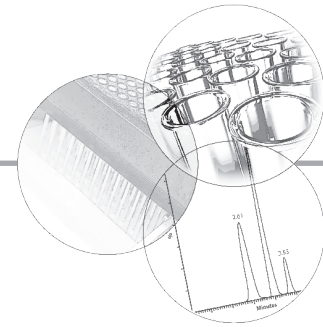

Bioanalytical method requirements and statistical considerations in incurred sample reanalysis for macromolecules

Background: Incurred sample reanalysis (ISR) is the most recent in-study validation parameter that regulatory agencies have mandated to ensure reproducibility of bioanalytical methods supporting pharmacokinetic/toxicokinetic and clinical studies. The present analysis describes five representative case studies for macromolecule therapeutics. **Method:** Single ISR acceptance criteria (within 30% of the averaged or original concentration) and a modified Bland–Altman (BA) approach were used to assess accuracy and precision of ISR results. General concordance between the two criteria was examined using simulation studies. **Results:** All five methods met the ISR criteria. The results indicated that thorough method development and prestudy validation were prerequisites for a successful ISR. The overall agreement between the original and reanalyzed results as determined by BA was within 20%. Simulation studies indicated that concordance between the ISR criteria and BA was observed in 95% of the cases. Dilution factors had no significant impact on the ISR, even for C_{max} samples where 1:100 or higher dilutions were used. **Conclusion:** The current ISR acceptance criteria for macromolecules was scientifically and statistically meaningful for methods with a total error of 25% or less.

Bioanalytical methods used to support toxicokinetic and clinical studies are developed and validated following regulatory agency guidelines for analytical method validation [1,2]. During prestudy validation and implementation (in-study validation), standards and quality controls (QCs) are prepared *in vitro* by adding the analyte to the matrix of intended study samples. The standard and QC preparations are intended to be as similar as possible to the study samples or *in vivo*-derived samples (incurred samples); however, it is always possible that differences may exist. Analytical methods designed to measure either small molecules or macromolecules each have their own challenges. Specifically, the presence of metabolites poses challenges for small molecules, while the specificity of reagents to measure analytes poses challenges for macromolecules. Thus, reagent selection and the strategy of eliminating matrix interference for each matrix type are crucial for method development. Furthermore, thorough method development, optimization and prestudy validation are critical in supporting all regulated studies.

The topic of **incurred sample reanalysis** (ISR) was first discussed at the *Crystal City Meeting III* in 2006, and a subsequent conference report was published [3]. On 7–8 February 2008, the first AAPS workshop on current topics in good laboratory practice bioanalysis, *Assay reproducibility for incurred samples*, was held at Crystal City, VA, USA. At this workshop, regulatory agencies from the USA presented cases where there were discrepancies in results between original and reanalyzed concentrations in multi-analyte assays for small-molecule drugs. The workshop focused on how to conduct ISR and, at the end of the meeting, the workshop committee presented a summary session with a consensus and recommendations [4]. The purpose of performing ISR was to reinforce confidence that bioanalytical methods produce valid and reproducible results. Recently, it has been suggested that ISR can serve as part of a regular process check [5]. Therefore, ISR should be conducted during in-study validation activities.

The workshop recommendations provided general principles for conducting ISRs in all bioequivalence studies and drug–drug interaction studies, as well as studies where pharmacokinetics is the primary end point [4]. These guidelines apply to both preclinical and clinical studies. The guidelines on ISR sample selection criteria recommended analyzing individual samples instead of pooled samples, including more subjects with fewer samples per

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Key terms

Incurred sample reanalysis: Experimental in-study validation parameter to demonstrate that the bioanalytical method will produce reproducible results from study samples when re-analyzed on a separate occasion.

Bland–Altman analysis: Statistical method useful in assessing the agreement between two data sets and in evaluating both systematic and random differences.

Total error: Method acceptance criteria that include the sum of random (measured by coefficient of variation [%CV]), and systematic (measured by %bias) errors.

Matrix effect: Direct or indirect alteration or interference in response due to the presence of unintended analytes or other interfering substances in the sample.

Selectivity: Ability of a bioanalytical method to measure the analyte of interest in the presence of other constituents in the sample. Selectivity is evaluated by spiking the analyte into the matrix at or near the lower limit of quantitation.

Specificity: Ability of reagents used in the bioanalytical method to bind the analyte of interest.

subject, and having at least one sample from C_{max} and one from near the end of the elimination phase. Most importantly, the workshop recommended ISR acceptance criteria for both small- and large-(macro)molecule therapeutics. At least two-thirds of reanalyzed samples must be within 20% of the originally reported results for small-molecule therapeutics, and within 30% of the originally reported results for macromolecules [4]. Later, regulatory agencies have recommended that at least two-thirds of reanalyzed samples must be within 30% of the averaged results for macromolecules (within 20% of the averaged results for small-molecule therapeutics) [6]. This criterion is based strictly on an evaluation of the accuracy of the reanalyzed concentrations and has an arbitrary limit of 67% of samples to be within 20 or 30% of averaged concentration. However, the relevance of these acceptance criteria in setting ISR in relation to assay variability (determined during prestudy validation) was not clear.

