Laboratory Procedure Manual

Analyte: **Human Papillomavirus**

Matrix: **Oral Rinse**

Method: **PCR**

Revised:

as performed by: *Ohio State University*
Columbus Ohio

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.

HPV in oral rinse
NHANES 2011-2012

Public Release Data Set Information

This document details the Lab Protocol for NHANES data.

A tabular list of the released analytes follows:

HPVOR_G	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
	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
   DNA concentration determination via the NanoDrop Instrument
4. Roche HPV DNA PCR Amplification
5. Validation of PCR Amplification via 1% Agarose Gel Electrophoresis (control wells only)
6. 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:

General Reagent List and Storage Conditions:

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 -20^{o}C 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 4^{o}C until use.
4. HPV-16 plasmid DNA and Salmon Sperm DNA used for serial dilution standards are stored at -20^{o}C 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^{o}C until use. Roche KIT POS and KIT NEG controls are kept at 4^{o}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

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

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.
Eg. 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 4°C 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 = 4°C **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 °C freezer

-Sample DNA = -20 °C 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.

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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 un 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.

APPENDIX A

Human Papillomavirus – Oral Rinse Samples
Standard Operating Procedure (SOP)

A. Sample Preparation

1. Keep the NHANES sample in the 14-mL snap top Falcon tube in which it was delivered.

2. Centrifuge (Eppendorf 5810R) sample at 800 x g/2000 rpm and 4°C for 10 minutes.

3. Decant the supernatant into a designate waste container, leaving the pellet in the tube.

4. Add 8 mL of 1X PBS and resuspend the pellet by pipetting the buffer up and down several times to break up the pellet.

5. Centrifuge sample at 800 x g/2000 rpm and 4°C for 10 minutes.

6. Decant the supernatant into a designate biohazard waste container leaving the pellet* in the tube.

***NOTE:** Visually inspect the pellet size and note when pellet is small and difficult to see.

7. Add 3mL of PBS and dislodge the pellet while pipetting up and down with the buffer.

8. Split sample into two aliquots of 1.5 mL each into two new-labeled 2 mL Sarstedt cryo-tubes.

9. Discard the empty 14-mL Falcon snap top tube into a designated Biohazard Waste Container.

10. Store first aliquot of processed samples from step 8 in a pre-labeled microtube freezer storage box at -20ºC (Rm. 1505, short term) until ready to proceed to **Digestion via Qiagen Cell Lysis Buffer, RNAse and Proteinase K.**

11. Store the second aliquot at -80°C (Rm. #1520) for long-term storage.

Refer to NHANES Database for detail on sample storage location.

Reagent(s)	Brand	Lot #	Expiration Date
1X PBS	Gibco (Cat # 10010-023)		
K562	ATCC	9/10/2009	2012

B. Digestion via Qiagen Cell Lysis Buffer, RNAse and Proteinase K

1. Thaw processed samples and HP-DNA either at room temperature or 4ºC.

2. To make stock HP-DNA (100ng/µL), refer to **Preparation of Human Placental DNA.** The Lot # is the same number as the date it was prepared.

3. FOR HP-DNA skip to step 4. For all other samples, centrifuge (Eppendorf 5810R) samples at 960 x g/3000 rpm for 10 minutes at 4 ± 2°C.

4. Carefully, remove the supernatant by pipetting into a designated waste container, leaving behind the pellet and any residual supernatant.

5. Add 1.4 mL of Cell Lysis Solution to each sample; vortex for 5 seconds on medium speed.

6. Incubate sample(s) at room temperature for 15 minutes.

7. Add 15 µL RNase (final concentration: 5 µg/mL) to each sample; vortex briefly.

8. Incubate sample in the heat block (Barnstead/Thermolyne Type 16500 Dri-Bath) at 37.0 ± 2°C for 30 minutes.

9. Add 37.5 µL of Proteinase K** (final concentration: 500 µg/mL); vortex briefly.

10. To make the 20 mg/mL stock Proteinase K solution, refer to **Preparation of Stock Proteinase K Solution (20mg/mL).** Lot # is the same number as the date it was prepared.

