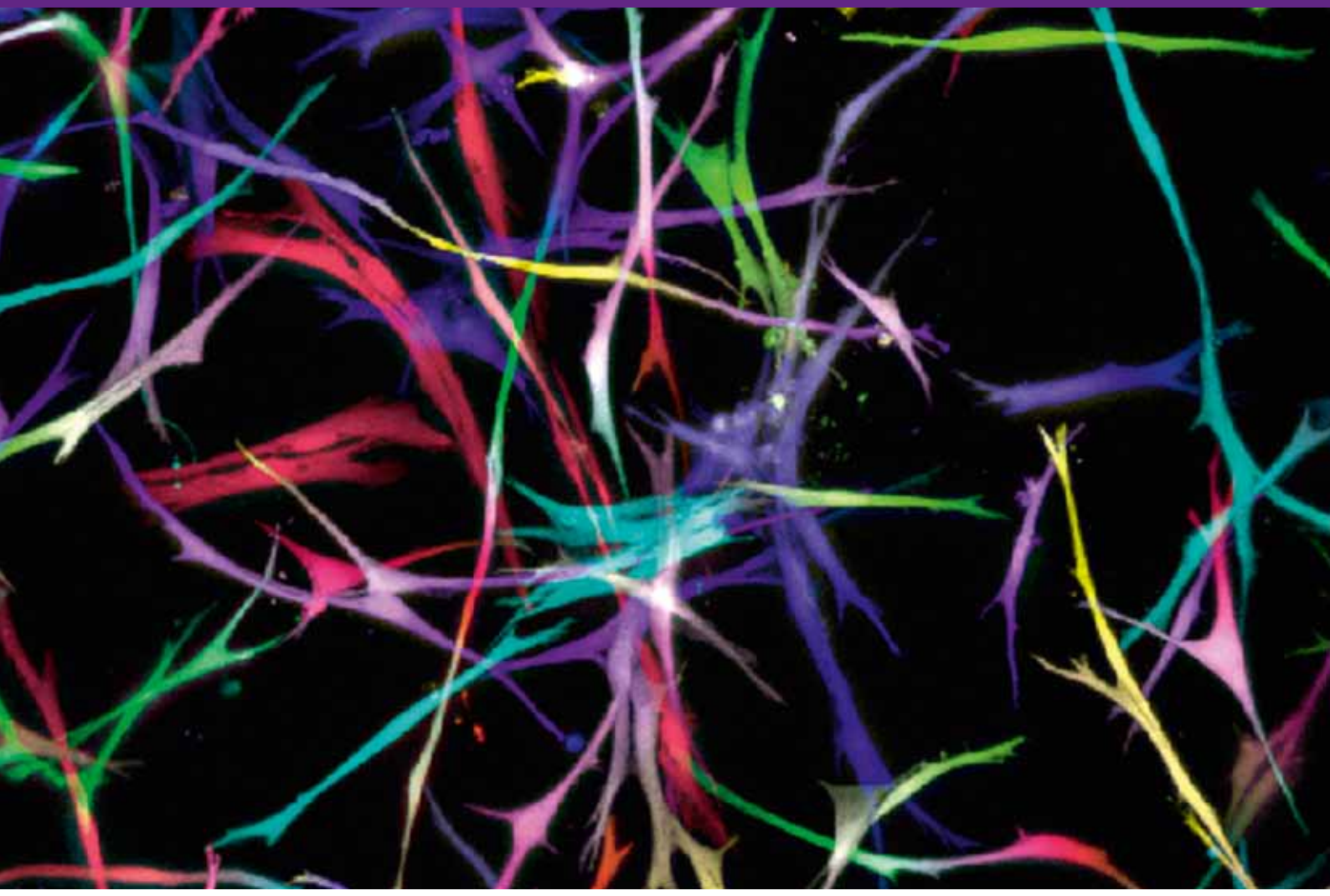
PAS 93:2011

Characterization of human cells for clinical applications

Guide



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Ministerial statement

I am delighted to introduce this new BSI publication that follows on from the successful previously published PAS 83 and PAS 84. It is of great importance to the UK that we not only provide new and innovative medicinal and healthcare products to an ageing population, but also develop the wealth creating industries that will serve this growing need.

The field of cell therapies, including regenerative medicine, is rapidly advancing and the UK has particular strengths in this area, as demonstrated by a recent report from the Office for Life Sciences "Taking Stock of Regenerative Medicine in the UK". The Government intends to bolster and grow the UK's global position in the technology and to support its translation into practical medical therapies.

PAS 93 is a crucial repository of knowledge that will guide developers of new cell therapy products towards successful commercialization and adoption of the technology. It is the first of its kind in the world and is a good example of how UK academia, industry and Government can work in partnership to deliver real economic and social benefits through standardization. BSI has a crucial role to play in harnessing and delivering such knowledge and I encourage all relevant stakeholders to consider how they could use standardization to deliver benefits such as this.

Rt Hon David Willetts MP
Minister for Universities and Science

Foreword

This Publicly Available Specification (PAS) was commissioned by the Department for Business Innovation & Skills (BIS) and its development was facilitated by the British Standards Institution (BSI). It came into effect on 31 August 2011.

Acknowledgement is given to Alison Wilson as technical author, Damian Marshall for developing the annexes on the cell characterization techniques, and the following organizations that were involved in the development of this PAS as members of the steering group:

- BioPharmaceutical Solutions Ltd
- Cell Data Services
- Consulting on Advanced Biologicals
- Department for Business Innovation & Skills (BIS) – Office for Life Sciences
- Department of Health
- LGC
- Loughborough University – Centre for Biological Engineering
- National Physical Laboratory
- University College London
- University of Sheffield – Centre for Stem Cell Biology

Acknowledgement is also given to the members of a wider review panel who were consulted in the development of this PAS.

Relationship with other publications

This PAS complements PAS 83, *Guidance on codes of practice, standardised methods and regulations for cell-based therapeutics – From basic research to clinical application* [1)], which exemplifies the place of characterization in the context of the overall development process for a cell therapy product.

The terms and definitions given in PAS 84, *Regenerative medicine – Glossary* [1)], apply to this PAS.

Presentational conventions

The provisions in this standard are presented in roman (i.e. upright) type. Its recommendations are expressed in sentences in which the principal auxiliary verb is "should".

Commentary, explanation and general informative material is presented in italic type, and does not constitute a normative element.

Contractual and legal considerations

This publication does not purport to include all the necessary provisions of a contract. Users are responsible for its correct application.

Compliance with a PAS cannot confer immunity from legal obligations.

[1)] Under revision.

Introduction

This PAS is designed to provide guidance on the characterization of human cells for clinical applications. It complements PAS 83, which exemplifies the place of characterization in the context of the overall development process for a cell therapy product.

An overview of the scope of PAS 93 is illustrated in Figure 1.

Terms used in this PAS are defined in PAS 84, a glossary for cell therapy and regenerative medicine.

For clarity, this PAS differentiates between the terms "cell therapy product", "medicinal product" and "advanced therapy medicinal product" (ATMP).

Cell therapy product is the broadest term used in the PAS and aspects discussed in the context of cell therapy products are applicable to all products consisting of or containing human cells.

In the EU, a medicinal product is one that is subject to the regulatory framework set out in European Directive 2001/83/EC [1] for the authorization, control and marketing of medicines. Medicinal products require prior approval from regulatory authorities before they can be used in clinical trials or sold in any country of the European Economic Area.

An ATMP is a cell-based (or gene-based) product that is subject to the general provisions of the regulatory framework for medicinal products and also to the specific provisions of Regulation (EC) No 1394/2007 [2].

The PAS also includes reference to US requirements. The US regulates most cell therapy products as biologics. This legal category is distinct from drugs (US) but the requirements are broadly comparable, and biologics require prior approval from the Food and Drug Administration (FDA) under Section 351 of the Public Health Services Act (PHS Act) [3] and relevant sections of the Food, Drug and Cosmetics Act (FD&C Act) [4] before they may be used in clinical trials or marketed in the US.

The US also recognizes human cell/tissue products (HCT/Ps) which are not subject to pre-marketing approval but are regulated solely under Section 361 of the PHS Act [3] and Title 21, Part 1271 of the Code of Federal Regulations (21CFR1271) [5] (establishment registration, donor eligibility requirements and good tissue practices). The information in this PAS could be of interest to developers of HCT/Ps, however, there is no current regulatory requirement for the characterization of HCT/Ps. Cell therapy products as covered in this PAS are not specifically defined by US legislation but are regulated under the category of biologics.

