Laboratory Procedure Manual

Analyte:	*Human papillomavirus (HPV) 6,11,16,18,31,33,45,52 and 58 Antibodies*
Matrix:	Serum
Method:	M9ELISA – Multiplexed VLP-based IgG ELISA on the Meso Scale Discovery Platform
Method No.:	
Revised:	
As performed by:	*Centers for Disease Control and Prevention* *1600, Clifton Road* *Atlanta, GA - 30329*
Contact:	*Dr. Elizabeth R. Unger and/or Dr. Gitika Panicker* *Email: eru0@cdc.gov; dhv1@cdc.gov*

Important Information for Users

NCEZID 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
HPVN_I_R	LBD9M6N	HPV 6 (IU/ml)
	LBD9M11N	HPV 11 (IU/ml)
	LBD9M16N	HPV 16 (IU/ml)
	LBD9M18N	HPV 18 (IU/ml)
	LBD9M31N	HPV 31 (IU/ml)
	LBD9M33N	HPV 33 (IU/ml)
	LBD9M45N	HPV 45 (IU/ml)
	LBD9M52N	HPV 52 (IU/ml)
	LBD9M58N	HPV 58 (IU/ml)
	LBX9M6	HPV6 9-plex coded result
	LBX9M11	HPV11 9-plex coded result
	LBX9M16	HPV16 9-plex coded result
	LBX9M18	HPV18 9-plex coded result
	LBX9M31	HPV31 9-plex coded result
	LBX9M33	HPV33 9-plex coded result
	LBX9M45	HPV45 9-plex coded result
	LBX9M52	HPV52 9-plex coded result
	LBX9M58	HPV58 9-plex coded result

1. SUMMARY OF TEST PRINCIPLE AND CLINICAL RELEVANCE

M9ELISA allows for simultaneous detection of antibodies to HPV 6,11,16,18, 31,33,45,52,and 58 in one microwell (Panicker et.al. 2021). Virus-like particles (VLP), self-assembled HPV L1+L2 protein capsids that resemble intact virions, are used as antigen in the assay. HPV-type specific antibodies present in test samples will bind to type-specific conformational epitopes on the VLPs adsorbed onto the surface of the microwell. Bound human IgG is detected with a SulfoTAG™ Anti-Human IgG using electrochemiluminescent detection on the MesoScale Discovery (MSD) plate reader that generates relative light units (RLU). The amount of antibodies in the sample is estimated relative to a reference sample using the parallel line method. Samples are scored as positive or negative using a pre-determined "cut-off" level. The assay is used for research purposes and does not have a clinical relevance.

2. SAFETY PRECAUTIONS

Observe universal precautions. Wear gloves, a lab coat, and safety glasses when handling all human blood specimens. Place all items like plastic tips, sample dilution plates, reservoirs etc. that contact blood in a biohazard waste container. Discard all disposable glassware into a sharps waste container. Place all liquid hazardous waste materials in closed containers labeled as hazardous waste and stating the composition of waste being contained. Handle neat serum in a biosafety cabinet if available. Protect all work surfaces by absorbent benchtop paper. Discard the benchtop paper into the biohazard waste container daily or whenever blood contamination occurs. Wipe down all work surfaces with 10% (v/v) sodium hypochlorite allowing appropriate contact time after work is complete.

3. COMPUTERIZATION; DATA SYSTEM MANAGEMENT

a. Each shipment of specimens received from the NHANES mobile unit contains a corresponding shipping manifest listing the samples in the box. An electronic data file (Excel worksheet), containing the specimen ID's, specimen locations in the box, collection dates and other relevant information concerning individual samples, is independently sent from Westat to the Human Papillomavirus laboratory via email.
b. From the data file, a worksheet for each assay run is generated. Each specimen is scanned and confirmed against the worksheet for correct sample ID on the label and acceptable condition of the specimen prior to the assay.
c. After the test results have been obtained and the final values approved by the reviewing supervisor for release, the result codes are transcribed into the data file originally sent from Westat. Data entry is proofed by the supervisor and clerk. The completed data file is then uploaded to the NHANES Westat laboratory data management website.
d. A copy is archived in the local computer, with weekly backup, to maintain an independent record. The new data are also appended to a local database, which includes all the specimens with results obtained in the project to-date; hardcopies of data are generated periodically and filed.
e. Documentation for data system maintenance is contained in hard copies of data records

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

a. No special instructions such as fasting or special diets are necessary.

b. Blood is collected in a red-top Vacutainer tube by standard venipuncture procedures.
c. Specimens for HPV analysis should be fresh or frozen serum.
d. A 0.5 mL sample of serum is preferable. The minimum sample volume required for analysis is 0.1 mL.
e. Specimens collected in the field should be processed for serum and frozen, and then shipped on dry ice by overnight mail. Once received, specimens are stored at ≤ -70°C until analyzed.
f. Portions of the specimen that remain after analytical aliquots are withdrawn should be refrozen at ≤ -70°C. Samples thawed and refrozen several times are not compromised, but extensively repeated freeze/thaw cycles should be avoided.
g. Specimens are rejected if insufficient quantity is available for analysis.

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

Not applicable

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

- Plate Reader (Sector Imager 6000 OR Quickplex SQ120, Meso Scale Discovery)
- Automated Plate washer (e.g. 405Select, Biotek)
- Orbital Plate Shaker (e.g. MTS2/4, IKA)
- Microplate incubator/shaker (e.g. Jitterbug 2/4, Boekel Scientific)
- Single and Multi-channel Pipettors, 20 μl, 100 μl, 200 μl, 1000 μl
- Automated Multichannel Pipettors, 1250 μl, 250 μl
- Vortexer
- Benchtop Centrifuge
- Weigh Balance
- Pipet-Aid
- Computer with Microsoft Excel® and program for PLL analysis
- Automated liquid handling system (e.g. JANUS®, Perkin Elmer) (Optional: Required only for automated serial dilutions)

Reagents (Room Temperature, RT):

- 10X PBS (0.01 M; pH 7.4) – (e.g. Hyclone, Cat. #4550)
- 10X PBST (pH 7.4)- (e.g. Hyclone, Cat. #0125)
- 4X Read Buffer T (MSD, Cat. #R92TC-1)
- ddH_2O

Reagents (4ºC):

- ECL blocking agent (GE Healthcare Life Sciences, Cat. #RPN2125)
- *Sulfo-tag™* Anti-Human IgG (Biotrend, Cat. # I-1193 labeled with *Sulfo-tag™* by Meso Scale Discovery)
- Diluent 100 (Meso Scale Discovery, Cat.# R50AA-3)

Preparation of Reagents

Note: Make all reagents on the day of testing. Do not store unless otherwise specified. Volumes shown are for one plate.

