16

Development of Metrology for Modern Biology

Sang-Ryoul Park, Jun-Hyuk Choi and Ji-Seon Jeong
Center for Bioanalysis,
Korea Research Institute of Standards and Science,
Republic of Korea

1. Introduction

1.1 Why metrology for modern biology

The full scale DNA information obtained from the Human Genome Project has enabled the revolutionary advances in biotechnologies (BT). Information on human genome is equal to a whole blueprint on human body. Although gigantic, such information can be comprehended and handled with the help of modern information technology. With the information, we can fully understand how our bodies are structured and functioning. Consequently, there will be tremendous advances in human health with innovations in modern medicine driven by BT. It is for sure that mankind will enjoy a healthy and extended life term in a way they never grasped before.

The bright sides of the advanced BT come only with dark sides. It is anticipated that disastrous mishaps could occur as we don't have experiences on such new products of BT. The great outcomes of modern BT can be enjoyed only with meticulous assurance on their safety and efficacy. As those directly touch the quality of life, they could be expensive ones that people have to pay over the financial burdens. Then, the fidelity of such claims will have grave importance to many customers. More important, it can greatly jeopardize lives unless all possible safety concerns have been exhaustively confirmed. Therefore, BT products are subject to extensive testing for quality control (QC) as well as for quality assurance (QA). An unavoidable question here is if the results of such testing are believable. There are numerous formats of bioassays that can be applicable to such testing. Some of those assays might be applied to critical tests for QC and QA without appropriate method validation. Consequently, different results can be produced at different laboratories, which will then create conflicts and disbelief among stake holders. Even worse, some assays that lack analytical reliability can be used to blur the undesirable characteristics of properties of some products.

There have been a number of prominent incidents that insufficient or inadequate testing for QC of biological products greatly risked public health. Among them, "tainted heparin" caused at least 80 deaths. Heparin is used as a blood thinner against formation of blood clotting during surgery or dialysis. The FDA's investigation concluded that heparin problem was due to contaminated raw stock from China with which Boxter Healthcare produced a prevailing portion of world heparin supply. Tight and adequate testing to detect such fatal contaminants before sales would easily avoid such incidents and save many lives. Similar

tragedy occurred with dairy products as baby meal. As a greedy adulteration of dairy products, melamine was added to milk products to fake high protein content in nitrogen analysis. At least 50 thousand babies were harmed by the melamine in their meal. This incident was also a prominent example of public health risked by insufficient or inadequate testing for quality control of biological products. If QC of dairy products was performed by a method capable of distinguishing real proteins and a nitrogen-rich toxic chemical, such disaster could be easily avoided.

Considering even more complicated products of modern BT, the need for rigorousness of such testing is out of question. There are and will be numerous new products from nucleic acids, proteins, and cells that are highly complex in structures and activities. Consequently, a number of novel analytical approaches are being developed to characterize and quantify such complex biological entities. However, what would be the standards for adequateness of such assessments? Proper answers to the question above should be attained before full-scale industrialization of modern biotechnology and eventually their routine commercial applications. In other areas undergoing earlier industrialization, the support of metrology was critical. The similar or even greater support of metrology is needed for industrialization of biotechnologies. In this area, inaccuracy is more critical, and uncertainty in measurement is much greater. Therefore, metrology for biology should be regarded as a critically important area of modern metrology.

1.2 Who work in this area

National metrology institutes (NMI) are on the first line in development of new areas of metrology. The efforts of NMIs are organized in international scale by Bureau International des Poids et Measures (BIPM, www.bipm.org). In 2000, BIPM arranged the first international meeting for the purpose of development of metrology in biology, which then led to creation of Bioanalysis Working Group (BAWG) as a member group of Consultative Committee on Quantity of Matter (CCQM). Since then, CCQM BAWG has been the most significant international organization to develop metrology for biology. Prominent international organizations related in biology have been working together with CCQM BAWG. World Health Organization-National Institute for Biological Standards and Control (WHO-NIBSC, www.nibsc.ac.uk) has been very active in CCQM BAWG activities. In addition, US-Pharmacopeia (www.usp.org) and International Federation of Clinical Chemistry (IFCC, www.ifcc.org) have been communicating closely with CCQM BAWG. A number of international measurement comparisons were made to assess international comparability or to investigate and pinpoint existing major problems in bioanalysis. Among them, quantification of an exogenous gene of genetically modified organisms (GMO) firstly attained interests of participants as it rose as an international concern in both food trade and environmental pollution. Accurate quantification of DNA was the first technical target as it is the most fundamental element in modern biology. As a natural advancement, quantification of proteins has become a central topic. Recently, dare movements toward cells or microorganisms are happening. This is a totally new area where technical advancement is rapid, and measurement issues come urgently. For this reason, proper projection and accordingly planning on future is of high importance. Recently, BIPM has published an investigative report (Marriott et al., 2011) on the current status and future directions of metrology development for biology that is of great value to oversee this particular area.

1.3 Approaches and technical issues

Establishment of biometrology is technically demanding. Biological materials are mostly large and complex in their chemical structure. Typical dimensions of important biological entities are illustrated in Fig. 1.1. Even with a highly advanced analytical technique such as mass spectrometry, biological materials are not readily quantifiable with a desired level of accuracy. There are substantial technical gaps between the demands and currently available techniques.

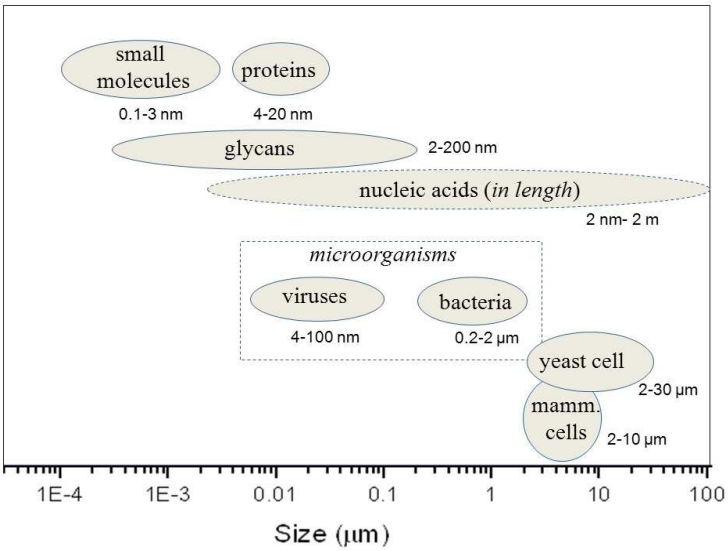


Fig. 1.1. Dimensions (Stokes-Einstein diameters) of biologically important materials. The dimensions of nucleic acids is indicated in length.

Then, approaches to overcome such technical gaps need to be sought out. The approaches suggested by Korea Research Institute of Standards and Science (KRISS, www.kriss.re.kr) scientists as basic approaches for establishment of metrology for biology (Fig. 1.2) would not be much disputable. The first coming idea is to reduce large biological molecules to smaller chemical units that can be accurately quantifiable with current analytical techniques. Reduction to chemical units can be achieved through chemical reactions or enzymatic reactions. The quantity of the original biological molecules is then calculated from the knowledge on their chemical compositions. Uncertainty of such analytical procedures mainly arises from the degrees of perfection of such reduction processes. As far as metrological assessment is made on the uncertainty of the reduction process, the overall analytical procedure can be regarded as of metrological quality. Quantitation of proteins by amino acid composition is a typical example of the reduction approach. Examples of this approach including metrology for protein quantification are described in detail in the following sections.

Large biological materials are discrete and countable. Quantification of cells (cytometry) is an excellent example of count-based quantitation of which history is now longer than

several decades. We can extend this approach whatever biological materials that have a discrete (countable) nature in principle. Quantification of large DNA particles is described in the following section as well as digital-polymerase chain reaction (digital-PCR) that can be also categorized into count-based quantification. In quantification of microorganisms, counting of cultured colonies is of a relatively long history.

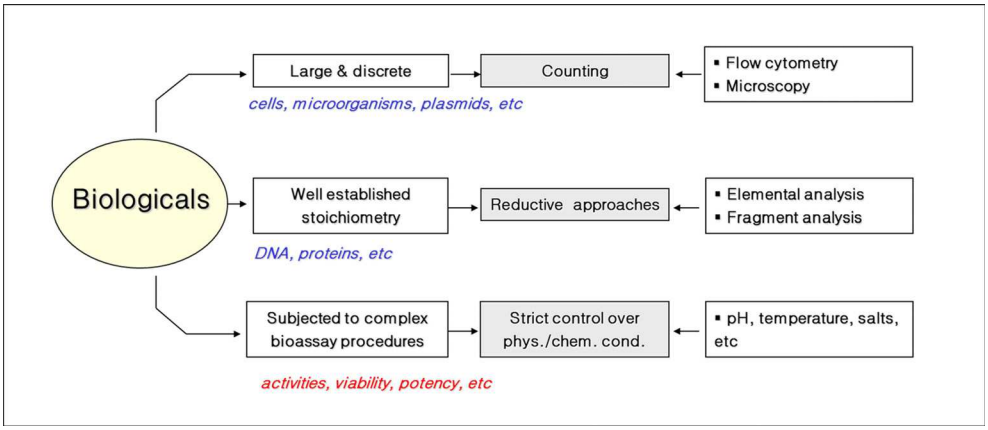


Fig. 1.2. Approaches for establishment of technical basis for metrology for biology suggested by KRISS scientists (from the presentation at 2005 CCQM workshop on "New Challenges for the development of Primary or Higher Order Measurement methods and Procedures").

Several important biological quantities such as activity, viability, efficacy, and toxicity are not the quantities of biological molecules as above. Instead, these quantities are defined by the experts group of the related fields. These quantities are often called "method-dependent quantities" as those are determined by following the analytical procedures agreed among the experts groups. Metrology for such quantities is therefore of complexity and is often thought to be out of the domain of metrology. However, comparability of the measurement of those quantities is of great importance, and a great deal of improvement can be made by applying metrological rigorousness to the physical or chemical conditions involved in each analytical procedure. An example of such aspect is demonstrated in PCR in the following section. Noticeable technical advances have been made in metrology for quantification of nucleic acids and also some for proteins, but not yet for other complex biological materials such as cells and glycans. For these materials, a great deal of research should be done to visualize the practical paths forward realization of metrology. Here, only currently conceivable technical issues are introduced.