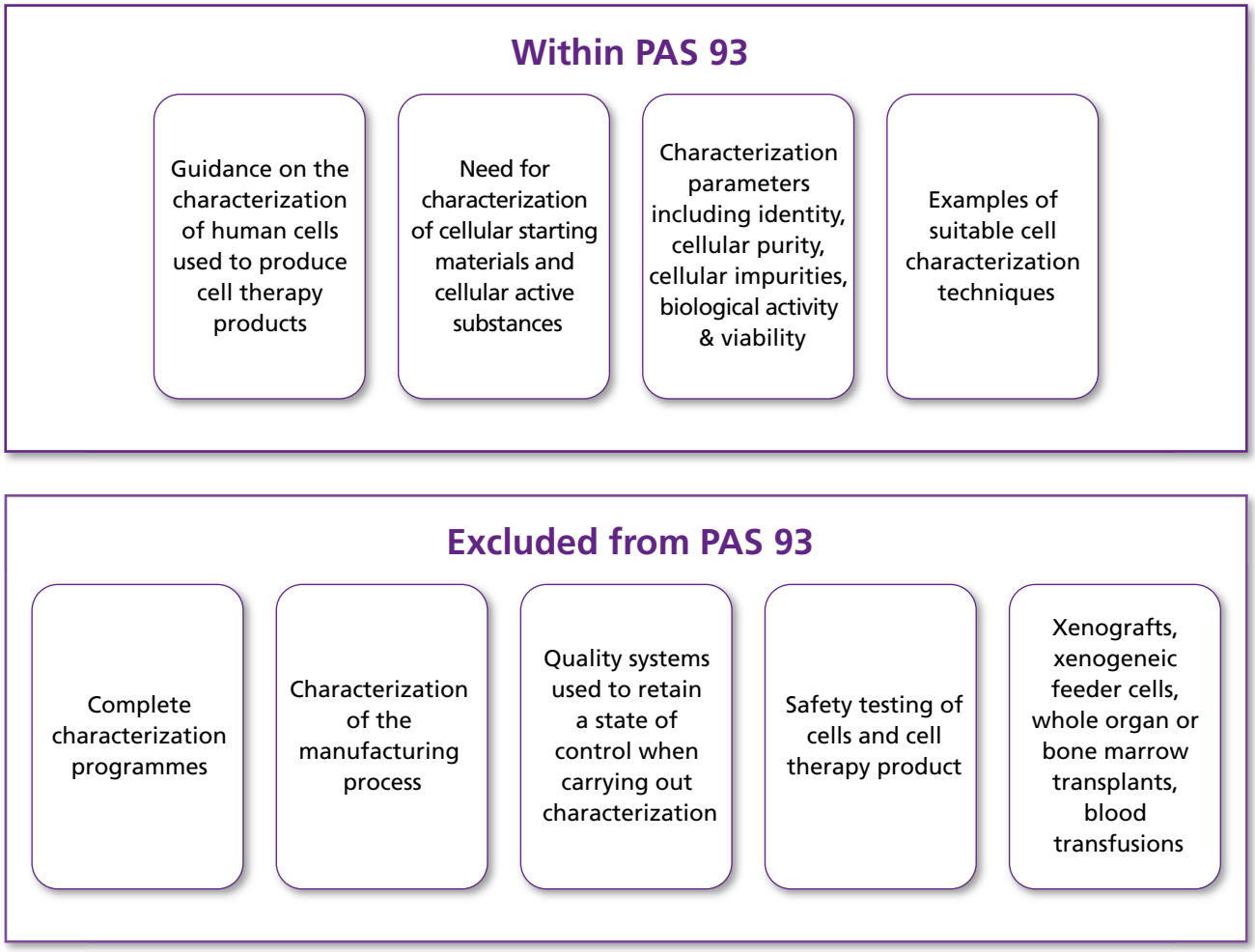
This PAS provides general guidance applicable to all cell therapy products and also guidance specific to licensed biologics and ATMPs.

Approval of a medicinal product requires the submission of a marketing authorization application to the appropriate regulatory authority. The application contains all of the known information concerning the quality, safety and efficacy of the medicinal product.

In the EU, the Marketing Authorisation Application (MAA) is submitted to the relevant authority: the European Medicines Agency is responsible for authorization of ATMPs in Europe.

In the US, the application is known as New Drug Application (NDA) for products regulated as drugs, and a Biologics Licence Application (BLA) for products regulated as biologics. The scientific information requirements are similar to those expected in the EU. The relevant authority is the Food and Drug Administration. Description of the regulatory frameworks in the EU and the US is beyond the scope of this PAS.

Figure 1 – Overview of PAS 93 scope



1 Scope

This PAS gives guidance on the characterization of human cells being developed for clinical applications.

This PAS is not a regulatory guideline, but gives guidance on the need for characterization of cells and key current, available cell characterization techniques. The choice of characterization techniques needs to be made by the developer on a case-by-case basis depending on the intended use of the cells.

This PAS covers the need for the characterization of cellular starting materials and cellular active substances and the characterization parameters (including identity, purity, cellular impurities, biological activity and viability).

This PAS is intended for use by organizations and individuals with an interest in the development of human cells for clinical applications including academic groups, small and medium sized enterprises (SMEs) and larger industrial manufacturers and the general public.

The guidance in this PAS is applicable to the characterization of human cells in the context of EU and US regulation of cell therapy products, but it will be broadly relevant to all regions/markets that accept or refer to International Conference on Harmonisation (ICH) guidance in the regulation of biological medicinal products including cell therapy products.

This PAS is not a manual for developing a cell therapy product and does not cover:

a) complete characterization programmes;

b) characterization of the manufacturing process;

c) quality systems used to retain a state of control when carrying out characterization;

d) safety testing of cells and cell therapy products; and

NOTE *Requirements for safety testing of cells and cell therapy products are addressed in detail in relevant legislation and guidance documents.*

e) xenografts, xenogeneic feeder cells, whole organ or bone marrow transplants and blood transfusions.

2 Terms, definitions and abbreviated terms

2.1 Terms and definitions

For the purposes of this PAS the terms and definitions given in PAS 84 apply.

2.2 Abbreviations

ATMP	advanced therapy medicinal product (EU)
BLA	biologics licence application (US)
CBER	Center for Biologics Evaluation and Research (US)
CBMP	cell-based medicinal products
CDER	Center for Drug Evaluation and Research (US)
CHMP	Committee for Medicinal Products for Human Use (EU)
EMA	European Medicines Agency
EU	European Union
FDA	Food and Drug Administration (US)
FD&C Act	Food, Drug and Cosmetic Act (US)
HCT/P	human cells, tissues and cell and tissue-based products (US)
HSC	haematopoietic stem cell
ICH	International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use
MAA	Marketing Authorisation Application (EU)
MSC	mesenchymal stem cell
NDA	New Drug Application (US)
PHS Act	Public Health Services Act (US)

3 The need for characterization

3.1 Scientific need for characterization

Characterization of cells used for clinical application involves an integrated assessment of the identification and properties of the cells, both as a cellular starting material and as the cellular active substance for a cell therapy product. The characterization of cellular starting materials and cellular active substances is a key part of the development of cell therapy products; its place in the overall development is exemplified in PAS 83.

There is a need to characterize both the cellular starting material and the cellular active substance, as a clear understanding of how the cells behave throughout the manufacturing process is essential in assessing the impact of processing changes or changes in the materials used on the cells and thus the finished cell therapy product. The quality, safety and efficacy of the product is intricately linked to the manufacturing process. Because cells cannot be fully characterized in the laboratory, developers should optimize product consistency, quality, and purity by ensuring that the manufacturing process remains the same over time and that any necessary changes are affected in a controlled manner. At the same time, the developer should develop tools (assays) that can be used as evidence towards confirming that the critical characteristics of the cells have not been altered.