Wash Buffer (PBS-0.1% Tween 20) (1L)
10X PBST= 100 ml
ddH_2O= 900 ml
Store at room temperature for 1 month. Buffer can be made fresh on day of assay as needed.

5% Blocking Buffer (30 ml)
ECL blocking agent = 1.5 g
1X PBST = 30 ml (QS)

Assay Diluent (50ml)
Diluent 100™ = 5 ml
1X PBST = 45 ml

Sulfo-tag™ Anti-Human IgG (3 ml)
Refer to antibody vial for stock concentration and dilute as needed to 1 µg/ml in Assay Diluent.

1X Read Buffer T (20 ml)
4X Read Buffer T = 5 ml
ddH_2O =15 ml

Supplies, Other Materials

- MSD pre-printed multiplex plates (10 spot plates)
- Costar 96-Well Untreated Round (e.g. Fisher, Cat. # 07-200-103)
- Pipettor tips
- Serological pipettes
- Adhesive plate seals (e.g.Thermo-Fisher, Cat. #AB-0580)
- Conical Tubes (e.g. BD Falcon, Cat. #352098)
- Solution Troughs (e.g. CDC Glassware, Cat. #98339)
- Paper towels

For automated serial dilutions
- Robopack tips 200 µl (Revvity, Cat# 6001250)
- Janus associated troughs (e.g. Seahorse Bioscience, Cat.# S30014)

7. CALIBRATION AND CALIBRATION VERIFICATION PROCEDURES

All equipment and instrumentation used were maintained and verified as per manufacturer instructions.

8. PROCEDURE OPERATING INSTRUCTIONS; CALCULATIONS; INTERPRETATION OF RESULTS

a. General notes:

i. A minimum of 3 dilutions were tested for each sample and control
ii. Reference sera along with positive and negative controls must be run on every plate.

iii. The MSD imager requires 30 min for the camera to warm up before the plate can be read.

iv. Avoid air bubbles when adding 1X Read Buffer. This is especially important at this step, since bubbles will cause an incorrect reading. Ensure that the bottom of each plate is devoid of moisture before reading the plate. Do not let the plate sit after adding the read buffer as it will affect the final reading.

b. Sample Preparation

i. Test specimens and assay controls were thawed on ice
ii. Serum samples vortexed to mix before performing serial dilutions

c. Assay procedure:

i. A plate diagram was created to identify location and dilution series of test sample and control sample wells on the microplate.

ii. Take out pre-printed plates from refrigerator and let them acclimate to Room Temperature (RT) (24°C± 2) on the day of assay.

iii. Add 150 µl/well of 5% blocking buffer. Seal plate and incubate for 1 h at RT on an orbital shaker at 650 rpm.

iv. Wash plate 4 times with 150 µl/well of PBS/T using plate washer. Tap the plate once against clean paper towels to remove residual liquid.

v. Prepare at least 3 serial dilutions of each sample in assay diluent in a new Costar 96 well dilution plate as described below. Serial 3.16-fold dilutions of human serum samples starting at 1:100 were prepared in assay diluent. Serial dilution can be performed by automated liquid handling workstation or manually.
Example: Add 11 µl sample to 99 µl assay diluent (1:10); from this dilution take 20 µl to 180 µl assay diluent (1:100), and from this dilution take 70 µl to 150 µl assay diluent (1:316).

vi. 50 µl of appropriate sample dilution was added to pre-printed multiplex MSD microplate according to the plate diagram. After adding all samples to the plate, plate was sealed and incubated at 37˚C in a plate incubator-shaker at 650 rpm for 1 h.

vii. Plate was washed 4 times with 150 µl/well of PBS/T using plate washer. Tap the plate once against clean paper towels to remove residual liquid.

viii. 25 µl per well of diluted Sulfo-tag™ Anti-human IgG was added to the plate. Plate was sealed and incubated at 37˚C in a plate incubator-shaker at 650 rpm for 1 h.

ix. Plate was washed 4 times with 150 µl/well of PBS/T using plate washer. Tap the plate once against clean paper towels to remove residual liquid.

x. 150 µl/well of 1X read buffer without any bubbles was added to the plate and plate read immediately.

xi. Read plate using the MSD plate reader.

xii. Save the .txt files in appropriate study folder for further calculations.

d. Calculations:

The parallel line method (PLL) as described in the WHO HPV Labnet Manual (Grabowska et al., 2002; Labnet, 2009) was used to calculate antibody concentration relative to the reference.

e. Interpretation of results:

Serostatus cut-off is established using children's sera (n= 200). Antibody concentration in a test sample below established sero-status cut-off value is negative and a value equal or above is considered positive.

HPV type	SeroStatus Cut-off value
HPV6	7.6 IU/ml
HPV11	5.2 IU/ml
HPV16	1.6 IU/ml
HPV18	3.5 IU/ml
HPV31	5.5 IU/ml
HPV33	6.8 IU/ml
HPV45	4.2 IU/ml
HPV52	6.2 IU/ml
HPV58	12.8 IU/ml

9. REPORTABLE RANGE OF RESULTS

a. Final qualitative reports express results as positive or negative for the presence of HPV6, or HPV11, or HPV16, or HPV18 or HPV31, or HPV33, or HPV45, or HPV52 or HPV58 in the sample.
b. Final quantitative reports express results as IU/ml (International units/mL) for HPV6, or HPV11, or HPV16, or HPV18, or HPV31, or HPV33, or HPV45, or HPV52 or HPV58 in the sample.
c. Below table shows the lower limits of quantitation for each HPV type. Antibody concentration above this limit have total error (imprecision + bias) ≤ 40%

Limits of quantitation

HPV Type	Lower Limits of Quantitation (Units)
HPV6	2.2 IU/ml
HPV11	2.6 IU/ml
HPV16	1.2 IU/ml
HPV18	0.4 IU/ml
HPV31	2.2 IU/ml
HPV33	3.6 IU/ml
HPV45	1.2 IU/ml
HPV52	4.3 IU/ml
HPV58	4.0 IU/ml

10. QUALITY CONTROL (QC) PROCEDURE

A reference sample, negative control, high and low controls were tested on each plate.

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

a. Repeat the test if the controls don't agree.
b. Do not report results from runs in which either the negative control or both high and low controls did not meet expected reactivity.

12. LIMITATIONS OF METHOD; INTERFERING SUBSTANCES AND CONDITIONS

a. Only about 60-70% of individuals seroconvert after natural exposure to HPV.
b. Positive results indicate type-specific sero-conversion, but correlates of protection are not known.

