HOW TO VALIDATE A MOLECULAR ASSAY

February 25, 2019

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Variant Database: Journal Article Review & Compile List with Clinical Significance
Follow-up Algorithm Development
LIMS: Modification & Validation
Stakeholders Input
Method Validation: Testing & QAO/Management Approvals
SOP: Development & QAO/Management Approvals
Method Development: Optimization & Scale-up
Molecular Testing Implementation
Communication: List Serv Announcement

Testing Method

- 1st tier or 2nd tier?
- Mutation panel or sequencing?
  - Common variants
- Any existing protocol/kit
  - Publication
  - Peer laboratory
  - Commercial vendor
  - FDA approval
- Existing methods or equipment

Why?

- Regulatory requirement
  - CFR Title 42 Public Health §493.1253 Standard: Establishment and verification of performance specifications.

When?

- Laboratories are required to perform analytical validation or verification of each nonwaived test, method, or instrument system before use in patient testing, regardless of when it was first introduced by the laboratory, including instruments of the same make and model and temporary replacement (loaner) instruments.
- Allow 2-3 months

What?

- **Analytical verification** - The process by which a laboratory determines that an unmodified FDA-cleared/approved test performs according to the specifications set forth by the manufacturer when used as directed.
- **Analytical validation** - The process used to confirm with objective evidence that a laboratory-developed or modified FDA-cleared/approved test method or instrument system delivers reliable results for the intended application.
- Verification and validation must assess the total testing process
  - Reagents
  - Instruments
  - Software
  - For all types of samples that will be tested
- Determination of calibration and control procedures based upon the performance specifications verified or established during validation.

Who?

Validation Plan: LDTs and modified FDA-cleared or approved test system

Establishment of performance specifications.

- Accuracy
- Precision
- Analytical sensitivity
- Analytical specificity
- Reportable range of test results for the test system
- Reference interval (normal values)
- Other performance characteristic
  - Stability – specimen, reagent
  - Carryover study

Samples Needed

- How many samples must be used for verification and validation?
  - Type of assay
  - Complexity of the assay
  - Prevalence of the target in the population
  - Data analysis required
  - Established accuracy of the reference method
- What types of samples must be included?
  - Collect in-house specimens needed for method development and validation
  - Request specimens from other states or laboratories
  - Positive and negative controls with known genotypes
- Same sample matrix

How?

Validation against:

- Manufacturer ranges and recommendations
- Expected results as previously established
- Parallel with accepted previous method

Accuracy

- To confirm that the assay can detect the targeted genotype/molecular marker
- Determined by testing ten normal and ten positive specimens, CDC Controls, and CDC Proficiency Testing (PT) specimens to assess for accuracy
- Accuracy is acceptable if 95% of the samples tested match the expected result interpretation with no false negatives

Precision

- To confirm that the assay can return the same result throughout the reportable range, regardless of variations in testing conditions that can cause random error
- Determined by assessing the day to day, run to run, and within run variation, as well as technician variability.
  - Testing 10-20 CDC controls (normal and positive) in triplicate over 5 days
- Precision is acceptable if repeat testing of the samples over time gives consistent clinical interpretation (e.g. >95% agreement or %CV less than 5%) and should not be time or technologist dependent.

Analytical Sensitivity

Limit of Blank (LoB)

- The highest TREC measurement result likely to be observed for a blank sample

- Determined by repeated analysis of SCID positive specimens using a minimum of 60 replicates

- Calculated as the mean value of the blank plus 1.65 times the standard deviation of the blank (the 95th percentile of the extrapolated TREC concentrations)

Analytical Sensitivity (Cont)

Limit of Detection (LoD)

- The lowest TREC quantity that can be detected with 95% probability.

- Determined by repeated analysis of the calibrator materials and their dilutions at TREC levels near the LoB. Test a minimum of 20 replicates at each dilution.

- Calculated by using direct probit analysis to determine the TREC concentration at which 95% of the replicate results are above LoB.

Analytical Sensitivity (Cont)

Limit of Quantification (LoQ)

- The lowest TREC quantity amplifies with at least 95% probability and has a coefficient of variation (CV) of no more than 50%.

- Determined by repeated analysis of the calibrator materials and their dilutions at TREC levels near the LoD. Test a minimum of 20 replicates at each dilution.

- Calculate the %CV at each dilution and select the lowest TREC quantity that amplifies with at least 95% probability and has a %CV of ≤50%.

Clinical Sensitivity

- Calculated by dividing the number of true positives by the sum of true positives plus false negatives.
- [TP/ (TP+FN)] x 100%
- Acceptable if clinical sensitivity is ≥95%.

Analytical Specificity

- The ability of a test or procedure to correctly identify or quantify an entity in the presence of interfering or cross-reactive substances that might be expected to be present.
- Components in clinical specimens and DNA extraction solutions have been reported to interfere with PCR
- No false positive/negative results - low or no amplification of the patient's reference gene reported as Unsatisfactory.