Characterization is required for:

a) product development – to ensure that the relevant properties of the cellular starting material and the cellular active substance are adequately understood and can be controlled; and

   NOTE *It is important to understand the attributes of the cells that are most important for the function of the cell therapy product, and the effects that changes in the manufacturing process may have upon them. The extent to which critical properties can vary without affecting the overall cell therapy product needs to be elucidated.*

b) comparability – to establish whether changes (for example, scale-up of manufacture, introduction of a new material) are likely to impact upon the safety or efficacy of the cell therapy product.

A detailed understanding of the properties of the cellular starting material and the cellular active substance is essential in order to control the quality, and therefore the safety and efficacy of the cell therapy product. Characterization data should be accumulated during the development process. This will allow the developer to define appropriate test methods and establish detailed release specifications controlling identity, purity and potency of the cellular starting material and the cellular active substance. If adequate characterization is not undertaken during development, it could be difficult to justify the choice of test methods selected for routine control of the cells, and demonstration of comparability following process changes might be problematic without non-clinical and/or clinical comparability studies.

The regulatory authorities in the EU, US and in other jurisdictions have indicated that reliable and comprehensive characterization data are essential to underpin many of the most critical aspects of control of medicinal products; this is a principle set out in ICH and regional guidelines. Owing to the immense structural and functional complexity of a single cell the regulatory authorities require detailed characterization of both the cells and the cell therapy product to which they contribute. The influence of culture processes and environment on viable cells requires that characterization of the cell therapy product is as necessary as that for the cellular starting materials and the cellular active substances. Furthermore, the range of possible cell therapy products is vast, and current formal guidelines do not (cannot) specifically cover every eventuality, so the developer has a considerable responsibility to develop and defend their individual understanding of the cells' characteristics and how they contribute to the finished cell therapy product.

Assessment of the impact of process or material changes should be undertaken using as much characterization data as exists at that point in development. The potential impacts should be considered in advance, with prospective assessment of likely impacts planned before the change is made. The acceptance criteria on which comparability will be made should be pre-defined. A detailed record of all changes made during development and an assessment of their impact on the quality, safety and efficacy of the product will be required for marketing authorisation.

Even the most fundamental concept such as the identity of the cells often cannot be confirmed by reference to the simple tests required for identification of chemical drugs or conventional biological drugs.

The regulatory requirements for medicinal products (EU) and biologics (US) include an obligation for the developer to identify that the cellular starting material or cellular active substance is the intended one and that it is present in the cell therapy product. This might include the ability to determine a range of specific parameters such as differentiation status, presence or absence of phenotypic markers, demonstration of functional abilities (differentiation, proliferation, expression of relevant proteins) and appropriate cell age. The identity test(s) should be specific: no other cell type that could be a potential undesirable contaminant should be capable of giving the same response as the desired cell population. Beyond identity, the cellular population requires characterizing in terms of purity (presence of unintended cell types, cellular fragments or DNA) and the specific biological activity(ies), which is/are necessary for the cell therapy product to be efficacious.

The cells used to produce cell therapy products can be sensitive to changes in the manufacturing process that could significantly affect the nature of the cell therapy product and its in vivo biological effects. Critically, these changes might not be readily detectable using routine control tests (for example the unexpected upregulation of a previously minor cytokine that is not assayed for in the cell therapy product), and therefore the developer should develop reference data, much of which will not be needed on a routine basis, against which to evaluate the impact of process changes. To ensure that a manufacturing process remains the same over time, developers of cell therapy products should tightly control the source and quality of cellular starting materials and cellular active substances, and consistently employ rigorous process controls, appropriate reference materials, and trending to assure consistent and predictable manufacturing outcomes.

A clear understanding of how the cells behave throughout the manufacturing process is essential in order to:

a) assess the impact of processing or material changes on the cells and thus the cell therapy product; and

b) demonstrate that changes to the process or materials have not adversely affected the cell therapy product.

NOTE *Characterization data may be used to show that studies performed with earlier iterations of the product or the process are still relevant for the assessment of the final product during the MAA/BLA process.*

The characterization of cells involves assessing their properties across a broad range of aspects. Information on cell characterization techniques is given in Annex A.

3.2 Regulatory need for characterization

3.2.1 General

Characterization is discussed in a number of legislative and guidance documents (see **3.2.2** to **3.2.4**).

EU law is represented by the text of the Directives and Regulations, and in the national transpositions of Directives by the Member States. Guidelines are produced by the European Medicines Agency (EMA) and other agencies to reflect the state of regulatory expectations in the interpretation of the laws themselves.

The US regulatory framework for biologics is established by the PHS Act [3] and the FD&C Act [4]. The Acts are supported by specific text published in the Code of Federal Regulations (CFR), which stipulate the requirements to be met in order to comply with the Acts, and by individual guidance documents published by the Center for Biologics Evaluation and Research (CBER) and Center for Drug Evaluation and Research (CDER).

Applications for authorization of medicinal products are required to include detailed scientific justifications in situations where the approach recommended in the EU and ICH guidelines is not adopted by the applicant.

Attention is drawn to additional US and EU published guidance documents that are specific to particular therapy areas and product types.

The ICH has issued several guidelines relevant to the development of biological medicinal products. These guidelines are applicable in all ICH regions (EU, US and Japan) and in other specific territories that have adopted them. Some of these guidelines specifically exclude cell-based products from their scope, however their general principles will be of relevance.

3.2.2 International guidelines

International Conference on Harmonisation
ICH Q6B Specifications: Test procedures and acceptance criteria for biotechnological/biological products [6]

ICH Q6B gives extensive guidance on the characterization of biological medicinal products. Its scope is limited to proteins and peptides and their derivatives, and it specifically excludes cells from the scope. However, the principles of this guideline are applicable in relation to characterization of cells for clinical application. ICH Q6B provides some background on how the regulators view the characterization of biological products.

3.2.3 EU requirements

European Communities

Directive 2001/83/EC of the European Parliament and of the Council of 6 November 2001 on the community code relating to medicinal products for human use [1]

Directive 2001/83/EC is the core directive on medicinal products. It includes an Annex that specifies the data requirements for approval of medicinal products in the EU. Specific requirements for ATMPs are included in Annex I Part IV of this directive. These are in addition to the general requirements for medicinal products (Part I) and any other specific requirements in other sections of Annex I.

NOTE *Directive 2001/83/EC has been amended on several occasions by additional Directives and Regulations, including the ATMP Regulation [2] itself. The effects of these amendments is cumulative and the developer needs to understand that all aspects of the EU medicinal product framework are applicable to ATMPs: the general requirements set out in Directive 2001/83/EC as well as the specifics of the ATMP Regulation.*

European Medicines Agency

Guideline on human cell-based medicinal products [7]

EMA's guideline is the basic starting point for adapting medicinal product data requirements to the specifics of cell therapy products. This guideline includes several references to requirements for characterization.

3.2.4 US requirements

US Department of Health and Human Services

Code of Federal Regulations, Title 21 – Food and Drugs Chapter I – Food and Drug Administration, Department of Health and Human Services – Subchapter F: Biologics – Part 601: Licensing **(21CFR601)** [8]

21CFR601 of this section of the US regulations establishes the requirements for the licensing of biologics. Subsequent sections establish general standards for biologics (Section 610).

US Department of Health and Human Services

Code of Federal Regulations, Title 21: Food and Drugs – Chapter I: Food and Drug Administration, Department of Health and Human Services – Subchapter F: Biologics – Part 610: General Biological Standards **(21CFR610)** [9]

21CFR610 relates specifically to the safety specifications to which licensed biological products in the US are submitted prior to release to the market. Attention is drawn to the requirement for pyrogen testing in 21CFR610.13b (Purity).

21CFR610.18 (Cultures) specifies requirements for cell cultures used in the production of biological medicinal products, and includes requirements for cell history, description of cytogenetic characteristics and characterization of in vitro growth characteristics and life potential.

US Department of Health and Human Services (FDA)

Guidance for Industry: Guidance for Human Somatic Cell Therapy and Gene Therapy [10]

This guideline covers the development and characterization of cell populations for administration, and the characterization and release testing of cell therapy and gene therapy products. Although it refers to somatic cell therapy in its title the main focus is on gene therapy products.

US Department of Health and Human Services (FDA)

Content and Review of Chemistry, Manufacturing, and Control (CMC) Information for Human Somatic Cell Therapy Investigational New Drug Applications (INDs) [11]

This guidance was written to help clinical trial sponsors and FDA reviewers to assess, for a given phase of clinical investigation, whether sufficient information is provided to assure the proper identification (identity testing), quality, purity, and strength (one aspect of potency) of the investigational product. It gives detailed discussion of the expectations of FDA when reviewing INDs for the completeness of the technical information provided by the sponsor.