Rocci *et al.* suggested that agreement between the original and reanalyzed results for ISR should be evaluated using the assay acceptance criteria and a modified **Bland–Altman** (BA) statistical approach to determine the method reproducibility [7]. An assay acceptance criterion for sample analysis is the **total error** (assay variability) of a bioanalytical method, which includes both random (measured by coefficient of variation [%CV]) and systematic (measured by accuracy) errors determined during prestudy validation using spiked or prepared samples [1]. When using incurred samples, the modified BA approach is particularly useful for determining method reproducibility because it evaluated both random and systematic differences in data sets.

Although a few studies on ISR for small molecules have been published [5,7], little or no information on ISR for macromolecules is available. Of 26 ISRs conducted using conventional criteria on macromolecules so far, herein we present five representative case studies, each associated with one of the following four modalities: humanized monoclonal antibody, peptibody, recombinant protein or fully human antibody. In addition, the case studies represent different assay formats or platforms and encompass various stages of drug development from preclinical to clinical. We then retrospectively evaluated the bioanalytical method reproducibility using a modified BA analysis and the 30% acceptance criteria. The concordance between the recommended ISR criteria (conventional) and the modified BA analysis approach was evaluated.

Experimental

■ Serum specimens

Serum samples used for each method were obtained from Bioreclamation Inc. (NY, USA) and were stored at -70° ± 10°C once received.

■ Bioanalytical methods

Modality and bioanalytical methods information are listed in **Tables 1 & 2**.

For the colorimetric methods (Method A, B, D and E), the intensity of the color (optical density [OD]) was measured at 450 nm minus the OD at 650 nm using a Molecular Devices Spectra (Danahar Corp., Washington, DC, USA) max 340PC microtiter plate reader equipped with SoftMax Pro software.

For the electrochemiluminescence methods (Method C), signals (electrochemiluminescence unit) were read on the MSD®

Table 1. Background summary of each method for each case study.

	Method A	Method B	Method C	Method D	Method E
Case study	***1***	***2***	***3***	***4***	***5***
Modality	Humanized IgG	Peptibody	Humanized IgG	Protein	Fully human IgG
Detection mechanism	Colorimetric	Colorimetric	Planar electrochemiluminescence	Colorimetric	Colorimetric
Species/matrix	Human serum	Human serum	Cynomolgus monkey serum	Rat serum	Human serum
Assay format	Sandwich Anti-Id/anti-Id	Bridging Anti-pep/anti-pep	Sandwich Anti-Id/anti-Id	Sandwich Anti-protein/ poly-anti-protein	Sandwich Ligand/poly anti-Id
Validated	Yes	Yes	Yes	Yes	Yes

Anti-Id: Anti-idiotypic antibodies against analyte of interest; Anti-pep: Antibody against the peptide region of the peptibody; Anti-protein/poly-anti-protein: Monoclonal antibody against protein as capture reagent and polyclonal antibody against protein as detection reagent.

Table 2. Assay parameters and performance results of each method during prestudy validations.

	Method A	Method B	Method C	Method D	Method E
Assay range	50–2498 ng/ml	15–1500 pg/ml	200–40,000 ng/ml	40–2000 ng/ml	20–2000 ng/ml
Sensitivity	50 ng/ml	15 pg/ml	200 ng/ml	40 ng/ml	20 ng/ml
Method accuracy (QC: %bias)	-9 to 3	20	-1 to 3	2 to 7	-3 to 1
Method precision (QC: %CV)	<13	<13 (<21% at LLOQ)	<11	<18	<13
Matrix effect and selectivity in total serum lot†	None (20 lots)	None (10 lots)	None (6 lots)	None (10 lots)	None (33 lots)
Assay criteria	20%	15% (30% at LLOQ)	15%	25%	15%

†Indicates that at least 80% of unspiked and spiked samples recovered within the assay acceptance criteria to conclude that there is no matrix effect, and the method is selective.
CV: Coefficient of variation; QC: Quality control.

Sector Imager 6000 equipped with Discovery Workbench™ software (MSD; MD, USA). The conversion of OD or electrochemiluminescence units for the study samples and the QC to concentrations was achieved through a computer software-mediated comparison against a standard curve assayed on the same plate, which was regressed according to a four- or five-parameter logistic (Auto-Estimate) regression model with validated weighting factor of 1, 1/Y or $1/Y^2$ using the Watson™ data-reduction package (Thermo Scientific; MA, USA) depending on each method.

Accuracy & precision

The robustness and ruggedness of each method were tested by having multiple analysts perform a total of at least six assays with minimum and maximum incubations over 3–7 days using different preparations of standard and validation/QC samples during prestudy validations. Final assay acceptance criteria for both standards and QCs were set based on the accuracy and precision results and are listed in **Table 2**.

Matrix effect & selectivity

For all methods, the **matrix effects** were examined during method development by comparing the standard calibrators prepared in the buffer with those of specific serum types. Matrix effects were minimized using various assay buffer types until the two standard calibrators almost overlapped. Then, the **selectivity** was examined by spiking analytes at the target lower limit of quantitation (LLOQ) and lower QC levels into the multiple matrix lots, which were then tested along with the unspiked matrix lots. The method was considered acceptable if 80% of the spiked samples recovered within 20% of the nominal concentration and the unspiked samples measured less than the target LLOQ level. The samples with recoveries within two standard deviations of the nominal concentrations were selected for pooling, and this pooled lot served as the blank matrix for standard preparation for prestudy and in-study validations. During prestudy validation, at least six additional lots of matrix were examined to confirm the selectivity and **specificity** of each method. **Table 2** shows the total number of matrix lots tested for selectivity during prestudy validations.

