

CHAPTER 1

Valid Analytical Molecular Biology: The Challenge

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1.1 Introduction

The last decade has seen a rapid increase in the pace of technological advancement and in the uptake of DNA analysis for a range of applications. The increased use of DNA as an analyte reflects its uniform presence in almost all cells of most organisms. In addition the greater stability of DNA, compared to RNA or protein molecules, is ideal for analysis of highly processed or aged samples.

Technical innovations include the development of more sensitive, quantitative, high-throughput and massively parallel analyses, all generating new applications and commercial opportunities and covering a wide range of uses. The complete DNA sequence of many genomes has been determined, opening the way for a plethora of new applications, including directed drug discovery and personalised genetic diagnostics and treatment. Forensic analysis, food testing and agriculture are just a few of the many other areas where DNA technology is being adopted, with concomitant changes in regulation and procedures. It is clear that there are significant advantages in using molecular methods, including reduced detection limits, greater speed and scale, lower cost and improved specificity. The potential of novel genetic diagnostic methods, directed drug discovery routes and the increased throughput of massively parallel array-based analyses are strong drivers for even greater uptake of this technology. However, to exploit fully the potential of these developments and remove barriers to wider uptake, there is a need to ensure that molecular analytical methods are reliable, consistent and fit for purpose, in order to avoid the use of biased or flawed techniques and resultant loss of confidence in the techniques.

The majority of technological development occurs in academic or medical research environments, where the main priority is innovation. Consequently little consideration is given to the more routine applicability, reliability and reproducibility of methods, particularly in the early stages of development. Despite evaluation of method performance characteristics and method validation being a prerequisite for the successful move of techniques from the research laboratory to the analytical laboratory, there is resistance to such formal evaluation in some sectors. There are also practical barriers to assessment of method performance, including the lack of reference materials which are necessary for the critical comparison of analytical approaches and the paucity of performance standards in the wider analytical community, as most regulation of analysis is carried out in-house. However, in the light of growing commercial and clinical application, consideration is increasingly being given to the reliability of the technology being used.

Although large volumes of analytical data may be produced from poorly applied methods, generation of dependable results usually requires careful and considered planning and validation. The aim of any experiment is to produce reliable results, and to avoid the need to repeat the analysis because of problems with the reagents, method or equipment used. Consistently ‘getting it right first time’ depends on a number of factors, including provision of a controlled laboratory environment with calibrated and regularly maintained instruments, use of an effective experimental design and performance of the work by an analyst with sufficient training and experience to correctly perform the method and interpret the result (Figure 1.1). Although it is difficult to estimate the actual cost of poor laboratory practice in wasted time and reagents, the benefits in avoiding repeating work are very clear.

This manual aims to introduce and address quality assurance and validation issues that arise in the application of DNA technology, and to provide a basis for the development of validated methods and experimental good practice. Specifically, Chapters 2 and 3 cover the benefits of formal laboratory management systems and method validation. The remaining chapters in the manual provide information on a range of commonly used techniques, from the initial extraction of DNA from analytical samples and quantification of the amount of DNA present, to a range of downstream processes including various forms of polymerase chain reaction (PCR) amplification and microarray-based analysis.

Analytical laboratories should work to produce quality analytical data, and reading the information presented here should provide a firm foundation for good experimental practice.

1.2 The Analytical Process

1.2.1 Analytical Requirements

Analysis is usually initiated by a ‘customer’, who can be a private individual or company, public organisation, research funding body or law enforcement