4 Provenance of the cells

Cell history does not constitute a part of characterization, however the provenance and culture of cells could have significant effects upon the subsequent behaviours and characteristics of the cells.

The provenance of the cells should be documented to demonstrate compliance with extant regulations concerning donor consent, tissue procurement and testing of donors and tissues.

NOTE *Attention is drawn to in the EU, Directive 2004/23/EC [12] and its implementing Directives 2006/17/EC [13] and 2006/86/EC [14], and in the US, Donor Eligibility Final Rule [15] on donor consent, tissue procurement and testing of donors and tissues.*

The following information on cell history should be documented:

a) the donor's age, sex and status (living or dead donor);
b) the donor's medical history and the results of tests performed on the donor for the detection of viral and bacterial infections; and
c) culture history of the cell line including:
    1) methods used for the isolation of the tissues from which the line was derived;
    2) passage history;
    3) media used and history of passage in animals, etc.;
    4) previous identity testing and the results of all tests for adventitious (contaminating) agents.

5 Characterization of cells

NOTE *Information on cell characterization techniques is given in Annex A.*

5.1 Identity

The cells required for the medicinal action of the cell therapy product should be proven to be present within the cellular starting material.

The identity test should be capable of distinguishing the cellular active substance from any other cells that might reasonably be present in the cellular starting material or final cell therapy product. The identity of the cells in the cellular starting material and cellular active substance should be demonstrated by specific data. A justification based on reasoning alone will be inadequate, and data should be provided to identify the cells.

It is unlikely to be acceptable to propose, for example, that "mesenchymal stem cells (MSCs) isolated from a bone marrow biopsy are MSCs just because they are adherent to tissue culture plastic and haematopoietic stem cells (HSCs) are not, and therefore the process of adhering the MSCs and washing off the HSCs can only produce a population of MSCs". Data would be expected to support this argument.

Demonstration of identity is likely to require a number of tests, not just one. Tests that result in negative responses are also useful, in that they may allow elimination of an alternative cell population from the possible options in the identity test panel, for example, two surface receptors, or a combination of expression and absence of expression of two or more receptors.

Demonstration of identity could include an evaluation of the phenotype of the cells, typically through confirming the presence or absence of established surface and intracellular markers characteristic of certain cells. The phenotypic evaluation should also include testing for markers of other cell types that could reasonably be expected based on the origin of the cells (for example keratinocytes in a dermal fibroblast population, fibroblasts in a chondrocyte population). Although this aspect of characterization falls more properly within the scope of purity of the population, they are clearly interlinked and the characterization strategy should take into account the need to determine the likely undesirable cell types that might have been introduced through the cellular starting material, e.g. tissue from a biopsy.

Phenotypic evaluation could also include markers based on gene expression, antigen presentation, biochemical activity, response to exogenous stimuli, capability to produce biologically active or otherwise measurable molecules, etc. For adherent cells, morphological analysis could be a useful tool in conjunction with other tests, although the use of morphometric analysis alone is unlikely to be accepted as adequate proof of identity. Many cell types can appear similar under different microscope conditions, and the same cell type can exhibit different morphologies depending on culture and plating conditions, so morphology alone is not a strong confirmation of identity. It may be useful to provide standard "reference" photomicrographs of the typical required appearance of the cells based on the established culture process as an ongoing in-process control tool.

The presence of the required cell phenotype does not always imply that the biological activity of the cells is appropriate, and therefore the biological activity should be confirmed as part of the characterization process (see **5.3**).

The specification for the cellular active substance should include one or a combination of identity tests necessary to confirm the identity of the cells. In accordance with the routine expectations for all medicinal products, identity tests should be used to confirm that the manufacturing process has delivered the correct cellular active substance.

5.2 Purity

Purity is closely linked to identity, and the spectrum of tests used to confirm the identity of the cellular starting material and cellular active substance might also yield information on the content of undesirable cellular impurities, such as contaminating or differentiated cells. The content of undesirable cell types in the cellular active substance used for clinical application should be defined and minimized. The specification limits for undesirable cell types should be based on a combination of data:

a) the percentage of each undesirable cell type typically seen; and
b) an assessment of the clinical safety implications of such a percentage.

The level of undesirable cell types should be monitored in development studies to determine the extent to which these cells are reduced over the course of the manufacturing process. The need for in-process controls on purity or cellular impurities should be considered.

A justification for purity based on culture conditions alone might not be satisfactory. The elimination of the undesirable cell type(s) over the culture period should be demonstrated with data during the characterization process. Physical purification techniques that isolate different kinds of cells, such as magnetic-activated cell sorting (MACS) or fluorescence activated cell sorting (FACS), can be used to separate the different cell populations in the culture of interest.

If more than one cell type is necessary for the required biological activity of the cell therapy product, the cellular starting materials may be isolated and undergo different manufacturing steps to each other, or may be manufactured together. The relative composition of the cellular starting materials should be studied where possible such that acceptable specifications can be defined.

The biological activity of the cell therapy product is assumed to be dependent upon the various activities of the cells, and therefore their viability should be assured. Inevitably the population will contain a percentage of non-viable cells, and the characterization work should aim to generate data that facilitates the setting of a maximum limit for the percentage of non-viable cells in the cellular starting material and cellular active substance to be used in the cell therapy product. In general, non-viable cells will be considered to be impurities. Viability of the cells is discussed further in **5.4**.

Impurities introduced early in the process, for example, antibiotics necessary to preserve the microbiological quality of the cellular starting material prior to initial processing, could be diluted out during subsequent processing. The absence of such impurities or residuals should be demonstrated during the characterization of the cellular active substance using appropriately qualified and sensitive detection methods. The generation of adequate characterization data showing that such residuals are undetectable may justify the absence of a routine test on the specification for the cellular active substance.

Any impurities arising from raw or starting materials, including break-down products, (e.g. from biodegradable materials, human or animal-derived proteins), should be thoroughly characterized in this respect and the impact of these impurities on the cellular active substance should be addressed.

5.3 Biological activity

Physical and chemical testing alone does not demonstrate that the cellular active substance has the required biological activity, therefore specific evaluations of biological activity should also be undertaken.

The biological activity of the cellular active substance should be characterized in terms of the properties or behaviours required to lead to a clinically functional cell therapy product. This could include the cells' ability to produce required functional proteins, change their phenotype, or exert effects on other cells (e.g. phagocytosis, cytotoxcity).

NOTE *As with other parameters, biological activity is dependent upon many different characteristics of the cells. Individual cells within the cellular population may be at different stages in the cell cycle or in differing differentiation states, leading to differences in functional characteristics such as protein expression, signalling capability, etc. Developers need to be aware of the potential effects of this variability when establishing the overall characteristics of the cell population.*

Characterization should include verification that the cells are capable of being expanded over population doublings beyond that required by the manufacturing process, and to maintain their genetic and phenotypic stability during expansion and frozen storage, if applicable. Cell behaviour should be monitored to detect cumulative changes in the biological activity of the cells over the course of the expansion process. Post-thaw viability and recovery rates should also be investigated if cells are cryopreserved during the manufacturing process. Such information facilitates the setting of in-process controls and maximum population doubling levels in production.

In the case of tissue engineered constructs, in which different cell types are combined, or during the production of which extracellular matrix is generated by the cellular active substance, it might not be possible to completely characterize the biological activity of the cells at the cell bank or cellular active substance stage because of the functions that may be affected by the presence of the other cell type. This should be addressed during characterization of the final cell therapy product.

Specifications relating to the relevant biological activity of both the cellular starting material and the cellular active substance should be set to ensure that they will perform as expected in subsequent manufacturing processes and ultimately lead to a functional cell therapy product.

5.4 Viability

The viability of the cell population is normally considered critical – at each stage the process needs to be able to produce a minimum number of viable cells capable of proliferation and/or production of proteins as required for the action of the cell therapy product.

Limits on the presence of non-viable cells should be established as a percentage of such cells in the population as part of the purity determination (see **5.2**). A lower limit on the number of viable cells yielded from a vial of the master cell bank or autologous cell lot should be established. Non-viable cells are generally considered an impurity except in the situation in which the cellular active substance itself is composed of non-viable cells.

