Date	Laboratory Director	Approval Signature

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Validation Protocol for the Quantitative analysis of Patient Samples for the Presence of specific DNA/RNA sequences via Real-Time PCR

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Purpose: The purpose of this document is to describe the experiments and acceptance criteria involved in the bioanalytical assay validation using DNA/RNA extraction techniques coupled with real-time PCR analysis. Note that experiments for matrix effects and interfering substances should be relevant to the sample type that is being tested and experimental design is up to the discretion of the lab director and technical supervisor.

Background: Real time PCR is a valuable diagnostic tool which allows for the quick screening of patient samples for the presence of target genomic sequences. This technique is suited for, but not limited to, pathogen detection, gene detection, mutation detection, and many other uses.

The presence of microbial pathogens within patient tissues can cause a variety of physiological disorders. Respiratory infections can result from any number of bacterial, viral and/or fungal species colonizing the respiratory tissues. Some infections have the potential to cause severe symptoms and can lead to hospitalization or death. Traditionally the presence and identification of microbial pathogens in these settings would be carried out using culture methods within a diagnostic laboratory. Technologists would attempt to culture (grow) portions of the pathogen in a controlled environment so that trained personnel could subject the organism to various conditions/reagents in combination with morphological tests to identify it.

Identifying organisms with a molecular detection method as opposed to traditional method has advantages. First, the difficulty of culturing some microbes in an artificial environment can lead to failure to grow, i.e. false negatives. Nucleic acid-based detection tests are intrinsically not susceptible to these types of false negatives. Secondly, a nucleic-acid based detection test is far more sensitive than traditional culture methods and can detect a minority microbial population much more easily. Nucleic acid-based detection methods also eliminate the need to culture live pathogenic organisms within the laboratory, as well as eliminating the

possibility of artificial growth environments affecting any treatment-resistance statuses of the pathogen.

In order for a bioanalytical assay to be deemed appropriate for use in a clinical laboratory, it must pass assay validation standards set forth by the FDA, CLIA, COLA, and CAP standards. This document describes the validation of a bioanalytical assay for the detection and qualitative analysis of RNA/DNA sequences in patient samples. During assay validation, assays are evaluated for the following parameters: assay dynamic range, limit of detection, sensitivity, specificity, matrix effects, matrix lot-to-lot, inter-day precision and accuracy, intra-day precision and accuracy, interfering substances/concomitant medications, and stability. Specificity is addressed during assay development.

Procedure Descriptions and Acceptance criteria

Matrix effects: A biological matrix refers to the raw materials collected from a subject for the purpose of testing. Biological matrices are complex fluids or homogenized solids from which genetic material can be extracted prior to analysis. The most common methods for extracting genomic material is organic extraction, chelation extraction, solid phase extraction, and direct lysis of cells without purification.

The purpose of sample preparation is to remove all components within the sample matrix that may hinder the polymerase chain reaction, hinder fluorescence, or fluoresce at a wavelength incompatible to the testing being performed. Real time PCR works by replicating specific portions of the genome being tested for. In the process of replicating the genetic material, a fluorophore is cleaved from the probe, a portion of the genomic material containing a fluorophore and a quencher molecule, and fluorescence occurs and increases over time. If the target genomic sequence is not present, the polymerase chain

reaction does not occur, and fluorescence does not increase over time. Ideally sample matrixes which do not require expensive DNA/RNA purification would be used, but we are often limited by the products available to healthcare professionals for sample collection. Additionally, OTC drugs, creams, sprays, and other products which come into contact with the sample collection area may have an adverse effect on PCR results or the ability to collect a sufficient sample.

