Laboratory Procedure Manual

Analyte: **Human Papillomavirus**

Matrix: **Oral Rinse**

Method: **Roche HPV DNA PCR Amplification/ Roche HPV Linear Array**

Revised:

As performed by: *The Ohio State University*
Innovation Center
2001 Polaris Parkway
Columbus, Ohio 43240

Contact: *Dr. Maura Gillison*

Important Information for Users

Ohio State University refines these laboratory methods. It is the responsibility of the user to contact the person listed on the title page of each write-up before using the analytical method to find out whether any changes have been made and what revisions, if any, have been incorporated.

Public Release Data Set Information

This document details the Lab Protocol for testing the items listed in the following table:

Data File Name	Variable Name	SAS Label
ORHPV_I And OHPV_I_R	ORXGH	HPV High Globulin Band result
	ORXGL	HPV Low Globulin Band result
	ORXH06	HPV Type 6
	ORXH11	HPV Type 11
	ORXH16	HPV Type 16
	ORXH18	HPV Type 18
	ORXH26	HPV Type 26
	ORXH31	HPV Type 31
	ORXH33	HPV Type 33
	ORXH35	HPV Type 35
	ORXH39	HPV Type 39
	ORXH40	HPV Type 40
	ORXH42	HPV Type 42
	ORXH45	HPV Type 45
	ORXH51	HPV Type 51
	ORXH52	HPV Type 52
	ORXH53	HPV Type 53
	ORXH54	HPV Type 54
	ORXH55	HPV Type 55
	ORXH56	HPV Type 56
	ORXH58	HPV Type 58
	ORXH59	HPV Type 59
	ORXH61	HPV Type 61
	ORXH62	HPV Type 62
	ORXH64	HPV Type 64
	ORXH66	HPV Type 66
	ORXH67	HPV Type 67
	ORXH68	HPV Type 68
	ORXH69	HPV Type 69
	ORXH70	HPV Type 70
	ORXH71	HPV Type 71
	ORXH72	HPV Type 72
	ORXH73	HPV Type 73
	ORXH81	HPV Type 81
	ORXH82	HPV Type 82
	ORXH83	HPV Type 83

Data File Name	Variable Name	SAS Label
	ORXH84	HPV Type 84
	ORXHPC	HPV CP 6108
	ORXHPI	HPV Type IS39
	ORXHPV	Oral HPV Result

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

The general outline of the NHANES project involves isolation of DNA from human SCOPE oral rinse samples (ORS) and analysis of DNA for 37 types of HPV via Roche Linear Array HPV Genotyping Test and Roche Linear Array Detection Kit.

Oral HPV infection is newly appreciated as a risk factor for a distinct type of oropharyngeal squamous cell carcinoma that is rising in incidence in the United States. It has been estimated that oral HPV16 infection confers an approximate 15-fold increase in risk for oropharyngeal cancer. Despite these strong risks, little is known about the epidemiology of oral HPV infection. In this study we will estimate the prevalence and determinants of oral HPV infection in a representative sample of the United States population.

The clinical implications of an oral HPV infection in terms of predictive value for subsequent development or oral cancer are unknown. This analysis is to be performed in the context of research studies only and is not available as a clinical diagnostic test at this time.

2. SAFETY PRECAUTIONS

All NHANES Samples are treated as Biohazardous Material and standard safety precautions for the handling of human body fluids in a BSL2 laboratory are taken by all personnel. Appropriate personal protection equipment (PPE) is worn at all times during sample handling. PPE includes but is not limited to: laboratory coat, gloves and hand sleeves. All laboratory work with samples is conducted in a certified biosafety cabinet.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

All results are stored in the NHANES Database excel spreadsheet. This database contains sample ID, vessel number, receipt date, location of Sample at different stages of processing (back-up SCOPE rinse, DNA location), Nanodrop values (DNA concentration, 260:280 ratios), HPV detection results, date of test procedures, etc. The database is updated weekly in order to reflect real time results. Access to the Database is limited to Gillison laboratory personnel only.

