Antibacterial Resistance Leadership Group (ARLG)

Performance of Nucleic Acid Amplification Tests for the Detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in Extragenital Sites (pNAAT)

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Signature Page

The signature below documents the review and approval of this protocol and provides the necessary assurances that this study will be conducted according to the protocol, including all statements regarding confidentiality, and according to national, regional, and local legal and regulatory requirements.

Site Principal Investigator Name (Print)

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Date

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All questions concerning this protocol should be sent via email to arlg.gc@mc.duke.edu. The appropriate team member will respond to questions via email, generally within 24 hours (Monday-Friday).

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List of Abbreviations and Definitions

ARLG	Antibacterial Resistance Leadership Group
ASIS	Anatomic Site Infection Status
CDC	Center for Disease Control and Prevention
CFR	Code of Federal Regulations
CT	*Chlamydia trachomatis*
DCRI	Duke Clinical Research Institute
DNA	Deoxyribonucleic acid
Equivocal	Final equivocal result from nucleic acid amplification test, as determined by manufacturer's guidance. This means the test was run and the result was neither positive nor negative
FDA	Food and Drug Administration
HIV	Human immunodeficiency virus
ICMJE	International Committee of Medical Journal Editors
ID	Identification
IFU	Instructions for Use
Indeterminate	Anatomic site infected standard cannot be determined as positive or negative
IRB	Institutional Review Board
ISRC	Independent Study Review Committee
LGBT	Lesbian, gay, bisexual and transgender
NAAT	Nucleic acid amplification test
Negative (-) NAAT reading	Result from the nucleic acid amplification test is negative
NR	This means no test was run and there is no result
NPA	Negative percent agreement
NPV	Negative predictive value
NG	*Neisseria gonorrhoeae*
NIH	National Institutes of Health
OHRP	Office of Human Research Protections
Positive (+) NAAT reading	Result from the nucleic acid amplification test is positive
PPA	Positive percent agreement
PPV	Positive predictive value
PI	Principal Investigator
RNA	Ribonucleic acid

rRNA	Ribosomal ribonucleic acid
SDMC	Statistical and Data Monitoring Center
STD	Sexually transmitted diseases
WHO	World Health Organization

Protocol Synopsis

Protocol Title:	Performance of Nucleic Acid Amplification Tests for the Detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* in Extragenital Sites
Study Design:	A cross-sectional, single visit study to evaluate the diagnostic accuracy of nucleic acid amplification tests for detection of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* from pharyngeal and rectal sites
Primary Study Objectives:	For each nucleic acid amplification assay, estimate the positive percent agreement (PPA) and negative percent agreement (NPA) for detecting: • *Neisseria gonorrhoeae* in rectal swabs • *Neisseria gonorrhoeae* in pharyngeal swabs • *Chlamydia trachomatis* in rectal swabs • *Chlamydia trachomatis* in pharyngeal swabs
Study Population	Patients presenting to an outpatient clinic for sexually transmitted disease (STD) testing for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections
Number of subjects	Up to 3,000
Study duration	Approximately 12 to 24 months
Number of sites	Up to 10 clinical sites (as needed)
Clinical Samples	Pharyngeal and rectal swabs

1.0 Background and Scientific Rationale

1.1 Background Information

Nucleic acid amplification tests (NAATs) have become the gold standard for diagnosing *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) infections in the urogenital tract but are not currently approved by the Food and Drug Administration (FDA) for use in extragenital sites, including the pharynx and the rectum.

The goal of this study is to determine the diagnostic accuracy of the following 3 NAAT platforms for the detection of NG and CT from the pharynx and the rectum:

- Company 1 Assay [Company 1] [1]
- Company 2 Assay [Company 2] [2]
- Company 3 Assay [Company 3] [3]

The assays being evaluated are intended for the direct qualitative detection of *Chlamydia trachomatis* and/or *Neisseria gonorrhoeae* in rectal or pharyngeal clinician-collected swabs from symptomatic or asymptomatic male or female patients.

1.2 Scientific Rationale

Infections due to NG and CT are a major public health threat. The World Health Organization (WHO) estimated more than 100 million new worldwide cases for each of NG and CT in 2008.[4] In the US alone, there were 333,000 NG and 1.4 million CT genitourinary infections in the year 2013.[5] Over the past 15 years, researchers have demonstrated an important burden of NG and CT infection in extragenital sites - the pharynx and rectum. Most CT and NG infections are asymptomatic, but infection can lead to serious sequelae, including infertility, chronic pelvic pain, adverse obstetrical outcomes, increased risk of human immunodeficiency virus (HIV) acquisition, and disseminated infection.[6-9] In addition to threats to individual health, there has been growing concern over antibiotic resistance, and in 2013, the US Centers for Disease Control and Prevention (CDC) classified drug-resistant NG as one of the three urgent-level resistant bacteria. Improved detection of extragenital NG is thought to be a crucial component of adequate treatment and thus prevention of further resistance.[10]

