Chemistry Specifications for Chemistry Support Contractors

Division of Translational Toxicology (DTT)

Biosample Analysis

1. ***Biosample Method Development and Analysis (BMDA)***

   1. <u>General Requirements</u>

      1. A biosample method development plan (BSMP), including a milestone schedule shall be developed and posted to the DTT IMS for approval by the COR.

         1. The BSMP shall describe the general analytical approach to the analysis of the chemicals, test articles, biotransformation products, biomarkers of exposure or effect, and/or other specified substances (analyte) in a specified biological matrix, or matrices, including, but not limited to the following.

            1. Literature reference(s) that serve as a starting point for the biosample method development.

            2. A list of the proposed analytical techniques, including method parameters, if known, e.g., chromatographic separation mechanism or polarity.

         2. A milestone schedule shall be developed and posted to the DTT IMS, which includes dates for one or more of the following milestones, at the direction of the COR:

            1. Commencement of lab work

            2. Completion of lab work

            3. Completion of Draft Final report

            4. Commencement of QC review

            5. Commencement of QA review

            6. Submission of Draft Final report

      2. BMDA work described in Part 2.2 *ff.*, cannot commence until COR-approval of the BSMP is received by the Contractor.

      3. All Quality Control (QC) standards shall be prepared and stored in containers similar to those used for storage of the samples to be analyzed.

   2. <u>Analysis Requirements</u>
   As directed by the COR, the Contractor shall perform one or any combination of the following activities:

1. *Method Development*

    1. The Contractor shall develop or adapt from another source an analytical method for an analyte(s) in a specified biological matrix.

    2. The Contractor shall employ an analytical method suitable for small sample sizes (typically 100 µL for biological fluids, typically plasma or whole blood, or 200 mg for tissues).

    3. The method shall be developed to quantitate the analyte(s) in a biological matrix with an LLOQ specified by the COR (typically 1-5 ng/mL for biological fluids, or 5-10 ng/g for tissues).

    4. Control matrix used to develop the method shall be from adult male Sprague-Dawley rats, unless otherwise specified by the COR. Secondary matrices shall be incorporated into the method via matrix evaluations (see BMDV section 2.3.6).

2. *Sample Analysis Requirements*

    1. Upon receipt of samples to be analyzed, or on a date specified by the COR, the Contractor shall prepare spiked QC stability standards in blank sample matrix.

        1. Blank sample matrix used for QC stability standards shall be the same tissue(s) from the same species, strain(s), and sex as the samples to be analyzed. If blank matrix that meets this criteria cannot be obtained an alternate matrix may be used with prior approval of the COR.

        2. QC stability standards shall be prepared at 150% of the lowest standard concentration in the analytical method range and stored with the samples.

        3. Sufficient QC stability standards shall be prepared to allow for at least duplicate analyses on each analysis day.

    2. The Contractor shall quantitate the analyte(s) in a biological matrix using the method developed in Part 1.2., above.

        1. The Contractor shall prepare QC matrix standards in the same matrix as the samples at two concentrations for each analytical run.

1. QC matrix standards shall be prepared at the low and at the high end of the method calibration range.

2. Sufficient QC matrix standards shall be prepared to allow for at least duplicate analyses on each analysis day.

3. If two calibration curves are needed to cover the expected analytical concentration range, QC matrix standards are to be prepared in each range.

2. The Contractor shall analyze a matrix standard curve, prepared at a minimum of six concentrations, in duplicate with the samples.

3. The Contractor shall analyze one set of three QC stability standards (Part 1.2.2) on each analysis day to assess the stability of the stored samples.

   1. On the first day of analysis the Contractor shall prepare and analyze one set of 3 QC stability standards to serve as Day 0 concentration standards.

   2. Day 0 QC stability standards shall be prepared from freshly prepared stock solutions, unless the stability of the stock solutions is known.

4. The Contractor shall analyze QC matrix standards covering the analytical concentration range during the analysis of each set of samples.

5. The Contractor shall analyze all submitted samples singly Samples found to be above the standard curve concentration range may be diluted into the range with blank matrix.

6. At the direction of the COR, the Contractor shall analyze 10% of the submitted samples for each dose group, in each matrix, as incurred samples.