13. REFERENCE RANGES (NORMAL VALUES)

An unvaccinated and unexposed person is negative for the presence of HPV6, or HPV11, or HPV16, or HPV18, or HPV31, or HPV33, or HPV45, or HPV52 or HPV58.

14. CRITICAL CALL RESULTS ("PANIC VALUES")

Not applicable

15. SPECIMEN STORAGE AND HANDLING DURING TESTING

Specimens are stored at ≤ –70°C until testing. After an aliquot of the thawed sample has been removed for testing, the residual is refrozen and stored at ≤ –70°C.

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

Other available methods have different performance characteristics (for example, lower sensitivity or specificity). If the analytical system fails, it is preferable to store specimens at ≤ –70°C until the system is returned to functionality.

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

Not applicable

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

Standard record keeping involves using the computerized database and the hard copy results themselves to track specimens. Records are maintained indefinitely. Only numerical identifiers (e.g., case ID numbers) should be used. All personal identifiers should be available only to the medical supervisor or project coordinator to safeguard confidentiality. For the NHANES study, residual serum >200 µl is retained at ≤ –70°C for 1 year and then returned to NCHS serum bank.

19. SUMMARY STATISTICS AND QC GRAPHS

Note that the QC data for HPV 6, 11, 31, 33, 45, 52, and 58 were reported in Arbitrary Units/mL. This does not affect trends illustrated.

If there is a need to compare these results to the reported data, a conversion factor for each type needs to be applied to the QC results.

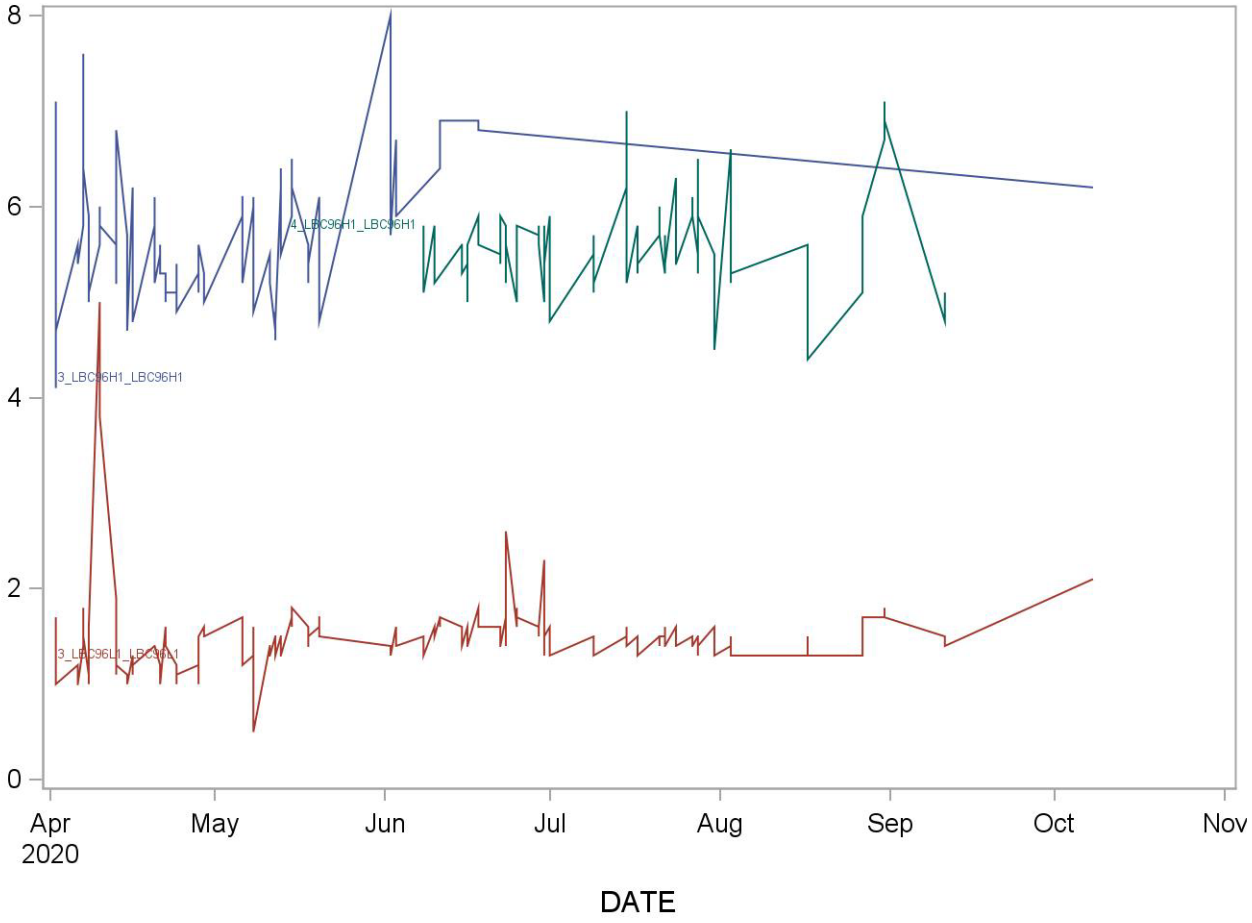
Example:
The HPV 6 AU/mL can be converted to IU/mL by multiplying by factor of 10.8

HPV6 3.2 AU/mL = 3.2 X10.8 = 34.6 IU/mL HPV6

ANALYTE NAME	Conversion factor
HPV6	10.8
HPV11	6.5
HPV16	1.2
HPV18	1.2
HPV31	1.4
HPV33	1.2
HPV45	0.5
HPV52	2.7
HPV58	1.8