2. Metrology in quantification of nucleic acids

2.1 Backgrounds

Nucleic acids consist of deoxyribose nucleic acids (DNA) and ribose nucleic acids (RNA). It is well known that chemically more stable DNA carries genetic information in long terms whereas RNA promptly response to physiological demands by transferring genetic information to protein productions. For DNA, its base sequences, the genetic codes, are of

primary concerns. Base sequencing of DNA is a full-blown technique these days. The trend in technical advancement was in increment of throughput of analysis. Multiple channel capillary gel electrophoresis has been the most effective and prevailing technique in this regard. Recently, next-generation sequencing (NGS) technology featuring markedly higher throughput than multiple capillary sequencers have been commercialized for more widespread investigation and application of genetic information (Mardis, 2008). Here, the potential bias in DNA sequencing or accuracy in base-calls could be an important issue (Harismendy et al., 2009). However, this area is not in the main scope of metrology as metrology primarily concerns on the quantity. On the other hand, RNA has to be assessed not only about their identities but also regarding their quantities. The expression levels of RNA responding to physiological demands are of critical importance in understanding life processes. Accurate determination of the levels of RNA is especially important to globally share the obtained information. In this regard, related communities such as the Functional Genomics Data Society (FGDS, www.mged.org) actively work for achieving satisfactory levels of global harmonization in RNA quantification.

However, accurate assessment of the quantity of DNA is often crucial (O'Connor et al., 2002). One of the simplest forms of DNA is oligonucleotide. Synthetic oligonucleotides are widely used as an essential component of PCR (Dieffenbach et al., 1993; Halford, 1999), probes for various detection schemes (Mitsuhashi et al., 1994), even therapeutic agents as anti-sense drug (Pirollo et al., 2003; Tewary and Iversen, 1997) and agents for RNA interference (Doran, 2004). Some of these applications demand accurate quantitation of oligonucleotides. For example, accurate quantitation of oligonucleotide preparations is one of the most important concerns in the field of DNA-chip technology, where quantitative information from the applied oligonucleotide probes is crucial (Peterson et al., 2001). Accuracy in the quantity of DNA may govern the confidence and inter-comparability of the results of various experiments. In regulations of genetically modified organisms (GMOs), the quantity of a modified gene relative to an endogenous gene is the basis of legal judgments, and technical feasibility in determination of the quantity has been a crucial point for the successful implementation of the legal systems. In modern forensics, revolutionary technical advance has been made by short-tandem repeats (STR)-based human identification. This technique itself is not in the category of DNA quantification, but its reliability is strongly dependent upon the quantity as well as the quality of the obtained DNA samples. Therefore, accurate determination of the quantity of DNA in the sample specimen is an important prerequisite to enable its powerful utility in courts (Brettell et al., 2009). Among various biological substances, nucleic acids come first as the target materials for establishment of metrology for biology. Success in establishment of quantitative metrology for nucleic acids would be the litmus paper for the future success on establishment of metrology for biology.

As discussed in the following section, conventional analytical methods such as UV-absorption and fluorescence measurements as well as recent PCR-based measurements lack measurement traceability to the SI unit of mole. Even the most advanced mass spectrometry (MS) is not suitable to accurate quantify nucleic acids. Numerous reports on applications of MS techniques, mostly matrix assisted laser desorption ionization-time of flight (MALDI-TOF) MS (Bruenner et al., 1996; Zhang and Gross, 2000) are found but are not of adequate accuracy. The complex nature of the ionization of poly-ionic DNA material causes

substantial uncertainty in quantitative analysis. Rigorous evaluations on quantitative performances of mass spectrometry in analysis of polymeric DNA molecules should be further carried out. Meanwhile, other forms of primary analytical methods for establishment of metrology in DNA quantification are demanded. In this regard, notable new approaches are discussed in the following sections.

2.2 Conventional methods

Measurement of UV absorbance is most widely used for quantification of DNA. UV absorbance is commonly described in the unit of optical density (OD) of which definition is absorbance of UV light through 1 cm absorption path-length. For DNA, OD for 260 nm (OD_{260}) is preferentially used since a local absorption maximum of DNA is at 260 nm. It is known that 1.0 OD_{260} corresponds to the absorption by a single strand oligonucleotide of 30-38 μg/mL, where the most commonly accepted value is 33 μg/mL. Although measurement of UV absorbance is quick and easy, the quantity of DNA estimated from OD is a crude approximation. Bases of DNA consist of four different kinds such as adenine (A), guanine (G), thymine (T) and cytosine (C), and the absorbance of a DNA string varies dependent upon the base composition. The purines of A and G featuring double hetero cyclic rings obviously show stronger molar absorption than the pyrimidine bases of T and C having one ring. The effect of base composition could be reflected in conversion of a UV OD value to a DNA quantity. However, such reflection is not simple due to hypochromicity effects caused by stacking among neighboring bases. A simple summing up method ignoring hypochromicity could lead to as highly as 24% overestimation of the extinction coefficient of a tested oligonucleotide (Cavaluzzi and Borer, 2004). Refinement in consideration of the sequence dependency of the extinction coefficient has been made to 'nearest-neighbor estimates' of extinction coefficients based on mono- and dinucleotide additivity rules (Kallansrud and Ward, 1996). Although quick and convenient, calculation of the concentrations of nucleic acids from the extinction coefficients and UV OD values can't be used as a definitive quantitation method for DNA analysis.

Measurement of fluorescence from dyes intercalated into DNA is known to be a highly sensitive quantitation method of DNA. The dyes are intercalated into DNA proportionally to the number of base units (Yan et al., 1999). Therefore, a linear relationship exists between the intensity of fluorescence and the quantity of the bases. However, the intensity of fluorescence depends on other parameters such as dye concentration, the degree of bleaching, intensity of the excitation light, the optics, and the geometry of a measurement cell as well as the quantity of DNA (Heid et al., 1996). For this reason, calibration using accurate measurement standards is indispensable to obtain accurate quantitative results in fluorometric determination of DNA.

Polymerase chain reaction (PCR), especially in real-time fluorescence detection mode (rt-PCR) or quantitative PCR (q-PCR), is widely used for quantification of specific genes in a DNA mixture for various purposes (Heid et al., 1996; Ponchel et al., 2003). The exponential amplification of PCR renders unprecedented sensitivity to the detection of DNA. If the degree of amplification is monitored at the early stage of the exponential amplification, a linear relationship between the logarithm of the quantity of DNA and the PCR cycle for a given amplification level is obtained (Heid et al., 1996). In PCR amplification, high

selectivity to a specific gene sequence is achieved by using the unique sequences of a primer pair. Therefore, the unknown amount of a target gene sequence can be determined from a calibration curve. However, q-PCR requires calibration standards as the amplification yield substantially depends upon various experimental conditions. Calibration materials of accurately determined quantity are not available yet, and the calibration curves are usually on the scale of the relative quantity of DNA. Although less precise in quantification (~ 10% RSD) and needs to be carefully performed (Halford, 1999), q-PCR is a prevailing quantification method for specific genes and requires urgent development of suitable calibration standards to have measurement traceability to the SI unit of mole.

2.3 Reductive approaches

Nucleic acids are polymers of nucleotide units which consist of base, carbohydrate, and phosphodiester bond (Fig. 2.1). As polymeric nucleic acids are not readily quantifiable even with advanced mass spectrometry due to the multiple charges, the components of nucleic acids could be measurement targets in reductive approaches. Firstly, it is noticeable that each unit of nucleotide bears one phosphorus atom in the phosphodiester backbone. Therefore, the quantity of a DNA sequence of a known length (base pairs: bp) can be accurately determined if the quantity of residual phosphorus is quantified accurately. Isotope dilution-mass spectrometry (IDMS) accepted by the metrology community is not an applicable option for determination of phosphorous as the isotope of phosphorus is radioactive (^{32}P and ^{33}P). Instead, inductively coupled plasma–optical emission spectroscopy (ICP-OES) may be chosen as it is capable of quantification of elemental phosphorus with reasonable precision and accuracy. KRISS scientists first tried this measurement approach where all substantial validation points were carefully examined (Yang et al., 2004). Phosphorus measured by ICP-OES should all come from the target DNA sequence. As a synthetic 20-mer DNA oligomer was quantified as a model DNA, all possible phosphorous contamination in the form of small molecules were removed by applying ultrafiltration of the sample solution. Complete removal was confirmed by the observation of complete disappearance of excessively spiked cytidyl monophosphate (dCMP) after repeated ultrafiltration. In phosphorus measurement, a certified reference material (CRM) of pure phosphorus was used as a calibrant, which provides measurement traceability. With the help of acid and microwave heating, DNA in the sample solution was digested to inorganic phosphates to avoid potential influences from the organic residues of the original molecular structure. An organic compound of a similar structure was excessively spiked into the sample solution to confirm the effect of the spiked organic additive disappears through the digestion process. Analytical performance of ICP-OES in measurement of phosphorus in element solutions has been within 0.2% of accuracy. For the nucleic acids it was estimated within 1% of expanded uncertainty. Sequence impurities of DNA oligomer was minimized by polyacrylamide gel electrophoresis (PAGE) purification, and confirmed using MALDI-TOF MS. As far as such validation points are well confirmed, the ICP-OES method seems to be a fine quantitative method for DNA with measurement traceability and assessable measurement uncertainty. This method has been used to determine the quantity of DNA materials for international comparison studies for DNA quantification. Several publications have been made in an attempt to establish the ICP-OES based phosphorous quantification as a primary analytical method for DNA quantification (English et al., 2006; Holden et al., 2007; Brennan et al., 2009).

One can target different moieties in DNA for the reductive measurement approach. Nucleotide could be the quantification target (O'Connor et al., 2002) whereas nucleoside can be also a measurement target (Donald et al., 2005). Phosphodiesterase produces single nucleotide from a DNA sequence with its activity for hydrolysis of phosphodiester bonds at the 5′-position. If the resultant nucleotides are further subject to phosphatase reactions, then nucleosides are produced. Dependent upon the suitability to available analytical methods, either form can be chosen for quantitation.

