

Laboratory Procedure Manual

Analyte: **Human Papillomavirus (HPV) Genotypes**

Matrix: DNA Extracted from Self-Collected Vaginal Swabs

Method: Linear Array HPV Genotyping Assay
(Roche Diagnostics)

As performed by: Chronic Viral Diseases Branch
Division of High-Consequence Pathogens
and Pathology
National Center for Emerging and Zoonotic
Infectious Diseases

Contact: Elizabeth R. Unger, MD, PhD

Important Information for Users

The HPV Laboratory of the Chronic Viral Diseases Branch/CDC periodically 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 Names	Variable Name	SAS Label
<p>HPVSWR_I</p> <p>and</p> <p>HPVS_I_R</p>	LBDRPCR	Roche HPV linear array summary result
	LBDRHP	Roche LA high positive globin control
	LBDRLP	Roche LA low positive globin control
	LBDR06	HPV type 6
	LBDR11	HPV type 11
	LBDR16	HPV type 16
	LBDR18	HPV type 18
	LBDR26	HPV type 26
	LBDR31	HPV type 31
	LBDR33	HPV type 33
	LBDR35	HPV type 35
	LBDR39	HPV type 39
	LBDR40	HPV type 40
	LBDR42	HPV type 42
	LBDR45	HPV type 45
	LBDR51	HPV type 51
	LBDR52	HPV type 52
	LBDR53	HPV type 53
	LBDR54	HPV type 54
	LBDR55	HPV type 55
LBDR56	HPV type 56	
LBDR58	HPV type 58	

Data File Names	Variable Name	SAS Label
	LBDR59	HPV type 59
	LBDR61	HPV type 61
	LBDR62	HPV type 62
	LBDR64	HPV type 64
	LBDR66	HPV type 66
	LBDR67	HPV type 67
	LBDR68	HPV type 68
	LBDR69	HPV type 69
	LBDR70	HPV type 70
	LBDR71	HPV type 71
	LBDR72	HPV type 72
	LBDR73	HPV type 73
	LBDR81	HPV type 81
	LBDR82	HPV type 82
	LBDR83	HPV type 83
	LBDR84	HPV type 84
	LBDR89	HPV type 89
	LBDRPI	HPV type IS39

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

This protocol describes procedures for HPV genotyping on DNA extracted from self-collected vaginal swabs (see laboratory methods – Cobas HPV detection vaginal swabs).

HPV Genotyping is achieved with the Linear Array HPV Genotyping Test that is based on L1 consensus Polymerase Chain Reaction (PCR) amplification of target DNA with biotinylated PGMY primers followed by nucleic acid hybridization of amplicons for the detection of thirty seven anogenital HPV genotypes [6, 11, 16, 18, 26, 31, 33, 35, 39, 40, 42, 45, 51, XR (52), 53, 54, 55, 56, 58, 59, 61, 62, 64, 66, 67, 68, 69, 70, 71, 72, 73 (MM9), 81, 82 (MM4), 83 (MM7), 84 (MM8), IS39, and 89 (CP6108)]. Detection of probe-bound amplified products is by colorimetric determination. The β -globin gene is amplified and detected concurrently to assess cellular adequacy, extraction and amplification for each sample. Detection (hybridization to the genotyping strips) is performed with a Beeblot instrument, which automates the hybridization and wash steps. Because the XR (52) probe cross-reacts with HPV 33, 35, and 58, the presence of HPV 52 is confirmed by a quantitative type-specific assay in XR-positive samples that are also positive for one or more of the cross-reacting types.

This is a research test and results should not be used for clinical management. The results are used for population monitoring of HPV.

