A Proposed Framework to Evaluate the Quality and Reliability of Targeted Metabolomics Assays from the UK Consortium on Metabolic Phenotyping (MAP/UK)

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EDITORIAL

SUMMARY: In this Perspective, authors from the UK Consortium on Metabolic Phenotyping propse a 'fit-for-purpose' 4 - tiered framework to evaluate the reliability of targeted metabolomics analyses, addressing the need for community-accepted, harmonised guidelines for tiers other than full validation.

TWEET: A New Perspective from the UK Consortium on Metabolic Phenotyping proposes a 'fit-for-purpose' 4-tiered Framework to Evaluate the Quality and Reliability of Targeted Metabolomics Assays

TEASER: 4-tier Framework to Evaluate Metabolomics Assays

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Abstract

Targeted metabolite assays that measure tens or hundreds of pre-selected metabolites, typically using liquid chromatography mass spectrometry (LC-MS), are increasingly being developed and applied to metabolic phenotyping studies. These are used both as standalone phenotyping methods and for the validation of putative metabolic biomarkers obtained from untargeted metabolomics studies. However, there are no widely accepted standards in the scientific community for ensuring reliability of the development and validation of targeted metabolite assays (referred to here as targeted metabolomics). Most current practices attempt to adopt, with modifications, the strict guidance provided by drug regulatory authorities for analytical methods designed largely for measuring drugs and other xenobiotic analytes. Here, the regulatory guidance provided by the European Medicines Agency, U.S. Food and Drug Administration, and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use are summarised.

In this Perspective, we have adapted these guidelines and propose a less onerous 'tiered' approach to evaluate the reliability of a wide range of metabolomics analyses, addressing the need for community-accepted, harmonised guidelines for tiers other than full validation. This 'fit-for-purpose' tiered approach comprises 4 levels – discovery, screening, qualification and validation – and is discussed in the context of a range of targeted and untargeted metabolomics assays. Issues arising with targeted multiplexed metabolomics assays, and how these might be addressed, are considered. Furthermore, guidance is provided to assist the community with selecting the appropriate degree of reliability for a series of well- defined applications of metabolomics.

Keywords: Metabolic phenotyping, metabolomics, LC-MS, multiplexed assays, validation, qualification, screening, discovery, regulatory, tiered framework.

Introduction

Metabolomics – or metabolic phenotyping - is a multidisciplinary field of research that investigates the metabolome. The metabolome refers to the terminal downstream products of the genome consisting of a repertoire of low molecular weight biomolecules (known as metabolites) involved in cellular metabolism and other biochemical processes in cells, tissues and bodily fluids, as well as those of exogenous xenobiotic and microbiome origin [1,2]. Metabolomics facilitates the characterization of a system from genomic to metabol(om)ic activity and interaction with the environment, and reveals dynamic insights into multiple metabolic pathways and networks that are the consequences of cellular activity, to understand molecular pathophysiology [3]. In addition, metabolomics aims to identify biomolecules (metabolite biomarkers) that modulate phenotype in physiological and/or disease status, reflective of biological processes as well as dysregulated pathways [4,5,6].

The analytical approaches applied in metabolomics research are generally categorised as either untargeted, targeted, or a hybrid approach (sometimes defined as a semi-targeted approach) that combines some aspects of both types of analyses [7]. Untargeted metabolomics is a discovery-based approach where the objective is to analyse as many detectable metabolites without biological bias, including unknowns, to determine which, if any, are significantly perturbed in the diseased phenotype, followed by post-hoc identification of those putative metabolic biomarkers [8]. Targeted approaches on the other hand, involve the (multiplexed) analysis of known metabolites, and such methods often focus on quantification of a subset of metabolites representative of key pathways, or of metabolites determined to be important from prior untargeted metabolomics [9]. Targeted metabolomics is hypothesis driven, with the significant advantage of quantifying known metabolites with greater sensitivity and selectivity [1], while untargeted metabolomics is hypothesis generating, with the advantage of increased metabolite coverage and potential of biomarker discovery [8]. The major disadvantage of untargeted approaches is that relative responses and not actual concentrations are reported, while

the major disadvantage of targeted approaches is their limited coverage of the metabolome [10]. The techniques that are most widely used for untargeted analysis include liquid chromatography high-resolution mass spectrometry (LC-MS), gas chromatography mass spectrometry (GC-MS) and ^{1}H nuclear magnetic resonance (NMR) spectroscopy, while liquid chromatography-triple quad-tandem mass spectrometry (LC-MS/MS) remains one of the traditional techniques for targeted analysis of a limited numbers of analytes, with another approach being GC-MS which involves fragmentation of the metabolite during electron ionisation [11,12]. One of the challenges in targeted metabolomics is that obtaining suitable internal standards is often difficult. On the other hand, one of the advantages of targeted biomarker assays is that the biology of the biomarker has often already been understood, so the anticipated levels, turnover rate, the intra- and inter-subject variability is known, thus enabling the analyst to develop the right assays with appropriate level of validation to generate quality data. However, for newly discovered biomarkers for which little is known, assay development should start with a focus on parallelism, selectivity and sensitivity. Then, at a later stage, the assay could be fine-tuned to the required acceptance criteria [13].