Study design & ISR selection

The total numbers of subjects and samples for each study and ISR analysis are summarized in **Table 3**. The procedure for ISR was implemented in 2006 and revised based on regulatory agency recommendations. With methods A–C, ISR evaluation was conducted using the procedure that specified to analyze ISR for the entire profile in at least three subjects/animals, one from each dose group. With methods D–E,

Table 3. Sampling statistic for each incurred sample reanalysis.

	Total no. in study	Total no. in incurred sample reanalysis	Percentage of total
Case study 1			
Subjects	72	4	6
Samples	1010	60	6
Case study 2			
Subjects	10	3	33
Samples	372	57	15
Case study 3			
Animals	54	34	63
Samples	736	80	10.8
Case study 4			
Animals	18	10	56
Samples	240	20	8
Case study 5			
Subjects	116	60	52
Samples	2381	120	5

ISR analysis was conducted using the procedure that specified selection of ISR from at least two time points, one from C_{max} and one from the elimination phase of at least 20 subjects/animals. For case studies 1, 2, 4 and 5, the percentage difference was calculated from original values using **Equation 1**:

$$(original\text{-}ISR) \times 100 \Big/ ISR$$

Equation 1

For case study 3, the percentage difference was calculated from averaged values using **Equation 2**:

$$(ISR - original) \times 100 \Big/ (original + ISR \times 0.5)$$

Equation 2

Computer software & statistical tool

The intra- and inter-assay performance characteristics, including total error (sum of %bias and %CV), were calculated from prestudy accuracy and precision experiments using a validated Ligand-Binding Assay's Excel Software. For in-study assay performance, the accuracy and precision result was generated by the validated Watson LIMS system. The modified BA statistical procedure/plotter followed the criteria described by Rocci *et al.* [7]. Specifically, reproducibility is confirmed if both the ratio limits (evaluation of accuracy) and the limits of agreement (evaluation of precision) are completely contained within the acceptance limits, which for macromolecules is ±30%. The modified BA procedure/plotter was performed using a validated utility created at Amgen using Excel. To investigate whether the two criteria for ISR differ in outcomes, the concordance between these two criteria is evaluated through the simulation of 980,000 studies, each with a total error between 15 and 40% and a sample size between 20 and 150 samples. SAS v 9.1.3 (Cary, NC, USA) was used for the simulation.

Results

■ Reproducibility in each method was demonstrated

Case study 1

Method A was used to support a Phase I clinical study. The method had no matrix effect in ten lots of normal human and disease state serum **(Table 2)**. All serum lots were recovered within the acceptable range during the selectivity test. During ISR analysis, the observed range

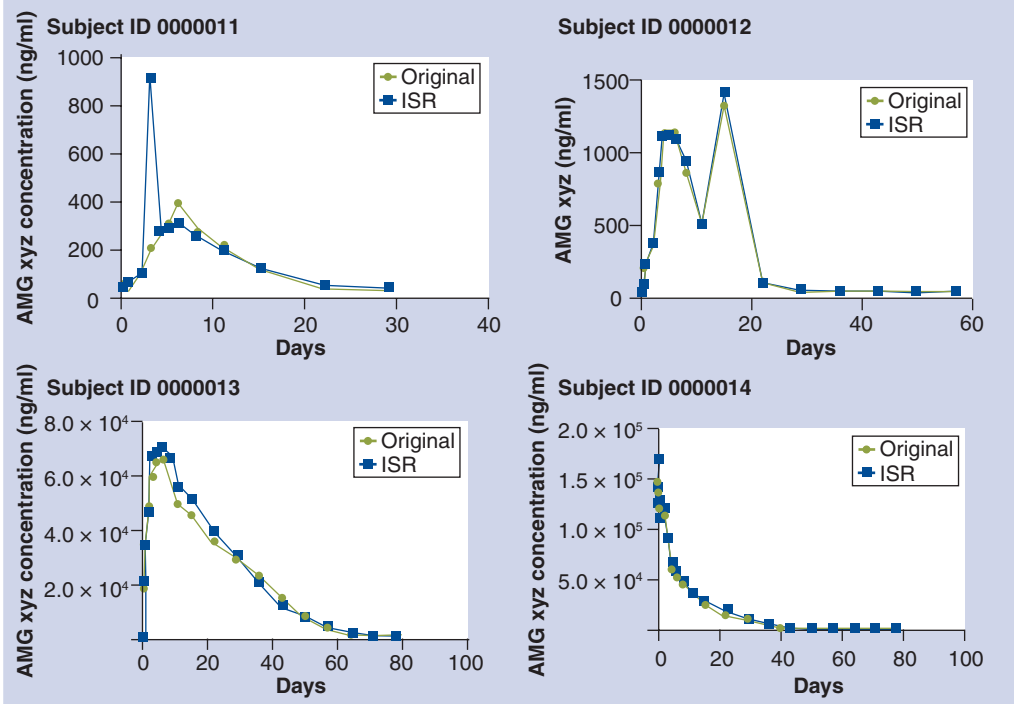

Figure 1. Incurred sample reanalysis was conducted with samples from four subjects using Method A. Entire profiles from four different patients were reanalyzed. An apparent technical error in one collection point during incurred sample reanalysis was noted.
ISR: Incurred sample reanalysis.

of difference between original and ISR results was -20 to 17% in 99% of the ISR samples analyzed from the entire profile of four subjects **(Figure 1)**. One sample from subject 11 (subject ID 0000011) had a difference of 332% at one time point before C_{max} **(Figure 1)** during ISR analysis. The maximal percentage differences at the C_{max} were 20, -1, -4 and -7% for subjects 11, 12, 13 and 14, respectively.