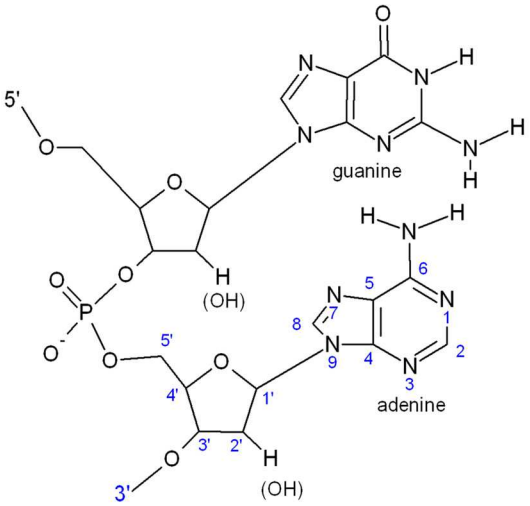


Fig. 2.1. Chemical structure of DNA (or RNA)

If the exact base composition of the target DNA is known, then its quantity can be readily deduced from the results of quantitation of nucleotides or nucleosides. O'Connor et al. first reported the use of HPLC-IDMS for accurate quantitation of nucleotides resulted from enzymatic hydrolysis of a DNA sequence (O'Connor et al., 2002). Later, they reported that analysis of the nucleosides instead of nucleotides resulted in better estimation of the quantity of the target DNA (Donald et al., 2005). For both analytical targets, isotope dilution-mass spectrometry (ID-MS) was applied to secure metrological quality of analysis. However, measurement traceability might not be well established due to the lack of high quality reference materials for preparation of nucleotides or nucleosides standard solutions. Some of those chemicals are too hygroscopic to accurately weigh the actual amounts in preparation of standard solutions. Instead, the exact quantity in the standard solutions can be determined by phosphorous measurement using ICP-OES where any phosphorus contamination of the nucleotide standard solutions can be determined using HPLC-ICP-MS in which different phosphorous-bearing species are separated and determined in terms of phosphorous quantity. Using a high resolution separation method, any partially degraded nucleotides can be also determined. Recently, Hong et al. reported a highly reliable capillary electrophoresis method for determination of nucleotides (Hong et al., 2011). The accurately determined nucleotide standard solutions will provide measurement traceability to this reductive approach. Utilizing those analytical techniques, several NMIs are preparing for the certified reference materials (CRMs) of the nucleotide solutions.

Regardless of the analytical methods applied for analysis of nucleotides or nucleosides, any imperfection of enzymatic hydrolysis will lead to bias in the final results. Therefore, the completeness of the enzymatic hydrolysis has to be carefully assured. Either an insufficient or excessive amount of hydrolysis enzymes may lead to abnormality in the final results. Fortunately, however, any substantial imperfection in hydrolysis can be unambiguously found by comparison of the quantitation results of four nucleotides or nucleosides. This aspect is a great advantage of the methods simultaneously determining four nucleotides or nucleosides. Compared to the ICP-OES method above, this group of analytical procedures requires substantially less amounts of samples, which dramatically expands the applicability. Therefore, the best approach in metrological quantification of nucleic acids will be as follows: 1) measurement traceability is established by the ICP-OES procedure; then 2) quantification of nucleic acids is performed by measuring nucleotides or nucleosides after enzyme hydrolysis where CRMs determined by the ICP-OES procedure is applied.

2.4 Count-based quantification

Another interesting approach in metrological quantification of DNA is a count-based quantitation of a trace level DNA. Two methods have been reported in this category. Firstly, several research groups have explored the potential of digital PCR (d-PCR) that aims amplify a single copy of a target gene in a microplate well (Sykes et al., 1992; Vogelstein and Kinzler, 1999). With appropriate dilution, the target gene is to be at the concentration level that only a single copy goes into a microwell or not (Fig. 2.2). Based on Poisson approximation of binomial distribution, at this concentration range, the population of wells that contain a single copy of the target gene among the given number wells will reveal the original concentration of the target gene if the dilution factor is properly considered. Occupation of each well can be determined by the fluorescence signal from the PCR amplicons stained with a fluorescent dye. The concept of digital PCR was first successfully transformed to a commercial instrumentation by Fluidigm (www.fluidigm.com), and commercialization was followed by several other companies. Interesting applications of digital PCR can be readily found (Zimmermann et al., 2008).

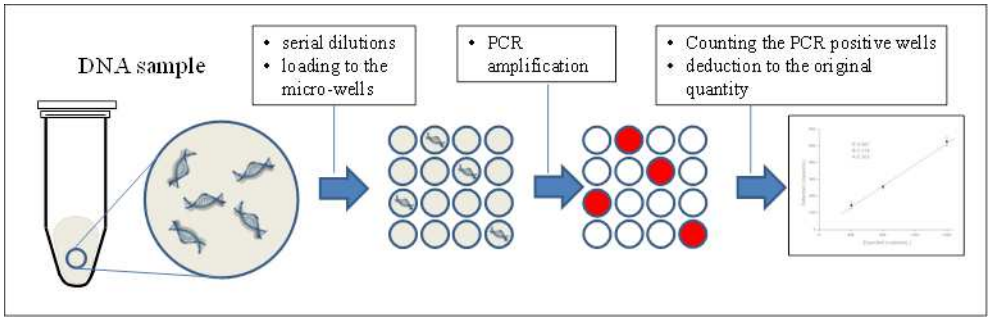


Fig. 2.2. Schematic illustration of the measurement principle of digital PCR for absolute quantification of DNA

Digital PCR, in principle, does not require calibration as the results of quantification comes from the fundamental distribution (Poission binomial distribution). Therefore, scientists at NMIs pay great attention to this particular measurement technique as it may accurately quantify the amount of DNA without calibration when no appropriate calibration materials are available. A number of publications exploring such possibility can be found (Bhat et al., 2010; Bhat et al., 2009; Corbisier et al., 2010; Sanders et al., 2011). However, there is an important validation point for the use of digital PCR as a metrological DNA quantification method. Calculation for the results of a digital PCR method assumes a 100% success rate for single-copy PCR amplification. Any deviation from 100% success rate will lead to underestimation. In practice, however, 100% success rate for a single-copy PCR is not readily achievable. As reported by Bhat et al., applied PCR conditions such as the status of template DNA or priming sites could result in variations in measurement results (Bhat et al., 2009). For this reason, digital PCR should be carefully validated to draw metrological determination of DNA quantity. Therefore, one would do his or her best to accomplish the 100% success rate for PCR amplification. The judgment for reaching the condition for 100% success rate can be surely made only if a certified reference material (CRM) with actual quantity of the target gene sequence is given.

As described above, digital PCR is a count-based DNA quantitation method for which commercial instruments can be conveniently utilized. However, its validity can be easily checked or demonstrated only if a suitable CRM is available. The other approach of the count-based quantitation of DNA may function in this purpose. KRISS scientists have worked on developing a method and instrumentation for counting individual DNA single molecules on a flow stream. DNA is stained with an intercalating dye and detected by laser induced fluorescence (LIF) detection. For securing enough fluorescence intensity, however, DNA particles need to be large enough. They succeeded in counting lambda phage DNA (45.8 kbp) single molecules (Fig. 2.3) (Lim et al., 2009) and now is capable of counting plasmid DNAs as small as 2 kbp. The LIF detection in this approach does not involve PCR but directly measure fluorescence intensity from the target DNA. Therefore, failure in PCR is not an issue. Direct measurement of fluorescence intensity is rather straight forward and less likely involves errors. In addition, the fluorescence intensity is proportional to the size of the DNA, which gives information on the size of each counted DNA particle. In this counting method, the molar concentration of target DNA is determined by the following simple equation:

$$C = (N_c / N_A) / V$$

Where C: molar concentration; N_c: counted number; N_A: Avogadro's number; V: sample volume.

As indicated by the equation above, measurement uncertainty may rise from uncertainty in counting of DNA particles (Nc) and estimation of sample volume (V). There are several check points for assuring accuracy of Nc as described in detail in the previous publication (Lim et al., 2009). All DNA particles should pass the detection points of a flow channel. Concentration of DNA should be limited under a certain level that simultaneous passing of a number of DNA particles is avoided. Occasional simultaneous passage of two DNA particles should be detectable and counted accordingly by the help of an algorithm for analysis of superimposed peaks. Signal to noise ratio should be large enough to reject background noise in counting. Validation of count-based determination of lambda phage DNA was attempted by comparison with the result of CE-dNMP analysis (Hong et al., 2011), for which the sample concentration was about 100 thousand-fold thicker than the sample for counting. The counting method resulted in about 30% less quantity for lambda phage DNA.