Experimental design and acceptance criteria for the determination of Matrix effects: There are multiple ways to determine if matrix effects exist for a bioanalytical assay utilizing real time PCR. For our purposes, we treat a sample taken from a willing participant in the new medium and run it via the real time PCR protocol for the pathogen in question. A blank sample, and a sample spiked with positive genomic material is also tested in tandem. If the positive spike sample yields positive results, the blank sample yields negative results, the willing specimen yields confirmation of extraction, and there is insignificant shift in CP value with respect to the DNA/RNA suspended in TE Buffer, then the matrix is suitable for testing. Insignificant shift in CP value means that no spiked samples CP value exceeds the CP value cutoff of the assay.

Sensitivity/LOD: Sensitivity of the assay refers to the ability to reliably produce a positive result at the lowest possible concentration of genetic material in copies/µL of sample. This is considered the Lower Detection Limit (LOD).

Experimental design and acceptance criteria for the determination of limit of detection:

An initial study should be conducted to determine the LOD range to be tested for each pathogen. This study should determine what the most likely LOD should be.

Dilutions of a known concentration of genomic material should be tested in triplicate until a concentration yields no positive amplification curves. The lowest concentration which yields positive results should then be determined and three solutions should be made using that concentration as a reference. A solution of the reference concentration should be made, a solution of +20% of the concentration of the reference should be made, and a solution of -20% of the reference should be made. Each solution should have 20 replicates tested, and the lowest concentration at which all 20 replicates yield positive results should be adopted as the LOD.

The latest CP value of the 20 replicates generated by the LOD should then be used to determine the CP cut-off value for the pathogen being validated.

Linear range: The linear range of the assay may determined using either characterized extracted genomic material or synthesized oligomer materials (example gBlock). You MUST know the starting concentration of these materials in order to perform these experiments. If you are using extracted genomic materials, you should have already determined the CFU/mL for the organism prior to extracting the DNA or RNA.

Experimental design for linear range and calibration curves: Dilute your genomic DNA/RNA or synthetic oligomers in Tris-EDTA (TE) buffer such that the highest concentration of DNA/RNA target is $1e^7$ copies/μL or CFU/μL, whichever is appropriate. Create up to 10 serial 1:10 dilutions using the $1e^7$ copies/μL or CFU/μL as starting material so that you have only 1 copy/μL or CFU/μL as your lowest dilution. Prepare and run these samples in triplicate using qPCR. Create a scatter plot of the Cp values versus the DNA/RNA or synthetic oligomer concentration. Be careful to convert copies/μL to copies/mL or CFU/μL to CFU/mL. Use the plotted data to create a linear regression with the regression formula and correlation values displayed on the plot. The linear range is defined as the concentration range in copies/mL or CFU/mL over which the reaction is linear as defined by a correlation coefficient of 0.99 or greater.

These calibration curves should be used to convert the Cp value determined by qPCR to a measure of the concentration of the organism in the original patient sample, reportable in copies/mL or CFU/mL, depending on what type of calibration material was used. Please document these curves and relay them to the QA department and to the LIS administrator in order to make sure that all calculations involved in testing are as accurate as possible. Linearity checks should be performed every 6 months for all assays involved in quantitative PCR.

Amplification efficiency. Amplification efficiency is a measure of how efficiently the qPCR reaction is performing. The efficiency should be determined from scatter plots of DNA/RNA or synthetic oligomer concentration versus the Cp values (the inverse of the calibration curves). The slope of the linear regressions of the above curve should be between -3.33 and -3.687 in order to be considered passing. This correlates to between 90% and 100% efficiency according to the Thermo Fisher qPCR efficiency calculator.

(https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html).

Precision and Accuracy: The precision of a measurement system, related to reproducibility and repeatability, is the degree to which repeated measurements under unchanged conditions show the same results. Simply put, it is determined from how close replicate determinations are to each other, and is used to estimate the amount of random error in a system.

The statistical accuracy of a measurement system defines how close replicate

determinations are to the actual target value. Accuracy is a determination of the amount of systematic error in the system.