   1. The Contractor shall use the incurred sample results to estimate the reproducibility for the assay in each matrix using a Bland-Altman plot including limits of

agreement (see http://en.wikipedia.org/wiki/Bland-Altman_plot)

3.   Additional Requirements

1.   The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

2.   Biosample Method Development and Validation (BMDV)

1.   *General Requirements*

1.   A biosample method development plan (BSMP), including a milestone schedule shall be developed and posted to the DTT IMS for approval by the COR.

1.   The BSMP shall describe the general analytical approach to the analysis of (an) analyte(s) in a specified biological matrix, or matrices, including, but not limited to the following.

1.   Literature references or previously developed methods that serve as a starting point for the biosample method development.

2.   A description of the proposed analytical techniques, including method parameters, if known, e.g., chromatographic system, column type, and sample preparation and cleanup.

2.   A milestone schedule that includes dates for one or more of the following at the direction of the COR:

1.   Commencement of lab work

2.   Completion of lab work

3.   Completion of Draft Final report

4.   Commencement of QC review

5.   Commencement of QA review

6.   Submission of Draft Final report

2.   BMDV work described in Part 2.2 *ff.*, cannot commence until COR-approval of the BSMP is received by the Contractor.

2. *Analysis Requirements*

At the direction of the COR, the Contractor shall perform the following biological sample development work.

1. *Method Development and Validation*

    1. Method Development

        1. The Contractor shall develop a method to quantitate a chemical, test article, biotransformation products, and/or other specified substances (analyte) in a specified biological matrix.

            1. Control matrix used to develop the method shall be from adult male Sprague-Dawley rats, unless otherwise specified by the COR. Secondary matrices shall be incorporated into the method via matrix evaluations (see Section 2.3.6).

        2. The Contractor shall employ an analytical method suitable for small sample sizes (typically 100 µL for biofluids, typically plasma or whole blood, or 200 mg for tissues).

        3. The Contractor shall evaluate analytical methods available from the literature or other sources, including previously developed methods, to determine the lowest feasible concentration that may be measured.

            1. Feasibility shall be defined as that concentration at which %RSD is < 20%. Higher values for %RSD may be accepted but require advanced approval from the COR.

            2. Typically, analytical methods will be developed with an LLOQ of 1-5 ng analyte/mL biofluid or 5-10 ng/g tissue or other matrix.

        4. It is acceptable to use multiple standard curves to cover the required range.

    2. Validation

        1. General Requirements

            1. The Contractor shall develop a validation plan and submit it to the COR for approval prior to starting a

method validation. The validation plan shall describe the work to be done and the acceptance criteria that shall be applied to the validation results.

2. The validation must be performed such that it provides the following information:

    1. An indication of the precision of the method at specified concentrations.

    2. Confirmation by statistical and visual inspection that the response versus concentration function is linear, or deterministically non-linear, over the specified concentration range.

    3. An indication of blank matrix contribution to responses seen at the lowest spiked matrix concentrations.

    4. An estimate of recovery (percent) of the analyte from the matrix at specified concentrations.

    5. A determination of the accuracy of the method.

    6. A determination of the matrix effect for mass spectrometry or other relevant detectors.

    7. A determination of the within day, between day, and total variability.

    8. Estimates of the measurement limits [i.e. Limit of Detection (LOD) and Lower Limit of Quantitation (LLOQ)].

3. When a validated method exists for a similar tissue or biological fluid in the same species, the Contractor may perform a partial validation (Part 2.2.1.5.3) in lieu of a full validation (Part 2.2.1.2). If the partial validation fails, a full validation must be performed.

2. Solvent Standard Preparation

    1. Prepare single standards, in addition to the blank, at a minimum of 6 concentrations in the extracting solvent for use as a solvent-standard calibration curve.

2. Use 2 independently prepared stock standards of different concentrations (Stock A and Stock B) to prepare the solvent-standards.

3. Solvent standards shall be prepared over a > 10-fold concentration range.

3. Matrix Standard Preparation

1. Matrix shall be prepared using primary control matrix (default: adult, male SD rat), or as designated by the COR.

2. Matrix Calibration Curve

1. Prepare triplicate spiked standards at a minimum of 6 concentrations, in addition to the blank, in the biological matrix for use as a matrix calibration curve.

2. The matrix standard curve shall cover the same concentration range as the solvent standards.

3. Use 2 independently prepared stock standards of different concentrations (Stock A and Stock B) to prepare the matrix standards.