Advances in metabolomics have led to new clinical and toxicological diagnostic biomarkers [14, 15, 16], which can contribute to stratified medicine and safety assessment of drugs [17, 18]. Metabolomics is also central to the screening of innate errors of metabolism [19]. However, there are several challenges in the translation of metabolomics research to clinical and toxicological applications under regulatory control. Issues include analytical reproducibility, accuracy, precision, metabolite identification/quantification, study design, sample handling, lack of harmonised reporting frameworks for published data and metadata, insufficient open-access data to enable data-mining by other researchers [20], lack of harmonisation in bio-banking, batch-to-batch variation, and between-methods bias [21]. Assessing the reliability of bioanalytical methods for metabolomics is challenging when compared to the validation of other types of bioanalytical methods. Data from the metabolomics field are variable, and heterogeneity among

data formats, data analysis pipelines, algorithms and applied statistical methods needs to be addressed. There is a need to define the extent to which assessing the reliability of these methods is required, and the scope of such assessments, as well as how the standards applied and methods for reporting should be chosen in order to ensure appropriate data quality for use in regulatory processes [22]. To eliminate some of these problems, communication between the research and regulated clinical and toxicological communities needs to be more fully developed, and the establishment of a system to assess and cross-correlate metabolic profiles obtained by different laboratories and instruments is needed [20]. The new Metabolomics Reporting Framework for regulatory toxicology, developed by multiple stakeholders from research laboratories, industry and government regulatory agencies and coordinated by the Organisation for Economic Co-operation and Development (OECD) provides evidence on how progress can be made to achieve harmonised reporting of methods, data, metadata and findings, and thereby advance the application of metabolomics within regulatory settings [23]. There are a plethora of publications that provide comprehensive guidelines for assessing the quality of untargeted metabolomics assays [24, 25, 26, 27, 28]. Whilst these guidelines provide the foundation for metabolomics system suitability and quality assurance/quality control (QA/QC) proficiency, a community-initiated approach towards harmonised guidelines that ultimately achieves acceptance via their consensus use for evaluating the reliability of targeted metabolomics within research, clinical and toxicological settings is still required.

Our scientific collaboration, the UK Consortium on Metabolic Phenotyping (MAP/UK, https://mapuk.org), is a partnership of eight specialised research laboratories and two Phenome Centres, which has been funded by the Medical Research Council to improve UK-wide metabolic phenotyping expertise and capabilities. The MAP/UK partnership brings together a critical mass of methodological, analytical, and computational platforms to develop, optimise, transfer, harmonise, and validate efficient, high-quality metabolomics research and training methods, specifically tailored to the growing need for biomedical studies that require robust

metabolic phenotyping. The overall aim of the MAP/UK partnership is to investigate new biomarkers within metabolic signatures of disease, novel targeted quantitative metabolomic and hybrid approaches, and developing untargeted metabolomics to meet gaps in molecular coverage of key disease-related pathways, alongside a variety of other factors, including diet, lifestyle/environment, microbiome and genetics. As a collective of scientists with the aim of harmonisation of metabolic phenotyping, existing regulatory guidelines have been reviewed to extract commonalities from these guidelines that can be adopted to 'fit-for- purpose' and tiered approaches for untargeted and targeted metabolomics.

The aim of this manuscript is to propose harmonised guidelines for evaluating the reliability of targeted (multiplexed) mass spectrometry-based metabolomics assays taking into consideration intra-laboratory precision, accuracy, reproducibility, and cross-laboratory harmonisation of methods and data acquired on different instrument platforms. First, existing guidelines for bioanalytical method validation, including an existing 4-tiered framework applied in drug discovery, are reviewed. Then, after introducing the applications of clinical and toxicological metabolomics in regulatory settings, a new 'fit-for-purpose' 4-tiered (discovery, screening, qualification and validation) framework for assessing analytical reliability that is suitable for targeted and hybrid untargeted metabolomics assays is proposed. In addition, a checklist for the bioanalytical process has been provided to facilitate better understanding and emphasise the importance of harmonisation at each step, as described in Box 1

****START BOX 1****

Checklist for bioanalytical assay process:

1- Pre-analytical:

- Hypothesis/study design/ sample size.

- Data acquisition of demographics for groups/individuals including clinical, diet, medications and life-style data.
- Sample type (plasma/serum/urine/feces), collection method, preservation, and timing.
- Sample storage.

2- Analytical:

- Sample preparation and purification.
- Authentic reference materials (external standards), quality control (QC) samples and suitable internal standards.
- Maintaining assay reliability and quality by selecting the right tier based on number of metabolites and assay purpose (consult Table 1).
- Select validation parameters and acceptance criteria for targeted assays (tier 1 and 2), by consulting Table 2. Note that Tier 1 parameters are the same as suggested by regulatory guidelines (FDA/EMA/ICH2019) for validation, and Tier 2 (qualification) has a wider range of acceptance criteria.
- Select appropriate instrumentation such as liquid chromatography high-resolution mass spectrometry (LC-MS), liquid chromatography-triple quad-tandem mass spectrometry (LC-MS/MS), and considerations regarding instrument calibration, settings, analytical batches, and quality assurance (QA)/performance.

****END BOX 1****

The concept of regulatory bioanalytical validation

An analytical assay starts with a definition of its purpose (i.e. intended application),

217 defining what is ‘fit-for-purpose’, followed by method development and optimisation, then
218 subsequently by assay validation (dependent upon the tier, as introduced above) and
219 documentation before it can finally be applied for the intended purpose. Validation is defined as
220 a process that provides proof of assay integrity within given specifications with the parameters
221 of an assay used for quantification being statistically reliable between assays over time. Prior to
222 initiating a validation study, a well-planned validation protocol should be written and reviewed
223 for scientific soundness and completeness. The protocol should describe the procedure in detail
224 and should include pre-defined acceptance criteria and pre-defined statistical methods, and
225 should be approved by all participants in the analytical pipeline.