In addition to determination of the percentage of viable cells in the cellular active substance produced for subsequent use in the cell therapy product, viability should also be considered as part of the evaluation of recovery of cells after any thawing step: the percentage of viable cells achieved at, e.g. 24 hours post-thaw.

5.5 Specification

The specification is considered to be an output of characterization. The understanding of cell properties and behaviours achieved during the characterization work allows for generation of specifications that are capable of confirming the consistency and biological activity of the cellular active substance. These specifications should be established for the acceptance of the cellular active substance such that they can be used in the manufacture of the cell therapy product. The development programme, guided by the risk analysis, should identify those parameters for which the cells need to be assessed, and establish limits on those parameters.

The characterization process is, in part, the process of gathering data on the characteristics of batches of cellular starting material and cellular active substance in order to establish what is necessary for the performance of the cells in the cell therapy product. This provides data on variability that might be related to the cells themselves. These data will normally form part of the justification for the final specification range.

The final specification for the cells should include aspects of identity, purity and biological activity, as well as microbiological quality requirements.

The characterization data should be used to select and justify those parameters that need to be assessed for each lot of cellular starting material and cellular active substance, and which characteristics can be kept in reserve in case a more thorough assessment is required.

The characterization data should be used to identify critical parameters that need to be assessed for each lot of cellular starting material. Where the cellular starting material is changed (e.g. a new working cell bank or different biopsy location or procedure), the additional characterization undertaken during development provides a basis for extended characterization to ensure the change has not altered the quality of the cellular starting material.

NOTE *It is not always necessary to include the full range of characterization assessments in the specification.*

The specification for the cellular active substance should be developed in sufficient detail to ensure:

a) consistency of the cellular active substance when manufactured on a routine basis; and
b) that the cellular active substance being produced for use in commercial lots of a cell therapy product is comparable to that used for safety tests or clinical trials.

For release purposes, any assay that is required to be performed before release of the cells should fit in with the timescale of their useable shelf life.

Annex A (informative)
Cell characterization techniques

Table A.1 – Application of cell characterization techniques – Constituent analysis

Technique		Application																													
		Identity											Purity				Biological activity								Stability					Other	
		Single protein marker	Multiple protein markers	Protein secretion	mRNA expression	MicroRNA expression	Morphology	Adherence	Differentiation potential	Genetic identity	Proliferation	Distribution	Absence of markers	Single marker expression	Multiple markers	Cellular impurities	Receptors	Metabolism	Secreted proteins	Migration	Proliferation (lack of)	Differentiation potential	mRNA expression	MicroRNA expression	Proliferation	Recovery	Chromosomal stability	Stress markers	Phenotypic stability	Cell concentration	Cell viability
Protein analysis	Mass spectrometry A)		O	O X									O		O	O X		O X	O X												
	Immunocytochemistry	O	O						O				O	O	O		O					O						O	O		
	Immunohistochemistry B)	X	X										X	X	X		X												X		
	ELISA A)	O	O	O X									O	O X		O X			O X			O						O	O		
	Electrochemiluminescence A)	O	O	O X									O	O X	O X	O X			O X									O			
	Flow cytometry	O	O				O		O		O		O	O	O	O X	O				O	O			O			O	O		O
	Protein ligation assay A)	O											O	O			O					O						O	O		
	Protein array	O	O	O X					O										O X												

Table A.1 – Application of cell characterization techniques – Constituent analysis *(continued)*

Technique		Application																														
		Identity										Purity				Biological activity								Stability					Other	
		Single protein marker	Multiple protein markers	Protein secretion	mRNA expression	MicroRNA expression	Morphology	Adherence	Differentiation potential	Genetic identity	Proliferation	Distribution	Absence of markers	Single marker expression	Multiple markers	Cellular impurities	Receptors	Metabolism	Secreted proteins	Migration	Proliferation (lack of)	Differentiation potential	mRNA expression	MicroRNA expression	Proliferation	Recovery	Chromosomal stability	Stress markers	Phenotypic stability	Cell concentration	Cell viability
Gene analysis	qRT-PCR [C]				O X	O X			O X													O X	O X	O X				O			
	Microarray [C]				O X	O X			O X													O X	O X	O X							
	Digital PCR [C]				O X	O X			O X													O X	O X	O X							
	PCR [C]				O X	O X			O X													O X	O X	O X							
Genome analysis	STR									O X																						
	CGH									O X									O X								O					
	FISH									O																						
	Karyology									O																	O					

Key
O Characterization techniques applicable to isolated cells
X Characterization techniques applicable to cells embedded in a 3D matrix

[A] When used with cells in a 3D matrix, the analyte can become entrapped making the characterization technique non-quantitative.
[B] Samples require sectioning prior to analysis, therefore compatibility of 3D matrix with paraffin, resin or cryo-embedding has to be considered.
[C] When used with cells in a 3D matrix, characterization techniques have to be optimized to allow the extraction of high quality intact RNA.

Table A.2 – Application of cell characterization techniques – Whole cell analysis

Technique		Application																													
		Identity											Purity				Biological activity								Stability					Other	
		Single protein marker	Multiple protein markers	Protein secretion	mRNA expression	MicroRNA expression	Morphology	Adherence	Differentiation potential	Genetic identity	Proliferation	Distribution	Absence of markers	Single marker expression	Multiple markers	Cellular impurities	Receptors	Metabolism	Secreted proteins	Migration	Proliferation (lack of)	Differentiation potential	mRNA expression	MicroRNA expression	Proliferation	Recovery	Chromosomal stability	Stress markers	Phenotypic stability	Cell concentration	Cell viability
Imaging	Bright field microscopy						O	O																		O					O
	Epifluorescence microscopy	O	O						O					O	O		O					O						O	O		O
	Confocal microscopy [A)]	O X	O X				O X		O			O X		O X	O X		O X					O						O	O		O X
	Fluorescence resonance energy transfer (FRET) microscopy																O X														
	Transmission electron microscopy (TEM) [B)]	X												X			X														
	Scanning electron microscopy (SEM) [B)]						X					X																			
	Optical coherence tomography						X					X																			
Viability assays	Colorimetric assay																														O
	Fluorescent assay																														O
	Membrane integrity assay																														O

Table A.2 – Application of cell characterization techniques – Whole cell analysis *(continued)*

Technique		Application																												
		Identity											Purity				Biological activity								Stability					Other	
		Single protein marker	Multiple protein markers	Protein secretion	mRNA expression	MicroRNA expression	Morphology	Adherence	Differentiation potential	Genetic identity	Proliferation	Distribution	Absence of markers	Single marker expression	Multiple markers	Cellular impurities	Receptors	Metabolism	Secreted proteins	Migration	Proliferation (lack of)	Differentiation potential	mRNA expression	MicroRNA expression	Proliferation	Recovery	Chromosomal stability	Stress markers	Phenotypic stability	Cell concentration	Cell viability
Cell counting	Flow cytometry																				O				O					O	
	Automated cell counter										O										O				O					O	
	Total DNA										O										O				O						
	Hoechst/DAPI/PI										O										O				O						
	Impedance spectroscopy										O										O				O	O					
Cell migration	Dunn chamber																			O										
	Boyden chamber																			O										
	Scratch assay																			O										
	Matrigel invasion																			O										
	Impedance chamber																			O										

Key

O Characterization techniques applicable to isolated cells

X Characterization techniques applicable to cells embedded in a 3D matrix

A) When used with cells in a 3D matrix, the optical properties of the matrix have to be considered as well as the barrier to mass transfer, which can affect the ability to incorporate fluorescent labels.

B) Requires sectioning to visualize cells embedded in a matrix.