11. Incubate sample in heat block (Barnstead/Thermolyne Type 16500 Dri-Bath) at 55.0 ± 2°C **overnight** (15-18 hours).

12. The next day: Denature Proteinase K by transferring samples to 95.0 ± 2°C heat block (Barnstead/ Thermolyne 16500 Dri-Bath) for 10 minutes.

13. Briefly quick spin down the digested samples in order to remove any liquid condensation from the sample caps and proceed **DIRECTLY** to **DNA Isolation via QIAsymphony SP Automation.**

Reagent(s)	Brand
Cell Lysis Solution	Qiagen (Cat. No 158908)
RNase – DNase free (from bovine pancreas)	Roche (#11-119-915-001); Stock: 500 ug/mL
Proteinase K	Sigma (#P6556-100MG): Stock: 20 mg/mL
HP-DNA	Sigma (# D7011-25MG) Stock: 100 ng/µL

C. DNA Isolation via QIAsymphony SP Automation

1. QIAsymphony Initialization:

(1) Close all drawers and hood.

(2) Push the blue button on lower left side of QIAsymphony SP to begin initialization. Wait until the “Sample Preparation” screen appears.

2. Inventory Scans:

(1) Open the “Eluate” drawer and perform the following actions:

(2) Scan the bar code of the elution slot (usually slot 2) that you want to use with a handheld bar code scanner connected to QIAsymphony via the USB jack

(3) Press the “Yes” button to the question “Do you want to add a rack on slot #”

(4) Select the type of elution rack: Select **Corning MTP 96 round bottom** from the list displayed in the touch screen for the chosen elution slot.

(5) Place the elution rack into the chosen elution slot.

(6) Press the “Add” button.

(7) Close the “Eluate” drawer and press “OK” to start the inventory scan of the “Eluate” drawer. A “clicking” noise indicates that the scan has finished and the “Stop Scan” button is no longer displayed.

(8) Press the “Close” button to move onto the Waste Drawer.

3. Open the “Waste” Drawer and perform the following actions:

(1) Make sure drawer is loaded with empty unit boxes and an empty liquid waste bottle located below the tip park station.

(2) Make sure there is an empty tip dispenser bag in holder below the tip disposal chute.

(3) Close the “Waste” drawer and start an inventory scan of the Waste drawer. A “clicking” noise indicates that the scan has finished and the “Stop Scan” button is no longer displayed.

4. Open the “Reagents and Consumables” drawer and load it as shown below:

Load 200ul and 1500ul filter tips, Sample Prep Cartridges and 8-Rod Covers.

(1) Load the ATL buffer* by:

a. Press “R+C” in the touch screen to open consumables status screen.

b. Press “Scan Bottle” to scan the barcode of the Buffer ATL with the handheld bar code scanner.

c. Press “OK” and place the opened bottle in B1 location

* Make sure ATL Buffer contains no protein precipitation. If necessary, dissolve by heating at 70.0 ± 2°C water bath.

(2) Load **QIAsymphony Virus/Bacteria Midi Kit** reagent cartridge(s). Each new reagent cartridge can process 48 samples Refer to Virus/Bacteria Midi Kit Handbook.

NOTE: All reagent cartridges placed into QIAsymphony SP should be fitted with a piercing lid, enzyme rack and magnetic beads. **ALL** caps and lids should be removed prior to loading into machine

NOTE: Make sure the magnetic particles are fully resuspended. Remove the magnetic-particle trough from the reagent cartridge frame and vortex it vigorously for at least 3 minutes. After vortexing, replace it in the reagent cartridge and make sure to remove the cover prior to run.

(3) When Reagents and Consumables drawer is fully loaded, close the drawer and start a full inventory scan. A “clicking” noise indicates that the scan has finished and the “Stop Scan” button will no longer be displayed.

NOTE: Samples can be loaded into the QIAsymphony SP while an inventory scan of the “Reagents and Consumables” drawer is occurring.