4. SPECIMEN COLLECTION, STORAGE, AND HANDLING PROCEDURES; CRITERIA FOR SPECIMEN REJECTION

Specimen collection is performed by the NHANES staff in the mobile units and samples are shipped weekly to the Oral HPV Detection Laboratory (OHD), Polaris Innovation Center, Polaris, Ohio. Specimen collection is accomplished with a 30 second oral rinse and gargle with SCOPE^{TM} mouthwash. NHANES subjects, age 14 - 69 years will alternate a series of three, five second rinses and five second gargles with 10 mL of SCOPE^{TM}. The Scope is then expectorated into a sterile collection tube, transferred to a 14-mL Falcon snap cap tube and refrigerated at 4^{o}C until shipping to the OHD Laboratory. Collected oral rinse specimens from survey participants (SPs) are assigned a 9 digit identification number referred to as the Sample ID.

Collected oral rinse specimens from SP's are shipped on ice per biological specimen collection shipping standards to the OHD laboratory on a weekly basis via Fed Ex. Each Fed Ex shipment also includes a hard copy shipping manifest which is kept for records. Laboratory personnel receive email verification from NHANES with a corresponding electronic "MEC Send" file and the FedEx tracking number. The "MEC Send" Excel spreadsheet contains information on samples included in each shipment, e.g. Sample ID, Analyte Type, Slot Number, Sample Collection date, etc. Refer to NHANES Contract and CDC Laboratory Manual for detail.

Upon arrival at the OHD laboratory, samples are stored in a designated 4^{o}C refrigerator until further processing within one week of receipt. Samples are treated as biohazardous material and standard safety precautions for the handling of human body fluids are taken by laboratory personnel (i.e. laboratory coat, gloves).

Any shipment(s) that arrives with evidence of severe damage or leak, which results in a complete loss of sample, will be discarded and the NHANES central office will be notified. All samples are processed regardless of sample volume but a note is recorded for samples containing ≤ 8ml of SCOPE.

5. PROCEDURES FOR MICROSCOPIC EXAMINATIONS; CRITERIA FOR REJECTION OF INADEQUATELY PREPARED SLIDES

Not applicable for this procedure.

6. EQUIPMENT AND INSTRUMENTATION, MATERIALS, REAGENT PREPARATION, CALIBRATORS (STANDARDS), AND CONTROLS

All NHANES Oral Rinse Samples undergo a series of seven sequential test procedures before final reporting of HPV testing results:

1. Processing of Oral Rinse samples from Scope mouthwash into PBS
2. Digestion via Qiagen Cell Lysis Buffer, RNAse and Proteinase K
3. DNA Isolation via QIASymphony SP Automation
4. DNA concentration determination via the NanoDrop Instrument
5. Roche HPV DNA PCR Amplification
6. Validation of PCR Amplification via 1% Agarose Gel Electrophoresis (control wells only)
7. Roche HPV Linear Array (line blot) for HPV detection.

A. Reagents/Supplies

Below is a general list of reagents/supplies used for each test procedure.

1. <u>Protocol: Reagents and Supplies</u>

a. **Processing of Oral Rinse samples from Scope mouthwash into PBS**: K562 cells (Quality Control = QC), 1X PBS, 2mL screw cap tubes
b. **Digestion via Qiagen Cell Lysis Buffer, RNAse and Proteinase K**: Qiagen Cell Lysis Buffer, RNAse, Proteinase K, HP DNA (QC)
c. **DNA Isolation via QIAsymphony SP Automation**: QIAGEN Virus/Bacteria

Midi Kit, AVE Buffer, 1X PBS, QIAGEN QIAsymphony SP instrument and consumables, Buffer ATL

d. **Obtaining DNA concentration via the NanoDrop Instrument**: Nanodrop 8000, AVE buffer, ddH2O
e. **Roche HPV DNA PCR Amplification**: Roche Linear Array HPV Genotyping Kit, DEPC treated water, SeraCare HPV DNA controls (QC), Applied Biosystems 9700 PCR machine.
f. **Validation of PCR Amplification via 1% Agarose Gel Electrophoresis**: Agarose, SB buffer, Ethidium Bromide, Loading Dye, 1Kb DNA Ladder, electrophoresis equipment, Alpha Innotech Alpha Imager (Gel imager)
g. **Roche HPV Linear Array**: Roche Linear Array Detection Kit, RBS35 Detergent, ddH2O

2. Labeling Requirements

The expiration date, date reagent is opened and date reagent was received must be clearly labeled on all reagents.