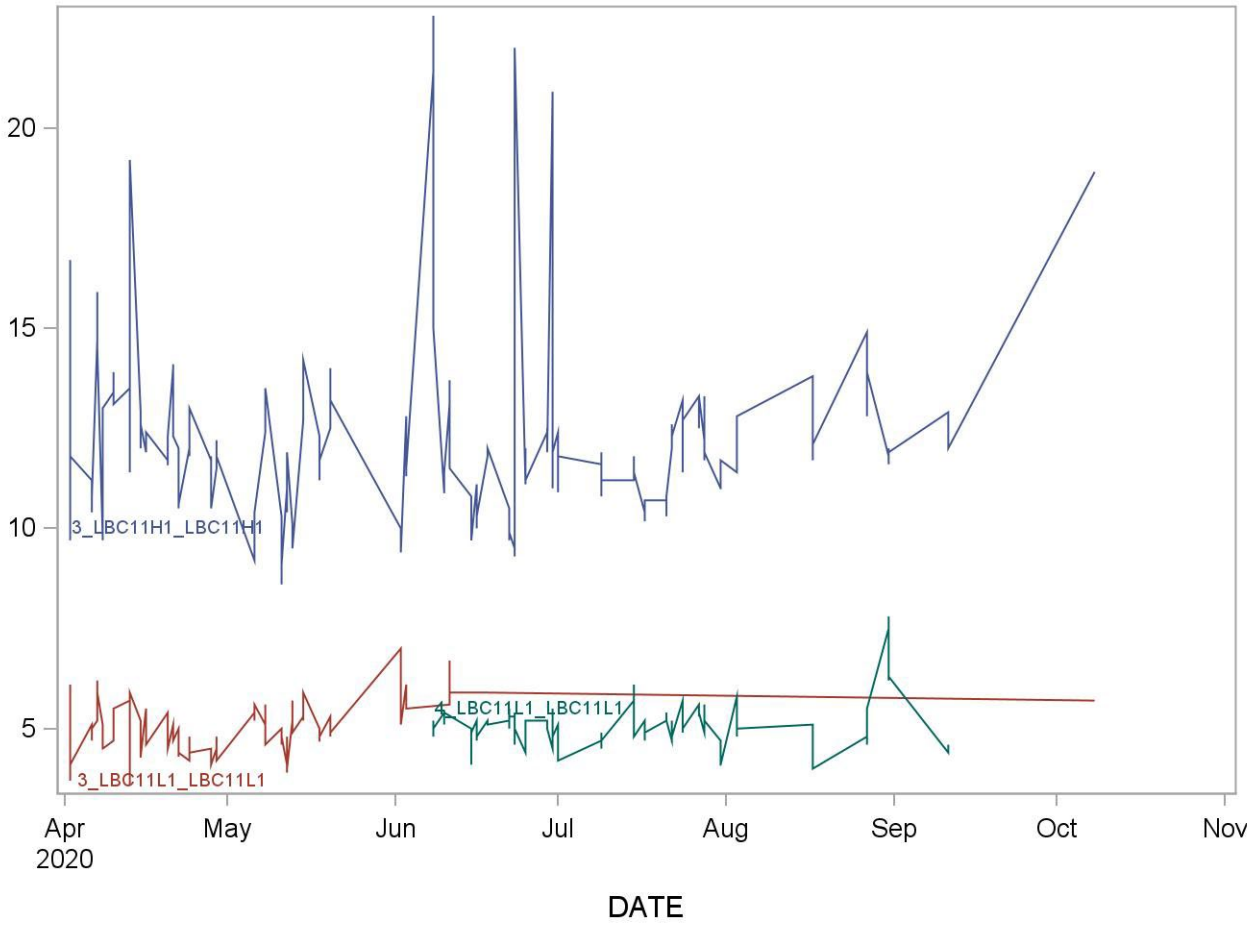
2015-2016 Summary Statistics and QC Chart
LBX9M6N (HPV 6 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC96H1	110	02APR20	08OCT20	5.64	0.67	11.9
3_LBC96L1	210	02APR20	08OCT20	1.50	0.47	31.3
4_LBC96H1	100	08JUN20	11SEP20	5.58	0.48	8.7



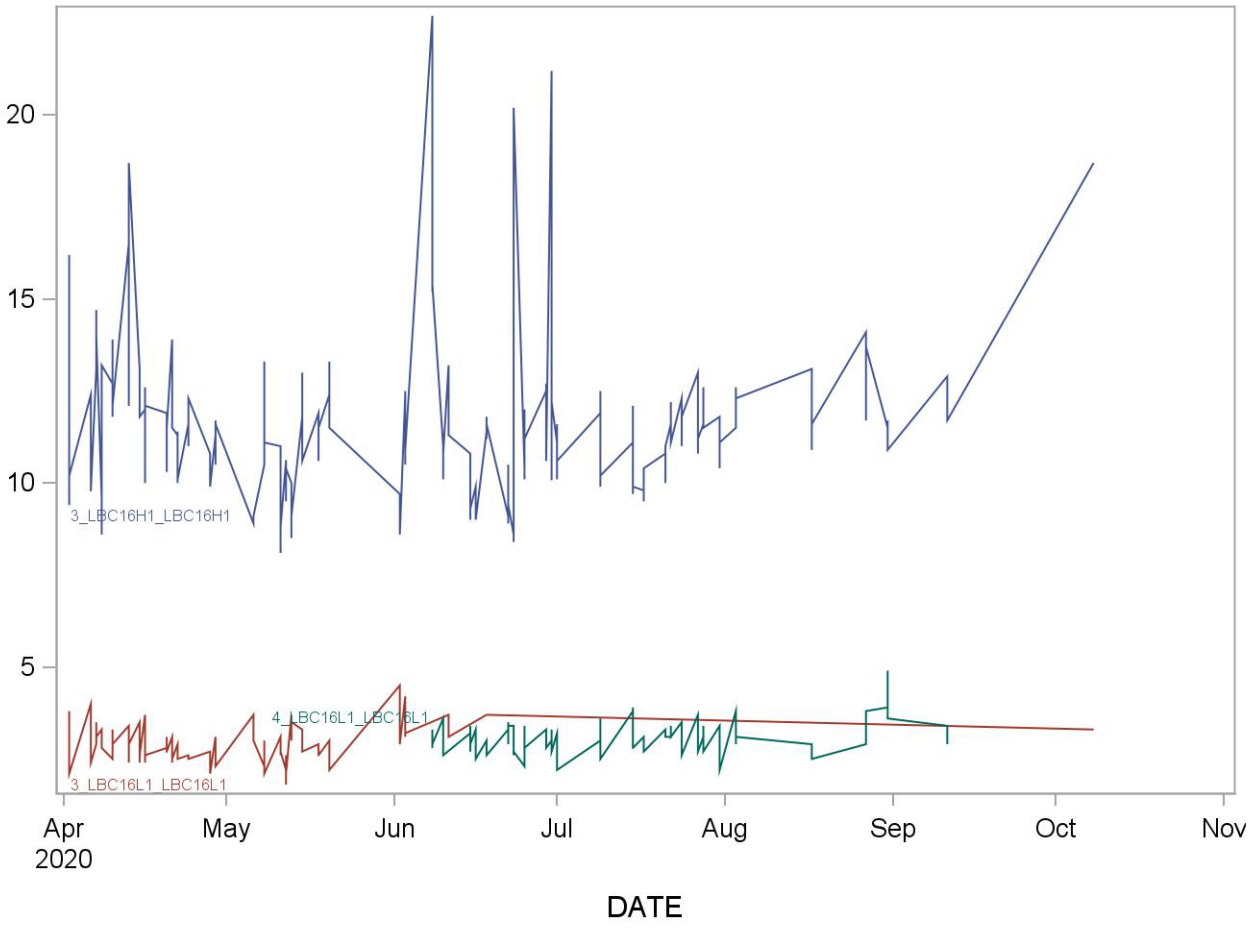
2015-2016 Summary Statistics and QC Chart
LBX9M11N (HPV 11 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC11H1	210	02APR20	08OCT20	12.11	2.09	17.3
3_LBC11L1	109	02APR20	08OCT20	5.01	0.64	12.7
4_LBC11L1	101	08JUN20	11SEP20	5.07	0.55	10.9