Case study 2

Method B was used to support a clinical bioequivalence study. The method had no matrix effect in study population **(Table 2)** despite the sensitivity of the assay. During ISR analysis, the observed range of difference between original and ISR results was -30 to 18% in 96% of the ISR results. The maximal percentage differences at the C_{max} among all three subjects were less than 15% **(Figure 2)**. Only two samples were outside of the acceptable range (>30%). These samples are from the time points near the terminal phase and had concentrations near the LLOQ. The highest percentage differences observed in these two samples were 32 and 34%.

Case study 3

Method C was applied in a preclinical study supporting toxicokinetic assessments. No matrix effect was observed in any of the six lots of serum tested **(Table 2)**. During ISR, 100% of the samples were within 30% of their respective original results **(Figure 3)**. The observed range of difference between original and ISR results was between -23 and 21%, which was also the maximal percentage difference between two subjects at the C_{max}. The method reproducibility met the predefined acceptance criteria.

Case study 4

Method D was used to support a preclinical study. This method has the highest assay variability, and the assay acceptance criterion was 25% during prestudy validation. However, no matrix effect was observed during prestudy validation **(Table 2)**. The observed range of difference between the original and ISR results in all samples was between -2 and 22% **(Figure 4)**. The method reproducibility met the predefined acceptance criteria.

Case study 5

Method E was used to support a clinical bioequivalence study. During ISR, 100% of the samples were within 30% of their respective original results **(Figure 5)**. No matrix effect was observed in 33 lots of serum tested **(Table 2)**. When the values were calculated from the original results, the observed range of difference