Currently, the CDC recommends the use of NAATs for NG and CT screening and diagnosis in the genitourinary tract due to superior sensitivity compared to traditional culture methods.[11] The sensitivity and specificity of commercially available NAATs for the detection of genitourinary NG and CT infection is estimated to be between 90-100%.[12] CDC also recommends the use of NAATs for extragenital sites.[11] Due to widespread use and interest in these tests for extragenital NG and CT infections, multiple studies have examined the test characteristics of NAAT tests in comparison to culture and to additional NAAT platforms (see Table 1). For diagnosis of CT, reported sensitivities are 80-100% (pharyngeal) and 46-100% (rectal), with significant variation by platform tested.[13-19] Reported specificities are >99% for pharyngeal site and 89-100% for the rectum. For diagnosis of NG, reported sensitivities are 72-100% (pharyngeal) and 75-100% (rectal), again with variation by platform.[13,14,16-18,20,21] Reported specificities are 72-100% (pharyngeal) and 95-100% (rectal).

Table 1. Prior Studies of extragenital testing for chlamydia and gonorrhea.

Screening test	Sensitivity/specificity* by specimen type (%)	
	Pharyngeal	**Rectal**
Neisseria gonorrhoeae		
Bachmann LH *et al,* 2010[16]	100 / 96.2	100 / 95.5
Cosentino LA *et al,* 2012[17]		76 / 100
Moncada J *et al,* 2009[19]		78.3 / 99.8
Ota KV et al, 2009[13]	95 / 99.6	100 / 100
Schachter J *et al*, 2008[14]	84.3 / 99.4	93.2 / 99.7
Walsh A ***et al***, 2011[22]	100/100	100/100
Goldenberg SD *et al*., 2012[23]		91.1 / 100
Cosentino LA *et al*, 2015[24]		100 / 100
Chlamydia trachomatis		
Bachmann LH *et al,* 2010[16]		100 / 88.8
Cosentino LA *et al,* 2012[17]		100 / 99.8
Moncada J *et al,* 2009[19]		71.2 / 99
Ota KV *et al*, 2009[13]	100 / 99.2	100 / 98.7
Schachter J *et al,* 2008[14]	100 / 99.6	93.5 / 97.7
Goldenberg SD *et al*., 2012[23]		86 / 99.2
Cosentino LA *et al*, 2015[24]		96.6 / 99.7

*Sensitivity and specificity as reported by authors, each using unique definitions for the reference standard. As discussed below, we will be using positive percent agreement (PPA) and negative percent agreement (NPA) for these calculations.

Despite the CDC recommendations, at this time, there are no FDA-cleared commercial NAAT tests for the detection of pharyngeal and rectal NG or CT infection. The FDA-clearance of such assays would increase the availability and uptake and subsequently lead to improved screening and enhanced control of NG and CT extragenital infections. The current proposed study will help provide the data required for FDA consideration to clear such assays for marketing.

Because of the multi-site nature of the ARLG, its strong laboratory network and inclusion of investigators with expertise in NG and CT detection and treatment, the ARLG is well-positioned to conduct such a study. Current commercial assays are “dual” assays and include targets for both NG and CT, so the goal of this study will be to evaluate the diagnostic accuracy for detection of both organisms at extragenital sites.

The molecular targets of the three NAAT platforms that will be tested differ, which will allow for comparative evaluation.[25]

- Company 1 Assay (Company 1) [redacted] [1]

- Company 2 Assay (Company 2) [redacted] [2]
- Company 3 Assay (Company 3) [redacted] [3]

Additional testing using the tiebreaker test [redacted] will be performed in cases of discordant results (see Section 3.5). This test utilizes [redacted] to identify the presence of the organism of interest in the clinical sample. [redacted]. Though the diagnostic accuracy of these assays for extragenital infections has not previously been evaluated, studies evaluating the performance of these assays for confirmation of other NAATs suggest excellent concordance.[28-31]

2.0 Objectives

2.1 Primary Objective

For each NAAT, estimate the PPAs and NPAs for detecting:

- *Neisseria gonorrhoeae* in rectal swabs
- *Neisseria gonorrhoeae* in pharyngeal swabs
- *Chlamydia trachomatis* in rectal swabs
- *Chlamydia trachomatis* in pharyngeal swabs

2.2 Secondary Objectives

2.2.1 Global analyses:

For each NAAT, positive predictive values (PPVs), negative predictive values (NPVs), positive likelihood ratios, negative likelihood ratios, and sensitivity analyses will be calculated for detecting:

- *Neisseria gonorrhoeae* in rectal swabs
- *Neisseria gonorrhoeae* in pharyngeal swabs
- *Chlamydia trachomatis* in rectal swabs
- *Chlamydia trachomatis* in pharyngeal swabs

2.2.2 Subgroup analyses:

For each NAAT, to estimate the PPAs, NPAs, PPVs, and NPVs for detection of NG and CT from rectal and pharyngeal swab specimens by sex and by anatomic site-specific symptom status.

2.3 Outcome Measures

The anatomic site infected status (ASIS) will be determined for each anatomic site (pharyngeal and rectal) and each organism (NG and CT), as shown below in Table 2.

