medical Condition Studies for Nucleic Acid-Based Diagnostic Assays**

PURPOSE: This SOP is a broad and generic template for determining and characterizing the effects of potential interferents on assay performance and is to serve as a foundation for developing a more specific and individualized evaluation plan for each assay received from a third party.

LEVEL: Principal Investigator/designee
Laboratory staff

SUPPLIES/ EQUIPMENT:
Policy and Procedure Manual
Third Party's Assay / Instrumentation
Third Party's Assay Software
Third Party's Test Procedure
AsTeC Consortium Laboratory Equipment
Test samples
Pipettes and pipette tips
Computer
Printer

REQUIREMENTS:

Interfering substances can be a significant source of error in diagnostic tests. This SOP is written in general terms to provide flexibility to accommodate the technology being evaluated. A specific SOP outlining test procedures according to manufacturer's recommendations will be written for each test method accepted for evaluation. The analyses described below will be conducted for each test method under evaluation following the individual test's specific SOP testing procedures. Interference studies will be performed for the overall system, from nucleic acid extraction to detection and quantitation.

Two approaches will be employed to evaluate the susceptibility of a diagnostic test to interfering substances. First, potentially interfering substances will be determined by adding those substances to samples or matrices and subsequently testing them with the assay or testing commercially manufactured reference materials (spiked pool testing). Second, representative patient specimens from the repository will be used to determine the bias introduced by the substance/medical condition by comparing test results with the EORTC/MSG classified disease status for each specimen.

A. *Interference Screen*

In order to screen for potentially interfering substances in a standardized format, "paired-difference" testing will be used. Potentially interfering substances identified in Table 1 will be chosen by the AsTec Review Committee for evaluation. The potentially interfering substance will be added to a sample pool at relatively high concentrations (to simulate worse case) and the bias relative to a non-spiked control portion of the same pool will be determined. Substances that show a clinically significant effect will be considered interferents and further evaluation will be performed to determine the relationship between interferent concentration and the degree of interference.

For each nucleic acid-based test method under evaluation, inhibitors of amplification will be detected by the use of internal control templates each time the test is performed in an AsTeC laboratory. The internal control failure rate for the test will be documented on QC sheets, including any remedial actions taken and the type of sample tested. The frequency of inhibition with any one specimen type will be expressed as a percentage of the total number of samples tested. This information will be summarized and reviewed by the AsTeC Review Committee in order to identify conditions/substances to screen as potential interferents as described below. (CLSI MM3-P2, page 11, MM6-A, page 16)

Experimental Procedure

1. Evaluate interference at two medical decision concentrations of the analyte. Record in Datasheet 1 the analyte concentration that determines a positive test result (medical decision point) and the high limit of detection for the analyte. From this range, select and record two medical decision concentrations of the analyte, one low positive and one high positive, to use for assessing potential interferents.
2. Determine criteria for a "clinically significant" difference (d_{max}) by either 1.) consensus of clinical experts (the AsTec Review Committee) or 2.) based on repeatability standard deviation (s) of the test method as determined from prior studies of reproducibility/precision-SOPPM 051.01. (CLSI EP7-A2, Section 5.1.2 and 5.1.3) As a reminder, the clinically significant" difference (d_{max}) is the allowable interference to be detected at the analyte test concentration.
3. Determine the number of replicates needed to detect various interference effects with 95% confidence and power using a two-sided test. For convenience, the interference criteria are expressed as multiples of the repeatability (with-in run) standard deviation (d_{max}/s) in Table 2. Where d_{max} is the maximum allowable interference to be detected at the analyte concentration and s is the

repeatability standard deviation of the diagnostic test being evaluated. (CLSI EP7-A2 Section 7.1.3.4)

