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Strengthening quality control of biopharmaceutical products through the establishment of structure- function relationships

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ABSTRACT

Establishing relationships between data from different analytical techniques supports the selection of a suitable set of tests for the characterisation of individual biopharmaceutical preparations. In this case study a portfolio of physicochemical and biological assays was developed that examined batch-to-batch-variability in Interferon-alpha preparations produced in a *Pichia pastoris* expression system, and in samples from external collaborators. Throughout the case study, the NPL material was compared to Chemical Reference Standards supplied by EDQM.

A review was undertaken of the industrial and regulatory debate on the application of structure-function relationships for biopharmaceuticals. The selection of key cell-based bioassays and physicochemical methods established a structure-function relationship for the class of model system used within this project.

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Approved on behalf of the Managing Director, NPL
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List of Abbreviations

ABPI	Association of the British Pharmaceutical Industry
ANS	8-anilino-1-naphthalene sulfonate
BMGY	Buffered Glycerol-complex Medium (Yeast)
BMMY	Buffered Methanol-complex Medium (Yeast)
CD	Circular Dichroism
CE	Capillary Electrophoresis
CHO	Chinese Hamster Ovary
CRS	Chemical Reference Substance
DLS	Dynamic Light Scattering
DMEM	Dulbecco Modified Eagle's Medium
EDQM	European Directorate for the Quality of Medicines
ED ₅₀	Effective Dose
ELISA	Enzyme-linked immunosorbent assay
EMA	European Agency for the Evaluation of Medicines
EP	European Pharmacopoeia
EPO	Erythropoietin
FBS	Fetal Bovine Serum
FDA	Food and Drug Administration
GCV	Geometric Coefficient of Variance
ICH	International Conference for Harmonisation
IFN	Interferon
IS	International Standard
IU	International Units
LB	Luria Broth

NMS	National Measurement System
PASG	Pharmaceutical Analytical Sciences Group
PBS	Phosphate Buffered Saline
PEG	Polyethylene Glycol
RP-HPLC	Reverse-phase High Performance Liquid Chromatography
RPMI	Roswell Park Memorial Institute
RT-PCR	Real-Time Polymerase Chain Reaction
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEC	Size-exclusion chromatography
SME	small to medium enterprise
SPR	Surface Plasmon Resonance
USP	United States Pharmacopoeia
UV	Ultra Violet
WHO	World Health Organisation
YPDS	Yeast Peptone Dextrose Sorbitol

1 Introduction

This project addresses the key issues associated with strengthening the quality control procedures for biopharmaceutical products through applying the FDA-led “well specified product” concept. The project was led and coordinated by the National Physical Laboratory on behalf of a consortium, which includes key stakeholders and beneficiaries in the quality control of biopharmaceutical products. The research consortium includes the National Institute for Biological Standards and Control (NIBSC), and an SME biopharmaceutical analytical service laboratory (Chiralabs Ltd). An industry steering committee comprising of GlaxoSmithKline, Lonza Biologics, Biacore, Adprotech and Novartis was convened at the beginning of the project to advise on the scientific direction of the project, and to work with the technical partners to ensure the project will remain relevant to, and focussed on, industrial requirements. The high degree of industry interest and participation shows the importance of this work in this sector, and the breadth of the consortium will help to ensure that the outputs of the project will be seen as independent. The areas that the project will disseminate to are as follows;

- The national and international regulatory and standards community – through the regulatory role of NIBSC.
- The national and international metrology community – through the role of NPL as a National Measurement Institute.
- The UK and international biopharmaceutical industry – through the participation of GlaxoSmithKline, Novartis and Lonza; together with representation within the Pharmaceutical Analytical Sciences Group (PASG) of the Association of the British Pharmaceutical Industry (ABPI).
- The UK analytical services industry – through Chiralabs Ltd, a laboratory dedicated to providing analytical services to the UK biopharmaceutical sector.

This project supported the Mission and Strategy of the NMS Measurements for Biotechnology Programme, to improve the comparability of biological measurements, and to remove barriers to the exploitation of Biotechnology by UK industry.

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3 The aims of the project

This exercise provides the opportunity carry out a case study of the development of different assays to be applied in the quality control of a biopharmaceutical product, without constraints of commercial confidentiality. Presentations of individual case studies at specialist meetings indicate that some issues are commonly encountered in the development of such a package.

Testing the quality of the final product requires the use of a number of analytical techniques providing complementary information that, together, will permit assessment of the properties of the product and comparison with the batches tested in clinical trials to determine clinical safety and efficacy. The biological activity of protein products depends on the primary, secondary, tertiary and quaternary structure of the molecule, its post-translational modifications and levels of aggregates. These properties can show variation between production batches. As a result, for most biological products, there is a regulatory requirement that the potency of individual batches for the majority of products is tested upon release of the batch. In most cases the product is not assessed adequately by physicochemical techniques alone to permit prediction of the activity of the batch, and a functional bioassay is required. In a few cases, when no suitable functional bioassay exists or when there is adequate data linking the measured product attributes to potency, surrogate assays for potency, such as receptor-ligand binding assays or physicochemical analyses may be used.

Therefore the bioassays used in quality control of the product should assess attributes that are relevant to the product's clinical action. The guidelines from the International Conference for Harmonisation (ICH) for the Technical Requirements for Regulation of Pharmaceuticals for Human Use states in section Q6B that;

“mimicking the biological activity in the clinical situation is not always necessary. A correlation between the expected clinical response and the activity in the biological assay should be established in pharmacodynamic or clinical studies”.

This correlation may be made late on in the product development cycle, after preclinical studies and Phase I trials. In addition, the relationship between structure and the mechanism of action of the molecule may be unknown, particularly if an animal model of the human disease is not available. When the mechanism of action is unclear, it may be difficult to link small changes in structure to a clinical outcome, in which bioavailability, processing and clearance of a molecule will play a role.

Through the interaction of several physiological systems, *in vivo* bioassays can assess some attributes of a product to which *in vitro* assays may be insensitive. However, *in vitro* assays offer a number of other advantages, and where possible their use is generally preferred. A wide variety of *in vitro* bioassay systems are used, in many of which the cell signalling pathway activated is relevant to the clinical mode of action (MOA) of the drug.

The response of a cell to external stimulus (the drug substance) involves a biological event which may be quantified, perhaps through the expression of a “reporter” molecule, cell proliferation or cell death. Advances in elucidation of cell signaling pathways and molecular biology techniques to engineer molecules for reporting cellular events, along with the advances in reagents and instrumentation to monitor them, now make it possible to more accurately quantify changes in the early biological events in a cell. As long as these events are in some way related to the overall mechanism of action of the molecule, then manufacturers can begin to address the guideline stated above.

With advances in analytical instrumentation to study the structural components of biopharmaceuticals, more emphasis is being placed on correlating events in a cell-based bioassay with the information from physicochemical techniques, provided that small changes to a molecule are reflected in the functional assay. The correlation relies on identifying the key characteristics of the molecule that are clinically relevant, to avoid wasting resources on examining structural details that have no influence on the clinical outcome. This implies that a wide range of product characteristics have to be screened before some can be ruled out. By developing appropriate cell-based bioassays such a link may be established early in the product development cycle before expensive and time consuming clinical studies are carried out. Combining data from bioassay and physicochemical approaches may also provide insights for the

development of other molecules in the same therapeutic class and speed the rational development of similar products.

Understanding the variability in the different types of analyses is a crucial step in developing meaningful structure activity relationships. Excessive variability in bioassay results would make it impossible to assess the influence of protein structure on biological activity.

Therefore, the aims and objectives of this project are:

- To underpin the use of the “well specified product” concept for product quality control and regulation.
- To establish a portfolio of physicochemical and biological assays with sufficient sensitivity that can be used to provide meaningful structure-activity relationships for biopharmaceutical products.
- To identify the current sources of uncertainty in cell based bioassays used to measure the potency of biopharmaceutical products, and identify approaches to reduce uncertainty.
- To investigate the relative merits of early and late stage readouts and correlate in appropriate model systems.

4 Background

4.1 Quality Control of biopharmaceuticals

Biopharmaceutical products for clinical use are licensed because they have a desirable effect on the human patient (or animal, in the case of veterinary products) that outweighs the inconvenience and risks of drug administration, side effects etc. The decision to licence, or not, is normally based upon data from one or more Phase III clinical trials, which are the “Gold Standard” assays defining clinical efficacy in man. These will have been preceded by lengthy pre-clinical investigations and Phase I and Phase II trials, which normally focus on product safety. Phase III clinical trials are

usually complex, expensive and lengthy, requiring administration of the potential product to relatively large numbers of patients under closely supervised conditions and with extensive follow-up of the patients' responses and statistical analysis. The drug product used in these trials usually derives from a small number of early production batches, and there is an ongoing requirement on manufacturers to demonstrate that the "quality" (comprising factors such as identity, potency and purity) of subsequent production batches is comparable with, or better than, material used in clinical trials. Repeating the clinical trials on successive production batches is clearly not feasible, and so alternative quality control approaches that do not measure clinical efficacy directly have been developed.

Two basic approaches to ensuring product quality are used. The first is to ensure that the production process is tightly controlled through adherence to well-defined Standard Operating Procedures, analyses of starting materials and consumables, and process control (ie. under Good Manufacturing Practice), carefully designed to deliver a consistent product. The second, confirmatory step is to carry out analyses of the drug intermediates and the final product, to ensure that these materials meet specifications developed during the optimisation of the manufacturing process. These assays cannot "analyse in" quality, they act to confirm that the production process has delivered a product of appropriate quality. This project deals with the final set of assays on the drug substance and drug product, rather than assays related to starting materials or intermediates.

Guidelines for ensuring the quality of pharmaceutical products are developed by the ICH (see <http://www.ich.org>), regulatory agencies in Europe (EMA) and the USA (FDA), and the World Health Organisation (WHO). Detailed standards are published by pharmacopoeias, including the European Pharmacopoeia (EP) and the United States Pharmacopeia (USP). The purpose of these assays is to demonstrate consistency of the individual production lots with the material used in clinical trials, for quantity, integrity and potency of active material, in terms of the profile of product-related and process-related impurities, and sterility. Frequently there will be no direct correlation between any single assay, or combination of assays, and clinical efficacy in man, although functional assays will usually be chosen to mimic as closely as possible the anticipated mode of action in the patient. Long term experience of a

product and other academic work is likely to lead to an understanding of those aspects which correlate most closely to its function. The ICH Guideline Q6B highlights the fact that lot-release specifications should highlight those factors which it is believed to reflect clinical efficacy and safety.

The assays used for lot-release should be validated to a very high degree, according to ICH Guideline Q2 (R1), and should be demonstrated to be accurate, robust and precise.

4.2 Different types of bioassay

The terms “bioassay” and “biological assay” cover the whole range of assays performed in biological systems, from whole animal, *in vivo*, test systems, through isolated organs, cell cultures and cellular fractions, to biochemical or enzymatic systems. In the biopharmaceutical industry, bioassays are used for a variety of purposes such as screening for potential active compounds, toxicology, preclinical efficacy, pharmacokinetic studies, testing for unwanted immunogenicity and measuring potency.

For the majority of products, a bioassay for potency measurement is a required part of the specifications for batch release. ICH Q6B, Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products, states that “A valid biological assay to measure the biological activity should be provided by the manufacturer”. Since the biological activity depends on the integrity of the molecular structure, often including higher order structure which cannot be assessed by physicochemical methods, in many cases, the bioassay is essential to assess these aspects of molecular structure and to predict the potency of the preparation. In addition to specifications for batch release, bioassays therefore play an important role in stability, formulation and comparability studies, in-process product monitoring, product monitoring following process changes and product characterization. Since development of a bioassay and maintenance of the necessary infrastructure and expertise demand a considerable investment, it is common for the bioassay used for product release to be used for additional other purposes.

4.2.1 Functional bioassays

It is necessary to make the distinction between “functional bioassays” – those in which an induced biological response is measured – and other types of bioassay, such as ligand- receptor binding assays and immunoassays, in which the measured change is not necessarily related to a biological function. Biological activity is defined in ICH Q6B as “the specific ability or capacity of the product to achieve a defined biological effect” and potency as “the measure of the biological activity using a suitably quantitative biological assay (also called potency assay or bioassay), based on the attribute of the product which is linked to the relevant biological properties”. A functional bioassay measures the potency of the product, which is the biological activity in that particular system. The potency measured in one system frequently, but not always, permits prediction of the potency in other biological systems. The bioassay used for product release does not necessarily have to measure the same biological response obtained in its clinical use: “Mimicking the biological activity in the clinical situation is not always necessary” but “... correlation between the expected clinical response & the activity in the biological assay should be established” (ICH Q6B). The measurement of potency for the purposes of product release is to demonstrate the similarity of the test batch to the batches used in clinical trials. As the potency is dependent on the integrity of specific aspects of the molecular structure of the product, the specific activity of a preparation is an important property for characterization of the product. If the biological action measured in the bioassay is close to clinical action of the product, the measured specific activity is more likely to assess integrity of parts of the molecule important in the clinical action, hence the desirability of having a bioassay that reflects the clinical mode of action (MOA).

4.2.2 Binding assays

Ligand-receptor binding measurements are a class of bioassay and can provide important information on the quality of products by demonstrating structural variations which affect the binding to the receptor. However, binding of a product to its receptor does not necessarily induce a biological response and so does not necessarily assess the potency of the product. Many molecules, including variants of the active ligand, may bind to a receptor without inducing the normal response, and by occupying the receptor, can inhibit the action of the active ligand. For some drugs,

such inhibition represents the desired action of the product. In such cases, if binding to the receptor is the sole action responsible for the activity of the drug, measurement of the binding may suffice as a surrogate potency assay for product characterisation and release.

Immunoassays, which measure the binding of antibodies to various epitopes of the product molecule, are sometimes classified separately from bioassays. The antibody binding provides information on the integrity of selected epitopes, but does not necessarily reflect the potency of the preparation. Binding assays or immunoassays often form part of a functional assay. If, for example, the functional assay involves stimulation of secretion of a protein by the test product, this protein may then be quantified by immunoassay or binding assay. This assay is classified as a functional assay since it has involved the induction of a biological response by the test product.

It is unlikely that any one bioassay alone will permit assessment of all properties of a biopharmaceutical product that will impact on its safety and efficacy in the patient.

4.2.3 Assay variability and reference standards

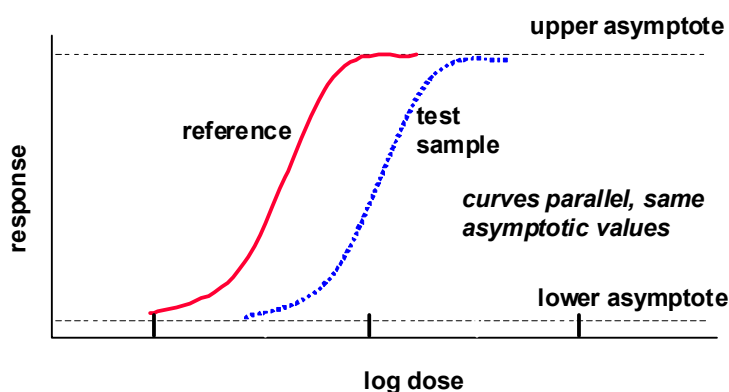
Because they are complex systems, bioassays are subject to many sources of variability. Some of these may be difficult, or impossible to control. Other factors may not even be identified as varying or as having an effect. For example, cell culture media contain biological components that vary from batch to batch. For factors that are identified as components, examination of certificates of analysis may permit use of batches of similar composition or adjustment of the composition. For unidentified factors however, no such remedial action is possible and the assay has to be designed to accommodate such variation as far as possible. Identification and assessment of the variables which can affect the performance of an assay are an important part of assay characterisation. The permissible range of these parameters can then be specified and the satisfactory performance of the assay within these ranges is demonstrated during validation.

Many analytical techniques, including physicochemical techniques, use a reference standard consisting of a material with properties similar to that of the test sample. The measured attribute is reported as a value relative to that of the standard. Because

bioassays are complex systems and subject to more sources of variability than are most physicochemical assays, any absolute measure of potency, such as ED50 (the dose giving 50% of the maximum response), can vary widely between assays so the activity of the sample has to be measured relative to that of a reference standard. For example, in a multi-lab study where labs used their own protocols for an assay based on a Chinese hamster ovary (CHO) cell line response, the ED50 for a common set of pertussis toxin samples varied about 1000-fold across all assays (1). Availability and use of appropriate reference standards are crucial to the utility of bioassays. Provided the standard and sample are sufficiently similar, any change in the system will affect them equally and their relative potency will remain constant. Potency measurements from assays using a common reference standard can then be compared – across time and between laboratories.

Functional similarity of the reference standard and sample is a fundamental condition for assay validity. In other words, the preparations have an identical action in the assay, and, as a consequence, the dose-response curves are identical except for a possible displacement along the concentration axis i.e. dose-response or transformed dose-response curves are parallel and reach the same asymptotes.

Functional similarity between reference standard and test sample



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World Health Organisation International Standards (IS) are the primary reference standards for biologicals. The IS for IFN- α 2a and IFN- α 2b codes 95/650 and 95/566, respectively, are intended for the calibration of biological activity. Their formulation precludes their use in many physicochemical analytical techniques (often including other protein molecules as carriers). This is a common situation for biologicals, for external and in-house reference standards, and is recognised in ICH guidelines:

“ while it is desirable to use the same reference material for both biological assays and physicochemical testing, in some cases, a separate reference material may be necessary” (ICH Q6B 2.2.1).

In this study, European Directorate on the Quality of Medicines chemical reference substances for IFN- α 2a and IFN- α 2b (CRS I03200300 and CRS I03200301 respectively), were obtained as reference standards for the physicochemical studies. These are supplied with the following statement from EDQM, “supplied exclusively.... for use as standards or reference substance in the tests and assays indicated (identification by peptide mapping, identification by electrophoresis, test for related substances by liquid chromatography, test for related substances by electrophoresis), in accordance with the official methods of the Ph. Eur, and for no other purpose”.

We were able to show that the EDQM reference standards had similar biological activity to the respective International Standards (IS) (2) in the bioassay systems used in the study, and could thus be used as reference standards for biological activity. This enabled us to use the same preparation for both physicochemical and bioassay analysis. We also investigated whether the standards were appropriate for a wider range of physicochemical assays than specified by EDQM.

4.2.4 Changing or transferring a bioassay system

At any stage during product development, it may prove desirable or necessary to change a bioassay. This may involve apparently minor modifications or a complete change of assay system. In such a case, it is essential to make a comprehensive comparison of the performance of the two assay systems using a panel of appropriate

test samples and reference standards, before validation of the new protocol. A similar exercise is necessary on transferring an assay between labs.

Development of a bioassay and its characterisation, also referred to as qualification, in order to determine the critical parameters and design an appropriate validation program, represent a significant investment. Failure in the timely development of an appropriate bioassay can delay the whole drug development program.

4.2.5 *In vivo* bioassays

Selection of an appropriate system is essential and many factors have to be considered. Considering functional bioassays, the various types of assay system each offer advantages and disadvantages.

Compared with *in vitro* assays, *in vivo* assays suffer from a number of disadvantages:

- Ethical considerations due to the use of live animals
- Legal constraints (including limiting the numbers of animals used and consequently the numbers of data points that can be obtained)
- High variability (intra- and inter-assay)
- Difficulties in transfer of the assay from one site to another
- High cost per assay
- Labour-intensive, not suitable for automation
- High cost of infrastructure – housing, staff, etc
- Low throughput

However, *in vivo* assays can provide more meaningful data than *in vitro* assays when interactions between multiple physiological systems are involved. For example, glycosylation of a molecule at a site distant from the receptor binding site may not affect the ligand-receptor binding and subsequent biological response in a cell culture-based assay. However, this glycosylation may affect the rate of clearance of the drug from the patient's system, and thus the time over which the drug can act in the patient. Similarly, a structural change in an epitope which does not affect the ligand-receptor binding may have no effect on the biological response *in vitro*, but could provoke an immune reaction in the patient. For some cases where assessment of biological activity depends on interaction of different cell types, there are now possibilities of

co-culture of two or more cell types or genetic modification of cell lines to combine multiple functions. While *in vivo* assays can sometimes be more physiologically relevant than *in vitro* assays, they suffer from the constraint that they are performed in non-human species. For biopharmaceuticals intended for human use, this may be a disadvantage if there are potential differences in the response to the drug by the test species compared with humans.

4.2.6 *In vitro* bioassays

In vitro cell-based assays are intermediate between *in vivo* and biochemical assays in many of the characteristics listed below. They are widely used (3) and, as a consequence, there is a large and expanding range of commercially available supporting technology. Within one cell-based functional bioassay system, there may be a choice of measurable responses (readouts), from the initial responses on ligand binding, through the signal transduction system changes (often called “early-stage” responses) to the “late-stage” or “end” responses such as cell division, secretion of a specific protein, or cell death.

In vitro assays, in general, offer the following advantages:

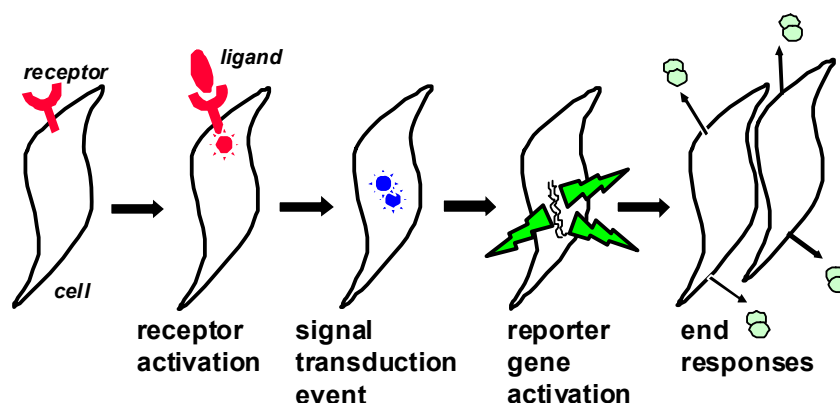
- fewer uncontrolled variables
- less expensive
- less labour-intensive
- avoid use of live animals
- higher throughput
- more suitable for automation
- more flexible, more rapid
- range of off-the-shelf technology available

For a few types of product, biochemical *in vitro* assays, generally measuring enzymatic activity, are suitable. In general, these offer the advantages of

- simplicity – few variables to control
- low intra-assay variability, often low inter-assay variability

- often relatively low cost
- logistically simple
- suitable for automation
- high throughput
- rapid

Cell-based bioassay readouts



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Figure 1. Cell-based bioassay readouts, ranging from ligand binding, signal transduction and the “late-stage” or “end” responses such as cell division, secretion of a specific protein, or cell death.

Both primary cells (freshly isolated) and cell lines are used in potency assays. Generally cell lines are preferred for their batch-to-batch consistency. Genetic engineering offers the possibility of building in a convenient artificial response and of making a cell-line responsive to further products by expressing exogenous receptors. It is not always necessary to insert or change a complete receptor molecule to make a cell line responsive to a further product. It can prove possible to create a chimeric receptor, where only the external, ligand-binding domain of the receptor is exchanged to give an assay a different specificity.

4.2.7 Late-stage *in vitro* bioassays

Late-stage assays may be more a stringent test of the integrity of the molecule and its biological activity. For example, the end response may depend on integration of signals from more types more types of ligand-receptor interaction. Late –stage assays

may require only generalised low-tech equipment, materials and protocols so may be used for several different products. This often means that the initial cost and time outlay are less, though possibly this difference is less marked as more experience, materials and protocols become available for various early-stage types of assay. These logistical issues will depend strongly on existing assay platforms within a company and may vary greatly between companies.

4.2.8 Early-stage *in vitro* bioassays

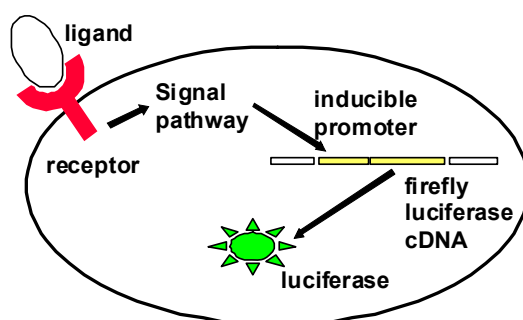
Early-stage assays offer some advantages and some disadvantages compared with late-stage assays. The cell-based part of the assay is usually of shorter duration (possibly minutes compared with days). As the cell-based part of assay is usually the part most susceptible to variables which are difficult to control, early-stage assays offer the possibility of lower total assay variability. The assay system may be able to tolerate unfavourable conditions (such as non-sterile conditions or suboptimal pH) for short periods giving flexibility in sample/dosing and a reduced requirement for a controlled environment. The total assay time of an early-stage bioassay (i.e from dosing the sample to obtaining the reportable value) can be, but is not always, shorter so data may be available more rapidly. It should be noted that the total assay procedure can include steps after the cell-based part which may require significant operator time.

Early-stage readout assays are more likely to require greater development, specialised equipment and materials so the initial cost and time outlay is often greater, but this difference may be decreasing as more experience, materials and protocols for early-stage assays become available. Early-stage assays are often (but not necessarily) developed in systems less obviously related to clinical mode of action.

Early-stage assay systems (cell-lines) are often unique; many systems have genetic manipulation (receptor expression or reporter gene readout); these may have less history than the established late-stage systems with regulators but there is an increasing awareness of and interest in early-stage assays.

An example of a system which gives an early-stage readout is one that utilises cells that are genetically modified by expression of the reporter gene luciferase. On binding

of the test product (ligand) the signal transduction system is activated and the inducible promoter drives expression of the luciferase. Upon addition of the substrate luciferin, a luminescent signal is generated, the amplitude of which is related to the potency of the test product.



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Figure 2. Schematic of an early-stage bioassay using genetically modified cells to give a luminescent signal.

In this project, a comparison was made of the use of early and late-stage bioassays in measuring the potency of preparations of the model biopharmaceutical.

4.3 Survey of industry attitudes and experience of physicochemical and bioassays

One of the frequent complaints about bioassays is that they are imprecise. They are susceptible to many sources of variability, and when these are not controlled, the results will be variable. However, when performed under well-controlled conditions, the precision can be much better than figures commonly quoted. The following tables show some examples quoted at recent meetings. Table 1 shows results obtained for repeatability (intra-assay precision) and intermediate precision (inter-assay precision) during validation exercises, which represent a snapshot of the assay performance and are unlikely to encompass the whole range of variables that will be encountered during the long-term use of the assay.

Table 1. Repeatability and intermediate precision of different types of bioassays. KIRA = Kinase Receptor Activation; PACE = Phosphospecific Antibody Cell-based ELISA.

Assay	Repeatability (CV)	Intermediate precision (CV)	Source
Reporter gene (luciferase)	3%	7%	V Mukku, Amgen, IBC Biological Assays, Munich 2006
KIRA	1.7%	1.5%	H-J Wallny, Novartis, IBC Biological Assays, Munich 2006
PACE	1%	3%	
Reporter gene (luciferase)	5%	7%	
Proliferation (ATP-luciferin)	2.4%	5.0%	

Table 2 shows the reproducibility of a range of assays. These values include the inter-laboratory variability.

Table 2. Reproducibility (inter-laboratory precision) of assays in use for lot release, described by H. Gazzano-Santoro (Genentech) at the CMC strategy forum at the Well Characterised Biological Products conference, January 28 2007.

	precision	n (approx)	labs
Complement-dependent cytotoxicity	7%	700	6
Inhibition cell adherence	7%	500	3
Inhibition cell proliferation	4%	1700	6
Inhibition binding to receptor	9%	510	3
Cell proliferation	10%	400	3
Clot lysis	5%	500	3

4.4 Industry attitudes

Expression of specific concerns associated with bioassay development and use is encountered widely throughout the biopharmaceutical industry. It is to the benefit of all parties concerned — industry, regulators, and consumers – to identify common problems and assess their impact in order to seek solutions. However, this is difficult without hard data. In order to gather such data, a survey of delegates' use of bioassays was made at the 2005 IBC European Biological Assays conference (Basel, 18–19 October). The results of the survey have been made available by publication - A Survey of Bioassays in the Biopharmaceutical Industry. C. Jane Robinson, Laureen E. Little, E. Clare Robinson, and Hans-Joachim Wallny, *BioProcess International* 4(6):24-36, June 2006), and the full report can be accessed online at www.bioprocessintl.com .

The report covers issues such as the types of bioassay used, at what stage of product development the assays are developed, the importance attributed to various criteria in selecting a bioassay system, consultation with regulators, availability of statistical support, adequacy of resources available for the bioassay and whether delegates would like to replace their functional bioassays with binding or physicochemical assays.

The most widely reported criteria considered of high importance in selecting a bioassay were availability of cells and critical reagents. Assay performance was considered of high or medium importance. Availability of statistical or technical advice was generally only of low or medium importance. Closeness to *in-vivo* mode of action was listed by relatively few, but classed as high or medium importance by those who did so. Generally, the items listed as criteria for selecting an assay system were the same as the issues causing concern in the use of bioassays, though availability of advice did feature slightly more prominently as an issue of concern than as a criterion in the choice of assay. It was found that cell-line-based assays and immunoassays were the most commonly used assay types and represented the greatest number of established assays.

Based on the results of the 2005 survey, a follow-up a survey concentrating on cell-based assays was made of the delegates at the 2006 IBC European Biological Assays conference (Munich, Germany, 16-18 October). 108 delegates and speakers representing 71 organisations were registered at the meeting. Delegates were asked that if colleagues from the same organisation were present, to collaborate and submit only one form per organization. 33 survey forms were returned, including 2 from consultants. The responses from the remaining 31 survey forms have been analysed and will be submitted for publication to make the data freely available.

Briefly, the survey revealed that more functional assays than binding assays are under development. More of the assays under development use cell lines than use primary cells, but assays are being developed with primary cells. A wide range of assay systems and readouts are used and genetically modified cell lines with exogenous receptors and/or reporter genes are used. The survey asked whether companies would like to replace functional bioassays with binding or physicochemical assays, whether they intend to attempt to do so, and whether they have done so for some products. A few of the results of the survey are summarised in the tables below.

Table 3. Cell-based assays under development (figures = number of companies reporting development).

		For GLP	For any GMP	specs for product release	stability studies
primary cells	binding	1			
	functional	3	7	4	5
any cell line	binding	5	6	4	5
	functional	12	11	9	10
lines + exogenous receptor or reporter gene	binding	5	2	2	2
	functional	6	5	5	5
<i>Positive responses from 25 companies. Bioassay Survey Oct 2006 IBC Biological Assays, Munich</i>					

Table 4. Established functional cell line assays for GMP (figures = number of companies reporting development).

Established functional cell line assays for GMP			
<i>no reporter gene</i>	no exogenous receptor	+ exogenous R (incl. Chimeric)	+Chimeric receptor
receptor activation	6	4	1
signal transduction	5	3	1
DNA- total	1	1	-
- specific	-	1	-
mRNA	1	1	-
protein- total	3	1	-
- specific	3	1	-
cell number	11	4	1
colony number	6	2	1
intracellular translocation	-	1	-
metabolism redox	4	1	1
- other	4	-	-
apoptosis marker	4	1	-
plaque	1	-	-
<i>+ reporter gene</i>	4	2	1
<i>Bioassay Survey Oct 2006, IBC Biological Assays, Munich</i>			

Table 5. Replacement of functional bioassays with binding or physicochemical assays

Companies would prefer / intend to / have replaced some functional bioassays	YES	19
	NO	3
Have replaced some		2
<i>Bioassay Survey Oct 2006, IBC Biological Assays, Munich</i>		

4.5 Physicochemical measurement of biopharmaceuticals

In general, physicochemical assays reflect four aspects of product quality (i) primary structure (amino acid sequence, glycan profiling, and including identification and quantification of product-related impurities), (ii) purity (quantification and possibly identification of process related impurities), (iii) higher order structure (secondary, tertiary and quaternary structures), and (iv) physicochemical measurements of binding

to a ligand or a receptor. Whilst measurement of primary structure is almost invariably carried out using physicochemical approaches, other aspects may be deduced from biological assays or from a combination of physicochemical and biological approaches. Experience with a specific manufacturing process, experimental testing of the process efficiency (e.g. spiking experiments to demonstrate clearance of impurities), may generate sufficient data to allow experimental verification of some non-critical specifications to be discontinued (to be “validated out”). Regulatory guidance is often available for this (EMEA Committee for Proprietary Medicinal Products, Biological Working Party position paper on DNA and host cell proteins Impurities, Routine testing versus validation studies).

4.5.1 Primary structure

The most common approach to determine amino acid sequence is peptide mapping, using High-Performance Liquid Chromatography (HPLC) or capillary electrophoretic (CE) separations, usually with UV detection but sometimes coupled to a mass spectrometer. The additional information available from mass spectrometry allows identification of minor peaks from product-related impurities, such as clipped forms, methionine oxidation etc. Mass spectrometric methods that determine the molecular weight of the intact protein or glycoprotein also support these measurements. Glycoprotein glycosylation can be determined from direct mass spectrometric analysis for simple materials, or by glycan cleavage and profiling. Where specific aspects of the glycosylation are known to have a key role in product efficacy (eg. the role of sialylation in determining the *in vivo* half life of rhEPO), the methodology may be optimised for that particular aspect of product structure (the use of CE and/or isoelectric focussing to monitor sialylation variants in rhEPO, for example).

4.5.2 Secondary and tertiary structure

Whilst a variety of optical methods, including UV absorption, circular dichroism, fluorescence spectroscopy and vibrational spectroscopy (infra-red absorption, Raman and vibrational circular dichroism spectroscopies) are sensitive to the secondary and/or tertiary structure of proteins, they are rarely applied in a batch release context, due to the problem of deriving a suitable specification from such complex multivariate data (for discussion of this topic, please see NPL report number DQL-AS

008, 2004, available from the Measurements for Biotechnology website). That a material has the correct secondary structure is usually assumed from the biological activity of the material. The biological activity can be impaired by multiple factors such as the presence of impurities and aggregate content. Measurement of aggregates is frequently carried out by size exclusion chromatography, although techniques such as analytical ultracentrifugation or light scattering may also be used.

4.5.3 Ligand and receptor binding assays

Most protein-based biotherapeutic products exert their biological function by binding to a receptor or another protein, either to activate it or to block activation by some other agonist. A number also require ligand binding (of which the heparin-binding proteins are probably the most widely distributed group). Another group of products are enzymes which operate by binding to and catalysing reactions upon small molecules, proteins or other biomolecules. If the molecule is not in the correct conformation with accessible active sites for such interactions, the biotherapeutic product will probably lack the desired biological activity. Whilst physicochemical methods, such as fluorescence titrations, surface-plasmon resonance and ELISA techniques can be used, their application to date has been best used for those which do not result in an “active” biological response (eg. monoclonal antibodies which block a response), rather than products which stimulate responses (eg. rhEPO, cytokines and growth factors). Generally, physicochemical methods can yield a measurable binding constant for a singly discrete process, whilst biological assays look at the activation of the whole pathway, for example, cell signalling events down-stream of the receptor binding event.

5 Choice of model system – Interferon-alpha

This project provides a case study of the development of a package of different assays to be applied for the quality control of a biopharmaceutical product, without constraints of commercial confidentiality. The rationale behind choosing interferon-alpha as a model system is outlined. Interferon-alphas (IFN- α) are currently one of the top selling biopharmaceutical drugs: as a result there has been extensive research into the gene sequence and variants, and the protein crystal structure. This information can

be correlated to clinical experience. European Pharmacopoeial (EP) monographs are designated to be appropriate to the needs of regulatory authorities, those engaged in the control of quality, and manufacturers of biopharmaceutical products. The EP and the European Directorate for the Quality of Medicines (EDQM) provide a set of reference methods and materials that can be readily accessed as a standard for the material produced in-house (4). This project reviewed late and early stage cell based bioassays that are well reported in the literature and in current usage, and identified the major factors that contributed to uncertainty in these assays. To assess the sensitivity and uncertainty in cell-based and physicochemical analysis, the consortium produced batches of IFN- α that differed in their molecular profile in an attempt to mimic the variation likely to be found between batches in biopharmaceutical production. Common chemical and biological post-translational modifications to proteins include glycosylation, oxidation of methionine residues, sulphation and deamidation, and N-terminal pyroglutamate formation. These can be caused by a range of factors, from the mode of production (culture vessel and control), choice of expression system, formulation and storage conditions. The aim of this experimental study is to evaluate both the sensitivity and uncertainty in techniques to monitor variability in biopharmaceutical products and to correlate the data to biological activity (potency). To achieve this aim the case study aimed to produce;

- A range of structural variants for the model biopharmaceutical, by producing material at NPL and engineering molecular variants or creating them by controlled modification.
- Rapid, precise and reproducible bioassays, developed at NIBSC, perhaps with desirable characteristics engineered into the cell line
- Rapid, precise and reproducible physicochemical assays
- Access to the appropriate receptor, either in the cells or expressed in soluble forms through recombinant technology

6 Clinical usage of Interferon-alpha

Interferons are proteins that elicit a virus-nonspecific anti-viral activity in immune cells, and have anti-proliferative and immunomodulatory activities that influence the metabolism, growth and differentiation of cells. As biopharmaceutical products, IFN- α 2a and α 2b are used in anti-viral treatment for Chronic hepatitis B and C and as a treatment for Chronic Myelogenous Leukemia and Hairy Cell Leukemia.

Recombinant interferon and interleukin products accounted for more than thirty percent of protein therapeutics in clinical studies in the last twenty years, with PEG-Intron A (pegylated IFN- α) from Schering Plough now in the top ten therapeutic proteins in terms of global sales (5). In the mid 1980's genetic engineering techniques enabled the production of a pure recombinant IFN- α in sufficient quantities for the market so that production of IFN- α by extraction from cultured white blood cells was replaced. Recombinant IFN- α was put on the market for the first time in 1986. An IFN- α called consensus (AlphaCon1), was created by Amgen after comparing the amino acid sequences of ten varieties of human IFN- α and by choosing, for each amino acid of the sequence, the most representative from the various IFN- α studied. Amgen licensed the product to InterMune in 2001 (6).

The therapeutic action of IFN- α is reviewed in an article published in Nature (7) that follows the therapeutic use of the molecule for the treatment of patients with hepatitis C from 1986 onwards. Once the hepatitis C virus (HCV) was identified and tests developed for its detection in serum, the mechanism of action for IFN activity was elucidated. IFN- α therapy leads to a rapid decline in HCV-RNA levels in serum, and long-term responses were marked by sustained loss of HCV RNA from the serum and liver and reduction in chronic infection. IFN- α has potent antiviral activity but does not act directly on the virus or replication complex. It acts by inducing IFN-stimulated genes (ISGs), which establish a non-virus specific antiviral state within the cell.

Circulating IFN- α binds to IFN cell-surface-receptor subunits, leading to their dimerization and the activation of the receptor associated Janus-activated kinase 1 (Jak1) and tyrosine kinase 2 (Tyk2). The activated kinases phosphorylate the signal transducer and activator of transcription proteins 1 and 2 (STAT1 and STAT2). The activated STAT1/2 complex is then translocated to the cell nucleus, where it combines with IFN-regulatory factor 9 (IRF-9) to form a complex that binds to IFN-stimulated response elements on cellular DNA, leading to the expression of the

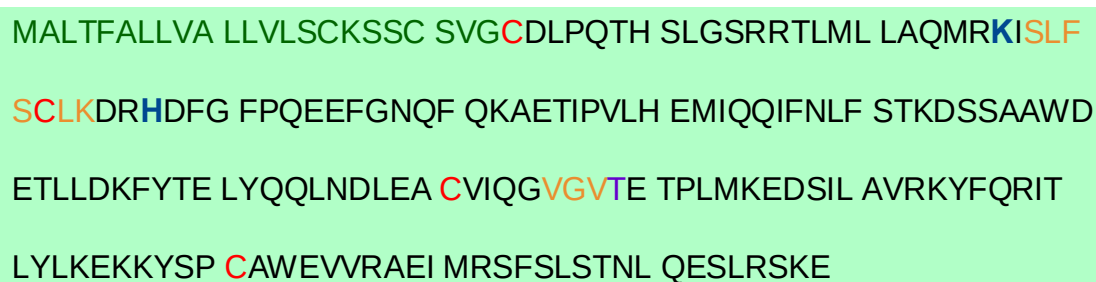
multiple ISGs. Microarray analyses show that hundreds of genes are induced by type-1 IFN, many related to antiviral activity but others involved in lipid metabolism, apoptosis, protein degradation and inflammatory cell responses. Recombinant IFN- α has been shown to bind to and activate cellular receptors, leading to the same response cascades that occur with endogenous production. This mechanism of action is mimicked *in vitro* by cell-based bioassays using cell that express receptors for type-1 interferons, such as Daudi cells.

