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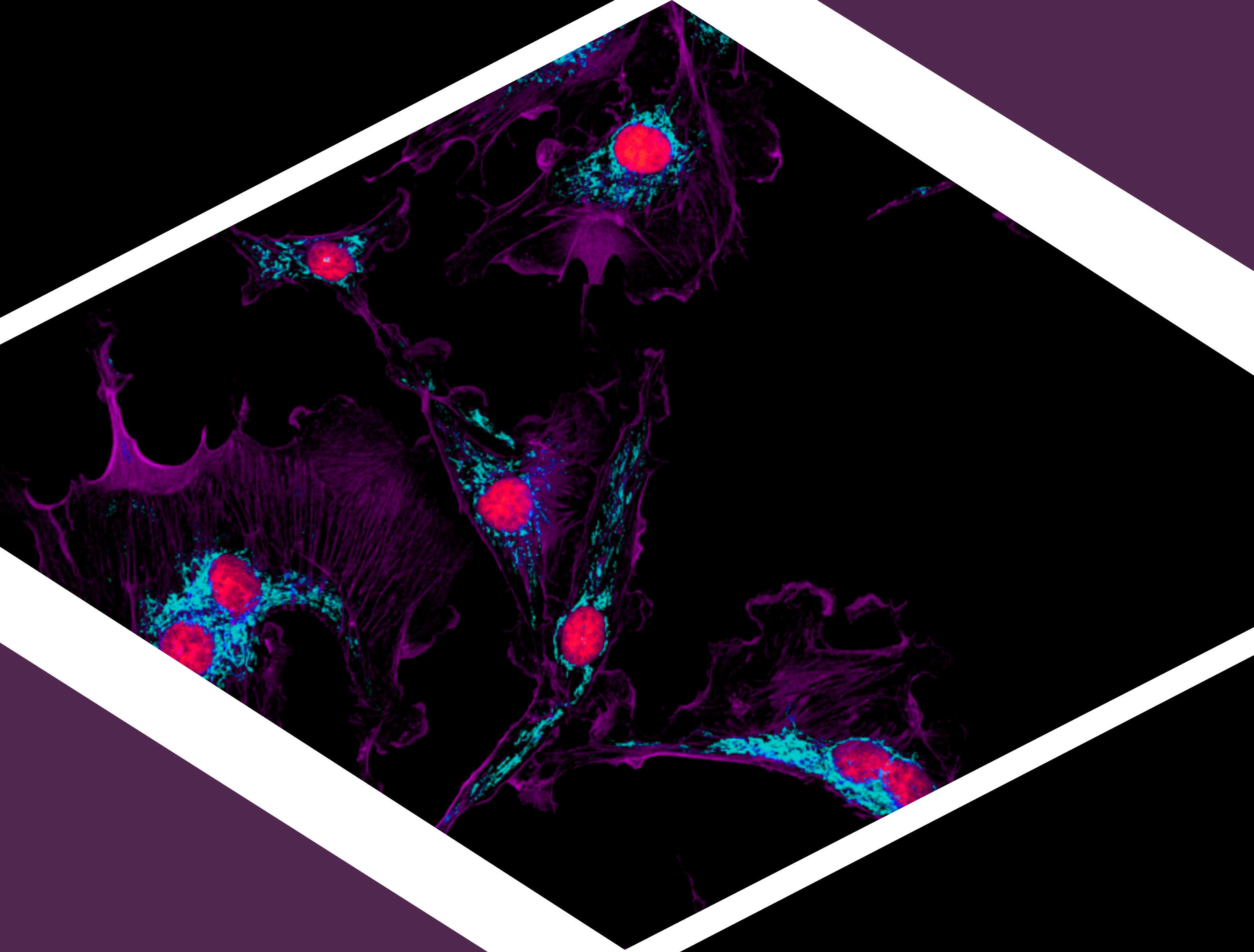