226 There are numerous validation parameters (accuracy, precision, calibration curve, lower
227 limit of quantitation, selectivity/specificity, carryover, analyte stability, recovery, dilution
228 integrity, system suitability test, matrix effect/factor, parallelism, incurred sample re-analysis,
229 quality control, robustness/ruggedness, hook/prozone effect, and minimum required dilution) to
230 incorporate into the validation process (please see Supplementary Table 1 for comparison of
231 validation parameters by multiple guidelines, and Supplementary Table 2 for definition of
232 validation parameters). The validation workflow has been summarized in visual format (Fig. 1).
233 This workflow is a modification of general validation workflow in combination with two extra
234 steps based on our proposed framework to advise analysts for choice of appropriate tier of the
235 assay, and depth of required validation. One should justify the required level of validation to be
236 ‘fit-for-purpose’ based on the differing applications of a particular method. Theoretically, there
237 are no limits to the extent of validation and verification procedures. However, in practice, there
238 are both time and economic constraints on what can be achieved. Therefore, it is crucial to have
239 optimised guidelines that are generally accepted, harmonised and cost-effective [29].

240 Multiple guidelines exist that describe the regulation of bioanalytical assays such as
241 those from the U.S. Food and Drug Administration (FDA) [30], the European Medicines Agency
242 (EMA) [31], the International Council for Harmonisation of Technical Requirements for

Pharmaceuticals for Human Use (ICH) [32], the Japanese Ministry of Health, Labour and Welfare (MHLW) [33], Chinese (State) Food and Drug Administration (CFDA, currently the National Medical Products Administration, NMPA) [34], Australian Therapeutic Goods Administration (TGA) [35], and Brazilian National Health Surveillance Agency (Anvisa) [36, 37, 38].

The two most well-used bioanalytical guidelines from the EMA and FDA are similar but not identical. The scientific basis for the evaluation of parameters is the same across both guidelines. However, there are also differences in terminology, recommended validation parameters, acceptance criteria and methodology, which can cause confusion amongst bioanalysts and/or pharmaceutical companies. Standards setting and harmonisation was advanced by the ICH, which is an international organisation with the mission to achieve greater harmonisation worldwide to ensure that safe, effective, and high-quality medicines are developed and registered in the most resource-efficient manner. The ICH consolidated best practices from the FDA and EMA guidelines in 2019 into a harmonised M10 bioanalytical method validation draft in order to clarify any areas of uncertainty between the two guidelines. A comparison between the FDA and EMA guidelines and the consolidated ICH M10 draft guideline are summarised in Supplementary Table 1.

Whilst these regulatory guidelines are comprehensive, they are largely developed for the measurement of drugs and other xenobiotic analytes. Endogenous biomarkers are often measured in metabolomics which requires different considerations including matrix effect. Matrix effect is referred to a phenomena usually encountered in LC-MS/MS where ionisation efficiency of target analytes are altered in the presence of co-eluting compounds in the same matrix. It could cause either ion suppression or enhancement. Quantitation of matrix effect is termed matrix factor (MF), and should be determined within the lowest limit of quantification (LLOQ), and upper limit of quantification (ULOQ) of a matrix-matched calibration curve.

Lower limit of quantification (LLOQ) represents sensitivity of the assay and determines the lowest concentration of analyte in a sample which can be quantified reliably with an acceptable accuracy and precision. LLOQ should be considered as the lowest point in the calibration curve where signal-to-noise (S:N) ratio should be at least 5:1. Evaluating these limits using standard solutions in neat solvent, and/or matrix deprived of specific classes of metabolites (such as stripped plasma) are not an ideal solution as what has been depleted is not defined. Furthermore, measurement of specificity/selectivity for endogenous metabolites is much more challenging due to presence of multiple isoforms. Recently, regulatory bodies have begun to address the requirements needed to achieve robust and reliable data in biomarker assays applying 'omics' data. To our knowledge, the Omics subgroup report [22] and C-Path report [39] are the only documents published by the regulatory agencies on assessment of biomarkers assays. The Omics subgroup report [22], on behalf of the EMA and Heads of Medicines Agencies (HMA), published in 2017 a checklist to introduce considerations for successful qualification of novel methodologies such as biomarker quantification, clinical outcome assessment, imaging methods and big data approaches. This checklist entails brief recommendations for context-of-use (CoU), selection of endpoints, statistical analysis plan, demonstration of clinical utility, standard of truth/surrogate standard of truth, suitability of the analytical platform, as well as a link to ICH E16 and ICH E18 guidelines that focus on pharmacogenomics biomarkers, and sampling and management of genomic data (EMA/750178/2017 document). Furthermore, the FDA in conjunction with the Path Institute (C-Path) published a document entailing broad scientific insight to biomarker assay challenges, and a complete description of necessary approaches that can be applied to biomarker qualification [39]. Before introducing our proposed framework to assist bioanalysts in selecting the appropriate tier of validation for a series of well-defined applications of metabolomics, a brief introduction to the existing tiered regulatory guidance for the targeted measurement of single drugs is discussed.

Existing tiered regulatory guidance for bioanalysis

A fundamental question is how stringently regulatory bodies view these guidelines as being hard rules, or whether they could be adopted as 'fit-for-purpose' for targeted metabolomics assays, and used within a 'tiered' framework. The concept of defensible scientific flexibility has been a debate within the bioanalytical community in the pharmaceutical industry. The Crystal City III workshop proposed the concept of 'fit-to-purpose' in 2006 as an alternative for the full validation workflow already described by the FDA regulatory documents in order to address uncertainties from the bioanalytical community as to what level of data scrutiny is required to generate quality data whilst optimising resources to meet study objectives with an adequate level of data quality and reliability [40]. Furthermore, the European Bioanalysis Forum (EBF) proposed the consolidation of tiered approaches to include three levels (or tiers) of quality standards for metabolite quantification for screening, qualified and validated assays [41]. Consequently, the MHLW and FDA allowed adjustments and modifications of their bioanalytical method validation guidelines to fit the intended use of the assay, and this perspective was extended to tiered approaches for metabolite quantification [42, 43, 44].

