Chapter 28

Self-Collection of Specimens for Nucleic Acid-Based Diagnosis of Pharyngeal, Cervicovaginal, Urethral, and Rectal *Neisseria gonorrhoeae* and *Chlamydia trachomatis* Infections

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Abstract

Commercially available nucleic acid amplification tests (NAATs) for *Neisseria gonorrhoeae* and *Chlamydia trachomatis* detection allow for self-collection including home-based collection from multiple anatomic sites such as the urethra, cervicovagina, rectum, and pharynx. Verification studies need to be done prior to processing pharyngeal and rectal specimens. We review specimen collection and test characteristics of NAATs at different anatomical sites.

Key words: Self-collection, Gonorrhea, *Chlamydia*, Nucleic acid amplification test, NAAT

1. Introduction

Nucleic acid amplification tests (NAATs) for *Neisseria gonorrhoeae* (NG) and *Chlamydia trachomatis* (CT) detection have revolutionized screening for sexually transmitted infections. NAATs have greatly improved sensitivity over culture and allow testing from noninvasive specimens (e.g., urine), and multiple anatomic sites such as the urethra, cervicovagina, rectum, and pharynx. Testing from any of these anatomic sites allows for self-collection of specimens making non-clinic-based specimen collection, including home-based specimen collection, feasible. The use of other sample types obtained through other noninvasive means (oral wash (1), glans swab (2)) has been evaluated. Those specimens, however, are not yet recognized as reliable specimens for testing. Self-collected vaginal swab specimens are equivalent in sensitivity and reliability compared to traditional clinician-collected endocervical swab

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specimens and more sensitive and reliable than first-catch urine samples for the detection of CT and NG (3). The test characteristics (sensitivity, specificity, and accuracy) of NAATs performed with self-collected rectal swab specimens were similar or slightly better than those of NAATs performed with clinician-collected rectal swabs (2).

Currently in the USA, there are three widely used and studied commercially available NAATs which use proprietary methods of target capture, amplification, and signal identification: Amplicor CT/NG using polymerase chain reaction (PCR) (Roche Diagnostics Corp., Branchburg, NJ), Probe Tec using strand displacement amplification (SDA) (Becton Dickinson Co., Sparks, MD), and Aptima Combo 2 using transcription mediated amplification (Gen-Probe Inc., San Diego, California) (4–8). NAATs detect and amplify bacterial DNA or RNA sequences specific for the targeted organism with each NAAT using a slightly different target and method of amplification (Table 1). False positive results for NG can occur with the current Roche and Becton-Dickinson assays as their targets may cross-react with other *Neisseria* species such as *N. cinerea* and *N. subflava*. The Aptima assay uses a ribosomal RNA target with minimal cross-reactivity with genes from nongonococcal *Neisseria* species. Other benefits of the Aptima assay include a target capture step which eliminates false negatives due to amplification inhibitors, and the presence of thousands of copies of the ribosomal RNA target in each bacterium in contrast to far fewer copies of the DNA target in the other assays. Due to potential false positive results and the lower specificity of certain NAATs, the Centers for Disease Control and Prevention (CDC) recommends confirmatory testing of specimens positive for CT and NG by NAATs when the positive predictive value is <90 % (9). Large-scale studies, however, have found that confirmatory testing using NAATs is not needed for genital specimens (10–12).

The CDC recommends annual CT screening of all sexually active women aged ≤25 years (13). Focus groups have voiced the need to "normalize" CT testing, favoring home-based specimen collection (14, 15). Recent efforts have successfully linked Internet-based educational sexual health information and self-collection test kits for STDs with partner notification. The Internet educational Website www.iwantthekit.org has tested over 1,200 women in Maryland, Washington, DC, and West Virginia using free test kits for self-collected vaginal samples with mailing to a laboratory for CT and NG testing. Women using this site described high acceptability of the service and had a high overall CT prevalence of 9 % (16). A study comparing results of cervico-vaginal swabs tested with Gen-Probe PACE 2 assay for CT sent to a state laboratory by courier in a relatively controlled environment to swabs transported by the US mail in hot summer months in a southern state found 99 % agreement between courier and mailed specimen results (17).