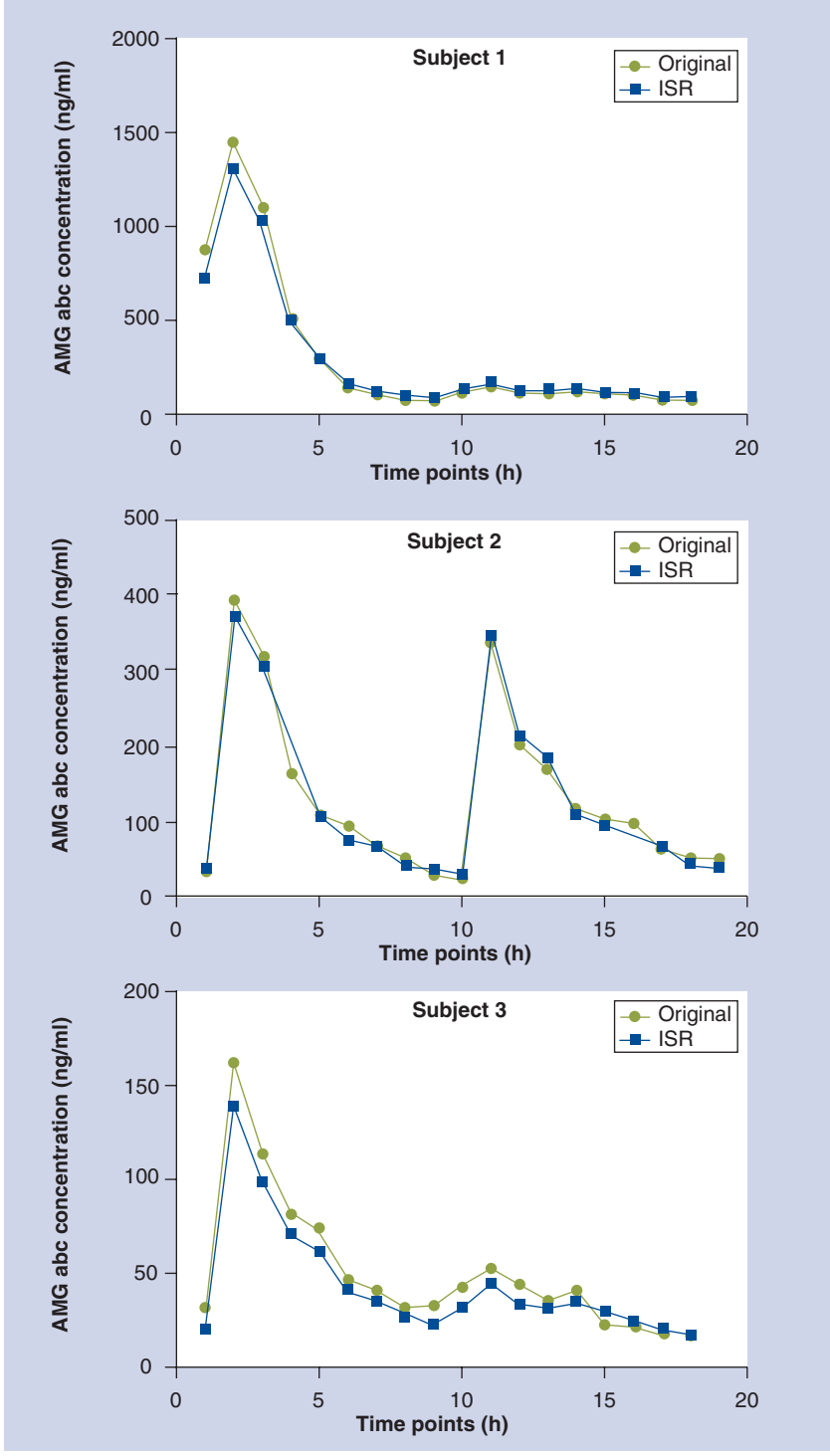

Figure 2. Incurred sample reanalysis was conducted with samples from three subjects using Method B. Entire profiles from three different patients were reanalyzed.
ISR: Incurred sample reanalysis.

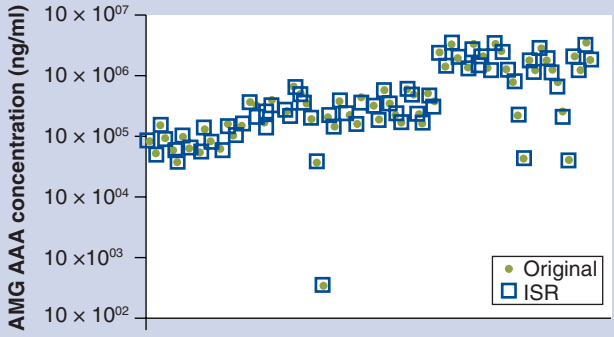

Figure 3. Incurred sample reanalysis was performed using 80 samples collected from 17 subjects using Method C. At least one sample from C_{max} and one from treatment-free period were used in ISR.
ISR: Incurred sample reanalysis.

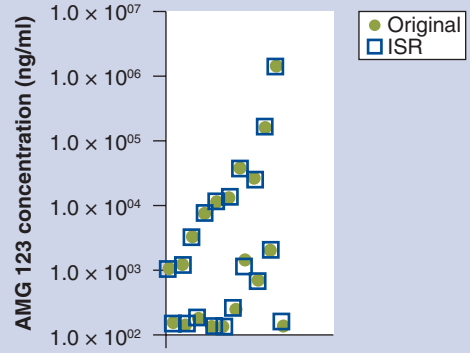

Figure 4. Incurred sample reanalysis was performed using 20 samples collected from ten animals using Method D. At least one sample from C_{max} and one from treatment-free period were used in ISR.
ISR: Incurred sample reanalysis.

between the original and ISR results was between -16 and 13% **(Figure 6)**, which was also the maximal percentage difference of the C_{max} in two subjects. The method reproducibility met the predefined acceptance criteria.

■ Statistical findings of ISR results

The conventional ISR criteria are based strictly on an evaluation of the accuracy of the reanalyzed concentrations and have an arbitrary limit of 67% of samples to be within 30% of averaged concentration. A modified BA approach was used to assess both the accuracy (ratio limits) and precision (limits of agreement) of the reanalyzed values in order to retrospectively evaluate the ISR data from a statistical perspective. Using the same threshold of 30% and applying the criteria to both the accuracy and precision measurements demonstrated the concordance between the conventional ISR criteria and the modified BA approach. The conventional ISR criteria and the modified BA approach provide concordant results in all five studies where the assay acceptance criteria ranged from 15 to 25% **(Table 4 & Figures 6 & 7)**. It is worth noting that the accuracy and precision between original and reanalyzed results are actually within 20% **(Table 4)**. A total of 26 ISR studies were conducted in-house and all 26 studies assessed met the conventional ISR criteria. To evaluate the statistical relevance of this discordance rate (4%), general concordance between the two criteria was examined using simulation. **Table 5** summarizes the results from 980,000 simulated studies and demonstrates that 95% of the time the results using conventional criteria and modified BA criteria were concordant and that 5% of the time the results were discordant. This provides statistical evidence for the observed discordance in the actual studies. Further evaluation is warranted to correlate the two criteria.

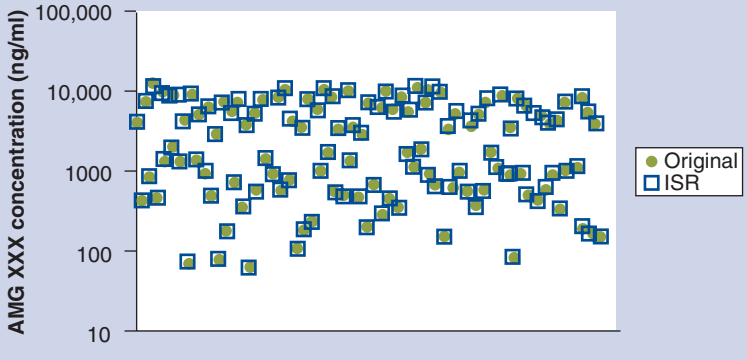

Figure 5. Incurred sample reanalysis was performed using 120 samples collected from 60 subjects using Method E. At least one sample from C_{max} and one from treatment-free period were used in ISR analysis.
ISR: Incurred sample reanalysis.