Experimental design and acceptance criteria for the determination of inter-day and intra-day precision and accuracy:

Inter-day precision and accuracy is determined using six samples spiked with positive genomic material at the LOD to 3x the LOD and six samples without spiked genomic material. All samples should be tested in triplicate.

Intra-day precision and accuracy is determined using six samples spiked with positive genomic material at the LOD to 3x the LOD and six samples without spiked genomic material. All samples should be tested in triplicate. Identical plates should be tested on three consecutive days.

Specificity: Demonstration of the specificity of an assay is an essential part of the validation process. For the purpose of this document, we consider a demonstration of specificity to be a critical component of assay development insomuch as you should not proceed with assay validation if you have not demonstrated that your assay is specific for your analyte of interest.

Demonstration of specificity mandates that you have a unique signal for each analyte and internal standard. The signal for the analyte should be negative in the absence of analyte and positive in the presence of analyte. Further, the signal should be unique for your analyte and internal standard.

PCR primer/probe sets should be tested against the genomic material of other pathogens to determine if the material yields false positive results. If this is the case, the primer/probe set may need to be re-evaluated.

Exogenous interfering substances:

While it is not possible to test for all products which may interfere with fluorescence and PCR, an effort should be made to test several OTC medications, sprays, creams, and other products which have the potential to be contaminants for the sample collection medium.

Experimental design and acceptance criteria for concomitant medications and interfering substances: Volunteers should use the product as directed and a sample should be taken from the volunteer immediately to be processed as a patient sample. The sample should be run with and without positive spike in triplicate. This will ensure that the product does not suppress PCR or fluorescence and will also ensure that no species fluoresce in a competing manor to the fluorophore of the probe being validated.

Lab-to-lab correlation study: Lab-to-lab correlation studies are necessary to establish equivalence between methods in different laboratories. This type of study should also be performed every 6 months for non-regulated analytes that are not included in routine proficiency testing programs.

Study Design:

A combination of patient samples spiked with QC material should be prepared and run by at least two labs. The concentration of the QC material should be from the LOD to 3x the LOD or at a concentration agreed upon by all labs participating so that each lab receives at least 1 negative and two positive QCs for each analyte. QCs should be spiked with positive genomic material at known concentration.

Acceptance Criteria:

All samples spiked with QCs should yield positive results and all negative QCs should yield negative results. Any analytes that do not follow this trend should be evaluated for error. A positive result will have a Cp value between 10 and 35 cycles. Any Cp values less than 10.0 or greater than 35.0 are considered negative.

Stability

Freeze-thaw stability: In order to determine if samples are stable for analysis after freeze-thaw cycles, samples spiked at the LOD were subjected to a series of up to six freeze-thaw cycles. Acceptance criteria are that the determined concentrations for these standards are that samples must yield positive amplification curves once thawed.

Temperature stability: Three levels of QC standards are subjected to storage at room temperature (22 ˚C), refrigerator temperature (4-8 ˚C), and freezer temperature (-20 ˚C) for at least 24 h. Acceptance criteria are that the determined concentrations continue to yield positive amplification curves when analyzed.

Acceptance Criteria for All Bioanalytical Runs

i. Plate Controls

Each plate must contain at least one positive amplification control, one negative amplification control, one positive extraction control, and one negative extraction control. Positive amplification controls consist of positive genomic material in TE Buffer. Negative amplification controls contain nuclease free water in place of patient control. Positive extraction control consists of a previously tested positive patient sample. Negative extraction control consists of an extracted sample media containing no patient sample. Each sample must also have verification of extraction in the form of an extraction control or an internal standard. A positive result will have a Cp value between 10 and 35 cycles. Any Cp values less than 10.0 are considered to be outside the reportable range. The amplification curves for these samples should be visually inspected and if they follow a normal sigmoidal amplification, they should be repeated at the original concentration and at a 1:10 and 1:100 dilution. If the sample continues to demonstrate a normal amplification curve and the dilutions are linear (eg the Cp values increase with dilution), then the sample may be reported out as positive. Samples with a Cp value greater than 35.0 are considered negative.

