Assay Development and Method Validation Essentials

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Fundamental to all aspects of drug development and manufacturing are the analytical methods. Analytical methods require development, validation and controls just as all other product and process development activities. Measurement of API characteristics, the factors that influence them and key impurities is at the heart of product development for efficacy and safety.

A systematic approach for analytical method development and validation is discussed in this paper and was developed in line with the International Conference of Harmonization (ICH) Q2(R1), Q8(R2) and Q9 guidelines.

Historically insufficient attention has been paid to assay development, how it impacts the product, on-going release testing and product control. Simple coefficient of variation (CV) calculations for assay precision is a necessary but insufficient measure of assay goodness and may be misleading as CV has no relationship to product acceptance and release testing limits.

Assays and measurement systems must be viewed as a process. The measurement process is made up of methods, software, materials and chemistry, analysts, sample preparation, environmental conditions and instrumentation/equipment. Quality risk management and statistical data analysis techniques should be used to examine the process of measurement and identify factors that may influence precision, accuracy, linearity, signal to noise, limits of detection and quantification and/or any other assay attributes to achieve optimal assay results.
Based on ICH guidance and the author’s experience the following ten steps are recommended for analytical development and method validation:

1. Identify the purpose of the analytical method (characterization/release) and all critical quality attributes (CQAs)
2. Select the appropriate analytical method aligned with CQAs and development objectives
3. Identify the process steps associated with the method
4. Determine all specification limits associated with release testing
5. Perform a risk assessment as to where assay development is needed
6. Characterize the method (accuracy/bias/linearity etc.)
   a. System design (right technology/ right chemistry)
   b. Parameter design (set points)
   c. Tolerance design (allowable variation)
7. Complete method validation tests
   a. Define the method validation requirements
   b. Make sure representative materials are used for the evaluation
   c. Conduct all method validation tests
   d. Achieve acceptable results for method validation of all analytical methods
   e. Determine if the analytical method is fit for use and ready to transfer
8. Define the control strategy for each method
9. Train all analysts on the method
10. Determine the impact of the analytical method on process variation, validation and product acceptance rates.

These steps are covered in detail below.

1. **Identify the Purpose**
   Make sure the purpose of the analytical method is clear. Will it be used for release testing and or for product/process characterization only? What are the target product profile parameters (ICH Q8(R2)) and CQAs the analytical method is associated with? Are there any CQAs that have no clearly defined measurement method? What impurities need to be measured and what is the risk of not measuring them? Is the assay correlated with other analytical methods? How orthogonal is each assay to other assays used to evaluate the product. How does the assay minimize or influence risk during drug development and manufacturing?
2. **Select the Analytical Method**
   There are many analytical methods. Make sure the method selected has appropriate selectivity and has high validity. Valid analytical methods measure the condition of interest. It is possible to have good precision with poor measurement validity. For example, it is possible to measure the quantity of a protein without knowing how active the protein is. Measures of activity and measures of quantity need to be correctly considered and balanced against other objective measures of the product.

3. **Identify all Steps in the Analytical Method**
   Lay out the flow or sequence used in the analytical method. Using Visio or some other process mapping software lay out and visualize the sequence and flow used in performing the assay. This will be used for development, documentation, risk assessment and training. Make sure all steps are listed and detailed as to the flow and use of plates, materials and chemistry. Identify steps in the process that may influence bias or precision.

4. **Determine Product Specification Limits**
   For those analytical methods that will be used for release testing, what are the specification limits that will be used to control the release of the product? Limits may be set from historical data, industry standards, based on statistical k sigma limits and/or tolerance intervals and or based on a transfer function. Limits need to reflect the risk to the patient, CQA assurance and control the flow of materials in the production of the drug substance and drug product.

5. **Perform a Risk Assessment**
   The analytical method risk assessment is used to identify areas/steps in the analytical method that may influence precision, accuracy, linearity, selectivity, signal to noise etc. Specifically the risk question is "Where do we need characterization and development for this assay?"

   FMEA and or other risk assessment methods may be used when performing a risk assessment. In addition to the traditional FMEA approach of failure mode, severity, probability and detectability, we need to add Influence on CQA and uncertainty to the risk ranking. Specific questions of what may influence precision and or what may influence bias or accuracy need to be examined. Each step in the analytical method should be looked at from this point of view.
6. Characterize the Method

Based on the risk assessment define the development/characterization plan for the assay. Determination of sample size and sampling method are key considerations. Assay development can be broken into three steps 1) system design, 2) parameter design and 3) tolerance design. System design is making sure we have the right chemistry, right materials, right technology, and right equipment. Parameter design is usually done by running DOEs and making sure we have the right parameters selected at their optimal design set point. Characterization of the design space for precision and accuracy is a key assay development outcome. Finally the allowable variation for key steps in the assay must be defined to assure a consistent outcome. Partition of variation (POV) (Little, T.A.) analysis is recommended to further breakdown precision variation into the factors that influence it. Plate variation for example must be considered when developing analytical methods. Failure to understand plate variation and other sources of assay error will directly mix into the total variation and will be linearly added to product variation and will increase limits of quantitation and detection effectively reducing the assay range and will add to out of specification rates for product acceptance testing.

