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Standardisation of Gene Therapy Products: *Practical Considerations and Perspectives*

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A prerequisite for producing medicinal products is ensuring their quality and safety. This requires appropriately controlled and standardised manufacture and testing procedures that result in consistent potency, safety, and efficacy. Assuring the quality and safety of gene therapy products in particular presents a great challenge because they are cell-based, multi-gene products which include viral and therapeutic proteins as well as modified cells. Although more than 860 gene transfer clinical trials are in progress and the first gene therapy product is already on the market (in China), the development of reference materials for gene therapy products is at an early stage with only a few accessible reference materials. Standardisation of gene therapy products to ensure their quality and safety is clearly necessary and has become increasingly important. Standardisation and other issues specifically related to gene therapy products are discussed in this article.

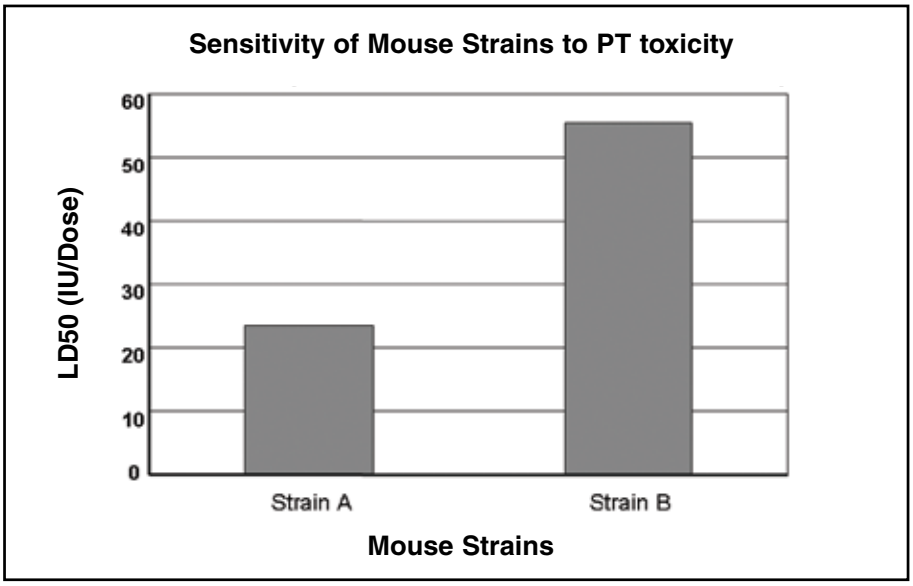

Figure 1. Adequate assay systems are essential for standardisation. An example of using two different mouse strains for testing the same batch of pertussis vaccines shows a significant variation of the mouse LD_{50} by pertussis toxins.

Standardisation of Biologicals

In 1897, Paul Ehrlich initiated biological standardisation by establishing a biological unit for diphtheria antitoxin. The first "Ehrlich" unit for diphtheria toxin was part of the content of a particular reference preparation of dried antitoxin, and its unitage was maintained by the use of cross-calibrated secondary standards. Before this, the potency of many medicinal products had been determined by measuring a response-based unit, such as the derived LD_{50} for mouse, rat, cat, or frog units. The variation in assays and incompatible unit systems compounded the problems of consistency for a wide range of biological substances. For example, using different mouse strains to test the safety of the same batch of pertussis vaccine could give a significant variation in LD_{50} results (Fig. 1). Using an inappropriate testing system may have a significant impact on the safety of biological products. Standardisation is necessary for meaningful data analysis and product comparison, but without valid reference materials and standardised assays and biological units, it is difficult, if not impossible, to compare results from different laboratories.

In 1922, the League of Nations adopted the Ehrlich unit as the International Unit (IU) for diphtheria toxin, initiating the concept of a worldwide status for standard preparations and their associated units. This activity has been maintained to the present day by the World Health Organisation (WHO) of

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the United Nations, which succeeded the League of Nations. Many other international standards have been produced for a wide range of biological products. Madsen, Dale, and Hartley can be credited with ensuring that such efforts were pursued with vigour and appropriate scientific thoroughness. The Expert Committee on Biological Standardisation (ECBS) of the WHO has established appropriately characterised preparations of biological materials, which are published as WHO International Standards or Reference Materials. The ECBS has set out criteria for selection of materials as international reference reagents, including requirements for (1) the stability of reference materials, (2) adequate performance in as wide a range of assay methods as possible, and (3) dose-response linearity and parallelism to compare and calibrate the primary standard and secondary standards or unknowns.