Analytical Specificity (cont)

EDTA and Heparin

- To determine if EDTA or Heparin interferes with the assay, a single source of blood is spiked with various concentrations of EDTA or Heparin, spotted onto a filter card, and tested using real-time qPCR.

- A one-way ANOVA test can be performed using the EDTA or Heparin concentration as the independent variable and RNaseP Ct value as the dependent variable. No interference exists if p value is > 0.05.

Clinical Specificity

- Calculated by dividing the number of true negatives by the sum of true negatives plus false positives. The acceptable criteria for diagnostic specificity must be ≥95%.

- [TN/ (TN+FP)] x 100%

- Acceptable if clinical specificity is ≥95%.

Reportable Range

- To confirm or establish Reportable Range
  - Qualitative tests would include all reportable outcomes (e.g. homozygous wild type, heterozygous or homozygous variant).
  - Quantitative tests, define the analytical measurement range (AMR)
- Run and graph the calibration curves with linear regression analysis
- The reportable range for TREC is defined as from the LoQ to the highest point on the standard curve. SCID screening results are reported qualitatively, with TREC values within reference range reported as normal. Values that are out of reference range are reported “Abnormal”, “Borderline” or “Unsatisfactory”.

Reference Interval

- To verify or establish Reference Interval

- Determined by testing and analyzing a minimum of 6,000 newborn DBS samples from the regular daily collection.
  - Mean, median, standard deviation, and percentile values from different birth weight and age categories.
  - Provisional cutoff values ~12.5% of population median

- Provisional cutoff is acceptable if at least 95% of the screening results match the previously determined result interpretation and 100% true positives remain positive.

Stability Study

- To determine if the TX newborn screening criterion of 13 days after Date of Collection is applicable for SCID screening and how long the TREC and RNaseP markers are stable at room temperature storage.
- Eighty eight newborn specimens received within 1 or 2 days after Date of Collection are tested on the same day (Day 1), Day 5, Day 8, Day 13, Day 19, Day 26, Day 40, month 3, month 6, month 9, and month 12.
- The average TREC and RNaseP Ct values are calculated and plotted against time to assess TREC and RNaseP stability.

Carryover Study

- Determine potential cross contamination caused by using the same puncher head to punch samples without cleaning between punches by punching 8 blank filter paper spots (CP) after punching 80 newborn DBS specimens
- No cross contamination has occurred during the punching if the TREC quantities for all CPs are below LoQ, and the RNaseP Ct values are above the cutoff.

	1	2	3	4	5	6	7	8	9	10	11	12
A		patient	patient	patient	patient	patient	patient	patient	patient	patient	patient	CP
B		patient	patient	patient	patient	patient	patient	patient	patient	patient	patient	CP
C		patient	patient	patient	patient	patient	patient	patient	patient	patient	patient	CP
D		patient	patient	patient	patient	patient	patient	patient	patient	patient	patient	CP
E		patient	patient	patient	patient	patient	patient	patient	patient	patient	patient	CP
F		patient	patient	patient	patient	patient	patient	patient	patient	patient	patient	CP
G		patient	patient	patient	patient	patient	patient	patient	patient	patient	patient	CP
H		patient	patient	patient	patient	patient	patient	patient	patient	patient	patient	CP

Carryover Study (cont)

- Determine potential cross contamination during extraction and assay by testing plates with alternating newborn specimens with no template control in a checker board pattern
- No crossover contamination has occurred if at least 95% of wells containing blank filter paper spots (CP) have TREC quantities less than LoD and RNaseP Ct values above cutoff.

	1	2	3	4	5	6	7	8	9	10	11	12
A		patient	CP	patient	CP	patient	CP	patient	CP	patient	CP	patient
B		CP	patient	CP	patient	CP	patient	CP	patient	CP	patient	CP
C		patient	CP	patient	CP	patient	CP	patient	CP	patient	CP	patient
D		CP	patient	CP	patient	CP	patient	CP	patient	CP	patient	CP
E		patient	CP	patient	CP	patient	CP	patient	CP	patient	CP	patient
F		CP	patient	CP	patient	CP	patient	CP	patient	CP	patient	CP
G		patient	CP	patient	CP	patient	CP	patient	CP	patient	CP	patient
H		CP	patient	CP	patient	CP	patient	CP	patient	CP	patient	CP

Precision – Instrument Comparison

- Testing five identical 384-well RT-qPCR plates containing DNA extract from ten normal and ten abnormal specimens, CDC Controls, and CDC PT specimens. Each instrument runs one of the five plates at the same time.