Table 1
Commercially available nucleic acid amplification test characteristics and performance in rectal and pharyngeal specimens compared with culture for the diagnosis of *Neisseria gonorrhoeae* and *Chlamydia trachomatis* infections[a]

	PCR (COBAS Roche Amplicor)	SDA (BD ProbeTec)	TMA (Gen-Probe Aptima)	Culture
Nucleic acid amplified	For *Chlamydia trachomatis* (CT), multicopy cryptic CT plasmid and major outer membrane protein For *Neisseria gonorrhoeae* (NG), chromosomal primer based and 16S rRNA	For CT, multicopy plasmid region For NG, a region within the multicopy pilin gene inverting protein homologue	For CT, specific region of the 23S rRNA For NG, a specific region of the 16S rRNA via DNA intermediates	
Amplification method	Primer binds to and amplifies DNA gene sequence. Oligonucleotide probe binds to the DNA copies (amplicons) which are detected by spectrophotometer	Primer binds to DNA and displaces one strand prior to amplification with amplified gene sequences detected by fluorescent probe	Primer-bound rRNA target binds to a magnet prior to amplification allowing substrate inhibitors to be cleansed from the sample. Amplified target detected using two different light-producing labels	
Sensitivity and specificity (mean), NG rectum	Sensitivity 54 % Specificity 99 %	Sensitivity 78 % Specificity 100 %	Sensitivity 100 % Specificity 100 %	Sensitivity 53 % Specificity 100 %
Sensitivity and specificity (mean), NG pharynx	Sensitivity 66 % Specificity 89 %	Sensitivity 75 % Specificity 100 %	Sensitivity 95 % Specificity 100 %	Sensitivity 42 % Specificity 100 %
Sensitivity and specificity (mean), CT rectum	Sensitivity 91 % Specificity 96 %	Sensitivity 77 % Specificity 100 %	Sensitivity 100 % Specificity 100 %	Sensitivity 76 % Specificity 100 %

[a]*PCR* polymerase chain reaction, *SDA* strand displacement amplification, *TMA* transcription-mediated amplification. Unable to determine sensitivity of NAATs for pharyngeal CT due to low prevalence of infection in available studies (26, 27)

Based on the significant prevalence of CT and NG infections among men who have sex with men (MSM) and the often asymptomatic clinical presentation of those infections, the Centers for Disease Control and Prevention (CDC) guidelines also recommend screening sexually active MSM using NAATs from genital and extra-genital sites for NG and CT at annual or more frequent intervals as influenced by risk (13). Among over 6,000 MSM in San Francisco, 85 % of rectal infections were asymptomatic. In this same study, 53 % of CT infections and 64 % of NG infections were at non-urethral sites. These data support the need for routine screening of genital and extra-genital sites (the pharynx and rectum) in MSM (18). Increasing evidence suggests that the pharynx might be an important sanctuary site for the development of cephalosporin-resistant *N. gonorrhoeae*, emphasizing the importance of routine pharyngeal screening and treatment particularly in MSM (19). For MSM diagnosed with gonorrhea at any site, 10–25 % have only pharyngeal infection (20).

2. Materials and Methods

1. NAATs used for the diagnosis of NG and CT infections can be performed on noninvasively collected specimens: urine, self-collected vaginal swab, and self-collected rectal swab and pharyngeal swab (see Note 1). Self-collected pharyngeal and rectal swabs are reliable for CT and NG detection (21). The collection kit sent home with the patient will depend on the NAAT assay being used. Each NAAT will have specifications for storage time and conditions as well as acceptable transport media (Table 2). Due to the ability to store these specimens without freezing for days to months, and the fact that the Roche Amplicor and the GenProbe Aptima Combo2 can be automated, the specimens can be accepted around the clock and the assay performed on specific days. This saves both time and expense as the microbiologist is using one set of internal and external controls to test numerous specimens.