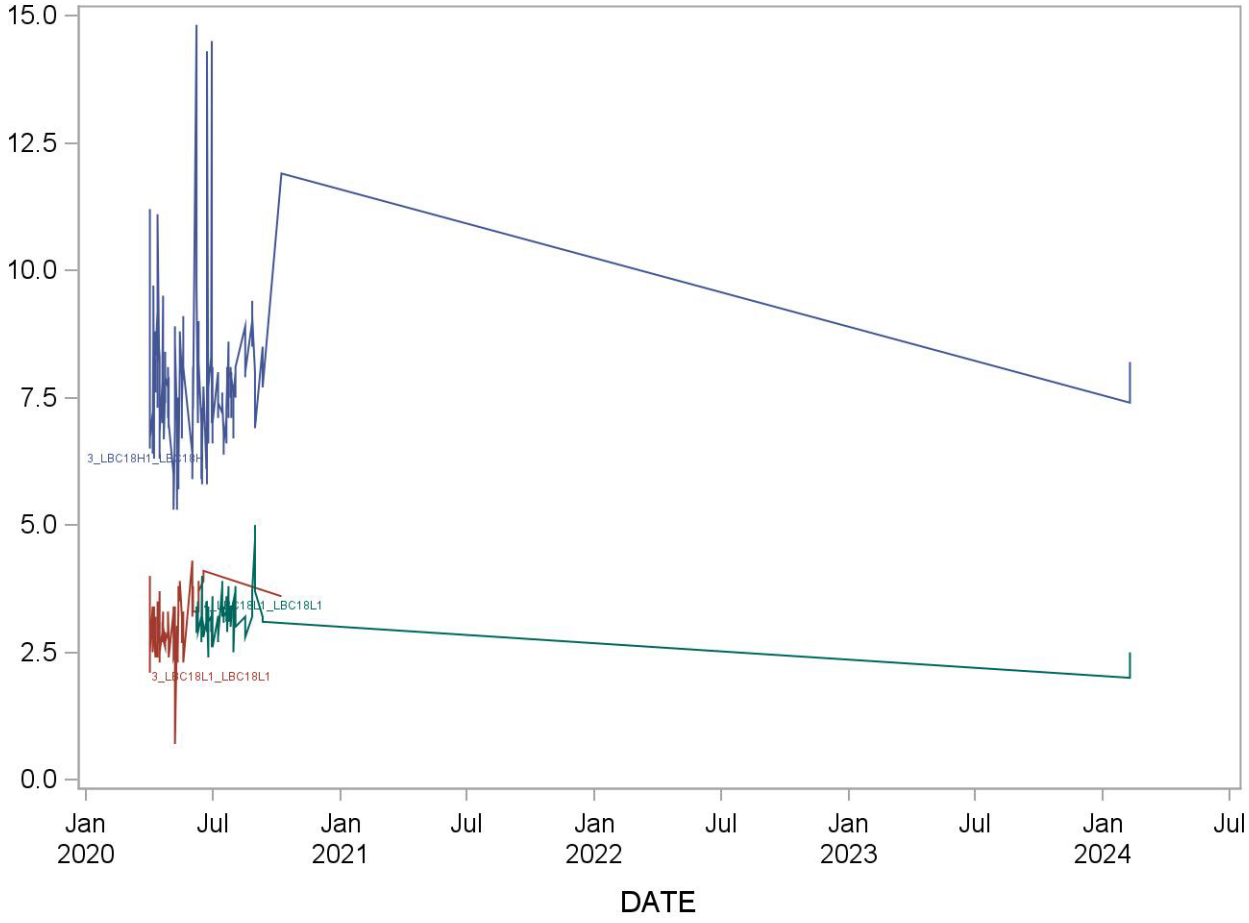
2015-2016 Summary Statistics and QC Chart
LBX9M16N (HPV 16 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC16H1	210	02APR20	08OCT20	11.58	2.19	18.9
3_LBC16L1	110	02APR20	08OCT20	2.90	0.51	17.5
4_LBC16L1	100	08JUN20	11SEP20	3.07	0.42	13.6



