Laboratory Procedure Manual

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

Matrix: Self-collected penile swabs

Method: Linear Array HPV Genotyping Assay (Roche Diagnostics)

First Published:

Revised:

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

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 Name	Variable Name	SAS Label
HPVP_I	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
	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

Data File Name	Variable Name	SAS Label
	LBDR82	HPV type 82
	LBDR83	HPV type 83
	LBDR84	HPV type 84
	LBDR89	HPV type 89
	LBDRIS	HPV type IS39

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

This protocol describes procedures for DNA extraction and HPV genotyping from self-collected penile swabs.

DNA extraction is performed with the Chemagic MSM1 (Chemagen, Perkin Elmer), based on a magnetic bead system. Exfoliating keratinized cells from the swabs are lysed with detergents and Proteinase K under heat. The released DNA is bound to paramagnetic beads which are transferred through a number of liquid purification solutions to wash away proteins, lipids and other cell debris. Through pH change the purified DNA is finally released and dissolved into an elution buffer.

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 ß-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 OID Safety Manual and Chemical Hygiene Manual.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

Final HPV genotyping results and dates for DNA extraction and 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

NHANES MEC personnel explains the process for self-collection of external genital sample to study participants and provides a individually packaged sterile polyester swab (Puritan Medical, Guilford, MA) and specimen collection vial with 1 ml specimen transport media (STM; Qiagen, Valencia, CA). Penile keratinized skin cells are collected by study participants using vigorous rubbing, and the swab in then placed in the STM vial and returned to NHANES personnel who cut end of swab to permit capping. The closed vial is kept at 4°C and shipped to CDC on dry ice in weekly or bi-weekly shipment. At CDC the STM sample is kept at 4°C until extraction (within 4 weeks).

DNA extracts can be stored temporarily at 4 ºC or for long term storage, in -80ºC freezer.

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 Cell Lysis and DNA extraction

6.1.1 Equipment

- Chemagic Magnetic Separation Module I (Chemagen, Baesweiler, Germany)
- Heat block (65º - 120º C) with 96-well insert and for 2.0ml tubes
- Pipettes, single channel: 20µl, 200µl, and 1000µl, 8-channels: 200 µl and 1000 µl (Rainin, cat. #L-20XLS, L-200XLS, L-1000XLS, L8-200XLS+, and L8-1200XLS)
- Multidispense pipette, Rainin Autorep or similar (Rainin cat.#AR-E1)
- Vortex mixer
- Tabletop centrifuge, Eppendorf 5415D or similar
- Centrifuge with dual plate rotor, Eppendorf 5804 or similar
- Orbital shaker, Benchmixer Multi-Tube Vortexer (Benchmark Scientific, cat.# BV1010 or similar

6.1.2 Reagents and Media

Reagents at room temperature:

- Viral NA/gDNA Kit special (Perkin Elmer, Cat # CMG – 1077): kits includes Lysis Blood Buffer, Lysis Buffer Tissue, Wash Buffer 2, Wash Buffer 4, Wash Buffer 5, Magnetic Beads, Elution buffer, lyophilized Proteinase K powder
- Pathogen NA Extension Kit (Perkin Elmer, Cat # CMG 466): Lysis Buffer EL1, Lysis Buffer EL2, lyophilized Proteinase K powder
- Nuclease-free water (Ambion, Cat. # AM9939)
- 70% Reagent alcohol (ethanol) in spray bottle (Fisher Scientific, cat.#2546-70-1)

Reagents at 4°C:

- Dissolved Proteinase K- refer to Reagent Preparation under Procedure

6.1.3 Supplies, Other Materials

- Aerosol barrier pipette tips: 1000µl, 200µl, 1000µl extended length, 200µl extended length (Glassware, cat. #99810, 91157, and 99930) (Extended length: Rainin, cat# RT-L250XF and RT-L1000XF)
- Simport Screw Cap Micrewtubes or similar storage tube with O-ring seal screwcap, 2ml and 0.5ml (Daigger, cat. #EF4219J and EF4219C)
- Biotube System, Microtiter format rack and tube system, 12 strips of 8 tubes (Daigger, cat.# KX3256DG)
- SepraSeal Cap mats (Matrix, Fisher Scientific, cat. #50823905)
- 1.5 ml microcentrifuge tubes, Eppendorf self-locking (Fisher Scientific, cat. #05-402-25)
- LC 96-well PCR plates and clear seals (Roche Diagnostics Cat.# 0510241001)
- Transfer Pipets, Sterile (Fisher Scientific, cat# 13-711-27)
- Absorbent bench pads (Fisher Scientific, cat. #15235101)
- Matrix screw cap tubes, 96 well plate, 1.0 ml, (Fisher Scientific, cat#NC9514468)
- Sterile syringes for multidispense pipette, 5.0 ml and 12.5ml (Rainin cat# ENC-5MLS and ENC-12MLS)
- Tube racks
- KayDry towels (Kimberly-Clark, cat# 34721)
- Gloves, latex or nitrile, powder-free (Glassware)
- Lab coat with ribbed knit cuffs (Daigger, cat. #EF1463)
- Autoclavable discard pan