Table A.3 – Practical considerations for cell characterization techniques – Constituent analysis

	Technique	General	Advantages	Disadvantages
Protein analysis	Mass spectrometry (MS)	MS is an analytical technique that can be used to aid the identification of unknown compounds, determine the isotopic composition of elements, and determine the structure of a compound by observing its fragmentation. The MS principle is based on ionizing molecules and/or atoms to generate charged species and measuring their mass-to-charge ratios.	• Offers higher specificity than other methods of protein analysis • Uses small sample size (µl volumes) • Attomole level detection • Can provide structural information on proteins	• Expensive cost per sample • Requires lengthy optimization for measurement of cell samples • Requires specialized staff • Sensitivity can be affected by matrix effects
	Immuno-cytochemistry (ICC)	ICC uses antibodies that target peptides or protein antigens in the cell via specific epitopes. Once bound to the target epitope the primary antibody is typically detected using a fluorescently labelled secondary antibody and imaged using either epifluorescent or confocal microscopy.	• Can be used to examine positive and negative expression • Allows protein markers to be detected within the sub-cellular compartments • Quantum dots can be used to increase resolution • Multiple markers can be detected within a single sample	• Reliant on the binding efficiency and specificity of the antibodies • Signals can be quenched when using multiple fluorophores • ICC is typically not quantitative
	Enzyme-linked immunosorbent assay (ELISA)	ELISA is an antibody-based diagnostic technique used for the detection of known analytes. For traditional ELISAs using chromogenic substrates, the enzyme-linked detection antibodies bind to the protein analyte immobilized to the assay plate. The enzyme (e.g. horseradish peroxidase or alkaline phosphatase) catalyses the hydrolysis of chromogenic substrates. Quantification of the protein analyte is achieved by measuring the intensity of the coloured products using spectroscopy at the appropriate wavelength.	• Assays are versatile, easy to design and to perform • ELISA kits are commercially available for popular protein targets • Detection can be via fluorogenic, chromogenic or luminometric measurements • Can have high sensitivity (pg/µl) • Requires small sample volumes (typically 25 µl to 50 µl)	• Traditional chromogenic substrates can limit sensitivity • Fluorogenic substrates can be subject to photobleaching • Sensitivity can be affected by matrix effects
	Electrochemi-luminescence	Electrochemiluminescence platforms (such as MSD) utilize the same underlying principles as ELISAs but with electrically induced luminescence detection. The immunoassay comprise stepwise incubations to capture the analyte to the assay plate with subsequent bounding by a ruthenium tagged detection antibody. A voltage is applied which stimulates a chemiluminescence reaction, catalysed by the ruthenium label on the detection antibody, thus emitting light. The signal output (light) is measured using a cooled charge couple device (CCD) camera close to the base of the reaction well, minimizing background noise. This enables the greater tolerance of complex matrix effects such that neat serological samples may be used in the assay.	• Assays are versatile, easy to design and to perform • There is scope for multiplexing assays for up to ten targets per reaction well • Platform tolerates complex matrix effects as only genuinely bound ruthenium label is detected by the CCD camera • Greater sensitivity and more dynamic range than ELISA • Low sample and reagent volumes are required (25 µl to 30 µl) • Can measure a 384 reaction well plate (up to 3 840 targets) within a minute	• Expensive set-up costs (>£100 000) • Increased cost compared to ELISA technology • Requires specialized staff to develop/optimize assays

Table A.3 – Practical considerations for cell characterization techniques – Constituent analysis *(continued)*

	Technique	General	Advantages	Disadvantages
Protein analysis *(continued)*	Fluorescent flow cytometry	Fluorescent flow cytometry is an optical-based analysis technique that allows the simultaneous multi-parametric measurement of cell size and granularity using forward and side scattering of light and the expression of cellular proteins using antibodies conjugated to fluorescent dyes. The use of multiple fluorochromes, each with similar excitation wavelengths and different emission wavelengths allows several cell characteristics to be measured simultaneously.	• Can measure multiple proteins in a single sample, theoretically >18 • Can be used for high-throughput analysis using an automated plate loader • High data acquisition rate (up to 70 000 cells per second) • Can provide morphological information (size and granularity)	• Measurements are affected by the binding efficiency and specificity of the antibodies • Is difficult to distinguish signals with similar emission spectra • Requires specialized staff to operate the system • Expensive set up costs, typically >£100 000 • Can only be used with cells in suspension
	Protein ligation assay (PLA)	PLA uses antibodies to bind to a single cognate protein, or to two distinct co-localized proteins. If secondary antibodies conjugated with oligonucleotides are in close proximity, the probes hybridize to form a closed circular template. The circularized probe undergoes rolling circle amplification (RCA) to generate a concatemic product that is subsequently labelled with a fluorescent tagged oligonucleotides. The fluorescence can be visualized using epifluorescent or confocal microscopy.	• Highly sensitive allowing single protein measurements within cells • Can detect and quantify protein-protein interactions • Can detect protein post-translational modifications (e.g. phosphorylation) • Can measure protein expression within subcellular compartments or within tissue sections	• Measurements are expensive compared to ELISA • Requires specialized staff to set up and optimize assays • Requires validated controls as background signals can also be amplified
	Protein array	A protein array typically consists of a capture protein on a matrix such as a glass slide, silicon chip, or set of coded microparticles. The same underlying principles as for ELISAs applies, i.e. the means to immobilize the target on to the matrix and to detect the protein by a labelled antibody. A primary advantage of microarray technology over conventional immunoassays is the ability to multiplex assays. For glass slide arrays, a highly ordered array of analyte or capture antibody is deposited on to a glass slide coated with poly-L-lysine, aldehyde or silane either by passive adsorption or via chemical coupling. Solution phase protein microarrays rely on chemical coupling of the protein to the microparticle.	• Assays are versatile, easy to design and to perform • Miniaturization technology with low reagent and sample consumption (µl volumes) • Ability to multiplex assays for simultaneous detection of multiple proteins • Can measure up to 40 000 targets per glass slide array • Improved sensitivity compared to ELISA's • Particle microarray platforms have also been developed to minimize steric constraints and thereby enhance reaction kinetics	• Expensive set-up costs (typically >£100 000) • Fluorophores tend to be used for measuring signal output which are subject to photobleaching • Requires specialized staff for assay design and data analysis • Instruments with integrated microfluidics have potential for blockages depending on the sample matrix • For glass slide arrays, a dedicated spotter (contact or piezoelectric) is required and has to be carefully calibrated

Table A.3 – Practical considerations for cell characterization techniques – Constituent analysis *(continued)*

	Technique	General	Advantages	Disadvantages
Gene analysis	qRT-PCR	qRT-PCR is a molecular method for quantifying the expression of genes of interest in a biological sample. The targeted mRNA or microRNA is reverse transcribed (RT) and then amplified by PCR using fluorescence probes or intercalating dyes. The number of PCR cycles needed is directly related to the amount of RNA in the sample.	• Works over a large dynamic range (1 cell to 1 000 000 cells) • Sensitive technology: can identify gene expression in a single cell • Data are quantitative (using standard curves or relative quantification) • High throughput, with a large number of commercial reagents and chemistries available (hydrolysis probes, SYBR, molecular beacons, etc.)	• Quantification can be affected by inhibition, matrix effects and poor experimental design • Relatively expensive set-up costs (>£50 000) • Quantification can be affected by the efficiency of the RT step • High background can mask the analyte of interest
	Microarray	A microarray is a multiplex technology which consists of an arrayed series of thousands of microscopic spots of DNA oligonucleotides. These can be a short section of a gene or other DNA element that are used to hybridize a cDNA or cRNA sample under high-stringency conditions. Probe-target hybridization is usually quantified by detection of a fluorophore to determine relative abundance of nucleic acid sequences in the target and can be used to measure changes in expression levels.	• Enables gene expression profiling at a global level • Can provide information on expression of splice variants • Offers a high degree of assay specificity • Uses small sample size (µl volumes) • Rapid hybridization times	• Lack of standardization can affect data comparability • There can be a bias in labelling and amplification of target • Non-specific hybridization of target to probes can occur • Requires knowledge of target genome sequence
	Digital PCR	Digital PCR involves diluting and partitioning a sample between many hundreds of individual PCR reactions such that a single molecule or less on average is present in each PCR reaction. Positive or negative PCR reactions therefore provide a digital signal, enabling template molecules to be counted.	• Does not require a calibrant for absolute quantification • Offers high sensitivity to detect copy number variations in, for example, circulating DNA	• Increased throughput requires specialist microfluidic PCR chips • Relatively expensive set-up costs (>£70 000)
	PCR	PCR amplifies template DNA exponentially based on doubling of the number of molecules with each thermal cycle corresponding to denaturation of the double-stranded template, annealing of primers and extension by DNA polymerase.	• Suited to endpoint analysis for presence/absence of a target • Cheap set-up costs (<£10 000) • Low cost per sample (typically <£5 including RNA extraction)	• Endpoint analysis might not reflect abundance of targets in starting material • Does not provide absolute or relative quantification