5. Sample Loading

Load Samples and 1X PBS **according to the QIAsymphony/Nanodrop Excel Spreadsheet** onto a tube rack (24 samples per rack)

(1) Unscrew the first set of 24 tubes (includes 1X PBS controls) in a biosafety cabinet. Discard caps into a Biohazard Waste Container.

(2) Place the tubes onto the tube rack in proper order according to QIAsymphony/ Nanodrop Excel Spreadsheet

(3) Open the “Sample” drawer. Place the rack onto the first slot line and wait until the green LED starts to flash.

(4) Slide the rack into the slot, steadily and continuously. After successful loading, the color of the LED changes to orange.

a. After loading the first rack, press the “Batch 1” button in the touch screen. The color of the button is now blue and the batch status is “Loaded.”

b. Assign “Batch 1” the correct tube type: **Sarstedt 2 mL tubes** and then press “Next”.

c. Assign “Batch 1” an Assay Control Set: Select **Complex 800 V3**

default IC. Then press “Next” button.

d. Assign “Batch 1” to an appropriate elution rack (same slot containing the Corning MTP-96 round bottom plate in “Eluate” drawer). Assign an **Elution Volume of 60 µL**. Press **“Queue”.** Batch 1 on the touch screen will now be green in color indicating proper loading.

e. Repeat steps **a-d** for the remaining samples. Load in sequential order (rack 2, 3 and/or 4) **according to QIAsymphony/ Nanodrop Excel Spreadsheet**. Assign all batches the same tube type, assay control set, elution rack and elution volume.

6. Load Internal Controls

In order to run the Pathogen 800 Complex program, you must load the appropriate amount of **AVE buffer** in lane A of Sample Drawer. For every 50 samples, load a total of 10 mL of AVE buffer split into 5 aliquots of Sarstedt 2 mL tubes.

(1) Load appropriate number of tubes containing AVE buffer into tube rack.

(2) Load tube rack into lane “A” of Sample Drawer.

(3) Press “IC” button on the touch screen. Assign the AVE tubes to the **Complex 800 V3 default IC program** and assign them as **IC tubes**. Press “OK” button. **No Elution slot is designated for the IC rack.**

7. Running Program

When all samples and IC tubes (AVE buffer) are loaded in the “Sample” Drawer, close the drawer and Press the “Run” button to start the purification. Run time is approximately 1.5 hrs for every batch.

NOTE: If the appropriate amount of AVE buffer and consumables & reagents are loaded, the QIAsymphony SP will begin the program. If an error is detected, then a message will appear notifying of the problem. Refer to troubleshooting guidelines highlighted in the QIAsymphony SP Manual and Virus/Bacteria Midi Kit Handbook for further assistance.

8. Post-Run Follow up

When run is complete, remove eluted DNA from “Eluate” drawer and measure DNA concentration. See **Obtaining DNA Concentration via the Nanodrop Instrument.**

Restock QIAsymphony SP:

(1) Discard all “used” reagents in the **Waste Drawer** and the tip disposal bag into a Biohazard Waste Container.

(2) Replace with empty unit boxes and new tip disposal bag. Decant the liquid waste bottle into a designated Waste Container.

(3) From the **Reagents and Consumables** drawer, discard empty tip holders into the Biohazard Waste Container. Restock all tips, sample-prep cartridges and 8-Rod Covers in designated areas for future QIAsymphony run.

(4) Re-cap any leftover ATL buffer for future runs and store at room temperature.

(5) If Virus/Bacteria Midi Kit reagent cartridge is empty, place caps over all reagents (including enzyme rack, magnetic beads, etc.) and discard into the Biohazard Waste Container.

NOTE: Partially used reagent cartridge(s) can be stored for a maximum of 2 weeks after date of initial use. Seal all reagents with provided caps and store at room temperature. The enzyme rack should be capped and placed back at 4^{o}C until later use.

NOTE: Record the date of use and total number of samples run on both the reagent cartridge, its corresponding enzyme rack and on the **QIAsymphony Virus/Bacteria Midi Kit Log Sheet**.