   Patients should be instructed on how to perform the swab collection. Vaginal swabs should be inserted 3 to 5 cm and once inserted rotated for 30 s. The swab is then placed into a tube and capped. Rectal swabs should be inserted 3 to 5 cm into the anus and rotated for 5–10 s. If needed prior to insertion, the swab can be moistened with water or saline solution. Self-collected pharyngeal swabs should make contact with five key anatomic landmarks: bilateral tonsils, bilateral posterior walls, and the uvula. Users should wash their hands between specimen collection if collecting a specimen from more than one anatomic site. Patient instructions for self-collection of

Table 2
Specifications for collection and transport of specimens for nucleic acid amplification testing

	PCR (COBAS Roche Amplicor)	SDA (BD ProbeTec)	TMA (Gen-Probe Aptima)
Sample collection	Dacron-, rayon-, or calcium alginate-tipped swabs with plastic or non-aluminum	*Chlamydia trachomatis/ Neisseria gonorrhoeae* amplified DNA assay endocervical specimen collection kit	Unisex swab specimen collection kit
Recommended maximum storage time prior to testing, room temperature[a]	1 h	4–6 days	60 days
Recommended maximum storage time prior to testing, 2–8 °C	7 days	30 days	60 days
Recommended maximum storage time prior to testing, < –20 °C	30 days	N/A	90 days
Transport media	2SP culture transport media, Bartels Chlamtrans, SPG, and M4 culture transport media	Included in kit	Included in kit

[a]Specimen must be processed for assay or frozen at conclusion of transport/storage time

pharyngeal, rectal, and vaginal swabs using the Gen-Probe Aptima kit are presented in Figs. 1–3 (22).

2. Verification studies need to be done prior to processing pharyngeal and rectal specimens. The currently available commercial NAATs are not currently FDA cleared for marketing for use on extra-genital specimens. To adhere to the US Clinical Laboratory Improvement Amendments (CLIA) regulations, clinical laboratories need to perform a verification study prior to reporting NG and CT test results from pharyngeal and rectal specimens. Verification is a one-time procedure, completed before extra-genital NAAT testing is offered and conducted on clinical specimens. The goal of a verification study is to evaluate a test system to determine whether the claims outlined in the manufacturer's package insert as they relate to product, process, results, or interpretation can be reproduced. A document published by the American Society of Microbiology (ASM) (www.asm.org), Cumitech 31- Verification and

Pharyngeal Swab Collection Instructions

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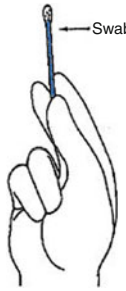
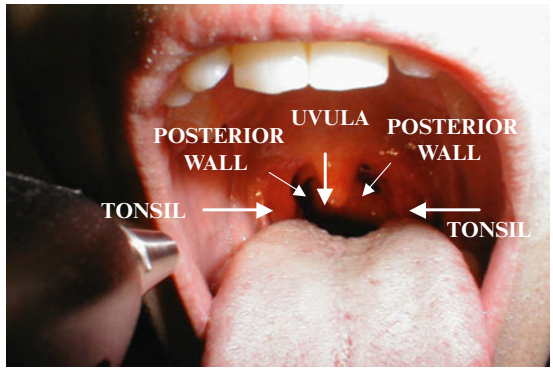


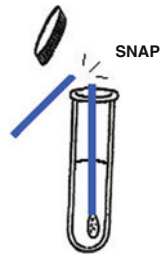
Step 1.

Open kit and remove tube and package with green writing. Remove the swab with the **BLUE** shaft. **USE BLUE SHAFT SWAB ONLY.**

Step 2.

Instruct patient to open mouth widely. Be sure to make good contact with 5 key areas of the throat (See below).





Step 3.

Remove cap from test tube. Place swab in test tube. Do not puncture the foil cap.

Break swab shaft at the score mark.

Step 4.

Put cap back tightly on test tube to prevent any leaking. Try not to splash the liquid out the tube.

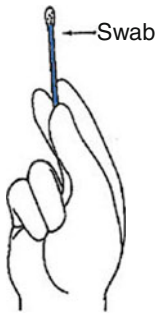
Step 5.

Discard wrapper and unused swab. **Wash your hands.**

Fig. 1. Pharyngeal swab collection instructions using the GEN-PROBE APTIMA kit for gonorrhea and chlamydia testing.