4. Prepare a *base pool* of clinical samples by obtaining appropriate type specimens (serum, urine, plasma, etc) from healthy controls.
5. Calculate the required pool volume, considering the test's sample volume requirements, the number of substances to be tested, and the replication requirements determined above.
6. Determine the level of analyte contained in the base pool and adjust the level of analyte (using suitably pure material) to the medical decision concentrations as determined in step 1 above.
7. Obtain a pure form of the potential interferent (for example, a pharmaceutical grade preparation of drug) and prepare a highly concentrated *stock solution* (20 times the intended test concentration [the highest concentration that a laboratory would expect to observe among patient samples submitted for analysis]) using a solvent in which the interferent is sufficiently soluble. Dilute the sample matrix as little as possible, preferably no more than 5%.
8. Prepare the *test pool* by pipetting a 1/20 volume fraction of the *stock solution* into a volumetric flask. Make the desired concentration by adding the appropriate amount of the base pool and mix well. Example: Add 0.5 mL of a 20x concentrated *stock solution* to a 10-mL volumetric flask. Make up to volume with the base pool and mix well.
9. Prepare the "*control" pool* by pipetting a 1/20 volume fraction of the solvent used to prepare the *stock solution* into a second volumetric flask. Make up to volume with the base pool and mix well.
10. Prepare *n* aliquots of the *test pool* and *n* aliquots of the *control pool* based on the number of required replicates as determined in Step 3.
11. Analyze the test (T) pool and control (C) pool samples in alternating order (e.g. $C_1\ T_1\ C_2\ T_2\ C_3\ T_{3....}\ C_n\ T_n$).
12. Record results for data analysis using the format of Datasheet 1 in Appendix A.
13. Data Analysis:
    Determine the "point estimate" of the observed interference effect as the difference between the means of the test and control samples and the cutoff value as described in CLSI EP7-A2, Pages 18-19, Section 7.1.5. If the point estimate is greater than the cut-off value, the substance is an interferent. If a substance is determined to be an interferent, proceed to characterize the interference effect as follows.

B. *Characterization of Interference Effects*

A dose response experiment using spiked specimens will be performed using the following procedure to determine the relationship between the interferent concentration and the magnitude of interference.

1. Determine the highest and lowest concentrations of the interferent to be tested. The high concentration should be at least 3 times the highest concentration reported following a drug therapeutic dosage or ingestion of a dietary substance, 5 times the recommended additive concentration for anticoagulants and preservatives, and the highest concentration expected in the patient population for an endogenous substance. The low concentration should be the average concentration of the interferent in the pool of clinical samples (e.g., a negligible concentration in the case of a therapeutic drug and a low, non-pathologic level in the case of endogenous substances such as hemoglobin or bilirubin).
2. Determine the difference that would be considered "clinically significant." This has been done in Step 2 of the experimental procedure for the *Interference Screen.*
3. Determine the number of replicates, *n*, to run at each concentration as described in CLSI EP7-A2, Page 87, Appendix F. (It is generally sufficient to conduct the dose-response series in triplicate at each concentration; however, the calculations in Appendix F will assure 95% confidence and power.)
4. Prepare a base pool as described in step 4 of the experimental procedure for the *Interference Screen.*
5. Prepare a high concentration pool to contain the concentration of potential interferents that was determined in Step 1. Label this ***concentration 1.***
6. Prepare a low concentration pool to contain the concentration of potential interferents that was determined in Step 1. Label this ***concentration 5***.
7. Prepare five concentrations of test pools that contain intermediate concentrations of the interferent using the following steps
    a. Mix equal volumes of ***concentration 1*** and ***concentration 5*** to create ***concentration 3*** which will be midway between the high and low concentrations.
    b. Mix equal volumes of ***concentration 1*** and ***concentration 3*** to create ***concentration 2*** which will be a quarter of the way between the high and the low concentrations.
    c. Mix equal volumes of ***concentration 5*** and ***concentration 3*** to create ***concentration 4*** which will be three quarters of the way between the high and the low concentrations.
8. Prepare *n* aliquots of each pool (as determined in Step 3 of this procedure, *Characterization of Interference Effects*).

9. Analyze the series of five pools within the same analytical run. The first set of replicates should be analyzed in ascending order, the second set in descending order, the third set in ascending order, etc., in order to average out any systematic drift effects. Record the results in Datasheet 2 Table A.
10. Calculate the average concentration for concentration 1 (low pool) and subtract it from all results obtained for replicates from concentrations 2 through 5 to determine the observed effect for each pool. Record the results in Datasheet 2 Table B.
11. Data Analysis:
    Plot the results from Datasheet 2 Table B with the observed effect on the y-axis and the interferent concentration of the x-axis and examine the shape of the dose-response relationship. If the data appear randomly distributed about a straight line, apply linear least squares regression analysis. Determine the slope, intercept and residual error from the individual observations (not averages). A 95% confidence band can be computed around the dose-response line, from which the 95% confidence interval for the interference can be determined at any interferent concentration. If the data appear non-linear, conduct a non-linear regression analysis to determine confidence intervals.