■ Dilution effect in ISR

We have examined the effect of higher dilution factors on reanalysis of all samples, including C_{max} samples. The average difference (%) between the original and reanalyzed results at different dilution factors was calculated for all the methods. The differences were 2.8, 2.2, -0.5 and 12.8 (p-values: 0.43, 0.18, 0.22 and 0.82, respectively) for dilution factors of 10, 100, 1000 and 10000 when comparing neat to each dilution factor. The results indicated

that dilution factors up to 1:10000 have no significant impact on ISR, even for C_{max} samples where 1:100 or higher dilutions are typically used **(FIGURE 8)**.

Discussion

The practice of whether to conduct ISR was heavily debated until the AAPS Workshop in February 2008. A survey was conducted indicating that only 54% of pharmaceutical companies were conducting ISR at that time [4], most of which involved the methodology for measuring small molecules. The case studies presented herein represent multiple modalities and assay platforms. Included methods were those using ligands, monoclonal or polyclonal anti-idiotypic antibodies, monoclonal antibodies against proteins or peptibodies as capture or detection reagents.

A few checkpoints throughout method development and prestudy validation were important for method robustness, which in turn is critical for successful ISR. First, elimination of the matrix effects is a critical factor in method development. Availability of specific reagents and selection of the right reagent pair are the foundation of bioanalytical method robustness. None of the methods had any matrix effects during prestudy validation experiments, indicating that the method was thoroughly developed and optimized. The results shown in **TABLE 2** demonstrated the selectivity of each method. Second, assessment of robustness and ruggedness for each analytical method during prestudy validation is important. Experimental design during prestudy validation should mimic the in-study design as closely as possible.

According to the European Bioanalysis Forum White Paper, half of failed ISRs are caused by method failure itself and the other half are due to human error for small-molecule ISR [5]. For large-molecule bioanalysis, dilutions are typically performed manually, and technical error could potentially affect method reproducibility. The potential impact of dilution factors on the results of ISR should be taken into consideration during method development. Therefore, the variability of validation samples after dilution should be rigorously tested during prestudy validation.

At a minimum, ISR must meet the predefined criteria required by regulatory agencies [6,101]. Other statistical approaches can be used to assess the quality of each bioanalytical