The Lot number and expiration date of all reagents used in each experimental protocol must be documented on the SOP for that experiment. Refer to NHANES SOPs and NHANES Results binders for detailed documentation.

3. Storage Requirements

All reagents used for each SOP must be stored at the appropriate storage condition(s) as stated by reagent manufacturer until use. A brief summary is provided below:

<u>General Reagent List and Storage Conditions:</u>

1) 1X PBS: Room Temperature
2) QC: Human Placental DNA: -20 ^{o}C
3) QC: SeraCare HPV DNA controls: -20 ^{o}C
4) QC: K562 cell aliquot (in PBS): -20 ^{o}C
5) Proteinase K: -20 ^{o}C
6) RNAse: -20 ^{o}C
7) Qiagen Cell Lysis Buffer: Room Temperature
8) DEPC treated Water: Room Temperature
9) Qiagen Virus/Bacteria Midi Kit: Room Temperature
10) ATL Buffer: Room Temperature
11) AVE Buffer: Room Temperature
12) Roche Linear Array HPV Genotyping Kit: 4 ^{o}C
13) Loading Dye: 4 ^{o}C
14) 1Kb DNA Ladder: 4 ^{o}C
15) SB Buffer: Room Temperature
16) Ethidium Bromide: Room Temperature
17) Agarose: Room Temperature
18) Roche Linear Array Detection Kit: 4 ^{o}C

19) RBS 35 Detergent: Room Temperature

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

A. Calibration Procedures/Daily Records

All equipment used in standard operating procedures will be properly calibrated every 6 months or annually (depending on type of equipment) in order to ensure proper working order. This includes all thermometers, single and multichannel pipettes, repeater pipette, Applied Biosystems 9700 PCR machine, Nanodrop 8000, QIAsymphony SP, Biosafety Cabinet, Mettler-Toledo Scale, OHAUS Harvard Trip Balance, centrifuge, shaking water bath(s) and Orbital Shaker Rotor Speed. Daily temperature readings will be taken when laboratory is in use for all refrigerators and freezers. When the biosafety hood is in use, the down-flow velocity and intake velocity will also be recorded and checked for proper working function. All digital and non-digital thermometers used for temperature readings will be calibrated yearly against a NIST certified thermometer to ensure proper temperature records.

B. Schedules for Performing Calibrations

Calibrations will either be conducted on a yearly or bi-annual schedule. It is the responsibility of lab personnel(s) to be knowledgeable of upcoming calibration deadlines and to adhere to the calibration schedule(s) assigned to each lab equipment. Contact information of calibration technicians for select equipment is provided in the Maintenance Binder.

1. Yearly Calibrations

Nanodrop 8000
Applied Biosystems 9700 PCR machine.
QIAsymphony SP (only if preventative maintenance is needed)
Digital and Non-Digital Thermometers against NIST certified thermometer.
Biosafety Cabinets
Rotor Speed on Eppendorf Centrifuge, Bellco Hot Shaker(s), Orbital Shaker
Mettler-Toledo Scale
OHAUS Harvard Trip Balance

2. Bi-annual Calibrations:

All single and multi-channel pipettes.
Repeat liquid pipette dispenser

3. Step-by-Step Instructions for performing calibration

All calibrations listed above (with the exception of the annual calibration of digital/nondigital thermometers against NIST and Mettler Toledo Scale calibration) are conducted by technicians certified by the manufacturer. Detailed protocols for thermometer calibrations and Mettler-Toledo Scale are provided in: **Protocol**: Digital Thermometer Calibration against NIST Thermometer, **Protocol**: Annual Calibration of Non-Digital Thermometers against NIST Thermometer (in

Maintenance Binder) and **Protocol:** Calibration of Mettler-Toledo Scale respectively.

4. Maintenance Procedure for Qiasympony SP instrument

A weekly preventative maintenance schedule is established for the Qiasymphony SP instrument. Detailed instructions for the QIAsymphony SP maintenance can be found in the QIAGEN QIAsymphony SP Manual. Record of the weekly maintenance which includes: O-Ring Test, UV-scan and wiping down of conveyor base tray, liquid waste container, waist tip guard, tip disposal chute, sample racks and reagent cartridge are kept in the Maintenance Binder.