Daily Testing Matrix for Assay Validation

This testing matrix should walk you through all of the experiments required in order to fully validate a bioanalytical method according to current FDA guidelines. These experiments are designed to validate a method that has already been through some degree of assay development. This testing matrix is a suggestion, and may be modified to fit your needs. Before you get to the validation steps, you should have already completed the following during assay development:

1. All primer/probe mixes should be tested to ensure that positive controls/genomic material yield positive results. Master mix recipe should be determined and optimized to the greatest extent achievable.

2. Have all instrument methods programmed. Note the file names for the final conditions.

3. Determine PCR conditions. The following parameters should be optimized to the analyst's satisfaction prior to the commencement of validation runs
    a. Master mix recipe
    b. Primer/probe composition
    c. Primer/probe combinations
    d. PCR temperatures
    e. PCR cycles
    f. Probe fluorophores
    g. Plate layout
    h. Controls layout.

4. Sample preparation should already be worked out.
    a. Determine sample preparation method: MagNA Pure extraction, manual extraction, extraction-free lysis, etc.

5. You should have already determined the approximate LOD of each pathogen.
    a. Preparation of LOD study with solutions at expected LOD and ± 20% of LOD.

6. **Specificity should be demonstrated prior to assay validation.** We consider this to be part of assay development, but it is critical that the specificity of the assay is demonstrated as part of this process. There should be no

significant cross reactivity with similar pathogens. There should also be notes of any significant inactivation with OTC sprays, creams, etc,.

7. Prior to commencing assay validation, you will likely have run through sample preparation and detection several times and have fair confidence in the robustness of the analytical method.

8. **Verify the DNA/RNA extraction efficiency using a 3 day precision and accuracy study with characterized control materials.**

    a. 6 positive controls and 6 negative controls daily for 3 days.
    b. Determine the purity of the extracted product using the 260/280 ratio
    c. Quantify the amount of DNA or RNA using the nanodrop
    d. Extract 20 patient samples and compare to another analogous method of extraction.

Day 1	LOD Study – may be done prior to validation
Day 2	Linearity Study – may be done prior to validation
Day 3	Matrix effects
Day 4	Day 1 Precision and Accuracy Intra-day Precision and Accuracy Inter-day Precision and Accuracy
Day 5	Day 2 Precision and Accuracy Intra-day Precision and Accuracy Inter-day Precision and Accuracy
Day 6	Day 3 Precision and Accuracy Intra-day Precision and Accuracy Inter-day Precision and Accuracy
Day 7	Freeze-thaw stability Temperature stability
Day 8	Lab-to-lab correlation study

Assay Validation Runs contain a minimum of the following:

1. Positive amplification control for each pathogen
2. Negative amplification control for each pathogen
3. Positive and negative extraction controls for each pathogen
4. Each pathogen in replicates of 6 positive controls and 6 negative controls

Day 1: LOD Study

Experimental Design: The LOD is the lowest concentration at which the real-time PCR instrument will consistently yield positive results, and it is a crucial data point utilized to determine the CP value cut-off for each pathogen. At this point in the validation process, there should be an expected LOD.

A standard validation run should be prepared as outlined above, with the following additions.

1. Prepare three solutions
    a. One solution at the expected LOD
    b. One solution at +20% of the expected LOD
    c. One solution at -20% of the expected LOD

2. Run at least 20 replicates of each solution
3. The lowest concentration at which the solution yields 100% positive results should be considered the LOD.
4. The CP value cut-off must then be determined by comparing the CP values of all 20 LOD samples. The CP value cut-off should include all CP values for the LOD meaning the CP value cut-off should be after the CP value of the latest LOD sample.

Acceptance Criteria For our purposes, we will prepare positive genomic material in TE Buffer at the expected LOD for each pathogen. Each result will be acquired by the acquisition method that is being tested. All 20 replicates for the LOD must yield positive results, and all positive and negative controls for the plate must pass.