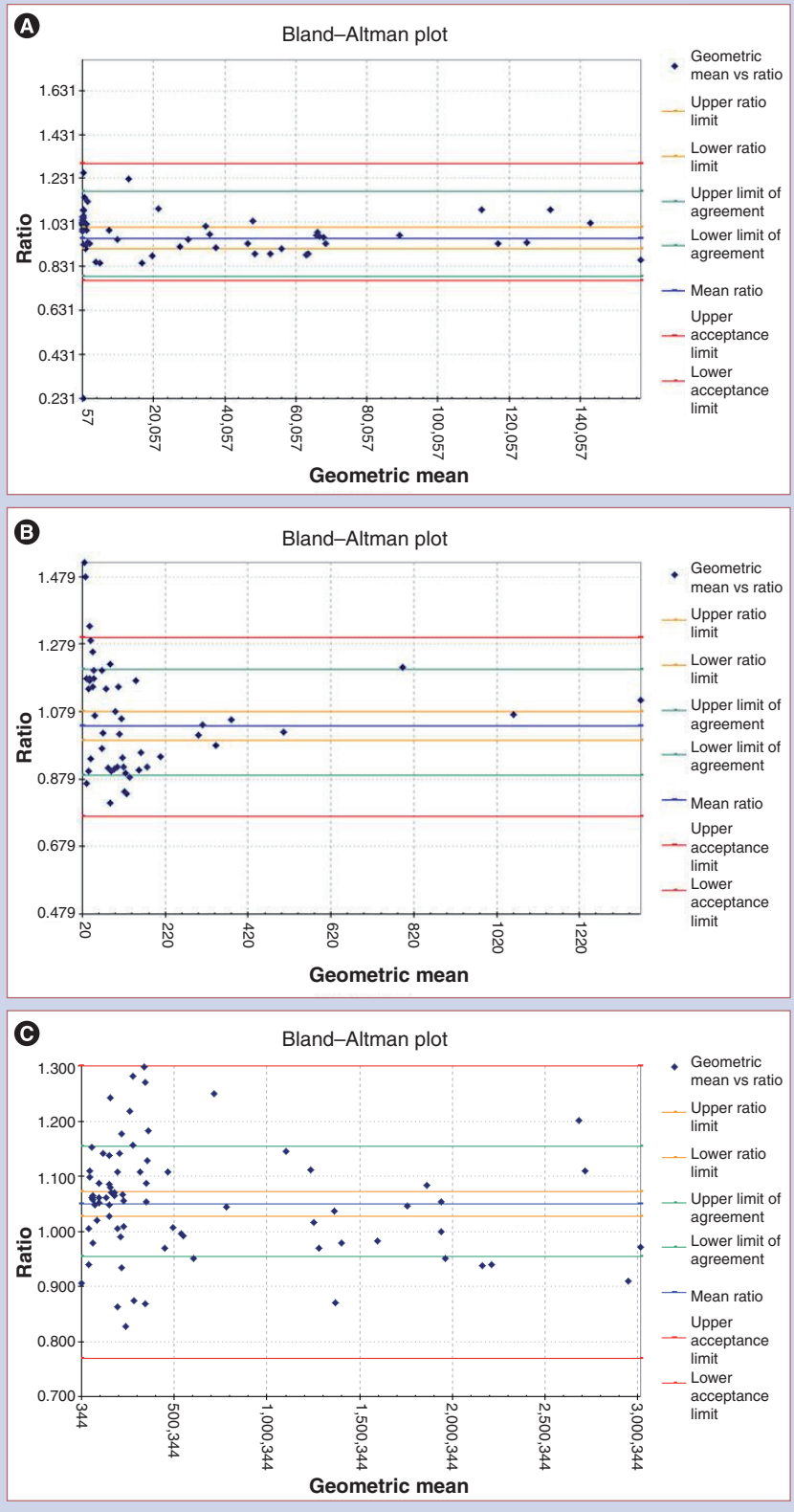

Figure 6. Modified Bland–Altman plots (A–C) for case studies 1–3, respectively. Ratio limit and limit of agreement lines were computed using the original and incurred sample reanalysis concentrations and plotted by the program. Acceptance limits were entered at 30% and plotted by the program.

Table 4. Summary of Bland–Altman approach.

	Method A	Method B	Method C	Method D	Method E
Mean ratio	0.960	1.040	1.050	1.10	0.981
Ratio limit	0.91–1.01	0.99–1.08	1.03–1.07	1.07–1.13	0.97–0.99
Limit of agreement	0.78–1.17	0.89–1.21	0.95–1.16	1.03–1.17	0.93–1.04
Pass/fail based on Bland–Altman analysis (at 30%/20%)	Yes/yes†	Yes/yes	Yes/yes	Yes/yes	Yes/yes

†*Excluding the one apparent outlier.*

method. A retrospective analysis using a modified BA procedure with the 30% criteria was conducted to provide scientific confirmation for setting the conventional acceptance criteria such that 67% of the reanalyzed values agree within 30% of the original values. In the five presented studies, the modified BA approach indicated that the overall agreement between the original and reanalyzed results was within 20%, even though the variability of the assay ranged from 15 to 25%. Thus, the conventional ISR criteria were reasonable for a method with total error of 25% or less. General concordance was observed between the conventional ISR criteria and the modified BA approach in 25 of 26 (96%) studies performed and in 95% of the simulated studies. Further assessments are needed in order to determine whether a modified BA approach should be used to determine the method reproducibility during ISR for macromolecules and whether the acceptance criteria should continue to be set at 30% for assays with total error of less than 25%.

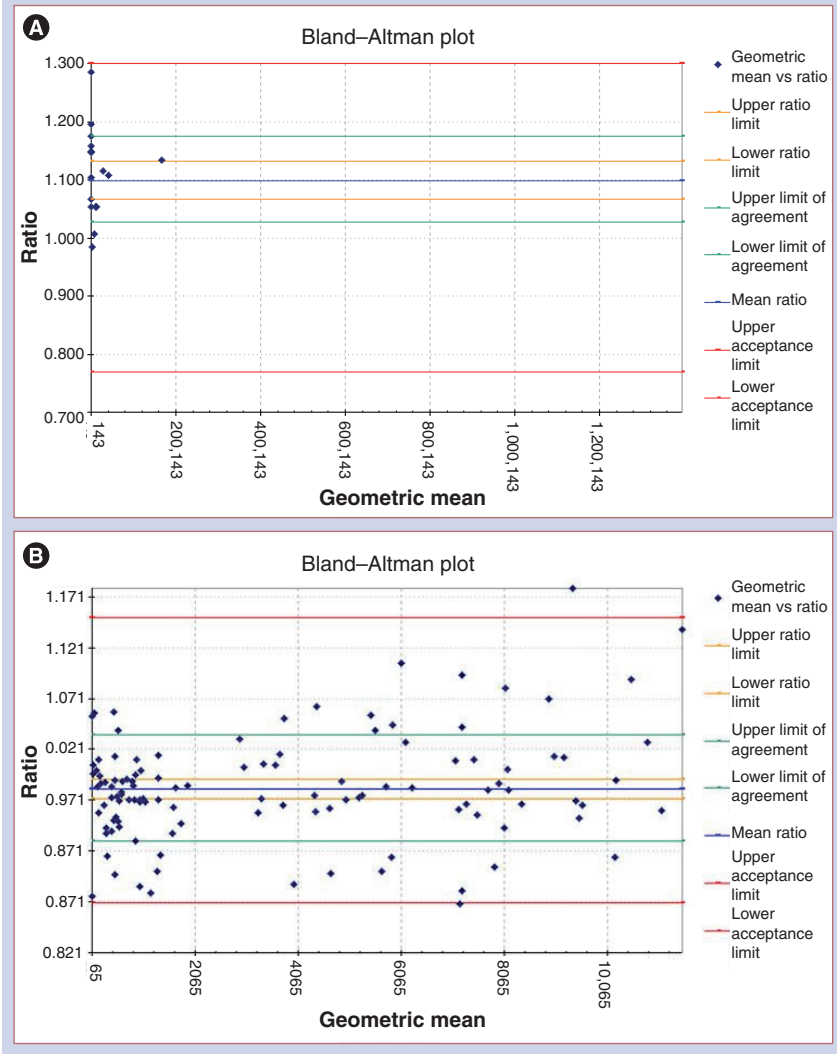

Figure 7. Modified Bland–Altman plots (A and B) for case studies 4 and 5, respectively. Ratio limit and limit of agreement lines were computed using the original and incurred sample reanalysis concentrations and plotted by the program. Acceptance limits were entered at 30% and plotted by the program.