The Crystal City VI workshop in 2015 [45] defined a less rigorous level of validation than the FDA guidelines for drug metabolite quantification at early stages of development. The Global Bioanalytical Consortium (GBC) assigned Team A2 with the objective of providing a framework to rationalise the level of bioanalytical methods for drug characterization and proposed a clear path for implementation and use of tiered approaches [42]. Furthermore, two globally recognised teams within the GBC (S1 and L1) provided acceptance standards for validation methods for small and large pharmaceutical molecules, respectively [46]. However, different terminologies have been used as part of the 'fit-to-purpose' concept, such as tiered assays, scientific validation, qualified assays or partial validation. Thus, it has been a source of

confusion for academia and the biotechnology/pharmaceutical industry due to a lack of clear guidance [42]. More recently, these alternative validation assay workflows in the bioanalytical industry have been categorised into four tiered levels of method performance and evaluation based on the final purpose of the derived analytical data ranging from the most to least stringent, as follows: level 1) validation, intended for regulatory studies; level 2) qualification; level 3) research; and level 4) the least stringent defined as 'screening' [42, 47, 48]. These four tiered levels are described in more detail below, and whilst these concepts have been designed for drug development and submission to regulatory authorities, they provide a framework that could be adapted for a range of assays used in metabolomics studies.

- Level 1) **validated bioanalytical assays** are designed for intended pharmaceutical products and thus require the highest level of confidence in analytical results as suitable for regulated good laboratory practice (GLP), pre-clinical/clinical, pharmacokinetic and/or toxicological studies, and identification of active metabolites in safety testing (MIST). These mandate that assay precision, accuracy, selectivity, sensitivity, and stability of the analytes should be determined throughout the bioanalytical measurement process. FDA recommended evaluations should be performed [41].

- Level 2) **qualified bioanalytical assays** do not need to demonstrate that the measurement methods are as robust as validated assays. This tier is suitable for non- regulated studies in the drug development process, with additional assessment of tissue concentrations or other matrices during preclinical or late discovery phases, and in decision-making for context of use (COU) statements. Single method performance with a statistically appropriate number of quality controls (QC) samples ($n \geq 5$) at each level and a suitable calibration range, precision and accuracy should be performed.

- Level 3) **research-grade bioanalytical assays** are suitable for mid- to late-discovery phases of drug development projects for decision-making evaluations and/or verification of additional biomarkers or metabolites for non-GLP regulated studies. They use limited characterization with calibration standards prepared using a comparator reference material such as an *in situ* (in solution) standard with the concentration estimated by radioactivity measurement, NMR or ultraviolet (UV) absorption as representative methods. The method provides semi-quantitative analyte concentrations within wider accuracy and precision limits than for the two higher tiers [42]. This approach enables the partial characterisation of an analytical method that may eventually move to a qualified or validated assay. It should provide sufficient scientific rigor to ensure that it is fit-for-purpose and that there is confidence in the data. Method evaluation should be conducted prior to sample analysis, with the precision and accuracy needed to achieve the more relaxed criteria of 20% relative standard deviation (RSD) and 30% reduction of error (RE) at the LLOQ (Lowest Limit of Quantitation).
- Level 4) **screening bioanalytical assays** apply a generic method (not specific to the analyte) to provide adequate results for the analyte of interest and are suitable for early discovery and qualitative (present/absent) analysis. Screening assays undergo limited characterization based on relative instrument analyte response when reference material is not available. The assay provides relative analyte measurements (i.e. response and not concentration) only but may still be suitable for decision-making processes. An abbreviated set of QCs with large margins of variability of 30% RSD and 40% RE is advisable. As such, screening bioanalytical assays are most similar to untargeted metabolomics assays.

Apart from the four-tiered levels approach in the bioanalytical industry, there is a

general concept of ‘full’ and ‘partial’ validation. Full validation is necessary when developing and implementing a bioanalytical method for the first time such as when analytes are added to a panel for bioanalytical quantification. In targeted metabolomics, full validation of a method by the accredited clinical laboratory is required when the result from that assay (e.g. concentration of a biomarker in terms of molarity for liquids or µg/mg for tissue) is used for making a clinical decision. Partial validation is required in the case of bioanalytical method transfers between laboratories or when the method parameters such as instrument and/or software platform change, such as changes in species (e.g. human plasma to murine plasma) or matrix (e.g. human plasma to human serum/urine). Partial validation can range from as little as one intra-assay accuracy and precision determination to nearly full validation [49] depending on the degree of change required being undertaken.

The sections above have introduced concepts and terminologies within bioanalytical validation as well as highlighting the need for the standardisation of guidelines for the validation of endogenous metabolite analysis with the aim of maximising the cross-comparability of generated data. In the next section, a flexible and practical framework to assist bioanalysts to select the appropriate tier of reliability for multiplexed metabolic biomarker assays, each with a defined use, is proposed.

Proposed tiered framework for assessing the reliability of metabolomics bioanalytical methods

Considering that there are a range of applications for metabolomics and new advances in LC-MS techniques for multiplexed measurement of metabolites, there is a clear need to propose a harmonized framework that describes which reliability tier is most ‘fit-for-purpose’ for different applications. Evaluation of being ‘fit-for-purpose’ involves questions such as: 1) what is the context of use for the assay (i.e. what will the data be used for); 2) should it be a quantitative, semi-quantitative or relatively quantitative assessment; and 3) what level of uncertainty can be

tolerated in the assessment. Consolidating the concept of 'fit-for-purpose' assists bioanalysts in decision-making on whether to qualify or validate a biomarker assay, and which parameters to choose in addition to the number of appropriate replicates [50]. The end-result of a 'fit-for-purpose' validation of an assay using relative quantification is a resource-effective and -efficient demonstration of the bioanalytical method's performance that is tailored to meet the objective of the application. This ultimately provides reliable study data to make important decisions. The decisions may involve further assay development and progression to a fully validated method.