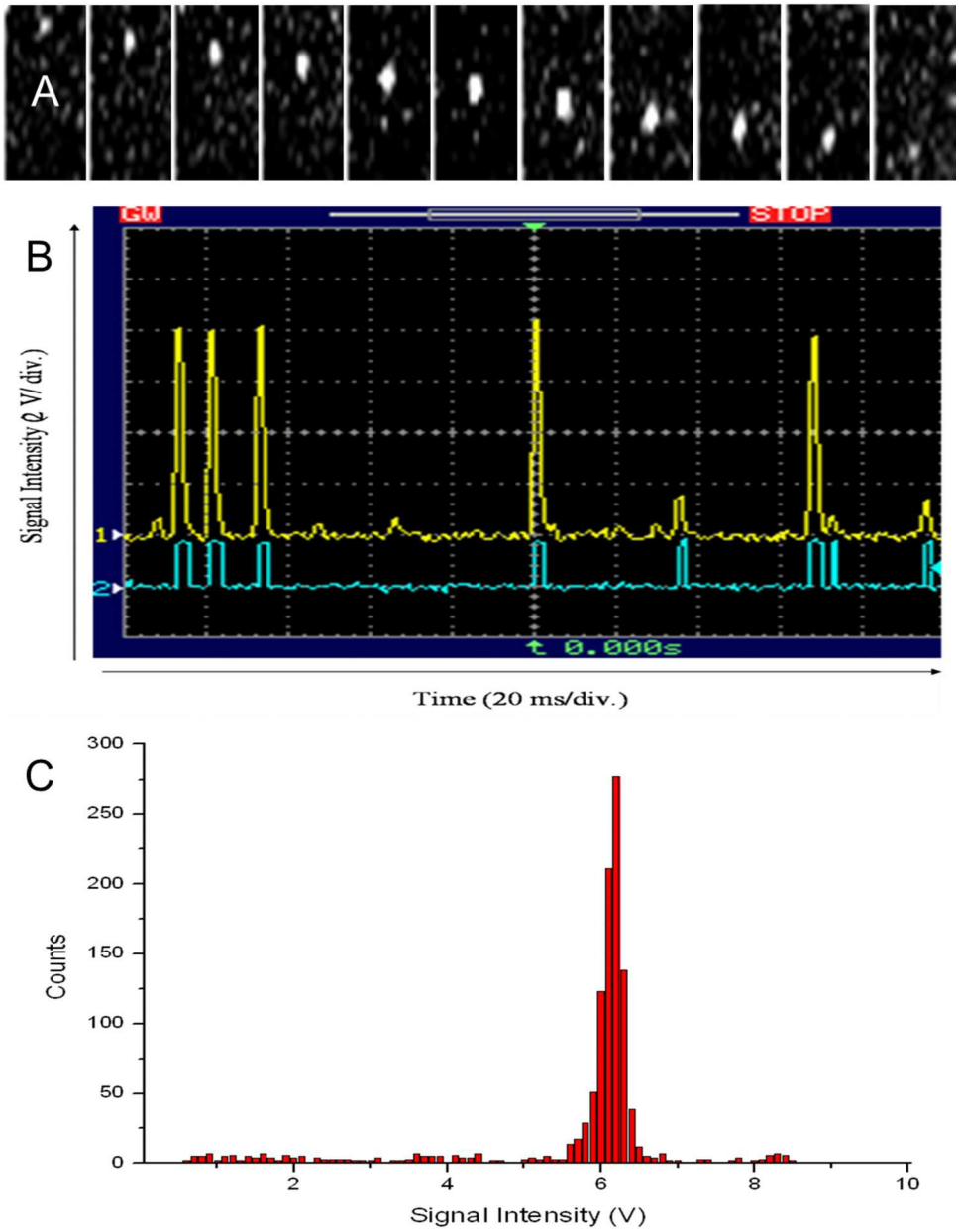


Fig. 2.3. A single lambda DNA captured at 1 ms intervals (A), signals displayed on an oscilloscope (B), and count result displayed in a histogram (C). In panel B, the upper trace indicates the fluorescence intensity, and the lower trace is the digital pulse to be counted (Lim et al., 2009).

In counting plasmid DNA, however, such underestimation disappeared. Signal intensity from plasmid DNA such as pBR322 (4.8 kbp) is 10-fold less than that from lambda phage DNA. Therefore, the underestimation for lambda phage DNA counting should not be due to the weak fluorescence signal for detection. Instead, the underestimation is highly likely due to the fragmentation of lambda viral DNA in the given sample. Small fragments (< 2 kbp) were not counted in counting of lambda phage DNA as the fluorescence signals were far less than that of lambda phage DNA. However, the CE method measures nucleotides from all DNA fragments regardless of their sizes. Therefore, it is possible that the result of CE analysis is substantially greater than the counting result. In contrast, pBR322 DNA that is smaller than lambda phage DNA is too small to be readily fragmented by shear stress (Yoo et al., 2011). The lack of small DNA fragments in pBR322 sample is concordant to the close agreement between the counting method and the CE method. This feature should be carefully considered in determination of what we want to measure. If we want to measure only the DNA molecules of whole integrity, then the counting method gives the right answer from its resultant histogram (count vs. size). On the other hand, if we are interested in the quantity of all DNA fragments, then what the CE method measures is the right answer. Digital PCR in this regard measures a portion of DNA sequences encompassed by a primer pair, which should not necessarily be the same as the number of integral DNA molecules. The "measurand" (exactly what to measure) of each method is significantly different, and this point should be well considered in comparing the results of such methods.

In conclusion, digital PCR will be widely used in attempts to quantify the copies of DNA sequences as commercial instrumentations have become available. However, such applications may have to be confirmed with certified reference materials (CRMs) that are accurately determined by other reliable methods like the direct counting method.

2.5 Summary

Metrology for biology has been pursued first in quantification of nucleic acids. Due to the polymeric nature of nucleic acids, conventional methods are not as accurate as a metrological standard should be. In addition, measurement traceability was hardly established. Efforts of many scientists, mostly scientists at various NMIs, have been poured for establishment of metrology for DNA quantification, and substantial achievements have been made. Reductive approaches have been well developed to establish measurement traceability, and count-based quantification methods for trace level DNA copies were also successfully progressed. Although there are some technical challenges still remained, the overall technical structure for metrology in this area is visible now (Fig. 2.4).

Soon, the products of metrology such as certified reference materials in DNA quantification will be available and their widespread uses are expected. Consequently, the credibility in data of DNA quantification will be dramatically improved in various areas such as R&D, industrial, medical, and regulation sectors. More important, the success in the effort to establish metrology for nucleic acid quantification will encourage the scientists who are gazing over the technical challenges in establishment of technical infrastructures for modern biology.

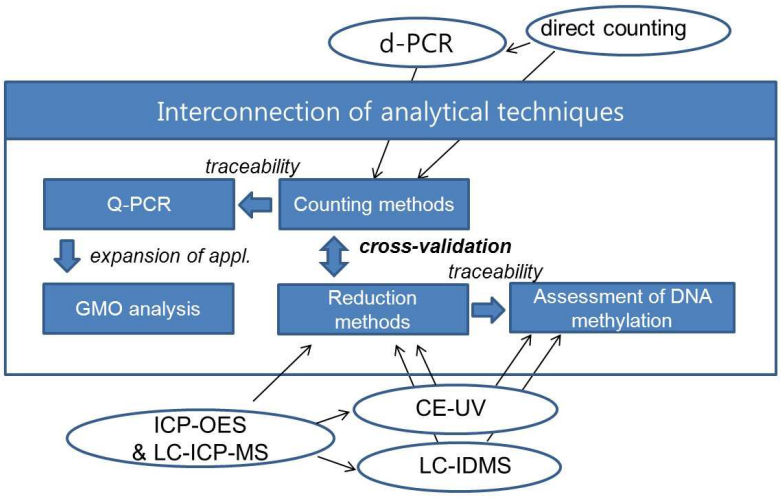


Fig. 2.4. Core analytical techniques developed for establishment of metrology in DNA quantification, and their links to validations or applications

3. Metrology in protein quantification

3.1 Backgrounds

Protein is a complex molecule composed of one or more amino acid chains and constitutes a large part of the body's cells, tissues, and organs. This building block of the body has been shown to be a potential biomarker of human health risk assessment. Over the years, therefore, substantial efforts have been made for the development of bioanalytical approaches for accurate quantification of proteins. Monitoring protein levels is important to understand the biological mechanism and regulation of targeted proteins. In clinical practice, such approaches for protein quantification are used to compare biological samples from healthy and diseased patients, providing more accurate diagnosis of human disease. Thus, technologies for accurate quantification of protein biomarkers has been a high priority for many public health related institutions.

Nevertheless, measurement traceability, the essential element of metrology, is hardly established at all in protein quantification. There are several bioassay-based or instrumental methods for protein quantification that are frequently used. All these methods have to be calibrated to produce accurate results, but are not supported with reference calibration materials of metrological grades. Therefore, those methods work at best for comparison of relative amounts. Certified reference materials of proteins to provide measurement traceability to the SI unit of mole is yet to be developed. Our analytical capability is not as advanced as to quantify a whole protein accurately. However, quantification of appropriately reduced forms such as amino acids or peptides using isotopic dilution mass spectrometry (IDMS) should be a feasible approach to quantify proteins in a metrological manner. Of course, such approaches should also be supported by certified reference materials of the reduced forms for accurate calibration. The technical issues in the reduction approaches combined with the use of IDMS are described below.

3.2 Conventional methods

There are a number of quantification methods for proteins either bioassays or instrumental methods. The most commonly used methodologies in bioassays are the Biuret (Savory et al., 1968), Bradford (Bradford, 1976), Lowry (Fryer et al., 1986), and bicinchoninic acid (BCA) (Smith et al., 1985) assays. These methods employ chemical reagents which specifically react with proteins to produce colored products which can be measured by UV spectrophotometer in a concentration-dependent manner. The absorbance of colored sample is compared to standard curves constructed with a known protein (frequently bovine serum albumin) in order to determine concentration in unknown samples. Biological methods are widely used owing to their simplicity and low cost, but are hampered by poor accuracy and reproducibility since their responses are either assay or calibrator-protein dependent, and also the results can vary with the residue composition of the target protein. Thus bioassays lead to relative differences in protein quantification and cannot give absolute values. On the other hand, instrumental analytical methods including chromatographic techniques (HPLC, GC), capillary electrophoresis (CE), and mass spectrometry (MS) are often favored because of higher precision. These methods may require time-consuming sample preparation, which leads to higher costs than biological methods. Nevertheless, both bioassay and instrumental analysis need to be calibrated with a highly reliable protein standard material to ensure accurate and comparable results. A higher order analytical method needs to be established for accurate determination of such protein standard materials.

The same is true for high throughput analysis formats such as 2D-PAGE gels (Smithies and Poulik, 1956) and enzyme-lingked immunosorbent assay (ELISA). These methods are particularly well fit the purpose of high throughput analysis for proteomics research and protein-chip analysis, respectively. Therefore, they have become powerful tools for screening effective protein markers. As such research progresses, it has become obvious that reliable quantification in an absolute manner is essentially required for comparability of data.

3.3 Metrology for protein quantification based on mass spectrometry

Over the last decade, mass spectrometry-based approaches has been shown to be the most powerful tool for protein characterization and become available in many laboratories (Cravatt et al., 2007). Although early biological studies using mass spectrometry merely provided lists of proteins identified in a particular sample, mass spectrometry-based proteomic strategies have been developed, improved, and used for many applications. With the respect to quantitative protein analysis, mass spectrometry alone does not provide much information. MS in general can't detect a whole protein in a quantitative manner. To improve quantification performance, a whole protein needs to be reduced to amino acids or peptides, which are done by chemical or enzymatic hydrolysis, respectively. Even so, repeatability of MS quantification is poor due to the complex nature of ionization prior to MS analysis, which can be dramatically mitigated by the format of isotope dilution-mass spectrometry (IDMS). For the reductive approaches, conversion of the target protein to amino acids or peptides while maintaining the original stoichiometry is a key issue. There could be much debate on the completeness of reduction. This issue is addressed in the following sections. In addition, the sound measurement traceability in quantification of proteins should be established as a very basic requirement for establishment of metrology,

which is highly likely to be achieved through preparation for high quality amino acids CRMs for amino acid based quantification. Nevertheless, the establishment of metrology for protein quantification seems to be a doable task based on advanced mass spectrometry, and the refinements of analytical strategies for reduced measurement uncertainties are pursued as below.