- Results from four instruments are compared to results from the previously validated instrument.
  - Comparison is acceptable if 95% of the clinical interpretations of all samples and controls match between the five instruments.
  - Comparison is acceptable and no significant difference exists among the instruments if the p value is > 0.05 when a two-tailed t-Test of the Ct values for controls and calibrators from each instrument is performed.

Documentation

- A written assessment of each component of the validation or verification study, including the acceptability of the data
- A signed approval statement - Lab Director review and approval
- If data include discordant results, there must be a record of the discordance and investigation of any impact on the approval of the test for clinical use

Verification Plan: FDA-cleared or approved test system

- Per manufacturer's instructions
- Demonstrate that it can obtain performance specifications comparable to those established by the manufacturer for the following performance characteristics:
  - Accuracy.
  - Precision.
  - Reportable range of test results for the test system.
- Verify that the manufacturer's reference intervals (normal values) are appropriate for the laboratory's patient population.

Determination of Control Criteria

- Pass/Fail Criteria – established during validation study
  - Parameters
    - Specific PCR product bands
    - Specific DNA fragments
    - Quantity or Ct of reference gene
    - Quantity or Ct of targeted marker
  - Threshold
    - Presence or absence of DNA bands
    - Above or below LoB
    - Above or below cut-offs
    - Within Mean± 2SD, Mean ± 3SD, or Mean± 10%
  - % of controls acceptable
  - Impact the entire run or only affected samples

Determination of Calibrator Criteria

- Within Mean± 2SD, Mean ± 3SD, or Mean± 10%
- Slope, R2, and Y-intercept of Calibrator curve

Mean TREC Standard Curve Ct's for all 41 assays							
Standard Points	STD 1	STD 2	STD 3	STD 4	STD 5	STD 6	STD 7
TREC copies/µL WB	3719	953	500	234	109	63	31
Mean TREC Ct	28.828	30.842	31.647	32.732	33.821	34.769	35.823
SD	0.213	0.235	0.250	0.270	0.350	0.466	0.608
%CV	0.74%	0.76%	0.79%	0.82%	1.03%	1.34%	1.70%

LIMS Validation

Number	Test Action	Pass	Fail & Accept	Date	Initials	Comments
1	**Login to the Test System, Result Viewer and Life Cycle**					
2	**Multipuncher - SCID Plates**					
2.1	Create plates with plate maps in puncher workstation, including dummy plates	❑	❑			n/a
2.2	Plates show up on Multipuncher with correct plate maps	❑	❑			n/a
2.3	Completed plates show up in Result Viewer - in Create Work list	❑	❑			n/a
2.4	Work list created successfully in Result Viewer	❑	❑			n/a
2.5	Work list imported into Biomek FX (L:Data>PCR_Jack>From SG folder)	❑	❑			n/a
3	**Panthera - SCID Plates**					
3.1	Login to the Test System for the Panthera	❑	❑			n/a
3.2	Create plates with plate maps in Panthera workstation, including dummy plates	❑	❑			n/a
3.3	Plates show up on Panthera with correct plate maps	❑	❑			n/a
3.4	Punch standards and controls and unload on Panthera	❑	❑			n/a
3.5	Punch samples on Panthera	❑	❑			n/a
3.6	Verify repunching wells on Panthera	❑	❑			n/a
3.7	Verify disabling/enabling wells & custom punches on Panthera	❑	❑			n/a
3.8	Verify plates started on Panthera can be loaded and modifed on the multipuncher & vice versa	❑	❑			n/a
3.9	Punched plates on Panthera show up in Result Viewer - in Create Work list	❑	❑			n/a
3.10	Work list created successful with Panthera plates in Result Viewer	❑	❑			n/a
3.11	Panthera plates Work list imported into Biomek FX (L:Data>PCR_Jack>From SG folder)	❑	❑			n/a
4	**Result Viewer - Assays**					

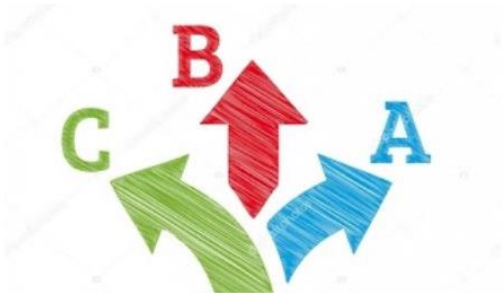
TEXAS Department of State Health Services

Decision Matrix

https://goo.gl/forms/TF4UDJcHZsMSev3t2

Newborn Screening Sequencing Decision Matrix

Considerations for Sequencing Decision Matrix Design:
Decision matrix begins at the level of 2nd (or 3rd) tier testing:



Take Home Messages….

- Be familiar with CLIA and CAP requirements and CLSI guidelines on validation
- Know your assay – FDA approved or not, quantitative or qualitative
- Planning is the most important step
- Need help? Just ask