Conjugation of the polymer polyethylene glycol (PEG) to proteins can significantly decrease their clearance from plasma, increasing their half-lives *in vivo*. This approach has been used to design cytokine products that can be administered to patients once a week, rather than on a daily or alternate-day schedule, and has been successful for PEG-IFN- α 2. The chemistry of attachment of PEG molecules must be considered as it may lead to loss of protein specific activity. Lysine amino groups of proteins are often used as substrates for PEGylation reactions because they are highly reactive and the conjugation reaction is mild enough not to damage the protein structure. However the reaction is non-specific and occurs predominantly at the N-terminus as well as internal lysine residues, which may be near active sites of the protein. The positional isomers of the protein with PEG at various sites can have distinct activities and lead to inconsistent therapeutic effects (8). Attempts to use site-directed PEGylation reactions have been successful in a number of biopharmaceutical products.

The pharmacokinetics of PEGylated IFN are greatly improved compared to standard IFN, where injections can lead to peaks and dips in blood concentrations. Clearance of PEGylated IFN is seven times slower and its administration is followed by a constant blood level concentration for a number of days. The use of PEGylated IFN means the number of weekly injections can be reduced (a single injection instead of three) and efficacy of the drug is improved, in particular for hepatitis C (due to the rapidity of division of the HCV) (6). More recently, therapeutic regimes include an antiviral agent called Ribavirin as a combination of a chemical and biopharmaceutical treatment for hepatitis C.

7 Description of the protein

In humans, viral infection triggers the production of IFN by the cell. The activating signal is the double-stranded RNA which appears during the viral replication cycle, whether it is an RNA virus or DNA virus. Some viruses are better inducers of IFN than others, by multiplying slowly and not disturbing protein synthesis of the host cell, with IFN synthesis lasting from 24 to 48 hours. IFN is processed in the endoplasmic reticulum and Golgi apparatus of the cell, where it is glycosylated and then secreted (6). The human IFN- α proteins are encoded by a family of over twenty genes that are clustered on human chromosome 9, and share 85-98 % sequence identity at the amino acid level. They are produced by peripheral blood leucocytes, lymphoblastoid and myeloblastoid cell lines and the natural genetic diversity of human IFN is a result of DNA “shuffling” of the IFN- α genes. A total of 28 different sequence variants have been described in the scientific literature, variants differing from each other in one to four amino acid positions. Many of the IFN- α gene variants have been cloned and sequenced from transformed cell lines and they can present mutations not present in the normal leucocyte genome (9) (10). IFN- α 2 protein consists of 165 amino acids and contains four conserved cysteine residues which form two disulphide bonds. The predicted molecular weight of IFN- α 2a from the amino acid sequence is 19,241 Da.



MALTFALLVA LLVLSCKSSC SVGCDLPQTH SLGSRRTLML LAQMRKISLF
 SCLKDRHDFG FPQEEFGNQF QKAETIPVLH EMIQQIFNLF STKDSSAAWD
 ETLLDKFYTE LYQQLNDLEA CVIQGVGVTE TPLMKEDSIL AVRKYFQRIT
 LYLKEKKYSP CAWEVWRAEI MRSFSLSTNL QESLRSKE

Figure 3. Amino acid sequence of human interferon- α 2. UniProtKB/Swiss-Prot entry P01563.

The signal sequence is depicted in dark green in the figure above, cysteine occurs at the N-terminus of natural secreted human interferon- α 2 and at three other positions in the protein (red residues), to form two intra-chain disulphide bonds. Three variants of the molecule exist and differ in the amino acid at positions 23 and 34 (blue residues), where interferon- α 2a contains K23 and H34, interferon- α 2b contains R23 and H34, and interferon- α 2c contains R23 and R34. The turns in the three dimensional structure

are shown in yellow. A number of studies have noted size and charge heterogeneity of human leucocyte IFN by SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE) and isoelectric focusing, which has been attributed to glycosylation (11). The sequence does not contain an *N*-glycosylation motif, but natural IFN- α 2 does carry an *O*-linked carbohydrate chain (purple residue). Sequence analysis of the protein by proteolytic cleavage followed by separation and analysis of peptide fragments has identified T106 as the *O*-glycosylation site. Therapeutic preparations of IFN- α 2a have been produced in *E.coli*, a prokaryotic organism that does not have glycosylation capabilities, which may lead to a difference in the biological activity of the molecule. However, in anti-viral bioassays the natural and recombinant interferons have not shown differences in their *in vitro* biological activity (12). In other bioassay formats however, such as those based on inhibition of cell proliferation, interferons from different expression systems have exhibited differences in potency (13). The molecule adopts a predominantly α -helical structure as determined by circular dichroism. Human IFN- α has 66% α -helix, 15% β -sheet (14) and is structurally similar to other cytokines in the “four-helical bundle” family (15). Nuclear magnetic resonance (NMR) spectroscopy of IFN- α in solution has identified four stretches of flexibility in the molecule, Cys1-Ser8, Gly44-Ala50, Ile100-Lys112, and Ser160-Glu165 which may be important in receptor interactions. Site-directed mutagenesis studies indicate that Tyrosine and/or Isoleucine at position 86 and Tyrosine at position 90 are very important in the anti-proliferative activity of human IFN- α , and that hydrophobic residue(s) at position 86 may be important for the interaction of the molecule with its receptor. IFN- α is functional as a monomer.

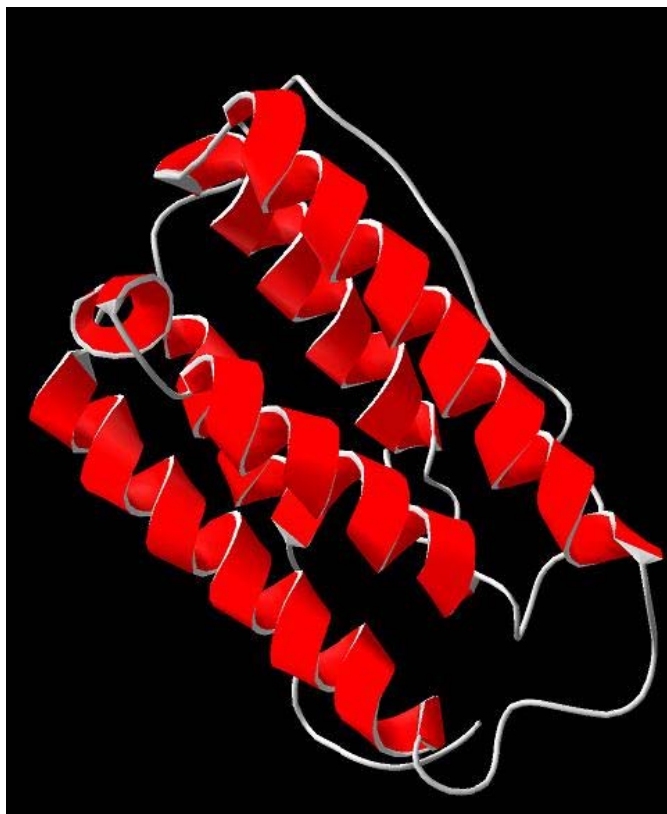


Figure 4. Model of interferon- α 2a, showing a predominantly α -helical structure (PDB code 1ITF).

The physicochemical tests carried out in this project referred to the available scientific literature in order to aid the interpretation of the structure-function information that we obtained.

7.1 Sources and variants of Interferon-alpha

There are numerous clinical grade preparations of IFN- α available on the market that are produced from natural or recombinant sources, including preparations from human, murine, rabbit and chick sources, that exhibit multiple molecular forms and biological activities. An international collaborative study was initiated by NIBSC and CBER to identify appropriate biological standards from the materials donated by companies and health organisations worldwide. The aims of the study were to assign potencies for the preparations and assess the influence of different bioassay systems on the estimates of potency (16). Titrations were performed on up to fifteen different IFN- α preparations and one IFN- ω preparation in a variety of assays, based upon the anti-viral, anti-proliferative, and other biological activities of IFN. There was an assay-dependent disparity between the relative activities of different IFN- α

preparations, even between similar IFN- α preparations, so that a single biological potency standard for all IFN- α subtypes and mixtures is inappropriate. Individual, homologous standards, are required for biological standardisation and potency determinations of individual IFN- α subtypes.

7.2 Production methodology

7.2.1 De novo production in *Pichia pastoris*

Pichia pastoris is a yeast cell expression system that is used to produce a number of biopharmaceutical products, and was chosen as the expression system for the material produced “in-house” at NPL for this case study. The cell line used to produce material for this study was the generous gift of Imperial College and was used solely for academic research. It is a methylotrophic yeast that has the capability to use methanol as the only source of carbon due to two genes *AOX1* and *AOX2* which code for the alcohol oxidase enzyme. The metabolism of methanol consists of oxidation of methanol to formaldehyde by the enzyme alcohol oxidase. This reaction takes place in the peroxisome where it generates both formaldehyde and hydrogen peroxide that is toxic for the yeast cell. Alcohol oxidase has a low affinity for oxygen so *Pichia pastoris* compensates for this by generating large amounts of the enzyme. The expression of the *AOX1* gene is controlled by a repression/ derepression mechanism; the presence of glucose represses transcription, even in the presence of the inducer methanol. One advantage of using this expression system is that *Pichia pastoris* can secrete protein into the growth medium. When using plasmids such as pPICZ α , the protein of interest is expressed as a fusion protein containing the secretion signal of the α -mating factor from *Saccharomyces cerevisiae* (Baker's yeast). It is induced by the addition of methanol to the culture medium. The α -factor sequence is processed in *Pichia pastoris* in two steps:

- The preliminary cleavage of the signal sequence by the *KEX2* gene product, with the final *KEX2* cleavage occurring between arginine and glutamine in the sequence Glu-Lys-Arg * Glu-Ala-Glu-Ala, where * is the site of cleavage.
- The Glu-Ala repeats are further cleaved by the *STE13* gene product.

The cleavage of the α -factor sequence is more efficient when the *KEX 2* cleavage sequence is followed by Glu-Ala repeats; also a number of amino acids are tolerated at site X instead of Glu in the sequence Glu-Lys-Arg-X. In some case the cleavage of the extra Glu- Ala repeats by *STE13* gene product is not efficient and leads to the presence of this extra amino acids at the N-terminus of the protein. The cleavage and the secretion of the mature protein into the media may vary from protein to protein. Small proteins are preferentially secreted compared to large molecules, but no major change in secretion efficiency was found for IFN in the presence or absence of Glu-Ala repeats (17). The construct used in this project was designed without the Glu-Ala repeats, the KEX2 cleavage site was cloned just in front of the mature IFN- α 2 sequence.

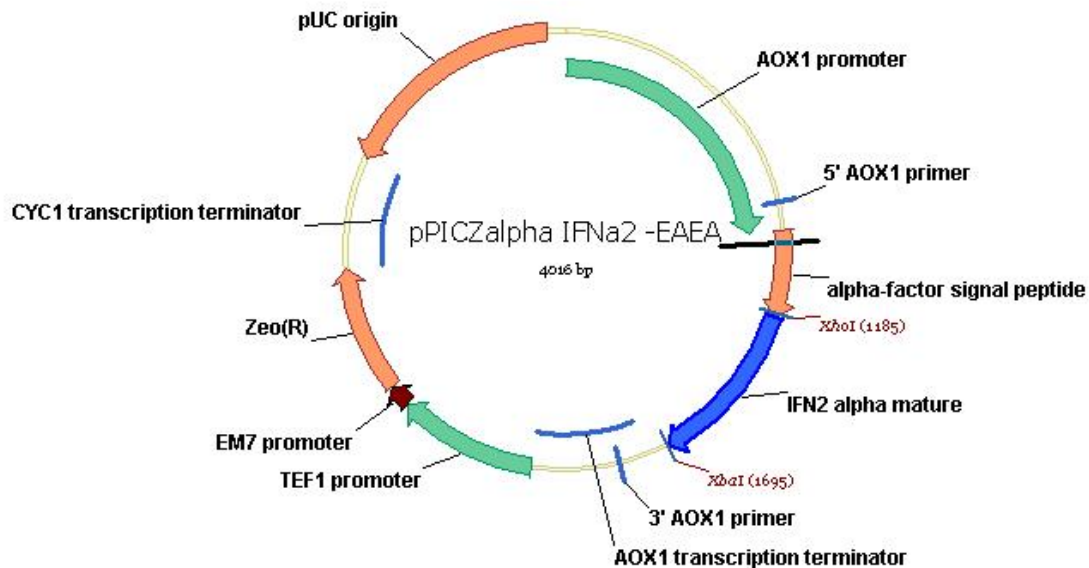


Figure 5. Vector for the expression of IFN- α 2 in *Pichia pastoris*.

7.2.2 Materials and methods

The IFN- α 2b cDNA was obtained from an in-house plasmid and cloned into the pPICZ α vector. The ligation product was transformed into JM109 competent cells after an overnight incubation at 37 °C. Four colonies were selected and grown in 2 mL of Luria Broth (LB) containing 100 mg/mL of Zeocin. Plasmids were prepared with the FastPlasmid® Mini kit (Eppendorf). Sequencing reactions were outsourced

to MWG (Germany), all four clones were sequenced using the alpha-factor (5'-TACTATTGCCAGCATTGCTGC-3') forward primer and 3AOX (5'-GCAAATGGCATTCTGACATCC-3') reverse primer.

Transformation and selection of the *Pichia pastoris* clone: A large plasmid preparation was performed from clone 1 using the PerfectPrep® Plasmid Midi kit (Eppendorf). The plasmid was linearised by PmeI digestion overnight at 37°C. The linear plasmid was transformed by electroporation into fresh X33 *Pichia pastoris* competent cells which were plated on YPDS agar plate containing 100 mg/mL of Zeocin. After three days of incubation, six colonies were grown in 5 mL of BMGY at 30 °C (180 rpm) for 72 hours. 1 mL of the BMGY culture was inoculated into 4 mL of BMMY and incubated at 30 °C (180 rpm) during 24 hours. Cells were harvested by centrifugation (3000xg, 20 min at 4 °C) and 10 mL of the supernatant from each culture was mixed with an equal volume of 2 x LDS sample buffer (Invitrogen) containing β -mercaptoethanol (5%), and heated at 95°C for 10 mins. Cooled samples were centrifuged (2 min, 16000xg, Eppendorf 5415 D centrifuge) and 10 μ L from each was analysed on a 12% Novex Bis-Tris gel with MES buffer (Invitrogen). The gel was stained using ProtoBlue Safe Colloidal Coomassie G-250 (National Diagnostics), according to the manufacturers instructions.

Mutagenesis of the IFN- α 2b cDNA to obtain IFN- α 2a cDNA: The point mutation that leads an amino acid change in the protein sequence (K23R) is due to a substitution of a guanine to an adenine at position 137 on the gene sequence (see appendix for sequence). The mutagenesis reaction used two mutagenic primers (sense: 5'-GCTCCTGGCACAGATGAGGAAAATCTCTCTTTCTCC-3' and anti-sense: 5'-GGAGAAAAGAGAGATTTTCCTCATCTGTGCCAGGAGC-3') purchased from Eurogentec. Mutagenesis was carried out with a QuickChange II site-directed Mutagenesis kit (Stratagene). The products of mutagenesis were transformed into JM109 competent cells and transformed and selected as described above.

7.2.3 Production of Interferon-alpha 2 batches in flask and fermenter

Five batches of IFN- α 2 were produced in different production systems to increase the chance of obtaining variability in the molecular profile of the material. IFN001 was a development batch to optimise the production system.

Table 6. List of IFN α 2 batches produced at NPL.

Batch Name	Interferon type	Expression system	Culture vessel
IFN001	IFN- α 2b	<i>Pichia pastoris</i>	Shaker flask
IFN002	IFN- α 2b	<i>Pichia pastoris</i>	Fermenter
IFN003	IFN- α 2b	<i>Pichia pastoris</i>	Shaker flask
IFN004	IFN- α 2a	<i>Pichia pastoris</i>	Fermenter
IFN005	IFN- α 2a	<i>Pichia pastoris</i>	Shaker flask

For the large-scale (fermenter) production of IFN- α 2 material, a BioStat B Plus system (Sartorius) was used and controlled by MFCS-DA software. pH, Dissolved Oxygen (DO) percentage, liquid level and foam formation were monitored during production. The pH, temperature and sparge rate were kept constant at 5.8, 30 °C and 2 L/min, respectively. The impeller speed was constant (400 rpm) during the biomass phase and adjusted to balance the amount of Dissolved Oxygen (DO) after methanol induction. The inoculum was prepared from 500 μ L of a glycerol stock of cells inoculated into 50 mL BMGY broth for 36 hours at 30 °C, 200 rpm (orbital 50 mm). 10mL of the pre-culture was then inoculated into 200 mL of BMGY broth and incubated at 30 °C for 36 hours at 200 rpm. 100 mL of the second culture inoculated the fermenter containing 1L of BMGY medium/ 0.1 % (v/v) of antifoam A reagent (Sigma). After inoculation, the biomass phase was maintained by the addition of 40 g/L of glycerol, 1x YNB, 1x Phosphate Buffer, 2 mL of 500x Biotin and 0.1 % (v/v) of antifoam A reagent (Sigma). After inoculation, we initiated a slow glycerol feed phase of 2.5 mL/h/L of 50% glycerol (w/v), supplemented with 12mL/L PTM solution. During the initial growth phase, the DO percentage was decreased from a starting point of 100% to 30%; at this stage the glycerol feed was increased to 18.5 mL/h/L. Induction of IFN- α 2 production was initiated when a DO peak appeared that is the result of a decrease in yeast growth when the glycerol has been consumed. After a short period of starvation, the induction was started by addition of pure

methanol. Fermentation was stopped after 36 hours and harvested by centrifugation at 4000 rpm for 30 min, followed by filtration through a 0.22- μ m membrane.

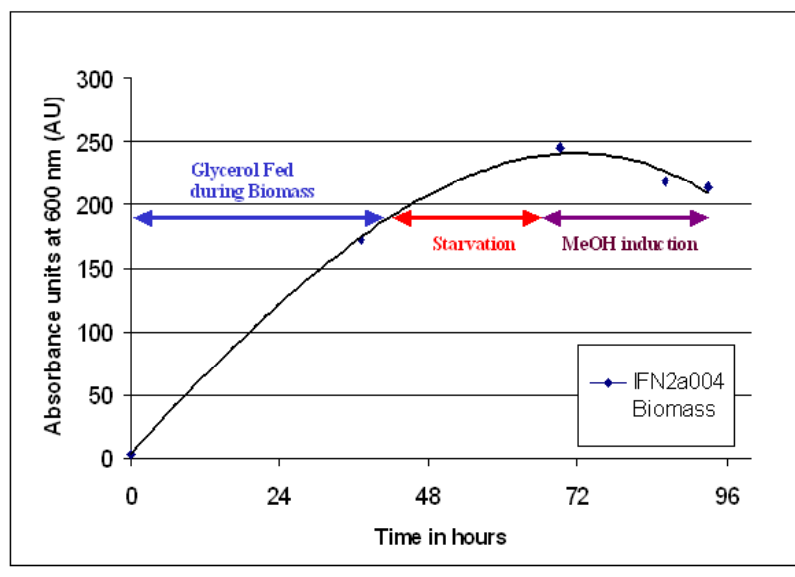


Figure 6. Example of Biomass measurement during production of a batch of IFN α 2. Exponential growth phase of yeast biomass during the glycerol feed step followed by induction of IFN α 2 production by the addition of methanol.

For the small-scale (shaker flask) production of IFN- α 2 material, 1 L baffled flasks were used. To ensure sufficient aeration the volume of the culture was maintained at 30% of the total flask volume. From a glycerol stock of IFN- α 2b competent cells 50 μ L were spread on Yeast Peptone Dextrose Sorbitol (YPDS) agar containing 100 μ M of zeocin, and incubated for 48 hours at 30 °C. The largest colony was selected and grown in 5 mL of BMGY broth at 30 °C for 60 hours. 2 mL of this was used to inoculate 200 mL of BMGY broth, with a further culture at 30 °C for 72 h at 200 rpm (orbital 50 mm). The culture was harvested by centrifugation at 4000 rpm for 20 min, and the pellet was re-suspended into a sterile baffled flask containing 20 mL of BMMY broth for induction. After 24 hours of induction, cultures were harvested by centrifugation at 4000 rpm for 30 min, followed by filtration of the supernatant through a 0.22- μ m membrane. In order to reduce the volume the supernatant was concentrated using a TFT Labscale system (Millipore) composed of a manifold with three Pellicon XL Biomax membranes of 5KDa molecular weight cut-off (MWCO). The concentrated supernatant was dialysed overnight against 2 L of 25mM Tris-HCl pH 8.5 at +8 °C to prepare the clarified bulk for purification.

Slight variations in the operating conditions were required over the five batches in order to obtain sufficient number of cells for inoculum or to maintain pH and dissolved oxygen concentration of cultures.

7.2.4 Purification of IFN alpha-2

All purification steps were performed using an AKTA™ purifier (GE Healthcare). IFN- α 2 was purified by affinity chromatography on a 1mL HiTrapBlue column eluted with 25 mM of Tris buffer pH 8.5 containing 2 M NaCl. Fractions containing the protein were pooled and concentrated using a Vivaspin column 10KDa MWCO (Sartorius). The preparation was desalted with a P10 desalting column (GE Healthcare) with PBS buffer. This initial protocol was optimised during production of the five batches due to the presence of high molecular weight contaminants of unknown origin in batches IFN004 and IFN005 (18). The PBS used during purification and for the final sample buffer was prepared from 10x concentrated PBS solution (Cat # P5493, lot 025K5402 from Sigma) - this solution is Biotechnology Performance Certified and cell culture tested. When diluted to a 1X concentration, this product yielded a phosphate buffer concentration of 0.01M and a sodium chloride concentration of 0.154M, pH 7.4.

Table 7. Purification steps for IFN- α 2 produced at NPL

Batch Name	Purification strategy	Quantity of material after purification (mg)
IFN001	Affinity, desalt	1.7
IFN002	Affinity, desalt	89.0
IFN003	Affinity, desalt	75.0
IFN004	Affinity, IEX, HIC, Size exclusion	5.5
IFN005	Affinity, IEX, Size exclusion	8.2

The protein concentration for each batch produced at NPL was determined by absorbance at 280 nm on a Lambda 850 spectrometer (PerkinElmer) using a 1cm path length, low volume quartz cuvette and PBS solution as a blank. To calculate the

concentration of IFN- α 2, we used the molar extinction coefficient value of $17780 \text{ M}^{-1} \text{ cm}^{-1}$ and an absorbance value of $= 0.924$ (calculated using the amino acid sequence of IFN- α 2a from ExPASy, accession number P01563 and the method of Edelhoch (19) to calculate the extinction coefficient). The concentration was adjusted to 1 mg/mL and filtered through a $0.1 \text{ }\mu\text{m}$ Anotop filter (Whatman), and the concentration was checked by absorbance at 280 nm . $100 \text{ }\mu\text{L}$ aliquots were prepared into 1.5 mL Eppendorf tubes and labelled using an alpha-numerical nomenclature: IFNXXX, where $X = 3$ a digit number that referred to the batch number. Aliquots were stored at $-80 \text{ }^{\circ}\text{C}$ at NPL or sent in dry ice to NIBSC to perform cell-based bioassays.

7.2.5 Chemical modification of purified materials

Oxidation of methionine residues in IFN- α 2 is known to decrease its biological activity. In order to create a directed physicochemical change to the material used in this project, oxidation of methionine residues in EDQM IFN- α 2a material was carried out as described in the method recommended by the European Pharmacopoeia (20). Some modifications were made to obtain a larger amount of oxidised product, by extending the oxidation reaction to 3 hours, and $0.1 \text{ \%}$ TFA (trifluoroacetic acid) in chromatographic buffer A and $0.085 \text{ \%}$ TFA in buffer B was used to improve resolution of oxidised material. A control sample of EDQM IFN- α 2a was exposed to the same conditions as the modified material, without the oxidising reagent (hydrogen peroxide).

7.3 Physicochemical characterisation of IFN alpha-2

Quality control of a biopharmaceutical requires the use of an appropriate set of analytical techniques to assess relevant properties of the product. A range of physicochemical tests were carried out on material produced at NPL and on commercially available reference material (CRS – chemical reference substance) from the European Directorate for the Quality of Medicines (EDQM); IFN- α 2a (CRS I03200300) and IFN- α 2b (CRS I03200301). The reference materials are supplied as reference standards for specified physicochemical tests, as described in the EP monograph “Interferon alpha-2 concentrated solution” (1110). These techniques are focussed on gel electrophoresis and peptide mapping. This was used as a guide, as

well as some orthogonal techniques to prove extra product quality information on primary, secondary and tertiary structure.

7.3.1 Confirmation of gene sequence

Sequencing of the plasmids created in the project was outsourced to MWG (Germany). IFN- α 2a and α 2b variants were confirmed.

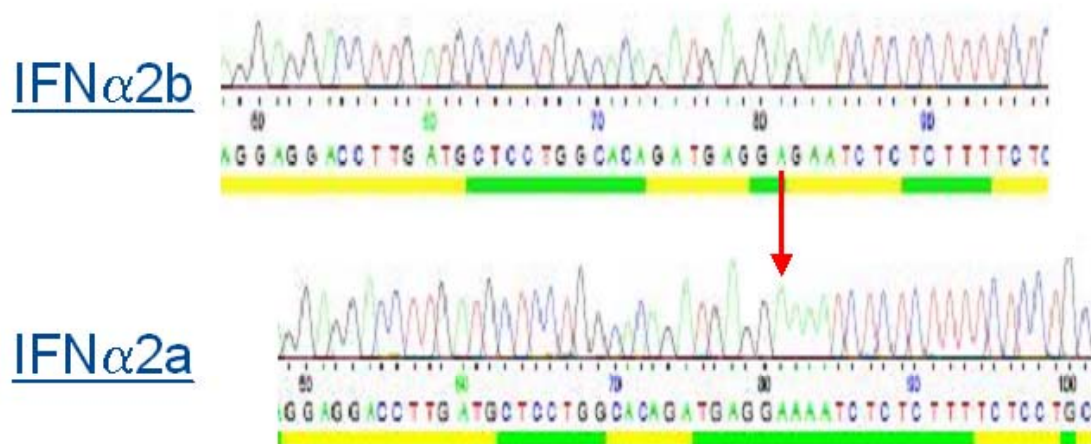


Figure 7.Forward sequencing results before and after mutagenesis, showing the substitution of Guanine by Adenine at position 137, this single point mutation leads to the amino acid change K23R.

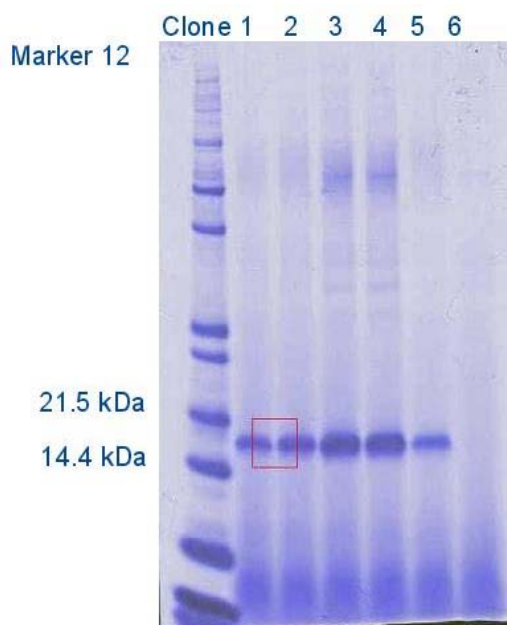


Figure 8. Screen of IFN α 2a clones. 10 μ L of culture supernatant after 24h of MeOH induction 4-12% reducing SDS-PAGE. Clones 1 to 5 showed the presence of protein between 14.4kDa and 21.5 kDa corresponding to the molecular weight of IFN α 2a. Clone 3 was selected for large scale expression.

7.3.2 pH measurement

The pH of the EDQM reference standards and NPL produced material was measured using a pH PocketFET, Sentron 501 pH meter, with an electrode suitable for small volumes (20 µL) and BDH indicator paper, pH 4-7 and 5-10.

Table 8. pH of the IFN- α 2 stock and diluted preparations used in the study.

Material	Concentration	pH measurement
EDQM IFN-α2a (CRS I0320300)	~1.46 mg/mL (stock) ~ 0.4 mg/mL (diluted <i>in-house</i> , in PBS)	pH 5 pH 6.4
EDQM IFN-α2b (CRS I0320301)	~7.19 mg/mL (stock) ~ 0.4 mg/mL (diluted <i>in-house</i> , in PBS)	pH 7 pH 7
IFN001, IFN- α 2b	14.2 µg/mL (diluted <i>in-house</i> , in PBS)	pH 7
IFN002, IFN- α 2b	35.9 µg/mL (diluted <i>in-house</i> , in PBS)	pH 7
IFN003, IFN- α 2b	35.9 µg/mL (diluted <i>in-house</i> , in PBS)	pH 7
IFN004, IFN- α 2a	35.9 µg/mL (diluted <i>in-house</i> , in PBS)	pH 7
IFN005, IFN- α 2a	1 mg/mL (stock) 35.9 µg/mL (diluted <i>in-house</i> , in PBS)	pH 7 pH 7.3
PBS (Gibco®, 14190)	-	pH 7

It is notable that the EDQM Chemical Reference Standards IFN- α 2a and IFN- α 2b are supplied at different protein concentrations and in different pH formulations. Information on the formulation components (buffer salts, excipients) were not supplied and not available at time of purchase.

7.3.3 SDS-PAGE

Purified IFN- α 2 material produced at NPL was compared to EDQM reference material using SDS polyacrylamide gel electrophoresis (SDS-PAGE) to estimate the size (molecular weight) of the main isoform, any high molecular weight species (such as aggregates) and any degradation products. 2 μ g of material was loaded into each lane and detection of resolved protein bands was by colloidal Coomassie Blue staining. Stained gels were scanned using a BioRad GS800 densitometer and percentage areas for the product bands were calculated.

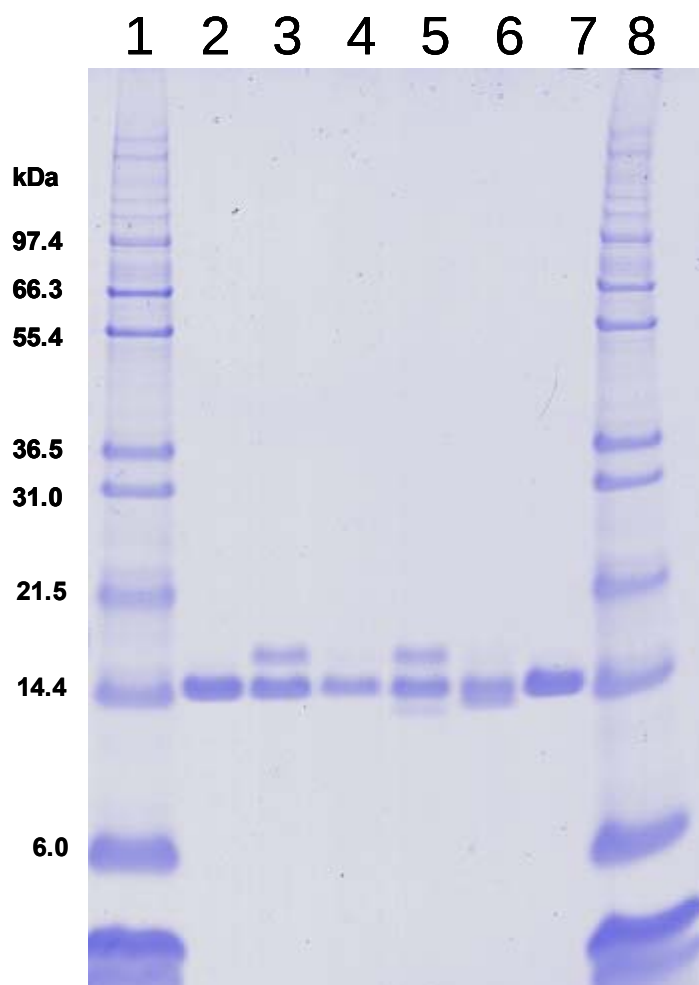


Figure 9. 4-12% reducing SDS-PAGE of IFN- α 2 batches. Lanes 1 and 8 are the molecular weight ladder Marker 12 (Invitrogen), EDQM IFN- α 2b (lane 2), IFN002 (lane 3), IFN003 (lane 4), IFN004 (lane 5), IFN005 (lane 6) and EDQM IFN- α 2a (lane 7).

The EDQM reference material exhibits one major isoform at the expected molecular weight for IFN- α 2a and α 2b (~19kDa). The material produced at NPL showed heterogeneity between batches, most noticeable in fermenter batches IFN002 and IFN004 which showed an additional band at a higher molecular weight, which

amounted to approximately 26 % and 21 % of the total product bands respectively. Some minor lower molecular weight bands are also visible in batches IFN004 and IFN005. In order to investigate the identity of the extra bands, amino acid sequencing was carried out.

7.3.4 N-terminal amino acid sequencing

N-terminal sequencing was carried out on the protein by excising bands from SDS-PAGE resolved material and conducting five cycles of Edman sequencing on the eluted material. This work was contracted out to the University of Birmingham. The additional higher molecular weight bands (lanes 3 and 5, Figure 9) compared to the EDQM reference material correspond to an uncompleted processing of the α -factor signal peptide that is placed upstream of the Kex2 cleavage site in the expression vector (Figure 5). This is a reported processing event of the Invitrogen *Pichia* expression system, when another upstream site is cleaved in addition to the correct signal cleavage site (21). The additional peptide sequence (-EEGVSLEKR) is indicative of this processing event and occurred in batches produced in the fermenter. The presence of the peptide sequence adds a molecular weight of 1046 Da to the mass of the protein which is resolved by the SDS-PAGE method.

Using the ExPASy (Expert Protein Analysis System) proteomics server of the Swiss Institute of Bioinformatics (SIB) for the analysis of protein sequences, it is also possible to predict the change to the pI and hydrophobicity (22) of the molecule due to the presence of the signal peptide. The grand average of hydropathicity index (GRAVY) can indicate the solubility of the protein: a positive GRAVY score indicates a hydrophobic protein, whilst a negative score indicates a hydrophilic protein. The aliphatic index of a protein is defined as the relative volume occupied by aliphatic side chains (alanine, valine, isoleucine, and leucine). It may be regarded as a positive factor for the increase of thermostability of globular proteins.

Table 9. Prediction of changes to IFN- α 2a due to the presence of the peptide signal sequence EEGVSLEKR

Property	IFN-α2a	IFN-α2a + peptide signal
Mw (Da)	19241.1	20269.2
PI	5.99	5.73
GRAVY	-0.336	-0.388
Aliphatic index	85.70	85.17

Theoretical predictions of how the peptide signal alters the property of the sample suggests that the fermenter batches (IFN002 and IFN004) have a different molecular profile which will include a population of larger, more hydrophilic protein species. Orthogonal physicochemical methods were used to confirm the extent of the heterogeneity and to investigate any relationship to a change in biological activity of the IFN- α 2 batches relative to the EDQM reference material.

7.3.5 Mass Spectrometry (MS)

Mass spectrometry was carried out at NIBSC on all IFN- α 2 materials produced at NPL and EDQM IFN- α 2 reference material. Liquid chromatography (LC, reverse phase C18) coupled with electrospray ionisation ion trap tandem mass spectrometry (MS/MS) was used for protein identification using peptide sequencing, and Matrix Assisted Laser Desorption Ionization Time-of-flight (MALDI-TOF) mass spectrometry was used in linear mode for intact protein mass analysis and reflectron mode for protein identification using peptide mass fingerprint.

A 12% reducing SDS-PAGE was performed for the IFN- α 2b NPL batches (IFN001, IFN002, IFN003) and EDQM IFN- α 2a, with 20 μ g total protein load. The gel was stained with Coomassie Blue and all bands were excised, digested with trypsin and extracted peptides subjected to mass spectrometry analysis, following the methods summarised in Figure 10. As more batches became available, the same methods of analysis were used for NPL batches IFN004, IFN005, EDQM IFN- α 2a and IFN- α 2b reference materials.

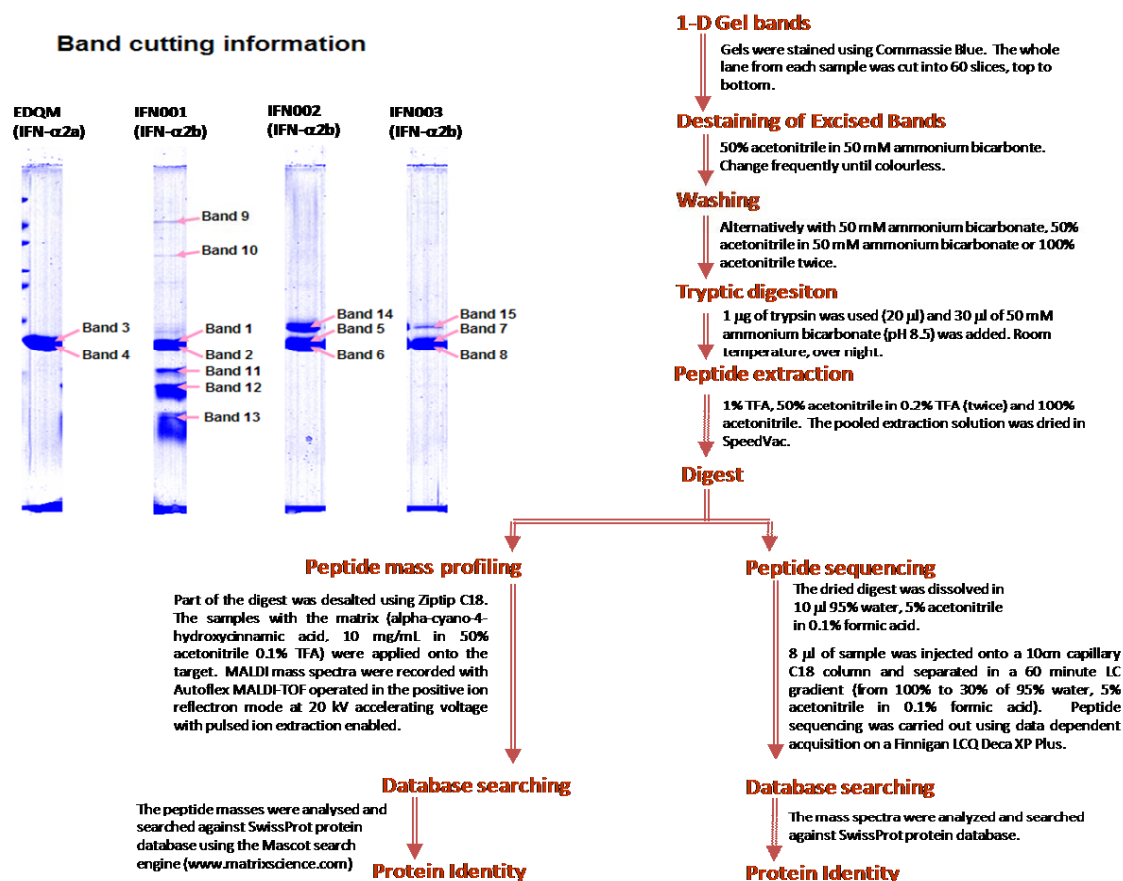


Figure 10. 12% reducing SDS-PAGE of IFN- α 2b NPL batches. All bands were excised for mass spectrometry analysis, for protein identification.