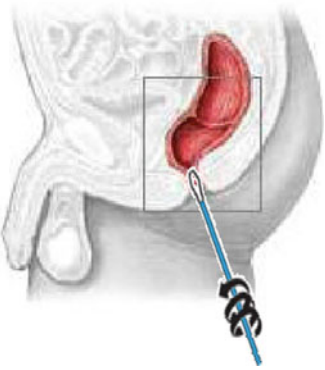
Rectal Self-Swab Collection Instructions

City Clinic
A landmark in prevention



Step 1.

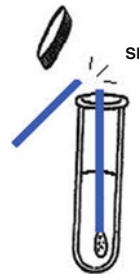
Open kit and remove tube and package with green writing. Remove the swab with the **BLUE** shaft. **USE BLUE SHAFT SWAB ONLY.**



Step 2.

Insert swab 1 inch into the anus and turn for 5 – 10 seconds.

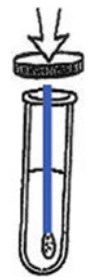
If needed, before inserting swab, wet swab with water or saline solution.



Step 3.

Remove cap from test tube. Place swab in test tube. Do not puncture the foil cap.

Break swab shaft at the score mark.



Step 4.

Put cap back tightly on test tube to prevent any leaking. Try not to splash the liquid out the tube.

Step 5.

Discard wrapper and unused swab. **Wash your hands.** Return the tube to the health worker.

Fig. 2. Rectal self-swab collection instructions using the GEN-PROBE APTIMA kit for gonorrhea and chlamydia testing.

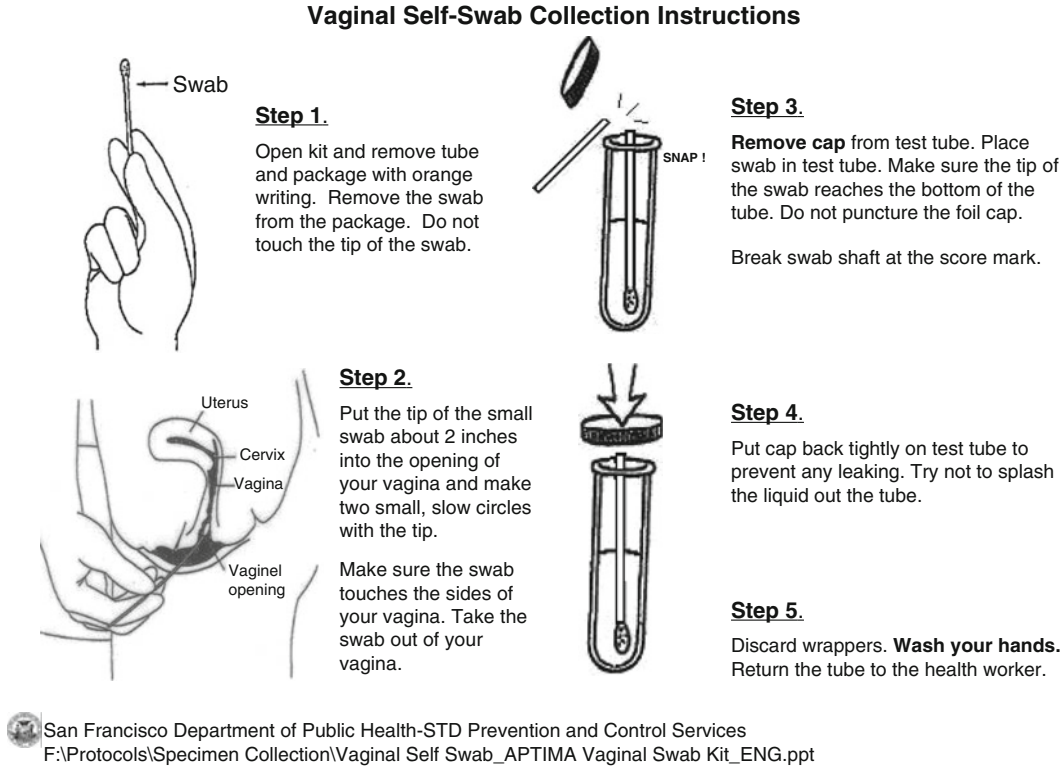


Fig. 3. Vaginal self-swab collection instructions using the GEN-PROBE APTIMA kit for gonorrhea and chlamydia testing.