The intended use (or application) of a metabolomics assay determines which level of reliability assessment should be used, not the type of assay. Selecting the most appropriate tier for measuring multiple metabolic biomarkers simultaneously for targeted metabolomics assays is challenging if the intended data use is not carefully defined. Hence, the first step in selecting an appropriate tier is to define the intended use of the data and which type of assay is needed, then the most appropriate reliability tier can be further defined.

The following framework is proposed as a guideline for the metabolomics community to assess the reliability of both targeted and untargeted metabolomics assays for different types of applications (i.e. from biomarker discovery by a research laboratory, transfer of a method to a different laboratory, through to the use of biomarkers within a clinical setting). The proposed framework is summarised in Table 1 (Tiers 1-4) to assist bioanalysts in selecting the most appropriate tier based on their purpose and assay type. Tiers 1 and 2 (targeted metabolomics) are the main focus of this manuscript, and all related parameters for safeguarding scientific rigor for robust validation and bioanalytical quantification for these two tiers (termed validation and qualification) are summarised in Table 2. These tiers differ in depth, robustness of parameters, and the number of replicates performed for each parameter (See Table 2).

Tier 1 - Validation

Diagnosis of disease/toxicity phenotype using traditional targeted metabolite analysis

with absolute quantification of typically one to a few (less than 10) metabolites. Tier 1 validation is required for compliance with regulatory agencies for clinical diagnostics. This requires an authentic standard (external standard) for each metabolite. The proposed procedure is in alignment with current FDA and ICH M10 bioanalytical method validation guidelines, and is applicable to quantitative analytical assays such as chromatographic, liquid chromatography-mass spectrometry (LC-MS and/or LC-MS/MS), and ligand binding assays (LBA) (see Table 2).

Tier 2 - Qualification

Diagnosis of disease/toxicity phenotype using a multiplexed targeted metabolomics assay with absolute quantification of more than 10 metabolites. This requires an authentic external standard for each metabolite. The criteria for qualifying a method are less strict than for tier 1 validation of a method (see Table 2).

Tier 3 - Screening

Screening for a disease/toxicity phenotype using a multiplexed targeted or hybrid metabolomics assay with relative or semi-quantification of a panel of hundreds of metabolites. This does not require an authentic external standard for each metabolite. The criteria to meet in a screening method are less strict than for tier 2 qualification of a method.

Tier 4 - Discovery

Discovery of putative metabolic biomarkers using untargeted or hybrid metabolomics with relative quantification in a research laboratory. Untargeted methods have the least strict criteria. Furthermore, the use of system suitability tests, intra-study QC samples, phenotyping QCs (healthy vs. disease), inter-laboratory QC samples, and dilution series of pooled QCs have been previously discussed [7, 51] and provide a dimension of semi-quantitative nature to these

untargeted assays.

Bioanalytical considerations for generation of quality data in targeted and untargeted or hybrid metabolomics assays

Targeted metabolomic studies often require the quantification (e.g. absolute, semi- and/or relative) of multiple analytes (e.g. multiplexing) in order to exploit putative biomarkers identified via untargeted metabolomics methods, and validate derived hypotheses. The gap between targeted and untargeted metabolomics is very narrow and often overlapping. For example, in assays for the quantification of hundreds of polar or lipophilic metabolites, authentic external standards and internal standards may not be available for all analytes. Many of these assays also satisfy the criteria for the accuracy and precision of metabolite measurements as defined by the FDA. However, they should be reported as semi-quantitative concentration rather than absolute concentrations mainly due to lack of standard and/or internal standard availability.

LC-MS multiplexing allows for the measurement of numerous analytes in the same analytical run, thus providing significantly more information about molecular biomarker signatures than measurements of single analytes. As the number of analytes increases, favourable accuracy and precision values are often more difficult to obtain. As noted by regulatory guidelines, all quantified analytes in the same assay need to meet the same acceptance criteria. If one of the analytes fails to meet acceptance criteria, the whole analytical run fails. However, in multiplexing assays, re-analysis of the whole panel of analytes should not be necessary if most of the analytes are within the pre-defined quality specifications.

Additionally, acceptance criteria should be widened [52], in which the variation at the LLOQ is increased from 20% to 30%-40%. One should bear in mind that increasing the number of replicates at the LLOQ will result in lower variation (RSD%). The degree of

analytical variability that can be tolerated depends on biological variation. Higher variation is often expected for large biomolecules compared to metabolites. Incurred sample reanalysis (ISR) of macromolecules as recommended by the FDA is within 30% of the average of original and reanalysed values compared to 20% for small molecules [53]. In the proposed framework, acceptance criteria for Tier 2 is more relaxed as size and number of replicates are lowered. However, increased calibration points for Tier 2 when the number of metabolites are increased are recommended. Furthermore, biomarkers should be simultaneously evaluated in both absolute and semi/relative quantification manners for multiplexed assays [52]. For instance, identification or presence of a particular compound (e.g. qualitative evaluation) alongside quantification of related metabolites or a precursor could provide better insight into metabolic phenotyping.