2. SAFETY PRECAUTIONS:

Appropriate PPE must be worn throughout all lab procedures. General precautions must be followed as outlined in the CCID Safety Manual (CDC). All specimen handling (pre-lysis) is to be performed in a biosafety cabinet. The reagent Substrate B from the Linear Array Detection Kit contains 40% (w/w) DMF. The chemical is harmful by inhalation and in contact with skin. It is also irritating to eyes and particular safety goggles must be worn. Follow procedure as outlined in the OI Safety Manual and Chemical Hygiene Manual.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Final HPV genotyping results and dates of HPV typing are recorded in a password-protected database on a secure server. Digital images of all HPV genotyping strips are archived. Additional observations and comments kit/reagent lot numbers and name of operator is recorded on a worksheet accompanying the entire procedure.

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

Specimen collection and processing are described in laboratory methods – Cobas HPV detection vaginal swabs. DNA extracts used for Linear Array HPV test are stored temporarily at -20°C. For long-term storage -80°C freezer is used.

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

N/A

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

6.1 Supplies for HPV Genotyping (Linear Array)

6.1.1 Equipment

- Vortex Tube Mixer
- Centrifuge (Eppendorf 5415D, capable of 15,700rcf) with swing-out bucket for PCR plates
- Thermocycler (GeneAmp PCR System 9700 with gold block, Perkin Elmer)
- Waterbath 280 Series (Precision Scientific)
- BeeBlot 48-3 (BeeRobotics Ltd., Gwynedd, U.K.)
- Pipettes, 12 channel and single channel: 20µl, 200µl and 1000 µl (Rainin Cat. #L12-200, L12-20, L-20XLS, L-200XLS, and L-1000XLS)
- PCR Cabinet
- Drummond Pipet-Aid or equivalent

6.1.2 Supplies, Other Materials

- Aerosol-barrier tips for volumes 20 µl, 200 µl, 1000 µl
- Kaydry towels (Kimberly-Clark, Cat.# 34721)
- Absorbent bench pads (Fisher Scientific, cat. #15235101)
- Solution basin for multichannel pipette (Heathrow Scientific, Cat. # HS20521C)
- PCR tube strips 0.2 ml (Applied Biosystems, Cat. # N0801-0534) or 96-well Optical reaction plate (Applied Biosystems, Cat. #4306737) with MicroAmp Clear Adhesive Film (Cat. #4306311)
- MicroAmp 96-Well Tray (Life Technologies, cat.# N8010541)
- Ziplock Bag 8 x 8 (CDC Labware, Cat. #94400)
- Serological Pipettes, 50 ml, 25 ml, 10 ml, 5 ml (Glassware, cat.#97720, 97865, 96075, and 90405)

- Graduated Cylinders (CDC Labware; 1000 ml Cat. #96900, 500 ml Cat. #94815, 250 ml Cat. #93810, 100 ml Cat. #94425, 50 ml Cat.#9475)
- Beakers (CDC Labware; 1000 ml 90165, 250 ml Cat. #96105)
- Labmarkers, waterproof and solvent resistant
- Lab coat with ribbed knit cuffs (Daigger, Cat. #EF1463)
- Gloves, latex or nitrile, powder-free (Glassware)

6.1.3 Reagents and Media

Storage at Room Temperature

- Nuclease-free water (Ambion, Cat. #AM9938)
- Virkon S Disinfectant and Virucide (Santa Cruz Animal Health, Cat. #sc-362041)
- Ethanol, 70%
- D.I. Water

Storage at 4°C

- Linear Array HPV Genotyping Test (Roche, Cat# 04472209)
- Linear Array Detection Kit (Roche, Cat# 03378012)

Storage at -20°C

- HPV16 plasmid DNA (containing 50 copies/10ul of HPV 16 DNA and 10⁵ copies of human placental DNA)

6.1.4 Quality Control

Nuclease-free water is used as negative control for the assay. The water should have no colorimetric signal during detection. Plasmid DNA consisting of 50 genome equivalents of HPV16 per 10 µl is utilized as positive control for the Linear Array HPV test. A colorimetric signal at the HPV16 probe on the genotyping strip should be visible after the test is completed.

