L1 Run Acceptance

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Interdependencies with other teams

- S1: Small Molecule Run Acceptance
- L2: Assay Specific Operation
- L3: Assay Formats
- A3: Method Transfer

In scope

- Identify the parameters to be used for monitoring validity of the data
  - Accuracy, precision for standard curve calibrators in pre-study validation and during sample analysis
  - Curve editing /Anchor Points
  - Accuracy, precision and total error for Quality Controls in pre-study validation and during sample analysis
- Preparation of Standards and QCs
  - Fresh or Frozen QCs/Standards during validation
  - Preparation of Controls
  - Preparation of Standard Curve Calibrator

Out of scope

- Stability of QC long term during sample analysis

Validation (Pre-Study) Recommendations

Standard Curve Calibrators: A&P Runs in Pre-Study Validation

- No changes to current practice
- Within a run: Evaluate each calibrator: Accuracy: RE = 75% of calibrators within 20% of nominal (LLOQ/ULOQ 25%) - this assumes the LLOQ and ULOQ are calibrator concentrations on the curve
- If fewer than 75% are not acceptable, reject and repeat the run to assess the performance QCs
  - Report the run and the reason it was rejected
- Cumulative table: RE and CV of replicates = ≤15% (≤ 20% at LLOQ concentration) at any standard level

Editing the Curve

- Editing/masking calibrators to obtain an acceptable curve is permitted - 75% of calibrators (minimum of 6) must be acceptable
- A step-wise approach to editing the curve - objective and documented
- First- assess precision as a measure of reliability (comparable to how unknowns are assessed), Second – assess accuracy.
- Re-run the regression after each editing - document each assessment
- Assess the accuracy of the remaining calibrators
- If you consistently have to mask standard calibrators, then the method warrants a critical examination
- Electronic capture to document audit trail is idea

Anchor Points

- Handle consistently and objectively

- Due to the nature of curve fitting, there may need to be calibrators (anchors) outside the range of the curve
  - Do not touch anchor points if curve is acceptable
  - If curve is not acceptable anchor points may be masked if there is some *a priori* approach defined
  - These are not held to the same criteria. In some cases, it may be helpful to mask anchor points, as long as there are criteria specified. In other cases, masking them does not help.

Pre-Study Validation A&P QCs

- At least 5 QC samples should be used to assess accuracy, precision and the total error of the method
  - anticipated LLOQ; Low QC = ≤ 3 times the LLOQ; Mid QC; High QC = 70-80% ULOQ; anticipated ULOQ
  - Suggestion - place the LQC above the second std, and the HQC below the second highest std, can serve to protect the asymptotes.

- Validation should be representative of the actual study samples analysis, i.e. if a study sample is reported as a mean of two replicates, then validation QCs should be treated similarly

- Measurements should be made across at least 6 independent assay runs over several days by more than one analyst
  - Balanced design
  - If only one analyst will conduct the study, then only one needs to qualify

Assessment of Validation Controls (QCs) (continued)

- Analyze all runs using an appropriate statistical method determining both intra and inter-assay precision and accuracy. (see table VIIa in DeSilva for an example)

- For a method to be considered acceptable, it is recommended that the inter-batch precision (%CV) and the absolute mean bias (%RE) both be </=20% (25% at LLOQ).

- The total error (i.e. sum of absolute value of the % relative error and % coefficient of variation) should not exceed 30% (40% at LLOQ and ULOQ).

Concept of Total Error

- TE expresses the closeness of agreement between a measured test result and it theoretical true value by describing the combination of systematic (mean RE) and random (%RE) error components (Findlay)
- ** Note - % CV is not on the duplicate wells of a single validation sample, but to the variability of the validation samples

Table VIIA. Precision and Accuracy Numerical Example. Replicate Results are Analytical Data from an Immunoassay for a Therapeutic Protein. Statistics were Calculated in a Excel Spreadsheet by an Analysis of Variance (ANOVA). Symbolic Notation for all Data Values are Listed in Table VIIB with Formulae Defined in Appendix A.

		Replicate results			Intrabatch (within-run) statistics					
Sample	Batch run	1	2	3	n	Mean	SD	%CV	%RE	Ancillary statistics
QC 4 50 (ng/mL)	1	47.6	48.1	52.2	3	49.3	2.52	5.0	−1.4	MS_w = 9.320
	2	42.0	41.4	43.7	3	42.4	1.19	2.4	−15.3	MS_b = 59.444
	3	72.4X	53.1	45.8	2	49.5	5.16	10.3	−1.1	MS_t = 24.984
	4	53.4	55.3	54.5	3	54.4	0.95	1.9	8.8	s_t = 4.998
	5	45.6	42.6	51.5	3	46.6	4.53	9.1	−6.9	s_b = 4.213
	6	46.5	42.3	40.8	3	43.2	2.95	5.9	−13.6	p = 6
	Intrabatch (within-run) statistics (Pooled):				2.88	47.4	3.05	6.1	5.1	
	Interbatch (between-run) statistics (ANOVA):				17	47.5	5.20	10.4	−5.0	

X—Analytical error, value omitted from computations.