5. Software Programming

Any up-to-date programming software for QIAsymphony SP and NanoDrop ND-8000 V2.0.0 programs will be provided by Qiagen/Barnstead technicians during annual calibrations, respectively. After a Software upgrade(s) has been conducted on the QIAsymphony SP, a test run will be conducted using PBS blanks and Human Placental DNA controls with the same Assay Control Program (Complex 800) used in the NHANES study. Following QIAsymphony run, DNA concentration will be measured for the samples in order to ensure proper working order/DNA isolation from QIAsymphony SP. For greater detail, refer to **Protocol: QIAsymphony Test Run with HP-DNA and PBS**. Protocol can be found in the NHANES Maintenance Binder.

6. Documentation Methods and Storage of Calibration Data

Records of all daily temperature readings, yearly and biannual calibrations, software upgrades and additional equipment calibrations will be kept in a designated Gillison Maintenance Binder. Results of any test runs conducted on QIAsymphony SP following software upgrades will also be kept in the NHANES Maintenance binder.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

Step-by-step procedures for conducting every step of the NHANES experimental procedures are highlighted in the NHANES SOPs and are attached as part of Appendix A at end of this document. As mentioned earlier, all NHANES Oral Rinse Samples undergo a series of seven sequential test procedures before final reporting:

1. Processing of Oral Rinse samples from Scope mouthwash into PBS
2. Digestion via Qiagen Cell Lysis Buffer, RNAse and Proteinase K
3. DNA Isolation via QIAsymphony SP Automation
4. DNA concentration determination via the NanoDrop Instrument
5. Roche HPV DNA PCR Amplification
6. Validation of PCR Amplification via 1% Agarose Gel Electrophoresis (control wells only)
7. Roche HPV Linear Array (line blot) for HPV detection

A. Interpretation of Results

HPV detection results (Line blot strips) are interpreted by two trained lab technicians on two separate days to ensure consistency in HPV detection results. If interpretations of the HPV detection results read by the two technicians do not match, Dr. Maura Gillison (lead PI) makes final interpretation of the conflicting results. Roche PCR/Line Blot may be repeated for a sample at the discretion of the PI in order to ensure correct result. Study PI reads the line blot strips for **ALL** samples and QC prior to reporting.

9. REPORTABLE RANGE OF RESULTS

The reportable range is a negative or positive result.

All NHANES samples are considered reportable if the sample contains the low and high B-globin bands (Roche Kit internal control bands). Presence of the B-globin bands indicate adequate sample amount necessary for HPV detection with the Roche kits. Samples that are not B-globin positive are still reported but are designated a certain number code which indicates the deficiency in the sample detection.

10. QUALITY CONTROL (QC) PROCEDURES

A. Quality Control Materials to be used and expected results:

Quality Controls (QC) have been introduced at critical points in the NHANES SOPs in order to ensure valid protocol procedures. Details of each QC are highlighted in each NHANES SOP.

The main QCs are:

1. NHANES LOG SHEET: Completed upon arrival of Sample(s) to Laboratory

   a. Verification that Samples shipped to NHANES are the same samples shipped to the OHD Laboratory (Hard copy Manifest shipped with samples matches the "MEC Send" Excel spreadsheet
   b. Record of SCOPE Oral Rinse Sample volume(s) less than 8mL under the Comments column of Delivery Receipt Log Sheet.

2. SAMPLE PROCESSING INTO PBS:

   a. Record of small pellets observed during processing of oral rinse samples from Scope mouthwash into PBS under the Comments column corresponding to Sample ID.
   b. A 5-mL aliquot of K562 cells (in PBS), which are HPV negative leukemia cell line, are included at every sample processing session. The K562 serve as a control for cross-contamination at the processing phase and allow us to distinguish whether cross-contamination occurred at initial phase of sample processing or during sample collection at NHANES mobile unit.

   Expected HPV detection result: K562 are HPV (-) but B-globin positive similar to HP-DNA and thus serve as an additional form of negative control when analyzing the Roche PCR/Linear Array results in the NHANES SOP.