6.2 Supplies for HPV 52 verification

6.2.1 Equipment

- LightCycler 480 Instrument
- Centrifuge with swing-out buckets for 96-well plates
- Pipettes for 20 and 200 µl ranges

6.2.2 Reagents and Media

Storage at Room Temperature

- Nuclease-free water (Ambion, Cat. #AM9938)

Storage at -20°C

- Lightcycler 480 Master Kit – Roche, Cat. #04707494001
- Purified HPV 52 PCR amplicon for standard curve (further described under 8.3)
- Purified globin amplicon for standard curve (further described under 8.3)
- Human genomic DNA, Roche Diagnostics, Cat. #11691112001
- PCR primers (CDC Biotechnology Core Facility) plus HPV and Globin probes (Integrated DNA Technologies, IDT)

Oligo	Sequence
HPV52 (forward)	GCTAACGCACGGCCATGT
HPV52 (reverse)	CCAGCACCTCACACAAKTCG
HPV52 probe	FAM – CCAGCAACAMGACCCGGACC – BHQ1
β-Globin (forward)	CAGGTACGGCTGTCATCACTTAGA
β-Globin (reverse)	CATGGTGTCTGTTTGAGGTTGCTA
β-Globin probe	HEX – TGCCCTGACTTTTATGCCAGCCCTG – BHQ1

(K = G or T, M = A or C)

6.2.3 Supplies, Other Materials

- LC 96-well plates and seals, clear, cat. #05102413001
- Pipette tips with aerosol barrier

6.2.4 Quality Control

All of the following criteria must be met for a valid assay:

No template (H₂O) controls must be negative for HPV52 and Globin.

The calibrators serve as positive control for each PCR. The unadjusted Cp value of the calibrators should not deviate more than 0.5 Cp from the original dilution that was used to create the standard curve. Deviation of more than 0.5 Cp will invalidate the assay and all results.

For HPV52 detection, a positive threshold of 5 copies of HPV52 DNA is required. If results indicate the presence of HPV52 DNA below 5 copies

should be tested again, but will only be accepted as positive if more than 5 copies are detected.

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

N/A

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

8.1 HPV Genotyping (Linear Array)

Process 48 or 96 samples in 96 well plates for the PCR and use multichannel pipettes. The strip tray in the BeeBlot is compatible with these standard devices.

- Prepare LA worksheet recording sample IDs and Lot numbers from all reagents that are processed.
- Switch on thermocycler (GeneAmp 9700), select “LA Assay” program. Sign name, date and time into Thermocycler log sheet.
- In the Reagent preparation area: Place vials of Linear Array HPV Master Mix (HPV MMX) and the same number of vials of Magnesium solution (HPV Mg²⁺) from two LA Genotyping kits into the hood.
- Label 0.2 ml PCR tubes or a 96 well plate for the reactions.
- Combine the entire content of each MMX with 125 µl HPV Mg²⁺ for each, invert 12 times and transfer mixed PCR reagent into a sterile solution basin using a pipette. With a 12 channel or single channel 200 µl pipette, dispense 50 µl working Master Mix to each of the tubes/wells in the PCR Plate. All 4 MMX and 4 Mg²⁺ vials will be needed for the 48 strips in one BeeBlot.
- Cover tubes/plate with a clean lid and transfer to the sample preparation area.
- In the sample preparation hood: Transfer 5 µl DNA template to the prepared PCR tubes/wells and add 45 µl nuclease free water so that the total volume will be 100 µl.
- Close tubes or seal the plate with MicroAmp adhesive film. Place into thermocycler and start program (approximately 3h and 15 min). If using

PCR tubes, set tubes into thermocycler block lined with a MicroAmp Tray. This prevents any damage to any PCR tube walls during high temperature cycles. If using the PCR plate with plastic seal, place an optical mat on top of the plate before placing the thermocycler lid on plate. This will prevent the seal from peeling during high temperature cycles and prevent any chances of well-to-well or instrument contamination.