4. Prepare at least 6 replicate matrix standards at the expected lowest quantifiable matrix standard concentration.

5. If uncertainty exists with respect to the lowest matrix standard concentration 6 replicates may be prepared at several, NTE 5, low concentrations.

6. Prepare 2 sets of 6 matrix blanks, one set with (method blank) and one set without (matrix blank) internal standard (IS). Replicate each of the 2 sets in 3 lots of blank matrix from at least 2 sources.

4. QC Sample Preparation

1. QC samples shall be prepared in the target matrix at three concentrations corresponding to 150% the LLOQ, a midrange concentration, and 80% of the high calibration standard.

2. QC samples may be prepared from a single stock, but the stock shall be a different stock than those used to prepare calibration standards.

3. At least 9 QC samples shall be prepared at each concentration to ensure that enough QC samples are available for analysis.

5. Analysis Sequence
Validation requires evaluation of the standard curve and short-term stability of the prepared samples during the analysis period. Experiments for evaluation of these components are described below. Components may be run concurrently whenever possible to minimize the time required to complete the validation.

1. Evaluate matrix effects using matrix QC standards prepared at a minimum of 3 concentrations, which cover the expected concentration range.

2. Evaluation of the Standard Curve (ESC)

1. ESC General Requirements

1. LOD and LLOQ values and reproducibility of the IS response must be determined.

2. Linearity and reproducibility of the standard curve must be determined over a minimum of 3 analytical runs on different days.

3. Precision, accuracy, and recovery of the standard curve must be established and matrix effects must be determined.

4. A minimum of 9 QC samples shall be incorporated into each run.

    1. QC samples shall be analyzed in triplicate.

    2. QC samples shall be incorporated at 3 concentrations covering the expected concentration range of the samples in the analytical run.

2. ECS Experiment 1

    1. Part 1: Establish LOD and evaluate selectivity

        1. Analyze the method blanks prepared under Part 3.2.6. Matrix Standard Preparation on a single analysis day.

        2. Analyze the matrix blanks prepared under Part 3.2.6. Matrix Standard Preparation on a single analysis day.

        3. Evaluate the response of the method blanks to determine LOD.

        4. Evaluate the response of the matrix blanks to determine selectivity.

    2. Part 2: Establish LLOQ

        1. Analyze the set(s) of low matrix standard replicates on a single analysis day.

        2. Evaluate the precision and accuracy of the replicate matrix standard set(s) to determine LLOQ.

3. Part 3: Evaluate the reproducibility of the IS

    1. Evaluate the IS response in the method blanks analyzed in Experiment 3, Part 1, and calculate the mean response and %RSD.

    2. Evaluate the IS response in Experiments 1 and 2 and calculate the within-day and between-day variability.

3. ESC Experiment 2
   Performed to establish the linearity, precision, and accuracy of the response; recovery of the analytes; carry-over; and provide a check on the stability of the analytical system.

    1. Analyze the solvent standard calibration curve prepared in Part 2.2.1.2.2, above.

    2. Split the processed matrix standard solutions prepared in Part 2.2.1.2.3, above into two sets. Run one set of matrix standards at the beginning of the analytical run and the other set at the end.

    3. Analyze 3 method blanks run after the high matrix standard.

    4. Evaluate the data for absolute recovery.

    5. Evaluate the data for instrument drift based on the split matrix curve.

    6. Evaluate the standard curve (matrix and solvent) for linearity. Non-linear regression models may only be used with prior approval of the COR.

7. Evaluate the replicate matrix standards for precision.

8. Evaluate the method blank response for carry-over.

4. ESC Experiment 3
Performed to establish the reproducibility of the matrix standard curve.

   1. Prepare and run a single matrix standard curve on two different analysis days.

   2. On one of the two analysis days, prepare and analyze a 6-point standard curve prepared in extracted matrix.

   3. Evaluate the data from Experiments 1, 2, and 3 to determine the reproducibility of the matrix standard curve.

3. Analysis-Period Stability (APS)
Performed to establish stability of the processed matrix samples over the period of analysis and under conditions of storage, which may be expected to occur during the analysis period

   1. Preparation of APS QC Samples

      1. Prepare 4 sets of at least 4 replicate matrix standards at each of 3 concentrations bracketing the calibration curve concentration range. Each set will consist of 12 matrix standards (4 replicates x 3 concentrations).