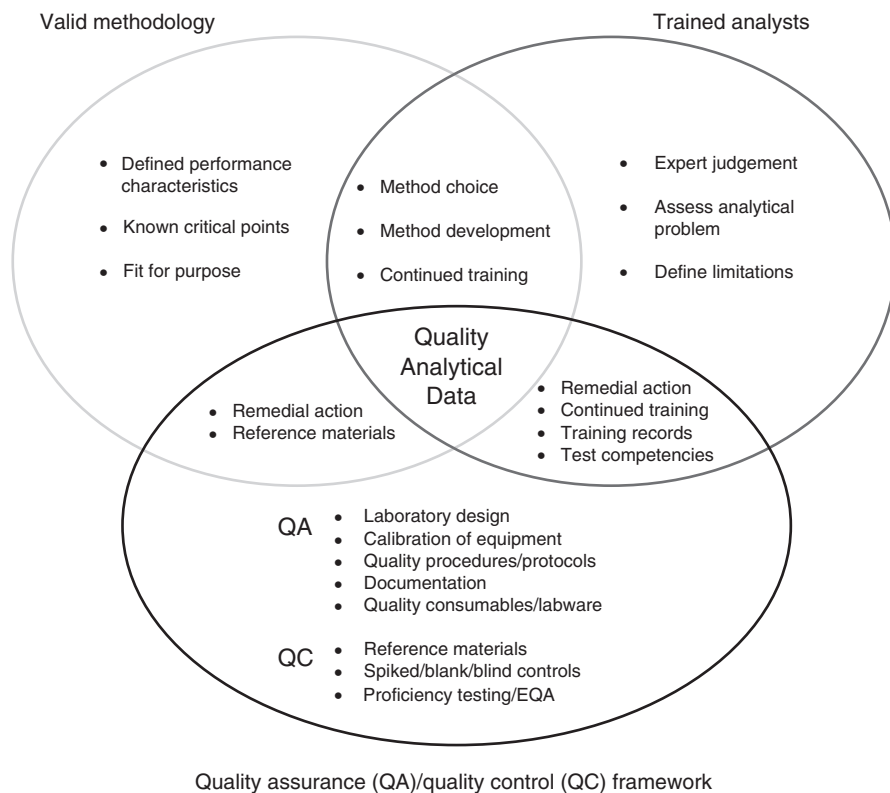


Figure 1.1 Schematic diagram showing the factors within the laboratory that contribute to the production of reliable data.

agency such as a police force or trading standards office. The results that are produced are usually required for a specific purpose, often as an independent source of information in order to gauge a situation, interpret evidence, determine whether action is required or to ascertain whether certain regulations are being adhered to. Increasingly, some indication of the level of confidence that can be placed in the result is also required, allowing the results of the experiment to be used or interpreted appropriately.

1.2.2 Stages in the Analytical Process

In undertaking an experiment or analysis to address a specific question, a complex procedure is undertaken, beginning with the initial researching of the questions and specific analytical requirements and ending with the interpretation of the analytical data produced and the reporting of results and conclusions. To ensure the process is efficient, careful planning of the work is required. Good experimental design, trained staff and use of suitable methods, equipment, standards and samples can save time in ensuring that sufficient and

Table 1.1 Stages of the analytical process.

Define the analytical enquiry	<ul style="list-style-type: none"> • Define type of data required (qualitative/quantitative) • Define use of the data and confidence required in the result • Define required performance of the method
Assess the sample	<ul style="list-style-type: none"> • Determine nature of the sample • Define storage, transport and preparation requirements • Use appropriate sampling procedures • Ensure trackability through unique sample ID system
Establish constraints	<ul style="list-style-type: none"> • Establish time available for analysis • Identify equipment and personnel resources available • Understand any financial limitations • Determine if there are special safety considerations • Is the analysis feasible?
Define technical approach	<ul style="list-style-type: none"> • Identify suitable techniques based on analytical requirement • Match analytical performance to requirements
Select or develop method	<ul style="list-style-type: none"> • Select suitable method from published literature, or commercial kits • If none suitable, develop in-house method • Finally, prepare a draft protocol
Validate method	<ul style="list-style-type: none"> • Ensure method performance meets analytical requirements • Demonstrate the method produces appropriate data • Document the suitability of the method
Apply the validated method	<ul style="list-style-type: none"> • Analyse the samples using the selected, validated approach • Use appropriate controls to enable confident interpretation of results
Interpret and report the data	<ul style="list-style-type: none"> • Interpretation of the data will depend on the results from QC materials included in the assay as well as test samples themselves • Any limitations of the method should be included in conclusions and interpretations of results

reliable results are produced first time. A flawed approach may produce experimentally valid data that do not directly address the enquiry, or insufficient data for confident interpretation. Incorrect sample collection or storage could produce erratic results even when a valid method is applied. In addition, use of uncalibrated equipment could generate biased results that do not allow correct judgement of the actual situation. An overview of the stages to consider when planning the experimental process is outlined in Table 1.1.