C. *Evaluate interferences using patient specimens*
Substances determined to cause a clinically significant effect as determined in the spiked pool testing studies will be considered interferents. At the discretion of the AsTec Review Committee, the impact of these interferents may be further evaluated using patient specimens from the repository as follows.

Experimental Procedure – Determining impact on sensitivity
1. Select a test group of specimens from the repository. These specimens are selected from patients diagnosed with proven invasive aspergillosis (IA), and who have a medical condition or who are receiving a substance identified as potential interferent in the spiked pool testing. The interfering medical condition or substance should be present or being administered at the time the specimen was collected.
2. Select the control group of specimens. These specimens are also selected from patients diagnosed with proven aspergillosis, however, these specimens are selected from a patient population that does not have the medical condition and who are not receiving the substance identified as a potential interferent in the spiked pool testing. Specimens selected from control patients should have been collected within a similar time period relative to the diagnosis of

IA as the "test specimens" in order to ensure the same range of analyte concentrations are represented.

3. Determine the sample size needed in each group (those with and those without the interfering condition) using 80% power (two-tailed $\alpha = 0.05$) and the Mean Standard Deviation test result for patients with proven aspergillosis. This information may be obtained from prior comparative studies evaluating accuracy-SOPPM 054.01 or the manufacturer.
4. Analyze each specimen from the test group and the control group in duplicate with the test procedure.
5. Divide the number of samples into 3-4 runs and test over several days rather than testing all the samples in one run on the same day to reduce the contribution of day to day imprecision. Alternate control and test samples within each run.
6. Record the results in Datasheet 3.
7. *Data Analysis*

   The mean test results from specimens with the interferent will be compared with the mean test result from specimens without the interferent using a two-sided level .05 unpaired t-test, and the point estimate and confidence interval for the difference in means will be calculated. Additionally, the proportion testing positive in specimens with the interferent will be compared to the proportion testing positive without the interferent using a two-sided level .05 Bernard's exact test, and the exact point estimate and confidence interval for the difference in proportions will be calculated.

   Note: Sensitivity is typically defined as the proportion of true positives by a gold standard that test positive with the novel test. Sample size is calculated for the test of means rather than the test of proportions because the latter would require prohibitively large numbers of samples from patients with invasive aspergillosis.

Experimental Procedure – Determining impact on specificity

Follow the same procedures used to determine the impact of the interferent on sensitivity except select specimens from patients without proven, probable or possible invasive aspergillosis. For example, the test group of specimens is selected from patients without aspergillosis but who have a medical condition or who are receiving the substance identified as a potential interferent in the spiked pool testing. The control group of specimens is selected from patients without invasive aspergillosis and who do not have the medical condition and who are not receiving the substance identified as potential interferent in the spiked pool testing.

REFERENCES:

1. CLSI. Interference Testing in Clinical Chemistry; Approved Guideline – Second Edition. CLSI document EP7-A2. 2005. CLSI, Wayne, PA.
2. FDA Guidance Document 1560 –Nucleic Acid Based In Vitro Diagnostic Devices for Detection of Microbial Pathogens December 08, 2005. U.S. Department of Health and Human Services Food and Drug Administration Center or Devices and Radiological Health.
3. CLSI. Molecular Diagnostic Methods for Infectious Diseases; Proposed Guideline – Second Edition. CLSI document MM3-P2. 2005.CLSI, Wayne, PA.
4. NCCLS. Quantitative Molecular Methods for Infectious Diseases; Approved Guideline. NCCLS document MM6-A. 2003. NCCLS, Wayne, PA.