      2. Extract all 4 sets of APS QC standards prepared above using the established sample preparation procedure for the method being validated.

      3. Analyze Set 1 extracts on the same day that it is prepared.

4. Place Sets 2, 3, and 4 extracts at one of three different temperature regimes as follows:

    1. Set 2: Store in autosampler vials at room temperature (RT) in the light.

    2. Set 3: Store in autosampler vials at 1–5°C in the dark.

    3. Set 4: Store such that the APS samples undergo 3 freeze–thaw cycles over a period of time equal to the longest expected storage period for extracted samples.

2. APS General Requirements

    1. Set 1 of the APS samples must be analyzed on the same day it is prepared.

    2. Sets 2– 4 of the APS samples may be analyzed in any order provided their storage requirements are met.

        1. Analyze Set 2 of the APS samples after they have been stored for a period of time equal to the longest expected analysis run time for the largest sample set anticipated to be run at one time.

        2. Analyze Set 3 of the APS samples after they have been stored for a period of time equal to the longest expected storage period for extracted samples.

        3. Analyze Set 4 of the APS samples on the same day that they were thawed for the third time.

3. All APS samples must be analyzed with a standard curve.

4. APS experiments may be run concurrently with the ESC experiments.

5. Evaluate Set 2 samples compared with Set 1 samples to establish the stability of the samples under ambient conditions in autosampler vials.

6. Evaluate Set 3 samples compared with Set 1 samples to establish the stability of extracted samples under refrigeration in the dark.

7. Evaluate Set 4 samples compared with Set 1 samples to establish the stability of samples that have been repeatedly thawed.

6. Additional Validation Requirements

    1. High Concentration Method Verification

        1. The Contractor shall demonstrate that matrix samples with concentrations at least 10-fold higher than the validated range, or at a concentration specified by the COR, can be diluted with matrix into the validated calibration range.

        2. The Contractor shall use matrix QC standards prepared near the highest anticipated concentration outside the validated range to perform the verification.

    2. Ruggedness (Intermediate Precision)

        1. Ruggedness, sometimes called Intermediate precision, is the precision obtained over time using the same method on identical test

material, and may involve different analysts, equipment, reagents, and laboratories.

2. When method transfer is anticipated as an issue, a second analyst, instrument, or column may be used as a check on the ruggedness of the method.

3. All ruggedness analyses performed must be discussed in the report and the data generated must be handled appropriately.

3. Method Evaluation

    1. Matrix evaluation: The COR may direct the Contractor to do additional studies to establish the use of a previously validated method (section 2.2.1.2, above) for analysis of a secondary matrix to assess the potential for interferences to be present.

    2. Method evaluation: The COR may direct the Contractor to assess the effect of a change, which does not fundamentally alter the method, but may result in an altered instrument response; e.g., similar column from different manufacturer or a different instrument model or type.

    3. Preparation of Matrix Standards and Blanks

        1. Prepare 6 replicate matrix standards at a concentration corresponding to 200% of the LLOQ for the validated method in the secondary matrix.

        2. Prepare 6 replicate matrix blanks using secondary control matrix from a minimum of 3 lots, or as specified by the COR.

        3. Prepare 6 replicate method blanks using secondary control matrix from a minimum of 3 lots, or as specified by the

COR to which internal standard has been added.

4.   Matrix Standard Curve Preparation

     1.   Prepare a minimum of three independent standards in duplicate in the primary matrix that bracket the nominal concentration of the secondary matrix samples (typically ½X, X, and 2X, where X is the nominal concentration of the secondary matrix sample prepared in step 2.2.1, above).

5.   Analysis Requirements

     1.   Analyze the primary matrix standard curve in duplicate.

     2.   Analyze the secondary matrix samples and blanks, along with the method blanks.

     3.   Evaluate the secondary matrix samples for precision and accuracy, and for interferences that alter the response of the analyte in the matrix.

     4.   Evaluate the secondary matrix blanks for interferences at the retention times of the analyte(s) and/or IS.

     5.   Evaluate the secondary method blanks for interferences that alter the response of the IS in the matrix.

     6.   If the matrix evaluation fails, a Partial Validation (section 5.4, below) is required.

4.   Partial Validation

     1.   The COR may direct the Contractor to do additional studies to establish the use of a

previously validated method (section 2.2.1.2, above) for a secondary matrix. Partial validation confirms that samples from a secondary matrix may be analyzed using a method that has been validated for a primary matrix.