Possible ASIS outcomes are:

- Infected
- Not infected
- Indeterminate
- Invalid, exclude from analysis

The anatomic site is considered to be infected if both of the reference test results are positive. The anatomic site is considered to be not infected when both reference test results are negative. If there is discordance between the reference tests, an additional NAAT will be done as a tiebreaker. In this case, agreement of 2/3 of the reference NAATs will determine the ASIS. If two tests are equivocal or one equivocal and one not run, the third test result will stand as the ASIS if positive or negative. If two tests are not run, the ASIS will be considered invalid and will be excluded from the analysis. All possible combinations are shown in Table 2. The tiebreaker test will be run by the lab if any NAAT is not concordant with the others and interpreted only in the case of discordant results between the two planned reference tests for each assay. [redacted].

To determine the ASIS, the test result for each respective site (pharyngeal or rectal) and each organism (NG or CT) for each NAAT platform will be used. Possible outcomes for each assay are:

- **Company 1 Assay (Company 1)[1]**: Not detected, detected, invalid (sample processing control or sample adequacy control failed), error (probe check control failed), or no result (insufficient data was collected, e.g. test aborted). Initial invalid, error, or no result tests will be repeated. If the repeat test returns invalid, error, or no result, the final result will be considered an invalid and will be categorized as no result (NR) for the ASIS determination and statistical analyses below. If the repeat test returns not detected (negative) or detected (positive), this will be the result used for the ASIS determination and statistical analyses.

- **Company 2 Assay (Company 2)[2]**: Negative, positive, equivocal (result between positive and negative), invalid (run status is FAIL or other technical failure), or error (sample was not tested due to an error detected by the instrument). Initial equivocal, invalid, and error test results will be repeated. If the repeat test result returns equivocal, the final test result will be considered an equivocal test result for the ASIS determination and statistical analyses below. If the repeat test result returns invalid or error, the final test result will be categorized as equivocal if the initial test was equivocal and as no result (NR) if the initial test was invalid or error for the ASIS determination and statistical analyses below. If the repeat test returns negative or positive, this will be the result considered for the ASIS determination and statistical analyses.

- **Company 3 Assay (Company 3)[3]:** For NG, possible results are positive (detected, with cycle number less than or equal to the assay cut-off), negative (no evidence of amplification or cycle number greater than the assay cut-off), and error. An equivocal interpretation does not apply. For CT, possible results are positive (detected, with cycle number less than or equal to the assay cut-off), negative (no evidence of amplification), equivocal (cycle number beyond the assay cut-off), and error. A sample with initial interpretation of error (both CT and NG) or equivocal (CT only) will be retested. If the repeat test returns negative or positive, this will be the result considered for the ASIS determination and statistical analyses. If the repeat

test result is equivocal (CT only), the final test result will be considered equivocal for the ASIS determination and statistical analyses below. If the repeat test result is error, the final test result will be categorized as no result (NR) for the ASIS determination and statistical analyses below if the initial test result was error and as equivocal if the initial test result was equivocal (CT only).

- **Tiebreaker assay:** ***Tiebreaker A*ssay (Company 4)**: Negative, positive, equivocal (result between negative and positive ranges), invalid (run status is FAIL or other technical failure), or error (sample was not tested due to an error detected by the instrument). Initial equivocal, invalid and error test results will be repeated. If the repeated test result is equivocal, it will be considered an equivocal test result for the ASIS determination and statistical analyses below. If the repeat test result returns invalid or error, the final test result will be categorized as equivocal if the initial test was equivocal and as no result (NR) if the initial test was invalid or error for the ASIS determination and statistical analyses below. If the repeat test returns negative or positive, this will be the result considered for the ASIS determination and statistical analyses.

Table 2. Determination of the Anatomic Site Infected Standard

Comparator NAAT 1 Result	Comparator NAAT 2 Result	Tiebreaker NAAT Result	Anatomic Site Infection status
+	+	Not indicated	Infected
+	-	+	Infected
+	E*	+	Infected
+	NR**	+	Infected
+	-	-	Not infected
+	-	E	Indeterminate
+	-	NR	Indeterminate
+	E	-	Indeterminate
+	E	E	Infected
+	E	NR	Infected
+	NR	-	Indeterminate
+	NR	E	Infected
+	NR	NR	Invalid, remove from analysis
-	-	Not indicated	Not infected
-	+	-	Not infected
-	E	-	Not infected
-	NR	-	Not infected
-	+	+	Infected
-	+	E	Indeterminate
-	+	NR	Indeterminate
-	E	+	Indeterminate
-	E	E	Not infected

-	E	NR	Not infected
-	NR	+	Indeterminate
-	NR	E	Not infected
-	NR	NR	Invalid, remove from analysis
E	+	+	Infected
E	-	-	Not infected
E	+	-	Indeterminate
E	+	E	Infected
E	+	NR	Infected
E	-	+	Indeterminate
E	-	E	Not infected
E	-	NR	Not infected
E	NR	+	Infected
E	NR	-	Not infected
E	NR	E	Indeterminate
E	NR	NR	Invalid, remove from analysis
NR	+	+	Infected
NR	-	-	Not infected
NR	NR	Not indicated	Invalid, remove from analysis
NR	+	-	Indeterminate
NR	+	E	Infected
NR	+	NR	Invalid, remove from analysis
NR	-	+	Indeterminate
NR	-	E	Not infected
NR	-	NR	Invalid, remove from analysis
NR	E	+	Infected
NR	E	-	Not infected
NR	E	E	Indeterminate
NR	E	NR	Invalid, remove from analysis

*E **=** equivocal result
****NR = no result. This can occur either because the test result was invalid or because the test could not be run (e.g. too little sample, improperly shipped, no sample received).