Processes for producing, evaluating, establishing, and distributing international standards involve three key stages: (1) establishing a single reference preparation for the calibration of assays, (2) establishing an assay independent, arbitrary biological unit, and (3) facilitating comparability of results obtained in different laboratories. This process was devised many years ago; it has stood the test of time and still remains in place.[1] Initially, candidate materials made available for standardisation purposes are evaluated for their suitability for calibration of appropriate assays. Selected materials are then aliquoted with a known precision of fill. Typically, approximately 4,000 ampoules are prepared and sealed under nitrogen. The ampouled material is then assessed for analyte recovery and stability. An international collaborative study involving a suitable number of expert laboratories from as wide a geographical area as possible is conducted to identify a suitable preparation that can be established by the WHO as the international standard. The biological activity (e.g., potency) of the standard is then assigned and an arbitrary IU defined as a proportion of the ampoule contents.

International standards are intended primarily for calibrating assays, although they are also valuable for assay validation. In some cases they can be used to compare the performance of different assays and to discriminate between or detect different or similar analytes. Suitable statistical procedures have been used to evaluate and establish a valid response in assays (i.e., that the dose response is linear and parallel responses are obtained for the standard and secondary standards and unknowns) (Fig. 2).

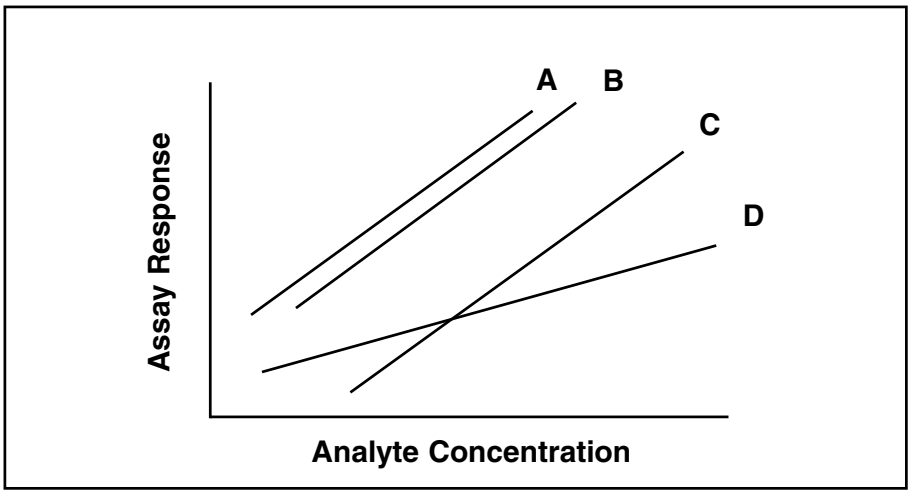

Figure 2. Parallel dose responses are required for direct comparison of results for different product preparations. The lines produced by preparations A, B, and C are parallel, showing that the assay is responding similarly to the activity in these preparations. The difference in displacement along the analyte concentration axis is proportional to the active analyte concentration in the samples. If one of these preparations is a standard, then relative analyte concentration can be calculated by comparison. It is invalid to attempt to derive a potency estimate for preparation D by comparison with the other samples, as D does not produce a dose response that is parallel to the other preparations.

For most therapeutic products, manufacturers commonly use internal reference materials to calibrate different batches of a biological product. However, in many cases, internal reference materials are product-specific and cannot be used for general assays or in different laboratories. These internal reference materials often do not meet the WHO criteria for standards and in some cases this impairs their usefulness and validity, except for the original manufacturer using them for calibration purposes. Figure 3a shows that results varied significantly — up to 2000-fold — when using an internal reference material in different laboratories; in contrast, a dramatic improvement in comparability was obtained by introducing an international standard in the same system (Fig. 3b). The use of a single worldwide standard facilitates the comparability of data.

Development of Reference Materials

Many international standards for biological products have been established under the auspices of the WHO. By 1974 there were more than 270 international standards for antigens, antibodies, antibiotics, vitamins, hormones, and enzymes. A general expansion in standardisation activities has occurred over the past 30 years but the general principles that are followed have not changed substantially. However, international standards for some small molecules, such as vitamins and some antibiotics, have been discontinued because these materials can be fully characterised using physiochemical procedures.