Table 1. Potentially interfering medical conditions noted or performed as part of routine clinical care

- Uremia (Urea)
- Hemoglobin (or Heme)
- Ammonia
- Bilirubin
- Neutropenia
- Additives
  - Heparin,
  - EDTA
  - Detergents
  - Guanidinium HCl
- Medications at time of sampling (+/- 7 days)
  - Antifungal agents
  - Antibacterial agents
  - Heparin
  - Others
- Concurrent bacterial bloodstream infection
- Close neighbor, non-target organisms (e.g., *Penicillium* species)
- Diarrhea
- Mucositis
- Rheumatoid factor
- Heterophile molecular …..Is this an error?
- Receipt of immunoglobulin products
- Receipt of blood components (transfusions)
- *Aspergillus* antibodies
- Plasmalyte
- Triglycerides
- Cholesterol
- Hemodialysis
- Plasmapheresis
- Receipt of hyperalimentation
- Sodium gluconate

Table 2. Number of Replicates Needed to Detect Various Interference Effects with 95% Confidence and Power

d_{max}/s*	No. of Replicates	d_{max}/s*	No. of Replicates
0.8	41	1.5	12
1.0	26	1.6	10
1.1	22	1.8	8
1.2	18	2.0	7
1.3	16	2.5	5
1.4	14	3.0	3

*d_{max} is the maximum allowable interference to be detected at the analyte concentration and s is the repeatability standard deviation of the diagnostic test being evaluated

APPENDIX A. INTERFERENCE TESTING DATA RECORDING SHEETS

DATASHEET 1: Interference Screen Results

RECORD TEST DETAILS:			
Date:		**Technologist:**	
Analyte:		**Test Substance:**	
Concentration:		**Concentration:**	
Precision (%):		**Acceptable Limit:**	
Method:		**Instrument:**	
Reagent Lot#:		**Calibrators:**	
RECORD RESULTS:			
Control Pool		**Test Pool**	
C1		**T1**	
C2		**T2**	
C3		**T3**	
C4		**T4**	
C5		**T5**	
C6		**T6**	
C7		**T7**	
C8		**T8**	
CALCULATE STATISTICS:			
Control Pool		**Test Pool**	
Mean	**X=**	**X=**	
Std. Dev.	**SD=**	**SD=**	
C.V.	**CV=**	**CV=**	
CALCULATE DIFFERENCE:			
Test Pool mean-Control Pool mean =			

DETERMINE 95% CONFIDENCE LIMITS:				
Control Pool			**Test Pool**	
Variance	**s^2 =**		**s^2 =**	
Average variance =				
Pooled standard deviation =				
Upper confidence limit [d+1.96 x (2 s^2 /n) $^{-1/2}$=				
Lower confidence limit [d-1.96 x (2 s^2 /n) $^{-1/2}$=				
CONCLUSIONS:				

DATASHEET 2: Characterization of Interference Effect

Table A: Raw Results for Five Level Dose Response Series

Pool	Interferent Concentration	Results			
		Rep 1	Rep 2	Rep 3	Rep ____*
1					
2					
3					
4					
5					

*Additional columns may be needed based on the number of replicates needed as determined in Procedure B.3 *Characterization of Interference Effects.*

Calculate Average (Mean) of Replicate Results for Pool 1:_____

Table B: Observed Effect for Five Level Dose Response Series

Pool	Interferent Concentration	Observed Effect (mean of results from pool 1 subtracted from the raw result of each replicate of pools 2-5)			
		Rep 1	Rep 2	Rep 3	Rep _____
1					
2					
3					
4					
5					

*Results for Replicates of Pool 1 will be the same as the Raw results for Replicates of Pool 1 recorded in Table A above.

DATA SHEET 3: Interference Testing Using Patient Specimens

Disease Status (circle one): Proven IA or No Proven, Probable or Possible IA						
Sample #	**Interferent Present**			**Interferent Absent**		
	Rep 1	**Rep2**	**Mean (x)**	**Rep 1**	**Rep 2**	**Mean (x)**
Total Mean X				**Total Mean X**		

ORIGINAL IMPLEMENTATION DATE: _____

APPROVED BY NIH NIAID Project Officer: _____ DATE _____

APPROVED BY PI/designee: _____ DATE _____

APPROVED BY Laboratory Coordinator: _____ DATE _____