Day 2: Linearity/ Calibration curves/ Amplification efficiency

Experimental design for linear range and calibration curves: Dilute your genomic DNA/RNA or synthetic oligomers in Tris-EDTA (TE) buffer such that the highest concentration of DNA/RNA target is $1e^7$ copies/μL or CFU/μL, whichever is appropriate. Create up to 10 serial 1:10 dilutions using the $1e^7$ copies/μL or CFU/μL as starting material so that you have only 1 copy/μL or CFU/μL as your lowest dilution. Prepare and run these samples in triplicate using qPCR. Create a scatter plot of the Cp values versus the DNA/RNA or synthetic oligomer concentration. Be careful to convert copies/μL to copies/mL or CFU/μL to CFU/mL. Use the plotted data to create a linear regression with the regression formula and correlation values displayed on the plot. The linear range is defined as the concentration range in copies/mL or CFU/mL over which the reaction is linear as defined by a correlation coefficient of 0.99 or greater.

These calibration curves should be used to convert the Cp value determined by qPCR to a measure of the concentration of the organism in the original patient sample, reportable in copies/mL or CFU/mL, depending on what type of calibration material was used. Please document these curves and relay them to the QA department and to the LIS administrator in order to make sure that all calculations involved in testing are as accurate as possible. Linearity checks should be performed every 6 months for all assays involved in quantitative PCR.

Amplification efficiency. Amplification efficiency is a measure of how efficiently the qPCR reaction is performing. The efficiency should be determined from scatter plots of DNA/RNA or synthetic oligomer concentration versus the Cp values (the inverse of the calibration curves). The slope of the linear regressions of the above curve should be between -3.33 and -3.687 in order to be considered passing. This correlates to between 90% and 100% efficiency according to the Thermo Fisher qPCR efficiency calculator.

(https://www.thermofisher.com/us/en/home/brands/thermo-scientific/molecular-biology/molecular-biology-learning-center/molecular-biology-resource-library/thermo-scientific-web-tools/qpcr-efficiency-calculator.html).

Day 3: Matrix effects

Experimental: Test 10 different lots of negative matrix. Use these lots to create negative and positive spiked controls. These materials should pass through the full extraction and amplification steps. *This experiment can be combined with a precision and accuracy experiment*.

Day 4: Day 1 Precision and Accuracy, Intra-day Precision and Accuracy, Inter-day Precision and Accuracy

Experimental: A standard validation run should be prepared as outlined above, with the following additions.

1. 6 positive QC and 6 negative QC should be run
    a. If the LDT is being developed via bridging study with an EUA the samples will also need to be ran via the EUA procedure.

Day 4: Day 2 Precision and Accuracy, Intra-day Precision and Accuracy, Inter-day Precision and Accuracy

Experimental: A standard validation run should be prepared as outlined above, with the following additions.

1. 10 positive spiked samples and 10 negative spiked samples should be run in triplicate

a. If the LDT is being developed via bridging study with an EUA the samples will also need to be ran via the EUA procedure.

Day 5: Day 3 Precision and Accuracy, Intra-day Precision and Accuracy, Inter-day Precision and Accuracy

Experimental: A standard validation run should be prepared as outlined above, with the following additions.

1. 10 positive spiked samples and 10 negative spiked samples should be run in triplicate
    a. If the LDT is being developed via bridging study with an EUA the samples will also need to be ran via the EUA procedure.

Day 6: Freeze-thaw stability and Temperature stability

Freeze-thaw stability: A standard validation run should be prepared as outlined above, with the following additions.