3. DIGESTION/QIAsymphony SP DNA ISOLATION and NANODROP READINGS:

a. Verification that the Samples are placed into the QIAsymphony in the proper order as stated in the QIAsymphony/Nanodrop Excel spreadsheet.

b. Human Placental DNA serves as a quality control for many of the NHANES SOPs

1) General control for cross-contamination as HP DNA does not contain HPV DNA

2) Control for documenting the efficiency of DNA isolation from QIAsymphony SP as determined by % yield. A set amount of HP DNA is digested with every QIAsymphony run. The output DNA eluted provides indication of DNA isolation efficiency.

3) **Expected HPV detection Results**: Human Placenta DNA is a Negative Control for HPV-DNA in Linear Array Protocol as it is positive for high and low B-globin (housekeeping gene) bands but HPV DNA negative.

c. The PBS “blanks” placed in QIAsymphony SP run serve as an internal test of cross-contamination. Nanodrop readings should be negative for DNA in these blanks.

d. 260/280 ratios are measured in order to determine the quality of DNA isolated. Samples are not excluded from further processing if aberrant ratios are observed, however if downstream results indicate a lack of B-globin bands, the ratios provide qualitative analysis of the isolated DNA. The target 260/280 ratio is 1.8 and the Nanodrop 8000 detection range is between 2-3700ng/ul of dsDNA. DNA yields near either end of this range begin to show aberrant 260/280 ratios.

4. ROCHE HPV PCR AMPLIFICATION and GEL ELECTROPHORESIS:

a. The Roche HPV Genotyping PCR kit includes KIT POS and KIT NEG controls supplied by the manufacturer. The KIT controls provide an indication of reagent integrity. Gel electrophoresis analysis of PCR reactions after amplification is conducted in order to verify presence of intended bands (KIT POS) or lack of bands (KIT NEG) as described in the SOPs

b. With every new LOT of Roche HPV Genotyping Test kit, HPV-16 DNA and Human Endogenous retrovirus-3 (ERV3) serial dilution standards will be run in order to measure the sensitivity and integrity of the kits. The PCR reaction will be followed by a linear array using the Roche HPV Detection kit. See standard protocols for details on HPV16 and ERV3 RT-PCR assays. Line blot reagents with ability to detect 16 or fewer copies of HPV16 are considered validated. Refer to **Protocol: Quality Control Test for HPV Detection Kits Using Standard HPV-16/ERV-3 DNA** for greater detail.

c. Plasmids containing either HPV-11, -16, -18 or -31 DNA serve as Positive Controls and are obtained from SeraCare (Milford, MA) and demonstrate the kit’s ability to amplify the DNA of multiple HPV types.

d. H2O blanks placed at Lane F of the PCR template serves as an additional Negative control and as a measure of cross-contamination during PCR setup.

e. As a measure of the reproducibility of results, 5% of samples from a previous run will be randomly picked and retested in both the Roche PCR and line blot steps.

5. ROCHE LINEAR ARRAY (line blot)

a. Detection of High and Low B-globin bands in NHANES samples is an indication of adequate Sample DNA.
b. As mentioned earlier, HP-DNA serves as a B-globin positive, HPV-Negative control.
c. KIT POS serves as a B-globin positive, HPV-16 DNA positive control.
d. KIT NEG and H2O only lanes serves as a B-globin negative, HPV-DNA negative controls and as indicators of any possible cross-contamination.
e. SeraCare HPV (-11, -16, -18, -31) DNA serve as B-globin positive, HPV type specific positive controls.
f. As mentioned above, K562 cells used during the processing phase of the NHANES SOP serve as B-globin positive and HPV(-) control.
g. As a measure of the reproducibility of results, 5% of samples from a previous run will be randomly picked and retested (PCR and line-blot).
h. As with the PCR reaction, new Roche detection kits will be tested with HPV-16/ERV3 standard serial dilutions in order to test the sensitivity and integrity of the reagents.

B. Quality Control Preparation Instructions

1. Human Placental-DNA is prepared as stated in **Protocol: Preparation of Human Placental DNA** (100ng/ul) and stored at -20ºC until use.
2. Type-specific (-11, 16, 18, 31) HPV DNAs were obtained from SeraCare (Milford, MA). Plasmid DNA is isolated using a phenol-chloroform extraction protocol which is documented in the **NHANES. Maintenance Binder**.
3. KIT POS/NEG controls are provided in the Roche Linear Array HPV Genotyping Test kits. They are stored at 4ºC until use.
4. HPV-16 plasmid DNA and Salmon Sperm DNA used for serial dilution standards are stored at -20ºC until use.
5. K562 cells are grown in IMDM with 10% FBS and 1x P/S as suggested by ATCC protocol. Detailed information on K562 propagation is available in **Protocol: K562 cells Production** of the NHANES SOP. Aliquots contain 1 x 106 cells in 5mL of PBS and are stored at -20 until date of sample processing (1 aliquot/ sample processing session)

C. Frequency

Quality controls are included in analysis of all samples per run. The HPV-16/ERV-3 standard serial dilution test will be conducted with each new LOT of Roche kits.