LC MS/MS and MALDI-TOF MS confirmed the identity of the major product bands as IFN- α 2. Results from both techniques have shown that peptide coverage was comparable for all of the bands at ~19 KDa. N-terminal amino acid sequencing confirmed that the higher molecular weight product bands (bands 14 and 15 in the figure above) were due to the presence of a peptide signal sequence. For IFN001 (particularly bands 12 and 13 in the figure above), less peptide coverage was achieved as some C-terminal peptides were missing. This indicated C-terminal fragmentation, resulting in lower mass isoforms of the product. The IFN001 batch was not extensively characterised as SDS-PAGE and early bioassay experiments indicated that there was significant product degradation and loss of biological activity at the start of the project, and its use was discontinued.

7.3.6 Circular dichroism

In order to examine the higher order structure of the IFN- α 2 material, CD spectra were acquired on JASCO spectrometers using cylindrical cuvettes of pathlength 0.01 cm (nominal) for the far-UV region (190-260 nm) and 0.2 cm for the near-UV region (240-360 nm). Prior to analysis, protein concentration was determined on a Lambda 850 UV spectrophotometer (Perkin Elmer). An absorbance value for IFN- α 2 of 0.924 was used, for the absorbance at 280nm of a 1mg/mL solution with a 1cm pathlength cuvette.

The sample concentration was adjusted to 1 mg/mL in PBS for both the far-UV and the near-UV analysis. A total of 3 spectra were acquired and averaged. After subtraction of the buffer spectrum the data were normalised and analysed using Spectra Manager™ software.

Far-UV data are normalised for the sample concentration and pathlength using the following equation;

$$\Delta\epsilon = \frac{MRW * \theta_{obs}}{3298 * 10 * d * c} \text{ M}^{-1} \text{ cm}^{-1}$$

Near-UV data were normalised using the following equation;

$$\Delta\epsilon = \frac{MR * \theta_{obs}}{3298 * 10 * d * c} \text{ M}^{-1} \text{ cm}^{-1}$$

MRW = mean residue molecular weight, *MW* = molecular weight, *d* = pathlength in cm, *c* = concentration in mg/mL.

The IFN- α 2 material produced at NPL exhibited spectra that were consistent with EDQM reference material in the far-UV wavelengths, which interrogate the arrangement of the peptide bonds in the protein to provide information on the amount of α -helix and β -sheet or random coil secondary structure in the protein molecule.

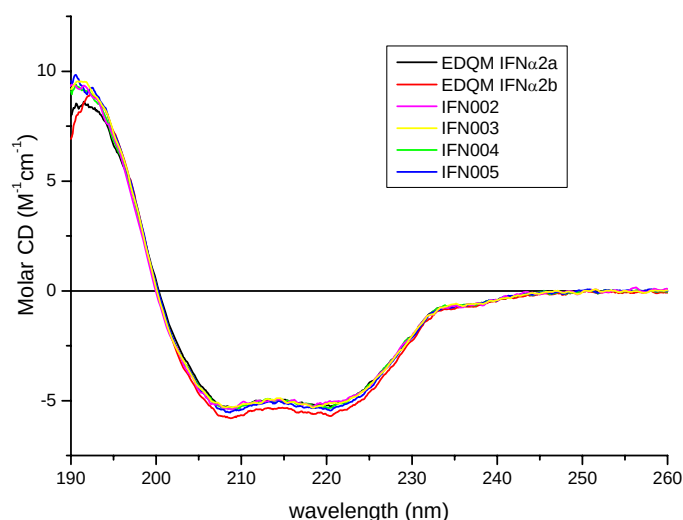


Figure 11. Far-UV spectra of IFN- α 2 material, indicating a predominantly α -helical secondary structure that is consistent between batches.

In the near-UV CD the signal arises from the aromatic amino acids and the disulphide bonds. The shape and intensity of the spectra is sensitive to the rigid tertiary environment these groups are in and represent a “fingerprint” that is characteristic for the particular protein (23) (24). For the near-UV CD spectrum we have identified a consistent difference in spectra between the α 2a and α 2b isoforms present. This difference could reflect the point mutation K23R but it could also be due to changes in pH: it has been reported that the near-UV CD spectrum of IFN- α is dependent on the pH (25), (26), (27). The pH of the working solutions was measured and showed that the stock solutions of IFN- α 2a and IFN- α 2b were different (pH 5 and pH 7 respectively - Table 8) which is the most likely explanation to the changes in spectra. A similar profile for the two materials is reported in other publications listed above. The working hypothesis is that the vicinity of two His residues (pKa approximately 6.0) to Phe36 and, especially, Tyr129 could determine a pH dependence of the near-UV CD spectrum (Figure 14).

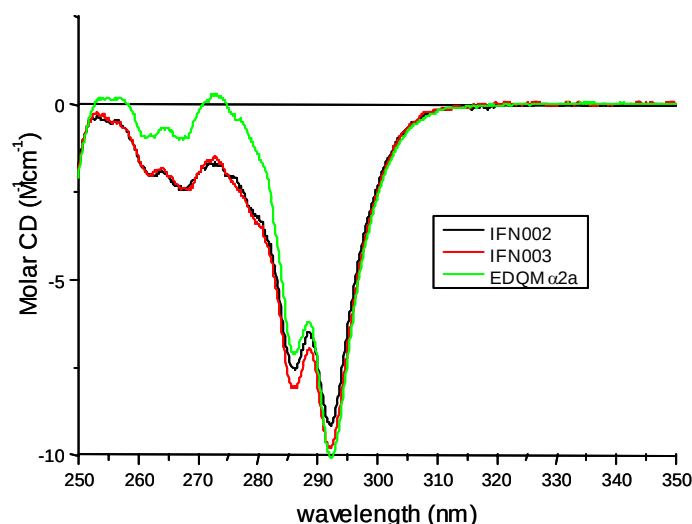


Figure 12. Near-UV CD spectrum of IFN- α 2a and -2b. NB – EDQM material supplied at 1.43mg/mL in unknown buffer (at pH5). IFN002 and IFN003 supplied at 1mg/mL in PBS.

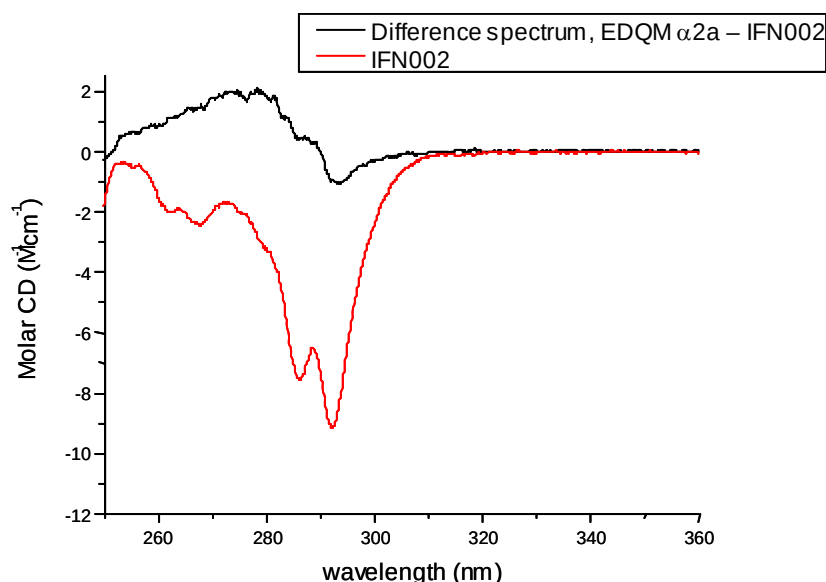


Figure 13. Difference spectrum, EDQM IFN- α 2a with the spectrum of IFN002 subtracted

The difference spectrum between EDQM IFN- α 2a and material produced at NPL was investigated, to confirm the likely structural changes between the two preparations. The shape of the difference spectrum shows no distinct peaks in the region below 285 nm which can reveal fine structure due to changes in phenylalanine and tyrosine residues. This supports the observation of a pH dependent change.

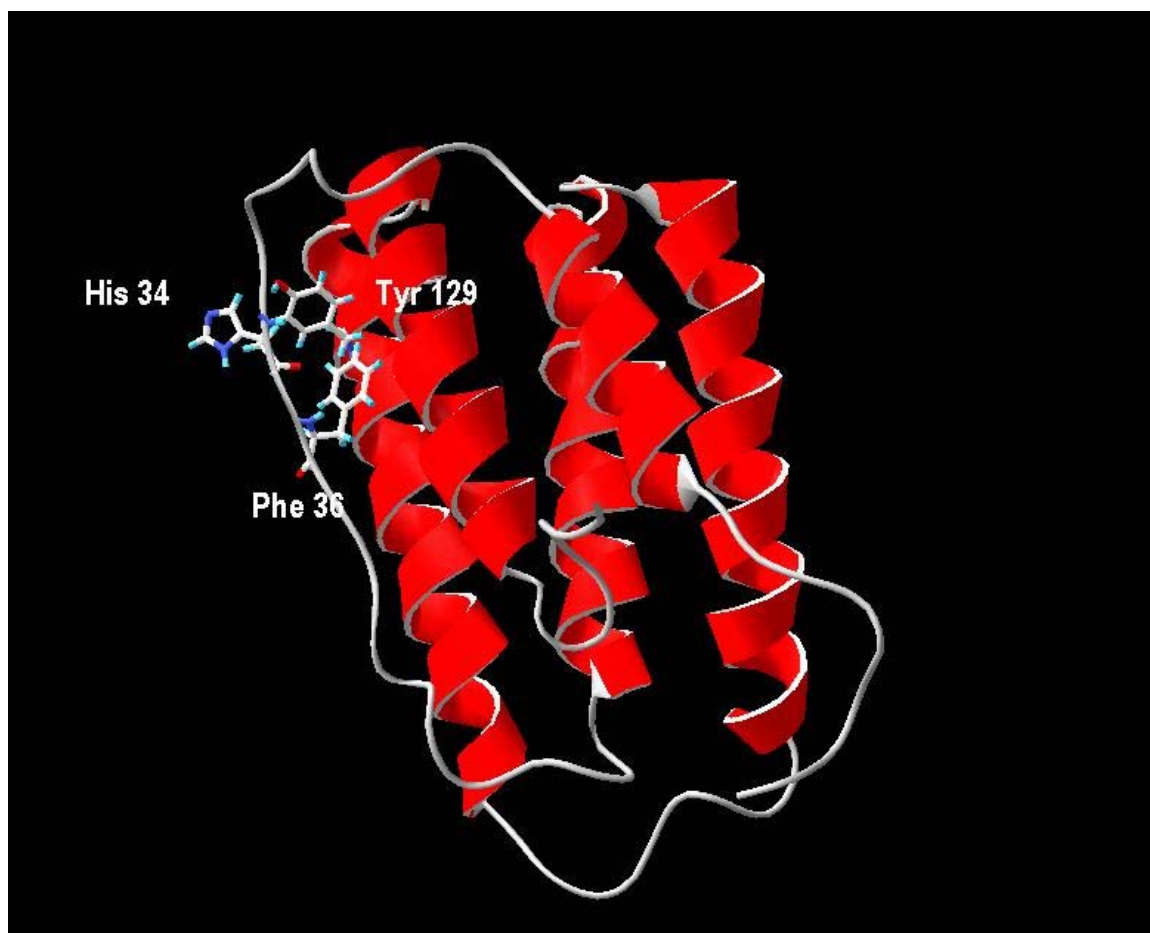


Figure 14. Protein database model (Pdb code: 1ITF) of IFN- α 2a: His 34, Phe 36 and Tyr129 are highlighted.

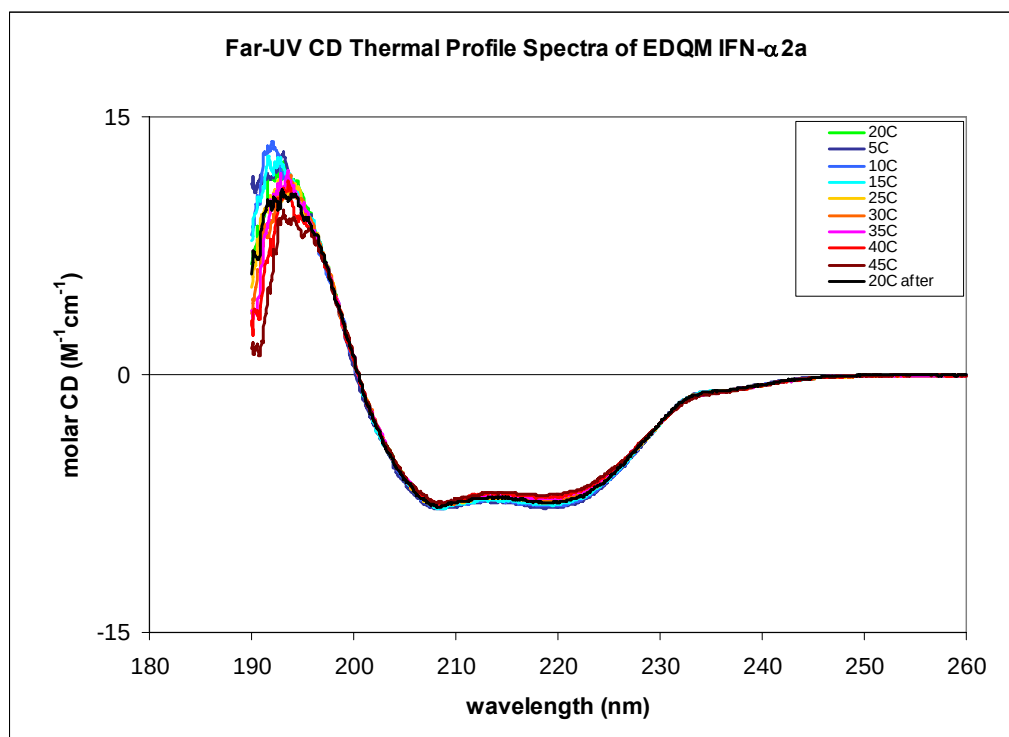


Figure 15. Far-UV CD thermal profile spectra of EDQM IFN- α 2a.

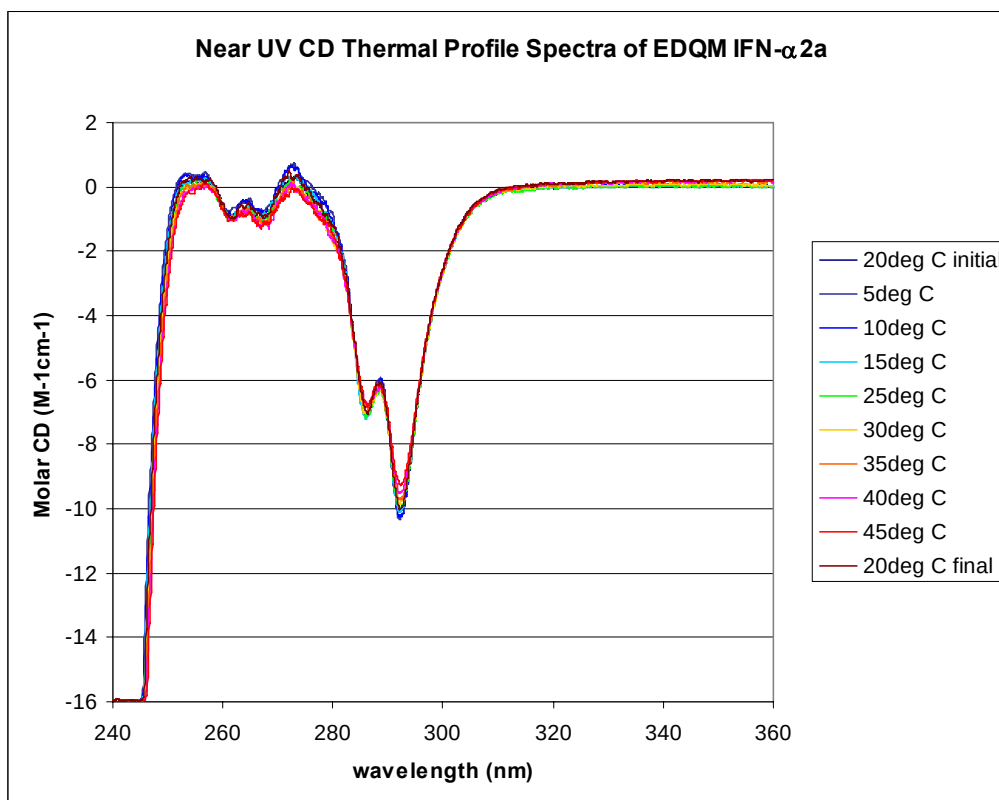


Figure 16. Near-UV CD thermal profile spectra of EDQM IFN- α 2a

Small changes to the secondary and tertiary structure of EDQM IFN- α 2a were observed between 5 to 45 °C, which were essentially reversible. This confirmed that there was flexibility in the operating temperature without significant change to the structure of the molecule (for example bioassays were carried out at 37°C whilst physicochemical assays were conducted at room temperature).

7.3.7 Steady-state tryptophan and ANS-fluorescence measurements

Tryptophan fluorescence gives an indication of the tertiary structure of the protein by revealing tryptophan solvent exposure, and information supplied by this method is complementary to that obtained from circular dichroism experiments. Tryptophan fluorescence analysis was performed on Perkin Elmer LS 55 equipment with the temperature maintained at 20 °C. The protein solutions were diluted to 0.1 mg/mL in PBS. In order to eliminate the contribution of other aromatic amino acids (tyrosine, phenylalanine) to the signal an excitation wavelength of 295 nm was used (excitation

slit width 10 nm). Data was acquired in the range 300-400 nm (emission slit 5 nm) using a scan speed of 50 nm/min. After buffer subtraction the data were displayed using Origin 7 software.

To correct for minor differences in protein concentration between the batches, the spectra were normalised using the maximum fluorescence value for each sample. When the spectra are normalised, shifts in wavelength (that indicate differences in tryptophan exposure and hence the tertiary structure) become easily visible. The reference and in-house materials show consistent peak maxima at 338 nm, leading to the conclusion that the tryptophan residues in all the batches are in a consistent, “buried” conformation, and that the tertiary structure is consistent across all of the batches as examined by this method.

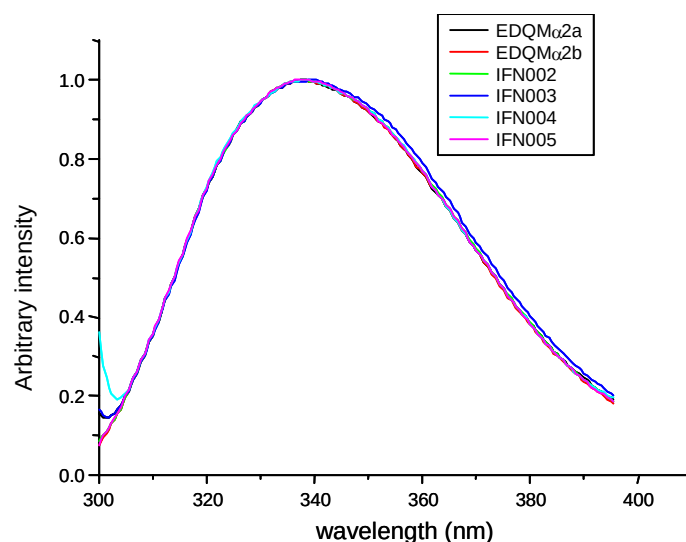


Figure 17. Tryptophan fluorescence (normalised for fluorescence intensity) for IFN-α2 reference and NPL material

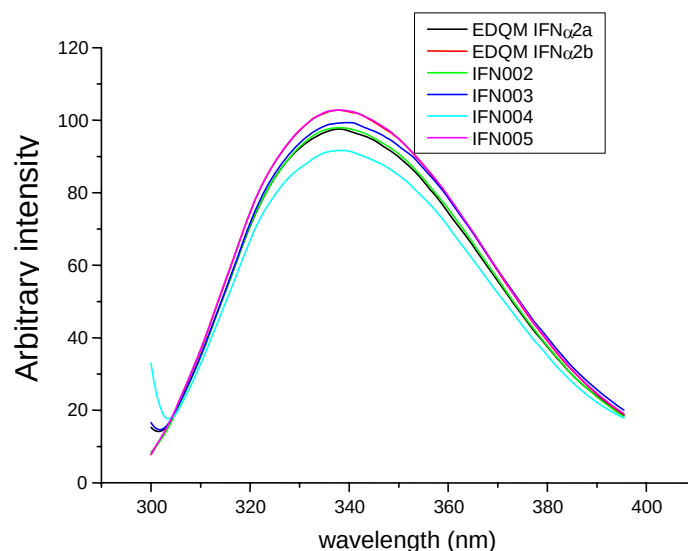


Figure 18. Tryptophan fluorescence (normalised for concentration) for IFN- α 2 reference and in-house material.

Another method for probing the tertiary structure of the protein is to conduct ANS (8-anilino-1-naphthalene sulfonate) fluorescent dye binding experiments. The dye binds to hydrophobic residues in the protein that are normally protected from the aqueous environment, and have been exposed when there is a lack of rigidity in the tertiary structure, such as the molten globule state (28) (29). The amount of fluorescence has been shown to correlate to the extent of unfolded protein. The protein samples were prepared by adding 20 μ l of a 40 μ M stock solution of ANS to the same solutions used for the intrinsic fluorescence measurements (molar ratio of dye to protein = 8:1). The fluorescence was measured after excitation at 380 nm (excitation slit 5 nm) and recorded in the range 400-600 nm (emission slit 10 nm).

ANS fluorescence can detect changes to molecules in change in two ways: one is a change in the peak intensity maximum (peak shift) and the other is the intensity. A change in intensity can be due to the interaction of the tryptophan residues with, for example, a quenching reagent which causes a shift of the peak towards the longer wavelengths. The spectra from these experiments indicate that batches IFN003 and IFN005 are different from batches IFN002 and IFN004 and the reference (Figure 19). The difference does not correspond to isoforms IFN- α 2a and IFN- α 2b, but may be a sensitive detection of differences in material produced in the flasks and fermenters. However, the ANS dye may bind to different regions of the protein if not in large

excess, as in this case. In addition, the composition of the EDQM sample buffers was unknown, and they may have contained molecules which quench ANS fluorescence, or other carrier molecules which may preferentially bind ANS. Therefore the utility of the method in this case study was limited.

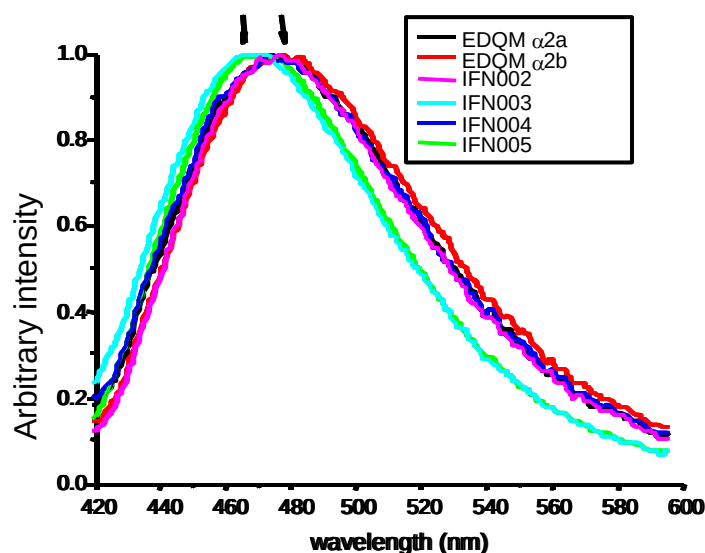


Figure 19. ANS fluorescence of IFN samples normalised by the maximum intensity

7.3.8 Reverse-phase HPLC

The RP-HPLC procedure recommended by the European Pharmacopoeia was used to analyse the batch consistency of recombinant IFN- α (20). Chromatographic analysis was carried out on a JASCO HPLC with PU-2080 Plus pumps equipped with a UV PDA detector (MD-2010 Plus), autosampler and column oven.

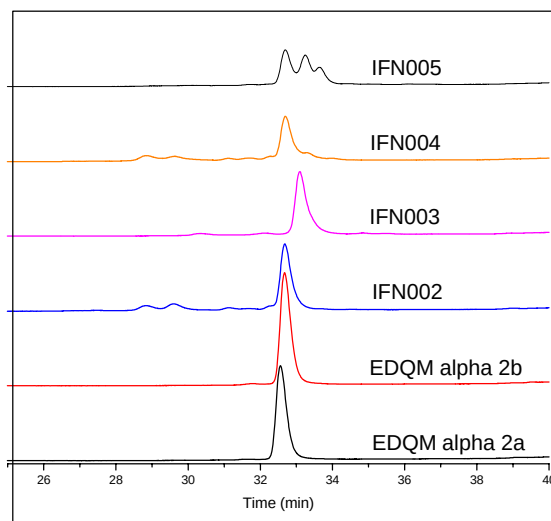


Figure 20. RP-HPLC of reference and NPL IFN- α 2.

The data reveals variation between batches which can be related to the production mode and the presence of the signal peptide that was incompletely cleaved, as identified by SDS-PAGE (section 7.3.3) and N-terminal amino acid sequencing (section 7.3.4). NPL IFN- α 2 batches that were produced in the fermenter (batches IFN002 and IFN004) contain minor variants that are less hydrophobic than the main isoform peak. This observed change in hydrophobicity is also supported by the predicted change as determined by an *in-silico* hydrophobicity plot. The trace for batch IFN005 reveals significant difference to the reference material, which requires further investigation.

Table 10. Comparison of bands identified on SDS-PAGE with RP-HPLC chromatograms.

Sample	Reducing SDS-PAGE		RP-HPLC		
	% main peak	% of HMW bands	% main peak	% of unknown peaks	% Oxidised products
<i>EDQM alpha 2a</i>	100.00	0.0	99.57	0.0	0.43
<i>EDQM alpha 2b</i>	100.00	0.0	99.32	0.0	0.68
<i>IFN002</i>	73.4	26.6	78.50	18.45	3.05
<i>IFN003</i>	100.0	0.0	96.37	0.0	3.63
<i>IFN004</i>	78.8	21.2	89.30	2.92	7.78
<i>IFN 005</i>	48.5	51.5 (mix of hmw & lmw)	43.44	55.87	0.68

Data from a study that subjected IFN- α 2 to site directed mutagenesis identified six “hotspots” in the molecule thought to be important in receptor IFNAR-2 binding, and therefore potency. These “hotspots” are on the AB loop and D-helix of IFN- α 2, containing the amino acids: L30, R33, R144, A145, M148 and R149 (30). Mutations to alanine (A) at these locations caused a decrease in binding affinity. Other methionine residues (M16 and M21) have been identified to be important for structural integrity and/or receptor binding after the crystallographic structure of IFN- α 2a was resolved (15). Methionine is an amino acid susceptible to oxidation, and is often the target of accelerated or forced degradation studies by formulation and quality control labs, who are concerned with the long-term storage and product quality for release of product. To confirm the effect of methionine oxidation on the physicochemical properties and potency of IFN- α 2, EDQM α 2a reference material was incubated with hydrogen peroxide (H₂O₂), following the protocol suggested by the European Pharmacopoeia (20). The untreated and oxidised sample were analysed

by reverse-phase HPLC to quantify the amount of oxidised product and to correlate this to a decrease in potency in two cell-based bioassays. The extent of oxidation was confirmed by the appearance of an earlier eluting peak in the RP-HPLC assay. The amount of oxidised material was estimated as 62.6 %, compared to the control sample (Figure 21).

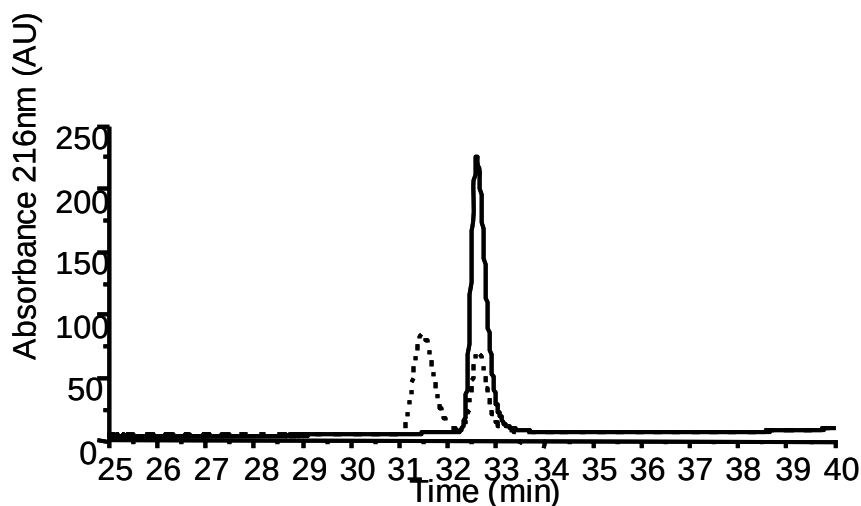


Figure 21. Reverse-phase HPLC trace of IFN- α 2a reference material (solid line) and oxidised material (dotted line)

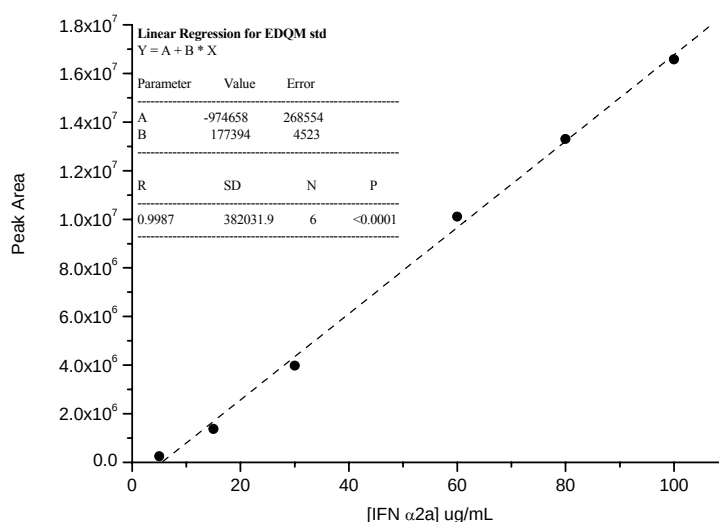


Figure 22. Calibration curve for the quantitation of oxidised IFN- α 2a

The assay is capable of detecting oxidised IFN- α 2a in our laboratory, the samples exposed to oxidising conditions were then tested for biological activity in the early and late-stage bioassays.

7.3.9 Size-exclusion chromatography and Dynamic Light Scattering

Size-exclusion chromatography (SEC-HPLC) and dynamic light scattering (DLS) are complementary techniques that examine the size of molecules in solution. SEC-HPLC can determine whether the molecules exist as a population of monomers, dimers or other oligomeric species, and may detect larger, covalently bound aggregates. However, during SEC-HPLC the samples are normally diluted which may cause non-covalently bound aggregates to disassemble. In addition, very large aggregates may be filtered out upon injection onto the HPLC column and escape detection.

DLS is very sensitive to trace amounts of very large aggregates and does not involve interaction with a chromatographic matrix. Concentrated protein solutions may be non-invasively monitored for both covalent and non-covalently bonded aggregate species. The resolution of DLS is lower than that of SEC-HPLC however, and cannot accurately distinguish between molecules differing by two or three-fold in hydrodynamic radius or up to twelve-fold in molecular weight. When taken together, the two techniques can provide valuable information on the species present in a biopharmaceutical product. Aggregation is of concern to the manufacturers of biopharmaceutical product as it may cause the loss of product during down-stream processing and long term storage, but more importantly aggregated molecules may display altered protein conformations upon administration to the patient, that may be immunogenic (31).

The SEC-HPLC analysis was carried out in PBS on a Phenomenex BioSep S2000 column, at 1 mL/min flow rate. 10 μ L injections of 1mg/mL sample solutions were interspersed with blank injections. The column was calibrated for molecular weight determination by injecting a 1mg/mL sample of myoglobin (17 KDa, RT= 8.45 min). The molecular weight of monomeric IFN- α 2a predicted by the amino acid sequence is 19,241 Da and 19,269 Da for IFN- α 2b. The chromatographic data (Figure 23) shows that the retention time of the major isoform present in all the batches (approximately 8.3 mins) is in the correct range for the molecular weight of monomeric IFN- α 2. The EDQM IFN- α 2a material contains species that are smaller in molecular weight than the monomer, but these are not detected in the SDS-PAGE or RP-HPLC analyses.

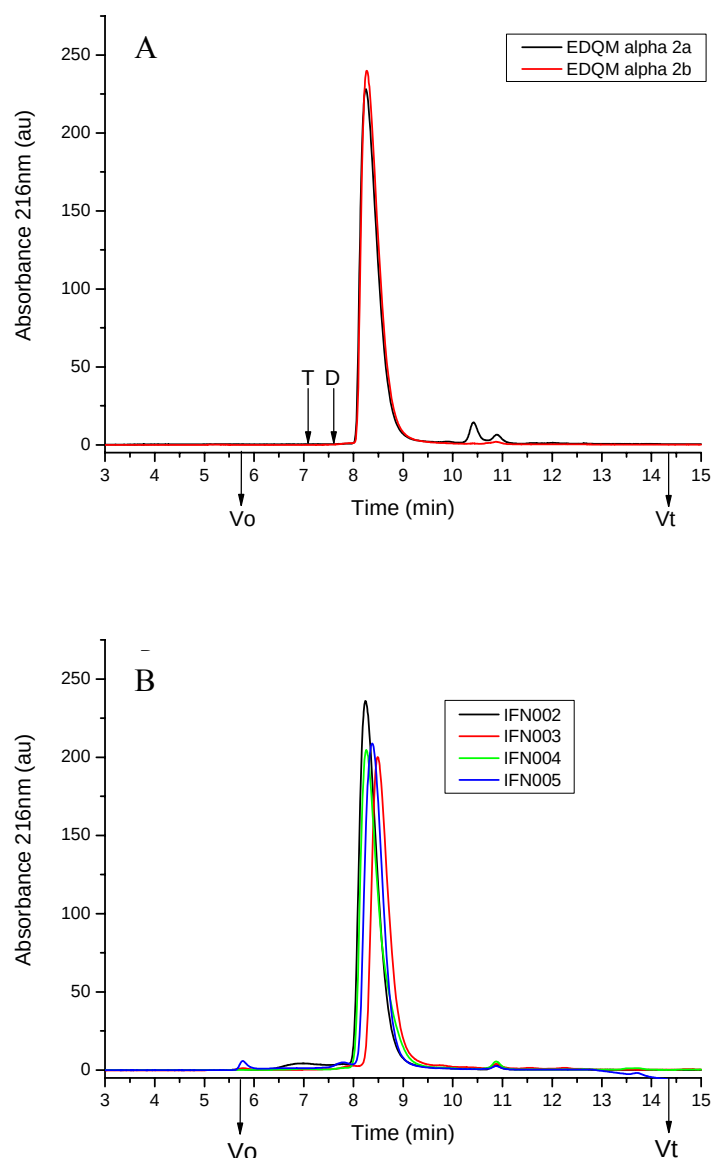


Figure 23. Overlaid SEC-HPLC chromatograms of EDQM reference material (A) and NPL material. The major isoforms are monomeric, aggregates are represented by peaks that elute before the main isoform, approximate elution positions for dimer (D) and trimer (T) are indicated. $V_o = 5.76$ mL, $V_t = 14.34$ mL.

The elution times from the Biosep-SEC-S2000 column suggest that IFN002 and IFN004 monomers are identical in molecular weight, whereas batches IFN003 and IFN005 are smaller. For the resolution of the column (2-5kDa in the IFN- α range), this data compares well to that obtained from SDS-PAGE (Figure 9) where batches IFN003 and IFN005 are resolved at a slightly lower molecular weight than the other batches. When comparing SEC data to the RP-HPLC data (Figure 20) the main isoforms of batches IFN002 and IFN004 have similar retention times whereas the

profiles for batches IFN003 and IFN005 differ. Taken together, the physicochemical data, despite arising from different separation modalities (size, hydrophobicity) highlight that there are consistent differences between the batches which warrants investigation of changes to biological activity. Integration of peaks eluting prior to the monomer show that there is less than 3% aggregate in each preparation (Table 11).

Table 11. SEC-HPLC analysis; retention times and % aggregate content in EDQM reference and in- house IFN- α 2a.

	RT (min)	Δ RT (ave)	Tot Impurities RT<main (%area)
IFN002	8.27	0.05	1.77
IFN003	8.52	0.29	1.32
IFN004	8.267	0.06	0.00
IFN005	8.39	0.18	2.67
EDQM a2a	8.25		0.00
EDQM a2b	8.27		0.00

Dynamic Light scattering (DLS) was carried out on a Nano-ZS (Malvern Instruments) in a low volume disposable cuvette at 25°C. The sample solutions, 1 mg/mL in PBS were analysed using the manufacturer's dispersion technology software. The hydrodynamic radius was estimated using the volume size distribution (multimodal analysis). The raw data, size distribution by intensity (Figure 24), revealed that the EDQM material differed from the NPL material in terms of the profile of large diameter species, and that all of the batches had a high polydispersity index indicative of the presence of particles heterogeneous in size (Table 12.). This may indicate differences in monomer, dimer or trimer content, although the resolution of DLS is not sufficient to determine this accurately. In order to account for the fact that large particles scatter more light, the distribution by volume for all the samples is shown in Figure 25, indicating that the samples consisted largely of the same size particle.

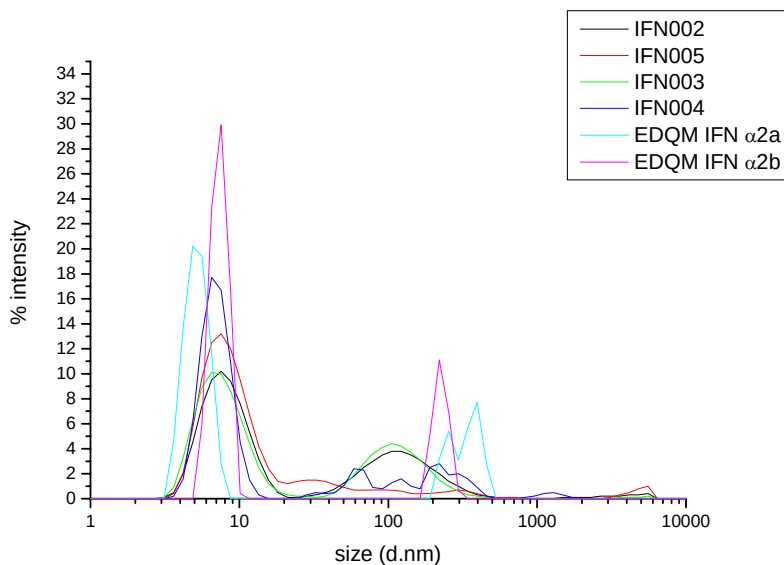


Figure 24. Size distribution by intensity. Material was filtered through 0.1um filters after production.

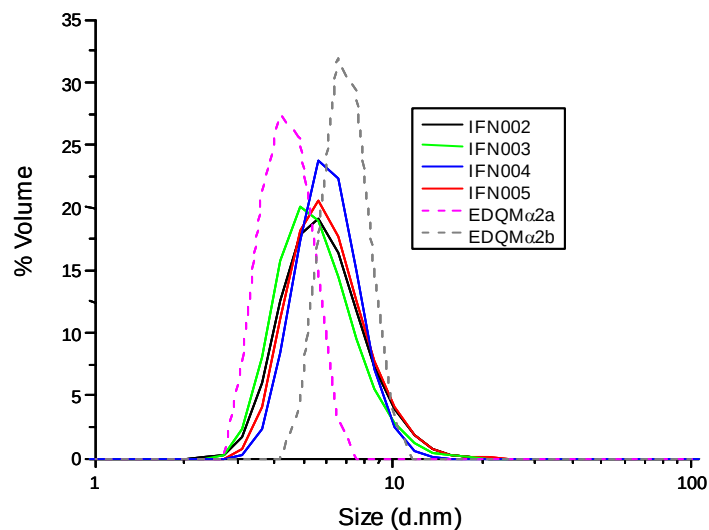


Figure 25. Dynamic light scattering data for reference and NPL IFN- α 2 material. (Size distribution by volume).