Thermocycler Program “LA Assay”

Step	Temp., Time	Number of Cycles
Hold	50°C, 2 min	1
Hold	95°C, 9 min	1
Cycle (set ramp rate at 50%)	95°C, 30 sec 55°C, 1 min 72°C, 1 min	40
Hold	72°C, 5 min	1
Hold	72°C, ∞	1

- Remove the tray within 4 h during the final HOLD step, and immediately add 100 µl of denaturation solution to each tube/well.

Note 1: *The denatured amplicon can be held at RT for no more than 2 h before proceeding with the Detection. The eternal 72°C hold at the end of the thermal cycling program prohibits degradation of fresh PCR product by the AmpErase. Denatured amplicon can be held at 4°C for 7 days.*

**Automated genotype detection with the BeeBlot:
(Performed in Post Amplification – Amplification/Detection Area)**

- Warm all detection reagents to room temperature
- Preheat SDS and SSPE containers from the Linear Array detection kit to 53± 2°C. (use beadbath). This step should not exceed 30 min, unless the precipitate in the containers is not completely dissolved. Record lot numbers from all reagents on the LA worksheet.
- Label HPV genotyping strips and separate into single strips.

Note 2: *Remove any fabric strings underlying the strips. Avoid any contact with the colored hybridization area.*

- Prepare all buffers according to the table below for 1 Beeblot instrument. Follow order from top to bottom. Use designated graduate cylinders to measure water, Sub A, SSPE, Sub B. Use serological

pipettes for volumes less than 25 ml (SDS and Citrate), 1000 µl pipette for SA-HRP. Mix thoroughly by stirring with serological pipette for 10 sec. Mix reagents in designated glass beakers. Use D.I. water to prepare all working buffers.

	Hybridization	Wash B.	Citrate	Conjugate	Substrate
D.I. water	233 ml	1418 ml	285 ml	284 ml	
20X SSPE	60 ml	75 ml		15 ml	
20% SDS	7.5 ml	7.5 ml		1.5 ml	
20X Citrate			15 ml		
SA-HRP				0.9 ml	
Sub A					240 ml
Sub B					60 ml

Reagent volumes for 48 strips (1 BeeBlot)

- Pour prepared reagents into the appropriate bottles and insert the lids with the supply tubes from the BeeBlot instrument: Hybridization – white; Citrate – green; Substrate – blue; Conjugate - yellow. The Wash Buffer bottle needs to be filled all the way (top off while bottle is in the instrument). Fill up white D.I. water bottles about 2/3.

Note 3: Check to verify the D.I. water and Ambient wash tubing are not pinched before the run and hoses are attached properly. Ensure line priming prior to run.

- Add Genotyping strips into the tray. Lock tray onto the BeeBlot (The tray has been designated in such a way that it can be positioned in one direction only): Lift the temperature sensor with one hand and slide the tray under, release the sensor all the way into the slot in the tray and lock the tray with the 3 clamps in the front.
- Switch on the instrument. Choose assay from the menu on the instruments display (LA_V2_5-25-16 = 01). Press “Start”. The following table lists subsequent messages that are prompted on the display and the required action by the user:

Message	Action (button)
HPV Linear Array	“Start”
AssayEdtr. V2.17b	“Start”
Cleaning cycle A	“Start” (priming pumps, soaking 1 min)
Cleaning cycle A complete	“Start”
Cleaning cycle B	“Start”
Cleaning cycle B complete	“Start”
Reagent preheat 15	“Start” (after completion)
Select no. well	use “>” and “<” to adjust, “Start”

Position sensor	“Start”
Begin Assay	“Start”
Please close lid	“Start”
Priming pumps	-
Dispense hyb buffer	-
Add amplicon	Visually verify that colored probe lines are gone. Add 75ul of denatured amplicon into round opening at the front of the slots. (See Note 4). Press "Start" (See Notes 5 & 6).
Dispensing Wash Buffer/Conj./Cit./Sub.	-
Aspirate DI water	(see Note 6). Remove strips and transfer to blotting papers to air dry. Press "Start
Remove Sensor	“Start”
Cleaning cycle A	“Start”
Cycle A complete	“Start”
Cleaning cycle B	“Start”
Cycle B complete	“Start”

After the hybridization buffer is dispensed, “Add amplicon” will be displayed. Add 75 µl of denatured amplicon to the rounded opening at the front of the slots into the hybridization buffer. Press “Start”.