Total Error

- Target acceptance for validation samples (QCs) in pre-study validation typically is a very liberal ± 20% RE and ≤20% CV (variability between the validation sample means).

- There is a disconnect between the liberal target acceptance for pre-study method validation and more stringent 4/6/20 acceptance for sample analysis.

- The 20% in the 4/6/20 rule possesses an inherent Total Error.

- Our acceptance for QCs monitored during in-study sample analysis is based on a fixed interval of +/- 20% of accuracy (nominal) and precision (the difference **between** the means of the replicates).

Bridging the Inconsistency

- Should a method *just* meet the validation criteria above - expect to see many failed runs during sample analysis when we apply the more stringent 4/6/20 rule (four out of six QC must be at or below 20% accuracy and precision

- To bridge this disconnect De Silva suggests that a method should target a total error of 30% i.e., if the method approaches both 20% accuracy and 20% precision (or a TE of 40%), one those parameters must be improved to a point where they total 30% (not 40 %)

- Moving forward - If you are willing to accept a method closer to the 30% or higher, you will likely fail a large number of sample analysis runs. Be prepared to defend why this method is acceptable

Sample Analysis

Standard Curve - Sample Analysis

No change from current practice:

- Minimum of 6 acceptable calibrators
- If either of the high or low standards is deleted, the assay range for this particular run will be limited to the next standard point and samples out of range must be repeated.
- Each run: >75% of calibrators within ≤ 20% on nominal (≤25%LLOQ/ULOQ) - this assumes the LLOQ and ULOQ are also calibrator concentrations on the curve.

- A standard point may be edited from a curve using the same criteria established during pre-study validation. (If one replicate of the standard point is “out” then the whole calibrator is lost)

Standard Curve - Sample Analysis

- Since we have validated the assay range, we may add calibrators within that range without re-validating the whole curve.

- Specific criteria for editing must be documented in method. In addition, it is recommended that the method total error (sum of the %CV and absolute %RE) be ≤30% (40% at the LLOQ) to be consistent with the pre-study validation acceptance criteria.

- All runs must be documented but only acceptable runs are included in the summary table and statistics.

Quality Controls - Sample Analysis

Again, no change from current practice:

- After the standard curve is assessed and passes then assess the QCs
- Each QC is reported and assessed as a mean of replicate wells
- Three concentrations (Low, Mid and High) are assayed twice, (4 wells = 2 reported values) which are used to assess the validity of a run (after the standard curve has passed)
- At least two thirds of all QC results from one run must be within 20% (accurate and precise) with at least one valid QC at each concentration

Best Fit, Preparation of Standards and QCs

Best fit

- Typically 4 or 5 PL sigmoidal regression
- Investigate during development and select best fit prior to validation; assessed using the back calculated values
- Cannot change *algorithm and weighting* for sample analysis after being validated

- Preparation of curves
- Each analyst spikes their own standards to support the balanced design (if one analyst prepared it the assays would not be independent of each other).
- While it could lead to higher variability it should reflect what is being done during sample analysis.
- In fact, it is a good idea to plan backwards from how samples will be analyzed during the clinical trial and apply that logic to the validation samples.

Best Fit, Preparation of Standards and QCs

- When standard calibrators are prepared in bulk, stability must be established.
- Test fresh and compare against frozen.
- If a sub-stock is needed, that must also be tested for stability.
- QCs are prepared, verified and frozen since they reflect the unknown samples.
- QCs prepared separately from standard calibrators

Assessing the Means of Replicates

- Some companies do not mean the replicate values of the calibrator curve but instead let Watson or other software package regress the curve. It is noted that, in some cases, the regression program does weight one value more than another.

- Some assays employ triplicates (not duplicates) and will assess the three values individually in order to have the option of dropping a value to “save” a calibrator point, prior to assessing the mean

- Alignment: It is agreed that there is no scientific reason to require the calibrator replicates be meaned prior to regression. There appears to be no significant difference in the outcome. It is necessary to be objective and consistent in the process, especially when masking a value.

Next Steps

The team still needs to:

Finalize the discussion on the use/acceptability of fresh or frozen Standards and QCs during validation

Understand the interdependencies of the L1 with:

- S1: Small Molecule Run Acceptance
- L2: Assay Specific Operation
- L3: Assay Formats
- A3: Method Transfer