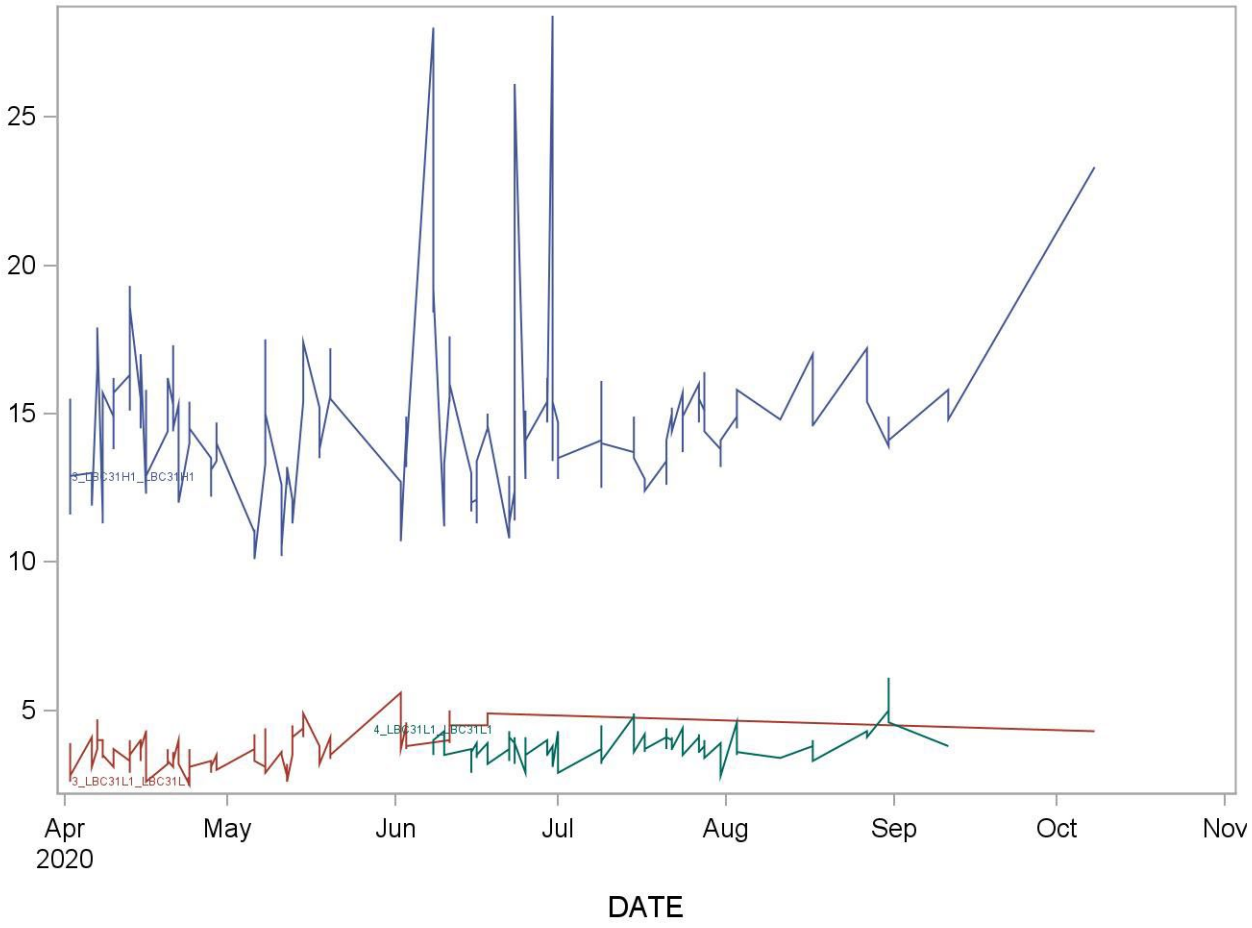
2015-2016 Summary Statistics and QC Chart
LBX9M18N (HPV 18 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC18H1	213	02APR20	09FEB24	7.71	1.42	18.5
3_LBC18L1	110	02APR20	08OCT20	3.01	0.51	17.1
4_LBC18L1	103	08JUN20	09FEB24	3.18	0.41	12.8



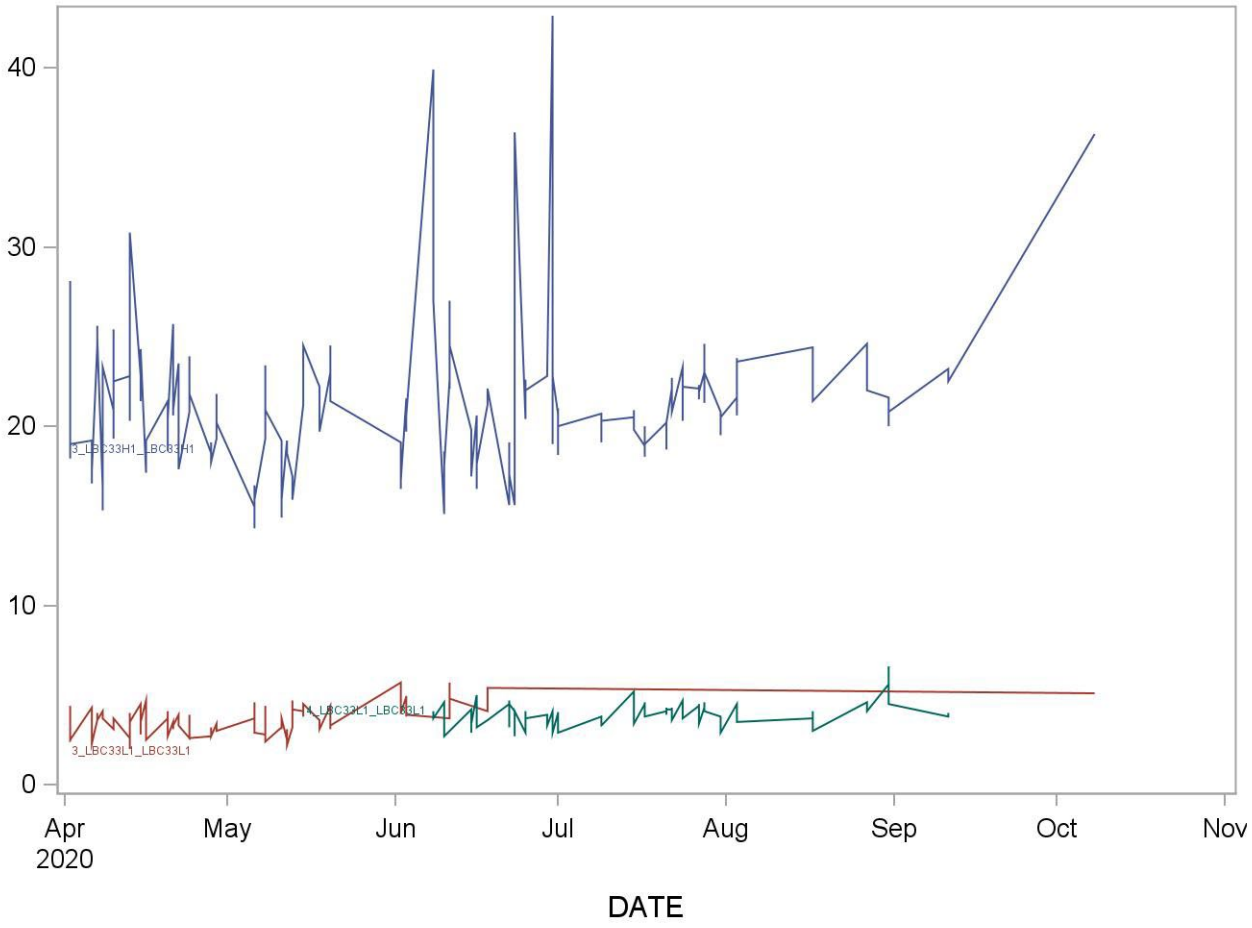
2015-2016 Summary Statistics and QC Chart
LBX9M31N (HPV 31 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC31H1	208	02APR20	08OCT20	14.47	2.65	18.3
3_LBC31L1	109	02APR20	08OCT20	3.62	0.59	16.3
4_LBC31L1	99	08JUN20	11SEP20	3.83	0.49	12.8