Note 4: *The amplicon should not be added directly onto the strip. If a strip has positioned itself too close to the front of the slot, push it back with a clean forceps or a new pipette tip.*

Note 5: *After closing the instrument cover, ensure that tubes for wash buffer and D.I. water move freely and are not pinched.*

Note 6: The assay will be performed automatically. Run-time is approx. 3 h, after the addition of the amplicon. After completion, the strips will remain in D.I. water of the last step with “Aspirate tray?” displayed on the screen of the instrument.

- Before aspirating the D.I. water from the tray, remove the strips and place them between clean blotting papers to air dry for 1 to 72 h (away from direct light) prior to interpretation. Once all strips are removed from plate, press the “Start” button to start aspiration of DI water from the tray.
- After all water is aspirated from tray, the instrument will display “Remove Sensor, Press Start to Continue”. At this point, the tray can be removed from the instrument. Press Start to continue with the cleaning cycle

programs.

- Run Cleaning cycle A and B when prompted.

Note 7: *Do not run the BeeBlot without liquids at any step. Serious damage may occur.*

- Empty general waste containers into the drain after each run.
- Empty DMF waste containers. Dispose the “special waste” with the blue substrate (DMF) as hazardous waste according to CDC procedure for hazardous chemical waste management. **Do not pour DMF waste down the drain.** Ensure that the tubes are placed back into the proper waste containers for BeeBlot_1, BeeBlot_2 and DMF-waste. Check off on the BeeBlot user log.
- Clean the tray: Add 5 ml of 70% Ethanol to each well used. Carefully rock tray 30-60 sec to dissolve any residual material and let stand for 5 min. Pour off ethanol into sink with running water and repeat washing steps. After second ethanol wash, rinse thoroughly with D.I. water. Make sure the D.I. water from the last wash is clear, indicating all DMF is removed from tray. Let tray air dry. The tray is ready for re-use after drying. Wipe down tray platform inside BeeBlot with 70% ethanol.
- Flush the pump reservoir next to the tray platform with 70% ethanol until the blue residue turns clear. Dry reservoir with KayDry towels. Remove instrument probes from reagent bottles and immerse briefly in D.I. water in clean beakers. Close the instrument lid and place probes on clean KayDry towels on top of the closed lid. Spray probes with 70% ethanol and cover with the bottom of the towel.
- Empty reagent bottles and wash with 1% Vircon. Let cleaning reagent soak for 2 to 10 min. Rinse thoroughly and finally with D.I. water. Clean reagent preparation beakers the same way.
- After overnight drying away from direct light, the strips are ready to be mounted on cardboard (blotting paper) and sealed in plastic. Affix to Linear Array worksheet.
- All observations and comments including date of testing, kit/reagent lot numbers, and name of operator are recorded.
- Any incidences or problems are documented on the worksheet and reported to the supervisor.

8.2 HPV 52 Verification

The Lightcycler 480 II software allows importing saved standard curves for quantification with adjustment of a standard samples that are included for both HPV52 DNA and hpDNA in every PCR assay. The standard curve files need to be prepared prior to using this assay and are described after the general assay setup in this document.

Worksheet

- Enter sample IDs into the HPV52 Assay worksheet HPVD.PM.J.727 to prepare the plate map (below) and reagent calculations. Information entered into the *Sample Input* worksheet automatically copies into *Sample Template* and *PCR Worksheet*. Print PCR Worksheet and use to record lot numbers and to assist in assay plate setup. Save the *Sample Template* worksheet as a tab-delimited text file.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	HPV52 10 ⁵	HPV52 10 ⁵	HPV52 10 ⁵	Globin 10 ⁵	Globin 10 ⁵	Globin 10 ⁵	Sample →				
B												
C												
D	NTC											
E												
F	NTC											
G												
H	NTC											

PCR Plate Setup

- Add primers and probes to the Lightcycler 480 master mix according to the reagent calculator on the worksheet (below). Add 40µl master mix to each plate well, then 10 µl DNA template.