D. Criteria for Accepting or Rejecting QC results and test date:

The criteria for accepting/rejecting QCs are highlighted on the NHANES SOPs. In general, if a QC does not give the expected result, that step of the NHANES SOP will be repeated. Greater detail is also provided in the Troubleshooting tab of NHANES SOPs

E. Recording Results/Documentation

The results of QCs are recorded on the individual NHANES SOP Reports. In addition, a summary QC sheet is placed at the beginning of the Results Reporting page. The ERV3/HPV-16 Standard Test results are kept in NHANES Maintenance

Binder.

F. Alternatives to Commercial Controls: None

G. Storage of Quality Controls:

Human Placental DNA, K562 and SeraCare DNA controls are kept at -20°C until use. Roche KIT POS and KIT NEG controls are kept at 4°C until use. DEPC treated water and 1X PBS are kept at room temperature until use. Storage conditions are maintained as recommended by manufacturers.

H. Calculation of % yield for HP-DNA Quality Control:

The percent yield (%) is calculated as a control for the efficiency of DNA isolation from the QIASymphony SP.

1. Step-wise Instructions

% Yield = [Actual DNA yield (ug) / Theoretical DNA yield (ug)] * 100

Actual DNA yield (ug) = X $\frac{\text{[HP-DNA Nanodrop value (ng/ul)] * 60 ul (Elution Volume)}}{\text{1000 ng/1ug}}$

Theoretical DNA yield: ≈ 10ug DNA
[20 ug of HP-DNA Digested on Day 1. However only about ½ of the total digestion volume is taken up by QIAsymphony SP. Thus THEORETICAL DNA yield is about 10ug of HP-DNA.]

Example

Nanodrop reading for HP-DNA sample from QIASymphony SP = 110 ng/ul
Actual DNA yield = (110 ng/ul * 60ul)/ 1000ng/ug = 6.60 ug total HP-DNA
Theoretical yield = 10ug HP-DNA
% yield = (actual DNA yield/theoretical yield) * 100….Thus (6.60ug/10ug)*100 = **66%**

11. REMEDIAL ACTION IF CALIBRATION OR QC SYSTEMS FAIL TO MEET ACCEPTABLE CRITERIA

Since quality controls are established at every step of the NHANES SOP, the step at which the QC fails helps us determine where a possible fault began. Typically, the step at which the QC fails is initially repeated to ensure that the QC failure was not a technique error. If repeat does not fix the issue, then the step previous to the QC failure step is repeated. For example, if one of the SeraCare control plasmids fails to produce a band during HPV detection (line blot), then the Roche PCR step is repeated.

The same is true for an NHANES test sample. If HPV detection (line blot) yields the sample to be B-globin negative (inadequate), then the PCR step is repeated for that sample. If the repeat PCR and line blot detection again yields a B-globin negative result, then the Nanodrop readings measured after DNA isolation step (QIAsympony SP) are looked at to

see whether there is insufficient DNA. If DNA levels are low, then the lack of B-globin is most likely attributed to low DNA yield and the Back-up sample is tested. If DNA levels seem high, then there could be PCR inhibitors in the DNA eluate that could be inhibiting the PCR reaction. The DNA is diluted and the PCR/line blot is repeated. If this also does not work then the back-up sample is tested. **Ultimately, for all samples, if the back-up sample does not work, then the sample is labeled as un-evaluable**.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

A. Adequate sample collection (high cell number) is important for ensuring comparable DNA yield. Given that the Roche HPV detection assay is a PCR based assay, PCR inhibitors present in the sample DNA may inhibit the reaction. However, the DNA isolation steps include treatment with Proteinase K and RNAse in order to eliminate possible PCR inhibitors. The Roche linear array is known to have variable sensitivity by HPV type. Additionally, the presence of multiple type infections can affect type-specific amplification. Border line positive samples scored at-1 are at the lower limit of sensitivity of the assay, and therefore are expected to have poor agreement on repeat testing. Interpretation of line blot results as + or – can be subjective due to the colorimetric style of the detection assay. To account for this, test interpretation is performed by two technicians and reviewed by joint consensus with PI prior to reporting. Furthermore, every new lot of HPV detection kit is tested using serial dilutions of DNA standard in order to guarantee an accepted lower limit of sensitivity.