Table 12. Diameter and estimated molecular weight values from DLS analysis of IFN- α 2.

	Diameter (nm)	% Intensity	Polidispersity index
EDQM α 2a	4.50	100	0.37
EDQM α 2b	7.01	100	0.277
IFN002	6.11	99.9	0.5
IFN003	5.76	100	0.469
IFN004	6.20	100	0.317
IFN005	6.31	100	0.347

7.3.10 ELISA

An enzyme-linked immunosorbant assay (ELISA) was developed in order to investigate non-functional binding of IFN- α 2 to anti-human IFN- α antibody. Assays of this type are often used as an identity test and to quantify the molecule of interest. In this case study, the physicochemical binding event did not reflect the mechanism of action of IFN- α 2 binding to its receptor. 96-well plates (Corning 9018, high binding polystyrene) were coated with 50 μ L of a 80 μ g/mL solution of anti-human IFN- α clone AE3 in PBS (Hytest) and incubated overnight at 4°C, followed by blocking with Superblock TBS (Pierce). Between steps the plates were washed three times with 200 μ L of washing buffer (50 mM Tris-HCl, 0.15 M NaCl, 0.05% Tween pH 7.5). IFN- α standard curves (0-100 ng/mL), NPL material from each production batch and EDQM reference material were diluted in assay buffer (50 mM Tris-HCl, 1 mM MgCl_2 , pH 8.0) and incubated at room temperature for 30 min on a rotating shaker. 50 μ L of a 1 μ g/mL solution of biotinylated anti-human Mab (Endogen, clone 7N41) was added for 1h at room temperature, shaking followed by 50 μ L of a 1/500 diluted solution of streptavidin alkaline phosphatase conjugate for 10 min. 200 μ L of a 1mg/mL PNPP substrate solution was added to each well for 30 min at RT, followed by detection at 405 nm on a Perkin Elmer Victor plate reader.

The ELISA was developed to examine product binding to anti-human IFN- α , and to determine if methionine oxidation resulted in a decrease in binding, which may correlate to a change in secondary or tertiary structure. The oxidised IFN- α 2a showed a decrease in binding, suggesting that the anti-human IFN- α binds a conformational epitope that is altered by methionine oxidation.

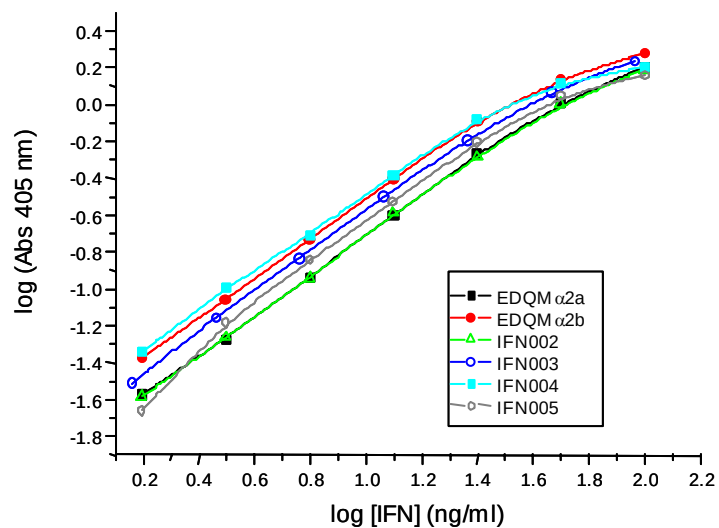


Figure 26. ELISA of IFN- α 2 reference and NPL material, showing binding of IFN- α 2 to anti-human IFN- α antibody.

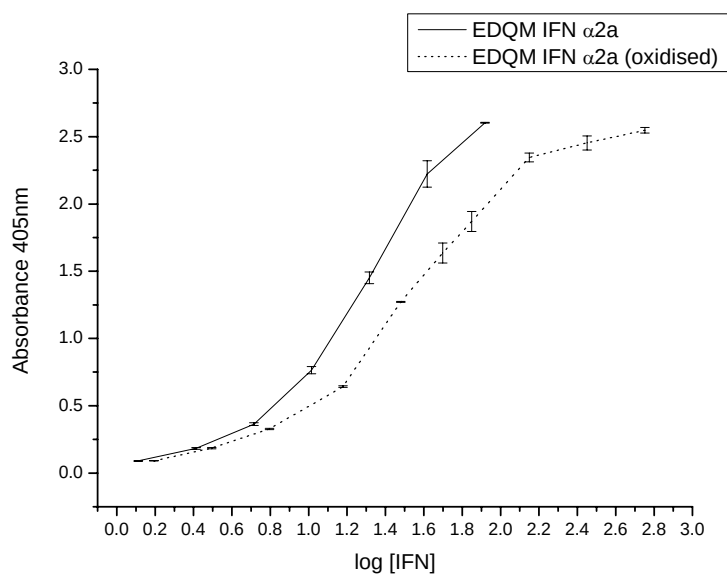


Figure 27. ELISA assay showing binding of EDQM α 2a (filled line) and oxidised EDQM α 2a (dashed line) to anti-human IFN- α antibody.

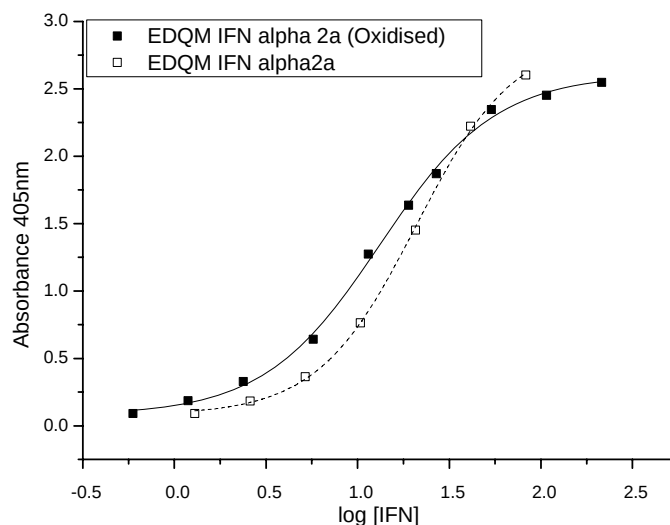


Figure 28. Binding of EDQM IFN- α 2a (filled symbols) and oxidised EDQM IFN- α 2a empty symbols) to anti-human IFN- α antibody, concentration normalised for amount of oxidised material.

The amount of oxidised material detected by RP-HPLC (62.6%) is not proportional to the reduction in binding to anti-human IFN- α antibody, for this example. Further method development would be required to determine the sensitivity and variability of the ELISA assay to IFN- α 2a containing different amounts of oxidised material.

7.3.11 Ellman assay for quantitation of free cysteine in product

A conserved feature of IFN- α 2 is the presence of intra-chain disulphide bonds between C1-C98 and C29-C138. Two of the domains thought to be involved in receptor binding are kept in proximity by the disulphide bond between C29 and C138. It has previously been demonstrated that mutation or modification of these cysteines results in a significant loss of biological activity (30). The quantitation of free cysteine (which can be correlated to unformed disulphide bonds) can be achieved by reaction with Ellman's assay reagent. Ellman's reagent (DTNB: 5,5'-dithio-bis(2-nitrobenzoic acid)) was prepared in Ellman's assay buffer (0.1 M NaH_2PO_4) at 4 mg/mL. The concentration of free cysteine can be calculated using a calibration curve of cysteine or by using the extinction coefficient of the Ellman's assay product, 2-nitro-5-mercaptobenzoic acid (TNB, extinction coefficient at 412 nm = $14,150 \text{ M}^{-1}\text{cm}^{-1}$). A cysteine standard curve was prepared in Ellman's assay buffer in the range 8.4 μM to

134.5 μM and measured after 15 mins incubation with the Ellman's reagent. The IFN batches and EDQM samples were diluted to a concentration of 26 μM , the concentration of TNB was measured after 15 mins incubation of the sample with the Ellman's reagent. In summary, the IFN- $\alpha 2\text{a}$ and $\alpha 2\text{b}$ samples produced by NPL or purchased from EDQM contained less than 5% free cysteine ($n=1$, data not shown) indicating that the disulphide bonds were correctly formed. The Ellman assay is a relatively imprecise method for determining disulphide bond formation, future work could utilise mass spectrometry to confirm correct positioning of the disulphide bonds.

7.3.12 Summary of physicochemical techniques

The physicochemical techniques used to monitor batch-to-batch variability in the material produced at NPL included electrophoresis, liquid chromatography, immunoassay and spectroscopic techniques that represent typical resources available to a quality control laboratory. Variability in the size and hydrophobicity of IFN- $\alpha 2$ material was detected by SDS-PAGE, SEC- HPLC and RP-HPLC, and were consistent with an extra peptide signal sequence being present in batches produced in the fermentor, rather than a point mutation (K23R) between IFN- $\alpha 2\text{a}$ and - $\alpha 2\text{b}$. The amino acid sequence change in the fermentor-produced batches was then confirmed by N-terminal sequencing. Oxidation of methionine residues was detected in each batch and in material exposed to accelerated stability conditions. The NPL materials separated on SDS-PAGE were identified as IFN- $\alpha 2$ using both peptide mass fingerprinting and sequencing mass spectrometry (MS) techniques, with high peptide sequence coverage. The mass of the intact protein was also confirmed with MALDI-TOF MS, which was similar to the SDS-PAGE estimation.

Circular dichroism highlighted a pH-dependent change to the molecule which had implications in the following stability study, and emphasised the importance of knowing as much as possible about the reference standard preparation if using it beyond the purposes for which it was intended. However, there was no detectable change to the secondary and tertiary structure of the molecule as a result of the peptide signal sequence being present in two of the batches, and the different isoforms IFN- $\alpha 2\text{a}$ and - $\alpha 2\text{b}$. In this case study, tryptophan steady state and ANS-fluorescence

were limited in their utility without further method development. The ELISA technique was utilised to confirm binding of IFN- α 2 to an anti-human IFN- α antibody, and served as an identity test as well as reflecting a change to the molecule caused by oxidation of methionine residues. The analysis was limited by not having routine access to a mass spectrometer at NPL, which would have aided characterisation of the disulphide bond arrangement and potential O-glycosylation profile of the material produced in *Pichia pastoris*. Glycosylated and non-glycosylated isoforms of the same cytokine have previously been distinguished in rigorously developed bioassays (16).

7.3.13 Stability study - physicochemical and potency assays

One of the most challenging tasks in the development of protein pharmaceuticals is to deal with physical and chemical instabilities of proteins. Quality control of a biopharmaceutical requires the use of an appropriate set of analytical techniques to assess relevant properties of the product over time (32). Establishing correlations and complementing data between different analytical techniques supports the selection of a suitable set of tests for each individual product. Biological assays are commonly used to measure potency together with selected physicochemical methods to monitor stability of biopharmaceutical products.

The purpose of the stability study was to create degradation products in IFN- α 2a and α 2b samples which may provide information on the structure-activity relationships for these therapeutic products. We have investigated the stability of preparations of IFN- α 2 diluted to 0.4mg/mL in PBS and stored at -70°C, +4°C, +20°C and +37°C, for periods up to 25 weeks using two *in vitro* biological assays (described in earlier sections) and SDS-PAGE. Studies under accelerated stress conditions may be useful in determining whether accidental exposures to unfavourable conditions (*e.g.* during transportation) are deleterious to the product, and also for evaluating which specific test parameters may be the best indicators of product stability.

Three different IFN preparations were selected for this study; the Chemical Reference Substances (CRS) for IFN- α 2a (CRS I03200300) and for IFN- α 2b (CRS I03200301), purchased from EDQM and the NPL IFN- α 2b preparation, IFN003. The

2nd International Standard (IS) for IFN- α 2a (code 95/650) and for IFN α -2b (code 95/566) were used as the controls. The pH and concentration of the samples are listed in Table 8.

At the end of each storage period, aliquots of each IFN preparation taken at a set time/temperature point, were analysed immediately by bioassay, using the IS for IFN IFN- α 2a or IFN- α 2b as the reference standards. Aliquots of the same samples were treated with a solution of Laemmli buffer for SDS-PAGE and stored at -70°C until the end of the 25 week period, to allow simultaneous analysis of all samples. SDS-PAGE was performed on 12% SDS homogeneous gels, in reducing conditions, followed by silver staining.

Three different stability studies were carried at different times, due to limited sample quantities and availability issues. As a result of the large number of time/temperature points, samples had to run on different SDS-PAGE gels, at different times, and some variation was observed in the staining of the silver stained gels and slight differences were observed for protein band migration (electrophoresis), within the normal variation. Therefore, it was difficult to select and combine the different time points for each sample to present a full picture of the entire stability study. Nevertheless, the gels with duplicates of each sample, for each time point, have shown reproducible results, with well-associated band patterns, which consolidated the results presented (Figure 29).

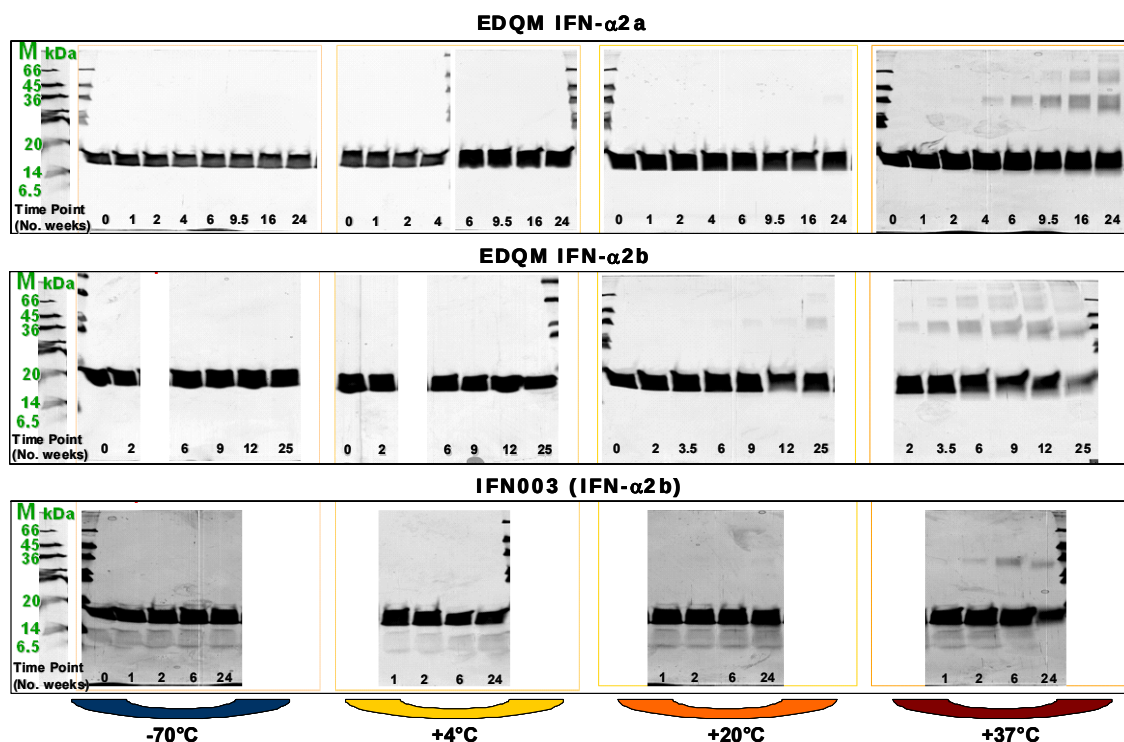


Figure 29. A selection of SDS-PAGE gels for EDQM IFN- α 2a and - α 2b and IFN003, at selected time/temperature points, as typical examples to illustrate the stability studies performed. M = M_w marker.

The results from the SDS-PAGE analysis are summarised in Table 13

Table 13. SDS-PAGE summary results for the stability study

IFN preparations	Storage temperature				
	-70 °C	+4 °C	+20 °C		+37 °C
EDQM IFN- α 2a	Main isoform band ~20kDa	Higher M_w band, ~35 kDa, from:	week 24	Higher M_w band, around 35 and 66 kDa, from:	week 4 and week 9.5
EDQM IFN- α 2b			week 9		week 2 and week 3.5
NPL IFN- α 2b (IFN003)			week 24	Higher M_w band, around 35 kDa, from week 2	

For the NPL IFN- α 2b, two low M_w bands, (around 14 and 7 kDa), were detected from Day 0 with intensity remaining constant up to week 24. The presence of these bands at Day 0 suggested that degradation of this preparation had already taken place at the start of the study. Degradation products were not seen in previous analysis of a vial shipped on a different date. This observation illustrates the desirability of an early

analysis of a sample in a long-term study to permit timely restarting of the study if necessary. This particular result would probably indicate the necessity of initiating a sampling homogeneity study.

At week 24 and week 25, the NPL IFN003 and EDQM IFN- α 2b stored at 37°C, showed a decrease in intensity of the main band and the higher M_w , and the lower M_w bands were no longer detected for the NPL IFN003. This reduction in the amount of protein detected may indicate aggregation, preventing migration into the gel.

The study showed loss of biological activity on storage for IFN- α 2a and IFN- α 2b, monitored by *in vitro* bioassays. This occurred over the same time and temperature range as the appearance of aggregates and low M_w degradation products on SDS-PAGE gels. This suggests the possibility that, for these molecules, detection of degradation products and aggregates could predict of loss of biological activity. This observation has also been made by Wang et al (33).

“Under certain conditions (or simply with time), the secondary, tertiary and quaternary structure of a protein may change and lead to protein unfolding and/or aggregation, a major event of physical instability. Protein aggregation may have no or reduced activity, reduced solubility and altered immunogenicity. Presence of any insoluble aggregates in a protein pharmaceutical is generally not acceptable for product release. Thermally-induced protein aggregation is often irreversible.”

Further characterisation was conducted of material stored at 37°C in a repeat of the study, using the physicochemical techniques that been demonstrated to detect changes in the size and hydrophobicity of the batches produced at NPL. The composition of the high molecular weight bands observed by SDS-PAGE after storage at elevated temperatures was investigated. Treatment of the sample with SDS will dissociate weak interactions between molecules, suggesting that the high molecular weight bands visible in SDS-PAGE were indeed irreversible aggregates or covalently associated molecules. The band profile suggests association of molecules corresponding to dimers, trimers and other oligomers of IFN- α 2a. SEC-HPLC was carried out which confirmed the presence of high molecular weight material, with

EDQM IFN- α 2b forming a higher percentage of aggregated material than EDQM IFN- α 2a, with some resolution of peaks at the elution times predicted for dimeric and trimeric species (Figure 30).

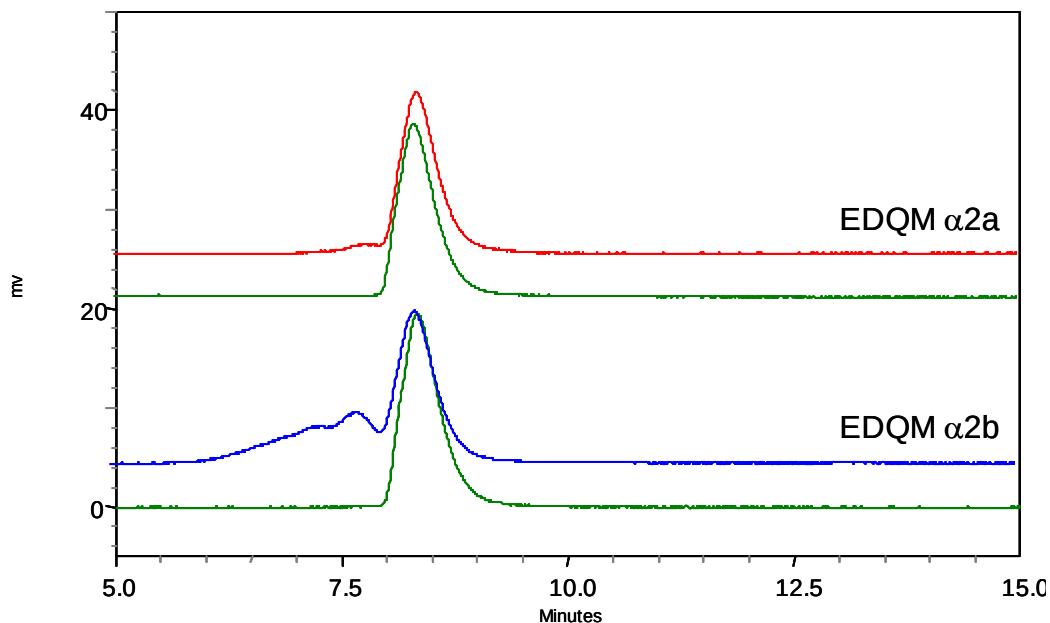


Figure 30. Representative SEC-HPLC chromatogram of stability study samples, stored at -70°C (green line) and 37°C (blue and red lines).

Table 14. SEC HPLC data 10 μL injection volume, 1mg/mL, n=2.

Sample	RT (min)	% Area
EDQM α 2a (-70°C)	8.31	100
EDQM α 2a ($+37^{\circ}\text{C}$)	7.78	5.0
	8.35	95.0
EDQM α 2b (-70°C)	8.33	100
EDQM α 2b ($+37^{\circ}\text{C}$)	7.30	21.0
	7.71	18.0
	8.37	61.0

The material was also examined using dynamic light scattering (Table 15) which suggested that the diameter of the particles in the samples was consistent and there

were no large molecular weight aggregates that would not be resolved by SEC-HPLC. The technique cannot resolve dimer, trimer or other oligomers of IFN- α 2.

Table 15. DLS size distribution by volume, stability samples. 0.2mg/mL, n=9

Sample	Diameter [nm]	% Volume	Width [nm]
EDQM α2a (-70°C)	4.58 (+/- 0.36)	100%	0.862
EDQM α2a (+37°C)	4.87 (+/-0.27)	100%	0.817
EDQM α2b (-70°C)	5.20 (+/-0.31)	100%	1.15
EDQM α2b (+37°C)	5.93 (+/-0.12)	99.6%	1.56

The material was then examined by RP-HPLC, which was shown in earlier experiments to detect oxidised IFN- α 2a. IFN- α 2 samples stored at 37°C showed a chromatographic profile characteristic of oxidised material, a peak eluting before the main isoform with a similar shift in retention time to material that had been exposed to accelerated oxidative conditions. EDQM- α 2b material showed the largest proportion of oxidative degradation at 37°C, possibly reflecting differences in storage conditions during the stability study, where EDQM IFN- α 2a stock was stored at pH 5 and IFN- α 2b was stored at pH 7.

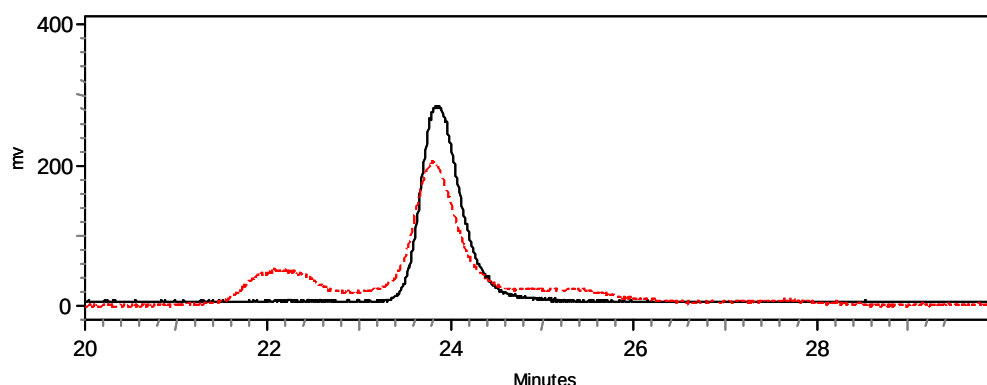


Figure 31. Representative RP-HPLC chromatogram of EDQM IFN- α 2a stability sample stored at -70°C (black line) and 37°C (red line).

Table 16. RP HPLC 0.2 mg/mL 50uL injection volume, n=2

Sample	RT (min)	% Area
EDQM α 2a (-70°C)	23.95	100
EDQM α 2a (+37°C)	22.08	22.5
	23.78	72.0
	27.81	5.5
EDQM α 2b (-70°C)	22.14	2.0
	23.72	98.0
EDQM α 2b (+37°C)	22.19	9.0
	23.98	39.5
	25.6	35.0
	27.62	16.5
MetOX	21.84	86.0
	24.37	14.0

In summary, the stability of IFN- α 2 stored at different temperatures was investigated using physicochemical techniques that had been previously demonstrated to detect variability in the molecular profiles of batches produced at NPL. SDS-PAGE, SEC and RP-HPLC techniques indicated that the degradation pathways involved oxidation and aggregation of IFN- α 2. The structural information obtained from these materials was then related to biological responses in two *in vitro* bioassay systems.

8 Linking structure to function- late-stage *in vitro* bioassay

8.1 Principle of the methods

An anti-proliferative assay “late-stage readout” was developed, measuring the anti-proliferative action of IFN- α 2a or α 2b on cultured Daudi cells (CCL-213, ATCC-LGC Promochem, UK) with a colorimetric readout. Changes in the metabolic activity

of the cell population, as a function of the IFN concentration, are measured through the reduction of the indicator alamarBlue™ (Serotec), which results in a colour change from blue to pink. There exist a number of assays for IFN- α 2 based on the inhibition of Daudi cell proliferation. These generally measure the incorporation of ^3H -thymidine into newly synthesized DNA. Increasingly stringent safety and environmental regulations are making the use of radioisotopes more difficult, so we developed this colorimetric assay protocol.

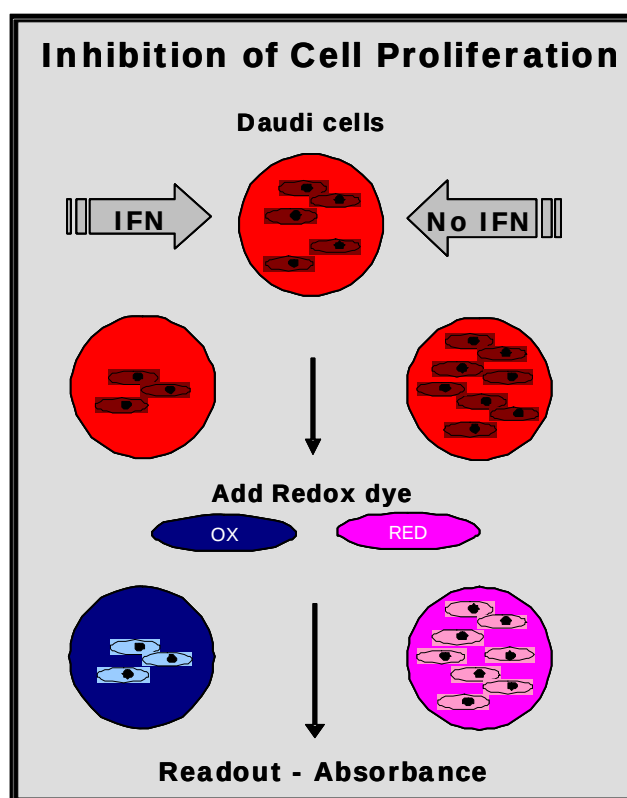


Figure 32. Schematic of an anti-proliferative, colorimetric cell-based bioassay for interferon.

The alamarBlue™ system incorporates a fluorometric/colorimetric, oxidation-reduction (Redox) indicator, resazurin, which both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth. As cells grow, innate metabolic activity results in a chemical reduction of the immediate surrounding environment. Continued growth maintains a reduced environment, while inhibition of the growth results in an oxidised environment. Reduction related to growth causes the redox indicator to change from oxidised (non-fluorescent, blue) to reduced (fluorescent, pink/red) form. Data may be collected using either fluorescence-

based or absorbance-based instrumentation. In this project, absorbance was monitored at 570 nm and 600 nm.

8.2 Methodology employed

Cells (CCL-213, ATCC-LGC Promochem, UK) were maintained in culture medium (RPMI-1640 supplemented with 10 % (v/v) Foetal Bovine Serum, both from LGC Promochem, UK) in 75 cm² flasks (Costar®, 3376) and passaged three times a week, seeding at approximately $1\text{--}3 \times 10^5$ cells/mL ($2.2\text{--}6.6 \times 10^6$ cells/flask). Under this maintenance regime, Daudi cells maintain their responsiveness over many passages (from P2 to greater than P60). For assay, cells are seeded in 96-well culture plates (Nunc, 167008) at a density of 5×10^4 cells/mL (1×10^4 cells/well) in culture medium and dosed on seeding with a 2-fold dilution series of stock solution of IFN- α 2a or IFN- α 2b in culture medium (*i.e.* working solution). After incubation (37 °C, 5 % CO₂ in air) for 72 hours, 40 µL/well of alamarBlue™ (Serotec, BUF012B) was added and the plates are incubated for a further 16 hours, before the final absorbance (A) readout ($A_{570\text{ nm}}$ minus $A_{600\text{ nm}}$) using a spectrophotometer, SpectraMax® 340PC from Molecular Devices. This method has been adapted from published and unpublished protocols and optimised following the validation approaches described in section 8.3.

The five batches of IFN- α 2 produced at NPL were assayed using EDQM IFN- α 2a and - α 2b as working reference standards. Despite the fact that no specifications as to their suitability for biological assays is provided by EDQM, a study carried out to assess the biological activity of EDQM CRS IFN- α 2a and IFN- α 2b, (work described in section 10.1 and accepted for publication to Pharmeuropa Bio, April 2007), has shown that these IFNs have comparable activity with the 2nd IS for IFN- α 2a and IFN- α 2b, the primary calibrant of the biological activity for IFN- α 2, defined in international units (IU), and the reference standard for the bioassay analysis in this project.

For comparison, note that IFN concentrations, for the working reference standards (*i.e.*, EDQM IFNs) and the 2nd IS for IFN- α 2a and IFN- α 2b, were based on the following information, as stated on their “Instructions for Use” datasheet:

- EDQM CRS IFN- α 2a, about 1.46 mg/mL

- EDQM CRS IFN- α 2b, 7.19 mg/mL
- 2nd IS IFN- α 2a, approximately 250 ng per ampoule (63 000 IU per ampoule)
- 2nd IS IFN- α 2b, approximately 500 ng per ampoule (70 000 IU per ampoule)

Different vials of the NPL IFN batches and EDQM IFN- α 2a and - α 2b were received as frozen solutions, (except batch 1, IFN2001, received at 4-8 °C), and sub-divided into several aliquots (i.e., stock solution) of 10 μ L, 20 μ L or 50 μ L. A few of these aliquots were further diluted in PBS for stock as single-use aliquots and stored at -70 °C before use. Several ampoules of the 2nd IS for IFN- α 2a and α 2b were independently reconstituted in 1 mL of sterile distilled water, as described in the instructions for use and the reconstituted solution from each ampoule was sub-divided into several aliquots (i.e., stock solution) of 20 μ L and stored at -70 °C before use. For assay, frozen aliquots of IFN stock solutions were quickly thawed in a water bath at +37 °C and prepared as working solutions by dilution in culture medium.

8.3 Validation approaches – parameters to be validated in the Daudi cell anti-proliferative assay

Development of a bioassay and its characterisation and validation represent a substantial investment in time and resources, and failure to complete any of these in a timely manner can delay a whole drug development program.

Validation of the bioassay, as for any assay, is the demonstration that the assay performs as claimed and that it is suitable for its intended use. It is a formal exercise. The desired performances (i.e., acceptance criteria) are specified before the validation begins. This requires extensive characterisation, or qualification, of the assay, investigating the effects of various parameters on assay performance, and defining their permissible ranges in order to design a protocol. The requirement for eventual validation should be kept in mind from the start of the assay development.

Bioassays have particular features that increase the complexity of their design, validation and interpretation compared with many physicochemical analytical systems. This is recognised in the ICH Guideline Q2 (R1): Validation of Analytical Procedures: Text and Methodology.

As with the validation of analytical techniques in general, there are a number of numeric, systematic and operational criteria that apply to bioassays. For instance, all data generated should be available to appropriate statistical analysis. Assays should be both as accurate and precise as possible (34).

One of the first steps in bioassay design is to identify parameters which could affect assay performance and to investigate the significance of their impact. Due to the complexity of bioassay systems they are susceptible to many variables, and failure to control adequately those, which affect assay performance, will cause high assay variability. One of the aims and objectives of this project was to identify sources of uncertainty in cell-based bioassays.

A full validation exercise is outside the scope of this project. However, in this study we have demonstrated how a systematic approach to the assessment of selected parameters is necessary to develop an assay. Strategies for reducing assay variability start by identifying causes of variability. Where possible, these are controlled and acceptable ranges defined. Appropriate assay design can then reduce the impact of unidentified sources of variability and sources of variability which cannot be tightly controlled. We show the results of this approach as applied to the anti-proliferative assay using Daudi cells.

Table 17 summarises the parameters investigated.

Table 17. Parameters assessed for validation of the cell-based bioassay

Cell Maintenance	Bioassay Parameters	Sample preparation Storage & Assay
Serum supplier Cell passage no.	Plate set up Cell seeding density Incubation times Redox indicator (alamarBlue™, AB) Concentration Cell density (end of assay)	Storage vials and concentrations (with or without protein carrier) Storage temperature/time Dilution buffer Sample dose-range

Other important parameters that could contribute to measure variability within the assay system include age of cell stock, composition source and batch of the growth medium, harvesting, processing, dispensing, source and batch of the plastic consumables used in cell culture, incubator and operator.

Several assays were performed for the late-stage bioassay, measuring the anti-proliferative action of EDQM IFN- α 2a on cultured Daudi cells, to characterise and optimise the assay system in order to reduce sources of variability. The parameters investigated and summarised in Table 17 are divided within cell maintenance, bioassay parameters and sample preparations sub-sections.

8.3.1 Cell Maintenance

Serum: The reduction in assay variability and stability of critical reagents such as serum is required in this and other bioassay systems to ensure that the measured signal is attributable to the cells. To address this issue, Foetal Bovine Serum (FBS) from ATCC-LGC Promochem was compared to FBS certified from Gibco-Invitrogen for both long term maintenance of cell stock and for bioassays. Both sera were manufactured from fetal bovine blood collected in inspected abattoirs located in the US with some variances on the quality control tests carried out. Information obtained from cell counts and observations made during long term cell stock maintenance, and from all dose-responses from bioassays performed using both sera, indicate that cells behave very similarly. As FBS from ATCC has been recommended for Daudi cells, as described on product description datasheet, FBS certified from Gibco-Invitrogen has been specified as an alternative reagent for the late-stage bioassay.

Cell passage number: The major source of variability in many cell-based assays is the cells themselves, all the manipulations during routine cell maintenance and their state when harvested for the assay. To access the impact of cell passage number on their response to the test material, Daudi cells were resuscitated from different stock vials, at different times, and grown for different lengths of time, under the same routine cell maintenance conditions and dosed with IFN on several assays. Information obtained from all dose-responses showed that Daudi cells can maintain their responsiveness over many passages (from P2 to greater than P60).

8.3.2 Bioassay parameters

Plate set up: Bioassays are normally performed in 96-well culture plates but non-uniformity and variable accuracy of the multi-channel micropipettes used can

contribute to uneven responses across the plates, in particular at the outer wells. In many cases, the outer wells of the plates appear to give the most uneven responses, where more rapid variations in temperature and pH can affect the cells. This issue of “edge effect” is discussed in more detail in Meager’s review on “Biologicals assays for interferons” (13). Therefore, only the inner 60 wells (middle 6 rows) were used for all bioassays performed throughout this project (including those assays in the section describing the early-stage *in vitro* bioassay). Since complete randomisation of samples in terms of plate positions is usually not feasible, a compromise between randomisation and systematic rotation, to reduce operator error, was set up for samples and standards by testing them on different rows across several plates, such that their positions relative to the standard were varied. To the extent possible, data were assessed for significant positional/order effects.

Cell seeding density and incubation times: The optimal conditions for this particular bioassay system, in terms of cell seeding density and incubation times on dosing, were assessed. Different sets of experiments were performed whereby Daudi cells were seeded at different cell densities, 2.5, 5, 10 and 20×10^4 cells/mL on the assay plate, dosed and incubated with IFN for different periods, 24, 48, 72 and 96 hours, and further incubated with AB for periods between 2 to 24 hours before absorbance readout. Information obtained from all dose-responses, indicated an optimal dose-response at 5×10^4 cells/mL (i.e., 1×10^4 cells/well) with 72 hours IFN incubation and a further 16 hours with AB. These incubation times were selected taking in consideration logistics of the assay system and convenience for the operator.

Redox indicator concentration: The concentration of alamarBlue™, redox indicator used on the late-stage bioassay, as a colorimetric readout platform, was tested at 20 and 40 μ L per well (no pre-dilution), with and without mixing on the assay plate prior to incubation. From the experiments performed, 40 μ L per well with no mixing has given the best results and fixed as an optimum for the set conditions so far investigated and optimised.

Cell density at the end of the assay: The Daudi late-stage bioassay is an anti-proliferative assay whereby the presence of IFN inhibits cell growth. To validate the

principle of this method, cell counts were taken at the end of several assays, i.e., after the final absorbance readout. The lower levels of AB reduction were shown to correspond to lower cell numbers, i.e., at zero or low on IFN concentration, cell number was shown to be higher than on initial seeding, while at higher IFN concentration there was a reduction in cell number. These observations are represented on Figure 33 and expressed in cell density after 88 hours dosing with IFN, at different concentrations.

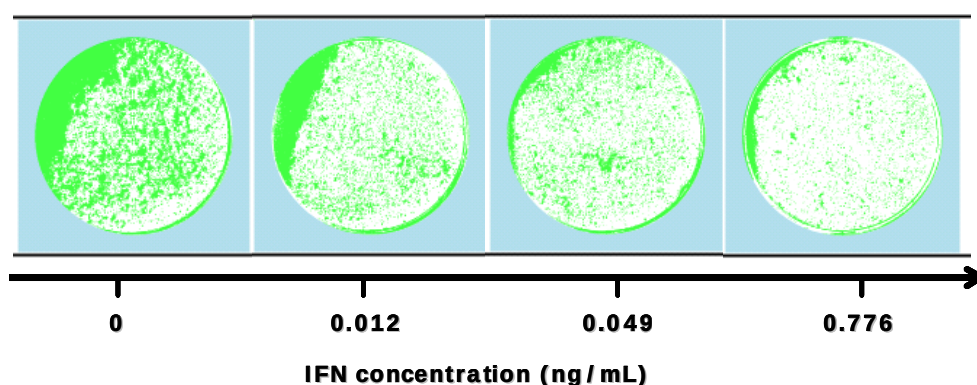


Figure 33. Decrease in cell density at 88 hours with increasing IFN concentration (data from Genetix CloneSelect imager).

8.3.3 Sample Preparation

Storage vials and concentrations: Proteins can be adsorbed to many surfaces such as the container surfaces used for storage. Preparing and stocking samples at the appropriate conditions, suitable for long term storage needs to be taken into consideration when developing or optimising an assay. If the right conditions are not met, chemical and physical degradation mechanisms can occur, affecting the proteins and compromising assay performance. To address this issue, bioassays were carried out using IFN samples that had been aliquoted at different concentrations, in the presence and absence of a protein carrier, and stored on different types of vials. Variability between vials/conditions in terms of relative biological activity was assessed.