3.3.1 Amino acid analysis

Amino acid analysis has been used in many applications as a conventional protein analysis technique. Until the emergence of mass spectrometry, amino acid analysis was essential to the identification of proteins. Nowadays, the analysis is known to be a powerful tool for determination of protein quantities based on detailed information regarding precise quantification of free amino acids. This quantitative analysis is based on the total amount of single amino acids, so the preparation of a highly pure sample is a prerequisite for an accurate analysis. The analysis consists of three steps; hydrolysis of the sample to amino acid constituents (Fig. 3.1), chromatographic separation of the target amino acid to be analyzed, and quantification by mass spectrometry with a labeled internal standard (Fig. 3.2). Of among, complete hydrolysis is critical for overall accuracy of the procedure (Albin et al., 2000; Anderson et al., 1977; Darragh et al., 1996; Fountoulakis and Lahm, 1998; Kinumi et al., 2010). Moreover, for the analysis of an unknown protein, optimization of hydrolysis conditions should be considered before conducting the whole procedure. Very recently, KRISS scientists have reported quantification of human growth hormone by amino acid composition analysis using isotope dilution liquid chromatography-tandem mass spectrometry (Jeong et al., 2011), which is briefly summarized below.

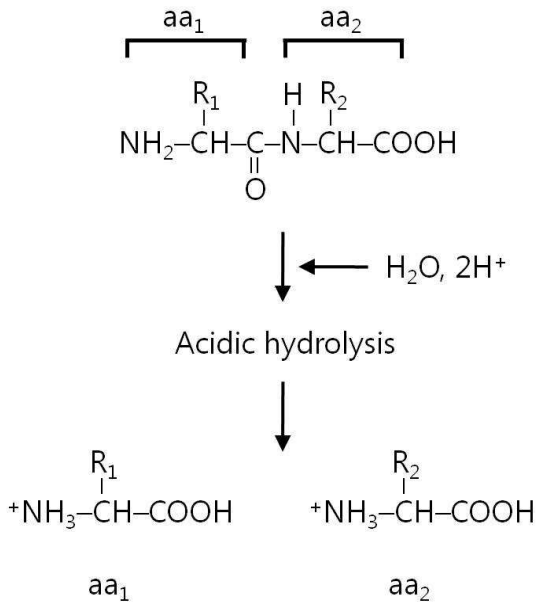


Fig. 3.1. Acidic hydrolysis for amino acid analysis

1. Sample purity assessment: As contaminant components in a protein sample can lead to overestimation of the target quantities, it is important to determine the precise sample purity. To this end, properly diluted human growth hormone (hGH) solution was subjected to capillary zone electrophoresis (CE). The analysis indicates that no significant impurities were present in the sample. The results were also confirmed by high-performance liquid chromatography (HPLC) and matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometry.

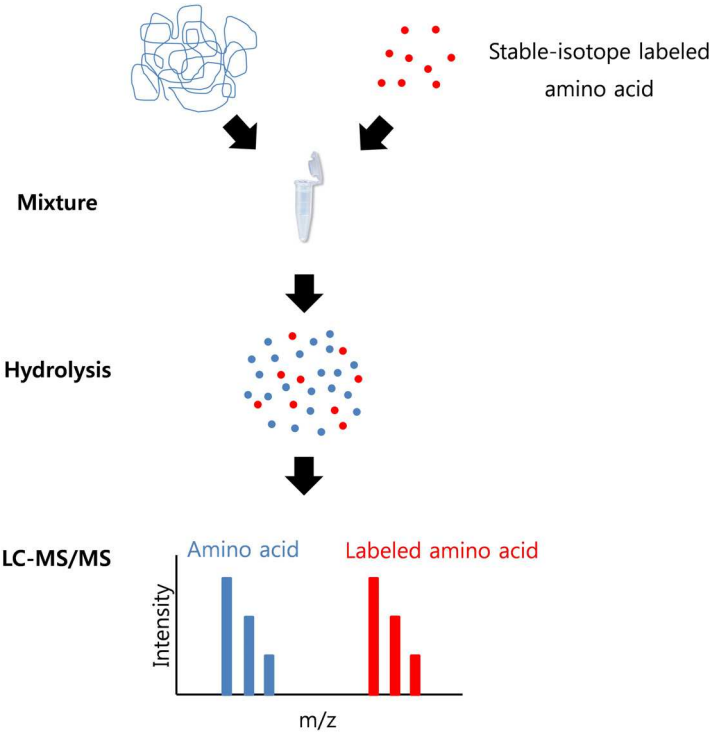


Fig. 3.2. Schematic of MS based amino acid analysis

2. Hydrolysis: As mentioned previously, optimizing the reaction conditions for protein hydrolysis is critical to minimize analytical variations. Through extensive optimization procedures, we found suitable reaction conditions for acidic hydrolysis with hydrochloric acid (HCl), which is the most common hydrolysis method to date. The experiments were performed to test the effects of various factors, including hydrolysis time, protein concentration, HCl concentration, and hydrolysis temperature. As the internal standards should be spiked to the sample prior to the hydrolysis step, the stability of the isotopically labeled free amino acids was also monitored under the identical reaction conditions. The results indicate that the defined hydrolysis step causes no substantial degradation of the internal standard candidates. Therefore, the hGH sample and isotope labeled amino acids were mixed, hydrolyzed, and subjected to HPLC-MS/MS.

3. HPLC-MS/MS: First of all, the optimized conditions for the instrumental analysis were established. Using a high-performance column and a simple isocratic elution, complete separation of the target AAs was observed. It should be noted that full baseline separation of isomeric AAs that might be indistinguishable by MS is often necessary depending on target AAs. In order to perform exact matching double ID-MS quantification, the expected AA concentrations of the hGH sample were calculated, and the same amounts of isotope labeled AAs were used as internal standards. In doing so, issues concerning measurement linearity of MS quantifications could be excluded. MS analysis was performed on a triple-quadrupole mass spectrometer using multiple reaction monitoring (MRM) mode. The ratio of peaks from unlabeled and isotopically labeled AAs was calculated, and doing so the quantity of hGH was determined.

3.3.2 Analysis using isotope labeled peptides

In 1980s, a research group reported the preparation and use of a stable isotope-incorporated peptide for measuring endogenous peptides in biological extracts (Desiderio and Kai, 1983; Desiderio et al., 1984). The pioneer work by this group seems to be the first attempt to use isotope labeled synthetic peptides as internal standards to measure the level of a specific peptide using mass spectrometry. After that, another research team described the use of this approach for the absolute protein quantification where a target protein was proteolyzed and quantified using LC-MS/MS with a stable isotope labeled peptide as an internal standard through (Barr et al., 1996). The rapid advances in mass spectrometry based proteomics have greatly improved the analytical sensitivity and made peptide based analysis possible to detect targets in a complex matrix (Anderson et al., 2004; Gerber et al., 2003; Kirkpatrick et al., 2005; Mayya et al., 2006; Putz et al., 2005; Stahl-Zeng et al., 2007). The principal of this approach is that the isotopically labeled synthetic peptide is spiked to the sample, and the mixture is subjected to enzymatic digestion followed by LC-MS/MS. Although this advanced technique is now commonly used for protein quantification, but this approach still has some issues. In addition to high costs of peptide synthesis, significant errors can be generated from sample preparation for mass spectrometry, probably from inefficient and/or inconsistent protease digestion of target proteins. The reaction conditions for protease digestion can be optimized, but the estimated values are likely to be less than the real concentrations of the target proteins. Recently, scientists from multiple NMIs collaboratively investigated the applicability of protein quantification by ID-MS using isotopically labeled synthetic peptides as standards (Arsene et al., 2008), which is briefly summarized below.

1. Purity assessment of the sample and peptide standards: The obtained hGH sample was further purified by liquid chromatography to remove impurities that might interfere with analytical data. Two peptides expected from trypsin digestion of hGH were synthesized in the isotopically labeled forms, where certain AAs were replaced with isotope labeled ones. Importantly, impurities of amino acid and/or peptide in the peptide solutions were carefully evaluated, the resulting factors were corrected.
2. Proteolysis: The defined amounts of the isotope labeled peptides were added to the hGH sample, and then the mixture was subjected to trypsin digestion according to the well-established protocol. In this study, a modified method for fast and complete proteolysis was developed and referred to as the rapid proteolysis protocol. The point of this method is the use of water-organic solvent digestion systems that increase protein solubility and retain high protease activities.

3. LC/MS: After proteolysis by trypsin, the sample containing the internal standards was subjected to LC/MS. The amounts of unlabeled and labeled peptides were monitored by revered-phase LC/ESI-quadrupole MS using certain ion traces. Comparing the signal ratio of unlabeled and labeled peptides for the standard solution of known concentration to that for the unknown sample, the quantity of hGH was calculated.

3.3.3 Analysis using isotope labeled intact proteins

Very recently, isotope-labeled intact proteins have been successfully used for MS based protein quantification (Brun et al., 2007; Janecki et al., 2007; Peng et al., 2004). This approach is based on the use of isotope labeled full-length proteins produced by recombinant DNA techniques as internal standards for quantification. The advantage of this approach is that it is unnecessary to take into account the efficiency of hydrolysis/proteolysis that is the main limitation of amino acid or peptide analysis approaches. As the target analyte and the internal standard are chemically identical, they act exactly the same way during not only pre-analytical treatments such as proteolysis, but also LC/MS measurement. For this reason, this analysis can provide more accurate quantitative analysis than the other MS-based approaches described above, minimizing the influences of experimental variations. However, the difficulty of preparation of isotopically labeled protein has been an obstacle. The following is one representative example of how to quantify a certain protein in a complex biological sample using an isotope-labeled intact protein. Janecki et al accurately measured the level of the human alcohol dehydrogenase (ADH1C1) in liver extracts using in-vitro expressed labeled ADH1C1 (Janecki et al., 2007).