Technology digest: reagent consistency

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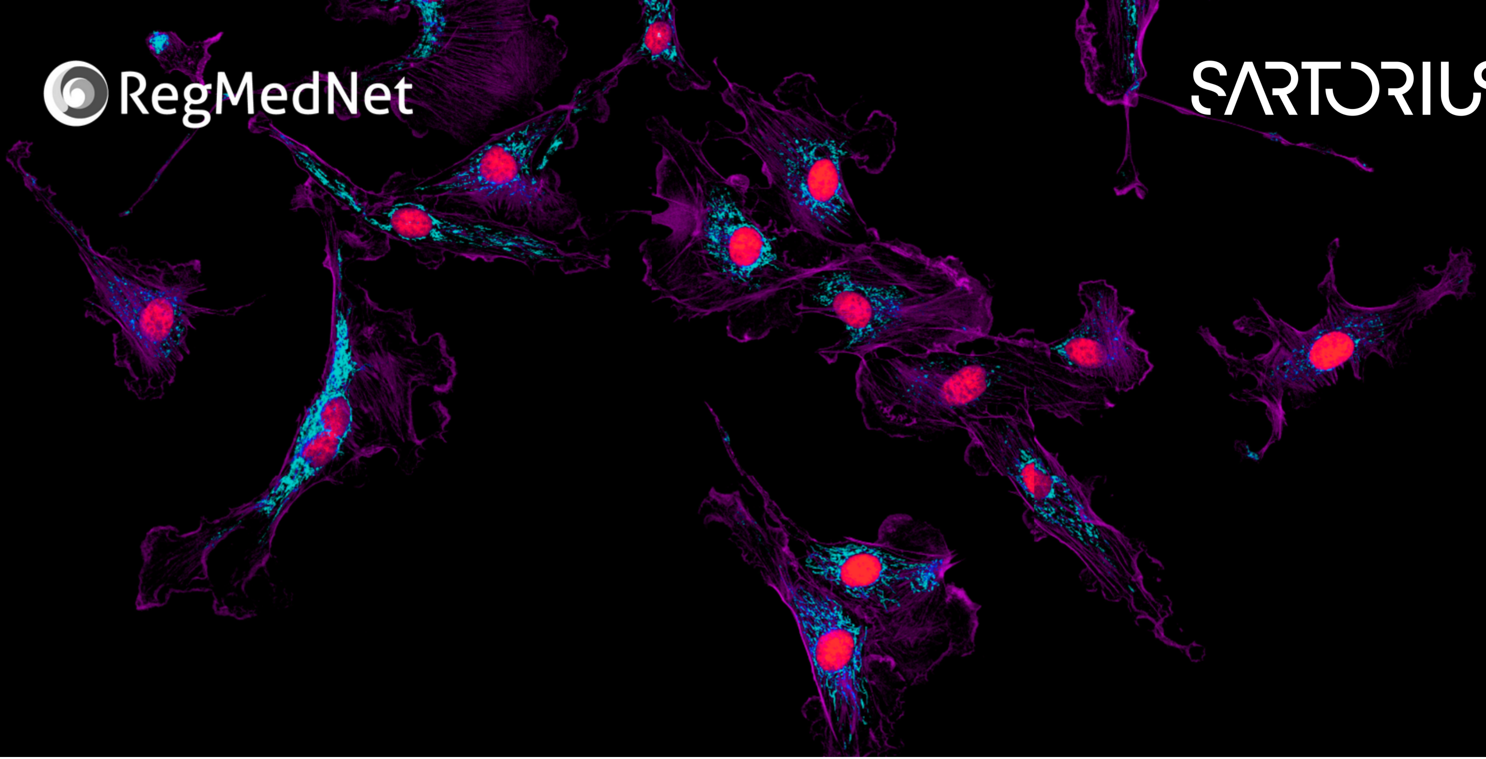

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Technology digest: The importance of reagent consistency in culturing cells for therapeutic applications

Introduction

The field of regenerative medicine has changed considerably within the past 10 years. As the industry has expanded, a growing sophistication in primary human cell culture has revolutionized our understanding of the pathological processes in many previously untreatable diseases. As a result, the increasing research and application of stem cells has paved a way for the commercialization of cell-based therapeutics, leading to a heightened awareness around the need for specialized materials used in their manufacture.

Sarah Rehman
Digital Editor
RegMedNet

Expansion of the field has fostered multidisciplinary collaboration across academic, industrial, and clinical sectors, all with the common goal of producing cells of high quality for the success of these novel therapies. To ensure high quality and reproducible results, reagent consistency is paramount.