Three IFN concentrations were prepared at 4.9 µg/mL, 29.96 µg/mL and 1.46 mg/mL in PBS, with and without protein carrier (bovine serum albumin, BSA) at 0.1% v/v in PBS, and stored in 1.5 mL Sarstedt® V bottom shape and 2.0 mL Corning® U

bottom shape polypropylene micro tubes. From the information obtained from all dose-responses, differences were observed at the lowest concentration without BSA, between the two types of vials under investigation. The Sarstedt® micro tubes were shown to retain the samples and their biological activity at low concentration in the absence of protein carrier but better results were obtained at high concentration. The presence of BSA, at the describe concentration, was shown not to interfere with the assays. However, if proteins can be stored in the absence of a carrier, no contamination issues can be raised from the presence of other proteins and their sources (e.g., BSA, is a blood-derived protein) (35).

Storage temperature/time: Freeze-thaw cycles can decrease protein stability so samples are prepared in single-use aliquots and stored frozen so that, once thawed, the protein solution will not have to be refrozen. A few experiments were performed where no apparent differences in relative activity were observed after five consecutive cycles of freeze (in dry ice) and thaw (37 °C water bath). However, whenever possible, repeated freezing and thawing of protein samples should be avoided, even when performed rapidly. For the various IFN preparations analysed throughout this project, the storage temperature was set at -70 °C, based on the recommended storage temperature for EDQM and 2nd IS for IFN. While manipulating the various preparations, IFN was always kept on ice prior to storage or assay. Since proteins are generally more stable at colder temperatures, maintenance at low temperatures even for shorter duration is recommended by suppliers, e.g., of EDQM IFN and 2nd IS IFN.

Dilution buffer: Concentrated protein solutions, prepared as single-use aliquots (e.g., in PBS or water), normally require further dilutions on dosing in a bioassay. These dilutions are commonly performed on assay medium to reduce any buffer interferences that may arise from the original solutions. However, the same aliquot may be required simultaneously on different assay systems, e.g., for economy of use. In that case, an initial buffer (e.g., PBS) would dilute the stock and increase volume to divide the sample for further dilutions on different assay media. To investigate, for example, if the presence of PBS in the assay medium would interfere with the final readout, experiments were performed whereby single-use aliquots were 1st diluted in PBS (i.e., stock solution) and then in assay medium for dosing (i.e., working

solution). The percentages of PBS found in the working solutions, ranging from 0.001% up to 8% PBS/mL medium did not seem to have a significant effect on the performance of the bioassays herein carried out. However, as a possible source of variability in an assay, dilutions and buffers should be taken into consideration when preparing samples for dosing.

Sample dose-range: This is a parameter most dependent on the samples expected or predictable biological activity. If unknown, preliminary assays need to be carry out to chose the best dose-range for each individual samples, on a one-to-one basis. For the samples and references used throughout this project, a preliminary assay was performed for each sample, testing different dose-ranges based on a 2-fold dilution, with 10-points including zero (i.e., no IFN), to assess the optimal dose-range for further analysis. The number of points on the dose-curve was selected based on the plate-set up described earlier and due to the fact that multi-channel micropipettes were used to deliver and dilute the samples directly across the assay plate.

8.4 Results

The potencies of the various IFN preparations produced at NPL were measured by bioassay and compared against the working reference standard EDQM CRS and the 2nd IS for IFN- α 2a and/or α 2b. All bioassay raw data, i.e., IFN dose-response curves of average absorbance (A570 nm minus A600 nm) versus IFN concentration (ng/mL), were plotted (Figure 34) and examined both graphically and statistically (using an in house software program (36) for any gross anomalies or outliers.

The IFN dose-response relations were assessed, and the responses were transformed to give approximately linear log dose-transformed response lines so that these could be compared using the methods of parallel line assay analysis. A four parameter logistic curve provided a satisfactory fit to the dose-response curve and an in house program (WRANL (37)) was used for analysis. Estimates of the relative activity of the various samples in terms of one another were obtained as the displacement of parallel log dose-logit response lines. Data for each 96 well have been analysed individually and relative activities of samples on the same plate have been determined. Estimates have been compared between plates within the same assay, and

between assays. Variances have been summarised as geometric coefficients of variation (GCV). Comparisons among the estimates of activity have been made using analysis of variance of the logarithms of the estimates. Combined estimates have been calculated as geometric means, with confidence limits (i.e. 95% limits) determined using the variance of the estimates combined. Evidence for trends in estimates over the time points was assessed by regression of logarithm of mean potency on length of storage in days.

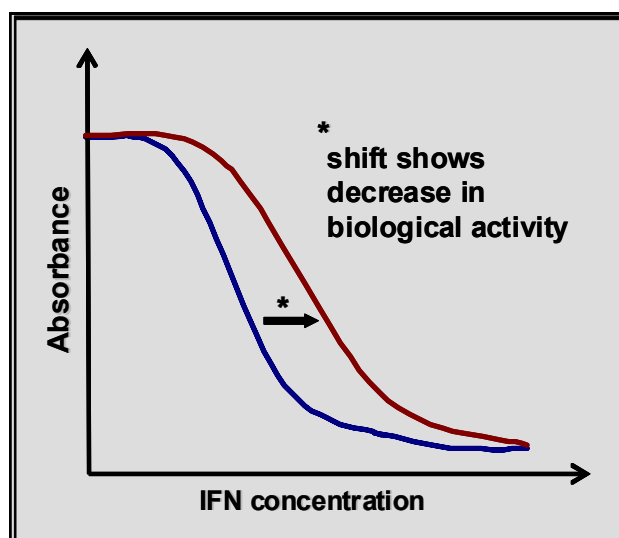


Figure 34. Schematic of an IFN dose-response curve, representing absorbance versus IFN concentration.

8.4.1 Biological activity of Interferon-alpha 2 batches

The five batches of IFN- α 2a and - α 2b produced at NPL were tested against EDQM and 2nd IS for their *in vitro* biological activity. A selection of dose-responses is shown in Figure 35.

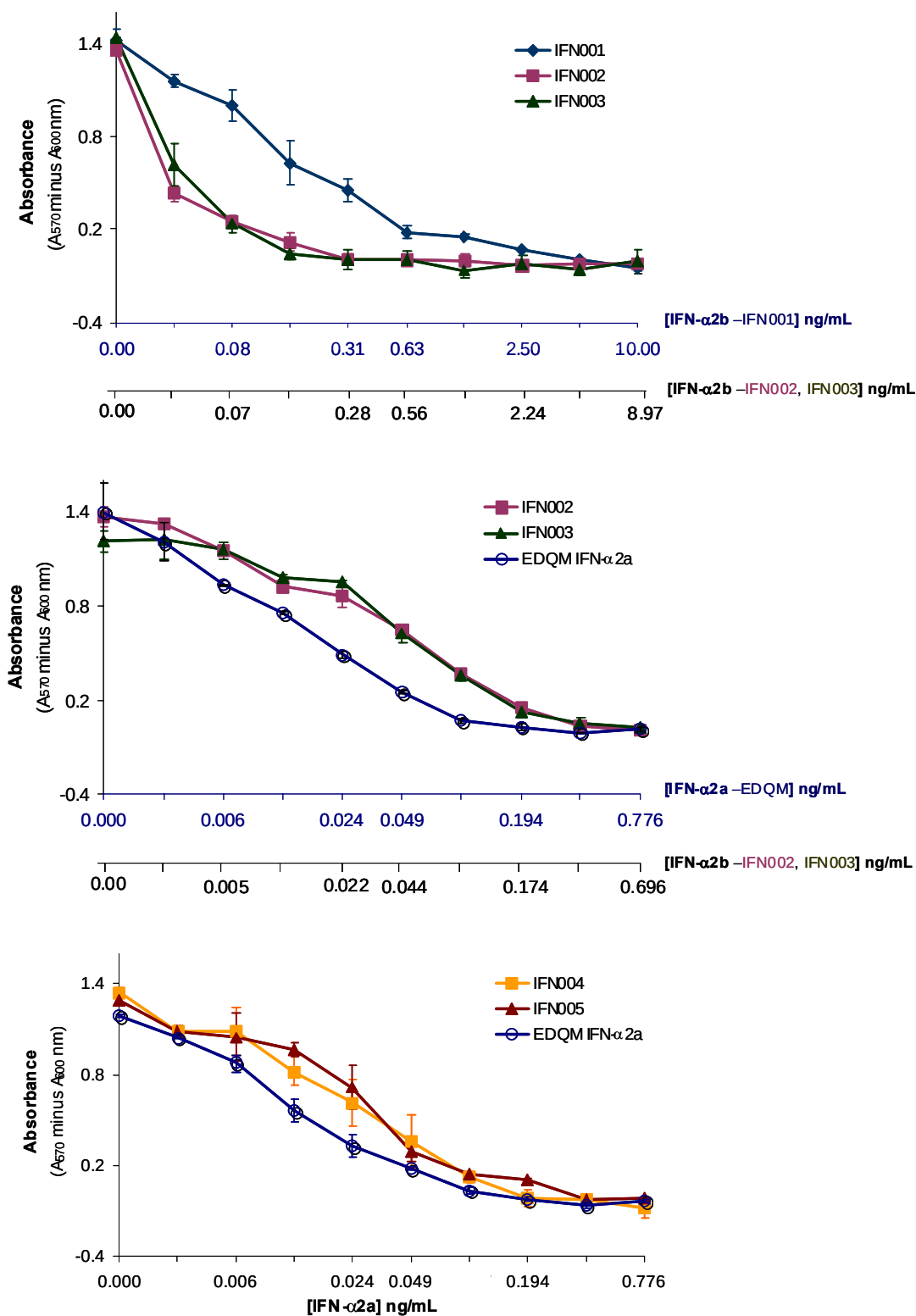


Figure 35. Selected IFN dose-response curves for all NPL batches and EDQM IFN-α2a. Data are the means $\pm$ standard error (n=2).

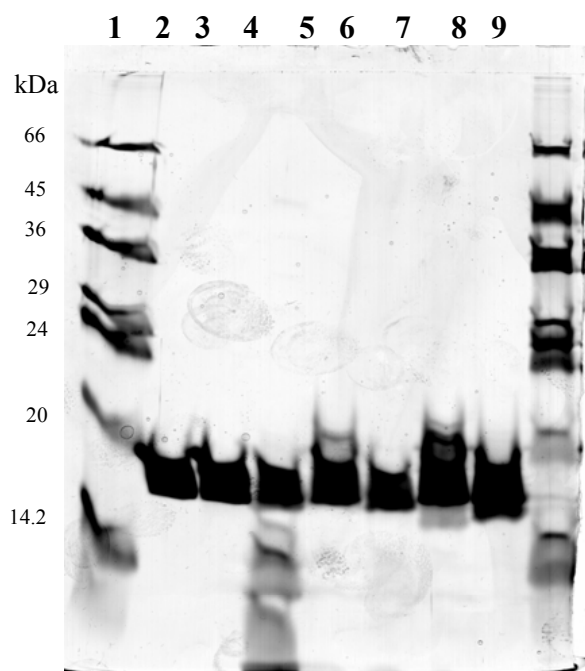


Figure 36. 12% reducing SDS-PAGE gel, silver stained, of all NPL IFN- α 2 batches and EDQM IFN- α 2a and - α 2b. Lanes 1 and 9 are the low molecular weight range markers (SigmaMarker™, M3913), EDQM IFN- α 2a (lane 2), EDQM IFN- α 2b (lane 3), IFN001 (lane 4), IFN002 (lane 5), IFN003 (lane 6), IFN004 (lane 7) and IFN005 (lane 8).

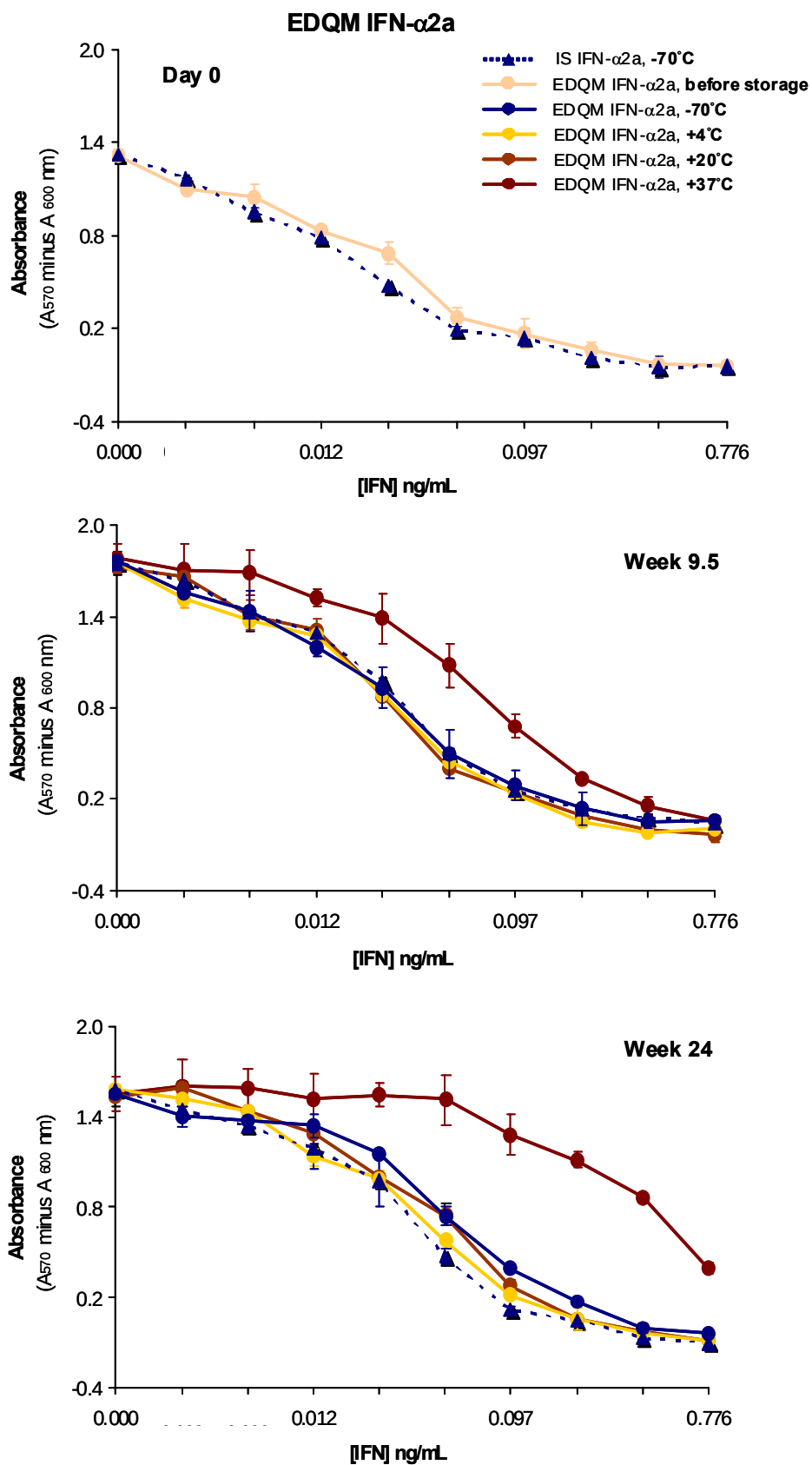
IFN001 was discarded from further use in the study due to extensive degradation of the material, as seen in Figure 36.

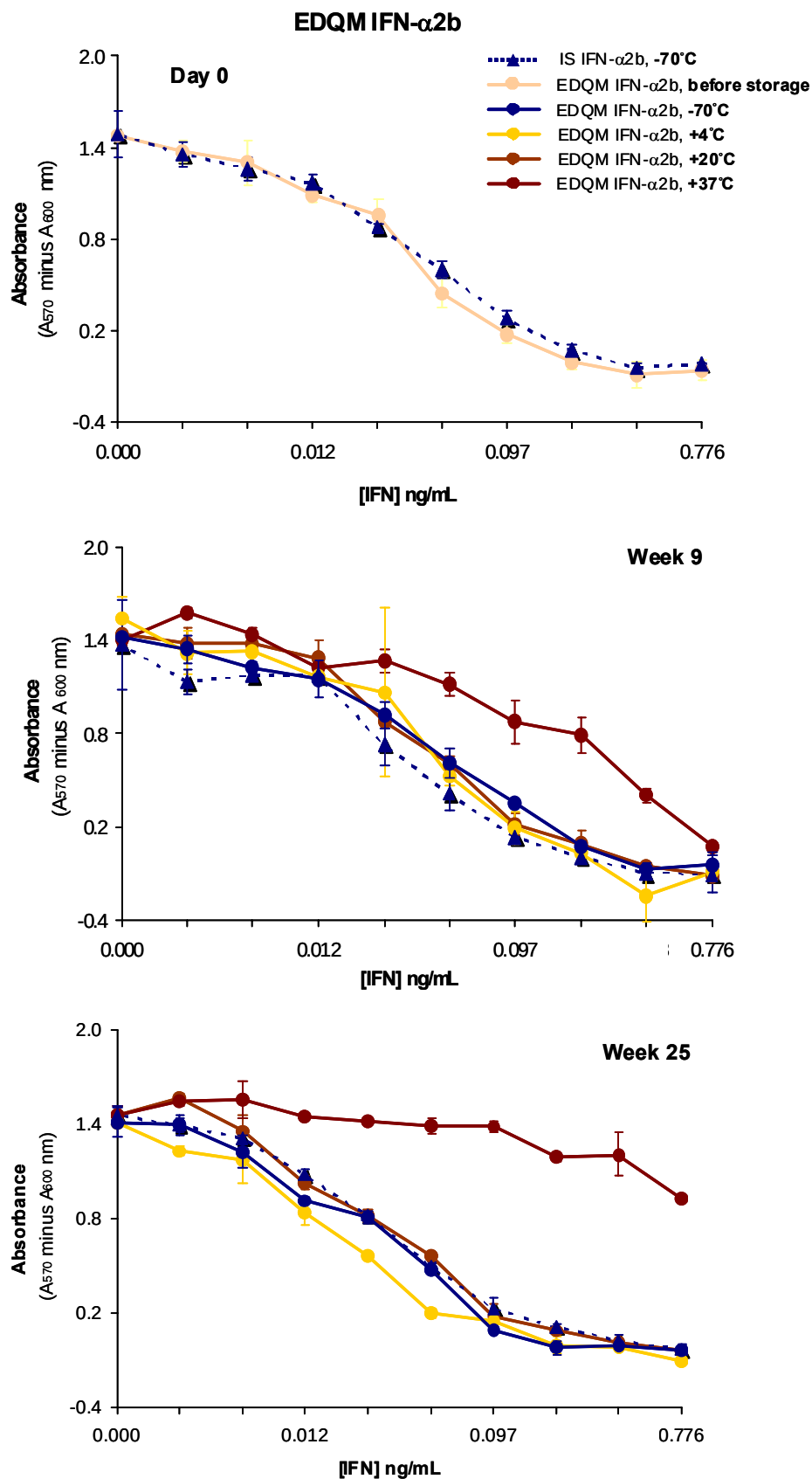
Table 18. Comparison by late-stage bioassay of specific activity of the *in-house* IFN- α 2 batches relative to EDQM IFN- α 2a. Values represent the mean of four ⁽⁴⁾ or six ⁽⁶⁾ estimates, i.e., 4 or 6 plates within 1 assay. Limits based on intra-assay variation.

Materials	<u>Relative Activity</u> compared to EDQM IFN-α2a (CRS I0320300)	95% confidence limits
IFN001, IFN- α 2b	0.18⁽⁴⁾	0.16 – 0.21
IFN002, IFN- α 2b	0.33⁽⁴⁾	0.29 – 0.38
IFN003, IFN- α 2b	0.34⁽⁴⁾	0.29 – 0.39
IFN004, IFN- α 2a	0.56⁽⁶⁾	0.49 – 0.64
IFN005, IFN- α 2a	0.45⁽⁶⁾	0.40 – 0.51

The material produced and characterised at NPL showed reduced biological activity compared to the EDQM IFN- α 2a, ranging from 0.33 to 0.56 relative activity (IFN002 to IFN005). There appears to be no relationship between relative activity and batches produced in the fermentor, IFN002 and IFN004, that contained the peptide signal sequence. There is a higher relative activity for NPL IFN- α 2a batches than for NPL IFN- α 2b batches. Subtle differences in potency between α 2a, α 2b and α 2c IFN subtypes in an anti-viral bioassay has previously been reported (16).

The loss of biological activity over changes in storage temperatures/times, as part of the stability studies, was monitored for EDQM IFN- α 2a and α 2b and the NPL IFN-IFN003. A selection of the results is shown in Figure 37.





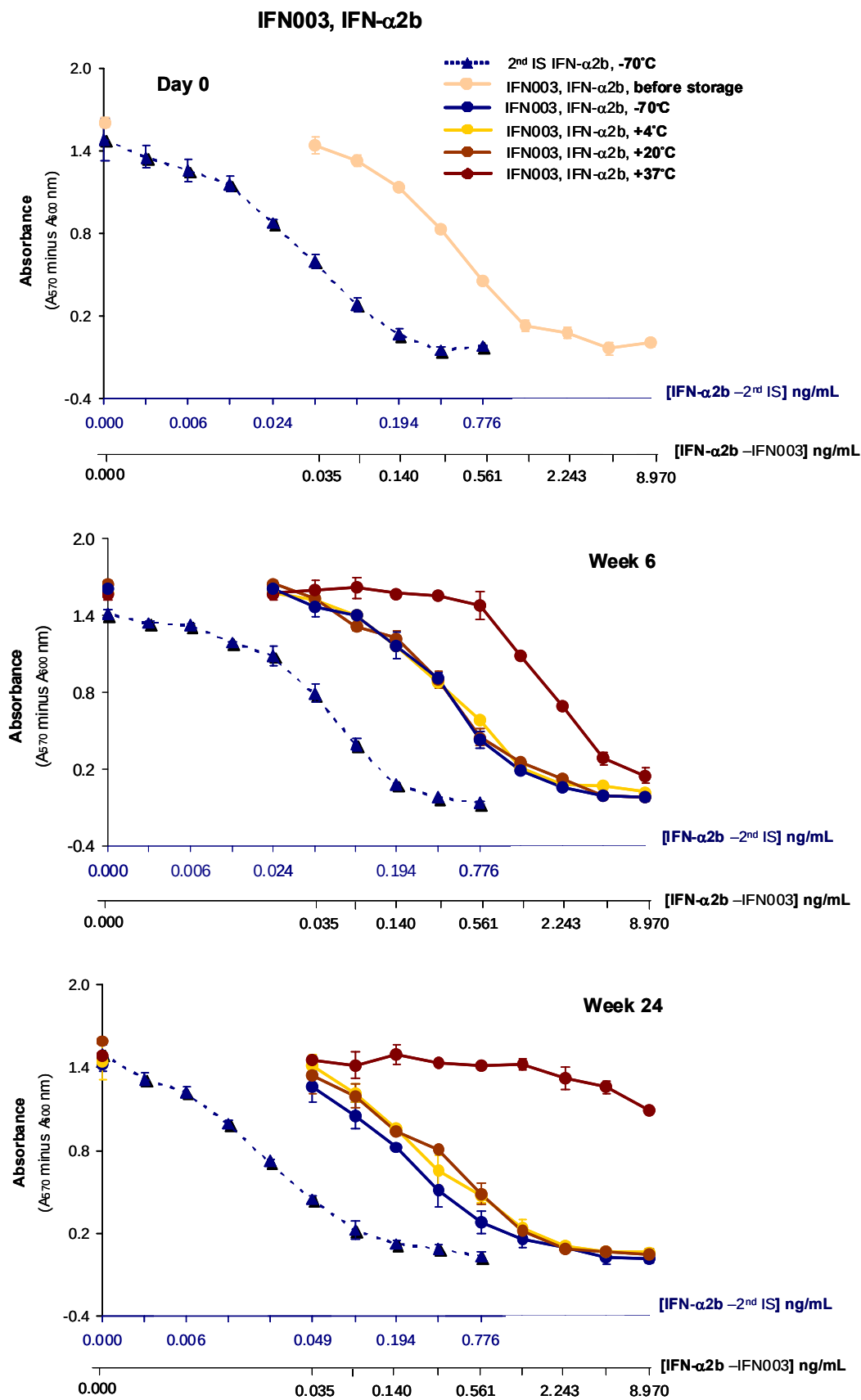


Figure 37. Selection of IFN dose-response curves for EDQM IFN- α 2a and - α 2ab and IFN003 (IFN- α 2b), at selected time/temperature points, as typical examples to illustrate the stability studies performed. The 2nd ISs at -70°C used as reference standards are plotted assuming 2.52×10^8 IU/mg (- α 2a) and 1.4×10^8 IU/mg (- α 2b). Data are the means $\pm$ standard error (n=2).

A decrease in biological activity was observed for all three IFN preparations at higher temperature and longer storage times. At +37 °C, all IFN preparations showed a decrease in biological activity by: week 9.5 for EDQM IFN- α 2a, week 9 for EDQM IFN- α 2b and by week 6 for the NPL IFN- α 2b. By week 24 and 25, at +37 °C, a greater reduction in the biological activity was observed for all IFN preparations, in particular for the IFN- α 2b preparations. The NPL IFN- α 2b has a lower specific activity than the EDQM CRS preparations at Day 0, as expected from the results shown in Figure 35.

8.4.2 Biological activity of methionine oxidised Interferon-alpha 2a

Oxidation of methionine residues in EDQM IFN- α 2a were tested against the working reference EDQM, prior to oxidation. Assays were carried out to measure changes in the biological activity of IFN following oxidation. A selected dose-response is shown in Figure 38.

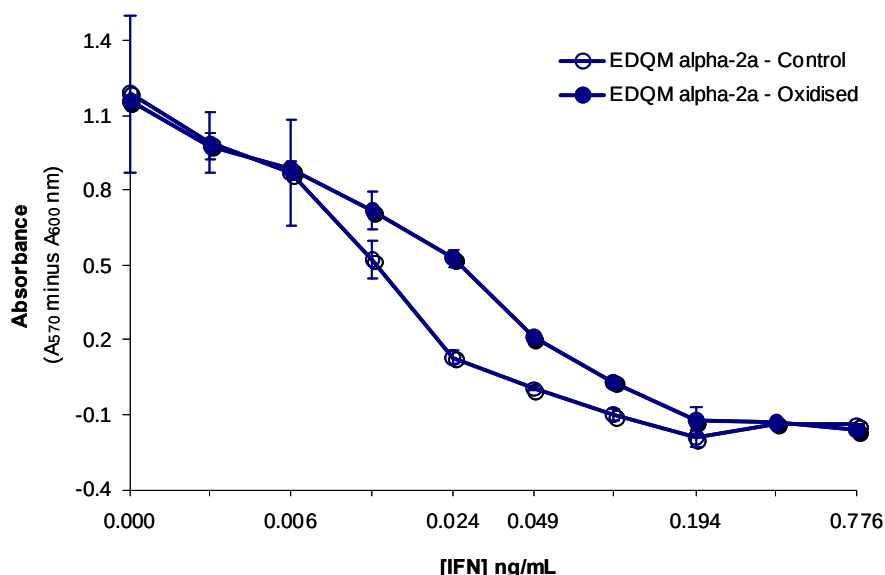


Figure 38. A selected IFN dose-response curve for methionine oxidised EDQM IFN- α 2a compared to a control. Data are the means $\pm$ standard error (n=2).

The dose-response curves indicate a decrease of *in vitro* biological activity of EDQM IFN- α 2a following the oxidation of methionine residues.

Table 19. Comparison of specific activity of the methionine oxidised EDQM IFN- α 2a relative to a control, i.e. sample of EDQM IFN- α 2a exposed to the same conditions as the modified material, without the oxidising reagent. Values represent the mean of relative activities from late-stage bioassay experiments calculated by the *in-house* program WRANL.

Material	<u>Relative Activity</u> compared to control EDQM IFN-α2a	95% confidence interval
Methionine oxidised EDQM IFN-α2a	0.55	0.45 – 0.68

These results show a clear relationship between the physicochemical data and a change in biological activity in the late-stage bioassay. IFN- α 2a material that had been shown to contain 62.6% of oxidised variant by RP-HPLC, and a reduced binding in the ELISA format, showed approximately 50% reduction in *in-vitro* biological activity. As with the ELISA result, the reduction in binding was not proportional to the amount of oxidised material, however the physicochemical assays had not been validated to demonstrate linearity of response depending on the amount of oxidised material. This could be within the scope of a full validation exercise.

9 Linking structure to function – early-stage *in vitro* bioassays

9.1 Principle of the methodology employed

A reporter gene assay “early-stage readout” was used, measuring the activation of the luciferase reporter gene in type I IFN-stimulated cells, with a luminescence readout. This assay was performed using a modified A549 cell line (A549/93D7 kindly donated by Dr G Adolf, Boehringer Ingelheim), which had been transfected with the promoter of the MxA gene (a type I IFN inducible protein) fused to a luciferase gene Figure 39.

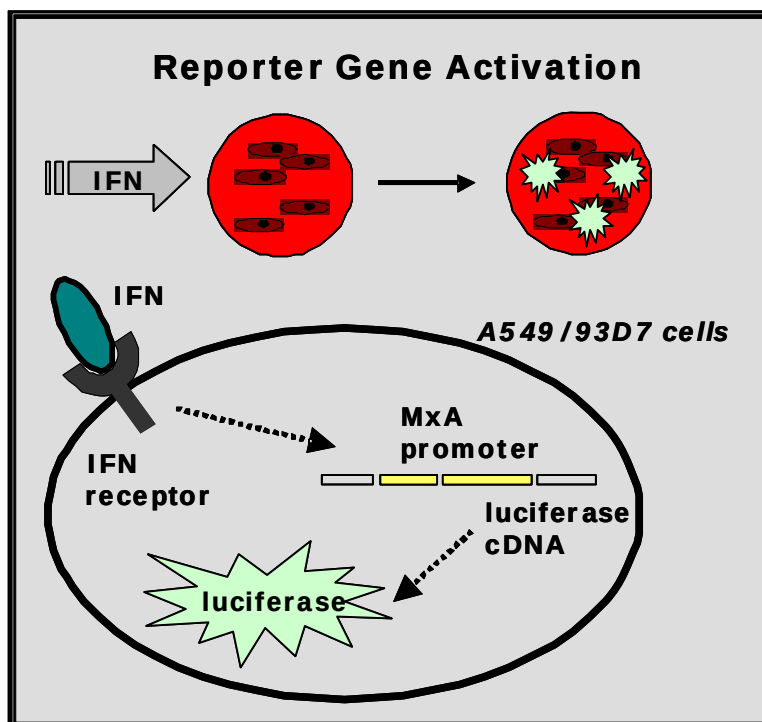


Figure 39. Schematic of a luminescence reporter gene activation cell-based bioassay for interferon (IFN).

Luciferase has been well defined as the reporter enzyme of choice in reporter gene assays due to the high sensitivity of detection (10^{-15} g). The sensitivity of luciferase based assays is a result of the high quantum efficiency observed with luminescent reactions. Light production results from the reaction of the active site of luciferin with ATP, in the presence of luciferase. Commercially available reagents are available, e.g., LucLite® (PerkinElmer) and Steady-Glo® (Promega), whereby Luciferin has been combined with a mixture of several substances that modify the enzymatic reaction to produce a long-lasting and linear light signal.

9.2 Methodology employed

Cells were maintained in culture medium (DMEM without phenol red supplemented with 10 % (v/v) heat-inactivated FBS, 50 U/mL penicillin, 50 µg/mL streptomycin, 500 µg/mL antibiotic G418 geneticin and 2 mM L-glutamine, from Sigma-Aldrich) in 75 cm² flasks (Falcon®, 353024) and passaged twice a week. Plates were seeded at

approximately 3.25×10^4 cells/mL (6.5×10^5 cells/flask). For assay, cells were seeded in white 96-well culture plates with shielded wells (PerkinElmer, 6005688) at a density of 5×10^5 cells/mL (5×10^4 cells/well) in culture medium and incubated (37 °C, 5% CO₂ in air) for 16 hours. After this period, supernatant was removed and cells were dosed with a 2-fold dilution series of stock solution of IFN- α 2a or IFN- α 2b in assay medium, (culture medium with 0.2 % (v/v) FBS instead of 10 %, i.e., working solution), and incubated (37 °C, 5% CO₂ in air) for 6 hours. The 2-fold dilution series is first prepared on polypropylene 96-well plates (ScreenMates™, Matrix, 4918) and then 100 µL/well transferred into the assay plate. After the 6 hours IFN incubation period, 100 µL/well of LucLite® (PerkinElmer, 6016911) was added and the plates were incubated at room temperature for 10-20 mins, before measuring the luminescence, using a liquid scintillation and luminescence counter, 1450 Wallac Microbeta® Trilux from PerkinElmer. This method has been adapted from published and unpublished protocols and optimised following the validation approaches described in section 9.3.

The five batches of IFN- α 2 produced at NPL were assayed using EDQM IFN- α 2a and α 2b as working reference standards and the 2nd IS IFN- α 2a and - α 2b as reference standards. For assay, stock solutions were prepared as described on section 9.2, and the working solutions for each IFN preparation were prepared in assay medium.

9.3 Validation approaches - parameters to be validated in the A549/93D7 reporter gene assay

Following the validation approaches described for the late-stage bioassay, and taking into consideration all the issues raised, other parameters that could contribute to measure uncertainty within the assay system were assessed for their effect on the A549/93D7 early-stage reporter gene assay, in preparation for validation for this assay system. Table 20 summarises some of the parameters investigated.

Table 20. Parameters assessed in preparation for validation of the early-stage bioassay.

Cell Maintenance	Bioassay Parameters	Sample Preparation Storage & Assay
Cell seeding density.	Plate set up Percentage of serum Luciferase reporter gene assay kits.	Storage vials and concentrations Storage temperature and time Dilution buffer Sample dose-range

At the extent possible, several assays were performed for the early-stage bioassay, measuring the activation of the luciferase reporter gene on A549/93D7 cells once stimulated with EDQM IFN- α 2a, to characterise and optimise the assay system in order to reduce sources of variability.

9.3.1 Cell Maintenance

Cell seeding density: As discussed previously, the cells themselves are a major source of variability in many cell-based assays. When cells are passaged during maintenance prior to an assay, cells can grow into a confluence or sub-confluence monolayer. Their state when harvested for the assay can be determinant and so experiments were performed to assess the optimal seeding density. Cells that had been grown for 3 days from high cell seeding densities (6.6×10^4 cells/mL) were shown to be 100% confluent and when seeded at low cell densities (3.3×10^4 cells/mL) were sub-confluent (approx. 70-80%). Bioassays were then performed using both set of cells and results have shown that cells grown to sub-confluency appear to perform better, i.e., higher expansion on the dose-response curves, when compared to those cells always grown to confluency.

9.3.2 Bioassay parameters

Plate set up: All bioassays were performed in 96-well culture plates, only using the inner 60 wells (middle 6 rows) as discussed for the late-stage bioassay.

Percentage of serum: The presence of serum used in the assay may have an effect on the measured signal that should only be attributable to the cells. Experiments were

performed at two percentages of FBS in the assay medium, used for dosing, i.e., DMEM medium, supplemented with 2% or 0.2% (v/v) FBS. From the dose-responses, no apparent differences were observed at 2% or 0.2%. Thus, 0.2% was selected for this early-stage bioassay.

Luciferase reporter gene assay kits: Two commercially available Luciferase Reporter Gene Assay kits, LucLite® from PerkinElmer and Steady-Glo® from Promega, were compared for their performance in the early-stage bioassay. Steady-Glo® was approximately 5 times more concentrated (i.e., higher signal) than LucLite®. However, both systems were very similar with LucLite® showing a slightly better expansion on the dose-response curves. In the reporter gene assay luminescence is normally measured after a 10-20 minute incubation period. When measured after a 2 hour incubation period, Steady-Glo® was shown to lose more signal when compared to LucLite®. Other specifications were taken into consideration (price, number of vials per kit, storage conditions, composition, etc) to select LucLite® for the early-stage bioassay with Steady-Glo® as the alternative kit.

9.3.3 Sample Preparation

As for the late-stage bioassay validation approaches on **Storage vials and concentrations, Storage temperature and time, Dilution buffer and Sample dose-range**, similar experiments were carried out for the early-stage in parallel to the late-stage bioassays.

9.4 Results

The potencies of the various IFN preparations produced at NPL were measured by bioassay and compared against the working reference standard EDQM CRS and the 2nd IS for IFN- α 2a and/or - α 2b. All bioassay raw data, i.e., IFN dose-response curves of luminescence (counts of 5 seconds per well) versus IFN concentration (ng/mL), were plotted (Figure 40) and examined both graphically and statistically as described for the late-stage *in vitro* bioassays.

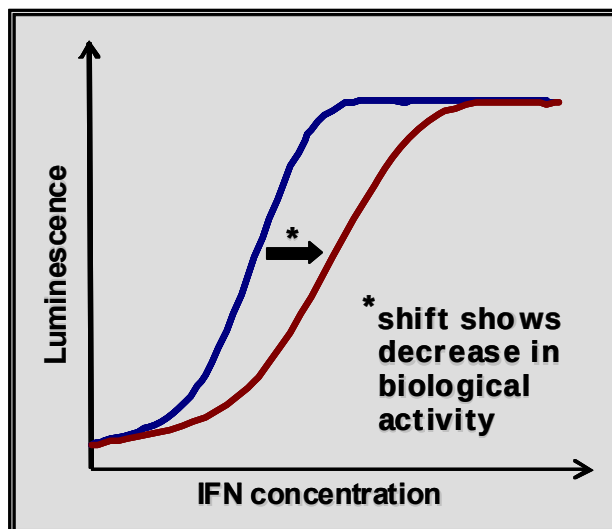


Figure 40. Schematic of an IFN dose-response curve, representing luminescence versus IFN concentration.

9.4.1 Biological activity of Interferon-alpha 2 batches

The five batches of IFN- α 2a and α 2b prepared at NPL were tested against EDQM and 2nd IS for their *in vitro* biological activity. A selection of dose-responses is shown in Figure 41.