As mentioned in the European Bioanalysis Forum White Paper, the practice of ISR can be considered as a way to check method reproducibility and process adherence [5]. Quality bioanalytical method development and validation can ensure a successful ISR, while adherence to the proper procedure during sample analysis is equally important. Nonetheless, the practice of ISR can provide assurance that the originally reported data are reproducible. From a scientific perspective, ISRs can be beneficial in the assessment of pharmacokinetic parameters for a therapeutic drug with a narrow exposure margin, and the increasing use of ISR in industry will help assess the broader application of ISR in the future.

Conclusions

The strategy of implementing ISR analysis was predefined for each case study, and each analysis was conducted according to the predefined plan or the standard operating procedure. Method reproducibility was demonstrated for five different therapeutic macromolecules and indicates that once the analytical method is

Table 5. Overall concordance evaluation between conventional incurred sample reanalysis criteria and Bland–Altman approach.

Concordance	Frequency count	Percentage of total	Denominator used for percentage calculation
No	49,713	5.1%	980,000
Yes	930,287	94.9%	980,000

thoroughly developed, reproducibility can be readily achieved and assurance provided for the originally reported data. The current ISR acceptance criteria for macromolecules appears to be scientifically meaningful, and reasonable for methods with a total error of 25% or less. Additionally, the BA approach for ISR evaluation may be applied to the same study data and using the same 30% criteria as a confirmatory evaluation of method reproducibility.

Future perspective

- Incurred sample reanalysis studies for methods with a total error of 30% will be of interest to the bioanalytical community.
- A large data pool for macromolecule ISR may be useful in teasing out the contributing factors involved in method robustness.
- Use of ISR will be a valuable practice for a therapeutic drug with a low therapeutic index/safety margin.

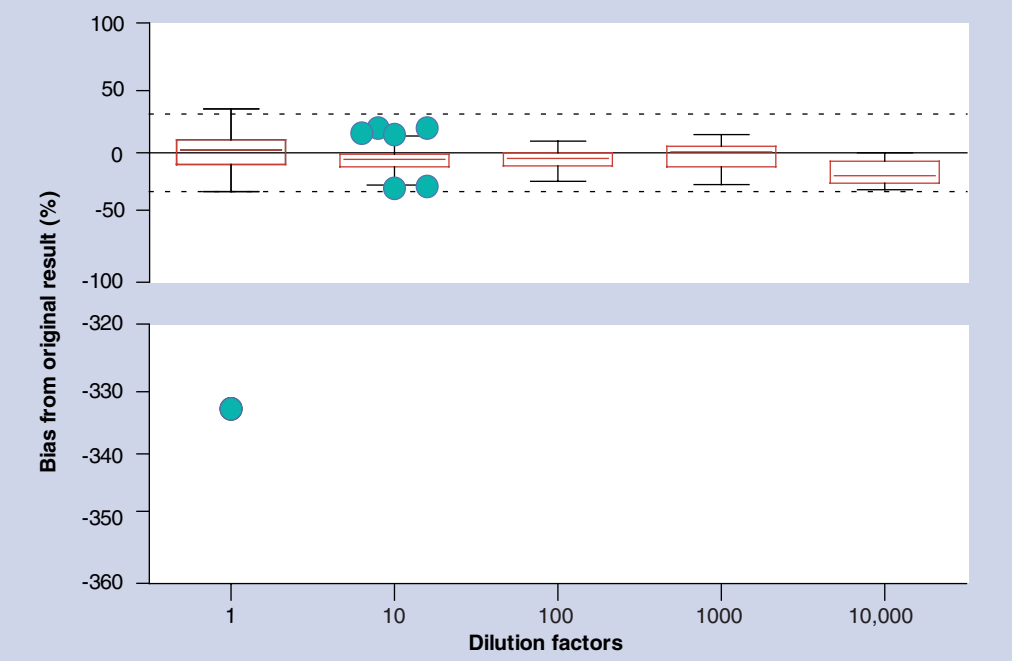

Figure 8. Dilution factors had no effect on reproducibility of results. The mean bias differences between original and repeat analyses in each dilution factor were 2.8, 2.2, -0.5 and 12.8 for 10, 100, 1000 and 10000 dilution factors when compared with those of undiluted samples data.

Executive summary

- Incurred sample reanalysis (ISR) is the most recent in-study validation parameter mandated to ensure reproducibility of bioanalytical methods.
- Five representative case studies are presented in this report.
- Method reproducibility was evaluated by using a single acceptance criterion for accuracy. Additionally, a modified Bland–Altman (BA) analysis was used to assess accuracy and precision of the ISR results.
- Reproducibility was demonstrated in all case studies presented.
- Conventional ISR criteria and a modified BA approach are concordant in all five studies.
- Dilution had no significant impact on the ISR even for C_{max} samples where 1:100 or higher dilutions were used.
- Thorough method development and prestudy validation were prerequisites for a successful ISR.

Acknowledgements

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

Financial & competing interests disclosure

Financial support for this study was provided by Amgen Inc. ***The authors have no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.***

No writing assistance was utilized in the production of this manuscript.

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