Validation of Procedures in the Clinical Microbiology Laboratory, provides general guidance. Verification studies to use NAATs for CT and NG diagnosis in rectal and pharyngeal specimens have employed a reference standard that includes a positive culture result, a second confirmatory commercially available NAAT, or a research NAAT using a different primer target (23). An alternative approach is to work with a reference laboratory that has previously verified an NAAT test for extragenital samples. The reference laboratory can (1) verify an adequate number of test samples and (2) provide a panel of samples previously tested and characterized by the reference lab to be tested at the lab undergoing verification. The minimum number of samples recommended for a verification of an FDA-cleared indication by the ASM is 20 positive and 50 negative samples and by the National Committee for Clinical Laboratory Standards is 50 positive and 100 negative (24). Given that the confidence interval of the sensitivity estimate will be broader with fewer positive specimens tested, use of fewer than ten specimens is not recommended. A typical verification protocol uses a reference panel of at least 50 positive and 50 negative specimens, obtained from a laboratory that has successfully completed verification. The positive, negative, and control samples are run on a CLIA-approved NAAT

system in the verifying laboratory. Specimens are tested by at least two microbiologists and by a single microbiologist on different days to show consistent results among different operators and from day to day. The goal is to obtain sensitivity and specificity estimates similar to that demonstrated in the reference laboratory that provided the samples (see Note 2).

3. Amplification inhibitors in NAATs using PCR and SDA can produce false negative results. Urine specimens are more commonly associated with inhibitors compared to male urethral or female endocervical/vaginal specimens. Assay inhibitors in urine include hemoglobin, glucose, nitrites, beta human chorionic gonadotropin, and crystals. Inhibitors can be detected in NAATs using PCR and strand displacement by means of an internal control (IC). The Roche Amplicor CT/NG Amplification kit includes an IC that permits the identification of processed specimens containing substances that may interfere with PCR amplification. The IC is a noninfectious recombinant plasmid containing primer regions identical to those of the CT target sequence, a randomized internal sequence of similar composition as the CT and NG target sequences, and a unique probe binding region that differentiates it from the target amplicon. The IC is introduced into each individual amplification reaction to be co-amplified with the target DNA from the clinical specimen.

   There are several options for detecting inhibition using the BD ProbeTec CT/NG amplified DNA assay. The positive controls supplied in the ProbeTec ET CT/NG amplified DNA assay can be used as ICs or a separate amplification control is available which can be used to detect inhibition. The users may develop their own ICs as described by CLSI C24-A3. These controls would be added to a separate aliquot of the suspect specimen and run in tandem.

   The Aptima Combo2 assay has a novel target capture step where the primer-bound nucleic acid target binds to a magnet prior to amplification, allowing inhibitors to be washed from the sample. This minimizes the effect of inhibitors on the specimen. In spiking studies conducted by Chong and colleagues, the false negative rate for specimens run using the Aptima assay was 0.48 % compared to 13 % for specimen tests run using ligase chain reaction. Repeat testing after overnight storage reduced the false negative rate to 0 % for Aptima and 5.4 % for ligase chain reaction tests. (24) It further helps to decrease the number of false negatives due to amplification inhibitors if the specimen is free of mucus and the patient has not recently used lubricant as some lubricants have been shown to inhibit PCR. Dilution, heat treatment, or freeze thawing of samples have been found to reduce amplification inhibition (25).

4. Care must be taken to avoid sample contamination as this can lead to false positive results. If it is necessary to pipette individual specimens, the microbiologist must be cautious not to cross contaminate samples through inadvertently touching more than one specimen with the same pipette tip or to allow any specimen to drip from the pipette tip once processing is finished. The microbiologist needs to be aware of the state of the specimen container. Improper seals and a buildup of pressure inside the specimen container can lead to aerosolizing of the specimen and potential contamination as well as a biohazard for the microbiologist.