The need for reagent consistency

The basic requirements for growing cells *in vitro* are simple: the cell culture requires both a substrate to grow on and nutrients to consume. However, with the growing sophistication of cell therapy, stem cells require more specific and finely tuned medium and substrate combinations to mimic their in vivo niche [1]. This is because different stem cells often require different culture conditions, demanding more specialized and complex materials for manufacturing reagents. "*Stem cells require much more effort to identify the specific nutrients, attachment proteins, growth factors and cytokines that best support their growth in culture,*" said Dan Haus, formerly an Application Development Scientist at Biological Industries (Beit HaEmek, Israel). Therefore, finely tuned reagents of high-quality are essential in stem cell applications.

The need for high cell quality in cell and gene therapy means that control of critical culture parameters for reproducibility is crucial. Compositional variations in cell culture reagents can result in changes in pH and salt concentrations, precipitates in solutions, or unit variations for critical enzymes [2]. Inconsistencies differ from others - can affect the efficacy of the final cell product, leading to wasted time and effort, and delayed treatment administration. Further, clinically significant variability between reagent lots can cause changes in results that pose a risk to patient care [3].

There are several factors that can reduce the efficacy of each reagent lot. One of the most common causes of reagent inconsistency are procedural errors in the laboratory, where the user makes mistakes preparing the reagent. Beyond this, other errors can include unclear manufacturer instructions, improper storage or transport that disrupts the stability of the reagent, using reagents past their expiration date and changes in critical reagent material during manufacturing.

Standardizing protocols

It is considered both good laboratory practice, as well as good laboratory regulations and accreditation standards to evaluate each new reagent lot before use [4,5], as every individual lot can affect quality control material and patient sample performance.

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Once specific and optimal culture conditions are established, researchers must ensure that these conditions are reproducible to be confident that they are working in identical compositions that will deliver products of high efficacy, from one experiment to the next.

The challenges associated with inconsistent reagents have highlighted a growing need for a standardized protocol or guideline to help laboratories ensure lot-to-lot consistency. To deal with this, the Clinical and Laboratory Standards Institute (PA, USA) published the document 'User Evaluation of Between-Reagent Lot Variation; Approved Guideline' in 2013 [5]. The EP26-A guidelines outline a standardized protocol that accounts for the resource constraints of the clinical laboratory, using the smallest possible patient samples.

The protocol describes two main steps in ensuring consistency. The first is to establish a threshold for rejection; this is based on data that defines the maximum allowance for difference between reagent lots, without having adverse impact on the final product. There should also be an appropriate statistical method applied to detect the significance of lot-to-lot differences. The second step is to test the two reagent lots on a determined number of patient samples – this verifies the adequacy of both lots by identifying any differences in performance. The results can be analyzed in accordance with the rejection threshold. The EP26-A guidelines provide the scientific community with a standardized protocol to ensure reagent consistency, strengthening reproducibility, quality, and confidence in the process.

Though undergoing the necessary steps for verification of reagents is paramount in ensuring final product quality, it can be a time-consuming task for laboratories – especially when processing products in bulk. For laboratories to streamline this process, it is important to source reagents from reliable manufacturers that provide highly specified products that meet qualified standards.

The role of the reagent manufacturer

Studies have found among all the sources that publish the composition of their reagents – from laboratories to manufacturers - commercial manufacturers were the most accurate in citation and formulation [2]. Producers of cell therapeutics can ensure reproducibility of culture conditions through reagent consistency. To achieve this, they must use sources of high-quality GMP reagents that are proven to be free of contaminants, are suitably qualified and display batch-to-batch consistency. There are a number of reagent manufacturers that fulfil these requirements, each seeking to provide superior performance and consistency of reagents for the benefit of the laboratory.

As the field of cell and gene therapy rapidly grows, the US Food and Drug Administration (FDA) regulatory guidelines call for greater control of raw materials. Serum-free media and reagents have been increasing in popularity over recent years – they are defined, reproducible and minimize the risk of introducing adventitious agents. "*By developing fully defined cell culture reagents, product developers can help reduce this variation and improve reproducibility across multiple laboratories*," advised Justin Colacino, John G. Searle Assistant Professor of Environmental Health Sciences in the School of Public Health, University of Michigan (MI, USA). Safety and reliability have made serum-free media and animal-free reagents an attractive target for cell and gene therapy manufacturers to deliver

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reproducible formulations with reduced batch-to-batch variability.