Progress in the field of biotherapeutics, particularly recombinant protein-based products, is very rapid, with new molecules being identified frequently. This has led to a modification of established procedures for producing international standards to include reference reagents, which are established following a more limited study. However, these are produced to the same high standard as other international standards and have a single associated arbitrary unit.

Over the past 80 years, more than 1,000 standards or reference materials have been produced for the standardisation of various biologicals by the National Institute for Biological Standards and Control (NIBSC). More than 60,000 vials of reference materials are distributed every year to laboratories in some 60 nations.

The nature of standards and reference materials has been continuously evolving. Initially, reference materials were derived primarily from crude "natural" preparations of materials (e.g., infectious viruses for measles, mumps, and rubella vaccines; coagulation factors like II, VIII, IX, and X; and hormones such as insulin). Advances in recombinant DNA technology have led to the rapid expansion in the need for standardisation of recombinant proteins (e.g., cytokines and growth factors). Because of the significant development in production methods, advances in assay systems, and increased information about products, forms of reference materials are becoming more diversified. In addition to a variety of established serological tests, highly sensitive nucleic acid test (NAT) methods, such as the polymerase chain reaction (PCR), have been widely used to detect blood-borne viruses (e.g. HCV and HIV). This has led to the increased development of reference materials for nucleic acid-based assays. For example, a PCR-based test, MAPREC (mutant analysis by PCR and restriction enzyme cleavage) has been recommended recently by the WHO as a pre-release test for oral poliomyelitis vaccines, which has significantly reduced the use of the traditional *in vivo* neurovirulence test in monkeys (MNVT) for control of polio vaccines.[2]

In terms of the intended functions, standards and reference materials can be divided into three major groups: (1) the determination of dosage toxicity of vaccine products, (2) the determination of therapeutic potency of recombinant proteins, and (3) the detection of adventitious agents. Accordingly, each group of reference materials has different requirements for measurement and precision of readout. These differences include threshold measurement of dosage toxicity, quantitative assessment of potency, and diagnostic detection of adventitious contaminants.

Reference Materials for Gene Therapy

Gene therapy has been under continual development for the past 13 years. Since 1989 there have been more than 860 clinical trials worldwide, including 17 premarket products currently in Phase III clinical trials and one product already on the market.[3,4] Therefore, to ensure the quality and safety of gene therapy products, standardisation is required and has become increasingly important. There is a professional and regulatory appreciation of the need for quality control of viral vectors used in gene therapy, which includes assuring the identity and potency of vector preparations and monitoring the pres-