1.3 Principles Underpinning Reliable Measurement

A series of six principles to underpin good experimental practice has been developed, known as the Valid Analytical Measurement (VAM) principles.

Although primarily directed towards chemical analysis, the principles are generic and fully applicable to biological measurement performed in both research and more routine laboratory environments. The described approach requires support and implementation at both a technical and management level, and the ethos needs to be understood and supported by all staff in the laboratory.

1.3.1 Understand the Experimental Requirements

Experiments or measurements are generally undertaken to answer a specific question or to provide a solution to a problem. Understanding the full nature of the enquiry enables an experimental approach to be developed to produce sufficient data to fully answer the question.

1.3.2 Use Methods and Equipment which are Fit for the Intended Purpose

Consistent production of reliable data requires that the methods, instruments, reagents and software used in an analysis have been tested and shown to perform as expected. Further information on fulfilling these requirements is given in Chapters 2 and 3.

1.3.3 Staff Undertaking Analysis Should be Both Qualified and Competent to Undertake the Task

To ensure that methods and equipment are used correctly, appropriate levels of staff training and support are required. Formal management schemes address the continued training and assessment of staff (Chapter 2), and even in laboratories where no formal system is in place, some level of training is advisable to avoid time wasted in repeating experimental analyses and the cost of equipment damage through misuse.

1.3.4 Regular Independent Assessment of Laboratory Performance

Independent assessment usually takes the form of proficiency testing (PT) or external quality assessment (EQA) schemes, where samples are distributed to participating laboratories for analysis. The results are returned and analysed by the scheme organisers, and a report detailing the performance of the participants is produced, usually without identifying individual participants. Such external assessments of performance are useful to confirm that procedures are producing acceptable in-house results, and results can be compared to those produced in peer laboratories.

1.3.5 Analytical Consistency

A primary aim of any researcher or analyst is consistently to produce reliable and valid results. The use of well-defined samples or certified reference materials (CRMs) can be used on a regular basis to demonstrate the consistent quality of measurements within a laboratory. In biological analyses few reference materials are available, but the use of previously characterised samples can be used to monitor performance over time.

1.3.6 Quality Control and Quality Assurance Framework

Formal management systems (Chapter 2) specify the need for laboratory management systems, including the use of trained staff, calibrated equipment, quality protocols and valid methodologies. This is the quality assurance (QA) framework, which can assist in preventing errors by ensuring the laboratory and analytical environment is fit for purpose. Quality control (QC) measures are used in parallel with QA systems, and confirm the quality of data obtained by the use of control samples and continual monitoring of performance.

1.4 Challenges to Measurement Quality

Despite the establishment of good measurement principles, many technical challenges remain, arising from a number of factors including the variety of available methods and platforms, the pace of technological development, lack of certified reference materials to establish comparability between approaches and few accessible EQA or PT systems to evaluate comparability between laboratories. In addition to the practical challenges there is also a number of administrative and management issues including pressure to publish results regularly, often high levels of staff turnover and lack of funds and resources for QC and QA activities.

The analysis of real samples often provides a further challenge, as low concentrations or inhomogeneous distribution of targets may pose problems. Difficult samples may originate from a variety of sectoral applications such as forensic, food or environment, where the DNA analyte may be in association with an organic matrix, for example, a blood stain on cotton fibre, bacterial species in milk or genetically modified (GM) soya in processed food products. A number of common problems that arise in the application of DNA technologies are considered here.

1.4.1 Low Concentration of Analyte Compared to Matrix

The need to detect, identify and/or quantify very low levels of the target in a large amount of sample matrix for various applications has led to the development of sophisticated DNA extraction and amplification methodologies to selectively isolate and concentrate the analyte of interest. Examples include low-level detection of environmental and food pathogens, non-invasive

prenatal diagnostic methods and the quantification of DNA contaminants in biopharmaceuticals.

1.4.2 Complex Matrices

Target analytes present in complex chemical or biological matrices can make sampling and DNA extraction a difficult undertaking. Challenging matrix components include naturally occurring secondary compounds which can interfere with enzyme activity and can cause total inhibition of biological reactions such as PCR and restriction enzyme digests. Certain components of biological samples, such as haem and urea, may also affect the analysis. There may also be difficulties in physically separating the analyte from the matrix, as clumping and adhesion can make uniform sampling and efficient DNA extraction difficult.