Unload Sample Drawer:

(1) Left over Digested Samples loaded in the “Sample” drawer should be capped with new screw-caps and stored in -80^{o}C.Discard Sarstedt tubes containing “used” AVE buffer and 1X PBS into a Biohazard Waste Container.

Turn **OFF** the machine by pushing the blue button on QIAsymphony SP.

Reagent	Brand
Virus/Bacteria Midi Kit(s)	Qiagen (Cat. No. 931055)
Buffer ATL	Qiagen (Cat. No. 939011)
1X PBS	Gibco (Cat. No. 10010-023)
AVE Buffer	Qiagen (Mat. No. 1049030)

D. Obtaining DNA concentration via the Nanodrop Instrument

NOTE: Perform in a PCR-FREE environment.

1. Click on the ND-8000 V2.0.0 icon to begin the program.

2. Choose the Nucleic Acid button for measuring DNA.

3. The prompt will ask you to initialize the instrument with water:

(1) Raise the lever on Nanodrop and wipe the top and bottom of the probes with a Kimwipe wet with water.

(2) Pipet 2 µL of ddH2O on the bottom part of the probes and shut the Nanodrop lever.

(3) Press OK and wait until the initialization step is complete

4. BLANK the instrument with AVE only buffer eluted from QIAsymphony SP.

(1) Clean the top and bottom of the probes with Kimwipe and ddH2O.

(2) Activate all 8-channels by clicking on: All Active On/Off. Indicator will turn Green.

(3) Pipet 2 µL of blanking buffer on the bottom part of the probes and shut the lever.

(4) Click on BLANK to begin program.

5. Now, you are ready to spec samples. For each row of samples:

(1) Pipette 2 µL of test sample onto bottom part of probes using multichannel pipette, shut the Nanodrop lever and click "Measure" DO NOT raise the lever until measurement is completed

(2) Repeat for remaining test samples

NOTE: Green indicator light designates which row you are measuring. Be sure to match the test samples with the indicator light.

*****Make certain to wipe the probe clean between samples and to change pipet tips with each sample.

6. Place caps over the 96 well plate containing eluted DNA and store at -20ºC (Rm. # 1505) until ready to proceed to **Roche HPV DNA PCR Amplification.** Refer to NHANES Database for DNA storage location.

7. Nanodrop readings are recorded and saved automatically by date of Nanodrop reading. To retrieve Data:

(1) Click on "My Computer".

(2) Click on "C-Drive".

(3) Click on "Nanodrop Data".

(4) Click on "Default"

(5) Click on "Nucleic Acid".

(6) Scroll over to the correct Nanodrop reading date and transfer data onto USB stick.

8. Record the DNA concentrations and 260/280 ratios in QIAsymphony/Nanodrop Excel Spreadsheet.

9. Calculations:

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

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

Theoretical DNA yield: **≈ 10ug DNA**

NOTE: 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%**

E. Roche HPV DNA PCR Amplification

NOTE: Create PCR Excel Spreadsheet prior to PCR run. Be sure to include appropriate samples, SeraCare HPV DNA controls, Kit Pos/Neg controls, H2O controls (Lane F).

Turn on the thermal cycler (Applied Biosystems GeneAmp PCR System 9700, Rm. # 1507) to warm up while preparing the amplification plate.

Take out Eluted DNA plate(s) and SeraCare DNA controls from -20ºC freezer (Rm. 1505) and thaw to room temperature.

1. REAGENT PREPARATION [Performed in a Biosafety Cabinet]

(1) Place MicroAmp Optical 96-well reaction plate (Applied Biosystems N801-0567) on a MicroAmp base.

(2) For every 12 samples, prepare Working Master Mix by adding 125 µL of HPV Mg2+ to one vial of HPV MMX. (You need 2 Vials of MMX for every 3 columns of samples). Recap the tube and mix well by inverting the tube 10-15 times. DO NOT vortex master mix!

(3) Add 50 µL of Working Master Mix into each reaction well using multichannel pipette and reagent reservoir.