2. A partial validation must assess the recovery, precision and accuracy of the method across the expected concentration range and determine the method limits (LOD and LLOQ) in the secondary matrix, characterize matrix effects on instrument response and/or recovery for the secondary matrix, and establish the blank response for the chemical or test article in the secondary matrix.

3. The partial validation is run on the secondary matrix(ces) using the procedures given in section 4.2.2.5.4.4. Partial Validation Procedure, below.

4. Partial Validation Procedure

    1. Solvent Standards Preparation

        1. Prepare solvent standards with the same final concentrations (i.e., after any extraction, dilution, or concentration step) as the primary matrix standards.

        2. Single replicates of each solvent standard shall be prepared from one stock solution. Use the same standard stock solution (Stock A or Stock B) prepared for the full validation, or a freshly prepared stock at the same concentration, to prepare the solvent standards.

        3. Measure the solvent standard responses using the same

analytical system used for the full validation in the primary matrix.

2. Matrix Standards Preparation

1. Sample preparation methods are typically based on those used for the primary matrix, but may deviate as needed to optimize extraction efficiency and method performance.

2. Prepare six primary matrix standards in triplicate, from 2 independently prepared stock solutions, at concentrations bracketing the proposed analytical range.

3. Prepare 6 replicate standards in the secondary matrix at a concentration corresponding to the LLOQ for the validated method in the primary matrix.

4. Using one of the 2 primary stock standards, prepare 3 replicate standards in the secondary matrix at each of three higher concentrations: 200% of the LLOQ for the primary matrix, 75% of the high primary matrix standard, and a concentration corresponding to the middle of the primary matrix standard curve.

5. If a matrix evaluation has not been performed and is not planned, Prepare 2 sets of 6 matrix blanks, one set with (method blank) and one set without (matrix blank)

internal standard (IS). Replicate each of the 2 sets in 3 lots of blank matrix from at least 2 sources.

3.   Analysis Requirements

    1.   Analyze the solvent standard curve.

    2.   Analyze the primary matrix standard curve in duplicate at the beginning and end of the run, using the validated method for the primary matrix.

    3.   Analyze the secondary matrix standards using the validated method for the primary matrix. Analytical parameters may be altered to optimize method performance, with COR approval.

    4.   Analyze the 6 replicate LLOQ secondary matrix standards.

4.   Partial Validation Evaluation Requirements

    1.   Evaluate the secondary matrix standards for accuracy, precision and recovery. Determine recovery compared to the solvent and primary matrix standards.

    2.   Evaluate the responses of the secondary matrix LLOQ standards to determine the LLOQ for the secondary matrix.

    3.   When applicable, evaluate the responses of the secondary method blanks to determine the LOD and the reproducibility of the

internal standard in the secondary matrix. Evaluate the response of the secondary matrix blanks to determine selectivity.

4. If the partial validation fails, a full validation may be required at the direction of the COR.

5. Partial Validation Method Verification

1. When the highest validated matrix concentration is less than 150% of the highest expected matrix concentration, perform a High Concentration Method Verification (Part 4.2.2.1.2.5.1, above) in the secondary matrix(ces).

7. See Part 4.3. Calculations for required validation calculations and Part 4.4. Acceptance Criteria for typical acceptance values for each calculated parameter.

1. *Stability Evaluation*

1. General Requirements

1. The stability of the target analyte(s) in the primary or secondary biological matrix shall be established for 60 days under the conditions at which the samples are to be stored prior to analysis.

1. Stability study time points shall be scheduled to give information about short-term and long-term (up to 60 days) stability.

1. Sufficient QC standards shall be prepared and stored to cover triplicate analysis at time points that include, but are not limited to 0, 15, 30, and 60 ± 2 days.

      2. Typical storage conditions include ≤ -70ºC, protected from light.

      3. Stability shall be evaluated against matrix QC samples prepared on Day 0.

  2. The recovery of the analyte from the matrix after a minimum of 3 freeze-thaw cycles shall be evaluated. The evaluation period shall not exceed 60 days without COR approval.

  3. Stability of the analyte solvent stock solutions shall be evaluated under their recommended conditions of storage, prior to their reuse.