	Tubes to set up (add 4 for excess)				10
	stock conc	final conc	units	1x	use
Lightcycler 480 Master	2	1	X	25	250
HPV 52 Primer	50	0.5	µM	0.5	5
HPV 52 Primer	50	0.5	µM	0.5	5
Globin Primer	50	0.5	µM	0.5	5
Globin Primer	50	0.5	µM	0.5	5
HPV Probe	10	0.1	µM	0.5	5
Globin Probe	10	0.1	µM	0.5	5
DI water				12	120
MASTER MIX Sub-total				40	400
Volume of template to be added to each tube				10	
Total Volume of reaction				50	

Reagent calculation table from the PCR worksheet for 10 test samples.

- Setup PCR plate according to the following scheme:

No-template controls (NTCs; 10µl dH₂O in place of DNA). NTCs in wells A1 and D1 should be used for all runs. If more than 52 samples are tested and wells on the F row are used, another NTC needs to be placed in F1. Greater sample number in row H will require an additional H1 NTC accordingly (see also plate map above).

A2-A4 – HPV52 standard; dilution at 1×10^5
A5–A7 – Globin standard; dilution at 1×10^5
A8 following – unknown sample DNA
A1, D1, F1, H1 – NTCs as needed.

Software Operation

After the PCR plate set up has been completed:

- Open the Lightcycler480 software and select “new Experiment from Template”.
- Select the templates:
 - Select the Run Template “HPV52 Dual Color Hydrolysis Probe UPL 50 ul Reaction”.
 - Select the Subset Template “HPV52 subset”.
 - Select the Sample Editor Template “HPV52 Sample List”.
 - Select ✓ to use the 3 selected templates.
- Review the protocol parameters in the Experiment window to confirm that the proper conditions were selected.
- Select Sample Editor window.
- Select extra columns for viewing the results.
 - Select Abs Quant
 - In Configure Properties under table column, select
 - Target Name
 - Dominant Channel
- Upload the *Sample Template* text file created from the Excel workbook.
- Click run under the “Experiment” tab.

Cycling program

Setup						
Detection Format			Block Type			Reaction Volume
Multi Color Hydrolysis			96			50ul
Programs						
Program Name			Cycles			Analysis Mode
Pre-Incubation			1			None
Amplification			45			Quantification
Temperature Gradient			1			Color Compensation
Cooling			1			None
Temperature Targets	Acquisition	Hold	Ramp Rate	Acquisitions		
Targets (°C)	Mode	(hh:mm:ss)	(°C/s)	(per°C)		
Pre-Incubation						
95	None	9:00	4.4	-		
Amplification						
95	None	00:00:15	4.4	-		
62	Single	00:01:00	2.2	-		
Temperature Gradient						
95	None	00:00:05	4.4	-		
65	None	00:01:00	2	-		
95	Continuous	-	0.06	5		
Cooling						
40	None	00:00:10	2	-		

Create Subsets for Analysis at the completion of the run:

- In the Subset Editor window, click on + to create subsets
- For HPV analysis, highlight the HPV standards (A2-A4) and unknown samples (A8 – H12) on the plate layout
- Name subset HPV Analysis
- Click “apply”
- Create New Analysis:
 - In the analysis window:
 - Click on + to create a new analysis run
 - Analysis Type: Abs Quant/2nd Derivative Max
 - Subset: HPV Analysis
 - Program: Amplification
 - Name:
 - Click ✓

Applying the external standard curve:

- Click on the “filter” button and select FAM for the HPV standard.