3.0 Study Design

3.1 Study Design

This will be a cross-sectional, single visit study evaluating the performance of three commercial NAATs to detect NG and CT in the rectum and pharynx.[1,2,32,33] A fourth NAAT is being used as a tiebreaker.[26,27] We are not evaluating the performance of this fourth NAAT.

The study will include specimens collected from adult subjects seeking sexually transmitted disease (STD) testing at the participating clinics, which include clinics focused on sexually transmitted diseases, women's health, student health, family planning clinics, and clinics specializing in lesbian, gay, bisexual, and transgender (LGBT) health. Potential subjects will be identified, assessed for eligibility and approached for informed consent. Both symptomatic and asymptomatic individuals will be included in the study population.

The study will enroll up to 3,000 subjects who will have four pharyngeal and four rectal swabs collected as part of a one-time study visit. Each manufacturer will have specific collection kit and transport media. Additional data collection will include, but is not limited to, eligibility screening questions, gender, age, and patient report of relevant pharyngeal, rectal, or other relevant symptoms, and swab collection completeness. Subjects will be classified as symptomatic or asymptomatic for each anatomic site. Results of testing at other anatomic sites and results of clinical testing will not be available.

Each swab will be used for a specific NAAT, which will be run at one of the two reference testing laboratories. The ASIS will be defined by the results of the two other nucleic acid amplification tests, and a tiebreaker test (if necessary), as shown in Table 2. Each anatomic site will be considered in isolation.

When accrual is complete, the study will be closed and an analysis plan will be enacted to finalize the database for analysis.

3.2 Inclusion Criteria

a) Attending a participating clinic for evaluation of STDs
b) ≥18 years of age at date of screening
c) Able and willing to provide informed consent
d) Willing to comply with study procedures, including collection of 4 swabs each from the pharynx and rectum for NG and CT testing

NOTE: Male, female, and transgender individuals will be included, and both symptomatic and asymptomatic individuals will be included.

3.3 Exclusion Criteria

a) Receipt of any systemic antibacterial drug in the past 14 days
b) Receipt of myelosuppressive chemotherapy in the past 30 days

3.4 Nucleic Acid Amplification Tests (NAATs)

This study will evaluate three distinct NAAT platforms. The selected platforms[25] are:

- Company 1 Assay (Company 1) [redacted] [1]

- Company 2 Assay (Company 2) [redacted].[2]

- Company 3 Assay (Company 3) [redacted] [3]

3.5 Tiebreaker Assay

In case of disagreement between reference NAAT results, a tiebreaker assay will be performed. This assay will use [redacted] (see Section 2.3). [redacted]

4.0 Study Procedures

4.1 Recruitment Plan

This study will enroll up to 3,000 participants who are seeking STD testing at one of the participating clinics. Potential subjects will be recruited without regard for risk behavior, symptom status, or gender. Participating clinics may include those designed for STD screening and management, family planning, student health, women's health, HIV management, and clinics focusing on the LGBT population.

4.2 Screening

Participants will be recruited from clinical sites performing routine STD testing. It is assumed that individuals presenting for routine STD testing at the participating clinics are at risk for such infections. Each person will undergo screening for eligibility and, if eligible, will be approached for oral informed consent.

4.3 Enrollment

Once enrolled, eight swabs will be taken: four swabs will be taken from the pharynx and four swabs will be taken from the rectum in addition to any swabs taken as part of routine clinical care. The swabs collected for routine clinical care will be taken first. The order of the research swabs will be randomized per subject to account for the possibility that the yield of the swab is affected by previous swabs at the same anatomic site. The swabs will be stored and transported per manufacturer guidelines. Results of those NAATs will <u>not</u> be reported back to the treating clinician for clinical purposes.

Data collection will include, but is not limited to, assessment of signs and symptoms of rectal and/or pharyngeal infection, as well as socio-demographic information such as age (not date of birth), race, ethnicity and gender, and swab collection completeness.

4.5 Incomplete Swab Collection

While required as an inclusion criteria for a potential subject to agree to both pharyngeal and rectal swabs, if only one anatomic site is swabbed after enrollment for whatever reason, and all four swabs are completed for that anatomic site, those swabs for that anatomic site will be included in the analysis. If fewer than four swabs at a site are completed, that anatomic site (and swab results) will be excluded from the analysis.