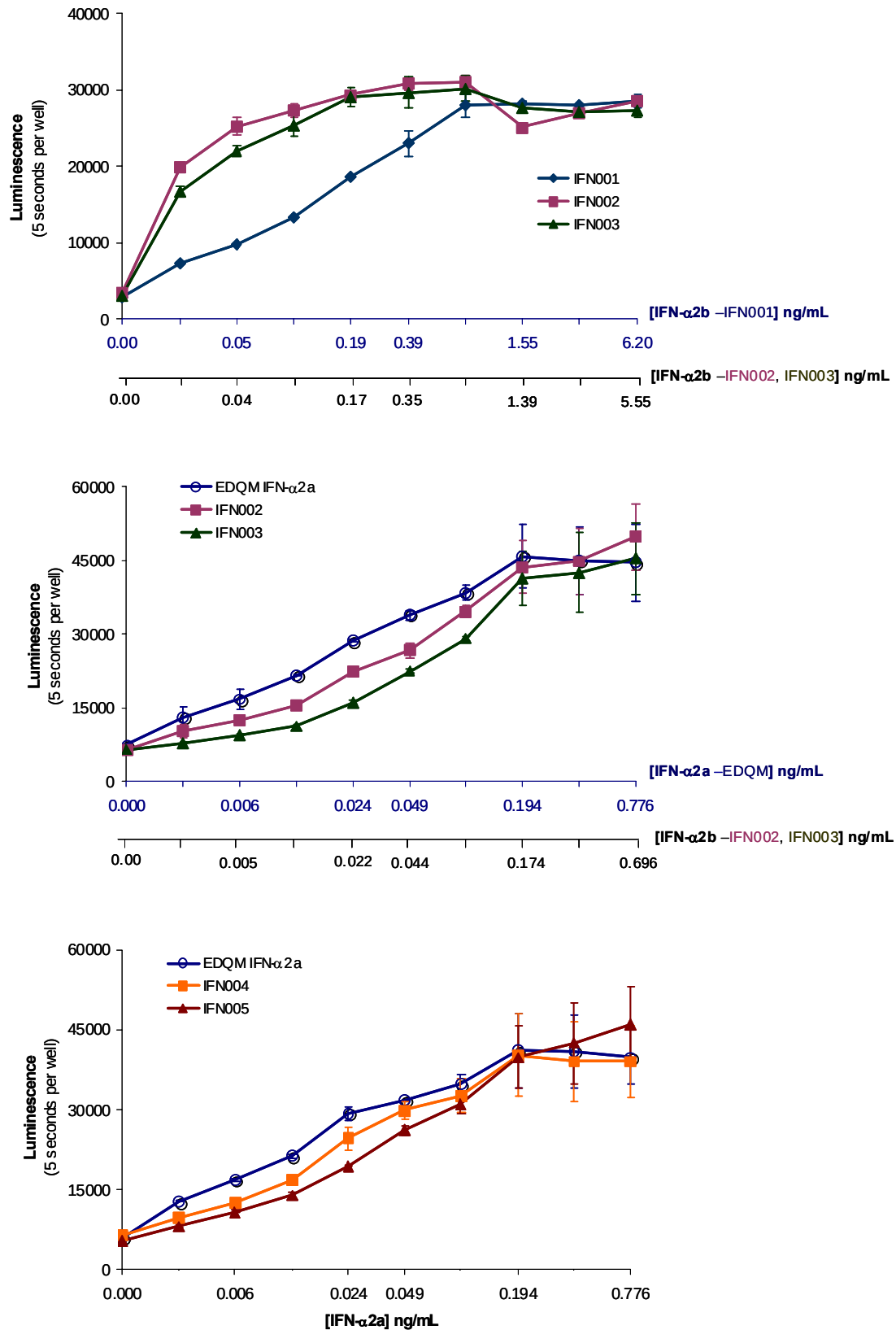


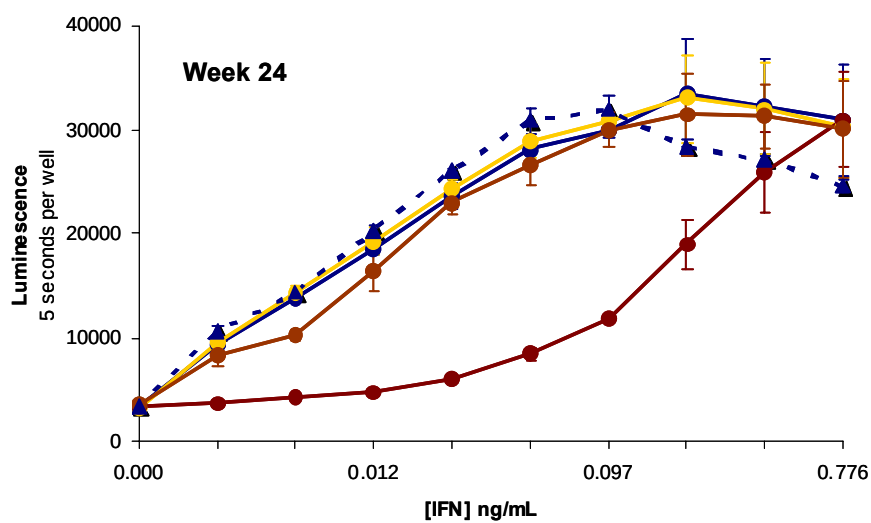
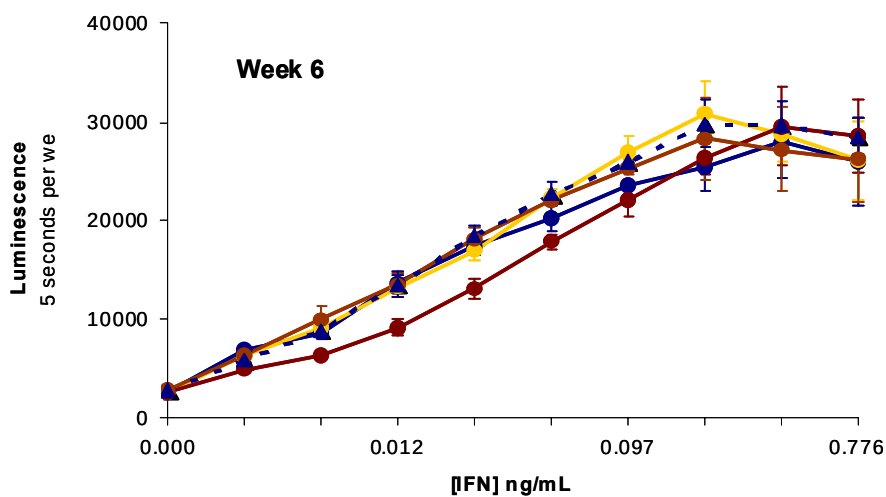
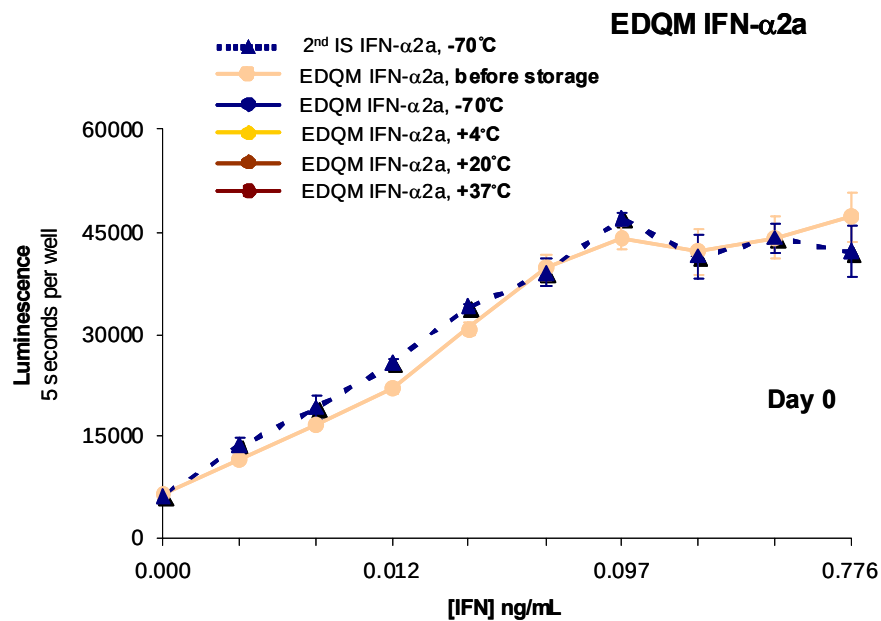
Figure 41. IFN dose-response curves for all NPL batches and EDQM IFN-α2a. Data are the means ± standard error (n=2).

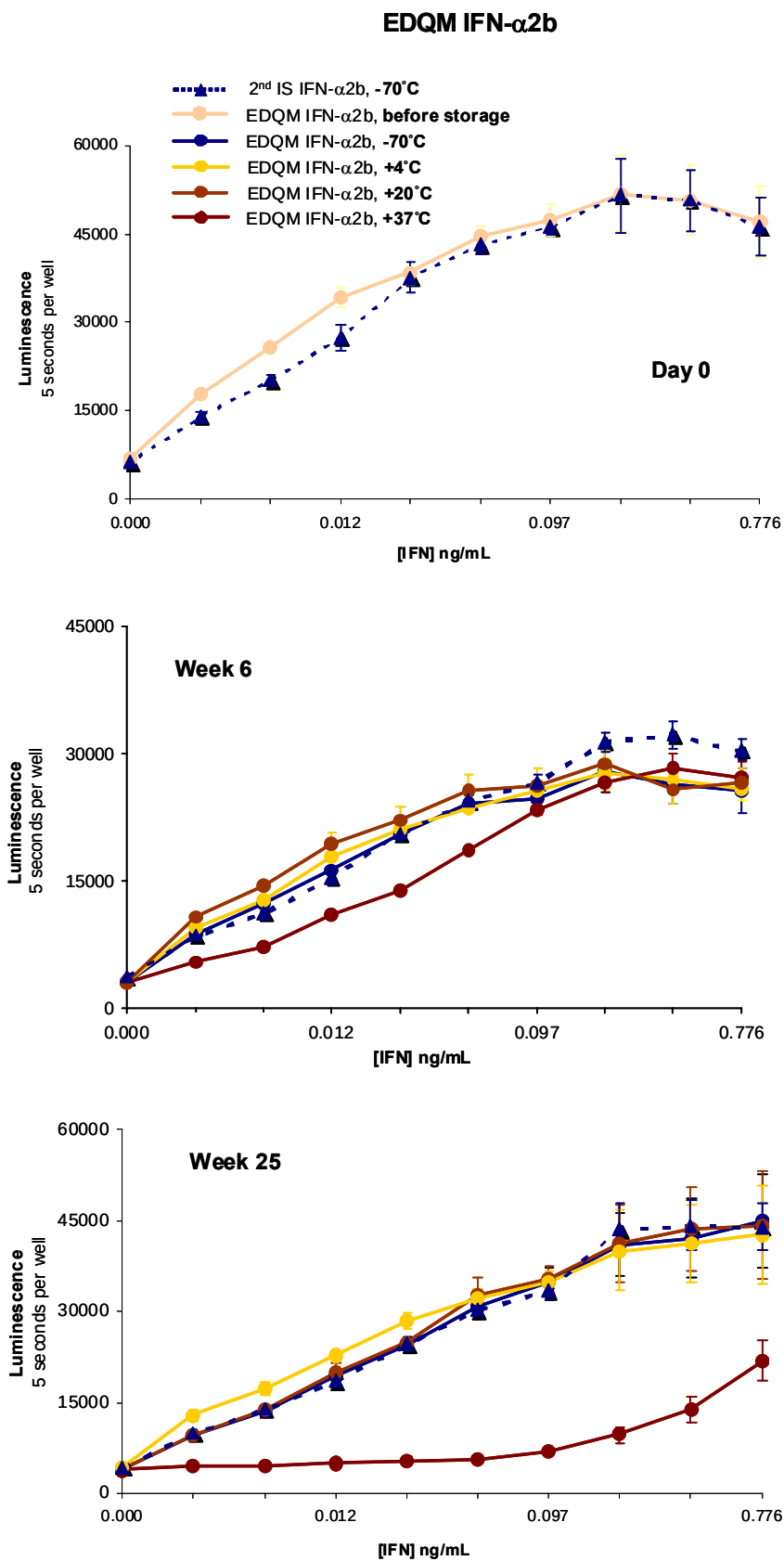
Table 21. Comparison by early-stage bioassay of specific activity of the *in-house* IFN- α 2 batches relative to EDQM IFN- α 2a. Values represent the mean of two ⁽²⁾ or four ⁽⁴⁾ estimates, i.e., 2 or 4 plates within 1 assay. Limits based on intra-assay variation.

Materials	<u>Relative Activity</u> compared to EDQM IFN-α2a (CRS I0320300)	95% confidence limits
IFN001, IFN-α2b	-	-
IFN002, IFN-α2b	0.47⁽²⁾	0.42 – 0.52
IFN003, IFN-α2b	0.23⁽²⁾	0.20 – 0.26
IFN004, IFN-α2a	0.69⁽⁴⁾	0.63 – 0.75
IFN005, IFN-α2a	0.51⁽⁴⁾	0.47 – 0.56

The early-stage bioassay data reflected the late-stage data for the NPL produced batches, which also showed a lower specific activity compared to the EDQM reference sample. Again, there was no relationship between biological activity and flask or fermentor-produced material, however the relative activity of batches IFN004 and IFN005 (α 2a) were higher than that of IFN-002 and IFN003 (IFN- α 2b).

The loss of biological activity over changes in storage temperatures/times, as part of the stability studies described in section 5.3.10, was monitored for EDQM IFN- α 2a and α 2b and the *in house* IFN- α 2b, IFN003. As with the late-stage bioassay, the early-stage bioassay also showed a decrease in biological activity for all three IFN preparations at higher temperature and longer storage times. A selection of the results is shown in Figure 29.





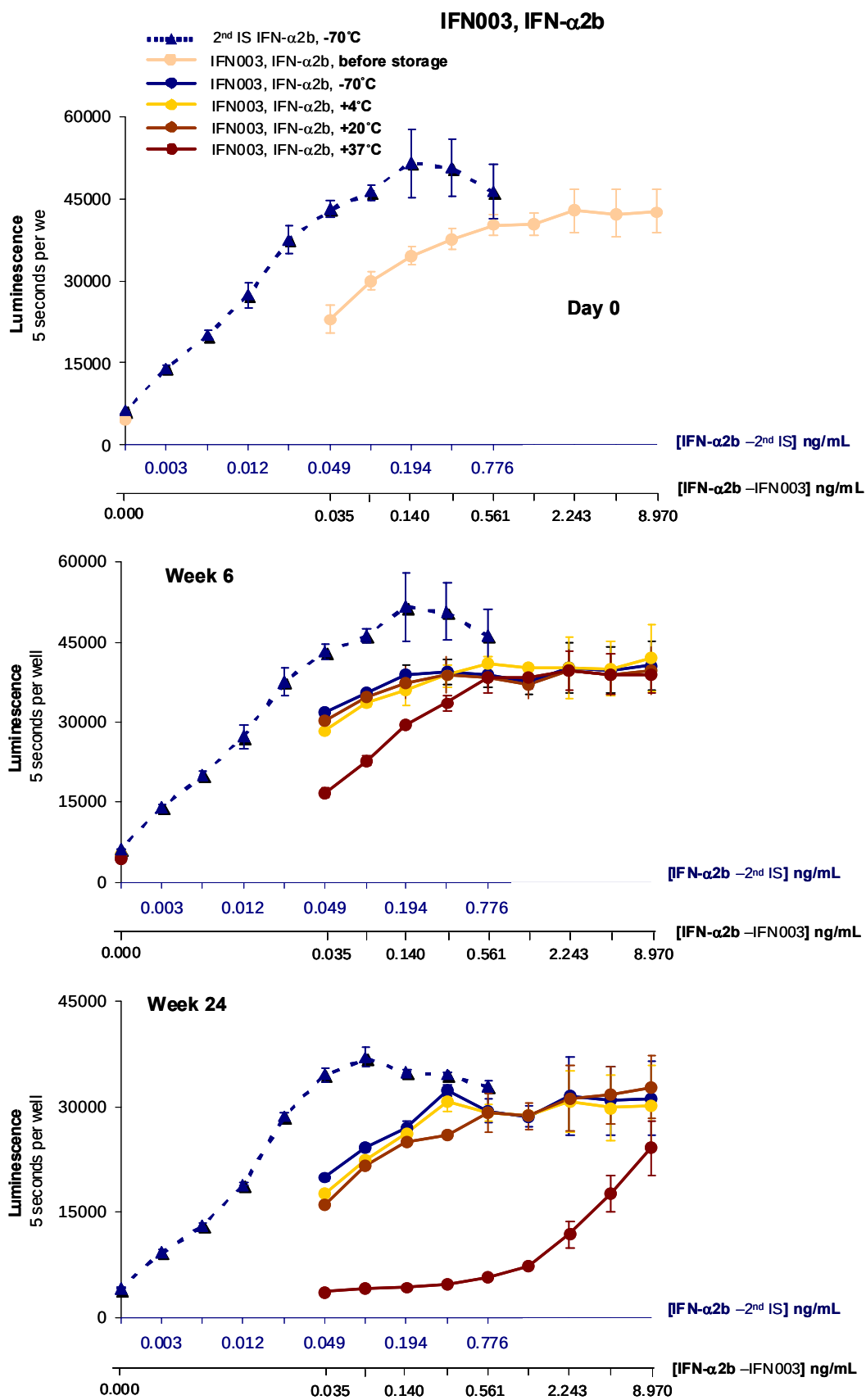


Figure 42. Selection of IFN dose-response curves for EDQM IFN- α 2a and - α 2b and IFN003 (IFN- α 2b), at selected time/temperature points, as typical examples to illustrate the stability studies performed. The 2nd IS at -70 °C used as reference standards are plotted assuming 2.52×10^8 IU/mg (- α 2a) and 1.4×10^8 IU/mg (- α 2b). Data are the means $\pm$ standard error (n=2)

9.4.2 Summary of late-stage and early-stage bioassay results – stability study

Table 22. Comparison by early- or late-stage bioassay of specific activities of EDQM IFN- α 2a, EDQM IFN- α 2b and the *in-house* IFN003 (IFN- α 2b), stored at +4 °C, +20 °C or +37 °C for periods up to 25 weeks, relative to a sample of the same IFN preparation stored at -70 °C, for the same periods. Mean of two estimates except as marked; (1) for one estimate, (3) for three estimates and (4) for four estimates, i.e. 1, 2, 3 or 4 plates within an assay. Note: (a), (b) and (c) represent three independent studies.

Materials	Storage temp.	Specific Activity																			
		Early-stage bioassay: Storage times (weeks)										Late-stage bioassay: Storage times (weeks)									
		1	2	4	6	9	12	16	24	25	1	2	3.5	4	6	9	9.5	12	16	24	25
EDQM IFN- α 2a relative to EDQM IFN- α 2a, stored at -70°C	+4°C	(a) 1.04	1.04	1.10	1.13	-	-	0.98	1.14	-	0.77	0.94	-	1.26	1.09	-	0.96	-	1.53	1.37	-
		(b) 0.95	1.04	-	1.25		0.77	-	1.13		0.96	0.88		-	1.12		-	0.80	-	1.11	
	+20°C	(a) 0.57	1.03	1.19	1.06	-	-	1.71	0.92	-	0.76	0.90	-	1.14	1.01	-	0.85	-	1.77	1.23	-
		(b) 0.92	1.10	-	1.11		1.09	-	1.01		0.83	1.09		-	1.12		-	1.13	-	1.18	
	+37°C	(a) 0.83	1.06	0.84	0.61	-	-	0.29 (3)	0.05 (3)	-	0.34	0.77	-	0.80	0.56	-	0.47 (3)	-	0.36 (3)	0.12 (3)	-
		(b) 0.91	0.91	-	0.43		0.14 (3)	-	0.07		0.86	0.77		-	0.50		-	0.19 (3)	-	0.07	
EDQM IFN- α 2b relative to EDQM IFN- α 2b, stored at -70°C	+4°C	(c) -	1.51	-	0.94	1.03	1.15	-	-	1.48	-	1.03	0.89	-	1.14	0.94 (1)	-	1.54	-	-	1.22
	+20°C	(c) -	1.08	-	1.40	1.22	1.28	-	-	1.12	-	1.14	0.93	-	1.50	1.19	-	1.54	-	-	0.86
	+37°C	(c) -	0.84	-	0.29	0.11 (3)	0.10 (3)	-	-	0.02 (3)	-	0.97	0.66	-	0.54	0.30	-	0.19 (3)	-	-	0.02 (3)
IFN003 (IFN- α 2b) relative to IFN003, stored at -70°C	+4°C	(b) 0.92	0.89	-	0.80 (4)	-	-	-	0.83 (4)	-	0.90	0.83	-	-	1.00 (4)	-	-	-	-	0.77 (4)	-
	+20°C	(b) 0.80	0.82	-	0.89 (4)	-	-	-	0.63 (4)	-	0.84	1.29	-	-	1.20 (4)	-	-	-	-	0.81 (4)	-
	+37°C	(b) 0.77	0.66	-	0.22 (4)	-	-	-	0.01 (4)	-	0.65	0.61	-	-	0.22 (4)	-	-	-	-	0.02 (4)	-

Table 23. Calibration by early- or late-stage bioassay of the various IFN samples, i.e. 2nd IS IFN- α 2a, 2nd IS IFN- α 2b, EDQM IFN- α 2a, EDQM IFN- α 2b and IFN003 (IFN- α 2b), stored at -70°C in terms of one another. The table shows the mean of assay means, mean of individual estimates, i.e. plates within an assay, respective geometric coefficients of variance (GCV) and 95% limits. n represents number of independent assays. (a), (b) and (c) represent three independent studies.

Reference	Materials (samples / study/ assay type)	Mean of assay means	GCV	n	95% limits	Mean of individual estimates	GCV	n	95% limits
2 nd IS IFN- α 2a	EDQM IFN- α 2a / (a) / early	0.77	29%	7	0.61 - 0.98	0.75	32%	14	0.64 - 0.88
	EDQM IFN- α 2a / (b) / early	0.79	24%	6	0.63 - 0.98	0.78	27%	11	0.66 - 0.91
2 nd IS IFN- α 2a	EDQM IFN- α 2a / (a) / late	0.59	36%	8	0.45 - 0.76	0.58	38%	18	0.49 - 0.68
	EDQM IFN- α 2a / (b) / late	0.65	23%	6	0.52 - 0.80	0.65	24%	12	0.57 - 0.75
2 nd IS IFN- α 2b	EDQM IFN- α 2b / (c) / early	1.27	21%	6	1.04 - 1.55	1.27	34%	12	1.06 - 1.53
	EDQM IFN- α 2b / (c) / late	1.08	40%	7	0.79 - 1.47	1.08	49%	14	0.86 - 1.36
2 nd IS IFN- α 2a	IFN003 (IFN- α 2b) / (b) / early	0.49	23%	5	0.38 - 0.64	0.46	24%	9	0.39 - 0.54
	IFN003 (IFN- α 2b) / (b) / late	0.10	19%	5	0.08 - 0.12	0.09	21%	10	0.08 - 0.11
EDQM IFN- α 2a	IFN003 (IFN- α 2b) / (b) / early	0.65	44%	5	0.41 - 1.02	0.61	37%	9	0.48 - 0.78
	IFN003 (IFN- α 2b) / (b) / late	0.16	24%	5	0.12 - 0.20	0.15	23%	10	0.13 - 0.17

Comparisons for estimates of the potency of one sample stored at -70°C in terms of another sample stored at -70°C , considered over all time points, showed no significant differences between the time points relative to the within assay variabilities of estimates, at the same time point. The overall means for these comparisons are shown in Table 23 both for the mean of the assay means, and for all individual estimates.

Comparisons of estimates for samples stored at $+4^{\circ}\text{C}$ in terms of the same sample stored at -70°C did not differ significantly between the different time points, and estimates showed no trend across the different time points (Table 22).

Comparisons of estimates for samples stored at $+20^{\circ}\text{C}$, in terms of the same sample stored at -70°C , showed marginal differences between the different time points ($p < 0.1$) for EDQM IFN- $\alpha 2a$ in the early- and late-stage assays of study (a), for IFN003 (but not for EDQM IFN- $\alpha 2a$) in the early- and late-stage assays of study (b), and for EDQM IFN- $\alpha 2b$ in the late-stage assays of study (c). However, in no case was there a significant regression of relative potency on time, suggesting that these differences are a reflection of between assay variability, and not of a consistent loss of activity.

Comparisons of estimates for samples stored at $+37^{\circ}\text{C}$, in terms of the same sample stored at -70°C , showed highly significant ($p < 0.01$) differences between estimates at the different time points in all cases, and in all cases there was a highly significant negative regression of logarithm of potency on time of storage. This indicates a clear relationship between the physicochemical data which shows the appearance of aggregates and oxidised species in material that had been stored at 37°C , and a loss in biological activity in both the early and late-stage bioassay formats.

Note the large differences (a factor greater than 4) between estimates by early- and late-stage assays when IFN003 is compared with 2nd IS IFN- $\alpha 2a$ or EDQM IFN- $\alpha 2a$. This contrast with comparisons of 2nd IS IFN- $\alpha 2a$ and EDQM IFN- $\alpha 2a$, and of EDQM IFN- $\alpha 2b$ and 2nd IS IFN- $\alpha 2b$, where there may be a difference between the two assays, although it is not clearly significant for these data.

9.4.3 Biological activity of methionine oxidised Interferon-alpha 2a

EDQM IFN- α 2a containing oxidised residues was tested against the working reference EDQM, prior to oxidation. Assays were carried out to measure changes in the biological activity of IFN following oxidation. A selected dose-response is shown in Figure 43.

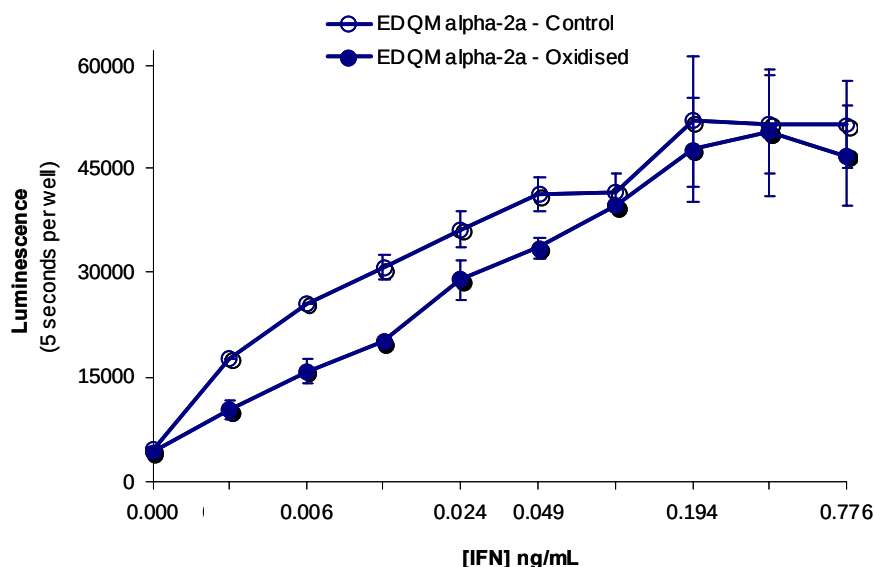


Figure 43. A selected IFN dose-response curve for methionine oxidised EDQM IFN- α 2a compared to a control. Data are the means $\pm$ standard error (n=2).

The dose-response curves indicate a decrease of *in vitro* biological activity of EDQM IFN- α 2a following the oxidation of methionine residues. For the late stage bioassay, the overall decrease in relative biological activity for oxidised material was 0.55 (0.45-0.68). This may indicate different sensitivities of the formats to oxidised variants of IFN- α 2a, however a full validation exercise is necessary to support this statement.

Table 24. Comparison by early-stage bioassay of specific activity of the methionine oxidised EDQM IFN- α 2a relative to a control, i.e. sample of EDQM IFN- α 2 exposed to the same conditions as the modified material, without the oxidising reagent. Values represent the mean of four estimates, i.e., 4 plates within 1 assay. Limits based on variation of estimates combined.

Material	<u>Relative Activity</u> compared to control EDQM IFN-α2a	95% confidence limits
Methionine oxidised EDQM IFN-α2a	0.35	0.31 – 0.39

9.5 Summary – linking physicochemical data to early-and late-stage bioassay data

IFN- α 2a and - α 2b are prone to aggregation and oxidation of methionine residues when exposed to long-term storage at 37°C. The degradation pathways were confirmed by exposing the molecule to stress conditions that accelerate these changes, and analysing the material in a range of physicochemical techniques. SDS-PAGE, SEC and RP-HPLC were demonstrated to detect changes to the molecule that were important for biological activity - a decrease in relative biological activity was observed in two cell-based biological assays with different biochemical endpoints. Sources of variability in the two assay formats were identified so that a statistically significant analysis was possible.

The same methods were used to analyse batch-to-batch variability in material produced at NPL, and allow comparison to an EDQM reference material. The electrophoretic and chromatographic (SEC, RP-HPLC) profiles showed differences between batches that would warrant further investigation in a biopharmaceutical product development schedule. All batches had a lower relative activity when compared to the reference standard, in both the late-stage and early-stage bioassays.

9.6 Quantitative PCR approaches

With scientific advances and the development of new techniques, new possibilities for functional bioassay systems are constantly arising. Quantitative real time PCR (qRT-PCR) appeared to have potential as an early-stage functional bioassay, so we have investigated its use. When responsive cells are dosed with IFN, several genes are rapidly induced while the expression of others is inhibited, resulting in changes in cellular concentrations of the corresponding messenger RNA (mRNA) species. Measurement of specific mRNA levels could provide a readout for early-stage assays, for the potency of IFN preparations and other biologically active compounds. In order to provide a suitable readout, the change in the specific mRNA level must be quantifiable with sufficient precision and related to the dose of IFN over an appropriate IFN concentration range. The measurement of changes in mRNA levels through synthesis of the corresponding complementary strands of DNA (cDNA) and use of Quantitative Real Time-Polymerase Chain Reaction (qRT-PCR) has been investigated as a possible early-stage functional bioassay for the potency of IFN preparations. Comparison with results from the bioassays measuring IFN-induced inhibition of Daudi cell proliferation and reporter gene activation in A549 cells demonstrate the feasibility of using qRT-PCR as an early-stage readout for IFN potency measurement. Further studies, using another biologically active molecule, vascular endothelial growth factor (VEGF) and responsive endothelial cells demonstrated that this technique could be applicable to a range of biopharmaceutical products.

The mRNA levels can be measured by synthesis of the corresponding cDNA followed by qRT-PCR. Quantitative RT-PCR is a technique for the *in vitro* amplification of specific DNA sequences, by the simultaneous primer extension of cDNA, measured by the detection and quantitation of a fluorescent reporter. This signal increases in direct proportion to the amount of PCR product in a reaction. By recording the amount of fluorescence emission at each cycle, it is possible to monitor the PCR reaction during exponential phase, where the first significant increase in the amount of PCR product correlates to the initial amount of target template. The exponential phase is the optimal point for analysing data. This is an advantage over traditional PCR methods, which use agarose gels for detection of PCR amplification at the final

phase or end-point of the PCR reaction. RT-PCR allows a highly sensitive quantification of transcriptional levels of the gene of interest in a few hours with minimal handling of the samples. As an analytical assay method, qRT-PCR could measure the biological activity of a product, offering a degree of specificity dependent on the specificity of induction of the gene being monitored.

The mRNA levels for several genes, from several cell lines, were screened for a quantifiable IFN-dose dependent change. The study concentrated on qRT-PCR experiments on the A549/93D7 cell line transfected with MxA promoter-luciferase (described on section 9). The use of this cell line enabled measurements of the MxA mRNA levels and reporter gene activation in the same cell stock, dosed with the same IFN preparations in the same experiment to facilitate comparison of the two assay techniques.

The mRNA was isolated from stimulated cells, after 90 min incubation with EDQM IFN- α 2a, and from control cells (no IFN), using a RNeasy Protect Mini Kit (Qiagen, 74126) according to the manufacturer's instructions. Oligo(dT)₁₅ and random hexamers were added to the mRNA. The mixture was then heated at 70°C for 5 min to remove the secondary RNA structure and cooled on ice. Molecular grade DL-dithiothreitol, deoxynucleotide triphosphates, recombinant ribonuclease inhibitor and reverse transcriptase MMLV-RT were added and the mixture incubated at 42°C for 50 min, followed by 15 min incubation at 70°C to inactivate MMLV-RT. The cDNA was stored at -40°C for future use or diluted with molecular grade water for PCR analysis. The remaining mRNA that was not reverse-transcribed was stored at -70°C.

Quantitative RT-PCR amplification was performed on a Rotor-Gene™ 6000 series real time rotary system (Corbett Research) using a SensiMix DNA kit (Quantace) containing nucleotides, heat activated DNA polymerase, MgCl₂ and SYBR® Green I. Selected forward and reverse PCR primers were used to detect the expression of genes such as MxA in the IFN stimulated and control A549/93D7 cells. The housekeeping gene GAPDH was amplified in the same samples to allow for normalisation of the initial amount of cDNA.

Figure 44 represents one of the qRT-PCR experiments carried out on A549/93D7 cells incubated with a preparation of EDQM IFN- α 2a. Results obtained indicated an increase in the MxA expression levels with an increase in the IFN dose concentration range, with maximal responses at 0.194 ng/mL. Other genes, such as the IFN inducible gene 6-16, were investigated and showed similar patterns of expression. The same IFN preparation analysed by qRT-PCR was also tested via the early-stage MxA-luciferase reporter gene assay for comparison. Activation of the reporter gene over the same IFN dose concentration range was observed as shown in Figure 44. These results indicate that qRT-PCR could serve as an early-stage bioassay to measure the biological activity of IFN preparations.

This work was performed as part of a collaboration with Dr. Chris Burns, Principal Scientist at NIBSC.

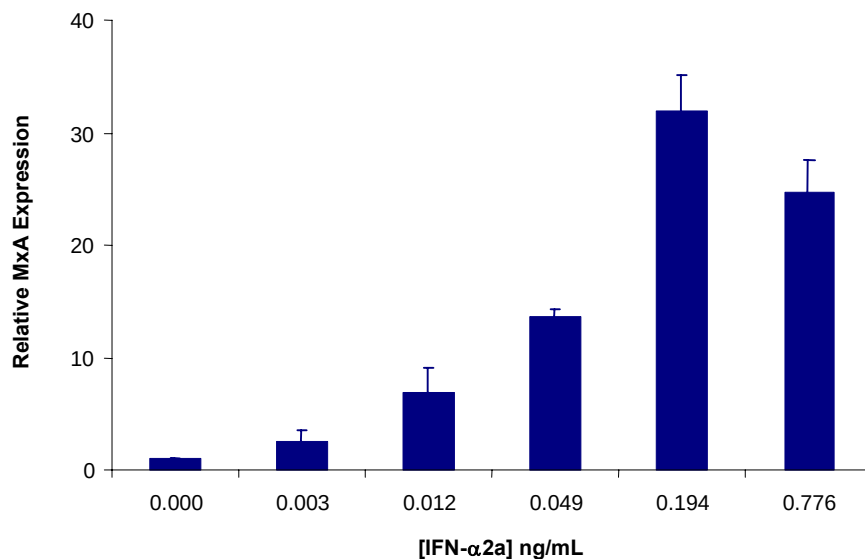


Figure 44. Mean relative expression levels for MxA gene induction in A549/93D7 cells after 90 min incubation with EDQM IFN-α2a. Data are the means ± standard error ($n = 2$).

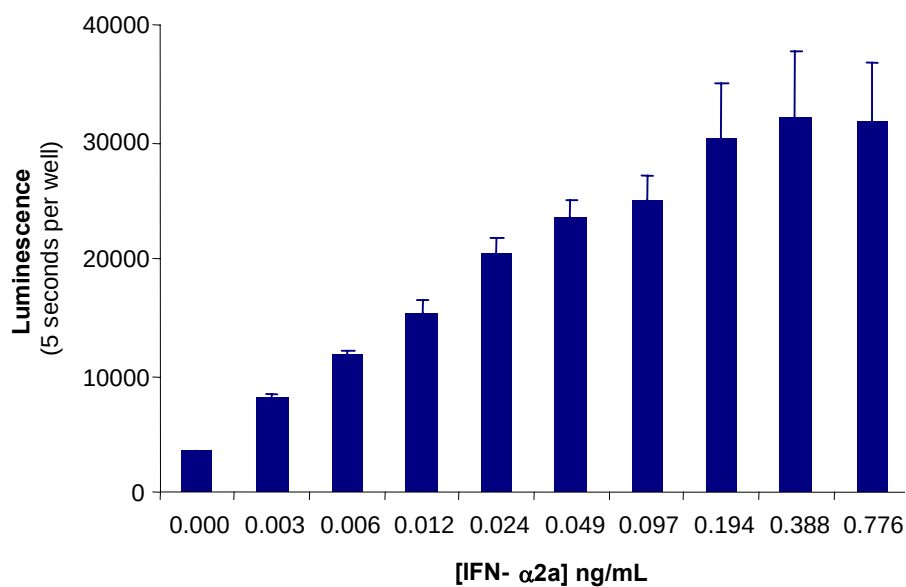
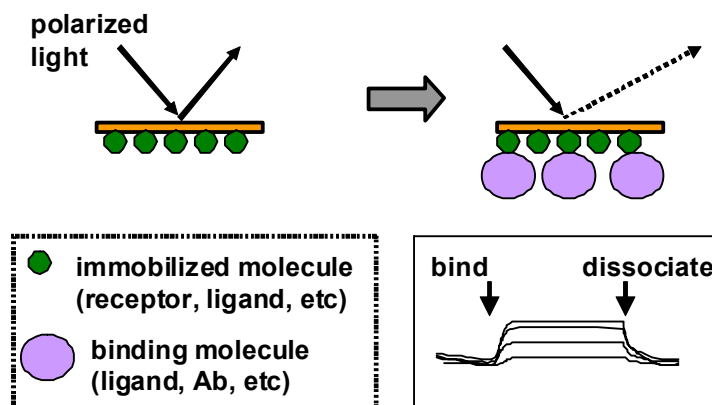


Figure 45. Selected IFN dose-response curve from the MxA-luciferase reporter gene activation assay on A549/93D7 cells, after 6 hours incubation with EDQM IFN-α2a. Data are the means ± standard error ($n = 2$).

9.7 Biacore measurement of protein-receptor binding

One of the objectives of the project was to develop an *in vitro* physicochemical protein binding assay for the interferon-alpha molecule, and to correlate the data to the cell-based bioassays for monitoring biological activity. As described in other sections of this report, cell-based assays are inherently variable and can report high (20-30%) coefficients of variation. The aim was to develop an assay that retained the initial receptor binding event that could be measured in a physicochemical method. If the data was comparable to that obtained from cell-based assays and could identify changes in receptor binding of different batches of material or populations of structural variants, the robustness and repeatability of the techniques was to be examined. A technique based on surface plasmon resonance (Biacore T100 equipment) was identified for this work package, which necessitated the expression and purification of a soluble receptor for the IFN- α molecule. In brief, one component of the receptor-IFN- α pair is immobilised onto a gold sensor surface, from which polarized light is reflected from a supporting glass plane. If binding occurs as the ligand passes over the surface, the interaction is monitored in real time, due to a change in angle at which polarized light is reflected. The change in angle, caused by binding or dissociation of molecules from the sensor surface, is proportional to the mass of bound material and is recorded in a sensorgram. The kinetics of association or dissociation can be recorded and information on the affinity of the interaction deduced (Figure 46).

Surface plasmon resonance (SPR) – eg. Biacore



cjr24a pr07

Figure 46. Schematic of Surface Plasmon Resonance technology.

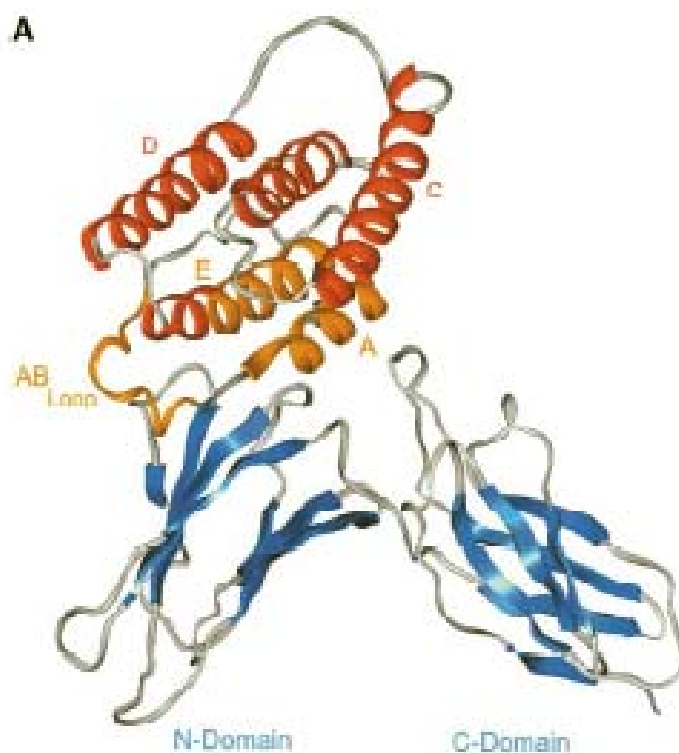


Figure 47. The IFNAR2 IFN binding ectodomain (IFNAR2-EC) binding to IFN- α (Chill et al, 2003)

9.7.1 Structure of the Interferon receptor

Type I interferons (of which human IFN- α 2a and IFN- α 2b are examples) share a common receptor, consisting of 2 transmembrane sub-units; IFNAR1 and IFNAR2, which associate to form a ternary complex with interferon. IFNAR2, which is the principle ligand-binding component of the complex, has a nanomolar affinity for IFN α (K_d 2-10 nM), while the IFNAR1 is of much lower affinity (K_d >100 nM). The affinity of the receptor for IFN is increased up to 20-fold on formation of the ternary complex. Formation of the complex activates a number of cellular signalling cascades, resulting in the characteristic functions of interferons – inhibition of viral replication and cell growth, and control of apoptosis (38).