6.1.4 Controls

Nuclease-free water is used as a negative quality control for the DNA extraction procedure as well as PCR assays. The water is processed in the same manner as specimens used in the assay. Four water controls are added per full 96 well

plate. The control and specimens processed are tested by downstream HPV genotyping assays.

6.2 Supplies for HPV Genotyping (Linear Array)

6.2.1 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

6.2.2 Supplies, Other Materials

- Aerosol-barrier tips for volumes 20 µl, 200 µl, 1000 µl Lab markers, waterproof and solvent resistant
- 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. #94745)
- 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)

6.2.3 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 HPV16/10 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.

6.3 Supplies for HPV 52 verification

6.3.1 Equipment

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

6.3.2 Reagents and Media

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):
HPV and Globin probes (Integrated DNA Technologies,IDT)

Oligo	Sequence
HPV52 (forward)	GCTAACGCACGGCCATGT
HPV52 (reverse)	CCAGCACCTCACACAAKTCG
HPV52 probe	FAM – CCAGCAACAMGACCCGGACC – BHQ1
ß-Globin (forward)	CAGGTACGGCTGTCATCACTTAGA
ß-Globin (reverse)	CATGGTGTCTGTTTGAGGTTGCTA
ß-Globin probe	HEX – TGCCCTGACTTTTATGCCCAGCCCTG – BHQ1
	(K = G or T, M = A or C)

6.3.3 Supplies, Other Materials

LC 96-well plates and seals, clear, Cat. # 05102413001

Pipette tips with aerosol barrier

6.3.4 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 DNA Extraction (automated)

- Using a wire rack and orbital shaker, shake STM tubes in upright position at maximum speed for 45 min at room temperature.

- In the biosafety cabinet: Transfer 400 µl of specimen into a 1.4 ml Biotube (refer for Chemagic plate layout worksheet for specimen well orientation). Prepare water blanks (400 µl) as negative control, four for each full plate of samples. Process along test samples.

- Add 380 µl Lysis Buffer Blood (LBB) and 20 µl Proteinase K. Mix briefly using a pipette.

- Close biotube with SepraSeal cap (best to work in columns).

- Incubate in a heat block at 65º C for 1 h.

- ***Note:*** *If Proteinase K and Lysis buffer are premixed for any of the cell lysis steps above, they must be aliquoted and mixed with the sample promptly. The Proteinase K activity will decrease after incubation longer than 10 minutes in Lysis Buffer. Lysed cells can be stored at 4°C over night (maximal 3 days) before the next step in the extraction procedure.*

<u>DNA Purification:</u>

- Touch-spin (using a centrifuge) the cell lysis mix to remove condensation. Transfer all liquid to a new deep-well plate according to the Chemagic plate layout worksheet and insert the deep-well plate into the plate rail of the Chemagen MSM1 instrument (see table on next page).

- Prepare reagents: Magnetic beads and elution buffer need to be manually aliquoted (highlighted in table below):

- For each sample tested, dispense 115 µl elution buffer into each well of a new deep-well plate, that will be occupied by a sample (use 6.5 ml for 48 and 13 ml for 96 samples and aliquot with 12 channel pipette or AutoRep).

- Prepare a flat 96-well plate with 150 µl paramagnetic beads in all used wells. (Use 8.25 ml for 48, 16.5 ml for 96 samples).

- *Note: Prior to use, vigorously shake or swirl the container with the paramagnetic beads. Ensure that the beads are fully suspended before opening - no brown sediment should be stuck to the bottom of the flask*
  - All other buffers will be dispensed by the instrument. Set up materials on the loading station (order from left to right):

Position	Label	Plate type
9	Waste	2 ml Deep-Well Plate (for used tips)
8	EB6	2 ml Deep-Well Plate filled with 110 µl elution buffer
7	Water	new 2 ml Deep-Well Plate
6	WE5	new 2 ml Deep-Well Plate
5	WB4	new 2 ml Deep-Well Plate
4	WB3	new 2 ml Deep-Well Plate
3	Lysate	2 ml Deep-Well plate with lysed cells
2	PMB	Flat plate with 150 µl paramagnetic bead solution
1		Rack with tips

- Ensure that each of the reagent bottles contains at least 125 ml and refill if necessary. Otherwise fill the buffers from the Viral NA/gDNA Kit into the corresponding reagent containers and close them tightly. The bottle on line 1 does not need to contain any liquid.