4.4 Subject Withdrawal and Replacement

Subjects may voluntarily withdraw their consent for study participation at any time and for any reason, without penalty. The primary reason for withdrawal from the study will be recorded on the Study Status case report form. Subjects who withdraw will not be replaced.

4.5 Study Discontinuation

This study may be terminated at any time by the Principal Investigator (PI) in consultation with the Antibacterial Resistance Leadership Group (ARLG) and/or the National Institutes of Health (NIH).

4.6 Follow-up Visit

There will not be a follow-up visit as part of this study. Subjects will continue with routine clinical care as directed by their medical providers.

4.7 Central Laboratory Procedures

There will be two reference testing laboratories, which will divide the processing and running of the NAATs for the study. Each clinical study site will be assigned to send swabs to one of the two laboratories. Swabs will be processed and run at the reference testing laboratories according to each manufacturer's Instructions for Use (IFU). Initial equivocal, invalid, or otherwise undetermined results will be repeated per manufacturer's guidelines before determining whether the result will be classified as positive, negative, equivocal, or no result. One of the reference testing laboratories will be responsible for running all of the tiebreaker assays, regardless of the location of original study site.

The reference testing laboratories will receive training for each of the study platforms from a representative from each of the manufacturers, in order to ensure adherence to the manufacturer's IFU procedures.

5.0 Device Monitoring

5.1 Specimen Collection Device Monitoring

Any complications with the specimen collection process, including device failure, significant patient discomfort, or inability to collect specimen, will be reported on the specimen collection case report form.

5.2 Laboratory Device Monitoring

The reference testing laboratories will maintain a log of all unanticipated device-related complications leading to no test, such as absence of transport media, quantity not sufficient, specimen transport collection system damage or incorrect transport system.

6.0 Statistics

6.1 Study Design

This is a cross-sectional, single visit study to evaluate the diagnostic accuracy of NAATs for the detection of NG and CT from pharyngeal and rectal swab specimens. Study participants will have swabs collected from both the rectum and pharynx, following any specimens taken for clinically indicated testing. Each of the swabs from each anatomic location will be tested for both NG and CT using three different NAATs, with a 4th test collected and tested as a tiebreaker, if needed. [redacted] Diagnostic accuracy will be calculated with respect to an anatomic site infected status (ASIS) reference standard described in Section 3.5. As the reference standard is imperfect, we will report positive percent agreement and negative percent agreement in place of sensitivity and specificity.

One potential limitation of this study is that all three of the tests in the reference standard are NAATs. However, all three tests [redacted]. So while, theoretically, there may be correlation in the errors produced by the three tests, the varied molecular targets and the different methods of capture, amplification and detection should mitigate this concern.

6.2 Endpoints

- Infection status for *Neisseria gonorrhoeae* in the rectum as determined by each NAAT
- Infection status for *Neisseria gonorrhoeae* in the pharynx as determined by each NAAT
- Infection status for *Chlamydia trachomatis* in the rectum as determined by each NAAT
- Infection status for *Chlamydia trachomatis* in the pharynx as determined by each NAAT
- Anatomic Site Infection status as determined by the reference standard (as described in Section 2.3)

6.3 Sample Size

For each assay, PPA and NPA[35] will be estimated for each of three diagnostic tests defined by pathogen - anatomical site combinations:

- *Neisseria gonorrhoeae* in the rectum
- *Neisseria gonorrhoeae* in the pharynx
- *Chlamydia trachomatis* in the rectum
- *Chlamydia trachomatis* in the pharynx

Each pathogen/anatomic site combination will be evaluated by constructing 95% Score confidence interval estimates for PPA and NPA and evaluating whether 90% can be ruled out by the lower bound of the CI with reasonable confidence.

NPA is expected to be greater than PPA and there will be considerably more not-infected than infected anatomic site, implying that there will be greater power for evaluating NPA. Thus it will suffice to size the study to evaluate PPA.

Assuming that the true PPA is 96%,[13] then 157, 174, and 196 evaluable participants that are infected are needed to have 80%, 85%, and 90% power respectively to demonstrate a PPA of greater than 90%. Since all of the participants will contribute to the evaluation of all three pathogen-anatomical site diagnostic tests, it will suffice to size the study for the diagnostic test with the lowest expected prevalence (except CT in the pharynx). The study will be more than sufficiently sized for more prevalent disease.

It is expected that the disease prevalence of NG in the rectum, NG in the pharynx, and CT in the rectum will each be greater than 7.5% in the population under evaluation.[13,14,16-20] Table 3 summarizes the required number of participants to have 80% and 90% probabilities of obtaining the 157, 174, and 196 evaluable participants that are disease-positive to have 80%, 85%, and 90% power when the true prevalence of disease is 7.5%, 10%, and 12.5%.

Table 3: Sample size required number to have 80% and 90% probabilities of obtaining the 157, 174, and 196 evaluable participants that are disease-positive when the true prevalence of disease is 7.5%, 10%, and 12.5% in order to have 80%, 85%, and 90% power (two-sided alpha=0.05) to conclude that sensitivity is greater than 90% when the true sensitivity is 96%.