1. Preparation of the stable isotope-labeled ADH1C1: Labeled ADH1C1 was expressed in *Eschericha coli* that were cultured in specific media containing isotopically labeled Lys. The protein was purified by affinity chromatography using His-tags at the N-terminus. The purity and activity of purified ADH1C1 were verified by sodium dodecyl sulfate (SDS)-PAGE and a specific activity assay.
2. Sample preparation: The labeled standard protein was added to liver samples in two defferent ways. One set referred to as experiment 1 contains various concentrations of the standard in a constant amount of liver extracts. The other set referred to as experiment 2 has a constant amount of the labeled protein in various concentrations of liver extracts. Both experiments were applied to quantification of ADH1C1 in liver extracts to generate statistically significant quantitative data. The mixture was then followed by trypsin digestion, and resulting peptides were recovered.
3. Quantitative analysis by HPLC-MS/MS: Prior to the actual analysis, preliminary experiments were performed using unlabeled ADH1C1 to optimize the multiple reaction monitoring (MRM) transitions , and a specific target peptide was selected. And then, the retrieved peptides from proteolysis were subjected to HPLC-MS/MS using MRM mode. For quantitative purpose, analyst quantification module software was used to calculate the target peaks generated from heavy and normal peptides.

3.4 Summary

Determination of the exact amounts of proteins gives valuable information about biological mechanisms of targeted proteins and becomes crucial for the evaluation of various biomarker candidates. Until recently, immunoassays such as ELISA were considered as the

reference methodology for protein quantification. However, these assays have some critical issues such as difficulty of antibody production and non-specific cross-reactivity of antibodies, and thus a significant investment in time and resources is often required for the accurate evaluation. On this regard, the recent development of MS-based protein quantification techniques using isotope labeled internal standards has become an alternative and compelling approach that provides high accuracy and specificity in protein quantification. Although such techniques are now essential for protein quantification, extensive validation of such procedures is necessary in order to establish metrologically sound analytical techniques.

4. Metrology for other biologicals

There are several other important categories of biological materials other than nucleic acids and proteins. They include cells, microorganisms and glycans. One may want to extend the scope to include lipids. Small molecules are also play important roles in biological processes as nutrients or metabolites, but it will be better to categorize them as chemicals instead of biologicals. In technical prospects, biologicals here are classified as large molecules that are not readily quantifiable by chemical analysis.

4.1 Cells

A cell is the functional basic unit of life and is much larger and complex than nucleic acids or proteins. Therefore, metrology for cells will be of substantially greater degree of complexity. Recent breathtaking advances in the attempt of utilization of cells in regenerative medicine (Mimeault et al., 2007) draw great attentions of metrologists in biology. Although unconceivable degree of technical difficulties are expected, metrology for cells need to be established to support the advances in regenerative medicine to turn into safe and effective means to restore health of many impaired or elder people. In fact, safety and efficacy of regenerative medicine are debated subjects at present (Ilic and Polak, 2011). In addition, many animal tests are being replaced by cell-based tests to avoid ethical problems, and the validity of cell-based testing will require rigorous examination of cells used for such tests to result in harmonious and valid outcomes. International cell depositories such as American Types of Cell Cultures (ATCC) are the major bodies performing R&D for collective testing of cells, which is necessary for QC and QA of reserved cell resources. In dealing with cells, authentication of the target cells is a key issue, which should be confirmed with effective and valid tests. For example, one of the ATTC R&D programs, Developmental Biology Program (http://www.atcc.org/Science/CollectionsResearchandDevelopment/DevelopmentalBiologyandStemCells/tabid/207/Default.aspx), employs state-of-the-art technologies for the authentication of human ES (embryonic stem) cell lines, mouse ES cell lines and mouse feeder cells. Five critical tests address the major concerns of researchers and clinicians working with human ES cell lines and are conducted routinely by ATCC:

1. **Sterility** — confirms the absence of contaminating organisms such as bacteria, fungi, mycoplasma and human viruses
2. **Identity** — confirms species and identity through STR analysis and HLA typing
3. **Stemness** — confirms the cells are in the undifferentiated state using immunocytochemistry, alkaline phosphatase assay, telomerase expression (TRAP), and gene expression (qRT-PCR and global gene expression)

4. **Stability** — confirms the integrity of nuclear genomes using cytogenetics and SNP profile
5. **Pluripotency** — confirms the ability of hESCs to generate various differentiated cell types using teratoma formation and characterization, as well as embryoid bodies formation.

Appropriateness of such tests is to be determined by experts in the field, and the quantities here are more likely method-dependent. As discussed in the later section, metrology for such quantities needs to be established to support 'metrological execution' of the given measurement protocols, where those protocols is not fully established yet. In this regard, the advancement of cell testing protocols should be closely tracked for prompt implementation of metrology in such testing protocols.

Counting of well specified cells is rather straight forward in terms of quantification. CCQM BAWG has executed or is preparing for two international comparisons: 1) flow cytometric determination (CCQM-P102) and 2) microscopic determination (CCQM-P123). It is noteworthy that physical parameters such as motility and morphology could be important in characterization of cells. Among NMIs, NIST scientists are in particular active in such investigations (Ni and Chiang, 2007).

4.2 Microorganisms

Although there are some useful microorganisms as essentially required in important biological processes such as fermentation, many microorganisms have been life-threatening enemies in long history. Especially, food-born infectious microorganisms are very near to our everyday life to often cause epidemics. The recent *E coli* O157-outbreak in Europe carried by fresh vegetables killed more than 50 people and infected about 4400 people. This incidence reminds that we are not well prepared against such outbreaks. Especially the insufficient technical levels in analysis of microorganisms for surveillance and investigation in a global network is of a great concern. While constructing or improving the global infrastructure against such outbreaks, adopting metrological principles as possible will reduce controversy or legal suits associated with the interpretation on the analytical data. In this regard, CCQM of BIPM moves in a swift manner to organize an international symposium and ad hoc Steering Group on Microbial Measurements to ensure food quality and safety. What to do for establishment metrology in microbial measurements are yet to be rummaged among experts in the field and metrologists. Measurements are for detection, identification, and characterization, and enumeration of target microorganisms. Microorganisms are proliferating dependent upon environmental conditions and frequently mutated. Due to these properties, none of quantification, identification, or characterization of a microorganism is straightforward. Characteristic properties and their relevance on infection should be carefully thought and selected for effective measurement. In addition, such measurements had better be in the domain of metrology, which will guarantee the comparability in a global network (Verhoef et al., 2011). Such a grand task is being organized among relevant experts groups and metrological institutes.

4.3 Glycans

Glycan is a polysaccharide, which is a linear or branched polymer of monosaccharide units linked by glycosidic bonds. Glycans exist in nature as free or glycoconjugates (e.g.

glycoproteins, proteoglycans, and glycolipids) with cell or tissue. Their function in living organisms is important. Especially the conjugated glycans in proteins and lipids affect physicochemical properties, such as solubility, stability as well as biological properties. Particularly, glycosylated biopharmaceuticals contain various glycoforms, and the heterogeneity of carbohydrate moiety is altered according to the manufacturing process. For this reason, glycan analysis is an essential part of protein characterization in development and quality control of biopharmaceuticals as well (Harazono et al., 2011). Therefore, the study of protein with post-translational modifications has increased explosively in recent proteomic research, and this phenomenon represents importance of glycans in this area (Wei and Li, 2009). Though the advanced mass spectrometry plays a versatile role in current glycan analysis, due to the complexity of structure and the difficulty of isolation of glycans, there is no single method which can clearly and efficiently analyze all of them (Zaia, 2010). The glycan analysis is processed by a combination of several methods, such as monosaccharide composition analysis after the hydrolysis (Harazono et al., 2011), profiling of liberated glycans (Pabst and Altmann, 2011), and structural assignment (Zaia, 2010). A number of methods have been developed for glycomics using either chromatographic, electromigratory or mass spectrometric method as qualitative and quantitative methods. Glycomics has been recognized as an emerging technology in this post-genome generation in worldwide. The term 'glycomics' includes the systematic study of all glycan structures as well as the comprehensive study in genetic, physiologic and pathologic areas. The dynamic complexity and variety, and the essential roles in physiology, drive the need of generally accepted methodology for glycoconjugates. However, the challenging task to implement metrological rigorousness in such complex analysis is yet to be launched. As new glycomics and glycoproteomics problems will require unique solutions, a wide range of methodological and instrumental techniques will be needed to address them.

5. Method-dependent biological quantities

Some important biological quantities are not described in the SI unit of mole. Quantities describing activity, viability, or potency used in biology communities are defined by the methods of assessment that are agreed among the experts in the related fields. Comparability of measurements of these quantities is governed by how precisely the measurement protocols are executed. Therefore, metrology for such quantities needs to be established to support 'metrological execution' of the given measurement protocols. In this regard, improvement of measurement comparability for these quantities can be achieved by somewhat more than providing a simple reference material or reference measurement procedure. In a typical measurement protocol of an enzyme activity, for example, several experimental conditions such as the quantity of the substrate, reaction temperature, pH and buffer composition influence the measurement results. Comparability of measurement of enzyme activity can be assured only when such experimental conditions are tightly controlled. In other words, measurement uncertainties of such quantities arise from the uncertainties in the control of such reaction conditions. It needs to be stressed that such experimental conditions are metrological quantities that can be precisely measured and controlled with clear uncertainty budgeting. Application of metrological rigorousness to such experimental conditions can dramatically improve the measurement comparability for method-dependent quantities. Although yet to be demonstrated, the use of modern computing power will also become essential in dealing with such complex measurement

procedures affected by multiple variables. Highly confusing measurement uncertainties of such procedures may be only correctly budgeted by correct computer- simulations of the procedures.

The potential impact of such notion for method-dependent quantities has been demonstrated in polymerase chain reaction (PCR) (Yang et al., 2005). As described in earlier section, PCR is frequently used as a way to quantify genetic materials, especially in the format of real-time PCR (or q-PCR). In a PCR process, there are several experimental conditions that need to be tightly controlled. Among them, reaction temperature can be a critical control parameter as melting, annealing, and elongation steps of PCR are all profoundly affected by temperature. Numerous commercial thermal cyclers for PCR are available. How well do those thermal cyclers operate if we observe them with a metrological criticism in terms of temperature control? In our investigation (Kim et al., 2008), most PCR instruments showed satisfactory accuracy in static temperature control. However, in dynamic temperature control for PCR, substantially different performances were observed (Fig. 5.1).

