Workshop on ICH M10

L01 - Dilutional Linearity & Parallelism

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Introduction to Round table C6

What's in ICH M10…?

4.2.6 Dilution Linearity and Hook Effect

- Due to the narrow assay range in many LBAs, **study samples may require dilution in order to achieve analyte concentrations within the range of the assay**. Dilution linearity should be assessed to confirm:
  - (i) that **measured concentrations are not affected by dilution within the calibration range** and
  - (ii) that **sample concentrations above the ULOQ of a calibration curve are not impacted by hook effect** (i.e., a signal suppression caused by high concentrations of the analyte), whereby yielding an erroneous result.

- The **same matrix as that of the study sample** should be used **for preparation of the QCs** for dilution.

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Introduction to Round table C6

What's in ICH M10…?

4.2.6 Dilution Linearity and Hook Effect

- Dilution linearity should be demonstrated by generating a dilution QC, i.e., **spiking the matrix with an analyte concentration above the ULOQ**, analysed undiluted (for hook effect) and **diluting this sample (to at least 3 different dilution factors) with blank matrix** to a concentration within the calibration range.
- For each dilution factor tested, **at least 3 independently prepared dilution series** should be performed using the number of replicates that will be used in sample analysis.
- The absence or presence of response reduction (hook effect) is checked in the dilution QCs and, if observed and unable to be eliminated with reasonable measures, steps should be taken to mitigate this effect during the analysis of study samples.
- The calculated mean concentration for each dilution should be within ±20% of the nominal concentration after correction for dilution and the precision should not exceed 20%.
- The **dilution factor(s) applied during study sample analysis should be within the range of dilution factors evaluated during validation**.

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Introduction to Round table C6

What's in ICH M10…?

7.2 Parallelism

- **Parallelism is defined as a parallel relationship between the calibration curve and serially diluted study samples to detect any influence of dilution on analyte measurement**.

- Although lack of parallelism is a rare occurrence for PK assays, **parallelism of LBA should be evaluated on a case-by-case basis**, e.g., where interference caused by a matrix component (e.g., presence of endogenous binding protein) is suspected during study sample analysis.

- **Parallelism investigation** or the **justification for its absence** should be included in the Bioanalytical Report.

Introduction to Round table C6

What's in ICH M10…?

7.2 Parallelism

- As parallelism assessments are rarely possible during method development and method validation due to the unavailability of study samples and parallelism is strictly linked to the study samples (i.e., an assay may have perfectly suitable parallelism for a certain population of samples, yet lack it for another population), **these experiments should be conducted during the analysis of the study samples**.

- **A high concentration study sample (preferably close to Cmax) should be diluted to at least three concentrations with blank matrix.**

- **The precision between samples in a dilution series should not exceed 30%. However, when applying the 30% criterion, data should be carefully monitored as results that pass this criterion may still reveal trends of non-parallelism**.

- In the case that the sample does not dilute linearly (i.e., in a non-parallel manner), a procedure for reporting a result should be defined a priori.

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Pre-meeting survey

	The question
Q1	Do you assess dilutional linearity in a single run with ≥3 dilution series, or over several different runs? Do you spike one control for all series, or separate controls for each series?
Q2	For DL assessment (and sample analysis) do you dilute samples in pooled matrix prior to MRD, or do you use alternative methods to conserve matrix (e.g., MRD then additonal dilutions in x% matrix buffer, use surrogate matrix, dilute in buffer, other...)?
Q3	In addition to the UHQC at ≥anticipated Cmax, do you include a second UHQC sample (e.g., 5-10x ULOQ) that will fall in range with lower dilution factors? If so , why?
Q4	Do you define the exact dilutions that can be applied in sample analysis, or a range of dilutions that can be used?
Q5	Are you assessing parallelism as a standard method validation parameter? If so, at what stage in the project? If not, how do you determine cases where parallelism assessment is needed?
Q6	How many samples are required to assess parallelism?
Q7	How do you handle (trends of) non-parallelism? What procedures for reporting results are used if samples do not dilute linearly?

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Key message from the pre-meeting survey comments

- **Dilutional Linearity & Hook Effect**
  - Reasonably well aligned in our approaches regarding number of assessments, spike levels, etc.
  - More diversity in some aspects:
    - Applying fixed dilution to sample analysis rather than range
    - Include second (lower conc) control to have low dilutions (e.g., 5, 10) falling into range
    - Dilution in 100% matrix pre-MRD vs %matrix buffer post-MRD

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Key message from the pre-meeting survey comments

- **Parallelism**
  - Is this the new ISR…? Appears to be run as a tick-box exercise, rather than case-by-case…

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Raw data from the pre-meeting survey comments

- In the next slides we provide the unredacted details from 56 survey files reaching us prior to the deadline.
- Surveys that have arrived after the deadline could not be included anymore, for logistic reasons. Please speak up if your comment wasn't already captured in the other 56 files…

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Questions on Dilutional Linearity (& Hook Effect)

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Q1: Do you assess dilutional linearity in a single run with ≥3 dilution series, or over several different runs? Do you spike one control for all series, or separate controls for each series?