Prevalence of Disease Positive	Power	Required Disease Positive to Obtain Power	Required Sample Size to have 80% probability of obtaining required Disease positive participants	Required Sample Size to have 90% probability of obtaining required Disease positive participants
7.5%	**80%**	157	2250	2325
	85%	174	2475	2575
	90%	196	2775	2875
10%	**80%**	157	1700	1750
	85%	174	1860	1925
	90%	196	2100	2150
12.5%	**80%**	157	1350	1400
	85%	174	1485	1530
	90%	196	1665	1715

6.4 Randomization of Swabs

Randomization will be employed to ensure that the swab order distribution is the same for 4 tests, the three NAATs under investigation in the study and the tiebreaker test. SAS software (Cary, NC) will be used to generate permuted blocks with a block size of 24, to ensure that each NAAT and the tiebreaker test would have their swab distributions consisting of 1/4 being first swabs, 1/4 being second swabs, 1/4 being third swabs and 1/4 being fourth swabs.

6.5 Monitoring

The core protocol team will receive monthly reports monitoring study accrual, study conduct and completeness of data and swab collection.

The study will be reviewed by an independent statistician, who will not have an association with the protocol or device companies. The independent statistician will review the progress of the study.

Additionally, the study statisticians will prepare reports for the independent statistician that will include baseline summaries (demographics and symptom status) and will address issues concerning the viability and appropriate execution of the protocol such as accrual, endpoint evaluability, and frequency of tests with equivocal results, invalid results, or no results. Issues with specimen collection will be summarized. The independent statistician will also review and evaluate the rates of disease. Toxicity is not expected given the nature of the study.

The study cannot be stopped at the interim for reaching regulatory goals at this time to preserve error rates / coverage probability and ensure enough data for subgroup analyses. Since the trial cannot be stopped for attainment of the regulatory goal, no adjustment to confidence levels are necessary.

Monitoring design assumptions for appropriate study sizing

Infection rates will be evaluated to determine whether sample size adjustments are warranted. The sample size will not be adjusted based on the observed PPAs and NPAs, which will not be reviewed while the study is ongoing, but may be adjusted based only on the infection rates to ensure enough infected participants to estimate PPAs with desired precision. If infection is more prevalent than expected, a smaller sample size may be accepted. If however infection is rarer than anticipated, then increases to sample size will be considered. The infection rate will be evaluated approximately every 500 participants or every 3 months after the enrollment of the first participant, whichever comes first.

6.6 Analyses

6.6.1 Primary Analyses

The result for each test will be compared with the ASIS for that anatomic site and organism. For each diagnostic test, PPA and NPA will be estimated using 95% Score confidence intervals. Confidence intervals will be estimated using the Score method.[34]

In the primary analysis, we will follow the FDA guidance document for incorporating indeterminate ASIS or test results for the test under consideration that are equivocal.[34] Indeterminate ASIS results will be counted against the results for the test under consideration, as described in Tables 4 and 5. Tables 4 and 5 will be presented in the analysis report, showing all combinations of Infected/Indeterminate/Not Infected with all outcomes from the test under consideration (Positive/Equivocal/Negative/No result or Invalid)

If the test under consideration has “no result” because the test was not run and no attempt was made to test the sample, it will be excluded from the primary analysis.

Table 4. Use of the ASIS in calculation of the positive and negative percent agreement for the Company 2 Assay (Company 2) and Company 3 Assay (CT only).

	ASIS		
	Infected	Indeterminate*	Not infected

Result of Test under Consideration**	Positive	A	D	G
	Equivocal	B	E	H
	Negative	C	F	I
	No result	Exclude from analysis		
	PPA	A / (A+B+C+F)		
	NPA			I / (G + H + I + D)

*The middle, indeterminate column will be included in the analyses as "Infected" or "Not infected" as described in the sensitivity analyses below.
**Per the ASIS definition in Section 2.3

Comprehensive sensitivity analyses will be conducted considering multiple scenarios. These include:

- Classify indeterminate tests on the basis of symptom status. Include indeterminate tests as infected if the participant is symptomatic in that compartment. Include indeterminate tests as not infected in the participant is asymptomatic in that compartment.
- Include all indeterminate tests as infected.
  - PPA=(A+D) / (A+B+C+D+E+F)
  - NPA=I / (G+H+I)
- Include all indeterminate tests as not infected.
  - PPA=A / (A+B+C)
  - NPA=(F+I) / (D+E+F+G+H+I)
- Considering indeterminate and equivocal test results as "missing", with the assumption of missing at random, and model the missing results.
- Additional sensitivity analyses, including the use of latent class models, will be explored. Details will be in the statistical analysis plan.

Table 5. Use of the ASIS in calculation of the positive and negative percent agreement for the Company 1 Assay (Company 1) and Company 3 Assay (NG only).