   Due to the molecular nature of the NAATs, it is very important to maintain a unidirectional work flow. The optimal arrangement is to maintain dedicated rooms for the pre-amplification and post-amplification stages. However, in an established laboratory this may not be possible and in this situation at least separate areas should be established for the pre-amplification and post-amplification procedures that are physically separated but with the ability to maintain a unidirectional flow. The processing should be designed to start with reagent preparation and move through product amplification without physically crossing back into a section of the lab used in an earlier part of the process. At each step, the work area must be thoroughly cleaned with at the very least a 1:1 bleach and water mix to avoid contamination from the previous batch of specimens. Maintaining work flow discipline is crucial as extremely large numbers of amplicons are created from a relatively small amount of nucleic acid. A very small number of amplified target accidentally crossing back into the pre-amplification area can cause false positives through several batch runs. Gloves should be changed after cleaning the area, pipetting specimens, mixing reagents, preparing the controls, and between the pre-amplification steps and post-amplification steps.

3. Notes

1. The preferred anatomic specimen for NG/CT testing for urogenital infection is urine in males and self-collected vaginal swab in women. NAATs are FDA cleared for use with male urethral, female endocervical, and urine specimens. The APTIMA assay is FDA cleared for use with self-collected vaginal swab specimens.
2. An indispensable part of any quality program is the use of controls. Each of the NAAT systems mentioned in this chapter is FDA approved and includes controls for continuous quality

maintenance. However, it is important to remember that the manufacturer's performance claims for these assays need to be verified in each laboratory environment before any system is used to test patient samples. Verification requires conformation of the test performance characteristics including sensitivity and specificity. Sensitivity is a measure of the test's ability to accurately detect patients with a specific disease. Mathematically, this is expressed as the number of true positive results divided by the number of true positive results plus false negative results multiplied by 100. Specificity is the measure of a test's ability to accurately identify all noninfected patients. Mathematically, this is expressed as the number of true negative results divided by the number of true negative results plus false positive results multiplied by 100. The clinical laboratory director is responsible for verification studies. The exact procedures for each verification study will vary depending on the lab. Consultation with local CLIA authorities is imperative. The general principle is to test a number of known specimens to assure that the results, that is the specificity and sensitivity, at least match the results published in the manufacturer's package insert. This can be accomplished using specimens spiked with a specific amount of organism procured from an institution such as the American Type Culture Collection (ATCC) and specimens that are not spiked. Once this basic level of accuracy is accomplished, then a larger number of known patient specimens—that is positive specimens that have not been manipulated and known negative specimens—need to be tested. It is from these results that the legitimate performance verification can be ascertained by the laboratory director or the decision made to do further testing with a larger number of samples. Documentation of the verification study must be maintained by the clinical laboratory director; it may be requested by CLIA inspectors.

In general, testing should be targeted to those at the highest risk. This general recommendation prevents excessive false positive results, which, regardless of the test, are always more likely in low-prevalence populations. The positive predictive value (PPV) is defined as the proportion of subjects with a positive test result who are correctly diagnosed.

$$\text{PPV} = \frac{\text{number of true positives}}{\text{number of true positives} + \text{number of false positives}}$$

For example, the Aptima Combo 2 assay package insert states that the test for *N. gonorrhoeae* has a sensitivity of 99.2 % and a specificity of 98.7 % when cervical swab specimens are used and a sensitivity of 91.3 % and a specificity of 99.3 % when urine specimens obtained from women are used. This would translate to a

PPV for the test in a female population with a 1 % prevalence of *N. gonorrhoeae* of 43 % and 57 % for cervical and urine specimens, respectively. The PPV increases to 87 % for NG in a hypothetical population with an NG prevalence of 5 %.

Despite the concern of a lower PPV using NAATs in low-prevalence populations, a study which tested 59,664 unduplicated cervical or urine specimens from women of which 280 (0.47 %) tested positive for NG directly estimated PPV for the APTIMA assay in this very-low-prevalence population. This group performed confirmatory tests on all specimens that yielded positive results and found a PPV of 97 %, far superior to that reported in APTIMA's package insert (12).

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