- Check waste container to ensure it is not full.

- Turn on the instrument with the green switch at the front. Start the PC. From the Desktop, launch the ‘Chemagen Chemagic’ user interface.

- From the program list, select ‘P8-Prime short 1-6 for 96 dispenser.che’ from the drop down menu located on the top right of the box; click on the ‘ID’ button to start.

- The program will prime each pump and dispense a small amount of all buffers through the dispensing manifolds. Watch the dispensing manifold and verify that liquid is dispensed from all nozzles. If any of the manifolds does not dispense liquid, select and run prime program for the specific manifold. Buffer 4 tends to evaporate from the pump and feeding tube and can be primed more extensively with the “prime 2 ml for manifold 4.che” program.

Note: *Do not start the run before buffer dispersal from all manifolds has been observed.*

- From the protocol list select the relevant program for keratinized epidermal skin cells in STM
- Click the 'IDs' button.
- Chose the number of samples and their position on the plate.
- Confirm that all consumables are provided and in place.
- Confirm that sample elution names are the same as in the lysis.
- Check fluid levels in buffer bottles; for a 96 well plate requires 125 ml as the minimum level.
- Start run by clicking the 'O.K.' button on the sample screen. A run takes about 50 min.
- After the run: Transfer DNA eluates (100 µl) from the deep-well plate at position 8 to tubes for further storage. To avoid transfer of residual paramagnetic beads in the Elution plate using the plate on the Chemagic magnetic stand to immobilize the beads.
- Discard all used consumables on the rail except the tip waste plate to the left. The tips in from the tip waste plate should be discarded into an autoclave pan. The empty tip waste plate can be inserted back on the plate rail for future extraction runs.
- Close lid and switch off instrument.

8.2 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" 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 Mg^{2+}) 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 Mg^{2+}, 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 Mg^{2+} vials will be needed for the 48 strips in one BeeBlot.

- Add nuclease-free water and template to each reaction so that the total volume will be 100 µl. (i.e. if 10 µl is used, add 40 µl of H_2O).

- Cover tubes/plate with a clean lid and transfer to the sample preparation area.

- In the sample preparation hood: Transfer appropriate amount of DNA template to the prepared PCR tubes/wells.

- 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: *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 genotyping with the BeeBlot:

- Preheat SDS and SSPE containers from the Linear Array detection kit to ≥ 53ºC. (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 to single strips.
  Note: *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.

- 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 (HPV Linear Array = 02). 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"
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	"Start"
Dispensing Wash Buffer/Conj./Cit./Sub.	-
Aspirate DI water	"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: *The amplicon should not be added directly onto the strip. If a trip has positioned itself too close to the front of the slot, push it back with a clean forceps or a new pipette tip. After closing the instrument cover, ensure that tubes for wash buffer and D.I. water move freely and are not pinched.*

- 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: *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.

- Rinse tray with water. Spray all slots generously with 70% Ethanol, rock tray about 30 sec and let stand for 10 to 20 min. Pour off ethanol, then rinse thoroughly wit tab water and finally with D.I. water. Let tray air dry.

- 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. Use designated clean beakers to rest the tubes from the BeeBlot instrument in the meantime. Clean reagent preparation beakers the same way. For complete instructions on cleaning and maintenance refer to CVDB.EQ.C.522

- After overnight drying away from direct light, the strips are ready to be mounted on card board (blotting paper) and sealed in plastic. Fix to LA 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.

8.3 HPV 52 Verification

The Lightcycler 480 software allows importing a saved standard curve for quantification with adjustment of a calibrator sample that is included in every PCR assay. The file with this standard curve file needs to be prepared prior to using this assay and is 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. Print PCR Worksheet and use to record lot numbers and to assist in assay plate setup.