The importance of good laboratory practice at different stages (e.g. sample collection, storage integrity) should be considered for bioanalysis. Sample, analyte and data integrity as well as basic laboratory record keeping are essential. Implementing a laboratory information management system (LIMS) is recommended. Routine calibration of laboratory instruments, pipettes and balances with well-written standard operating procedures (SOPs), as well as selection of suitable blank matrices, internal standards, system suitability test and intra-study QCs are essential. Intra-study QCs should be placed in the analytical run in such a way that the precision of the whole run is ensured by taking into account that study samples should always be bracketed by QCs [7]. Phenotyping QCs (e.g. healthy *vs*. diseased) are recommended. A QC is typically produced by pooling a small aliquot of all study samples, and these are analysed throughout the analytical run. For untargeted metabolomics, a dilution series of the intra-study QC is highly recommended to help differentiate features of biological origin from LC-MS chemical background [12]. Application of isotopically-labelled standards can provide a generalised measure of precision across the study. Furthermore, use of isotopically labelled internal standards helps to compensate for matrix-induced ionisation effects, thereby

enhancing the accuracy of the assay when quantification/semi-quantification is applied [26]. Choice of suitable surrogate matrices are recommended to improve sensitivity and selectivity of biomarker quantification [54, 55, 56, 57]. Blank matrices with the minimum level of endogenous analyte should be used wherever possible. This approach is suitable for multianalyte assays (spiked with appropriate concentration of each analyte), but matrix effects and stability should be investigated for each analyte. In the absence of blank matrices or surrogate matrices, standard addition approaches which take into account the native concentration of the targeted analyte(s) can be used for recovery and matrix effect checks; and the use of QCs or standards prepared only in solvent and/or buffer considered for accuracy and repeatability/reproducibility tests represents the approach that makes the least assumptions. Artificial blank matrices may be used. A solution of 4% fatty acid-free bovine serum albumin (BSA) in saline buffer that represents the same concentrations of salts and electrolytes in human plasma is an example of blank matrix for human plasma (artificial surrogate matrix). Normalisation strategies to correct for differences in sample amount should be considered. For example, urinary creatinine is often used to adjust the concentration of urinary biomarkers.

All targeted assays should have a clearly defined limit of detection (LOD) and limit of quantitation (LOQ). A clearly discernible peak must be visible above clearly visible baseline noise and should be comprised of a specified number of data points (often 6 or above is used). As a general rule, LOQ of S:N (signal-to-noise ratio) of at least 5:1 is used by research laboratories, with an LOD of around 3:1. This approach is fully in line with guidelines from international bodies [30, 58, 59, 60, 61, 62, 63, 64, 65, 66]. For targeted assays, all peaks should be checked to ensure they reach the specified S:N ratio as well as the required number of data points. However, for large scale metabolomics, manual checking is not feasible for all peaks, but if certain metabolites or features are judged to be discriminatory (e.g. predictive of sample type), then those should be prioritised for manual post-processing checks to ensure that the

differences are real and the data is of good quality.

Discussion

Validation is defined as the process of proving that any procedure, process, equipment, material, activity or system performs as expected within defined acceptance criteria under a given set of conditions, and that the performance characteristics of the procedure meet the requirements for the intended analytical applications [67, 68]. Although implementing fail/pass criteria advised by bioanalytical method validation guidelines have provided a useful degree of standardisation and consistency between regulated laboratories, new advances in technology, multiplexing, and metabolomics studies require tiered and/or 'fit-for-purpose' approaches [69] for pragmatic/practical use.

Pre-determined or fixed acceptance criteria are established and appropriate for validated assays (Tier 1); however, for qualified, research, and screening methods (Tiers 2-4), it may be appropriate to define these after the method performance experiments have been conducted to fine-tune the assay to the required acceptance criteria. Minimally, it is expected that *a priori* acceptance criteria can be relaxed for the higher tiers if such method performance still supports the intended use of the data and ultimately supports the necessary decisions that will be made [42].

Validation beyond the intended use of the data means significant re-work, loss of time and increased cost in the blind pursuit of absolute requirements. For metabolomics at its current state of development, what is required is the definition of a simple, pragmatic and easy- to-follow framework that reflects realistic and practical needs that allow for the most efficient practices. For instance, an assay that does not pass the criteria for full validation but, nevertheless, fulfils the essential requirements for linearity, accuracy, precision, LLOQ and carryover criteria may be devised. In that case, guidance should focus on minimum requirements. Specifications of

549 merit might include: linearity with an LLOQ set as first calibrant, accuracy, precision and
550 carryover.

551 Overall, the guidelines for assays developed for drugs that have been devised by
552 regulatory authorities to ensure safety and efficacy in humans represent a 'gold standard' that
553 may not be required for many types of targeted and untargeted metabolomics applications.
554 This is not to suggest that metabolic phenotyping methods should not be developed to the
555 standards necessary to provide reliable and scientifically valid data but to suggest that the
556 use of tiered approaches linked to the type of investigation (i.e. discovery, hypothesis
557 validation, biomarker/panel, and/or qualification stages) should drive the level of validation
558 performed. A number of intricate analytical factors (e.g. pre-analytical factors) defining core
559 assay expectations, and setting acceptable assay performance criteria, should be taken into
560 account for assessing the reliability and quality of metabolomics assays. Our MAP/UK
561 consensus framework provides a bench guide for the two major categories of validation and
562 qualification of targeted metabolomics analysis that have been described in Table 2.

563 **Conclusions**

564 Metabolomics has the potential to lead advances in the discovery of clinically and
565 toxicologically relevant biomarkers, yet the lack of harmonisation at different levels throughout
566 the whole metabolomics pipeline from study design, sample handling, biobanking, metabolite
567 quantification to data analysis remains an issue that needs to be addressed. Metrological
568 tracability and future development of certified matrix reference materials similar to National
569 Institute of Standards and Technology reference standards (NIST SRM 1950) [70], and standard
570 calibration mixtures should be established and harmonized within both the research and
571 regulatory communities. The MAP/UK consortium proposes the pragmatic development of a
572 'fit-for- purpose' 4-tiered framework for assessing the reliability of metabolomics assays via a

decision-making process and adaptation of existing drug regulatory guidance. The required level of analytical rigour and/or qualification that bioanalytical methods need to show in order to achieve scientifically valid studies in metabolomics has been considered. This framework is intended to guide bioanalysts and to facilitate improved communication between the research and regulatory communities, in order to enable the establishment of appropriately qualified targeted metabolomics assays to meet the needs of multiple applications of this technology in the regulatory sciences. Ultimately, we hope that such a community-initiated framework can accelerate the application of metabolomics in regulatory applications and achieve acceptance via its consensus use.