1. Set aside enough PC material to prepare three replicate samples of each pathogen.
2. Label one set of three replicate samples as '1', label the second set of three replicate samples as '2', and label the third set of three replicate samples as '3'.
3. Place all of the PCs in the freezer for at least 1 hr, or long enough to freeze.
4. Remove the two sets of three replicates of the QC material labeled '2' and '3', and allow to thaw at room temperature.
5. Place sets '2' and '3' back in the freezer for at least an hour, or long enough to freeze.
6. Remove the set labeled '3' and allow to thaw at room temperature.

7. Place the set labeled '3' back in the freezer for at least an hour, or long enough to freeze.
8. Remove all three sets from the freezer and allow to thaw at room temperature.
9. Mix all samples by vortexing to ensure that the samples have note separated into layers.
10. Prepare and run each aliquot as you would a normal patient sample.

Acceptance criteria: In order to pass, each PC must yield positive amplification curves with a CP value less than that of the CP cutoff. If the experiment fails, then this must be annotated in the assay validation report.

Temperature stability

Experimental: A standard validation run should be prepared as outlined above, with the following additions.

1. Prepare and run the triplicate sets of PC standards stored at each of three temperatures (room temperature, refrigerator temperature, and freezer temperature) according to your normal protocol.

Acceptance criteria: In order to pass, each PC level must have a determined mean that is below that of the CP value cutoff. All PCs must also yield positive amplification curves. If the experiment fails, then this must be annotated in the assay validation report.

Day 7: Lab-to-lab correlation study. A minimum of 20 samples comprised of patient samples and spiked QC materials covering the entire test menu should be

run by the laboratory validating the new assay and a partner laboratory. We recommend splitting the samples between a minimum of three labs, so if there is disparity between labs, it will be clear which lab had a problem. Further, patient samples will allow you to test the entire process while the spiked QC materials will reveal how accurate and specific the method is. Furthermore, it is extremely difficult to find patient samples to cover the entire test menu, so spiked materials are good for this purpose.

Acceptance Criteria:

Results generated from patient samples and spiked QC material were compared to the value obtained for participating laboratories. All 20 samples should agree between all laboratories participating.

Validation Report Contents

This document outlines the appropriate sections that should be present in a qualitative bioanalytical assay validation.

Once you have completed the initial assay validation, prepare your validation reports. All validation reports should contain a minimum of the following sections:

1. **General Information**
    a. Lab name, test menu, and LOD
    b. Instrument models and serial numbers
    c. Validation dates, validation scientist information, and lab director signature page.

2. **Instrument settings**
    a. Extraction method (and instrument settings if applicable)
    b. Real-time PCR settings
        i. Plate Layout
        ii. Detection Mode
        iii. Run template
            1. Temperatures
            2. Hold times
            3. Cycles
    c. CP value Cut-offs

3. **SOP** outlining how specimens were prepared during validation

4. **Assay Validation SOP** outlining experiments performed for validation

5. **Specificity** – Demonstrating lack of cross-reactivity between like pathogens and warnings for any OTC products that hindered testing.

6. **LOD** lower limit of Detection

7. **Matrix effects** – Verification that sample medium is suitable for collection of samples and yields true positives when spiked with genomic material and true negatives when processed as a blank.

8. **Inter-day precision and accuracy** – determined with replicates of QC standards across 3 days of validation.

9. **Intra-day precision and accuracy –** determined with replicates of QC standards within a single run (single validation run, n=3 replicates).

10. **Freeze-thaw stability** – QCs through 3 freeze-thaw cycles.

11. **Sample stability** – Store QC standards (in matrix) under the same conditions as your patient samples. Suggest refrigerated, frozen, and room temperature. Run them daily until a single analyte fails two consecutive runs. The date of the first failing run is the time-limit for storage of samples under those conditions.

Stability Addendum

This stability addendum was created to encompass some of the major sources of specimen/analyte instability that we encounter in clinical labs. Not all stability studies outlined below are necessary for all assays. Consult with your lab director and technical supervisor to determine which stability studies are relevant to your assay.