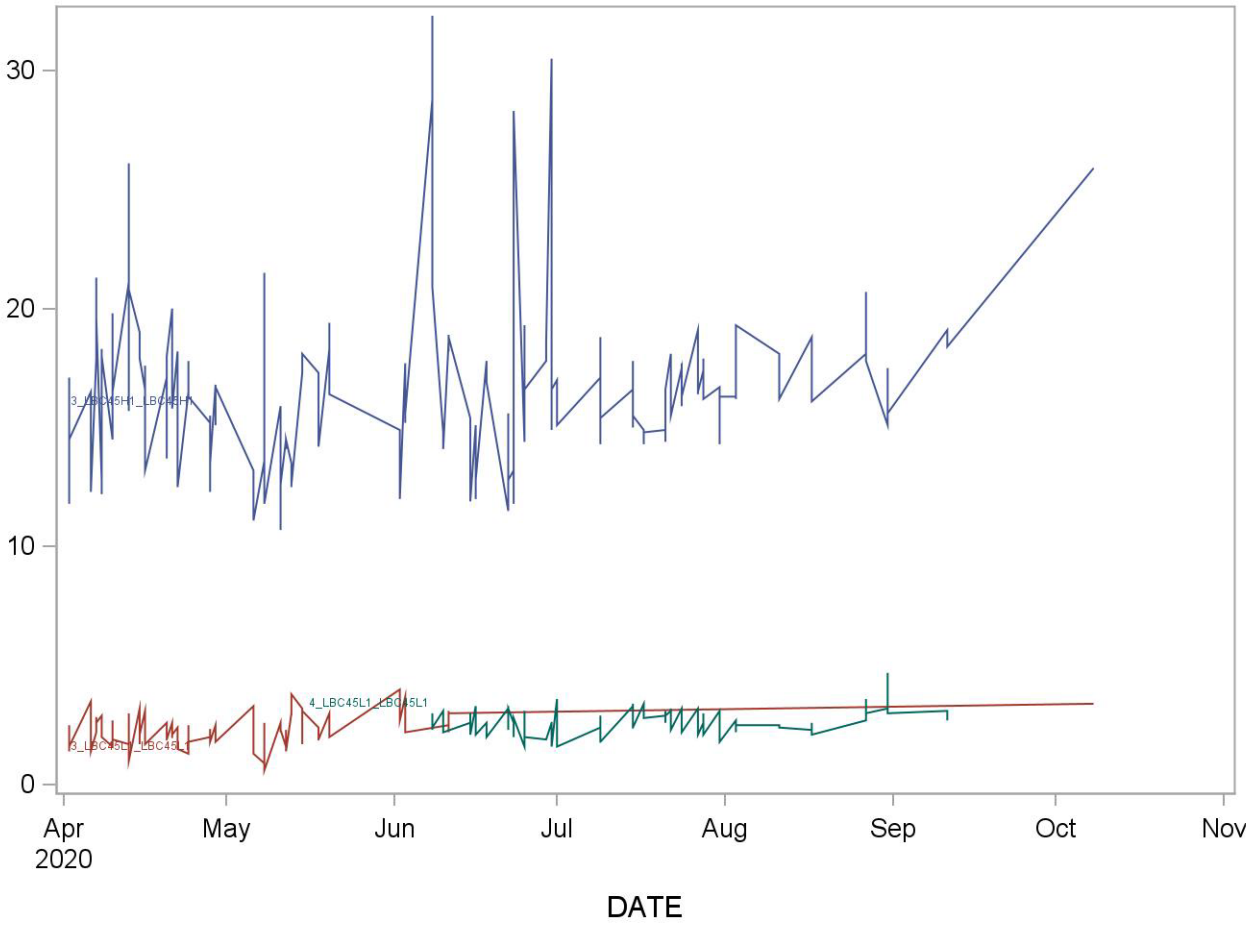
2015-2016 Summary Statistics and QC Chart
LBX9M33N (HPV 33 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC33H1	203	02APR20	08OCT20	21.12	4.04	19.1
3_LBC33L1	108	02APR20	08OCT20	3.56	0.74	20.7
4_LBC33L1	95	08JUN20	11SEP20	3.92	0.62	15.8



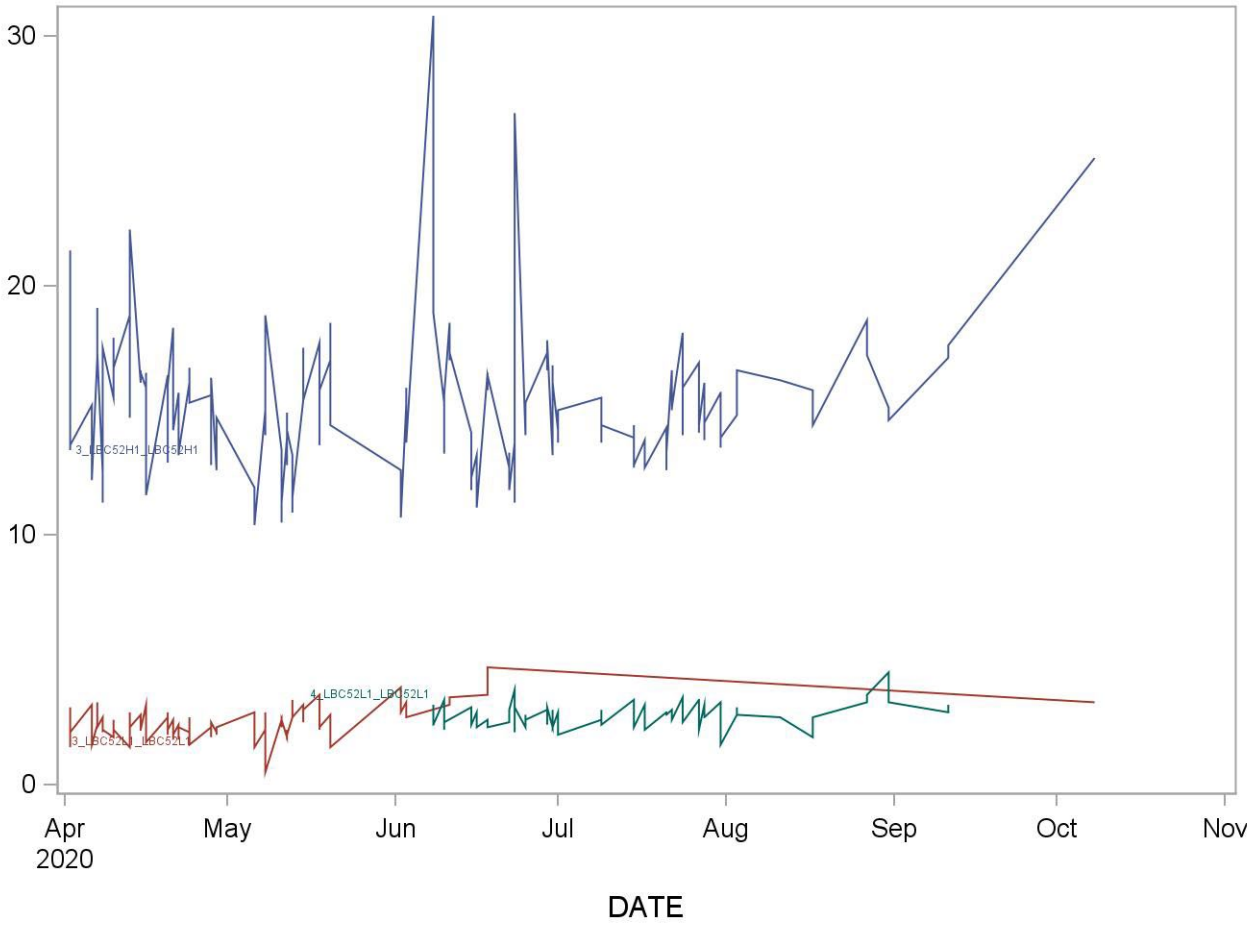
2015-2016 Summary Statistics and QC Chart
LBX9M45N (HPV 45 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC45H1	203	02APR20	08OCT20	16.40	3.15	19.2
3_LBC45L1	105	02APR20	08OCT20	2.31	0.66	28.7
4_LBC45L1	98	08JUN20	11SEP20	2.62	0.53	20.3