For example, Sartorius (Göttingen, Germany), a biopharmaceutical manufacturing company, offers a range of serum-free and xeno-free cell culture media and reagents [6] designed and tested to provide enhanced performance and maintain consistency during cells' culture, expansion and passaging. These include NutriStem® media [7] for the expansion and maintenance of human stem cells (human pluripotent stem cells and human mesenchymal stem cells), 4Cell® Nutri-T media [8] for the expansion and activation of immune cells, NutriFreez® solutions [9] for the cryopreservation of various cells in animal-component free conditions, as well as cell attachment and dissociation reagents. All products are chemically defined and vary in the range between xeno-free and serum-free to animal component free, therefore laboratories can leverage the benefits of increased safety, lot-to-lot consistency and simplified regulatory submissions.

As the reagent manufacturer aims to produce reliable products to streamline success of cell and gene therapies later in the pipeline, it is important that they follow standardized guidelines to guarantee quality and consistency of products. Sartorius' products are manufactured under cGMP conditions with a drug master file under FDA, attesting to their greatly reduced variability. This provides detailed information about the facilities and processes used in manufacturing, processing, packaging, and storage of their reagents, offering an invaluable insight into the end-to-end process.

Concluding remarks

The rapidly expanding commercialization of cell and gene therapy requires consistency in all areas, from laboratory protocols to the manufacture of reagents. To ensure quality and reproducibility of results, a well-informed approach must be taken when verifying lot-to-lot consistency and choosing a trusted reagent manufacturer. It is not only the laboratory's responsibility, but also the manufacturer's, to follow standardized guidelines for overall consistent results across the industry.

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Financial & competing interests disclosure

S Rehman is an employee of Future Science Group and Editor of RegMedNet. The author has no other relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.
No writing assistance was utilized in the production of this manuscript.

References

1. Zakrzewski W, Dobrzyński M, Szymonowicz M *et al*. Stem cells: past, present, and future. *Stem Cell Research & Therapy.* 10 (68), (2019).
2. Burke C.N, Croxall G. Variation in composition of media and reagents used in the preparation of cell cultures from human and other animal tissues: Dulbecco's, Earle's, and Hanks' balanced salt solutions. *In Vitro* 19, 693–698 (1983).
3. Thompson S, Chesher D. Lot-to-Lot Variation. *Clin Biochem Rev.* 39(2), 51–60 (2018).
4. Algeciras-Schimnich A. Tackling Reagent Lot-to-Lot Verification in the Clinical Laboratory. Clinical Laboratory News. (2014). https://www.aacc.org/cln/articles/2014/july/bench-matters
5. Person N. B, Budd J. R, De Vore K *et al.* User Evaluation of Between-Reagent Lot Variation; Approved Guideline. *CLSI document EP26-A*. Wayne, PA: Clinical and Laboratory Standards Institute. 33(12), (2013).
6. Sartorius. Stem Cell Culture Media and Reagents. https://www.sartorius.com/en/products/cell-culture-media-buffers/stem-cell-media-reagents
7. Sartorius. NutriStem® XF Media. https://www.sartorius.com/shop/ww/en/gbp/applications-laboratory-cell-culture-nutristem%C2%AE-xf-media/c/M_NutriStem_XF_Media
8. Sartorius. 4Cell® Nutri-T Medium. https://www.sartorius.com/shop/ww/en/gbp/applications-laboratory-cell-culture/4cell-nutri-t-medium/p/05-11F2001-1K
9. Sartorius. NutriFreez® Cryopreservation Solutions. https://www.sartorius.com/shop/ww/en/gbp/applications-laboratory-cell-culture-nutrifreez%C2%AE-cryopreservation-solutions/c/M_NutriFreez_Cryopreservation_Media

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Chapter 8

Critical reagent stability for immunogenicity assays

Arron SL Xu & Jessica Weant

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doi:10.4155/EBO.13.422

With the increase of biological drug candidates and biosimilars entering the drug development programs in the past decade, the need for robust and sensitive bioanalytical assays and critical reagents to support bioanalysis of biological drug candidates in both nonregulated (exploratory) and regulated laboratory environment throughout an entire drug-development program has highlighted the critical role bioanalytical support plays from *in vitro* screening, pharmacokinetics, safety and efficacy biomarkers development, immunogenicity assessment to product release pharmacovigilance. Biological drugs include monoclonal antibodies, antibody fragments (e.g., minibodies) and antibody–drug conjugates, nonantibody protein drugs (e.g., growth hormones), and peptide drugs [1]. Biologics are generally expressed in transfected or hybridoma cell culture with a significant post-translational modification (e.g., diverse glycoslation of the biological molecules). They may undergo chemical conjugation to generate a protein–small-molecule conjugate drug (e.g., antibody–drug conjugate). Immunogenicity assessment of biological drugs is part of the critical safety and efficacy assessment of the biological