Stability studies that are recommended for all assays:

1. **Specimen storage stability**
2. **Temperature stability**
3. **Long-term stability**
4. **Shipping stability**
5. **Elevated temperature stability**
6. **Freeze-thaw stability**

Assessment of the Two-Week Stability of Patient Samples

If patient samples can be procured that contain all of the analytes in your test menu, then use patient samples. It is often the case that patient samples are not available to cover the entire test menu, so QC materials often suffice for these studies.

Patient samples, spiked QC materials, or a combination of both are stored at room temperature, in the refrigerator (2°-8°C), and in the freezer (≤-10°C). The initial validation typically tests them for up to a week. For this experiment, the samples should be stored for up to 2 weeks and then prepared in triplicate as per your SOP. Acceptance criteria are the same as QC for your analytical run.

Assessment of light exposure

patient samples are often collected at a location remote to the lab, typically a medical clinic or collection site and then prepared for shipment to the receiving laboratory. The laboratory has no control over either the amount of time that the specimens are held, or the conditions under which they are held (typically refrigerated or room temperature).

Temperature Stability

The following experiment was designed in order to test the stability of analytes tested in this laboratory under the conditions that specimens are likely to encounter at a clinical site. For the sake of this experiment, QC standards prepared in matrix are used to simulate the condition of patient samples when stored under refrigerator or room temperature conditions for up to two weeks.

Since specimens may be exposed to light during this timeframe, a set of light-exposure QC samples may also be assessed at room temperature.

Materials required: QC standards prepared in matrix.

On day 0, place triplicate sets of PCs at each of the following temperatures:

1. Triplicate sets in the refrigerator
2. Triplicate sets at room temperature in an opaque container
3. For light exposure, a third set of samples may be kept at room temperature in a container or rack that permits exposure to ambient light, and assayed at the same timepoints as the stability samples.

Day 0: Run PC samples as per your normal protocol

Days 7, 10, and 14: Run PC samples from both storage temperatures, refrigerated and frozen, as per your normal protocol. Add the specimens stored in the dark, if desired, for light exposure stability.

Acceptance criteria: At the end of the 14-day study, determine the results generated by QC samples and determine if the shift in CP values causes any curves to be outside of the cutoff range of the assay. If all PC levels show positive amplification curves and yield CP values less than that of the CP cutoff of the assay, then they are considered to be stable for 14 days.

The light exposure samples can be compared to the dark, room-temperature exposure samples for an assessment of the effects of light on analyte stability. When the percent difference of the means of these groups of QC standards is compared, presence of positive amplification curves and CP values less than that of the CP cutoff indicates no degradation due to light exposure.

Long-term stability: In order to assess the stability of standards for prolonged periods in storage, they should be periodically assessed. Prepare enough QC standards to perform long-term stability studies. The recommended schedule for testing is as follows:

1-month
2-month
3-month
6-month
9-month
12-month

Acceptance criteria: Acceptance criteria are the same for this run as for a normal analytical run. If QCs fail during this time, then samples will be considered stable through the previous passing timepoint.

Shipment Stability

Sample specimens are typically collected at a remote clinical site and then shipped via overnight courier service to the laboratory. The ability of the sample to withstand the rigors of the shipping process should be assessed. During the process of shipping, the samples may experience extremes of temperature, humidity, and light exposure. Furthermore, specimens shipped on a Friday may not be delivered until the following Monday, so care must be taken to assess the quality of the samples at 1 and 3-day timepoints.

QC materials prepared in sample media will behave as a surrogate for patient samples. Shipping stability studies should be performed any time that there is a new container in use for specimen shipping.

Elevated temperature: A location should be established that simulates the elevated temperature environment that is likely to be encountered when shipping samples in the summertime. The back of a delivery truck in August is likely to experience temperatures in excess of 120°F (~50°C). In order to assess the stability of specimens subjected to this extreme of temperature, care should be taken to identify a water bath or incubator capable of maintaining the high temperature.