We do DL in a single run. We spike one control for all series.	in a single run with ≥3 dilution series. Separate controls for each series	we adapted our approach based on M10	Single run. One sample is spiked, aliquoted, and different aliquots are used for independent dilution series. - Comment: "in a single run with ≥3 dilution series", dilution factors are measured, not the dilution series	dilutional linearity in a single run with ≥3 dilution series

≥3 independent dilution series analyzed in a single run	One OQC as 3 independently prepared dilution series in one run.	a single run with 3 dilution series	Single run. Same.	single run with at least 3 dilutions	single run, 3 separate dilution series

Q1: Do you assess dilutional linearity in a single run with ≥3 dilution series, or over several different runs? Do you spike one control for all series, or separate controls for each series?

separate preps	single run, one control	Y - We will use this approach with single run from now on; separate spike for each dilution series	same assay, separate controls	LBA not implemented in our lab	single run, 3 separate dilution series

depending on space (one or more runs), separate controls	Typically, a single run is used to assess dilution linearity. Multiple spikes are done.	single run	Single run. Several controls (as we use multiple dilution factors) or one, depending on protocol.	same assay, separate controls

depends on plate wells availability, one control	Y, one run, one control	A Single run with 3 different dilution factors. Use one spike control (the Dilution QC) for all dilution factors. However, all Dilution factors are independent prepared and not diluted in a series.	Ideally over several runs with separate spiked controls where possible	minimum of 2 runs. One preparation of the controls diluted separately

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Q2: For DL assessment (and sample analysis) do you dilute samples in pooled matrix prior to MRD, or do you use alternative methods to conserve matrix (e.g., MRD then additional dilutions in x% matrix buffer, use surrogate matrix, dilute in buffer, other...)?

We spike in matrix and dilute with MRD during sample prep.	dilute samples in pooled matrix prior to MRD	dilution in buffer	In general: first MRD, then additional dilutions in MRD% pooled matrix; rare disease matrices are tested and replaced by surrogate (HV) matrix, if possible, but in same manner - Some assays require surrogate matrices or specifically the MRD step as last dilution	Test both approaches. Only use the second approach in bioanalysis if first approach is working

Preferred option (larger pipetting volumes, thus better accuracy) is a first dilution to MRD in buffer, then all following dilutions in buffer plus x% matrix to maintain a stable matrix conc.	Serial dilutions in pooled matrix and final dilution in buffer to MRD also possible	Both	Depending on method, surrogate is not always possible.	MRD then additional dilutions in buffer

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Q2: For DL assessment (and sample analysis) do you dilute samples in pooled matrix prior to MRD, or do you use alternative methods to conserve matrix (e.g., MRD then additional dilutions in x% matrix buffer, use surrogate matrix, dilute in buffer, other...)?

pooled matrix	dilute before MRD using pooled matrix	combination of both, case by case	Dilute to MRD and additional dilutions in x% matrix buffer	N - Depends per vendor but the preference is MRD followed by matrix buffer

prior to MRD	pooled matrix prior to MRD	Depends on the method. Preferred choice is first MRD, followed by additional dilutions in sample dilution buffers. However, if this fails the alternative method is employed. This is assessed at R&D stage and best path forward determined.	Yes, pooled matrix	Dilute in buffer (if justified in validation).

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Q2: For DL assessment (and sample analysis) do you dilute samples in pooled matrix prior to MRD, or do you use alternative methods to conserve matrix (e.g., MRD then additional dilutions in x% matrix buffer, use surrogate matrix, dilute in buffer, other...)?

prior to MRD	pooled matrix prior to MRD	No, dilute samples to MRD and then in matrix adjusted buffer	Prepare one Dilution QC - prepare dilution factors in matrix - apply MRD before adding to plate. However, if rare matrix MRD can be done prior to dilutions or the use of buffer(surrogate matrix). Which method to use should also be tested in the validation and not just shifted to during the sample analysis.	both pooled matrix and a % matrix in buffer.

Q3: In addition to the UHQC at ≥anticipated Cmax, do you include a second UHQC sample (e.g., 5-10x ULOQ) that will fall in range with lower dilution factors? If so, why?

Not sure would need to check	According to sponsor request, but not generally	Yes, this ensures dilution, not just procedure control	only one at ≥anticipated Cmax	High QC is set at Cmax concentration

Yes, to cover minimum and maximum dilutions, as per historic FDA guidance.	Y, As dilution factors needs to be validated for the actual sample concentrations.	High QC is set at Cmax concentration	Yes - However It depends on the study. If a Preclinical Tox Study I often include more than one Dilution QC in order to have the 5x or 10x dilution factor validated. If diluting the Cmax conc 10x it will often be read above ULOQ. Same also for a First-human-dose study. If a Phase II study and the assay is moved to another lab then most often only 1 dilution QC.	Yes, we've used a UHQC at the estimated Cmax

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Q3: In addition to the UHQC at ≥anticipated Cmax, do you include a second UHQC sample (e.g., 5-10x ULOQ) that will fall in range with lower dilution factors? If so , why?