- Click the “efficiency” button; select “standard curve (external)” apply **External curve (FAM)**.
- Click “calculate” and export results table.
- Right click on the sample list. The window will appear blue and the Export Table tab will appear. Click on the Export Table tab and save file to a CDC approved encrypted USB flash drive.
- Repeat for Globin analysis with the HEX filter, apply the standard **External curve (HEX)**.

Formatting the Results:

- Transfer the FAM and HEX results table to another computer with the the HPV52 Assay workbook HPVD.PM.J.727 that was used to prepare the *Sample Input*, *Sample Upload* and *PCR Worksheet* sheets.
- Copy and paste the FAM results table into the *RAW HPV52 FAM* sheet and HEX results table into the *RAW Globin HEX* sheet with the top-left corner of each table pasted into cell A1 of the respective sheets. FAM and HEX results are automatically merged and copies over into the *RAW* sheet.
- Review the *DATABASE output* sheet for the following messages to confirm the run has passed:
 - HPV52 Calibrators: “In Range!”
 - β -Globin Calibrators: “In Range!”
 - NT Controls: all “PASS”

Preparation of the Standard Curve (templates and reference file):

- DNA templates to establish this standard curve only need to be run once or on a new instrument and can be saved. Each subsequent assay setup will only contain a 10^5 calibrator in triplicate for HPV52 and β -globin to adjust the imported standards for the run.
- HPV52 amplicons are prepared from a molecular clone of Human Papillomavirus Type 52 (Cat# VRMC -29, ATCC Manassas, VA. 20108) using the primer listed under 6.2.2 and cycling conditions for the HPV 52 TaqMan assay.
- Globin amplicons are prepared from human placenta DNA (Cat#D7011-25MG Sigma Aldrich, St. Louis, MO 63178).
- All amplicons were purified using Centricon Centrifugal Filter Devices and the concentration determined using a Nanodrop

Spectrophotometer.

- The HPV52 plasmid is diluted to contain 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 30, 10, copies per 10 μ L aliquot in the background of 10^5 copies of placenta DNA. All template dilutions were prepared in 0.1 μ g/ μ L tRNA (Life Technologies, Gaithersburg, MD, USA) to limit degradation of low copy number templates.
- Globin templates are diluted to contain 10^7 , 10^6 , 10^5 , 10^4 , 10^3 , 10^2 , 30, 10, copies per 10 μ L aliquot with 0.1 μ g/ μ L tRNA.
- All dilutions of the prepared templates are subjected to the qPCR assay as specified above to create the importable external standard curve-file.

Files with validated standard curves for the Light Cycler 480:

- For HPV52: External curve (FAM): HPV52/ Special Data/External curve (FAM)
- For β -Globin: External curve (HEX): HPV52/ Special Data/External curve (HEX)

9. REPORTABLE RANGE OF RESULTS

The LINEAR ARRAY HPV Genotyping Strip is read visually by comparing the pattern of blue lines to the LINEAR ARRAY HPV Genotyping Test Reference Guide. The presence of a blue line indicates specific binding of DNA to an analogous probe sequence. Strips are evaluated and results for each HPV type and globin band are recorded on the LA worksheet by two technologists independently within 72 hours. In the event of discordant readings that cannot be resolved, the technical supervisor is consulted. Consensus data is entered into Access database by the technologist who performed the assay. Double entry is required for data validation in the Access database.

HPV 52 Verification Assay: The Light Cycler 480 produces an output file listing detector, Cp value, quantified target copies etc. for all sample IDs in the run. Each file is exported and pasted into the HPV52 Assay Workbook that was used for the PCR run, as explained above in *Formatting the Results*. The Excel template file automatically interprets each individual sample as “Positive”, “Negative” for HPV52 or “Inadequate” based on the following criteria:

- Samples with 5 copies of HPV52 per 10 μ l are considered positive.
- Samples with less than 5 copies of HPV52 and at least 5 copies of β -Globin are interpreted as “Negative”.
- Samples with less than 5 copies of HPV52 and β -Globin are Inadequate.