7. Complete Method Validation and Transfer

Define the method validation requirements. There are many measures of measurement performance (for example amount of API, activity of API and impurities) that may be used in method validation (see figure 4). Make sure there is a clear identification of the requirements for each method when organizing the validation plan. Figures 4, 5 and 6 are adapted from Q2(R1) and identify the requirements to complete a method validation.

Representative DS and DP materials should be used during method validation. Representative materials and standards will assure the limits of detection and quantitation have been correctly calculated and validated and will perform well when measuring and testing actual product.

Conduct all method validation tests with the correct sample size and sampling method as defined in the method SOP. Achieve acceptable results for method validation of all analytical methods. Make sure acceptance criteria have been defined for each validation method variable, modify/improve aspects of the assay so it will pass the validation testing criteria. Finally, it is necessary to determine whether the analytical method is fit for use and ready to transfer to other internal organizations or to external CRO/CMOs. This is
determined by meeting all acceptance criteria for precision, bias, linearity etc. Equivalence tests are typically used for method transfer.

<table>
<thead>
<tr>
<th>Method Validation List</th>
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</thead>
<tbody>
<tr>
<td>Specificity</td>
</tr>
<tr>
<td>Linearity</td>
</tr>
<tr>
<td>Range</td>
</tr>
<tr>
<td>Accuracy</td>
</tr>
<tr>
<td>Precision</td>
</tr>
<tr>
<td>Repeatability (Intra Assay)</td>
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<tr>
<td>Intermediate Precision (Inter)</td>
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<tr>
<td>Reproducibility (Inter Lab)</td>
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<tr>
<td>Detection and Quantitation Limits</td>
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<tr>
<td>Visual</td>
</tr>
<tr>
<td>S/N</td>
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<tr>
<td>LOD, LOQ (RMSE*K)/Slope</td>
</tr>
<tr>
<td>Robustness</td>
</tr>
<tr>
<td>System Suitability</td>
</tr>
<tr>
<td>Method Transfer Equivalence</td>
</tr>
<tr>
<td>ATP OOS Impact</td>
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</tbody>
</table>

**Figure 4. Method Validation List**

<table>
<thead>
<tr>
<th>Assay Characterization</th>
<th>Specificity</th>
<th>Linearity</th>
<th>Range</th>
<th>Accuracy</th>
</tr>
</thead>
<tbody>
<tr>
<td>Definition</td>
<td>Understanding of the factors that influence the mean and standard deviation/CV of the assay result which allows an accurate statement on the content or potency of the analyte in a sample.</td>
<td>The linearity of an analytical procedure is its ability (within a given range) to obtain test results which are directly proportional to the concentration (amount) of analyte in the sample.</td>
<td>The range of an analytical procedure is the interval between the upper and lower concentration (amounts) of analyte in the sample (including these concentrations) for which it has been demonstrated that the analytical procedure has a suitable level of precision, accuracy and linearity.</td>
<td>The accuracy of an analytical procedure expresses the closeness of agreement between the value which is accepted either as a conventional true value or an accepted reference value and the value found.</td>
</tr>
</tbody>
</table>

**Figure 5. Method Validation Quick Reference Guide**
8. Define the Control Strategy
Once the assay has been developed and validated a clear control strategy needs to be put in place (PAT). What materials will be used for control or reference materials? How do you know the standards are stable? What will be used for tracking and trending the assay so the true assay/plate variation is known over time? What will be used to adjust/correct the assay once drift is detected? How will you transition from one set of reference materials to another?

9. Train All Analysts
Train all analysts using the validated analytical method. If there is concerns that the analyst may have an effect on the results of the analytical method make sure each analyst runs qualification tests using known reference standards in order to qualify and certify the analyst on the method. Analyst method errors may include sample selection, sample prep, weighing, mixing, diluting, concentrating, location of peak, injection method, time variation, examination of gels, etc.

10. Determine the Impact of the Analytical Method
Total variation is expressed in the following equation:

\[
\text{Stdev Total} = \sqrt{\text{Product Variance} + \text{Assay Variance}}
\]

As the assay error rises the total standard deviation also rises. Using the accuracy to precision (ATP) model it is possible to visualize the relationship of precision and accuracy on product acceptance rates. The ATP model shows how changes in precision and accuracy impact product acceptance rates and the assay error design space. CV
calculation is a good measure of assay error; however, it is not scaled to the acceptance limits, it is scaled to the mean. Rescaling the variation to the release limits helps to clarify if the variation in the assay is fit for use. The number 5.15 is used in the equation to represent 99% of the assay error. Generally a percent of tolerance of less than 20% is considered an acceptable result; more than 20% will result in a high level of out of specification release failures and should be considered for further development.

\[
\% \text{ Tolerance Measurement Error} = \frac{\text{Stdev Measurement Error} \times 5.15}{\text{USL} - \text{LSL}}
\]

where USL=Upper specification limit and LSL = lower specification limit.

Figure 7. Accuracy to Precision Modeling

The attention paid to method development, validation and control will greatly improve the quality of drug development, patient safety and predictable, consistent outcomes.

References:

ICH Q2(R1) Validation of Analytical Procedures: Text and Methodology, 2005
ICH, Q8(R2) Pharmaceutical Development, 2009
ICH Q9 Quality Risk Management, 2006