IFNAR1 and IFNAR2 receptors belong to the family of helical cytokine class II receptors. Homology modelling with other receptors of this class, such as the IFN γ R, gave the first indications of the possible 3-dimensional structure of the receptor. However, the expression and purification of the 25 kD soluble extracellular domain of IFNAR2 (IFNAR2-EC) (39) has allowed a detailed picture of the interaction of IFN with this domain to be established. Evidence that the binding site for IFN resides in this domain has been provided by showing that this domain retains the IFN-binding activity of IFNAR2, and identification of the specific residues involved in IFN binding has been obtained by mutagenesis and immunoblocking studies.

The molecular basis of the IFNAR2-EC / IFN interaction has been extensively investigated, and a number of models of the interaction of IFNAR2-EC and IFN have been published. A detailed NMR-based model (40) showed that the IFNAR2-EC domain is comprised of two fibronectin-III domains connected by a helical hinge region. The IFN α -2 binding site is a contiguous surface on the N-terminal domain, including the S3-S4 loop (residues 44-53), S5-S6 loop and S6 β -strand (residues 74-82), and the S7 β -strand and hinge region (residues 95-105). The C-terminal domain does not contribute significantly to ligand binding. D₂O-exchange experiments indicated that ligand-binding induces pronounced tightening of the N-terminal domain of IFNAR2-EC.

The detailed multidimensional NMR structure of IFNAR2-EC was published in 2003 (40) together with a model of the IFNAR2-EC / IFN- α 2 complex using, additionally, the NMR structure of IFN- γ 2 solved previously (15). This model showed that the receptor binding site is formed by an extensive and predominantly hydrophobic patch on the receptor, which interacts with a matching hydrophobic surface on IFN- α 2. It was suggested that an adjacent area of alternating charged residues on each molecule guides IFN to the receptor binding residues. The lack of involvement of the C-domain in ligand binding was confirmed.

A number of spectroscopic techniques employing immobilised IFNAR2-EC or IFN have been employed to determine the binding constants for the receptor / ligand interaction. Surface plasmon resonance (SPR) measurements using a Biacore SPR instrument (39) have generated K_d values of between 8 nM and 10 nM for the IFNAR2-EC / IFN- α 2 interaction – values confirmed by the same authors using reflectometric interference spectroscopy (RifS). Measurements using SPR-Phase Imaging for IFN- α 2a, IFN- α 2b and IFN- α 2c (41) have given K_d values substantially lower (1.6 nM, 1.5 nM and 0.7 nM, respectively).

9.7.2 Expression of IFNAR2-EC in *E.coli*

Attempts were made to express IFNAR2-EC as both inclusion bodies (using the pET21d vector) and as a soluble periplasmic protein (using the pET20b vector). The experimental details are included in Appendix 1.

Experiments to solubilise and re-fold the IFNAR2-EC from inclusion bodies were unsuccessful. It is often difficult to obtain soluble and active proteins from expression in *E.coli*. Overexpression can lead to the production of inclusion bodies which are insoluble aggregates of misfolded protein. These inclusion bodies can easily be purified, but the solubilization of the expressed protein can usually only be obtained using strongly denaturing conditions and a major problem is then to achieve an efficient folding *in-vitro*. As a result, the physicochemical binding assay was not developed as part of this project. Instead, the focus of the functional studies was the early and late stage cell-based bioassays. A number of other functional assays were investigated but the project did not have adequate time or resource to develop these

fully. As an alternative binding assay, a small feasibility study using FACS analysis was conducted.

9.8 FACS analysis approaches

Fluorescence-activated cell sorting (FACS) is a flow cytometry technology that simultaneously measure and then analyses multiple physical characteristics of single cells, as they flow in a fluid stream through a beam of light. It uses laser light to excite fluorescent labelled molecules (such as antibodies or proteins) attached to or located inside cells and subsequently detects the emitted fluorescent labelled molecules to measure cell surface and intracellular antigens and receptors, cell cycle status, intracellular ion flux and cell proliferation.

Preliminary experiments using FACS, in an attempt to analyse how the different preparations of IFN bound to Daudi cells through their receptors (i.e., specific or non-specific binding), indicated the need for extended optimisation of the method. Several parameters, such as concentration and incubation periods were shown to be a major source of variability due to issues related to receptor down regulation/internalisation. Furthermore, the lack of a commercially labelled anti-IFN- α 2a or α 2b suitable for FACS required extra experimental steps that could otherwise be avoided. The time and resources of the project precluded extensive development of this approach.

10 Results from peripheral studies

10.1 The use of the EP reference standard in biological assays

The use of the same preparation as a working standard for both biological assays and physicochemical tests could prove advantageous in the characterisation of biopharmaceuticals. As previously discussed, the EDQM supplies CRS for IFN- α 2a and for IFN- α 2b for specified physicochemical tests. However, no information is provided as to their biological activity. In contrast, the WHO provides the 2nd IS for IFN- α 2a and for IFN- α 2b, with activity defined in IU for calibration of biological activity of preparations of IFN. However, due to their formulation (ampoules containing human serum albumin in PBS) and the amount per ampoule (described in

previous sections), these standards are not suitable for most physicochemical assays, with no defined mass content for such assays. Using two different bioassay systems, work was carried out to find that EDQM CRS IFN- α 2a and IFN- α 2b had comparable activity with the 2nd IS for IFN- α 2a and IFN- α 2b. Preparation of both types of IFN were shown to have similar dose response curves (Figure 48), consistent with a similar biological activity in these assay systems. Estimates of relative activity for each CRS, in terms of the respective IS, showed specific biological activity for the CRSs of the same order as the nominal specific activity for the ISs (Table 25). However, the estimates of relative activity were not consistent between the two assays systems, emphasizing the need for a multi-laboratory calibration of these preparations within each system, if the CRS were to be used as a working standard for bioassays. For structure-activity studies, both physicochemical and biological activity characterisation are required for the same biopharmaceutical preparation. EDQM IFN- α 2a (CRS I03200300) and EDQM IFN- α 2b (CRS I03200301) may prove useful as working reference standards for some biological assays as well as for physicochemical tests, which could contribute complementary data towards the characterisation of biopharmaceutical preparations. As discussed earlier, if EDQM CRS are to be used as standards in physicochemical tests beyond the purposes for which they are supplied, it is desirable to have as much information from the suppliers regarding the formulation of the storage buffers.

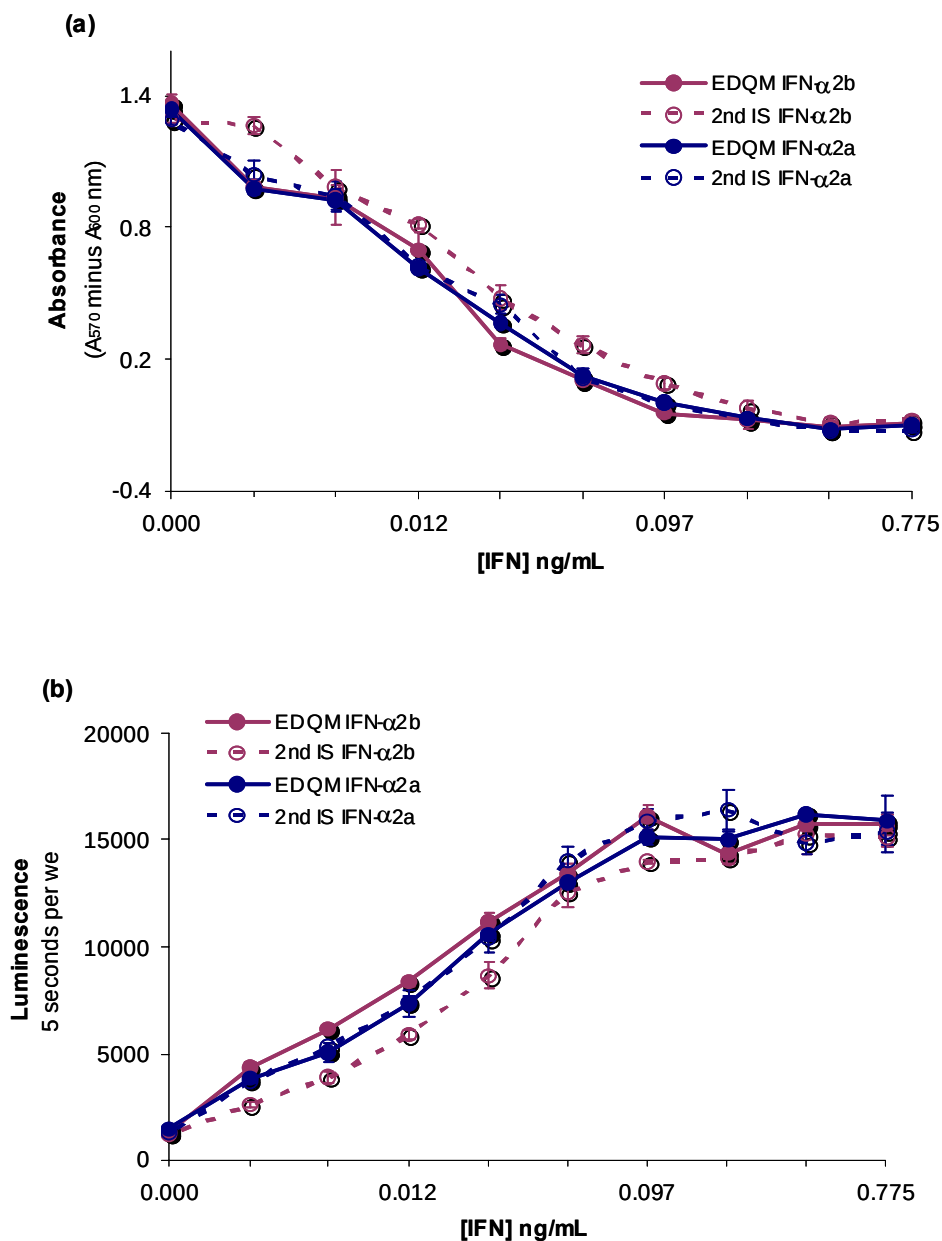


Figure 48. Typical IFN dose-response curves for EDQM and 2nd IS for IFN-α2a and -α2b: (a) from the late-stage bioassay; (b) from the early-stage bioassay. Data are the mean ± standard error (n=2). Note: 2nd IS plotted assuming 2.52×10^8 IU/mg for IFN-α2a and 1.4×10^8 IU/mg for IFN-α2b.

Table 25. Summary of the geometric mean estimates of relative activity for IFN- α 2a and IFN- α 2b, respective 95% limits and GCV, based on combined data for all plates within an assay system, i.e., late-stage anti-proliferation (AP) and early-stage reporter gene (RP) bioassay. n: number of independent assays.

Materials Compared	Assay System	Relative Activity (geometric mean)	n	95 % Limits	GCV %
Comparison of nominally identical samples of IS IFN-α2a (IU)	<i>AP</i>	0.98	4	0.73 – 1.31	20
	<i>RG</i>	0.90	4	0.74 – 1.11	14
Comparison of nominally identical samples of EDQM IFN-α2a (ng)	<i>AP</i>	1.08	7	0.98 – 1.18	10
	<i>RG</i>	1.05	4	0.89 – 1.23	11
EDQM IFN-α2a compared with IS IFN-α2a (ng)	<i>AP</i>	1.31	10	1.16 – 1.48	19
	<i>RG</i>	1.05	5	0.97 – 1.13	6
IS IFN-α2b compared with IS IFN-α2a (IU)	<i>AP</i>	0.90	3	0.80 - 1.02	8
	<i>RG</i>	0.99	1	-	-
EDQM IFN-α2b compared with EDQM IFN-α2a (ng)	<i>AP</i>	1.07	3	0.73 - 1.57	27
	<i>RG</i>	1.46	1	-	-
EDQM IFN-α2b compared with IS IFN-α2b (ng)	<i>AP</i>	1.58	3	1.29 - 1.94	14
	<i>RG</i>	1.46	1	-	-

10.2 Different sources of material – including PEGylated IFN- α 2

The bioassays developed in this case study were used to analyse native and pegylated IFN- α 2 sources, in order to investigate the sensitivity of the assays to other molecular forms. Late- and early-stage bioassays were performed on IFN- α 2a, pegylated IFN- α 2a and IFN- α 2b preparations provided by external collaborators. IFN- α 2a and pegylated IFN- α 2a samples were received in solution (at 20 °C), sub-divided into several aliquots (i.e., stock solution) and stored into 1.5 mL Sarstedt® V bottom shape polypropylene micro tube at -70 °C before use. Samples of IFN- α 2b were received lyophilised, reconstituted in 1 mL of sterile distilled water and sub-divided into several aliquots (i.e., stock solution) for storage at -70 °C before use. For assay, frozen aliquots of IFN stock solutions were quickly thawed in a water bath at +37 °C and prepared as working solutions by dilution in each assay media. SDS-PAGE and MS analysis were carried out for the IFN- α 2a and pegylated IFN- α 2a preparations (data not shown).

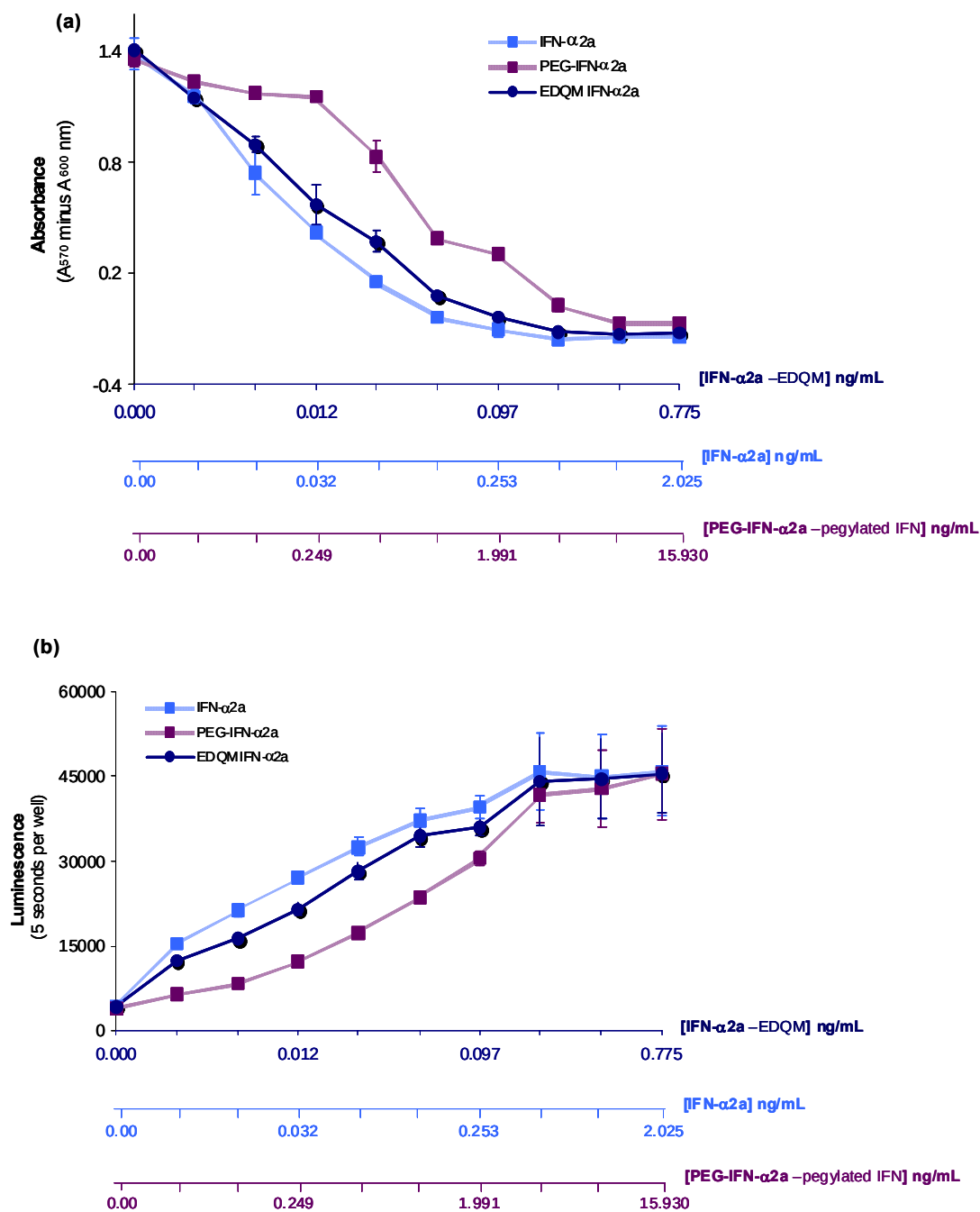


Figure 49. Selected IFN dose-response curves for 2 preparations of IFN- α 2a; native pure (IFN- α 2a) and pegylated (PEG-IFN- α 2a), and EDQM IFN- α 2a: (a) from the late-stage bioassay; (b) from the early-stage bioassay. Data are the mean $\pm$ standard error (n=2).

Table 26. Comparison by late- or early-stage bioassay of specific activities of 2 preparations of IFN- α 2, i.e., IFN- α 2a and pegylated IFN- α 2a (PEG- IFN- α 2a), relative to the EDQM IFN- α 2b. Values represent one estimate ⁽¹⁾ or the mean of two estimates ⁽²⁾, i.e., 1 or 2 plates within 1 assay. ⁽⁺⁾ Limits based on single estimate with GCV of approximately 30%. ⁽⁺⁺⁾ Limits based on two estimates with expected assay GCV of approximately 30%.

Materials	Late-stage bioassay		Early-stage bioassay	
	<u>Relative Activity</u> compared to EDQM IFN-α2a (CRS I0320300)	95% confidence limits	<u>Relative Activity</u> compared to EDQM IFN-α2a (CRS I0320300)	95% confidence limits
IFN-α2a	0.60⁽²⁾	0.42 – 0.86 ⁽⁺⁺⁾	0.51⁽¹⁾	0.31 – 0.86 ⁽⁺⁾
PEG-IFN-α2a	0.015⁽²⁾	0.010 – 0.022 ⁽⁺⁺⁾	0.018⁽¹⁾	0.011 – 0.030 ⁽⁺⁾

This native IFN- α 2a, like the NPL produced materials, showed lower biological activity than EDQM IFN- α 2a, in both early- and late-stage bioassays. The PEG-IFN- α 2a showed much lower biological activity than the native IFN. Pegylation has been reported (42) (43) to decrease *in vitro* activity through decreased receptor binding as a result of steric interference. However, for this sample physicochemical assays should be carried out to confirm integrity of the molecule after the PEGylation reaction. Although the conjugation of PEG molecules to proteins may be associated with a lower *in vitro* specific activity, it may not be associated with less activity *in vivo*. This modification increases plasma half-life and *in vivo* stability.

11 Conclusions and Recommendations

The deliverables for the project are based around the following work packages, which are contained within this report.

A report on the use of cell-based bioassays in the quality control of manufactured biopharmaceuticals, identifying generic sources of uncertainty. This incorporates a review of the industrial and regulatory debate on the application of structure-function relationships and recommendations on how to improve the efficiency of this work.
A section detailing the physicochemical and biological assays used to characterise the components of the model system.
A section detailing the development of any new assays required in order to deliver the project, e.g. cell-based assays or assays for the measurement of protein-protein interactions.
A section on the ability of cell-based assays and physicochemical assays to detect product variation, including statistical analysis of the data indicating the robustness of the systems and the contribution from measurement uncertainty.
Recommendations for the selection of key cell-based bioassays and physicochemical methods to establish a structure-function relationship for the class of model system used within this project.

Establishing relationships between data from different analytical techniques supports the selection of a suitable set of tests for the characterisation of individual biopharmaceutical preparations. In this case study a portfolio of physicochemical and biological assays was developed that examined batch-to-batch-variability in IFN- α 2a and IFN- α 2b preparations produced in a *Pichia pastoris* expression system, and in samples of IFN- α 2a and in IFN- α 2b from external collaborators. Throughout the case study, the NPL-produced material was compared to Chemical Reference Standards supplied by EDQM.

The feasibility of using EDQM as a physicochemical reference standard in a wide range of techniques was investigated. The reference standards are supplied by EDQM as “standards or reference substance in the tests and assays indicated ... (identification by peptide mapping and identification by electrophoresis) ..., in accordance with the official methods of the Ph. Eur and for no other purpose”. This study extended the physicochemical analyses of the reference materials to include reverse phase and SEC chromatography, LC and MALDI- TOF mass spectrometry, ELISA, dynamic light scattering, circular dichroism, steady-state tryptophan and ANS fluorescence methods. Early investigation did not discern the nature of the buffers that the EDQM

references were supplied in, in terms of the salt and excipient content, or the pH of the buffers. The physicochemical characterisation carried out in this project has shown that one should proceed with caution when using the standards beyond the purpose for which they are supplied. Changes in pH of buffers and excipient content can cause dramatic changes to the structure of the molecule. Where there is this information about the preparation, there are likely to be a greater number of applications for the standards.

Batch-to-batch variability in the NPL batches was observed, showing a change in the molecular profile that could be attributed to an incomplete processing of the peptide signal sequence in batches that were produced in a fermentor. Statistical correlation of changes in the molecular profile, as examined by physicochemical methods, to bioassay data was not possible, due to the extensive number of repeats that would have been required. However, the physicochemical data from SDS-PAGE, SEC and RP-HPLC in particular did reveal changes to the molecule that would prompt further examination in a product development scenario. Within a pharmaceutical company setting, resources are available for rigorous correlation of such data.

Sources of uncertainty in two cell-based bioassay systems were investigated and recommendations were made for plate set-up, serum concentrations, sample preparation, seeding density and passage number and cell maintenance procedures. Freedom from commercial pressures of development of an actual drug permitted the deliberate inclusion of some commonly encountered factors with potential for adverse consequences in order to illustrate the impact of these factors. In a commercial environment, economic criteria would preclude testing some factors of suspected high risk. The relative merits of early-and late-stage readouts were investigated and the study demonstrated a consistent response to IFN- α 2 measured by distinct endpoints.

For the stability study data, storage of the IFN- α 2 material revealed degradation pathways for this class of molecule, identified as aggregation and oxidation in the physicochemical assays. The changes to the molecule were also reflected in both the early and late-stage bioassay data and enforces the importance of early characterization of the product, in terms of structural and functional information.

11.1 Knowledge transfer activities

A wide range of dissemination activities were undertaken throughout the project.

Surveys conducted by NIBSC:

A survey of delegates' use of bioassays at the 2005 IBC European Biological Assays conference (Basel, 18–19 October)

A follow up survey on use of bioassays at the 2006 IBC European Biological Assays conference (Munich, 16-18 October)

Peer reviewed publications:

A Survey of Bioassays in the Biopharmaceutical Industry. C. Jane Robinson, Laureen E. Little, E. Clare Robinson, and Hans-Joachim Wallny, *BioProcess International* 4(6):24-36, June 2006)

Biological Activity of EDQM CRS for interferon alfa-2a and interferon alfa-2b, Assessment in two *in vitro* Bioassays. Silva M. M. C. G., Gaines-Das R. E., Jones C., Robinson C. J. Accepted to *PharmEuropaBio* (April 2007).

Data from the study, with acknowledgement of NMS MfB funding, included in invited presentations:

Oral presentations:

Customising Bioassays, C J Robinson. IBC's Validation for the Production of Biologicals (25-26 October 2005, Amsterdam, Netherlands).

Bioassays – where things can go wrong, C J Robinson. HPA external seminar, 20 June 2006, Centre for Emergency Preparedness and Response, Porton Down, UK

Measurement of Biological Molecules, Anna Hills. European Society for Animal Cell Technology UK meeting (4-5th January 2007, Wyboston, UK)

Protein Structure Measurement, Jonathan Moore. Pharmaceutical Analytical Sciences Group meeting (16-17 April 2007, Warwick, UK)

Trends in Bioassays, C J Robinson

IBC's 14th International Intensive Symposium Biological Assay Development & Validation, Techniques for Accelerating Product Approval, (23-25 April 2007, Boston, MA, USA)

Posters:

Strengthening Quality Control of Biopharmaceutical Products through the Establishment of Structure Function Relationships. Anna E. Hills; Marta Silva; Eleonora Cerasoli; Baptiste Lamarre; Jonathan Moore; Phil Nugent; Paulina Rakowska; Neelam Kumar swami; Jun Wheeler; Chris Jones; C Jane Robinson; Marc Bailey (WCBP 25-27 January 2006, Washington DC, USA)

Strengthening Quality Control of Biopharmaceutical Products through the Establishment of Structure Function Relationships. Anna E. Hills; Marta Silva; Eleonora Cerasoli; Baptiste Lamarre; Jonathan Moore; Phil Nugent; Paulina Rakowska; Neelam Kumar swami; Jun Wheeler; Chris Jones; C Jane Robinson; Marc Bailey (WCBP 2007 29-31 January 2007, Washington DC, USA)

Monitoring Stability of Interferon alfa-2a and Interferon alfa-2b by Bioassays and SDS-PAGE. Marta M. C. G. Silva; Jun Wheeler; Rose E. Gaines-Das; Anna Hills; Baptiste Lamarre; Eleonora Cerasoli; Phil Nugent; Neelam Kumarswami; Paulina Rakowska; Marc Bailey; C Jane Robinson; Chris Jones; (WCBP 2007 29-31 January 2007, Washington DC, USA)

A simple colorimetric cell-based bioassay for Interferon alfa 2a. M Silva, C Jones, C J Robinson. 196th Meeting of the Society for Endocrinology. (7-8 November 2005, London, UK)

Workshops:

Measurements for Biotechnology Programme dissemination workshop, 9th March 2007. The workshop was attended by over one hundred delegates, which included representatives from the biopharmaceutical industry, regulators, the DTI and academia. The day of presentations and posters on all projects in the 2004 to 2007 programme included an oral presentation on this project and two posters.

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13 Appendix

13.1 Appendix 1

13.1.1 Expression as inclusion bodies with pET21d:

pET21d_(NheI)_R2-transformed *E. coli* BL21(DE3) cells were grown at 37°C in LB broth containing 0.1mg/mL ampicillin. Protein expression was induced at A600=0.6 by the addition of IPTG to 1mM. Induction was performed overnight at 30°C and cells were harvested by centrifugation (2926 x g, 30min, rotor 5.3JA, Avanti™ J-20 XP Centrifuge) at different time points, and stored at -20°C until needed. Frozen sample were thawed on ice and mixed with an equal volume of 2 x LDS sample buffer (Invitrogen) containing mercaptoethanol (5%), and heated at 95°C for 10 mins. Cooled sample were centrifuged (2 min., 16000xg, Eppendorf 5415 D centrifuge) and 10µL from each analysed on a 12% Bis-Tris gel with MOPS buffer (Invitrogen). Gels were stained using Protoblue Safe Colloidal Coomassie G-250 (National Diagnostics), according to the manufacturers instructions.

13.1.2 Expression as a periplasmic protein with PET20b:

pET20b-transformed *E. coli* BL21 cells were grown at 37°C in LB broth containing 0.1mg/mL ampicillin. Protein expression was induced at A600=0.6 by the addition of IPTG to 1mM. Induction was performed overnight at 30°C and cells were harvested by centrifugation (2926 x g, 30min, 5.3JA rotor, Avanti™ J-20 XP Centrifuge) at different time points and stored at -20°C until needed. Whole cells and soluble protein/insoluble protein sample were prepared separately. Whole cell samples (1mL) were mixed with an equal volume of 2 x LDS sample buffer (Invitrogen) containing mercaptoethanol (5%), and heated at 95°C for 10 mins. Other sample (1.5 mL) were sonicated (Sonics Vibracell sonicator, 10 x 10 sec. pulses, amplitude 22%) and centrifuged (16000xg, 30 min, Eppendorf 5415 D centrifuge) to separate the insoluble protein pellet from soluble protein supernatant. The insoluble pellet was resuspended in water (1.5 mL), and samples (50µl) from soluble and insoluble protein were mixed with an equal volume of 2 x LDS sample buffer (Invitrogen) containing mercaptoethanol (5%), and heated at 95°C for 10 mins. Cooled samples were centrifuged (2 min., 16000xg, Eppendorf 5415 D centrifuge) and 10 µl from each was

analysed on a 12% Bis-Tris gel with MOPS buffer (Invitrogen). Gels were stained using Protoblue Safe Colloidal Coomassie G-250 (National Diagnostics), according to the manufacturers instructions.

13.1.3 Results

Expression of IFNAR2-EC as inclusion bodies was successful (Figure 50). After 2 hrs induction IFNAR2-EC protein (approx 25kD) was clearly visible in transformed and induced BL21(DE3), and continued to be present at 5 hrs and 21 hrs induction. At no time was the receptor expressed in untransformed, or transformed but non-induced, cells.

Expression of IFNAR2-EC as a soluble periplasmic protein was not so successful. Receptor was clearly expressed in transformed and induced BL21 cells at 4 hrs induction and 21 hrs induction (Figure 51A), and was absent in all non-transformed, or transformed but non-induced, cells. Interestingly, expression appeared to less after 21 hrs induction than at 4 hrs induction. However, analysis of the soluble and insoluble protein fractions from the same cells showed that the receptor was completely absent from the soluble protein fraction (Figure 51B), but was found in the insoluble protein fraction of 4 hr and 21 hr induced cells (Figure 51C).

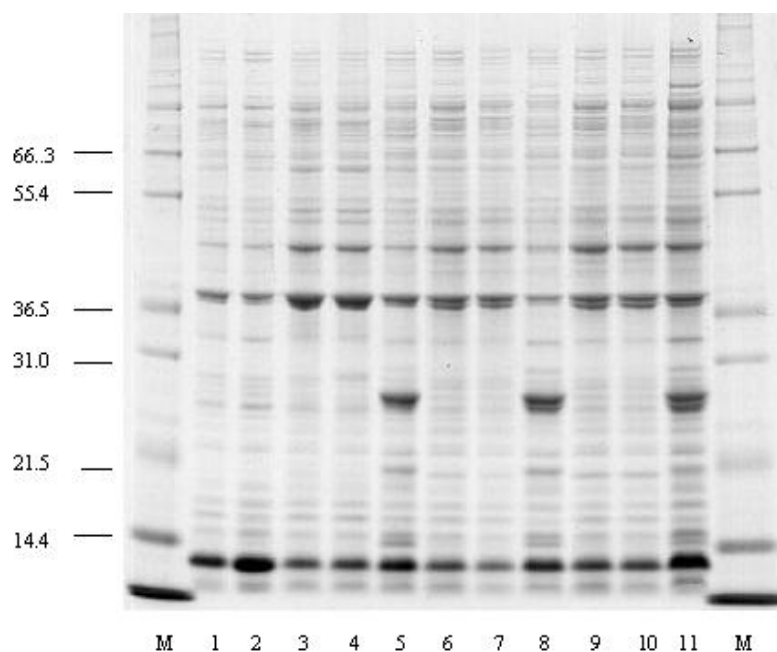


Figure 50. Time course of IFNAR2-EC expression as inclusion bodies in *E.coli* BL21. All gels were stained with Colloidal Coomassie G-250 stain. IFNAR2-EC is clearly visible in transformed and induced BL21 cells after 2 hrs induction.

Lane	Sample
1	Non-transformed and non-induced BL21 cells at t=0
2	Transformed and non-induced BL21 cells at t=0
3	Transformed and non-induced BL21 cells at t=2hr
4	Non-transformed and induced BL21 cells at t=2hr
5	Transformed and induced BL21 cells at t=2hr
6	Transformed and non-induced BL21 cells at t=5hr
7	Non-transformed and induced BL21 cells at t=5hr
8	Transformed and induced BL21 cells at t=5hr
9	Transformed and non-induced BL21 cells at t=21hr
10	Non-transformed and induced BL21 cells at t=21hr
11	Transformed and induced BL21 cells at t=21hr
M	Mark 12 Molecular weight standards (Invitrogen)

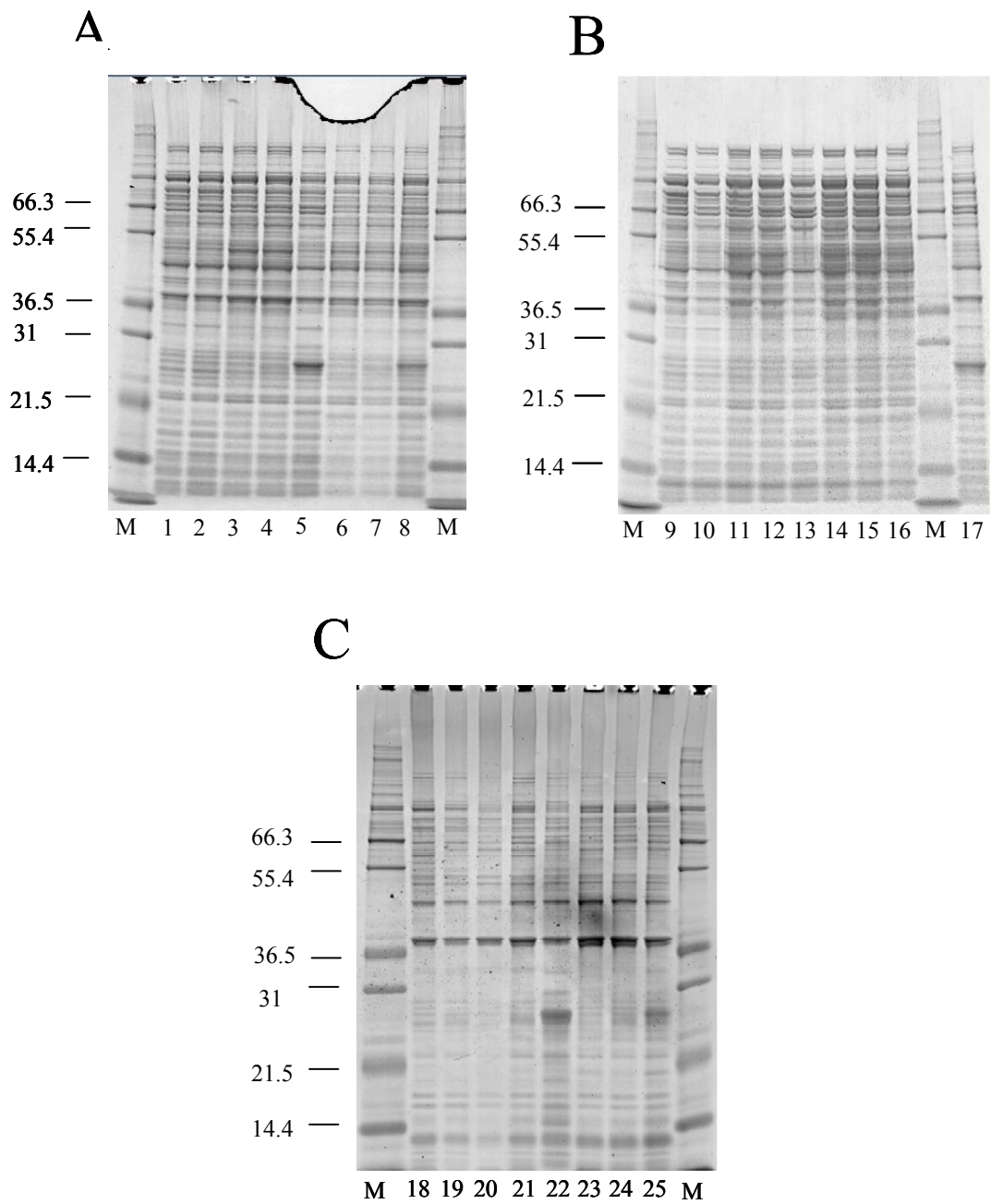


Figure 51. Time course of IFNAR2-EC expression from pET20b in *E.coli* BL21.

Panel A: Whole cell fraction: Expression of IFNAR2-EC visible at 4 and 21 hrs induction.

Lane	Sample
1	Non-transformed BL21 cells at t=0
2	Transformed, non-induced BL21 cells at t=0
3	Non-transformed BL21 cells at t=4 hrs
4	Transformed, non-induced BL21 cells at t=4 hrs
5	Transformed and induced BL21 cells at t=4 hrs
6	Non-transformed BL21 cells at t=21 hrs
7	Transformed, non-induced BL21 cells at t=21 hrs
8	Transformed and induced BL21 cells at t=21 hrs
M	Mark 12 Molecular weight standards (Invitrogen)

Panel B: Soluble protein fraction: Expression of IFNAR2-EC not visible.

Lane	Sample
9	Non-transformed BL21 cells at t=0
10	Transformed, non-induced BL21 cells at t=0
11	Non-transformed BL21 cells at t=4 hrs
12	Transformed, non-induced BL21 cells at t=4 hrs
13	Transformed and induced BL21 cells at t=4 hrs
14	Non-transformed BL21 cells at t=21 hrs
15	Transformed, non-induced BL21 cells at t=21 hrs
16	Transformed and induced BL21 cells at t=21hrs
M	Mark 12 Molecular weight standards (Invitrogen)

Panel C: Insoluble protein fraction: Expression of IFNAR2-EC visible after 4 and 21 hrs induction.

Lane	Sample
18	Non-transformed BL21 cells at t=0
19	Transformed but non-induced BL21 cells at t=0
20	Non-transformed BL21 cells at t=4 hrs
21	Transformed, non-induced BL21 cells at t=4 hrs
22	Transformed and induced BL21 cells at t=4 hrs
23	Non-transformed BL21 cells at t=21 hrs
24	Transformed, non-induced BL21 cells at t=21 hrs
25	Transformed and induced BL21 cells at t=21 hrs
M	Mark 12 Molecular weight standards (Invitrogen)

13.2 Appendix 2

Full Statistical Analysis of the Bioassays of the Stability Studies

Key: **asty**, assay type; **e**, early-stage bioassay; **l**, late-stage bioassay; **ptemp**, preparation and temperature; **ea**, EDQM IFN- α 2a; **na**, IFN003 (IFN- α 2b); **eb**, EDQM IFN- α 2b; **ia**, 2nd IS IFN- α 2a; **ib**, 2nd IS IFN- α 2b; **naea**, IFN003 (IFN- α 2b) stored at -70°C relative to EDQM IFN- α 2a stored at -70°C; **00**, -70°C; **04**, +4°C; **20**, +20°C; **37**, +37°C; **S000**, stored for 0 days; **S007**, stored for 7 days; **S014**, stored for 14 days; **S025**, stored for 25 days; **S028**, stored for 28 days; **S042**, stored for 42 days; **S063**, stored for 63 days; **S067**, stored for 67 days; **S084**, stored for 84 days; **S168**, stored for 168 days; **S175**, stored for 175 days. Note that Stability Study 1 has been represented as **(a)**, Stability Study 2 as **(b)** and Stability Study 3 as **(c)** on the main report section.