Positive copy counts may be invalidated by the LC software, placing alert messages in the Status column of the output file. These may be non-exponential signal curves, signal detection at very low cycle numbers ($C_p < 15$) or other unexpected results indicative of false signals.

Additionally, if manual inspection of the amplification curves identifies false positives, an "M" should be placed in the exceptions-field in the row of the corresponding sample ID on the Run Report tab.

10. QUALITY CONTROL (QC) PROCEDURES

For a valid run of the Linear Array assay, the negative control for DNA extraction (water blank carried through extraction procedure) and the water blank control for PCR reagents must be negative for globin and all HPV types. The positive PCR control (HPV16 plasmid DNA) must be positive for HPV16 and negative for all other HPV types. An individual sample result is valid if HPV or beta-globin is detected.

For the HPV 52 assay, the calibrator reaction serves as positive control. It must be positive for HPV52 and within the C_p range specified under Section 9. All negative controls (no-template) included must produce no valid signals and result in 0.0 copies. In some cases, erroneous signals may be identified in irregular amplification curves and acceptance can be manually overridden.

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

A valid run requires that the positive and negative control samples show results as detailed under section 10. If either condition is not met results cannot be recorded until problem is resolved. Usually, DNA extraction and or the Linear Array HPV test needs to be repeated.

If a sample is negative for all HPV types and for both beta-globin probe lines, the specimen is considered inadequate for evaluation. This situation may be due to insufficient or degraded cellular material, poor extraction or the presence of PCR inhibitors. Without additional testing the sample is report as "inadequate".

HPV-positive samples that are beta-globin negative may be reported. The beta-globin amplification is designed to be less efficient than the HPV amplification. This situation may occur with a low cellular yield and/or high HPV copy number.

If the sample is XR-probe-positive and HPV33, 35, and 58 negative, then the sample can be reported as HPV52-positive. If the sample is XR-probe-positive and either HPV33-, or 35-, or 58- positive, then status for HPV52

should be verified by the type-specific real-time PCR assay (see 8.2).

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

Detection of HPV is dependent on the number of viral genomes present in the sample and may be affected by sample collection methods, particularly by self-collected samples.

Certain body fluids or other specimen contaminants have been shown to inhibit PCR amplification and may give false negative or invalid results.

A negative result does not preclude the presence of types not included in the probe set.

The Linear Array assay is not quantitative. Results are not to be used for clinical management.

13. REFERENCE RANGES (NORMAL VALUES)

N/A

14. CRITICAL CALL RESULTS ("PANIC VALUES")

N/A

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

All specimens are stored in 4°C conditions during processing and testing unless specified differently by the procedure.

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

No alternative test method is available. In the event that the DNA extraction or the Linear Array test fails specimens are stored at 4°C if the procedure can be repeated within 2 weeks or at -80°C if longer storage is required.

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

HPV genotyping results are submitted to Westat electronically after testing of all specimen has been completed for the 2-year cycle. Result files in the format of the NHANES shipping manifest are uploaded to the Westat ftp server. Unexpected delays will be communicated to Westat.

18. TRANSFER OR REFERRAL OF SPECIMENS; PROCEDURES FOR

SPECIMEN ACCOUNTABILITY AND TRACKING

Original biological specimens are collected at the NHANES Mobile Examination Clinics and shipped to the HPV laboratory at CDC via FedEx. At the HPV lab, all specimens and resulting DNA extracts are tracked via a LIMS system.

19. Summary Statistics and QC graphs

N/A

REFERENCES

- Linear Array HPV Genotyping Test (Application manual 04462268001) Roche Molecular Systems, Inc., Branchburg NJ, USA.
- Gravitt PE, Peyton CL, Alessi TQ et al., Improved amplification of genital human papillomaviruses. *J Clin Microbiol* 2000;38:357-361.
- LightCycler 480 Manual – Roche (2008)
- Onyekwuluje et al. A real-time PCR assay for HPV52 detection and viral load quantification. *Clin Lab.* 2012;58:61-6.