	1	2	3	4	5	6	7	8	9	10	11	12
A	NTC	Globin 10^5	HPV 10^5	*sample*	*sample*	*sample*	→					
B												
C	NTC											
D												
E	NTC											
F												
G	NTC											
H												

PCR Plate Setup

- Add primers and probes to the Lightcycler 480 master mix according to the reagent calculator on the worksheet (below). Add 20µl master mix to each plate well, then 5 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	12.5	125
HPV 52 Primer	50	0.5	uM	0.25	2.5
HPV 52 Primer	50	0.5	uM	0.25	2.5
Globin Primer	50	0.5	uM	0.25	2.5
Globin Primer	50	0.5	uM	0.25	2.5
HPV Probe	10	0.1	uM	0.25	2.5
Globin Probe	10	0.1	uM	0.25	2.5
DI water				6	60
MASTER MIX Sub-total				20	200
Volume of template to be added to each tube				5	
Total Volume of reaction				25	

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

- Setup PCR plate according to the following scheme:

  A1 - no-template controls (5µl dH_2O in place of DNA). If more than 21 samples are tested and wells on the C row are used, another NTC needs to be placed in C1. Greater sample number in rows E and G will require additional NTCs accordingly (see also plate map above).
  A2 - globin Calibrator; dilution at $1x10^5$
  A3 - HPV52 Calibrator; dilution at $1x10^5$
  A5 following - unknown sample DNA

Software Operation

After the PCR plate set up has been completed:

- Open the Lightcycler480 software and select “new Experiment from Template”.
- Select the Run Template “HPV52 exp”.
- Select the Subset Template “HPV52 subset”.

- Select the Sample Editor Template “HPV52 sample”.
- Add sample names in the “Sample Editor” tab.
- Click run under the “Experiment” tab.

Cycling program: 95°/9m then 40 cycles of (95°/15s, 62°/1m) then hold 6o.

Applying the external standard curve (after the run is complete):

- In the analysis window, click the analysis tab.
- Click on the “filter” button and select FAM for the HPV standard.
- Click the “efficiency” button; select “standard curve (external)” apply **HPV25_05172013** (std).
- Click “calculate” and export results table.
- Repeat for Globin with the HEX filter, apply the standard **HPV52_GLOBIN_0521**2013 (std).

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 one calibrator for each target 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 7.0 and cycling conditions for the HPV 52 TaqMan assay.
- Globin amplicons were prepared similarly using the appropriate primers and the same PCR conditions.
- All amplicons were purified using Centricon Centrifugal Filter Devices and the concentration determined using a Nanodrop Spectrophotometer.
- The purified amplicons are diluted to contain 107, 106, 105, 104, 103, 102, 30, or 10 copies per 10 µL in the background of 105 copies of human genomic DNA.

Globin templates are diluted to final copy numbers

- To limit degradation of low copy number templates, all dilutions are prepared in 0.1 µg/µL tRNA (Life Technologies, Gaithersburg, MD, USA).
- All dilutions of the prepared templates are subjected to the qPCR assay as specified above to create the importable external standard curve-file:
- After the PCR is completed, select the analysis page and select “2nd derivative max”. Highlight the HPV standard and hit “ok”. Click “Color Comp” filter, then “In Database”, select HPV52 ColorComp and then click the “Calculate” button to generate the standard curve. Click the “Standard Curve” button and select “Save as External”. Save with the name: HPV52_ followed by the date; i.e. “HPV52_03112014” . Repeat the following for the Globin curve after selecting the HEX “Color Comp” filter.

Files with validated standard curves for the Light Cycler 480:

- For HPV52: HPV52 05172013 (std): C/Roche/Templates/Special/Standard Curves

- For ß-Globin: HPV52 GLOBIN_05212013 (std):
  C/Roche/Templates/Special/Standard Curves

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 by two technologists independently within 72 hours. In the event of discordant readings that cannot be resolved, the technical supervisor is consulted.

Positive HPV types and Globin control status are recorded on the LA worksheet. A second technologist interprets and records the types for each sample. Resolve any discrepancies by consultation and discussion with laboratory supervisor as needed.

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 “RAW” tab of the “HPV52 LC Template” file that also contains the Worksheet that was used for the PCR run. The Excel template file automatically interprets each individual sample as “Positive”, “Negative” for HPV52 or “Inadequate” based on the following criteria:

- Samples with 5 HPV52 copies per 10 µl are considered positive.
- Samples with less than 5 copies of HPV52 and at least 5 ß-Globin copies are interpreted as “Negative”.
- Samples with less than 5 copies of HPV52 and ß-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 (Cp < 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 (SiHa) 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 Cp range specified under Method Performance Specifications. 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 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. Repeat testing with more or less material added to the PCR reaction may be tried. Without additional testing the sample is reported as “inadequate”.

HPV-positive samples that are beta-globin negative may be reported. The beta-globin amplification is designed to be less efficient that 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 8.3).

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

20. REFERENCES

Manual Chemagic Magnetic Separation Module I and Chemagic Dispenser. Ver.-No. 060515

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.