Assay Mean Potencies Stability Study 1

Relative to Ia00 for ea00, and all else relative to ea00

Mean of two estimates except as marked + for one estimate, * for 3 estimates and ** for 4 estimates.

asty	ptemp	S000	S007	S014	S028	S042	S067	S112	S168	Mean of Assay Means
e	ea00	0.78*	0.77	1.02	0.67	1.06+	.	0.49	0.74	0.77
e	ea04	.	1.04	1.04	1.10	1.13	.	0.98	1.14	1.07
e	ea20	.	0.57	1.03	1.19	1.06	.	1.71	0.92	1.03
e	ea37	.	0.83	1.06	0.84	0.61	.	0.29*	0.05*	NV
l	ea00	0.50**	0.61	0.66	0.68	0.63	0.93	0.31	0.55	0.59
l	ea04	.	0.77	0.94	1.26	1.09	0.96	1.53	1.37	1.10
l	ea20	.	0.76	0.90	1.14	1.01	0.85	1.77	1.23	1.05
l	ea37	.	0.34	0.77	0.80	0.56	0.47*	0.36*	0.12*	NV

Assay Mean Potencies Stability Study 2

Relative to Ia00 for ea00 and na00 and else all ea relative to ea00 and all na relative to na00, except naea denotes na00 relative to ea00
Mean of two estimates except as marked + for one estimate, * for 3 estimates and ** for 4 estimates.

asty	ptemp	S000	S007	S014	S042	S084	S168	Mean of Assay Means
e	ea00	0.67**	0.97+	0.95	0.59+	0.92	0.70+	0.79
e	ea04	.	0.95	1.04	1.25	0.77	1.13	1.02
e	ea20	.	0.92	1.10	1.11	1.09	1.01	1.04
e	ea37	.	0.91	0.91	0.43	0.14*	0.07	NV
e	na00	0.39**	0.46+	0.46	0.68+	.	0.52+	0.49
e	na04	.	0.92	0.89	0.80**	.	0.83**	0.86
e	na20	.	0.80	0.82	0.89**	.	0.63**	0.78
e	na37	.	0.77	0.66	0.22**	.	0.01**	NV
e	naea	0.59**	0.47+	0.49	1.16+	.	0.73+	0.65
l	ea00	0.57**	0.78	0.73	0.68+	0.74	0.45+	0.65
l	ea04	.	0.96	0.88	1.12	0.80	1.11	0.97
l	ea20	.	0.83	1.09	1.12	1.13	1.18	1.06
l	ea37	.	0.86	0.77	0.50	0.19*	0.07	NV
l	na00**	0.08	0.12	0.08	0.12+	.	0.09+	0.10
l	na04	.	0.90	0.83	1.00**	.	0.77**	0.87
l	na20	.	0.84	1.29	1.20**	.	0.81**	1.01
l	na37	.	0.65	0.61	0.22**	.	0.02**	NV
l	naea	0.15**	0.15	0.12	0.17+	.	0.21+	0.16

Assay Mean Potencies Stability Study 3

8

Relative to Ib00 for eb00, and all else relative to eb00

Mean of two estimates except as marked + for one estimate, * for 3 estimates and ** for 4 estimates.

asty	ptemp	S000	S014	S025	S042	S063	S084	S175	Mean of Assay
e	eb00	1.61	1.38	.	0.96	1.08	1.44	1.28	Means 1.27
e	eb04	.	1.51	.	0.94	1.03	1.15	1.48	1.20
e	eb20	.	1.08	.	1.40	1.22	1.28	1.12	1.21
e	eb37	.	0.84	.	0.29	0.11*	0.10*	0.02*	NV
l	eb00	1.43	1.36	1.50	0.85	0.59	1.14	1.03	1.08
l	eb04	.	1.03	0.89	1.14	0.94+	1.54	1.22	1.11
l	eb20	.	1.14	0.93	1.50	1.19	1.54	0.86	1.17
l	eb37	.	0.97	0.66	0.54	0.30	0.19*	0.02*	NV

Slopes Stability Study 1

1

Assay type	Storage days	Assay date	Assay plate	Sample and temperature				
				Ia00	ea00	ea04	ea20	ea37
early	S000	060309	MS1	1.10	1.22	.	.	.
			MS2	1.17	1.14	.	.	.
			MS4	1.11	1.15	.	.	.
early	S007	060316	MS1	0.92	0.92	.	.	.
			MS2	0.98	1.02	.	.	.
			MS6	.	0.92	0.93	0.98	.
			MS7	.	0.85	0.95	.	0.92
			MS8	.	0.88	.	0.96	0.92
early	S014	060323	MS1	0.80	0.90	.	.	.
			MS2	0.92	0.86	.	.	.
			MS3	.	0.93	0.91	0.91	.
			MS4	.	0.87	0.95	.	0.92
			MS5	.	0.84	.	0.80	0.84
early	S028	060406	MS1	0.88	0.94	.	.	.
			MS2	1.04	0.93	.	.	.
			MS3	.	0.89	0.80	0.87	.
			MS4	.	0.90	1.08	.	0.91
			MS5	.	0.86	.	0.73	0.77
early	S042	060420	MS1	0.94	1.10	.	.	.
			MS2	.	0.86	0.91	0.93	.
			MS3	.	0.78	0.98	.	0.93
			MS4	.	0.97	.	1.03	1.05
early	S112	060629	MS1	0.85	1.03	.	.	.
			MS2	1.17	1.23	.	.	1.26
			MS3	.	0.93	0.91	0.83	.
			MS4	.	1.02	0.94	.	1.09
			MS5	.	0.85	.	0.84	0.94
early	S168	060824	MS1	0.75	0.86	.	.	.
			MS2	1.20	1.24	.	.	1.13
			MS3	.	0.95	0.92	1.03	.
			MS4	.	0.87	0.90	.	0.97
			MS5	.	0.88	.	0.91	0.99

Slopes Stability Study 1

2

Assay type	Storage days	Assay date	Assay plate	Sample and temperature				
				Ia00	ea00	ea04	ea20	ea37
late	S000	060310	MS1	-1.14	-1.14	.	.	.
			MS2	-1.03	-1.33	.	.	.
			MS3	-1.07	-1.06	.	.	.
			MS4	-0.86	-1.26	.	.	.
late	S007	060317	MS1	-1.03	-1.22	.	.	.
			MS2	-1.22	-1.22	.	.	.
			MS6	.	-1.01	-1.12	-.	.
			MS7	.	-1.19	-1.16	.	-1.06
			MS8	.	-1.18	.	-1.06	-0.88
late	S014	060324	MS1	-1.21	-1.24	.	.	.
			MS2	-1.16	-1.25	.	.	.
			MS3	.	-1.32	-1.25	-.	.
			MS4	.	-1.30	-1.35	.	-1.21
			MS5	.	-1.12	.	-1.28	-1.24
late	S028	060407	MS1	-1.12	-1.19	.	.	.
			MS2	-1.31	-1.16	.	.	.
			MS3	.	-1.19	-1.01	-1.27	.
			MS4	.	-1.19	-1.23	.	-1.10
			MS5	.	-1.17	.	-1.13	-1.29
late	S042	060421	MS1	-1.27	-1.24	.	.	.
			MS2	-1.11	-1.33	.	.	.
			MS3	.	-1.34	-1.18	-1.25	.
			MS4	.	-1.31	-1.15	.	-1.22
			MS5	.	-1.23	.	-1.37	-1.32
late	S067	060515	MS1	-1.25	-1.12	.	.	.
			MS2	-1.30	-1.23	.	.	-1.32
			MS3	.	-1.17	-1.33	-1.34	.
			MS4	.	-1.12	-1.22	.	-1.18
			MS5	.	-1.22	.	-1.21	-1.09
late	S112	060630	MS1	-1.18	-1.06	.	.	.
			MS2	-1.22	-1.22	.	.	-0.92
			MS3	.	-1.15	-1.38	-1.19	.
			MS4	.	-1.44	-1.27	.	-1.28
			MS5	.	-1.35	.	-1.26	-1.03
late	S168	060825	MS1	-1.28	-1.20	.	.	.
			MS2	-1.40	-1.40	.	.	-1.04
			MS3	.	-1.16	-1.50	-1.22	.
			MS4	.	-1.28	-1.31	.	-1.15
			MS5	.	-1.11	.	-1.24	-1.07

Potencies Stability Study 1

3

Assay type	Storage days	Assay date	Assay plate	Reference Sample	Sample and temperature			
					ea00	ea04	ea20	ea37
early	S000	060309	MS1	Ia00	0.78	.	.	.
			MS2	Ia00	0.57	.	.	.
			MS4	Ia00	1.08	.	.	.
early	S007	060316	MS1	Ia00	0.64	.	.	.
			MS2	Ia00	0.92	.	.	.
			MS6	ea00	.	0.96	0.56	.
			MS7	ea00	.	1.13	.	0.86
			MS8	ea00	.	.	0.58	0.81
early	S014	060323	MS1	Ia00	1.13	.	.	.
			MS2	Ia00	0.92	.	.	.
			MS3	ea00	.	1.04	1.16	.
			MS4	ea00	.	1.03	.	1.04
			MS5	ea00	.	.	0.91	1.09
early	S028	060406	MS1	Ia00	0.68	.	.	.
			MS2	Ia00	0.67	.	.	.
			MS3	ea00	.	1.03	1.26	.
			MS4	ea00	.	1.17	.	0.74
			MS5	ea00	.	.	1.12	0.96
early	S042	060420	MS1	Ia00	1.06	.	.	.
			MS2	ea00	.	1.11	1.20	.
			MS3	ea00	.	1.15	.	0.57
			MS4	ea00	.	.	0.94	0.65
early	S112	060629	MS1	Ia00	0.46	.	.	.
			MS2	Ia00	0.53	.	.	0.17
			MS3	ea00	.	1.01	1.37	.
			MS4	ea00	.	0.95	.	0.24
			MS5	ea00	.	.	2.14	0.32
early	S168	060824	MS1	Ia00	0.76	.	.	.
			MS2	Ia00	0.71	.	.	0.04
			MS3	ea00	.	1.16	0.88	.
			MS4	ea00	.	1.11	.	0.05
			MS5	ea00	.	.	0.97	0.07

Potencies Stability Study 1

4

Assay type	Storage days	Assay date	Assay plate	Reference Sample	Sample and temperature			
					ea00	ea04	ea20	ea37
late	S000	060310	MS1	Ia00	0.72	.	.	.
			MS2	Ia00	0.40	.	.	.
			MS3	Ia00	0.58	.	.	.
			MS4	Ia00	0.37	.	.	.
late	S007	060317	MS1	Ia00	0.52	.	.	.
			MS2	Ia00	0.70	.	.	.
			MS6	ea00	.	0.51	0.70	.
			MS7	ea00	.	1.16	.	0.39
			MS8	ea00	.	.	0.83	0.30
late	S014	060324	MS1	Ia00	0.68	.	.	.
			MS2	Ia00	0.63	.	.	.
			MS3	ea00	.	0.93	1.22	.
			MS4	ea00	.	0.94	.	0.91
			MS5	ea00	.	.	0.66	0.66
late	S028	060407	MS1	Ia00	0.60	.	.	.
			MS2	Ia00	0.77	.	.	.
			MS3	ea00	.	1.23	1.01	.
			MS4	ea00	.	1.29	.	0.80
			MS5	ea00	.	.	1.29	0.80
late	S042	060421	MS1	Ia00	0.66	.	.	.
			MS2	Ia00	0.61	.	.	.
			MS3	ea00	.	1.03	1.21	.
			MS4	ea00	.	1.15	.	0.61
			MS5	ea00	.	.	0.84	0.50
late	S067	060515	MS1	Ia00	0.91	.	.	.
			MS2	Ia00	0.95	.	.	0.83
			MS3	ea00	.	0.87	0.81	.
			MS4	ea00	.	1.06	.	0.37
			MS5	ea00	.	.	0.88	0.32
late	S112	060630	MS1	Ia00	0.28	.	.	.
			MS2	Ia00	0.35	.	.	0.10
			MS3	ea00	.	1.29	1.79	.
			MS4	ea00	.	1.82	.	0.38
			MS5	ea00	.	.	1.74	0.41
late	S168	060825	MS1	Ia00	0.58	.	.	.
			MS2	Ia00	0.52	.	.	0.07
			MS3	ea00	.	1.29	1.28	.
			MS4	ea00	.	1.45	.	0.11
			MS5	ea00	.	.	1.19	0.12

Slopes Stability Study 2

1

asid	plate	Ia00	ea00	ea04	ea20	ea37	na00	na04	na20	na37
e-000-060511	MS1	0.76	0.86	.	.	.	0.97	.	.	.
	MS2	0.88	0.91	.	.	.	0.68	.	.	.
	MS3	0.86	0.85	.	.	.	0.74	.	.	.
	MS4	0.89	0.87	.	.	.	0.76	.	.	.
e-007-060518	MS1	0.74	0.87	.	.	.	0.53	.	.	.
	MS3	.	.	.	.	.	0.97	0.79	0.89	.
	MS4	.	.	.	.	.	0.78	0.70	.	0.90
	MS5	.	.	.	.	.	0.81	.	0.91	0.94
	MS6	.	0.77	0.81	0.84	.	.	.	.	.
	MS7	.	0.76	0.84	.	0.86	.	.	.	.
	MS8	.	0.79	.	0.84	0.85	.	.	.	.
e-014-060525	MS1	0.78	0.78	.	.	.	0.41	.	.	.
	MS2	0.93	0.92	.	.	.	0.56	.	.	.
	MS3	.	.	.	.	.	0.81	0.75	0.50	.
	MS4	.	.	.	.	.	0.71	0.61	.	0.60
	MS5	.	.	.	.	.	0.84	.	0.38	0.74
	MS6	.	0.89	0.93	0.84	.	.	.	.	.
	MS7	.	0.84	0.76	.	0.80	.	.	.	.
	MS8	.	1.12	.	.	0.94	.	.	.	.
e-042-060622	MS1	0.84	0.91	.	.	.	0.54	.	.	.
	MS10	.	.	.	.	.	1.06	.	1.10	1.09
	MS2	.	.	.	.	.	0.24	0.59	0.40	.
	MS3	.	.	.	.	.	.	0.99	.	0.88
	MS4	.	.	.	.	.	0.78	.	0.72	0.98
	MS5	.	0.89	0.93	0.88	.	.	.	.	.
	MS6	.	0.95	0.92	.	1.01	.	.	.	.
	MS7	.	.	.	0.98	0.98	.	.	.	.
	MS8	.	.	.	.	.	1.08	1.05	0.90	.
	MS9	.	.	.	.	.	0.99	1.04	.	1.20
e-084-060803	MS1	0.81	0.87	.	.	.	.	.	.	.
	MS2	0.92	1.03	.	.	1.03	.	.	.	.
	MS3	.	0.92	0.95	0.84	.	.	.	.	.
	MS4	.	0.88	0.96	.	1.05	.	.	.	.
	MS5	.	0.90	.	0.87	1.03	.	.	.	.
e-168-061026	MS1	0.74	0.91	.	.	.	0.56	.	.	.
	MS10	.	.	.	.	.	0.90	.	0.93	1.31
	MS2	.	.	.	.	.	0.70	0.72	0.77	.
	MS3	.	.	.	.	.	0.68	0.80	.	1.19
	MS4	.	.	.	.	.	0.64	.	0.76	1.21
	MS5	.	0.99	0.88	0.85	.	.	.	.	.
	MS6	.	0.83	0.79	.	1.16	.	.	.	.
	MS7	.	0.84	.	0.83	1.24	.	.	.	.
	MS8	.	.	.	.	.	0.91	0.93	.	.
	MS9	.	.	.	.	.	0.75	0.77	.	0.54

Slopes Stability Study 2

2

asid	plate	Ia00	ea00	ea04	ea20	ea37	na00	na04	na20	na37
l-000-060512	MS1	-1.08	-1.21	.	.	.	-1.11	.	.	.
	MS2	-1.25	-1.12	.	.	.	-1.14	.	.	.
	MS3	-1.32	-1.59	.	.	.	-1.22	.	.	.
	MS4	-1.28	-1.47	.	.	.	-1.18	.	.	.
l-007-060519	MS1	-1.31	-1.60	.	.	.	-1.16	.	.	.
	MS2	-1.26	-1.23	.	.	.	-1.15	.	.	.
	MS3	.	.	.	.	.	-1.07	-1.14	-1.21	.
	MS4	.	.	.	.	.	-1.08	-1.13	.	-1.11
	MS5	.	.	.	.	.	-1.10	.	-1.15	-1.09
	MS6	.	-1.16	-1.23	-1.19	.	.	.	.	.
	MS7	.	-1.43	-1.30	.	-1.27	.	.	.	.
	MS8	.	-1.17	.	-1.24	-1.31	.	.	.	.
l-014-060526	MS1	-1.20	-1.21	.	.	.	-1.06	.	.	.
	MS2	-1.23	-1.40	.	.	.	-1.11	.	.	.
	MS3	.	.	.	.	.	-1.26	-1.11	-0.98	.
	MS4	.	.	.	.	.	-1.16	-1.28	.	-1.15
	MS5	.	.	.	.	.	-1.09	.	-1.21	-1.15
	MS6	.	-1.33	-1.09	-1.32	.	.	.	.	.
	MS7	.	-1.05	-1.29	.	-1.24	.	.	.	.
	MS8	.	-1.22	.	-1.12	-1.21	.	.	.	.
l-042-060623	MS1	-1.22	-1.28	.	.	.	-1.19	.	.	.
	MS10	.	.	.	.	.	-1.24	.	-1.49	-0.89
	MS2	.	.	.	.	.	-1.30	-1.18	-1.10	.
	MS3	.	.	.	.	.	-1.31	-1.43	.	-1.17
	MS4	.	.	.	.	.	-1.29	.	-1.19	-1.57
	MS5	.	-1.34	-1.38	-1.33	.	.	.	.	.
	MS6	.	-1.37	-1.46	.	-1.22	.	.	.	.
	MS7	.	-1.45	.	-1.57	-1.22	.	.	.	.
	MS8	.	.	.	.	.	-1.49	-1.64	-1.53	.
	MS9	.	.	.	.	.	-1.22	-1.62	.	-1.02
l-084-060804	MS1	-1.31	-1.41	.	.	.	.	.	.	.
	MS2	-1.41	-1.54	.	.	-1.33	.	.	.	.
	MS3	.	-1.77	-1.65	-1.44	.	.	.	.	.
	MS4	.	-1.49	-1.60	.	-1.22	.	.	.	.
	MS5	.	-1.47	.	-1.63	-1.41	.	.	.	.
l-168-061027	MS1	-1.11	-1.22	.	.	.	-1.02	.	.	.
	MS10	.	.	.	.	.	.	.	-1.09	(1)
	MS2	.	.	.	.	.	-1.24	-1.09	-1.24	.
	MS3	.	.	.	.	.	-1.15	-1.20	.	-0.82
	MS4	.	.	.	.	.	-1.08	.	-1.07	-0.75
	MS5	.	-1.29	-1.26	-1.18	.	.	.	.	.
	MS6	.	-1.06	-1.26	.	-1.22	.	.	.	.
	MS7	.	-1.20	.	-1.11	-1.06	.	.	.	.
	MS8	.	.	.	.	.	-1.08	-1.06	-1.14	.
	MS9	.	.	.	.	.	-1.15	-1.18	.	-0.79

Potencies Stability Study 2

3

asid	plate	pnstd	ea00	ea04	ea20	ea37	na00	na04	na20	na37
e-000-060511	MS1	Ia00	0.79	.	.	.	0.48	.	.	.
	MS2	Ia00	0.48	.	.	.	0.33	.	.	.
	MS3	Ia00	0.62	.	.	.	0.44	.	.	.
	MS4	Ia00	0.87	.	.	.	0.36	.	.	.
e-007-060518	MS1	Ia00	0.97	.	.	.	0.46	.	.	.
	MS3	na00	.	.	.	.	.	0.81	0.78	.
	MS4	na00	.	.	.	.	.	1.06	.	0.83
	MS5	na00	.	.	.	.	.	.	0.82	0.71
	MS6	ea00	.	0.83	0.89	.	.	.	.	.
	MS7	ea00	.	1.09	.	0.89	.	.	.	.
	MS8	ea00	.	.	0.95	0.93	.	.	.	.
e-014-060525	MS1	Ia00	0.92	.	.	.	0.45	.	.	.
	MS2	Ia00	0.97	.	.	.	0.48	.	.	.
	MS3	na00	.	.	.	.	.	0.72	0.85	.
	MS4	na00	.	.	.	.	.	1.10	.	0.90
	MS5	na00	.	.	.	.	.	.	0.79	0.49
	MS6	ea00	.	1.07	1.28	.	.	.	.	.
	MS7	ea00	.	1.01	.	1.22	.	.	.	.
	MS8	ea00	.	.	0.95	0.67	.	.	.	.
e-042-060622	MS1	Ia00	0.59	.	.	.	0.68	.	.	.
	MS10	na00	.	.	.	.	.	.	0.87	0.25
	MS2	na00	.	.	.	.	.	0.68	0.94	.
	MS3	na00	.	.	.	.	.	0.66	.	0.20
	MS4	na00	.	.	.	.	.	.	0.82	0.18
	MS5	ea00	.	1.23	1.13	.	.	.	.	.
	MS6	ea00	.	1.28	.	0.41	.	.	.	.
	MS7	ea00	.	.	1.08	0.46	.	.	.	.
	MS8	na00	.	.	.	.	.	0.96	0.93	.
	MS9	na00	.	.	.	.	.	0.95	.	0.26
e-084-060803	MS1	Ia00	0.90	.	.	.	.	.	.	.
	MS2	Ia00	0.94	.	.	0.11	.	.	.	.
	MS3	ea00	.	0.70	1.04	.	.	.	.	.
	MS4	ea00	.	0.83	.	0.15	.	.	.	.
	MS5	ea00	.	.	1.14	0.15	.	.	.	.
e-168-061026	MS1	Ia00	0.70	.	.	.	0.52	.	.	.
	MS10	na00	.	.	.	.	.	.	0.62	0.02
	MS2	na00	.	.	.	.	.	0.74	0.58	.
	MS3	na00	.	.	.	.	.	0.79	.	0.01
	MS4	na00	.	.	.	.	.	.	0.68	0.01
	MS5	ea00	.	1.03	1.02	.	.	.	.	.
	MS6	ea00	.	1.25	.	0.06	.	.	.	.
	MS7	ea00	.	.	1.01	0.07	.	.	.	.
	MS8	na00	.	.	.	.	.	0.84	0.67	.
	MS9	na00	.	.	.	.	.	0.95	.	0.02

Potencies Stability Study 2

4

asid	plate	pnstd	ea00	ea04	ea20	ea37	na00	na04	na20	na37
l-000-060512	MS1	Ia00	0.46	.	.	.	0.07	.	.	.
	MS2	Ia00	0.53	.	.	.	0.07	.	.	.
	MS3	Ia00	0.73	.	.	.	0.09	.	.	.
	MS4	Ia00	0.58	.	.	.	0.10	.	.	.
l-007-060519	MS1	Ia00	0.70	.	.	.	0.12	.	.	.
	MS2	Ia00	0.88	.	.	.	0.12	.	.	.
	MS3	na00	.	.	.	.	.	0.94	0.93	.
	MS4	na00	.	.	.	.	.	0.86	.	0.76
	MS5	na00	.	.	.	.	.	.	0.75	0.56
	MS6	ea00	.	1.04	1.04	.	.	.	.	.
	MS7	ea00	.	0.88	.	0.99	.	.	.	.
	MS8	ea00	.	.	0.67	0.75	.	.	.	.
l-014-060526	MS1	Ia00	0.85	.	.	.	0.09	.	.	.
	MS2	Ia00	0.62	.	.	.	0.08	.	.	.
	MS3	na00	.	.	.	.	.	1.02	1.52	.
	MS4	na00	.	.	.	.	.	0.67	.	0.66
	MS5	na00	.	.	.	.	.	.	1.09	0.56
	MS6	ea00	.	1.05	0.99	.	.	.	.	.
	MS7	ea00	.	0.74	.	0.78	.	.	.	.
	MS8	ea00	.	.	1.19	0.75	.	.	.	.
l-042-060623	MS1	Ia00	0.68	.	.	.	0.12	.	.	.
	MS10	na00	.	.	.	.	.	.	1.51	0.26
	MS2	na00	.	.	.	.	.	1.32	1.14	.
	MS3	na00	.	.	.	.	.	0.86	.	0.17
	MS4	na00	.	.	.	.	.	.	0.96	0.16
	MS5	ea00	.	1.03	1.15	.	.	.	.	.
	MS6	ea00	.	1.20	.	0.49	.	.	.	.
	MS7	ea00	.	.	1.09	0.50	.	.	.	.
	MS8	na00	.	.	.	.	.	0.91	1.26	.
	MS9	na00	.	.	.	.	.	0.99	.	0.34
l-084-060804	MS1	Ia00	0.72	.	.	.	.	.	.	.
	MS2	Ia00	0.76	.	.	0.15	.	.	.	.
	MS3	ea00	.	0.88	1.21	.	.	.	.	.
	MS4	ea00	.	0.74	.	0.21	.	.	.	.
	MS5	ea00	.	.	1.06	0.17	.	.	.	.
l-168-061027	MS1	Ia00	0.45	.	.	.	0.09	.	.	.
	MS10	na00	.	.	.	.	.	.	0.80	0.02
	MS2	na00	.	.	.	.	.	0.81	0.73	.
	MS3	na00	.	.	.	.	.	0.64	.	0.01
	MS4	na00	.	.	.	.	.	.	0.65	0.01
	MS5	ea00	.	1.19	1.19	.	.	.	.	.
	MS6	ea00	.	1.03	.	0.05	.	.	.	.
	MS7	ea00	.	.	1.16	0.08	.	.	.	.
	MS8	na00	.	.	.	.	.	0.86	1.13	.

Slopes Stability Study 3								1
type	stored	asdate	plate	Ib00	eb00	eb04	eb20	eb37
early	S000	060525	MS10	0.90	0.86	.	.	.
			MS9	0.91	0.80	.	.	.
early	S014	060608	MS1	0.80	0.78	.	.	.
			MS2	0.87	0.86	.	.	.
			MS3	.	0.89	0.91	0.77	.
			MS4	.	1.02	0.95	.	0.88
			MS5	.	0.94	.	0.78	0.82
early	S042	060706	MS1	0.85	0.87	.	.	.
			MS2	0.94	0.85	.	.	.
			MS3	.	0.83	0.76	0.83	.
			MS4	.	1.04	0.80	.	0.95
			MS5	.	0.76	.	0.74	0.85
early	S063	060727	MS1	0.89	0.92	.	.	.
			MS2	1.12	1.41	.	.	1.54
			MS3	.	0.98	0.94	0.95	.
			MS4	.	0.95	0.93	.	1.01
			MS5	.	0.99	.	0.93	1.11
early	S084	060817	MS1	0.86	0.79	.	.	.
			MS2	1.07	1.18	.	.	1.56
			MS3	.	0.94	0.97	0.82	.
			MS4	.	1.17	0.90	.	1.11
			MS5	.	0.92	.	0.86	1.17
early	S175	061116	MS1	0.74	0.69	.	.	.
			MS2	1.26	0.99	.	.	1.24
			MS3	.	0.66	0.65	0.67	.
			MS4	.	0.74	0.66	.	1.30
			MS5	.	0.76	.	0.79	1.05

Slopes Stability Study 3								2
type	stored	asdate	plate	Ib00	eb00	eb04	eb20	eb37
late	S000	060526	MS10	-1.25	-1.46	.	.	.
			MS9	-1.28	-1.45	.	.	.
late	S014	060609	MS1	-1.58	-1.51	.	.	.
			MS2	-1.62	-1.42	.	.	.
			MS3	.	-1.59	-1.44	-1.35	.
			MS4	.	-1.61	-1.76	.	-1.45
			MS5	.	-1.53	.	-1.56	-1.48
late	S025	060619	MS1	-1.35	-1.12	.	.	.
			MS2	-1.54	-1.44	.	.	.
			MS3	.	-1.43	-1.42	-1.33	.
			MS4	.	-1.17	-1.18	.	-1.18
			MS5	.	-1.21	.	-1.39	-1.28
late	S042	060707	MS1	-1.51	-1.45	.	.	.
			MS2	-1.68	-1.50	.	.	.
			MS3	.	-1.41	-1.55	-1.53	.
			MS4	.	-1.80	-1.50	.	-1.46
late	S042	060707	MS5	.	-1.81	.	-1.54	-1.44
late	S063	060728	MS1	-1.39	-1.48	.	.	.
			MS2	-1.15	-2.38	.	.	-1.01
			MS3	.	-1.09	-1.07	-1.09	.
			MS4	-1.03	.	-1.24	.	-1.15
			MS5	.	-1.13	.	-1.45	-1.04
late	S084	060818	MS1	-1.45	-1.31	.	.	.
			MS2	-1.37	-1.64	.	.	-1.10
			MS3	.	-1.30	-1.43	-1.24	.
			MS4	.	-1.46	-1.45	.	-1.26
			MS5	.	-1.40	.	-1.75	-1.32
late	S175	061117	MS1	-1.35	-1.31	.	.	.
			MS2	-1.11	-1.39	.	.	-1.34
			MS3	.	-0.98	-1.25	-0.98	.
			MS4	.	-1.22	-1.20	.	-0.91
			MS5	.	-1.22	.	-1.27	-0.83

Potencies Stability Study 3

3

type	stored	asdate	plate	pnstd	eb00	eb04	eb20	eb37
early	S000	060525	MS10	Ib00	1.52	.	.	.
			MS9	Ib00	1.69	.	.	.
early	S014	060608	MS1	Ib00	1.53	.	.	.
			MS2	Ib00	1.24	.	.	.
			MS3	eb00	.	1.25	1.20	.
			MS4	eb00	.	1.83	.	1.21
			MS5	eb00	.	.	0.98	0.58
early	S042	060706	MS1	Ib00	1.53	.	.	.
			MS2	Ib00	0.61	.	.	.
			MS3	eb00	.	1.01	1.57	.
			MS4	eb00	.	0.87	.	0.29
			MS5	eb00	.	.	1.24	0.30
early	S063	060727	MS1	Ib00	1.03	.	.	.
			MS2	Ib00	1.14	.	.	0.12
			MS3	eb00	.	1.12	1.40	.
			MS4	eb00	.	0.94	.	0.10
			MS5	eb00	.	.	1.06	0.12
early	S084	060817	MS1	Ib00	1.48	.	.	.
			MS2	Ib00	1.39	.	.	0.14
			MS3	eb00	.	1.35	1.08	.
			MS4	eb00	.	0.98	.	0.08
			MS5	eb00	.	.	1.50	0.12
early	S175	061116	MS1	Ib00	1.62	.	.	.
			MS2	Ib00	1.01	.	.	0.02
			MS3	eb00	.	1.67	1.14	.
			MS4	eb00	.	1.31	.	0.02
			MS5	eb00	.	.	1.10	0.02

Potencies Stability Study 3

4

type	stored	asdate	plate	pnstd	eb00	eb04	eb20	eb37
late	S000	060526	MS10	Ib00	1.72	.	.	.
			MS9	Ib00	1.19	.	.	.
late	S014	060609	MS1	Ib00	1.22	.	.	.
			MS2	Ib00	1.51	.	.	.
			MS3	eb00	.	0.99	1.17	.
			MS4	eb00	.	1.07	.	1.10
			MS5	eb00	.	.	1.11	0.86
late	S025	060619	MS1	Ib00	1.73	.	.	.
			MS2	Ib00	1.31	.	.	.
			MS3	eb00	.	0.91	1.01	.
			MS4	eb00	.	0.86	.	0.70
			MS5	eb00	.	.	0.87	0.62
late	S042	060707	MS1	Ib00	1.06	.	.	.
			MS2	Ib00	0.68	.	.	.
			MS3	eb00	.	1.16	1.55	.
			MS4	eb00	.	1.12	.	0.56
			MS5	eb00	.	.	1.44	0.51
late	S063	060728	MS1	Ib00	0.96	.	.	.
			MS2	Ib00	0.36	.	.	0.14
			MS3	eb00	.	0.94	1.35	.
			MS4	Ib00	.	1.35	.	0.15
			MS5	eb00	.	.	1.04	0.23
late	S084	060818	MS1	Ib00	1.18	.	.	.
			MS2	Ib00	1.11	.	.	0.23
			MS3	eb00	.	1.66	1.91	.
			MS4	eb00	.	1.43	.	0.17
			MS5	eb00	.	.	1.24	0.20
late	S175	061117	MS1	Ib00	1.01	.	.	.
			MS2	Ib00	1.04	.	.	0.02
			MS3	eb00	.	1.14	0.91	.
			MS4	eb00	.	1.32	.	0.02
			MS5	eb00	.	.	0.81	0.02

Full Statistical Analysis of Bioassays

Key: **type**, assay type; **asdate**, assay date; **All** or **ComPl**, plate identification for the assays; **pnstd**, identifies the preparation used as reference for the potency estimates shown; **Ea** or **E**, EDQM IFN- α 2a; **Ia**, 2nd IS IFN- α 2a; **N1**, IFN001 (IFN- α 2b); **N2**, IFN002 (IFN- α 2b); **N3**, IFN003 (IFN- α 2b); **N4**, IFN004 (IFN- α 2a); **N5**, IFN005 (IFN- α 2a); **Eox**, methionine oxidised EDQM IFN- α 2a; **EoxC** or **EoxC2**, control from methionine oxidation of EDQM IFN- α 2a; **npl**, NPL (National Physical Laboratory); **ifn**, Interferon (IFN); **G3_1** and **G4_1**, two preparations of IFN- α 2a; **G4_2** and **G2**, two preparations of PEG-IFN- α 2a.

Note:

Analysis has followed the usual pattern for data of this type, with preliminary graphical assessment to detect gross outliers (for which a generally conservative policy is adopted), assessment of positional effects, and analysis of each assay plate individually. In addition, for several of these studies, a combined analysis of all plates in an assay has also been included, using the responses transformed on the basis of the individual plates. Positional effects are typical for this type of assay.

Analysis of the transformed responses gives statistically valid assays apart from a limited number of inconsistent and apparently erratic deviations.

Slopes Study of npl preparations

1

type	asdate	plate	Ea	Ia	N1	N2	N3	N4	N5
early	050926	MS1	1.16	1.12	.	.	.	.	.
		MS3	.	.	1.03	1.02	1.05	.	.
early	051020	MS2	.	.	1.09	1.11	0.81	.	.
early	061102	All	0.74	.	.	0.86	0.97	0.82	0.89
		MS2	0.76	.	.	.	.	0.48	0.57
		MS5	0.77	.	.	0.85	0.99	.	.
		MS6	0.70	.	.	.	.	0.81	0.88
		MS7	.	.	.	0.88	.	0.87	0.89
		MS8	.	.	.	.	0.96	0.80	0.94
late	050701	All	-1.06	-1.07	-0.99	.	.	.	.
		MS1	-1.04	-1.21	-0.94	.	.	.	.
		MS2	-1.10	-0.97	-1.02	.	.	.	.
		MS3	-1.11	-1.12	-0.91	.	.	.	.
		MS4	-1.03	-1.07	-1.17	.	.	.	.
late	051013	MS1	.	.	-1.04	-1.05	-1.16	.	.
		MS5	.	.	-1.22	-1.19	-1.37	.	.
late	060227	MS6	.	.	-1.02	-0.84	-1.53	.	.
late	061030	All	-1.20	.	.	-1.01	-1.08	-1.13	-1.03
		MS1	-1.25	.	.	-1.03	-1.09	.	.
		MS2	-1.28	.	.	.	.	-1.28	-1.24
		MS3	.	.	.	-0.96	.	-0.84	-0.91
		MS4	.	.	.	.	-1.08	-1.36	-1.05
		MS5	-1.23	.	.	-1.06	-1.12	.	.
		MS6	-1.17	.	.	.	.	-1.14	-1.20
		MS7	.	.	.	-1.12	.	-1.13	-0.90
		MS8	.	.	.	.	-1.12	-1.09	-0.94

Potencies				Study of npl preparations						2
type	asdate	plate	pnstd	Ia	N1	N2	N3	N4	N5	
early	050926	MS1	Ea	0.81	.	.	.	.	.	
		MS3	N1	.	1.00	9.74	6.17	.	.	
early	051020	MS2	N1	.	1.00	7.43	7.21	.	.	
early	061102	All	Ea	.	.	0.47	0.23	0.69	0.51	
		MS2	Ea	.	.	.	.	0.88	0.65	
		MS5	Ea	.	.	0.49	0.27	.	.	
		MS6	Ea	.	.	.	.	0.61	0.39	
		MS7	N2	.	.	1.00	.	1.42	1.01	
		MS8	N3	.	.	.	1.00	2.86	2.45	
late	050701	All	Ea	1.10	0.18	.	.	.	.	
		MS1	Ea	1.24	0.20	.	.	.	.	
		MS2	Ea	0.97	0.20	.	.	.	.	
		MS3	Ea	0.94	0.13	.	.	.	.	
		MS4	Ea	1.30	0.20	.	.	.	.	
late	051013	MS1	N1	.	1.00	5.91	5.49	.	.	
		MS5	N1	.	1.00	7.20	3.98	.	.	
late	060227	MS6	N1	.	1.00	7.69	6.09	.	.	
late	061030	All	Ea	.	.	0.33	0.34	0.56	0.45	
		MS1	Ea	.	.	0.45	0.49	.	.	
		MS2	Ea	.	.	.	.	0.48	0.32	
		MS3	N2	.	.	1.00	.	1.95	1.66	
		MS4	N3	.	.	.	1.00	1.64	1.42	
		MS5	Ea	.	.	0.38	0.36	.	.	
		MS6	Ea	.	.	.	.	0.50	0.43	
		MS7	N2	.	.	1.00	.	1.51	1.32	
		MS8	N3	.	.	.	1.00	1.36	0.97	

Slopes Study of ifn methionine oxidized

1

type	asdate	plate	E	EoxC	Eox
early	061205	MS1	0.76	0.64	0.77
		MS2	.	0.88	0.95
		MS3	0.75	0.74	0.85
		MS4	.	0.74	0.87
late	061124	MS1	-1.39	-1.60	-1.14
		MS2	.	-1.73	-1.25
		MS3	-1.00	-1.00	-1.07
late	061204	MS1	-1.29	-1.56	-1.10
		MS2	.	-1.23	-1.15
		MS3	-1.43	-1.27	-1.31
		MS4	.	-1.17	-1.28

Potencies Study of ifn methionine oxidized

2

type	asdate	plate	pnstd	E	EoxC	Eox
early	061205	MS1	E	1.00	1.55	0.56
		MS2	Eox	.	2.61	1.00
		MS3	E	1.00	1.45	0.48
		MS4	Eox	.	3.05	1.00
late	061124	MS1	E	1.00	1.41	0.78
		MS2	Eox	.	1.77	1.00
		MS3	E	1.00	1.06	0.56
late	061204	MS1	E	1.00	1.35	0.95
		MS2	Eox	.	1.61	1.00
		MS3	E	1.00	1.25	0.84
		MS4	Eox	.	2.23	1.00

Potencies Study of ifn methionine oxidized, with reference shown in brackets. That is, Eox and EoxC are in terms of E, and Eox2 is the potency of Eox relative to EoxC.

type	asdate	plate	Eox (E)	EoxC (E)	Eox2 (EoxC)
early	061205	MS1	0.56	1.55	0.36
		MS2	.	.	0.38
		MS3	0.48	1.45	0.33
		MS4	.	.	0.33
late	061124	MS1	0.78	1.41	0.55
		MS2	.	.	0.56
		MS3	0.56	1.06	0.53
late	061204	MS1	0.95	1.35	0.71
		MS2	.	.	0.62
		MS3	0.84	1.25	0.68
		MS4	.	.	0.45

Slopes Study of ifn from external collaborator G							1
type	asdate	plate	E	G3_1	G4_1	G4_2	G2
early	061205	MS5	0.70	0.60	0.62	.	.
		MS6	0.68	.	.	1.00	1.13
late	061120	MS1	-0.98	-1.31	-0.81	.	.
		MS2	-1.34	.	.	-1.08	-1.15
		MS3	-1.15	-1.53	-1.41	.	.
		MS4	-1.31	.	.	-1.25	-1.30

Potencies Study of ifn from external collaborator G							2
type	asdate	plate	pnstd	G3_1	G4_1	G4_2	G2
early	061205	MS5	E	0.774	0.514	.	.
		MS6	E	.	.	0.018	0.0068
late	061120	MS1	E	0.603	0.668	.	.
		MS2	E	.	.	0.017	0.0093
		MS3	E	0.809	0.542	.	.
		MS4	E	.	.	0.014	0.0075