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Biological drug: commonly includes monoclonal antibodies (e.g., infliximab), proteins (e.g., EPO), peptides (e.g., human insulin), protein–drug conjugates (e.g., trastuzumab emtansin), and fusion proteins (e.g., entanercept). Other classes of biological drugs are vaccines and siRNAs, and are out of the scope of this review.

Ligand-binding assay: refers to the detection of analytes (e.g., drugs, anti-drug antibodies [ADAs] or biomarkers) by specific interaction (e.g., binding) between the analyte and its ligand. Enzyme-linked immunosorbent assay in multiple formats is one type of ligand-binding assay commonly used to measure biological drugs and ADAs.

drug development. Various assays and formats are employed in immunogenicity assessment for anti-drug antibody (ADA) screening, confirmatory assays and antibody neutralizing assays [2]. Ligand-binding assays (LBAs), comprising of direct (e.g., direct sandwich format) and indirect (e.g., bridging format) methods, are most commonly used for initial screening, titering and confirmatory assay of ADA. Critical reagents used in the LBAs and functional assays of a biological drug are often specifically developed for the drug molecule and expressed in biological systems. For example, positive control ADA for immunogenicity assay may be developed by hyperimmunizing suitable species such as rabbit and mouse. The complex process of generating and managing the critical reagents sustainable throughout the entire biological drug-development program and their impact in the analytical performance of the bioassays highlights the importance of critical reagent stability in the success of immunogenicity assessment. This chapter will review briefly the commonly used assays for immunogenicity assessment and discuss issues of critical reagent stability with respect to assay performance.

Common critical reagents of immunogenicity assays may include:

- The biological drug in study (e.g., monoclonal antibodies, fusion protein, antibody–drug conjugate, peptide and intact proteins);
- Positive control of ADA (polyclonal antibody raised in preclinical species and used for assay development);
- Species specific or anti-idotypic antibodies with or without label of detection signal molecules;
- Biological matrices required for establishing cut point of ADA detection;
- Pimary cells and cell lines having the drug target and being used for neutralization assay;
- Drug target (receptor) and functional ligand that are used for competitive ligand-binding assay to assess the presence of the neutralizing antibody
- Other 'off-the-shelf' reagents are not considered as critical reagents in the present scope.

In vitro immunogenicity assays

LBAs

LBA is the most common assay for the screening and confirmation of ADA. The commonly used LBA assay is label-based ELISA in multiple readout modalities (e.g., absorbance, fluorescence intensity, chemiluminescence, electrochemiluminescence, fluorescence polarization, and time-resolved fluorescence polarization). Various ELISA technologies and instrumentations such as microplate-based, microbead-based, microcapillary and other homogenous formats of different detection modalities and throughput are available [2–9]. In addition, a number of label-free platforms (e.g., surface plasma resonance, biolayer interferometry or resonant wave-guide

sensing) have been used for detection of ADA [10–12]. The selection of a suitable technology and assay format depends on considerations such as the availability of critical reagents, detection sensitivity, throughput, regulatory and industry acceptance and guidance, assay transferability from preclinical laboratories to regulated clinical laboratories, and established experiences and capability of individual laboratories [3,13]. In a direct LBA format, a drug is immobilized on the solid surface of a microplate or microbead and used to capture the ADA. The detection of the ADA may be achieved by labeled secondary antibody (e.g., horse radish peroxidase; HRP) appropriate for the detection modality. This assay format offers the simplicity in design, but often suffers from nonspecific interference by biological matrices. Alternatively, a drug may be immobilized indirectly on a microplate through a linker (e.g., biotin–streptavidin coupling) to capture the ADA. The detection of ADA is then achieved commonly by the labeled (e.g., biotinylated) drug coupled with strepatavin conjugated with HRP or ruthenium, or a digoxigenin labeled drug coupled with an anti-digoxigenin antibody conjugated with HRP for conventional ELISA or chemiluminescence detection of ADA. The bridging format may be constructed using labeled anti-idotypic antibody against the drug antibody for ADA detection. Indirect ELISA ADA assays may offer improved sensitivity and specificity due in part to reduced nonspecific matrices interference. Once ADA is detected from the screening assay per an established 'cut point' of ADA using a selected 'negative' (or naIve) population, the presence of ADA is further confirmed by a confirmatory assay in a competition format, and a neutralization assay. Neutralization assays are generally employed in immunogenicity assessment of clinical samples, but not consistently of preclinical samples. The assessment of immunogenicity and the decision tree has been described elsewhere and in guidance documents of regulatory agencies [14–16].