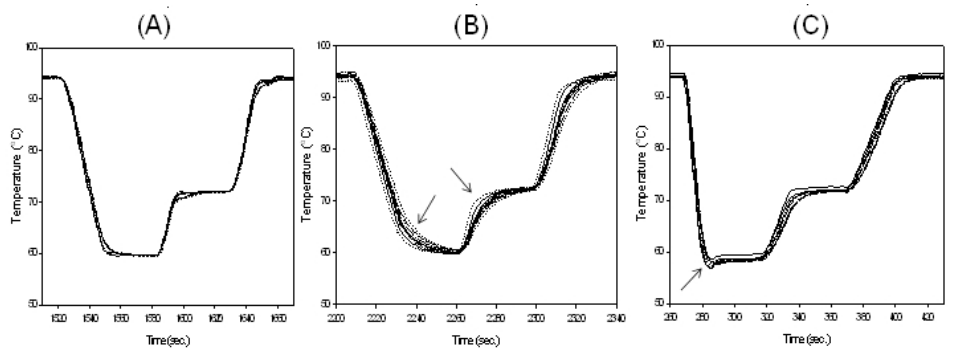


Fig. 5.1. Real-time monitoring of in tube-temperature during PCR cycles for different commercial thermal cyclers. Unlike the ideal case (A), shortened annealing time due to slow response (B), or overshooting (C) and undershooting of temperature were observed (data from Bioanalysis Laboratory at KRISS).

Slower acting instruments did not reach the programmed temperature in a fast PCR. On the other hand, some fast acting instruments showed under- or overshooting, which could lead to drastically different results. Such imperfection of instruments is not well known, and proper measures against such imperfections are not well taken, which would cause substantial disparity in performing bioassays based on PCR. It will be very helpful if the degree of imperfection of instrumentation is analyzed, and is considered in preparation of a measurement protocol. For example, an extreme PCR speed needs to be avoided for users of slower-acting thermal cyclers.

Various bioassays of method-dependent quantity are crucial in QC or QA of biological products. Confidences on biological products are heavily dependent on the credibility and

comparability of such bioassays for QC and QA. Therefore, the establishment of metrology for method-dependent quantities needs to be promptly established. Although brief, an example of how to achieve metrology for such quantities is described above.

6. Conclusions

It is certain that metrology for biological measurement will be a critical prerequisite for successful industrialization of modern biotechnology. It will be the fundamental basis of accurate and confident quality control and quality assurance of new products from biotechnology. This notion has been well acknowledged by the international community of metrology, mainly BIPM and CCQM as well as related professional world organizations such as WHO, IFCC, and USP. Because of the extraordinary complexities of biological materials, there were strong doubts about realization of metrology in this area. However, the scientists in national metrology institutes have devoted themselves to construct technical pathways toward the metrology for biology. A number of monumental achievements have been made, and metrology for certain areas especially quantitation of DNA has been established at a highly consistent level. These achievements have a strong symbolic value supporting that we could eventually establish metrology for biology even against many technical difficulties. At this stage, such efforts are now spread toward various important biologicals such as proteins, cells, microorganisms, and glycans. For those biologicals, extensive characterization of exactly what agreed to measure (the measurands) is first coming before quantification, which needs for the attention of not only the experts in metrology but also the experts in the related professional communities. Close communications and active collaborations among scientists in the various disciplines seem to be essential to make significant advancement in metrology for biology. The technical issues in metrology for biology need to be shared among many scientists to invite them to this challenging but interesting area. In this regard, we hope this chapter becomes a little window to look into the world of metrology for biology.

7. References

Albin, D.M., Wubben, J.E., and Gabert, V.M. (2000). Effect of hydrolysis time on the determination of amino acids in samples of soybean products with ion-exchange chromatography or precolumn derivatization with phenyl isothiocyanate. J Agric Food Chem *48*, 1684-1691.

Anderson, N.L., Anderson, N.G., Haines, L.R., Hardie, D.B., Olafson, R.W., and Pearson, T.W. (2004). Mass spectrometric quantitation of peptides and proteins using Stable Isotope Standards and Capture by Anti-Peptide Antibodies (SISCAPA). J Proteome Res *3*, 235-244.

Anderson, R., Annette, D., and Jackson, N. (1977). Ion-exchange chromatographic study of amino acid degradation during hydrolysis of avian protein. J Chromatogr *135*, 447-454.

Arsene, C.G., Ohlendorf, R., Burkitt, W., Pritchard, C., Henrion, A., O'Connor, G., Bunk, D.M., and Guttler, B. (2008). Protein quantification by isotope dilution mass spectrometry of proteolytic fragments: cleavage rate and accuracy. Anal Chem *80*, 4154-4160.

Barr, J.R., Maggio, V.L., Patterson, D.G., Jr., Cooper, G.R., Henderson, L.O., Turner, W.E., Smith, S.J., Hannon, W.H., Needham, L.L., and Sampson, E.J. (1996). Isotope dilution--mass spectrometric quantification of specific proteins: model application with apolipoprotein A-I. Clin Chem *42*, 1676-1682.

Bhat, S., Curach, N., Mostyn, T., Bains, G.S., Griffiths, K.R., and Emslie, K.R. (2010). Comparison of methods for accurate quantification of DNA mass concentration with traceability to the international system of units. Anal Chem *82*, 7185-7192.

Bhat, S., Herrmann, J., Armishaw, P., Corbisier, P., and Emslie, K.R. (2009). Single molecule detection in nanofluidic digital array enables accurate measurement of DNA copy number. Anal Bioanal Chem *394*, 457-467.

Bradford, M.M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal Biochem *72*, 248-254.

Brennan, R.G., Rabb, S.A., Holden, M.J., Winchester, M.R., and Turk, G.C. (2009). Potential primary measurement tool for the quantification of DNA. Anal Chem *81*, 3414-3420.

Brettell, T.A., Butler, J.M., and Almirall, J.R. (2009). Forensic science. Anal Chem *81*, 4695-4711.

Bruenner, B.A., Yip, T.T., and Hutchens, T.W. (1996). Quantitative analysis of oligonucleotides by matrix-assisted laser desorption/ionization mass spectrometry. Rapid Commun Mass Spectrom *10*, 1797-1801.

Brun, V., Dupuis, A., Adrait, A., Marcellin, M., Thomas, D., Court, M., Vandenesch, F., and Garin, J. (2007). Isotope-labeled protein standards: toward absolute quantitative proteomics. Mol Cell Proteomics *6*, 2139-2149.

Cavaluzzi, M.J., and Borer, P.N. (2004). Revised UV extinction coefficients for nucleoside-5'-monophosphates and unpaired DNA and RNA. Nucleic Acids Res *32*, e13.

Corbisier, P., Bhat, S., Partis, L., Xie, V.R., and Emslie, K.R. (2010). Absolute quantification of genetically modified MON810 maize (Zea mays L.) by digital polymerase chain reaction. Anal Bioanal Chem *396*, 2143-2150.

Cravatt, B.F., Simon, G.M., and Yates, J.R., 3rd (2007). The biological impact of mass-spectrometry-based proteomics. Nature *450*, 991-1000.

Darragh, A.J., Garrick, D.J., Moughan, P.J., and Hendriks, W.H. (1996). Correction for amino acid loss during acid hydrolysis of a purified protein. Anal Biochem *236*, 199-207.

Desiderio, D.M., and Kai, M. (1983). Preparation of stable isotope-incorporated peptide internal standards for field desorption mass spectrometry quantification of peptides in biologic tissue. Biomed Mass Spectrom *10*, 471-479.

Desiderio, D.M., Kai, M., Tanzer, F.S., Trimble, J., and Wakelyn, C. (1984). Measurement of enkephalin peptides in canine brain regions, teeth, and cerebrospinal fluid with high-performance liquid chromatography and mass spectrometry. J Chromatogr *297*, 245-260.

Dieffenbach, C.W., Lowe, T.M., and Dveksler, G.S. (1993). General concepts for PCR primer design. PCR Methods Appl *3*, S30-37.

Donald, C.E., Stokes, P., O'Connor, G., and Woolford, A.J. (2005). A comparison of enzymatic digestion for the quantitation of an oligonucleotide by liquid

chromatography-isotope dilution mass spectrometry. J Chromatogr B Analyt Technol Biomed Life Sci *817*, 173-182.

Doran, G. (2004). Oligonucleotides make sensible strides. Drug Discov Today *9*, 114-116.

English, C.A., Merson, S., and Keer, J.T. (2006). Use of elemental analysis to determine comparative performance of established DNA quantification methods. Anal Chem *78*, 4630-4633.

Fountoulakis, M., and Lahm, H.W. (1998). Hydrolysis and amino acid composition of proteins. J Chromatogr A *826*, 109-134.

Fryer, H.J., Davis, G.E., Manthorpe, M., and Varon, S. (1986). Lowry protein assay using an automatic microtiter plate spectrophotometer. Anal Biochem *153*, 262-266.

Gerber, S.A., Rush, J., Stemman, O., Kirschner, M.W., and Gygi, S.P. (2003). Absolute quantification of proteins and phosphoproteins from cell lysates by tandem MS. Proc Natl Acad Sci U S A *100*, 6940-6945.

Halford, W.P. (1999). The essential prerequisites for quantitative RT-PCR. Nat Biotechnol *17*, 835.

Harazono, A., Kobayashi, T., Kawasaki, N., Itoh, S., Tada, M., Hashii, N., Ishii, A., Arato, T., Yanagihara, S., Yagi, Y., *et al.* (2011). A comparative study of monosaccharide composition analysis as a carbohydrate test for biopharmaceuticals. Biologicals *39*, 171-180.

Harismendy, O., Ng, P.C., Strausberg, R.L., Wang, X., Stockwell, T.B., Beeson, K.Y., Schork, N.J., Murray, S.S., Topol, E.J., Levy, S., *et al.* (2009). Evaluation of next generation sequencing platforms for population targeted sequencing studies. Genome Biol *10*, R32.

Heid, C.A., Stevens, J., Livak, K.J., and Williams, P.M. (1996). Real time quantitative PCR. Genome Res *6*, 986-994.

Holden, M.J., Rabb, S.A., Tewari, Y.B., and Winchester, M.R. (2007). Traceable phosphorus measurements by ICP-OES and HPLC for the quantitation of DNA. Anal Chem *79*, 1536-1541

Hong, N.S., Shi, L.H., Jeong, J.S., Yang, I., Kim, S.K., and Park, S.R. (2011). Rapid and accurate determination of deoxyribonucleoside monophosphates from DNA using micellar electrokinetic chromatography with a cationic surfactant additive. Anal Bioanal Chem *400*, 2131-2140.