(4) Add DEPC treated water to PCR plate: **FOLLOW THE PCR EXCEL SPREADSHEET**

a. Add 50 µL of DEPC treated water to H2O control wells (Lane F).

b. Add 38 µL of DEPC treated water into each sample well(s) using multichannel pipette.

c. Add 48 µL of DEPC treated water into SeraCare HPV-DNA control wells.

d. Do **NOT** add any DEPC treated water for the KIT Pos/Neg wells.

(5) Cover the plate with a sheet of parafilm.

(6) Add DNA to PCR plate: **FOLLOW THE PCR EXCEL SPREADSHEET**

a. Uncap the first column of Eluted DNA plate.

b. Slide the parafilm to expose the first column of wells on PCR plate.

c. Add 12 µL of each sample into its appropriate well. **DO NOT** add DNA to the H2O only control well (LANE F).

d. When finished allocating a column of samples, cap that column with MicroAmp 8-cap strips (Applied Biosystems Cat. # N801-0535).

e. Repeat steps above until all samples are in the plate. Place Eluted DNA plate back in appropriate storage location in -20°C.

f. Add 2ul of each SeraCare HPV-11, -16, -18, -31 DNA control into appropriate wells.

g. Add 50 µL of KIT Positive and KIT Negative controls into the appropriate wells containing Working Master Mix only.

(7) Cap all wells tightly, gently vortex and spin down plate. Then, transfer PCR plate to the Amplification/Detection Area.

2. AMPLIFICATION [Performed in Amplification/Detection Area]

(1) Place plate into the 96-well block of the Applied Biosystems GeneAmp PCR System 9700 machine and amplify under the following conditions:

HOLD Program: 2 min 50°C

HOLD Program: 9 min 95°C

CYCLE Program (40 Cycles): 30 sec 95°C, 1 min 55°C, 1 min 72°C

HOLD Program: 5 min 72°C

HOLD Program: 72°C Indefinitely

Program Name: Roche HPV-PCR

Press F1 [Run] on machine.

Under Methods: choose **hpv-pcr**, user: **xiao**. Reaction Volume should be set to **100 µL** and speed ramp should read **Max.**

Push F1 [Start] to start program. The program runs approximately 3 hours.

(2) When Run is complete, push STOP twice, remove the plate from the thermal cycler and place in the MicroAmp base.

(3) For samples **USED** for PCR Gel Validation:

a. Uncap the column(s) containing KIT POS/NEG, H2O control well and SeraCare DNAs. Use forceps to avoid creating aerosols of the amplification products. Discard the caps into a Biosafety Waste Container.

b. Remove 10ul of designated sample(s) into a Greneir U-shaped 96-well plate for gel analysis. Refer to **Protocol: Validation of PCR Amplification via 1% Agarose Gel Electrophoresis** for further detail.

c. Add 90ul of DN solution into appropriate wells USED for gel analysis using a multichannel pipettor, aerosol barrier tips and Reagent Reservoir. Mix by pipetting up and down five times.

d. Recaps the columns with new caps.

(4) For samples **NOT USED** for PCR Gel Validation:

a. Remove the cap from the reaction plate using forceps. Discard the cap into a Biosafety Waste Container

b. Pipette 100 µL of DN Solution to reaction wells. Mix by pipetting up and down five times.

c. Recap the wells with a new MicroAmp 8-cap strip.

d. Repeat **a-c** for remaining columns.

(5) Label the plate and store the denatured amplicon at 4 ± 2°C (Rm. # 1507) until ready to proceed to **Protocol: Roche LINEAR ARRAY Detection**.

NOTE: Denatured Amplicon can be stored at 4 ± 2°C for up to 7 days only.

(6) Discard used PCR kit reagents (MMX, KIT POS/KIT NEG, Mg2+) in biohazard waste container.

Reagent(s)	Brand
DEPC treated Water	Quality Biological(# 351-068-721)
HPV-Mg 2+	Roche (REF # 04472209 190): Linear Array HPV Genotyping Test
HPV MMX	Roche (REF # 04472209 190): Linear

	Array HPV Genotyping Test
HPV(+) Control	Roche (REF # 04472209 190): Linear Array HPV Genotyping Test
HPV(-) Control	Roche (REF # 04472209 190): Linear Array HPV Genotyping Test
DN Solution	Roche (REF # 04472209 190): Linear Array HPV Detection Kit

F. Validation of PCR Amplification via 1% Agarose Gel Electrophoresis

NOTE: This protocol uses Ethidium Bromide; use gloves and Kimwipes to handle stock solution.