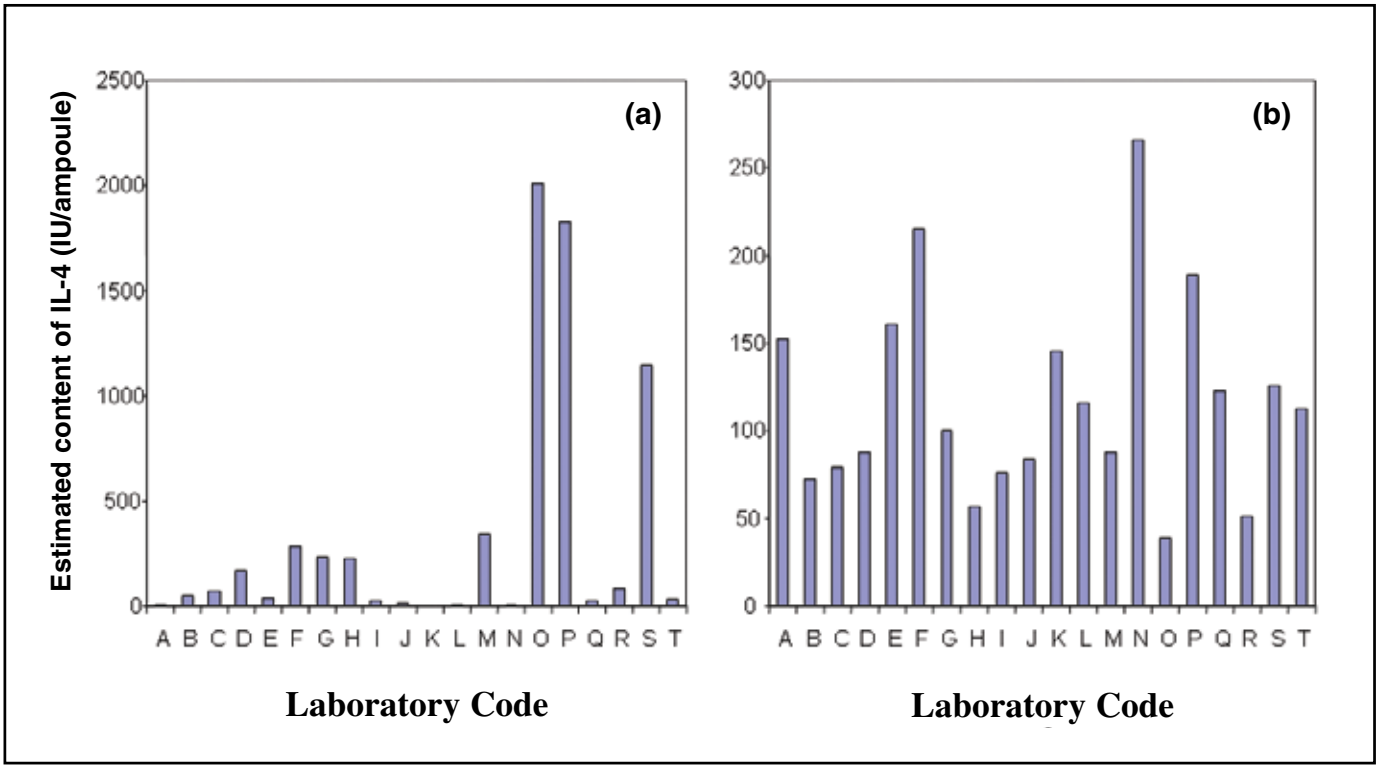

Figure 3. A single preparation of reference materials is beneficial for direct comparison of results. This figure shows estimates of the bioactivity of a preparation of IL-4 by bioassays carried out in twenty different laboratories as part of an international collaborative study. Panel a shows results obtained when the participating laboratories used different "in house" reference materials for assay calibration (all calibrated in mass units). Panel b shows results obtained by the same laboratories using the WHO International Standard for IL-4 (NIBSC 88/656) to calibrate bioassays in international units (IU). The dramatic improvement in comparability shown in b is the result of all laboratories using a single standard calibrated in arbitrary units, thus avoiding the problems of multiple reference materials.

Table 1. Experience from Preliminary AAV Reference Materials

WilBio Conference Data[7]

Centres	Physical Titre		Biological Titre	
A	$1.5x10^{13}$	$6.0x10^{13}$	$3.6x10^{11}$	$8x10^{10}$
B	$1.3x10^{13}$	$2.5x10^{13}$		
C	$8.9x10^{11}$	$1.4x10^{12}$		$1.3x10^{11}$
D	$1.0x10^{13}$			
E	$1.9x10^{12}$			$3x10^{10}$
Mean	**$8.2x10^{12}$**	**$2.9x10^{13}$**	**$3.6x10^{11}$**	**$8.0x10^{10}$**
F	$1.1x10^{13}$	$1.1x10^{13}$	$1.5x10^{12}$	$2.2x10^{12}$
Variation	**1:28-67**		**1:4.5-73**	

ence of replication-competent viruses (RCVs). However, development of reference materials for gene therapy is still in its infancy, with only two recognised reference materials available for testing replication-competent retroviruses and adenoviruses. Development of reference materials and assays for RCV testing began in 1997, as RCV generation is a major concern in the application of retrovirus based vectors.[5] Two reference materials for testing RCV are now available (ATCC VR-1488 and L/01B005) and testing for RCV is required by European authorities and FDA for all clinical trial materials.[6,7]

The lack of appropriate reference materials for adenoviral vector potency has been a concern because the data from the OCT trials (delivery of a functional ornithine carbamoyl transferase gene) suggested that the observed fatality was the consequence of dose-related toxicity.[8] As a result, development of reference materials for adenovirus-based vectors was actively pursued under the direction and auspices of FDA's Center for Biologic Evaluation and Research (CBER). The first preparation of 2,000 vials of adenoviral reference material (ARM; ATCC VR1516) has been established and is being used by laboratories worldwide.[9,10] FDA has recommended that the ARM be used in product characterisation and assay validation.