Obtain 6 aliquots of each of three levels of QC sample (6-at LOD, 5x LOD, and 10x LOD). Label one set of 3 aliquots of each level as 24 hours, and the second set of three aliquots as 72 hours. Place both sets of samples in the incubator or water

bath. Assay a third set of three each of LOD, 5x LOD, and 10x LOD QC samples that have not experienced the temperature extreme on Day 0.

At 24 hour and 72 hour timepoints, remove one set of QC samples from the incubator and perform the assay using your normal protocol.

Once all of the data has been collected, evaluate the shift in CP value for the samples if any. If the CP value has shifted past the CP cutoff for the assay, degradation has occurred.

Pre-validation checklist

- ☐ All primer/probe mixes should be tested to ensure that positive controls/genomic material yield positive results. Master mix recipe should be determined and optimized to the greatest extent achievable.
- ☐ Have all instrument methods programmed. Note the file names for the final conditions.
- ☐ Determine PCR conditions. The following parameters should be optimized to the analyst's satisfaction prior to the commencement of validation runs
    - Master mix recipe
    - Primer/probe composition
    - Primer/probe combinations
    - PCR temperatures
    - PCR cycles
    - Probe fluorophores
    - Plate layout
    - Controls layout.
- ☐ Sample preparation should already be worked out.
    - Determine sample preparation method: MagNA Pure extraction, manual extraction, extraction-free lysis, etc.
- ☐ RNA/DNA extraction steps must be verified as per the verification protocol
    - Limit of detection verified
    - Precision and accuracy verified
    - Linearity verified
    - Correlation study performed
- ☐ You should have already determined the approximate LOD of each pathogen.
    - Preparation of LOD study with solutions at expected LOD and ± 20% of LOD.
- ☐ **Specificity should be demonstrated prior to assay validation.** We consider this to be part of assay development, but it is critical that the specificity of the assay is demonstrated as part of this process. There should be no significant cross reactivity with similar pathogens. There should also be notes of any significant inactivation with OTC sprays, creams, etc,.
- ☐ Prior to commencing assay validation, you will likely have run through sample preparation and detection several times and have fair confidence in the robustness of the analytical method.

- ☐ **Verify the DNA/RNA extraction efficiency using a 3 day precision and accuracy study with characterized control materials.**
    - 6 positive controls and 6 negative controls daily for 3 days.
    - Determine the purity of the extracted product using the 260/280 ratio
    - Quantify the amount of DNA or RNA using the nanodrop
    - Extract 20 patient samples and compare to another analogous method of extraction.
- ☐ Verify that your assay if a fully validated lab developed test or 510k approved IVD device?
- ☐ Initiate communication with LIMS administrator
    - Create the test in the LIMS
    - Build the instrument interface
    - Create the report format in the LIMS
        - Are units correct?
        - Name, address, phone number, and CLIA number are on the report
        - Lab Director's name is on the report?
        - Normal range is displayed on the report?
        - Comments about LDT are on the report?
    - Use the specificity study to test the instrument interface. Verify that single analyte results cross into the proper channels on the report
    - Document all of this great work in a LIMS validation report for this assay.
- ☐ Perform a specifity study
- ☐ Validate the limit of detection for the assay
- ☐ Validate the linear range of the assay
- ☐ Validate matrix effects
- ☐ Validate intraday precision and accuracy
- ☐ Validate interday precision and accuracy.
- ☐ Validate amplification efficiency
- ☐ Validate temperature stability
- ☐ Validate freeze-thaw stability
- ☐ Validate shipping stability
- ☐ Validate specimen storage stability
- ☐ Perform lab-to-lab correlation study

- ☐ Create your validation report
  - Must be signed and approved by the lab director
- ☐ Document training and competency for testing personnel
- ☐ Set a go live date! Also must involve the lab director in communications.

Document History			
Archived Document	Version #	Effective Date	Archive Date