Can we please use the M10 wording and not invent new wording for the Dilution QC ? M10 says 'Dilution linearity should be demonstrated by generating a dilution QC, i.e., spiking the matrix with an analyte concentration above the ULOQ', and do not say Ultra High QC at ≥ anticipated Cmax. It just say Above ULOQ. Cmax is only mentioned in M10 in connection with ISR and Parallelism. Not in connection with Dilution QC.

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Q4: Do you define the exact dilutions that can be applied in sample analysis, or a range of dilutions that can be used?

Range of dillutions is performed	A range of dilutions	Range	exact	Range of dilution

Range	definition of MRD then application of higher dilutions if necessary to fall within the range of standard curve	Defined. More can be proven.	Not a lot of effort	Range

Exact	range of dilution	range	Y - N See free text	range

no, a range is defined	A range of dilutions.	Y, defined by the highest dilution validated	A range	only the max dilution/ max concentration we can measure after dilution

Range of dilutions, MRD till max validated dilution factor	Yes - define the exact dilution factors upfront based upon modelling data.	Exact dilutions

Questions on Parallelism

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Q5: Are you assessing parallelism as a standard method validation parameter? If so, at what stage in the project? If not, how do you determine cases where parallelism assessment is needed?

Y, after routine sample analysis.	Not as standard. We included this experiment in the ISR.	Yes, as standard during sample analysis. Only exceptions are further sample analyses with same analyte/matrix.	N - depends on the availability of study samples	Yes, dilution parallelism is assessed for all methods as soon as non-clinical or clinical samples are available
Assay validation	Y - During sample assay study	yes, then done with incurred samples as/if needed.	when samples are available, usually in first clinical trial	Yes, first clinical trial
PK end of study, PD start of study	Y at validation	N - depends on the molecule. Currently it is done during sample analysis stage on request of the vendor	only when FIH project or first disease population study, during sample analysis	yes, during sample analysis

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Q5: Are you assessing parallelism as a standard method validation parameter? If so, at what stage in the project? If not, how do you determine cases where parallelism assessment is needed?

Not as standard. We tend to test parallelism only if the method has an endogenous cross reactant or for troubleshooting purposes.	N, not standard but is applied by certain CROs we work with (even when endogenous and standard the same)	As part of justifying surrogate matrix in calibrators. During development or tech transfer.	only when FIH project or first disease population study, during sample analysis
in-study, if not we put an indication why it was not needed	N, only endogenous compounds	No - Add in parallelism if there are many ADA or other matrix related interference (in example if Selectivity is borderline accepted) .	Depend on the project

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Q5: Are you assessing parallelism as a standard method validation parameter? If so, at what stage in the project? If not, how do you determine cases where parallelism assessment is needed?

Yes, after routine sample analysis.	Not as standard. We included this experiemtn in the ISR.	Yes, as standard during sample analysis. Only exceptions are further sample analyses with same analyte/matrix.	No - depends on the availability of study samples	Yes, dilution parallelism is assessed for all methods as soon as non-clinical or clinical samples are available

Assay validation	Y - During sample assay study	yes, then done with incurred smaples as/if needed.	when samples are available, usually in first clinical trial	Yes, first clinical trial

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Q6: How many samples are required to assess parallelism?

so far, 6 samples, will be discussed in future	We test minimum 3 study samples for this parameter.	At least three study samples will be used to create pooled sample	10

6 high concentration study samples from 6 separate subjects	6 individuals	at least 5

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Q7: How do you handle (trends of) non-parallelism? What procedures for reporting results are used if samples do not dilute linearly?

not occured until now	NA, we let the CROs guidelines/SOPs control this	If trends are noticed, more samples are tested for parallelism. If non-parallelism is identified the source of error needs to be evaluated, and if necessary, assay needs to be re-defined and re-validated. So far created sample results need to be evaluated case-by-case.	Up to know we did not have studies where parral;ism failed. if you know reason for failure you can apply fixed dilution	Case by case decision, our SOP states that a procedure for reporting should be defined a priori in the validation report

Example for free assay: Report the concentration determined at lowest dilution factor	Continue method development	Not yet this case	you do not have a method right? Change range?	No fixed criteria for trends of non-parallelism, in this case start investigation to assess impact on results

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Q7: How do you handle (trends of) non-parallelism? What procedures for reporting results are used if samples do not dilute linearly?

case by case - never experienced	No experience Only one case, where in study parallelism on PK was performed, and not linear, these non-linear results were discussed in the report, results at only one dilution were reported.	never happened	Depends on the method. We would typically assess dilutions / concentrations at which point there is lack of parallelism and select sample dilutions to avoid this region of the curve.	Find an MRD that works. Or re-optimize assay.

never happened	our procedure states that we should correct range of dilutions	There are several options. The most conservative is that data are not reported	We would try and understand why the parallelism isn't performing by gaining as much information as possible from troubleshooting. This helps us understand the impact it will have on the data generated.