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Author Contributions

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Competing Financial Interests

The authors declare no competing interests as defined by Nature Research, or other interests that might be perceived to influence the interpretation of the article.

Figure legend

Figure - Validation workflow steps and positioning of the suggested tiered framework within the general workflow to select the most appropriate tier and degree of validation.

Supplementary Information

Supplementary Table 1. Comparison of the U.S. Food and Drug Administration (FDA), European Medicines Agency (EMA) and International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use (ICH) M10 guidelines.

Supplementary Table 2. Definition, methodology, and acceptance criteria of validation parameters.

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Table 1. Four-tiered framework for assessing the reliability of metabolomics assays

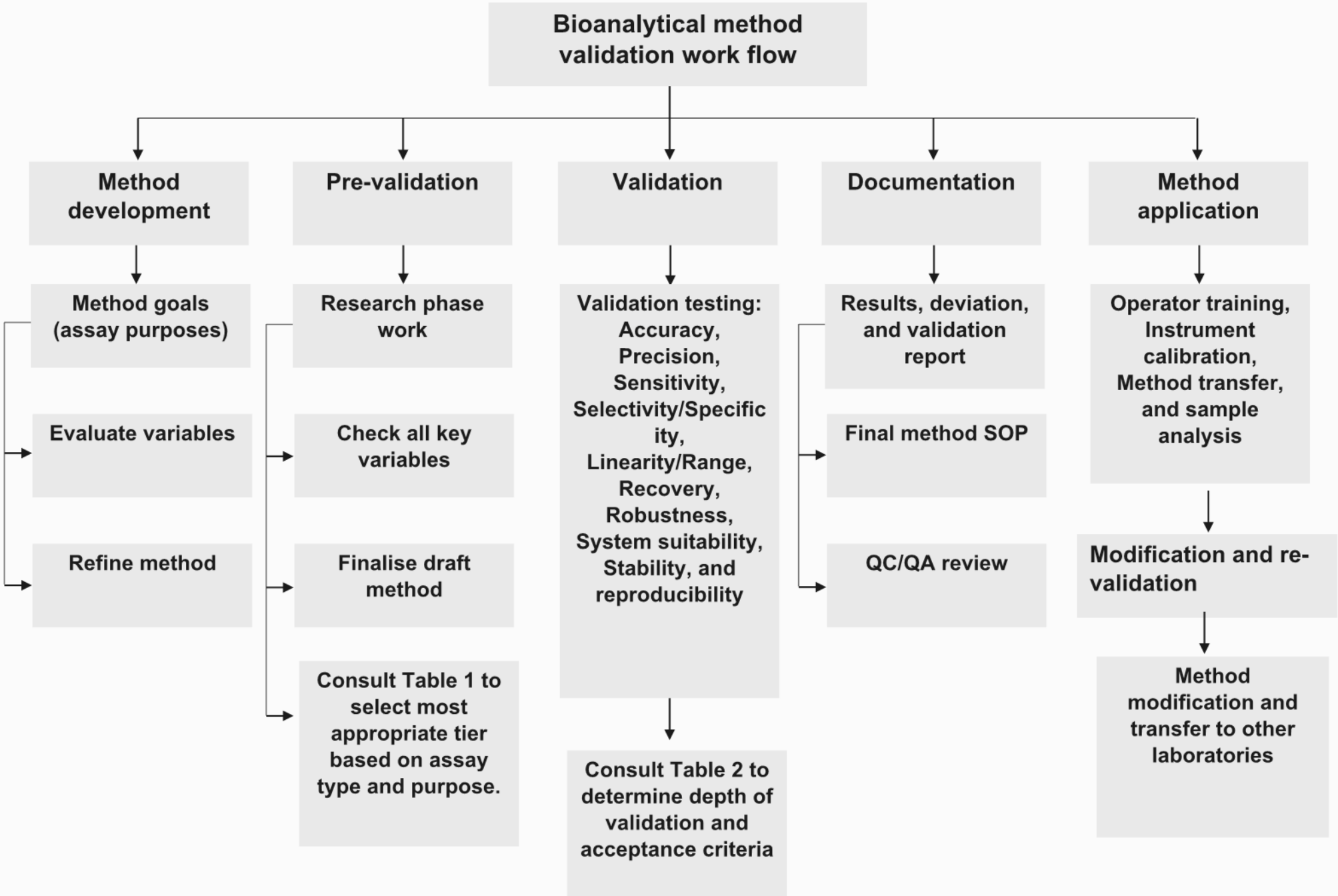
Tiers of framework to evaluate reliability	Purpose (example)	Assay type	Assay quantification
1- Validation	Diagnosis of disease/toxicity phenotype	Targeted metabolite analysis of 1 to < 10 metabolites	Absolute quantification with authentic standard(s)
2- Qualification	Diagnosis of disease/toxicity phenotype	Multiplexed targeted metabolomics analysis of > 10 metabolites	Absolute quantification with authentic standards
3- Screening	Screening for a disease/toxicity phenotype	Multiplexed targeted metabolomics analysis of panel of hundreds of metabolites	Relative or semi-quantitative; does not require an authentic standard for each metabolite
4- Discovery	Discovery of putative metabolic biomarkers	Untargeted metabolomics	Relative quantification