Development of reference materials for adeno-associated viruses (AAVs) began in 1998 and the first preliminary AAV reference material was made at the University of Florida in 2000. The preliminary preparation of AAV reference materials was not successful, with significant data variations between the laboratories and assay systems involved (Table 1). An AAV reference material Working Group has been convened by the Williamsburg BioProcessing Foundation (WilBio), which is working with FDA, academia, industry, regulatory organisations, and the NIBSC to help establish reliable reference material for AAV. In 2003, WilBio called for proposals from academia and industry for the production, purification, and characterisation of the AAV Reference Material, as well as for a repository and distribution of the ampouled virus genomes.[11,15] Currently, there are neither available reference materials nor international efforts to develop reference materials for vectors based on other viruses (e.g., herpes, poxvirus), although vectors based on poxviruses are the third most popular vectors modified for gene therapy and are used in 6.1 percent of current clinical trials. (WilBio formed a Lentivirus Reference Material working group in 2002, but reference material is not yet available.)

Development of reference materials presents a great challenge for gene therapy products because they are not only multi-gene products, but they are also cell-derived. All types of reference materials, including those that monitor the toxicities of vaccines (such as for viral proteins expressed by vectors), the potency of recombinant proteins (transgene products), and adventitious viruses in blood products (RCVs), can be applied to a single gene therapy product. The challenge is further compounded by the diversity of assay systems. A number of biochemical, virological, and molecular biology-based assays (e.g., ELISA, rtPCR, infection assays), have been used to quantify vector particles and transgene expression levels. Multiple reference materials are required for standardisation of a single gene therapy product.

A considerable number of reference materials can be needed to calibrate assays and control the quality, potency, and safety of gene therapy products. For example, this number may reach as many as five for a single HIV-1-based vector product: p24 antigen reference (NIBSC90/636), vector particle reference preparation, integrating DNA reference, transgene (e.g., FIX NIBSC95/544), and RCV reference materials. Furthermore, available gene therapy products are significantly diversified. Current clinical trials involve more than six types of vector backbones based on retrovirus, adenovirus, herpes virus, and lentivirus and more than 13 transgene types (antigens, cytokines, or receptors).[12] Out of 73 clinical trials being conducted in the United Kingdom, there are 62 different product candidates which vary within their vector backbones or transgenes, as well as in the modifications of promoters or transcriptional elements.[13] Diversity of future gene therapy products is likely to increase with current efforts to produce safe, site-specific, integrating vectors.

Although gene therapy has been under significant development as an indispensable augmentation to conventional medicines, most gene therapy products and clinical studies are still at an early stage of development. Therefore, standardisation and development of product-specific reference materials for gene therapy is currently unpredictable and unfocused. Generic approaches need to be developed to devise reference materials that are not product-specific. This will support the quicker development of products in clinical trials and also establish a foundation for future standardisation.

Since the first international reference materials for insulin were produced by NIBSC eighty years ago, the organization has gained extensive experience with the development of international standards and reference materials for biological products. There are already a number of international reference materials available at NIBSC that can readily be applied to the potency and purity testing and assay validation of gene therapy products.[14] Apart from the need for reference materials for vector components, a number of reference materials will be required for validating assays to detect mRNA and viral DNA or to integrate viral sequences. For instance, oligonucleotide primers, fluorescent probes, and synthetic DNA/RNA templates with well-characterised performance, together with Taq polymerase with consistent activity, are clearly required for PCR-based assays. Availability of reference monoclonal antibodies specific for the viral proteins or transgene proteins (e.g., anti-HIV-1 p24) would improve laboratory-to-laboratory comparability of results obtained using immunological methods such as ELISA or flow cytometry analysis.

The requirement for reference materials in the gene therapy field will continue and increase along with the rapid modification and expansion of existing vector systems. Furthermore, most assay systems used in gene therapy involve measurement of assay-dependent parameters, such as fluorescence intensity for flow cytometry and real-time PCR or absorbance of light for ELISA. The only reliable approach for calibration and validation of such procedures is comparison with a reference material that has a defined analyte content. Therefore, it is crucial that a panel of well-characterised reference materials be developed and made available for worldwide use to ensure the reliability, consistency, and comparability of existing and future gene therapy products.

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