		ASIS		
		Infected	Indeterminate*	Not infected
Result of Test under Consideration**	Positive/Detected	A	C	E
	Negative/Not detected	B	D	F
	No result	Exclude from analysis		
	PPA	A / (A+B+D)		
	NPA			F / (C+E+F)

*The middle, indeterminate column will be included in the analyses as "Infected" or "Not infected" as described in the sensitivity analyses below.
**Per the ASIS definition in Section 2.3

If the test under consideration has "no result" because the test was not run and no attempt was made to test the sample or because of invalid ASIS results it will be excluded from the primary analysis.

Comprehensive sensitivity analyses will be conducted. These include:

- Classify indeterminate tests on the basis of symptom status. Include indeterminate tests as Infected if the participant is symptomatic in that compartment. Include indeterminate tests as not infected in the participant is asymptomatic in that compartment.

- Include all indeterminate tests as infected
  - PPA = (A+C) / (A+B+C+D)
  - NPA = F / (E+F)
- Include all indeterminate tests as not infected
  - PPA = A / (A+B)
  - NPA = (D+F) / (C+D+E+F)
- Additional sensitivity analyses that consider indeterminate results as "missing" and utilize imputation strategies, and graphical techniques
- Additional sensitivity analyses, including the use of latent class models, will be explored. Details will be in the statistical analysis plan.

There are pros and cons to the manner in which indeterminates are handled and the resulting calculations of PPA and NPA. The primary definition is the most conservative but is biased downwards. If PPA > 90% under this scenario, then the conclusion of PPA > 90% is clear. The second and third sensitivity analyses count indeterminates (treat all as infected or not infected, respectively) but may not be conservative for calculations of PPA and NPA.

6.6.2 Secondary Analyses

Global Analyses

For each diagnostic test:

- Positive and negative predictive values (PPV and NPV) will be estimated. PPV and NPV will each be plotted as a function of prevalence (point estimates and 95% pointwise confidence bands)
- Positive and negative likelihood ratios will be estimated using 95% Score confidence intervals
- A sensitivity analysis will include calculating equivocal predictive values and likelihood ratios
- An additional sensitivity analysis will calculate PPV, NPV, and positive and negative likelihood ratios for the sensitivity analyses where indeterminates are counted as Infected and Not Infected

Subgroup Analyses

For each of the three diagnostic tests, subgroup analyses will be conducted for males, females, symptomatic participants, and asymptomatic participants by pathogen and anatomic site. The 95% Score confidence interval estimates of PPA and NPA will be displayed using forest plots (one plot per test). The 95% Score confidence interval estimates of positive and negative likelihood ratios will be displayed using forest plots (one plot per test). PPVs and NPVs will each be plotted as a function of prevalence (point estimates and pointwise confidence bands).

The sensitivity analyses where indeterminates are counted as Infected and Not Infected will be repeated for the four subgroup analyses.

Exploratory Analysis

Developing methods for diagnostic benefit:risk analyses (e.g., diagnostic yield) will be applied.

7.0 Quality Control

7.1 Quality Controls and Study Monitoring

The ARLG will provide direct access to the dataset for the purposes of monitoring and auditing by the DCRI, Harvard School of Public Health and inspection by local and regulatory authorities. The local site PI will ensure that study personnel are appropriately trained and applicable documentations are maintained.

The DCRI will implement a Quality Plan to ensure that protocol training, data quality and data security are being undertaken.

7.2 Source Documents and Access to Source Data

Source documents will not be removed from the sites. Study monitoring will occur remotely, unless there are quality or performance concerns at a site, in which case an on-site visit will occur.

8.0 Ethics / Protection of Human Subjects

8.1 Ethical Standards

The investigator will ensure that the study will be conducted in accordance with all applicable national, regional, and local regulations.

8.2 Institutional Review Board and Informed Consent

This protocol and any subsequent modifications must be reviewed and approved by the IRB responsible for oversight of the study, as listed on its FWA. To minimize risks associated with loss of privacy and loss of confidentiality, oral informed consent will be obtained from the subject. The informed consent process will describe the purpose of the study, the procedures to be followed, and the risks and benefits of participation. A copy of a patient information form may be given to the subject (per local policy).

8.3 Subject Confidentiality

Subjects will have code numbers and will not be identified by name. Subject confidentiality is strictly held in trust by the participating investigators, their staff, and the Sponsor(s) and their agents.

The study protocol, documentation, data, and all other information generated will be held in strict confidence. No information concerning the study or the data will be released to any unauthorized third party without prior written approval of the ARLG.

All information provided by the ARLG and all data and information generated by the participating clinical site as part of the trial will be kept confidential by the site PI and other study personnel. This information and data will not be used by the site PI or other study personnel for any purpose other than conducting the trial. These restrictions do not apply to: (1) information which becomes publicly available through no fault of the site principal investigator or other study personnel; (2) information which is necessary to

disclose in confidence to an IRB solely for the evaluation of the trial (3) information which is necessary to disclose in order to provide appropriate medical care to a study subject; or (4) study results which may be published as described in Section 10.

The study monitor or other authorized representatives of the sponsor may inspect all documents and records required to be maintained by the PI. The clinical study site will permit access to such records.