B. **Mix Positive Samples**
The Roche HPV Genotyping and Detection kits cannot differentially test for HPV 52 infection. Instead, a Mix Positive Band is present which could account for infections from HPV52 33, 35, and/or 58. In order to test for the presence of HPV52, all NHANES samples that are shown to be Mix Positive are tested via Real-Time PCR for HPV52 DNA. If real-time PCR shows the presence of HPV52 DNA in sample, then the sample is reported as HPV52 positive, if sample is HPV52 negative then the Mix Positive band is attributed to infection with another HPV type(s) which is accounted for on the line blot strip. Greater Detail Protocol is provided in the **NHANES HPV52 protocol**.

13. REFERENCE RANGES (NORMAL VALUES)

A normal value for HPV oral rinse would be a negative result.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable to this procedure.

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Once received by the contractor, the specimens can be stored at -20° C after sample processing and before testing takes place. Store the 2nd aliquot at -80°C for long term storage.

16. ALTERNATIVE METHODS FOR PERFORMING TEST OR STORING SPECIMENS IF TEST SYSTEM FAILS

No alternative HPV detection system is used for this study. General Storage conditions if

test systems fail are initial storage at 4ºC to prevent unnecessary freeze-thaws. If the system failure is going to be a long-term issue, then the following storage conditions should be used:

- Sample in SCOPE = 4ºC. **NOTE**: Do NOT freeze. ORS should never be in SCOPE for longer than 7 days. ALL samples should undergo processing with PBS after 7 days.

-Back-up Sample(s) in PBS = -80 ºC freezer

-Sample DNA = -20 ºC freezer

17. TEST RESULT REPORTING SYSTEM; PROTOCOL FOR REPORTING CRITICAL CALLS (IF APPLICABLE)

HPV detection results for all samples that meet the QC standards will be reported to the NHANES. For samples that do not meet QC criteria, reasons and comments will be noted as indicated in the **NHANES Contract and CDC Laboratory Manual**. The study PI reviews all QC standards and samples prior to reporting results. All Samples tested (NHANES samples, KIT controls, and QCs) are batched into a Run # and are summarized initially in 2 Excel spreadsheets. The excel spreadsheets are a summary spreadsheet and a reporting spreadsheet for each Run. These initial results spreadsheets are used as a reference during the final submission of results into the NHANES database and FTP Reporting site. Results are summarized by vial in a reporting spreadsheet that provides values for fields listed in the NHANES Laboratory Information Sheet. Each reporting spreadsheet's name includes the vessel number and upload date, and is submitted by secure password-protected upload to the FTP website.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR SPECIMEN ACCOUNTABILITY AND TRACKING

Each FedEx shipment of samples will include a hard-copy manifest. The Gillison lab also receives an email with a corresponding electronic "MEC Send" file and the FedEx tracking number. Upon receipt of samples, the technician logs the samples in by verifying that the sample ID matches the hard copy manifest and MEC send file. The Technician also notes any SCOPE volume differences (<8mL) and notes if any samples are broken and cracked. If samples do not match to the MEC Send and hard copy file or if any leaks or damage occurred to samples during shipment, then NHANES is immediately contacted to resolve the issue.

19. Summary Statistics and QC graphs

There are no summary statistics or QC graphs for this type of testing. These assays are PCR assays with a positive, negative or not evaluable result.

References:

For greater detail, refer to NHANES Research Proposal, NHANES Contract and CDC Laboratory Manual, NHANES SOPs (Appendix A below), QIASymphony SP Handbook, QIAGEN Virus/Bacteria Handbook and Roche Linear Array HPV Genotyping Test Manual. For a detailed protocol of the annual thermometer calibrations refer to the Calibration SOP section of the Maintenance Binder.