1. Assemble gel apparatus by inserting smaller gel compartment into the center of the larger buffer compartment, making sure that the gel compartment's rubber sides face the buffer compartment's walls and fit together tightly.

2. Insert comb(s) into smaller gel compartment.

3. Make 1X SB Buffer with deionized water from 20X stock SB buffer.

4. Weigh out 1 gram of agarose powder.

5. Add 100mL of 1X SB Buffer into flask of agarose and swirl to mix.

6. Bring agarose mixture to boil in microwave. Approximate time is about 1:30 mins.

7. Add 15 µL of ethidium bromide into agarose mixture and swirl to mix.

8. Pour agarose mixture into the gel compartment and let solidify at room temperature (~20 mins).

9. When ready for use, turn smaller gel compartment 90 degrees so that the comb is near the black electrode.

10. Pour 1x SB Buffer into gel compartment, making sure to fill all the chambers and to completely cover the gel (~800 mL).

11. Remove comb(s) from gel.

12. Prepare samples for loading into gel using a Greneir U- shaped 96-well plate:

Samples: Mix 2 µL of 6x loading dye + 10uL sample

Ladder: 1kB ladder is already diluted in loading dye. Use 4 µL of stock solution for loading.

13. Load 4 µL of 1kB ladder into the far left well of gel and 12 µL of **prepared** samples from step 12 into remaining wells.

14. Place lid on gel apparatus making sure that red and black electrodes match up and run gel at 120 Volts for approximately 30 minutes.

15. Use Alpha Innotech Alpha Imager to view gel and take picture of gel for record.

16. Gel/SB Buffer Disposal:

(1) Dispose of 1% agarose gel in Biohazard Waste Container

(2) Dispose of "used" 1X SB buffer in designated plastic WASTE container.

17. Rinse gel apparatus and combs with deionized water and air dry for later use.

Reagents:

Agarose Fisher Scientific (BP160-100)
20X SB Faster Better Media Cat. No. SB20-1
Ethidium Bromide Solution (Stock: 10mg/mL)
Invitrogen Cat. No.15585-011
1KB DNA Ladder Fermentas Gene Ruler # SM0313
6X Loading Dye Fermentas #R0611

G. Roche LINEAR ARRAY Detection

NOTE: Prepare a **Line Blot Summary Spreadsheet** before beginning protocol.

ROCHE LINE BLOT [Performed in Amplification/Detection Area

1. Pre-warm water bath (Rm. # 1507) and shaking water baths to 53°C ± 2°C.

NOTE: Be sure that there is sufficient water in the shaking water baths to heat the 24-well tray, but not too much water such that it splashes into the 24-well tray. Water should cover no more than 1/4 of exterior well depth (approximately 0.5 cm) to prevent leaking into tray while shaking.

2. Warm all detection reagents to room temperature.

NOTE: Examine the 20X SSPE, the 20% SDS, and 20X CIT, if necessary, warm to 53°C ± 2°C water bath to re-dissolve any precipitated solids.

3. Prepare Working Hybridization Buffer and Working Ambient Wash Buffer.

4. Prepare Working **Stringent** Wash Buffer as follows:

For each strip being tested, remove 5 mL of Working Ambient Wash Buffer and add it to an appropriately labeled, clean media bottle (about 500 mL for 96 samples).

5. Warm the Working Hybridization Buffer and Working **Stringent** Wash Buffer in a pre-warmed 53°C ± 2°C water bath until use (minimum 15 minutes). **DO NOT** warm up the Working Ambient Wash Buffer.