Table A.3 – Practical considerations for cell characterization techniques – Constituent analysis *(continued)*

	Technique	General	Advantages	Disadvantages
Genome analysis	Short tandem repeat (STR) Also referred to as variable number tandem repeat (VNTR)	STR is a location in a genome where a short nucleotide sequence is organized as tandem repeats. These can be found on many chromosomes, and often show variations in length between individuals. Each variant can act as an inherited allele, which allows use for personal or parental identification, and in genome wide association studies.	• Genome wide distribution of target repeats • Amenable to high throughput assay technologies • Relatively cheap analysis (typically <£10 per sample)	• Requires genomic sequence information • Requires specialized staff for assay set-up and data analysis • Can be unstable between cellular generations
	Comparative genomic hybridization (CGH) Also referred to as chromosomal microarray analysis (CMA)	CGH is a molecular-cytogenetic method for the analysis of copy number changes (gains/losses) in the genomic DNA of a test sample compared to a control. The technique involves the hybridization of a fluorescently labelled target to either normal metaphase chromosomes or, with array-CGH, to a slide containing hundreds or thousands of defined DNA probes. Regional differences in the fluorescence ratio of gains/losses vs. control DNA can then be detected and used for identifying abnormal regions in the genome.	• Maps changes in sequence copy number to genomic location • Array-CGH has high level of resolution (down to 5 kb) • Utilizes samples genomic DNA, does not require cell culture	• Time consuming assays (takes 2 days) • Requires specialized staff for data analysis • Array CGH requires target genome sequence information • Structural chromosome aberrations such as balanced reciprocal translocations or inversions cannot be detected, as they do not change the copy number
	Fluorescence in situ hybridization (FISH)	FISH is a cytogenetic technique used to detect and localize the presence or absence of specific DNA sequences on chromosomes. FISH uses fluorescent probes that bind to only those parts of the chromosome with which they show a high degree of sequence similarity. Fluorescence microscopy is used to identify the chromosomal location.	• Rapid identification of alteration in ploidy (<5 hours) • Enables identification of taxonomic relationships • Can be used to detect and localize specific mRNAs within tissue samples • Can be used to define spatial-temporal patterns of gene expression within cells	• Requires specialized staff to perform assays and interpret data • Sensitivity can be affected by matrix
	Karyology	Karyology is an analysis of the number and appearance of chromosomes in the nucleus of a eukaryotic cell. The technique uses chromosome staining with a suitable dye, such as Giemsa, which is applied after cells have been arrested during cell division. Karyotyping allows basic characteristics such as differences in chromosome size, centromere position, basic chromosome number, and distribution of heterochromatin to be analysed.	• Rapid identification of alteration in ploidy (<5 hours) • Enables identification of taxonomic relationships • Relatively cheap (typically <£10 per sample)	• Low throughput • Lower sensitivity than other genetic analysis methods • Lower resolution than other genetic analysis methods • Requires specialized staff to interpret results

Table A.4 – Practical considerations for cell characterization techniques – Whole cell analysis

	Technique	General	Advantages	Disadvantages
Imaging	Bright field microscopy	Bright field microscopy is the simplest form of optical microscopy. Sample illumination is from transmitted white light and contrast in the sample is caused by absorbance of some of the transmitted light in dense areas of the sample. Improved image contrast can be achieved by changing the path length of the transmitted light using a phase ring and a matching annular ring, a technique known as phase contrast microscopy.	• Simplicity of set-up with only basic microscopy equipment required • Image features can be highlighted through the use of a coloured (usually blue) or polarizing filter on the light source • Phase contrast microscopy allows unlabelled live cells to be viewed	• Most biological samples have low contrast making imaging difficult • Low optical resolution compared to other imaging techniques
	Epifluorescence microscopy	Epifluorescence microscopy uses light passed through a wavelength selective excitation filter to irradiate a specimen with light of a specific band of wavelengths. This light excites fluorophores causing them to emit light at a longer wavelength than the original excitation light. The emitted light passes though a dichroic mirror and barrier filter to block unwanted excitation wavelengths and passes to a detector where the light is superimposed with high contrast against a very dark (or black) background.	• Can be used with live cells to measure biological events in real time • Is easy to set up and perform • Can be combined with techniques such as PLA to allow single molecule detection	• In live cell imaging, the excitation light might damage the living tissue • UV light causes the fluorophore to move into an excited triplet state, and undergo a reaction with molecular oxygen to release free radicals • In the excited triplet state, fluorophores can undergo a covalent modification that destroys their ability to fluoresce (photobleaching)
	Confocal microscopy	Confocal microscopy is a fluorescent imaging technique that is used to increase optical resolution and contrast by using lasers at specific wavelengths as the excitation source for point illumination and a spatial pinhole to eliminate out-of-focus light in specimens that are thicker than the focal plane. This creates optical sections of the specimen which can be combined to create a three-dimensional reconstruction from the obtained images.	• Allows control over the depth of field imaged • Eliminates or reduces background information away from the focal plane (that leads to image degradation) • Has the capability to collect serial optical sections from thick specimens	• Is a digital microscopy technique so the effect of signal sampling on contrast and resolution has to be considered • Requires specialized staff to optimize analysis • Expensive set-up costs (typically >£100 000)

Table A.4 – Practical considerations for cell characterization techniques – Whole cell analysis *(continued)*

	Technique	General	Advantages	Disadvantages
Imaging *(continued)*	Fluorescence resonance energy transfer (FRET) microscopy	FRET is a type of fluorescent imaging that can provide high resolution information about the spatial location of proteins. During FRET a donor fluorophore is excited and transfers its energy to an acceptor fluorophore which then emits light and is detected. This can only occur when the fluorophores are very close together (less than 50 angstroms), and hence the proteins only undergo FRET when direct binding is taking place. Consequently, this technique provides information about the locality of the proteins that is much higher than the resolution of the microscope.	• Provides information about the locality of proteins that is much higher than the resolution of the microscope • Measurements are obtained very rapidly (minutes) • Can be used to measure changes in distance between proteins	• Requires external illumination to initiate the fluorescence transfer, which can lead to higher levels of background noise • Only works if fluorophores are in correct orientation • Free fluorophores can mask energy transfer • FRET pair labelling needs to be bright with the donor completely saturated by acceptor • Can be pH sensitive
	Transmission electron microscopy (TEM)	TEM uses a beam of electrons transmitted through an ultra thin specimen. An image is formed from the interaction of the electrons transmitted through the specimen that are first diffracted by the specimen and then re-focused by the microscope lenses into a Fourier-transformed image of the diffraction pattern for the selected area of investigation. The image formed is magnified by a factor ranging from a few hundred to several hundred thousand times.	• Nanometre level resolution • Can magnify samples up to 100 000 times • Enhanced depth of field	• Expensive set-up and maintenance costs (typically >£250 000) • Requires specialized staff for sample preparation and analysis • Requires lengthy sample processing and sectioning • Requires an extremely stable high-voltage supply
	Scanning electron microscopy (SEM)	SEM produces images by probing the specimen with a focused electron beam that is scanned across a rectangular area of the specimen (raster scanning). At each point on the specimen the incident electron beam loses energy producing signals that contain information about the sample's surface topography, composition, and other properties such as electrical conductivity which can be used to create an image.	• Sub-micron level of resolution • Provides information on surface structures • Can be used to examine cell-matrix interactions	• Expensive set-up and maintenance costs (typically >£100 000) • Requires specialized staff for sample preparation and analysis • SEM samples typically viewed under vacuum so not compatible with live cells • Lower resolution than TEM • Non-conductive SEM samples require coating with gold
	Optical coherence tomography (OCT)	OCT is an interferometric technique, typically employing near-infrared light for optical signal acquisition and processing. OCT creates three-dimensional images from matrices with good optical scattering properties such as biological tissue where the relatively long wavelength can penetrate into the scattering medium.	• Higher penetration depth than tradition optical microscopies (>1 mm) • Can capture real time image data • No labelling so samples not damaged	• Can only image into biological matrices • Captures micrometer-resolution which is lower than optical microscopy

Table A.4 – Practical considerations for cell characterization techniques – Whole cell analysis *(continued)*