All laboratory specimens, case report form data, reports and other records will be identified only by a coded number to maintain subject confidentiality.

8.4 Specimen Handling

Specimens will be collected for this study and will be maintained by the ARLG at least until all companies have received their FDA approval for their assay.

Use of samples for future research will be part of the consent process. There will **not** be any opt-in/opt-out language. Samples may be shared with other investigators at other institutions. No human genetic tests will be performed on samples. Each sample will be encoded (labeled) only with a barcode and a unique tracking number to protect subject's confidentiality.

There are no benefits to subjects in the collection, storage and subsequent research use of specimens. Because the subject enrollment will be done anonymously, reports about future research done with subject's samples will NOT be reported into their health records.

9.0 Data Handling and Record Keeping

9.1 Data Collection

This study will use eClinical OS (eCOS), a Web-based e-CRF database used by the DCRI. The investigator's site staff who will be entering data will receive training on the system, after which each person will be issued a unique user identification ID and password.

For security reasons, and in compliance with regulatory guidelines, it is imperative that only the persons who own the user IDs and passwords access the system using their own unique access codes. Access codes are nontransferable. Site personnel who have not undergone training may not use the system and will not be issued a user ID and password until appropriate training is completed.

9.2 Inspection of Records

The DCRI will implement a Quality Plan to, at a minimum, ensure that activities proposed by the PI to ensure protocol training, data quality and data security are being undertaken.

Source Documents and Access to Source Data

Source documents will not be removed from the sites.

9.3 Retention of Records

Records will be maintained during the investigation and for a period of 2 years after the latter of the following dates: The date on which the investigation is terminated or completed, or the date that the records are no longer required for purposes of supporting a premarket approval application or a notice of completion of a product development protocol. Records may not be destroyed without the prior approval of the ARLG.

9.4 Confidentiality

The study protocol, documentation, data, and all other information generated by this study will be maintained in a secure manner and will be kept confidential as required by law.

The EDC database (eClinical OS data) used by the DCRI will be hosted by Merge which uses a Peak 10 datacenter located in Morrisville NC. Database access will be limited to study personnel who are issued a unique user identification and password. Data will be entered at each site by study personnel. No information concerning the study or the data will be released to any third party without prior written approval of the Sponsor. Study records may be reviewed in order to meet federal or state regulations. Reviewers may include the IRBs, the DCRI and the NIH.

10.0 Publication Policy

Following completion of the study, the investigator may publish the results of this research in a scientific journal under the oversight of the Publication Committee of the ARLG.

The ARLG Publication Committee comprises representatives of the network cores, thought-leaders, statistics and data management center (SDMC), and is responsible for generation and coordination of the publications that report scientific findings of the network. All public presentations (abstracts, manuscripts, slides and text of oral or other presentations, and text of any transmission through any electronic media) by participating investigators, participating institutions, SDMC, and ARLG that use ARLG data and are intended to represent the ARLG or are supported by the ARLG will be reviewed by the Publication Committee per the Publication Committee charter and must include the following statement: “Research reported in this publication was supported by the National Institute Of Allergy And Infectious Diseases of the National Institutes of Health under Award Number UM1AI104681. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.”

The Publication Committee will ensure that the study results are presented by experts in the field that have working knowledge of the study design, implementation, data synthesis/analysis, and interpretation. The committee goals are to ensure that any confidential or proprietary information is protected, and that all appropriate statistical analyses have been included.

The ARLG Publication Committee will adhere to the trials registration policy adopted by the International Committee of Medical Journal Editors (ICMJE) member journals. This policy requires that all applicable clinical trials be registered in a public trials registry such as ClinicalTrials.gov, which is sponsored by the National Library of Medicine. Other biomedical journals are considering adopting similar policies.

In June 2007, the ICMJE adopted the WHO's definition of clinical trial: "any research study that prospectively assigns human participants or groups of humans to one or more health-related interventions to evaluate the effects on health outcomes."

- Health-related interventions include any intervention used to modify a biomedical or health-related outcome (for example, drugs, surgical procedures, devices, behavioral treatments, dietary interventions, and process-of-care changes).
- Health outcomes include any biomedical or health-related measures obtained in patients or participants, including pharmacokinetic measures and adverse events.

All investigators funded by the NIH must submit or have submitted for them to the National Library of Medicine's PubMed Central an electronic version of their final, peer-reviewed manuscripts upon acceptance for publication, to be made publicly available no later than 12 months after the official date of publication. The NIH Public Access Policy ensures the public has access to the published results of NIH-funded research. It requires investigators to submit final peer-reviewed journal manuscripts that arise from NIH funds to the digital archive PubMed Central upon acceptance for publication. Further, the policy stipulates that these papers must be accessible to the public on PubMed Central no later than 12 months after publication.

Refer to: http://publicaccess.nih.gov/

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APPENDIX A - Schedule of Events

	Enrollment
Clinical Evaluations	
Review of eligibility	X
Verbal consent	X
Baseline CRFs	X
Collection of Specimens	
Rectal swabs	X
Pharyngeal swabs	X