Ilic, D., and Polak, J.M. (2011). Stem cells in regenerative medicine: introduction. Br Med Bull *98*, 117-126.

Janecki, D.J., Bemis, K.G., Tegeler, T.J., Sanghani, P.C., Zhai, L., Hurley, T.D., Bosron, W.F., and Wang, M. (2007). A multiple reaction monitoring method for absolute quantification of the human liver alcohol dehydrogenase ADH1C1 isoenzyme. Anal Biochem *369*, 18-26.

Jeong, J.S., Lim, H.M., Kim, S.K., Ku, H.K., Oh, K.H., and Park, S.R. (2011). Quantification of human growth hormone by amino acid composition analysis using isotope dilution liquid-chromatography tandem mass spectrometry. J Chromatogr A *1218*, 6596-6602.

Kallansrud, G., and Ward, B. (1996). A comparison of measured and calculated single- and double-stranded oligodeoxynucleotide extinction coefficients. Anal Biochem *236*, 134-138.

Kim, Y.H., Yang, I., Bae, Y.S., and Park, S.R. (2008). Performance evaluation of thermal cyclers for PCR in a rapid cycling condition. Biotechniques *44*, 495-496, 498, 500 passim.

Kinumi, T., Ichikawa, R., Arimoto, H., and Takatsu, A. (2010). Traceable amino acid analyses of proteins and peptides by isotope-dilution mass spectrometry using precolumn derivatization reagent. Anal Sci *26*, 1007-1010.

Kirkpatrick, D.S., Gerber, S.A., and Gygi, S.P. (2005). The absolute quantification strategy: a general procedure for the quantification of proteins and post-translational modifications. Methods *35*, 265-273.

Lim, H.-M., Yoo, H.-B., Hong, N.-S., Yang, I., Han, M.-S., and Park, S.-R. (2009). Count-based quantitation of trace level macro-DNA molecules. Metrologia *46*, 375-387.

Mardis, E.R. (2008). The impact of next-generation sequencing technology on genetics. Trends Genet *24*, 133-141.

Marriott, J., O'Conner, G., Parkes, H. (2011). Final Report: Study on Measurement Services and Comparison Needs for an International Measurement Infrastructure for the Biosciences and Biotechnology: Input for the BIPM Work Programme, Rapport BIPM-2011/02, Reprot Number: LGC/R2011/123 Number, BIPM.

Mayya, V., Rezual, K., Wu, L., Fong, M.B., and Han, D.K. (2006). Absolute quantification of multisite phosphorylation by selective reaction monitoring mass spectrometry: determination of inhibitory phosphorylation status of cyclin-dependent kinases. Mol Cell Proteomics *5*, 1146-1157.

Mimeault, M., Hauke, R., and Batra, S.K. (2007). Stem cells: a revolution in therapeutics-recent advances in stem cell biology and their therapeutic applications in regenerative medicine and cancer therapies. Clin Pharmacol Ther *82*, 252-264.

Mitsuhashi, M., Cooper, A., Ogura, M., Shinagawa, T., Yano, K., and Hosokawa, T. (1994). Oligonucleotide probe design--a new approach. Nature *367*, 759-761.

NI, Y., Chiang, Y.M. (2007). Cell Morphology and Migration Linked to Substrate Rigidity. Soft Matter *3*, 1285-1292.

O'Connor, G., Dawson, C., Woolford, A., Webb, K.S., and Catterick, T. (2002). Quantitation of oligonucleotides by phosphodiesterase digestion followed by isotope dilution mass spectrometry: proof of concept. Anal Chem *74*, 3670-3676.

Pabst, M., and Altmann, F. (2011). Glycan analysis by modern instrumental methods. Proteomics *11*, 631-643.

Peng, J., Kim, M.J., Cheng, D., Duong, D.M., Gygi, S.P., and Sheng, M. (2004). Semiquantitative proteomic analysis of rat forebrain postsynaptic density fractions by mass spectrometry. J Biol Chem *279*, 21003-21011.

Peterson, A.W., Heaton, R.J., and Georgiadis, R.M. (2001). The effect of surface probe density on DNA hybridization. Nucleic Acids Res *29*, 5163-5168.

Pirollo, K.F., Rait, A., Sleer, L.S., and Chang, E.H. (2003). Antisense therapeutics: from theory to clinical practice. Pharmacol Ther *99*, 55-77.

Ponchel, F., Toomes, C., Bransfield, K., Leong, F.T., Douglas, S.H., Field, S.L., Bell, S.M., Combaret, V., Puisieux, A., Mighell, A.J., *et al.* (2003). Real-time PCR based on SYBR-Green I fluorescence: an alternative to the TaqMan assay for a relative quantification of gene rearrangements, gene amplifications and micro gene deletions. BMC Biotechnol *3*, 18.

Putz, S., Reinders, J., Reinders, Y., and Sickmann, A. (2005). Mass spectrometry-based peptide quantification: applications and limitations. Expert Rev Proteomics *2*, 381-392.

Sanders, R., Huggett, J.F., Bushell, C.A., Cowen, S., Scott, D.J., and Foy, C.A. (2011). Evaluation of digital PCR for absolute DNA quantification. Anal Chem *83*, 6474-6484.

Savory, J., Pu, P.H., and Sunderman, F.W., Jr. (1968). A biuret method for determination of protein in normal urine. Clin Chem *14*, 1160-1171.

Smith, P.K., Krohn, R.I., Hermanson, G.T., Mallia, A.K., Gartner, F.H., Provenzano, M.D., Fujimoto, E.K., Goeke, N.M., Olson, B.J., and Klenk, D.C. (1985). Measurement of protein using bicinchoninic acid. Anal Biochem *150*, 76-85.

Smithies, O., and Poulik, M.D. (1956). Two-dimensional electrophoresis of serum proteins. Nature *177*, 1033.

Stahl-Zeng, J., Lange, V., Ossola, R., Eckhardt, K., Krek, W., Aebersold, R., and Domon, B. (2007). High sensitivity detection of plasma proteins by multiple reaction monitoring of N-glycosites. Mol Cell Proteomics *6*, 1809-1817.

Sykes, P.J., Neoh, S.H., Brisco, M.J., Hughes, E., Condon, J., and Morley, A.A. (1992). Quantitation of targets for PCR by use of limiting dilution. Biotechniques *13*, 444-449.

Tewary, H.K., and Iversen, P.L. (1997). Qualitative and quantitative measurements of oligonucleotides in gene therapy: Part II in vivo models. J Pharm Biomed Anal *15*, 1127-1135.

Verhoef, L., Kouyos, R.D., Vennema, H., Kroneman, A., Siebenga, J., van Pelt, W., Koopmans, M. (2011). An integrated approach to identifying international foodborne norovirus outbreaks. Emerging Infectious Diseases *17*, 412-418.

Vogelstein, B., and Kinzler, K.W. (1999). Digital PCR. Proc Natl Acad Sci U S A *96*, 9236-9241.

Wei, X., and Li, L. (2009). Comparative glycoproteomics: approaches and applications. Brief Funct Genomic Proteomic *8*, 104-113.

Yan, X., Grace, W.K., Yoshida, T.M., Habbersett, R.C., Velappan, N., Jett, J.H., Keller, R.A., and Marrone, B.L. (1999). Characteristics of different nucleic acid staining dyes for DNA fragment sizing by flow cytometry. Anal Chem *71*, 5470-5480.

Yang, I., Han, M.S., Yim, Y.H., Hwang, E., and Park, S.R. (2004). A strategy for establishing accurate quantitation standards of oligonucleotides: quantitation of phosphorus of DNA phosphodiester bonds using inductively coupled plasma-optical emission spectroscopy. Anal Biochem *335*, 150-161.

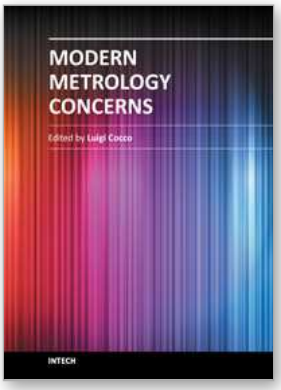
Yang, I., Kim, Y.H., Byun, J.Y., and Park, S.R. (2005). Use of multiplex polymerase chain reactions to indicate the accuracy of the annealing temperature of thermal cycling. Anal Biochem *338*, 192-200.

Yoo, H.-B., Lim, H.-M., Yang, I., Kim,. S.-K., and Park, S.-R. (2011). Flow cytometric investigation on degradation of macro-DNA by common laboratory manipulations. J. Biophysical Chemistry *2*, 102-110.

Zaia, J. (2010). Mass spectrometry and glycomics. OMICS *14*, 401-418.

Zhang, L.K., and Gross, M.L. (2000). Matrix-assisted laser desorption/ionization mass spectrometry methods for oligodeoxynucleotides: improvements in matrix, detection limits, quantification, and sequencing. J Am Soc Mass Spectrom *11*, 854-865.

Zimmermann, B.G., Grill, S., Holzgreve, W., Zhong, X.Y., Jackson, L.G., and Hahn, S. (2008). Digital PCR: a powerful new tool for noninvasive prenatal diagnosis? Prenat Diagn *28*, 1087-1093.



Modern Metrology Concerns

Edited by Dr. Luigi Cocco

ISBN 978-953-51-0584-8
Hard cover, 458 pages
Publisher InTech
Published online 16, May, 2012
Published in print edition May, 2012

"What are the recent developments in the field of Metrology?" International leading experts answer this question providing both state of the art presentation and a road map to the future of measurement science. The book is organized in six sections according to the areas of expertise, namely: Introduction; Length, Distance and Surface; Voltage, Current and Frequency; Optics; Time and Relativity; Biology and Medicine. Theoretical basis and applications are explained in accurate and comprehensive manner, providing a valuable reference to researchers and professionals.

How to reference

In order to correctly reference this scholarly work, feel free to copy and paste the following:

Sang-Ryoul Park, Jun-Hyuk Choi and Ji-Seon Jeong (2012). Development of Metrology for Modern Biology, Modern Metrology Concerns, Dr. Luigi Cocco (Ed.), ISBN: 978-953-51-0584-8, InTech, Available from: http://www.intechopen.com/books/modern-metrology-concerns/development-of-metrology-for-modern-biology

INTECH
open science | open minds

InTech Europe
University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
www.intechopen.com

InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
Phone: +86-21-62489820
Fax: +86-21-62489821