	Technique	General	Advantages	Disadvantages
Viability assays	Colorimetric assay	The majority of colorimetric cell viability assays measure the activity of enzymes that reduce tetrazolium salts into a coloured formazan product. The amount of formazan produced can be quantified and related to the number of cells in a sample by measuring the absorbance at specific wavelengths using a spectrophotomer. The most commonly used colorimetric assays include the MTT, MTS, XTT and WST.	• Well established and commonly used set of cell viability assays • No transfer of the cells required; the entire assay is performed in a single microplate • Assays such as MTT can be used with all eukaryotic cell types • Normal or highly metabolic cells can be used in low numbers • Low cost per assay (typically <£1)	• Might be affected by the availability of NADH, redox status of the cells and/or the presence of reactive oxygen species • Other viability tests such as automated cell counters sometimes can give different results • Some assays such as MTT produce an insoluble reaction product which requires resolubilization • Cells with low metabolic activity (e.g. lymphocytes) have to be used in high numbers • Some tetrazolium salts such as XTT and WST are not metabolized by all cell types
	Fluorescent assay	Fluorescent cell viability assays work in a variety of ways. For example, the acetomethoxy derivate of calcein (calcein AM) is transported across the cellular membrane of live cells where intracellular esterases remove the acetomethoxy group trapping the molecule which then gives out strong green fluorescence. As dead cells lack active esterases, only live cells are labelled. Other fluorescent cell viability assays such as alamar blue measure the number of live cells through their metabolism of the non-fluorescent compound resazurin to the highly red fluorescent compound resorufin.	• Assays such as alamar blue are well established and commonly used • High sensitivity, can be linear with as few as 50 cells • Calcein-AM can be used with fluorescent cell imaging or flow cytometry • Can be used with all eukaryotic cell types • Low cost per assay (typically <£2)	• Fluorescent signals can be reduced due to photobleaching • Intracellular environment can quench some fluorophores • Can be problems with background fluorescent noise (reduces sensitivity)
	Membrane integrity assay	Measurement of cell membrane integrity is one of the most common ways to measure cell viability. Vital dyes, such as trypan blue or propidium iodide, which are normally excluded from healthy cells, can cross the cell membrane and stain intracellular components if the membrane has been compromised. Alternatively, membrane integrity can be assessed by monitoring the loss of substances such as lactate dehydrogenase (LDH) which leaks from the cells when the membrane is compromised.	• Well established assays for measuring viability • Can be used with all eukaryotic cells • Not reliant on compound metabolism • Depending upon the assay the measurement can be made using imaging, automated cell counters, flow cytometry or spectrophotometry • Low cost per assay (typically <£1)	• Lower sensitivity than other assays

Table A.4 – Practical considerations for cell characterization techniques – Whole cell analysis *(continued)*

	Technique	General	Advantages	Disadvantages
Cell counting	Automated cell counter	Automated cell counters typically work on one of two principles. They either use microchannels that separate two chambers containing electrolyte solutions so that electrical impedance can be measured as cells move between the chambers. This resistance change is recorded as electric current or voltage pulses, which can be correlated to the size, mobility, surface charge and concentration of the cells. Other methods use imaging combined with viability assays, such as trypan blue, to count cells within a defined area and give a measure of viability, mean cell size and concentration.	• Impedance-based cell counters don't require cell labelling • Image-based cell counters store image data for cells • Provides an electronic report on the cell counting analysis • Fast counting method, typically takes less than 1 minute • Requires minimal sample handling • High level of reproducibility	• Problems counting agglomerated cells • Can't measure different populations in mixed cell samples • Image-based systems require optimization for each cell type
	Total DNA	Measurement of total DNA can be made using fluorescent dyes which bind cellular nucleic acids. As DNA quantity per cell is stable, a measure of cell number can be made by measuring the total fluorescence using spectrophotometry and compared against a standard curve.	• Assays have wide dynamic range, typically from 50 to 250 000 cells • Fluorescent dyes have high level of specificity for DNA • Moderately fast procedure, typically less than 1 hour	• Total DNA does not discriminate between live and dead cells • Calibration curves need to be generated for each cell count
	Hoechst/DAPI	Hoechst/DAPI are fluorescent dyes excitable under ultraviolet (UV) wavelengths which bind double stranded DNA. The dyes can cross the cell membrane of both live and dead cells and once bound to the DNA undergo a ~20-fold fluorescence enhancement due to the displacement of water molecules.	• Specific for eukaryotic double-stranded DNA • The dyes have a high quantum yield and are relatively photo-stable • UV excitation allows them to be used in conjunction with other fluorophores	• Cell counts typically have a measurement uncertainty >10% • Don't distinguish between live and dead cells • Dyes are potentially mutagenic and carcinogenic so cells cannot be reused
	Impedance spectroscopy	Cells are grown on the surface of gold electrodes in a multi-well assay plate. A low voltage current is passed across the array at defined intervals and the presence of cells affects the local ionic environment at the electrode/solution interface, leading to an increase in the electrode impedance. The more cells that are attached on the electrodes, for example as cells divide, the larger the increases in electrode impedance.	• Can be calibrated against cell numbers in a linear manner • Label-free method so allows growth/ proliferation kinetics to be measured • High throughput using multi-well assay plates • Requires minimal handling, allowing cells to be monitored in undisturbed states	• Not compatible with imaging techniques • Assay plates can suffer from "edge" effects • Expensive set-up costs and consumables (typically >£40 000)

Table A.4 – Practical considerations for cell characterization techniques – Whole cell analysis *(continued)*

	Technique	General	Advantages	Disadvantages
Cell migration	Dunn chamber	The dunn chamber consists of two circular chambers connected by a narrow bridge across which a chemotactic gradient is stably established. It allows directed migration of cells to be monitored using a time lapse microscope imaging system and cell tracking software.	• Allows kinetic measurement of chemotactic behaviour in linear gradients • Compatible with thin cover slips for optical measurement at high magnification	• Requires automated or time lapse imaging system, as well as image processing and analysis software
	Boyden chamber	The boyden chamber is a trans-migration assay, in which cells are placed in an upper compartment and migrate through a membrane filter into a lower compartment. Test compounds, such as chemotactic agents, may be placed in the lower compartment. The number of migrated cells is assessed using the Trypan blue assay, metabolic assays, or fluorochromes.	• Can discriminate between chemo-kinetic and chemotactic influences • Offers versatility in conducting mobility experiments • Systems exist in a wide variety of formats and can be modified	• Does not allow the observation of cell behaviour during chemotaxis • Cell migration assessed in the absence of flow conditions • Local chemotactic gradients in and around filter pores are unknown and variable and can influence cell-cell and cell-extracellular matrix interactions
	Scratch assay	The scratch assay measures the migration rate of cells. A "scratch" is made on a confluent cell monolayer and imaged using time lapse or video microscopy to quantify the rate at which the cells migrate into and close the scratch.	• Particularly suitable for studying the effects of cell-matrix and cell-cell interactions • Mimics cell migration during wound healing in vivo • Is compatible with imaging of live cells during migration to monitor intracellular events • Simple and cheap to set up	• Requires time-lapse microscopy and image analysis software • No chemical gradient is established for chemotaxis • Assays can take several days
	Matrigel invasion	Matrigel, an extract secreted by Engelbreth-Holm-Swarm mouse sarcoma cells, is rich in laminin and collagen IV and is used as a surrogate basement membrane for investigating cell behaviour. For invasion assays, a thin layer of Matrigel is coated onto a porous membrane in a boyden or transwell chamber and cell penetration into the gel is assessed.	• Suitable for highly invasive cells, such as cancer cells • Assays performed in more "normal" ECM cellular environment	• Batch variability with matrigel • Potential issues with matrigel creating a barrier to mass transfer
	Impedance chamber	The impedance chamber works on a similar principal to the boyden chamber but measure the cells directly so it does not require them to be labelled for analysis. Cells are placed into an upper compartment and can migrate through a semi-permeable membrane into a lower compartment which contains a gold electrode array and detects the presence of cells through their impedance to a low voltage current which is passed across the array at set intervals.	• Can be calibrated against absolute cell numbers • Label-free monitoring method so can be used for kinetic studies • High-throughput multiwell plate format • Requires minimal handling, allowing cells to be monitored in undisturbed states	• Not compatible with imaging techniques • Expensive set-up costs and consumables

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