2. Analysis Requirements

  1. Quality Control (QC) stability samples shall be prepared at 75% of the highest standard and 150% the LLOQ in the validated concentration range.

  2. Sufficient QC stability samples shall be prepared and stored to allow for at least triplicate analyses for each storage condition at each scheduled time point.

  3. QC stability samples shall be analyzed in triplicate using the same validated analytical method as that used for study samples.

  4. Calibration for the analysis of QC stability samples shall be performed using a minimum of 3 independent matrix standards that bracket each of the concentrations of the stability samples. The default for the matrix curves is the primary matrix, unless the secondary matrix does not pass partial validation.

  5. The 3-point matrix standard curve shall be analyzed in duplicate at the same time as the QC stability samples with one replicate curve analyzed at the beginning and end of an analytical sample set.

  6. Analyte solvent stock standards shall be analyzed over a time point range that brackets their period of use

       7. When storage of samples for periods > 60 days is anticipated, the COR may direct that an Extended Stability Study be run (Part 2.2.4.4. Extended Stability Study).

2. *Calculations*

    1. The Contractor shall use curve-fitting techniques employing a linear regression model with or without weighting. Under certain circumstances it may be possible to demonstrate that the analytical system is reproducibly non-linear. In these cases, with the approval of the COR, the Contractor may utilize a non-linear regression model (e.g., power curve).

    2. Once the model has been chosen, the Contractor shall perform the following calculations:

       1. Calculate the regression equations for the matrix and solvent standard curves. Do not correct the response for experimental blank values. For spectrophotometric determinations, zero the instrument using the solvent only, and then compare the blank value with the Y-intercept obtained from the regression equation.

       2. Calculate the correlation coefficient r for the solvent- and matrix-standard curve data.

       3. To estimate precision, calculate the mean and percent relative standard deviation (%RSD) for each matrix standard analyzed in at least triplicate.

       4. Assess within day, between day, and total variability.

       5. Determine the percent recovery at each concentration according to the following equation:

$$\%\text{Recovery} = [(Ym_{ij} - Ym_{\text{Bar-blank}}) \div (Ys_{ij} - Ys_{\text{Bar-blank}})] \times 100 \qquad [1]$$

where Ym_{ij} is the response for each of the matrix standards and Ys_{ij} is the response for each of the solvent standards, $Ym_{\text{Bar-blank}}$ is the mean response for the matrix blank and $Ys_{\text{Bar-blank}}$ is the response for the solvent blank. Do not calculate recovery for blanks.

       6. Determine the accuracy (relative error) of each found (calculated) concentration compared with each nominal (prepared) concentration as follows:

          1. Calculate the concentration from the measured responses for the matrix standards using the regression equation.

2.   Calculate the relative error using the following equation:

%Relative Error = $[(Xm^f_{ij} - Xm^n_{ij}) \div Xm^n_{ij}]$ X 100   [2]

Where Xm^n_{ij} is the nominal concentration of each matrix standard and Xm^f_{ij} is the found concentration of each matrix standard calculated from the regression equation.

3.   For matrix standards prepared and analyzed in at least triplicate, compute the relative error for the mean response in addition to that for the individual standards.

7.   Determine the measurement limits as defined below:

1.   LOD shall be defined as 3 times the standard deviation of the blank expressed as concentration. If the blank does not give a meaningful response, use 3 times the standard deviation of the lowest standard, expressed as concentration.

2.   LLOQ shall be defined as the concentration of the lowest standard that meets acceptability criteria of the assay and should correspond to the low matrix standard.

3.   *Acceptability Criteria*

1.   *Linearity or Conformance to the Model*

1.   Standard curve fitting is determined by applying the simplest model that adequately describes the concentration–response relationship using appropriate weighting and statistical tests for goodness of fit.

2.   Requirements

1.   The correlation coefficient r shall be ≥ 0.99 for linear fits.

2.   When coefficient of determination r^2 is an appropriate measure of goodness of fit, it must be ≥ 0.98.

3.   To achieve an acceptable fit, a data point may be discarded <u>only after</u> it has been shown to be statistically valid to do so. Normally this involves a Q-test of all the replicates at that concentration.