1.4.3 DNA Degradation

In some instances samples may be subjected to harsh environmental, transport or storage conditions that can damage the analytes significantly. Poor conditions include industrial processing such as freezing, dyeing, heating, grinding, tanning, drying and forms of weathering such as those caused by the sun or rain. Ageing of a sample can also cause physical degradation of the DNA analyte, and in such instances the use of short DNA targets can enable even highly degraded materials to yield results. However, it is important to use calibrators and controls that are in an equivalent physical state to the test sample, as otherwise the results of the analysis may be affected by any disparate performance of the intact and degraded materials in the experiment.

1.4.4 Biological Contamination of the Sample

Often the test sample has been contaminated before arrival at the testing laboratory, so nucleic acids from a variety of sources may be present. The contamination may be due to environmental insult (for example, bacterial or fungal contamination), or may inherently be a mixture of target and non-target material (such as food samples containing a proportion of genetically modified ingredients). A further problem may arise if the contaminating material contains chemicals or enzymes that are able to damage DNA within the sample, such as low pH fruit juices or DNases from contaminating micro-organisms. In this situation it is not always possible to utilise controls in a similar physical state to the test samples, and so the likely effect of the contaminant on the results should be taken into account when interpreting the results of the experiment.

1.4.5 Degradation of Matrix Components

Various components within an analytical matrix can sometimes produce breakdown products, such as polyphenols, that cause the degradation of

nucleic acids. In such circumstances, some assessment of the level of DNA degradation is helpful to enable the results of the test to be interpreted correctly. Tests of the effect of known contaminants may also be performed, using appropriately spiked samples as controls.

1.4.6 Limited Availability of the Sample

A sample may be limited because the sample represents a unique moment in time (for example, a particular stage in disease progression) or is limited by quantity (such as a scene-of-crime swab or patient biopsy). Samples that are difficult to replace require extreme care in processing and analysis, as by definition the analysis cannot easily be repeated.

1.4.7 Lack of Suitable Controls

One of the main challenges in molecular analysis is the lack of certified reference materials (CRMs), which are a key component in validation and QA procedures. The majority of physical and chemical measurements are underpinned by suitable standards but, because of the complexity of the analytes and the wide range of materials under test, such standards are not available for most biological applications. In addition, there are very few characterised reference samples that can be employed to ensure the accurate calibration of equipment, the correct handling of samples or applicability of methodologies. Alternatives to the use of CRMs include comparison of the results from several techniques, or analysis of the same material by several laboratories, followed by comparison of the results. However, as there are no materials for which a 'true value' is known, objective assessment of method, equipment and analyst performance is not straightforward.

1.5 Focus on Data Quality

As mentioned already, most QA and QC activity takes place in-house, and may sometimes be compromised because of resource constraints. An exception is in analyses where the results may be reported and used in a court of law, such as short tandem repeat (STR) forensic profiling and the quantitative determination of GM ingredients in foodstuffs. In such cases, the validity of the analytical result must be demonstrated absolutely, and is subject to stringent questioning and challenge by defence lawyers. The presence of an equivalent pressure is not always evident in other areas of analytical molecular biology. The quality of data is reliant on the professionalism of the analytical laboratory and the analysts involved, requiring continual questioning and re-evaluation of the analytical approach, procedure, staff capabilities and applicability of the test. Individuals within the laboratory need to look beyond the data that are produced by the instrument or method to the wider experimental context in order to correctly interpret results. For example many quantitative PCR (qPCR) instruments provide quantification results with an apparent accuracy

of several decimal places. However, consideration of the likely errors introduced into the process through the various stages including preparation of standards and reaction set-up indicates that reporting results with this level of precision may be misleading.

Exercising critical judgement in laboratory set-up, experimental design and practice and in interpretation of results is central to ensuring consistent production of reliable data and, most importantly, is in your hands.

Acknowledgements

The author would like to thank Ginny Saunders for information and illustrations, and Lyndsey Birch for many helpful discussions.