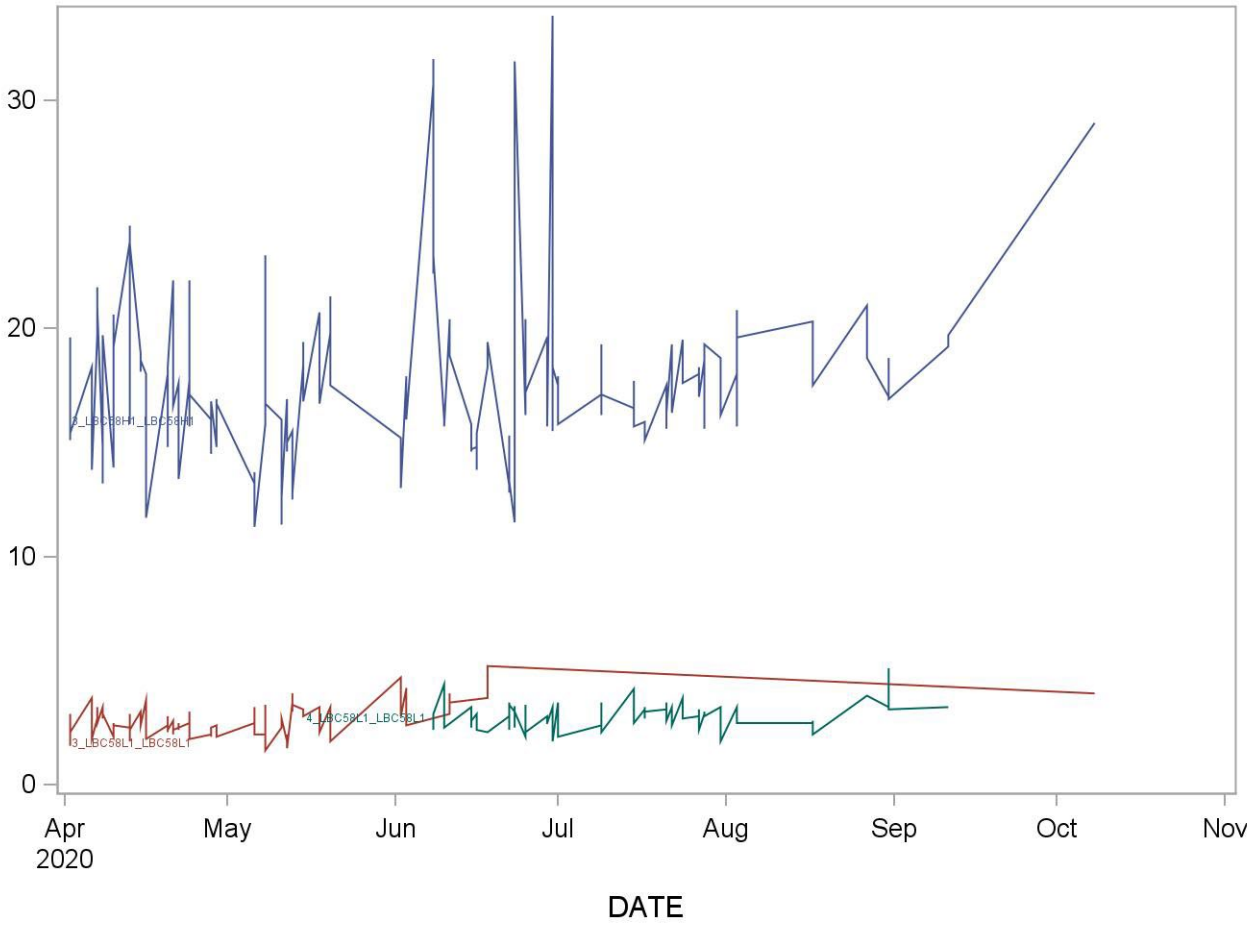
2015-2016 Summary Statistics and QC Chart
LBX9M52N (HPV 52 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC52H1	202	02APR20	08OCT20	15.22	2.83	18.6
3_LBC52L1	109	02APR20	08OCT20	2.46	0.60	24.2
4_LBC52L1	93	08JUN20	11SEP20	2.76	0.44	15.9



2015-2016 Summary Statistics and QC Chart
LBX9M58N (HPV 58 (Quantitative))

Lot	N	Start Date	End Date	Mean	Standard Deviation	Coefficient of Variation
3_LBC58H1	204	02APR20	08OCT20	17.47	3.40	19.4
3_LBC58L1	107	02APR20	08OCT20	2.80	0.65	23.3
4_LBC58L1	97	08JUN20	11SEP20	3.04	0.55	18.0



20. REFERENCES

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