2 **Table 2. Parameters for validation (Tier 1) *vs*. qualification (Tier 2) of a metabolomics assay**

Parameters	Tier 1- Validation	Acceptance criteria	Tier 2- qualification	Acceptance criteria
Calibrators/linearity	• 5 independent calibration lines, minimum of 6 non-zero calibrators covering the range of incurred samples	• R^2 >0.98, closer to 1 is better • Setting LLOQ as lowest acceptable standard	• 3 independent calibration lines, minimum of 8 non-zero calibrators covering the range of incurred samples	• R^2 >0.98, closer to 1 is better • Setting LLOQ as lowest acceptable standard
Assay range - lower/upper limit of quantification (LLOQ/ULOQ)	• Over 6 runs	• R^2 >0.98	• Over 3 runs	• R^2 >0.98
Calibration Quality Control (QC) levels	• Prepare LLOQ, low, medium and high QCs in 5 replicates	• RSD<15%, except for LLOQ (RSD<20%)	• Prepare LLOQ, low, medium and high QCs in 5 replicates	• RSD<20%, except for LLOQ (RSD<25%)
Intra-study QC (pooled QC) levels	• After every 6 unknown samples with the minimum number of 6 per assay	• At least 67% (e.g. at least four out of six) of the QC concentration results should be within CV<15 %	• After every 6 unknown samples with the minimum number of 6 per assay	• At least 67% (e.g. at least four out of six) of the QCs concentration results should be within CV<20 %
Precision (within-day/intra-precision)	• Over 1 Run, 5 replicates, 4 levels (LLOQ, low, medium and high)	• Should not exceed 15% of the coefficient of variation (CV% or RSD%) except for the LLOQ, where it should not exceed 20% of the CV	• Over 1 Run, 5 replicates , 3 levels (low, medium and high)	• RSD<20-25%
Precision (between-day/inter-precision)	• Over 6 runs, 5 replicates, 4 levels (LLOQ, low, medium and high)	• RSD <20%, at LLOQ RSD<25%	• Over 3 runs, 5 replicates, 3 levels (low, medium and high)	• RSD<30%
Accuracy (within-day/intra-accuracy)	• Over 1 Run, 5 replicates, 4 levels (LLOQ, low, medium and high)	• Within 15% of nominal value, except for LLOQ within 20%	• Over 1 Run, 5 replicates, 3 levels (low, medium and high)	• Within 20-25% of the nominal value
Accuracy (between-day/inter-accuracy)	• Over 6 runs, 5 replicates, 4 levels (LLOQ, low, medium and high)	• Within 20-25% of the nominal value	• Over 3 runs, 5 replicates, 3 levels (low, medium and high)	• Within 25-30% of the nominal value
Selectivity/specificity/ matrix effect	• Perform the test	• Absence of interfering compound accepted where the	• Not applicable.	• Not applicable.

Parameters	Tier 1- Validation	Acceptance criteria	Tier 2- qualification	Acceptance criteria
		response is less than 20% of LLOQ and/or less than 5% for IS		
Carryover	• Perform the test	• Absence of interfering compound accepted where the response is less than 20% of LLOQ and/or less than 5% for IS	• Perform the test	• Absence of interfering compound accepted where the response is less than 20% of LLOQ and/or less than 5% for IS
Parallelism	• Perform the test, depending on availability of sample with high endogenous analyte from 6 individual sources of blank matrix	• Precision between samples in a dilution series should not exceed 30%	• Perform 1 or 2 tests depending on availability of sample with high level of endogenous analyte	• Precision between samples in a dilution series should be 30-40%
Dilutional Linearity/integrity	• Perform the test	• Spike blank matrix to concentration above ULOQ and dilute it down with blank matrix (5 determinations per dilution) • Accuracy: ± 15% of nominal concentrations • Precision: ± 15% CV • R^2 >0.98	• Perform the test if applicable	• Spike blank matrix to concentration above ULOQ and dilute it down with blank matrix (1 determination per dilution) • R^2 >0.98
Prozone (hook) effect	• Perform the test, as applicable	• The calculated concentration for each dilution should be within ± 20% of the nominal concentration after correction for dilution and the precision of the final concentrations across all the dilutions, should not exceed 20%	• Not applicable.	• Not applicable.
Stability - room temperature	• Perform the test	• The accuracy (% nominal) at each level should be ± 15%	• Recommended	• The accuracy (% nominal) at each level should be ± 25%
Stability - 4◦C	• Perform the test	• The accuracy (% nominal) at each level should be ± 15%	• Recommended	• The accuracy (% nominal) at each level should be ± 25%
Stability - freeze/thaw	• Perform the test	• The accuracy (% nominal) at each level should be ± 15%	• Recommended	• The accuracy (% nominal) at each level should be ± 25%
Stability - long-term (-20◦C and/or -80◦C)	• Perform the test	• The accuracy (% nominal) at each level should be ± 15%	• Not applicable.	• Not applicable.

Abbreviations: LLOQ, lower limit of quantification; ULOQ, upper limit of quantification; IS, internal standard.


Bioanalytical method validation work flow
Method development
Pre-validation
Validation
Documentation
Method application
Method goals (assay purposes)
Evaluate variables
Refine method
Research phase work
Check all key variables
Finalise draft method
Consult Table 1 to select most appropriate tier based on assay type and purpose.
Validation testing: Accuracy, Precision, Sensitivity, Selectivity/Specificity, Linearity/Range, Recovery, Robustness, System suitability, Stability, and reproducibility
Consult Table 2 to determine depth of validation and acceptance criteria
Results, deviation, and validation report
Final method SOP
QC/QA review
Operator training, Instrument calibration, Method transfer, and sample analysis
Modification and re-validation
Method modification and transfer to other laboratories