6. Prepare Working Citrate Buffer (formulation attached)

7. With a Sharpie Extra-Fine Tip permanent marker, label each LINEAR ARRAY HPV Genotyping Strip(s) according to the location of the sample in the **PCR Excel spreadsheet.**

8. Place each strip with the probe lines facing forward into the appropriate well of the 24-well tray(s). Order the strips from A1-H1, A2-H2, A3-H4, A4-H4, etc.

9. Add 4 mL of **pre-warmed** Working Hybridization Buffer into each well that contains a labeled strip.

10. Using a multichannel pipettor and aerosol barrier tips, pipette 75 µL of denatured amplicon into the appropriate well containing its corresponding labeled HPV Genotyping strip. Use a new tip for each amplicon addition. Gently rock the tray to mix denatured amplicon with pre-warmed working hybridization buffer.

11. Cover the 24-well tray with the lid and place tray in a 53°C ± 2°C shaking water bath. Place a 1 lb. lead ring weight on the tray lid in order to hold the tray in place in the shaking water bath. **Hybridize for 30 minutes.**

12. During hybridization (step **11**) prepare Working Conjugate Solution (formulation attached)

13. Remove tray from the shaking water bath and remove the Working Hybridization Buffer from the wells by vacuum aspiration.

14. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Gently rock tray 3-4 times to rinse the strips and immediately vacuum aspirate the Working Ambient Wash Buffer from the wells.

15. Add 4 mL of **pre-warmed** Working Stringent Wash Buffer to each well containing a strip. Wipe off condensation from the tray lid with a clean Kimwipe, place the lid on the tray, and return tray to the 53°C ± 2°C shaking water bath. Place a 1 lb. lead ring weight on the tray lid and **incubate for 15 minutes.**

16. Remove tray from the shaking water bath and remove the Working Stringent Wash Buffer from the wells by vacuum aspiration.

17. Add 4 mL of Working Conjugate Solution to each well containing a strip. Wipe off condensation from the tray lid, place the lid on tray, and incubate **for 30 minutes** at room temperature on orbital shaker.

18. Remove tray from the orbital shaker and remove the Working Conjugate from the wells by vacuum aspiration.

19. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Gently rock tray 3-4 times to rinse the strips and immediately vacuum aspirate the Working Ambient Wash Buffer from the wells.

20. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Place the

lid on the tray and incubate for **10 minutes** at room temperature on orbital shaker.

21. Remove tray from the orbital shaker and remove the Working Ambient Wash Buffer from the wells by vacuum aspiration.

22. Add 4 mL of Working Ambient Wash Buffer to each well containing a strip. Place the lid on the tray and incubate for **10 minutes** at room temperature on orbital shaker.

23. Remove tray from the orbital shaker and remove the Working Ambient Wash Buffer from the wells by vacuum aspiration.

24. Add 4 mL of Working Citrate Buffer to each well containing a strip. Place the lid on the tray and incubate for **5 minutes** at room temperature on orbital shaker.

25. Prepare Working Substrate solution.

26. Remove tray from the orbital shaker and remove the Working Citrate Buffer from the wells by vacuum aspiration.

27. Add 4 mL of Working Substrate solution to each well containing a strip. Place the lid on the tray and incubate for approximately 5 minutes at room temperature on orbital shaker.

NOTE: Carefully watch the trays as to not over-develop. Stop developing when background color begins to appear blue.

28. Remove the 24-well tray from the orbital shaker and remove the Working Substrate from the wells by vacuum aspiration.

29. Add 4mL of deionized water to each well containing a strip.

30. Remove the strips from trays using clean forceps and place in order (A1-H1, A2-H2, etc.) on top of a clean, dry tray lid. Allow the strips to air-dry for a minimum of one hour or up to 72 hours at room temperature prior to interpretation.

24-WELL TRAY CLEANING

NOTE: This cleaning procedure must be followed after every use.

(1) Prepare a 2% solution of Roche RBS35 Detergent Concentrate; by adding 1 part RBS35 to 50 parts deionized water.

(2) Fill each tray well with the 2% solution and let soak overnight at room temperature.

(3) Thoroughly rinse the tray to remove detergent with deionized water.

(4) Dry tray completely before use; can also wipe with 70% Ethanol to remove any colored stains.