2.   *Accuracy (Relative Error)*

1.   Relative error shall be ≤ ± 15% of the nominal value for all concentrations above the LLOQ.

2. Higher relative error values may be accepted but will require the approval of the COR

3. *Precision (Relative Standard Deviation)*

    1. Relative standard deviation shall be ≤ ± 15% of the mean value for all concentrations above the LLOQ.

    2. Higher relative standard deviation values may be accepted but require the approval of the COR.

4. *Absolute Recovery*

    1. The method shall be considered to be acceptable when recovery at each matrix standard concentration is > 50% relative to the solvent standard curve.

    2. The lowest percent recovery value shall not differ from the highest percent recovery value by > 20 percentage points.

    3. When recovery is ≤ 50% at any matrix standard concentration, the COR must be consulted for approval.

5. Relative Recovery

    1. The method shall be considered to be acceptable when recovery at each matrix standard concentration tested is > 80%.

6. *Selectivity*

    1. Any peak eluting at the retention time of the analyte or internal standard shall be < 30% of the LLOQ response.

7. *Carry-over*

    1. Non-zero carry-over values must be approved by the COR.

8. *Measurement Limits*

    1. Acceptability criteria for measurement limits are dependent on the study and shall be provided by the COR on a case-by-case basis.

    2. Typically, the relative error shall be ≤ ± 20% of the nominal value at the LLOQ with a relative standard deviation (%RSD) ≤ 20%.

9. *Stability*

    1. Samples shall be considered to be stable when their measured precision and accuracy is not statistically different from the established validation criteria.

10. QC Standards

　　1. At least 67% of the QC samples' analyzed during an analytical run shall have concentration results within 15% of their nominal values (for QC samples prepared at the LLOQ this requirement may be set to 20% with approval of the COR).

　　2. At least 50% of the QC samples analyzed at each concentration level shall be within 15% of their nominal concentrations (for QC samples prepared at the LLOQ this requirement may be set to 20% with approval of the COR).

　　3. A confidence interval approach yielding comparable accuracy and precision in the run is an acceptable alternative to the requirements set forth in Parts 10.1 and 10.2 (above).

4. *Additional Requirements*

　　1. SOPs pertaining to the analysis of biological samples from a study shall be submitted once the biological sample method development work is completed.

　　2. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

3. **Biological Sample Analysis (BSA)**

1. *General Requirements*

　　1. A milestone schedule shall be developed and posted to the DTT IMS before BSA work (described below in Part 3.2 *ff*.) can commence. The milestone schedule shall include dates for some or all of the following at the direction of the COR:

　　　　1. Commencement of lab work

　　　　2. Completion of lab work

　　　　3. Completion of Draft Final report

　　　　4. Commencement of QC review

　　　　5. Commencement of QA review

　　　　6. Submission of Draft Final report

2. *Analysis Requirements*
    As directed by the COR, the Contractor shall perform the following
    activities:

    1. Upon receipt, biological samples to be analyzed shall be stored
        under conditions that have demonstrated stability. If stability has not
        been demonstrated, samples shall be stored at $\leq -70°C$ unless prior
        approval is obtained from the COR to store samples at a different
        temperature.

    2. Upon receipt of biological samples to be analyzed, the Contractor
        shall prepare QC stability standards in blank sample matrix at 75% of
        the highest standard concentration and 150% of the lowest standard
        concentration (LLOQ) in the validated analytical method range and
        store them with the samples. If the validated analytical method range
        is not known, QC stability standards shall be prepared at
        concentrations specified by the COR.

        1. QC stability standards shall be stored in the same containers,
            or containers made of similar material and under the same
            conditions as the study samples.

        2. Sufficient QC stability standards shall be prepared to allow for
            triplicate analyses on each analysis day.

        3. Blank sample matrix used for QC stability standards shall be
            the same tissue(s) from the same species and strain(s) as the
            samples to be analyzed. If blank matrix that meets this criteria
            cannot be obtained an alternate matrix may be used with prior
            approval of the COR.

    3. The Contractor shall prepare matrix[1] QC standards in the same matrix
        as the samples at two concentrations for each analytical run.

        1. The low concentration matrix QC standard shall correspond to
            the second-lowest matrix standard concentration in the
            validated analytical method.

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[1] If the method has shown that there is no matrix effect, QC and calibration standards may
be prepared as solvent standards, with the prior approval of the COR.

2. The high concentration matrix QC standard shall correspond to the second-highest matrix standard concentration in the validated analytical method.

3. If two calibration curves are needed to cover the expected analytical concentration range, matrix QC standards are to be prepared in each range.

4. Sufficient matrix QC standards shall be prepared to allow for at least duplicate analyses on each analysis day.

5. Matrix QC standards shall be stored in the same containers, or containers made of similar material and under the same conditions as the study samples.

4. The Contractor shall analyze one set of QC standards at each concentration on the day of their preparation to serve as a Day 0 reference point.

   1. Analysis shall employ a validated method when available.

   2. If no validated method is available, a qualified method may be used.

5. The Contractor shall quantitate chemicals, test articles, biotransformation products, and/or other specified substances in biological sample matrices, using previously developed and validated methods.

   1. Matrix QC standards, as defined in Section 3.2.3, above, are to be processed with each batch of study samples. The total number of QC matrix standards analyzed shall be ~ 10% of the number of the study samples.

   2. Matrix QC standard results shall be considered acceptable if they meet the variability parameter established in the method validation (i.e., when the validated method has shown a variability of 20%, the QC's run using that method, shall also have a variability that does not exceed 20%).

   3. If QC matrix standards fail to meet the acceptance criteria, the contractor shall immediately notify the COR.

6. The Contractor shall analyze one set of three QC stability standards (see Part 3.2.2.) at each concentration on each analysis day to assess the stability of the stored samples.

7. The Contractor shall analyze a 6-concentration matrix standard curve in duplicate with the samples.

8. The Contractor shall analyze 10% of the submitted samples for each dose group, in each matrix, as incurred samples.

    1. The Contractor shall use the incurred sample results to estimate the sample reproducibility for the assay in each matrix using a Bland-Altman plot including limits of agreement (see http://en.wikipedia.org/wiki/Bland-Altman_plot).

3. *Additional Requirements*

    1. The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.

4. *Extended Stability Study (EXS)*

1. *General Requirements*

    1. When stability at ≤ –70ºC has been established for short time periods (≤ 60 days), QC extended stability standards shall be stored at –20ºC. If stability is not known, or long-term instability is suspected, QC extended stability standards shall be stored at ≤ –70ºC.

    2. Blank sample matrix used for extended stability QC standards shall be the same tissue(s) from the same species and strain(s) as the samples to be analyzed. If blank matrix that meets this criteria cannot be obtained an alternate matrix may be used with prior approval of the COR.

2. *Analysis Requirements*

    1. At the direction of the COR the contractor shall conduct extended stability studies for chemicals, test articles, biotransformation products, and/or other specified substances in a variety of biological matrices.

2. The Contractor shall prepare spiked QC extended stability standards in blank sample matrix[2].

    1. Extended stability QC standards shall be prepared at 2 concentrations, 75% of the highest standard concentration and 150% of the lowest standard concentration (LLOQ) in the validated analytical method range. If the validated analytical method range is not known QC stability standards shall be prepared at concentrations specified by the COR.

    2. Sufficient extended stability QC standards shall be prepared to allow for at least triplicate analyses at each time point over the anticipated storage period.

3. Extended stability QC standards shall be stored in the same containers, or containers made of similar material and under the same conditions as study samples.

    1. When the container to be used for study samples is unknown, vial selection depends on the sample type:

        1. Store spiked tissue QC extended stability standards in capped vials of an appropriate size and composition for the tissue.

        2. Store spiked plasma QC extended stability standards in vials as small as possible, vial materials to be used are chemical dependent and shall be approved by the COR prior to use.

4. Extended stability QC standards shall be analyzed using the same validated analytical method as that used for study samples.

5. Calibration for extended stability QC standards shall be performed using a minimum of 3 independent matrix standards that bracket the concentration of the stability samples.

6. The matrix standard curve shall be analyzed in duplicate at the same time as the extended stability QC standards with one replicate analyzed at the beginning and end of an analytical sample set.

7. To establish stability analyze triplicate extended stability QC standards at each concentration, using a validated method, on days 0, 60, 90 ± 1-week

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[2] If the method validation has shown that there is no matrix effect, QC and calibration standards may be prepared as solvent standards, with the prior approval of the COR.

and at least 90-day intervals thereafter, up to the maximum anticipated storage time for the study samples.

3.   *Additional Requirements*

   1.   The contractor shall report the work done in this functional activity following the reporting requirements given in Section 4. Reporting Requirements.