Neutralizing antibody assays

Neutralizing antibody (Nab) assays are used to confirm the presence of neutralizing ADA. Nab assays can be derived from a potency assay of the drug and is commonly in the form of a cell-based functional assay as well as a non-cell-based competitive binding assays, which is discussed later. *In vitro* cell-based assays or bioassays are chosen for their ability to mimic more closely the *in vivo* pharmacology of the drug and is recommended by regulatory agencies US FDA and EMA as part of the immunogenicity assessment of a biological drug [15,16]. ADA neutralization can occur either through direct binding of ADA to a receptor binding site or

Neutralizing antibody assay: commonly refers to cell-based functional or competitive ligand-binding assays typically derived from potency assays of a biological drug, and used to ascertain the neutralizing effect of ADA to the drug.

modulation site of the biological drug, thus rendering the therapeutic drug ineffective [17]. The sensitivity of a cell-based Nab assay is determined with respect to immunogenicity by the neutralizing effectiveness of the positive control ADA on the potency of the biological drug [17]. A desirable Nab assay is one in which the cells provide a robust, specific and sensitive cellular response upon treatment by the therapeutic drug in the presence of neutralizing ADA. Depending on the mode of action of a therapeutic drug, the neutralizing response may be detectable by monitoring the change of cell growth, differentiation, apoptosis, and release of cytokines or expression of other cellular markers. In addition to cell-based Nab, non-cell-based competitive LBAs are also employed to detect neutralizing ADA by an observed decrease in a drug to its target (e.g., receptor) or other functional ligands in the presence of neutralizing ADA [14,17,18]. Competitive LBAs may be constructed by using the drug target as a capturing ligand whereby the presence of neutralizing ADA leads to competition of the binding of the labeled drug to the target (receptor), or by using the drug as a capture for the labeled drug target (receptor). The pros and cons of each configuration may depend on assay background and matrix interference as well as the assay signal window of an individual assay [18,19].

Critical reagents for immunogenicity assays

In general, critical reagents of immuinogenicity assays may include the biological drug in study, ADA, labeled drug for ADA capture or detection, secondary ADA used for detection, primary cells and cell lines, and drug target receptors used for neutralizing ADA assay, respectively. Transfected or hybridoma cell lines used for generation of biological drugs are often considered part of the critical reagents as variations of these generally lead to significant change to the analytical performance of the ADA assays, thus, potentially adversely affecting the efficacy and safety assessment of a biological therapeutics drug. Critical reagents used in various immunogenicity assays may evolve from small-scale generation for preclinical studies to large-scale production for clinical programs requiring multiphase quality management. Longitudinal multiyear clinical studies present unique challenges on critical reagent quality and stability. The inclusion criteria of critical reagents for immunogenicity assays may vary among different phases of drug development and different laboratories. The authors will briefly discuss each category of the critical reagents and approaches to address of their stability on immunogenicity assays.

Biological drug & ADA

While during the preclinical phase the generation of a biological drug may be on a small scale, this process may undergo a scale-up and process changes involving cell lines, feed stock or purification process changes. Ensuring the molecular and structural integrity is critically important throughout the drug development and analytical life cycle [20–22]. A minor change on glycosylation could lead to significant functional change to a protein, and thus the assay performance in part due to altered binding affinity and characteristics with the respective antibodies [20–22].

Physical and chemical degradation of proteins is a critical part of the reagent stability consideration. Chemical degradation occurs most commonly by deamidation, which is the hydrolysis of the asparagine and glutamineside chain amino acids. This can occur due to prolong storage as well as temperature and pH [20–22]. Other chemical deterioration processes include fragmentation, oxidation, isomerization and polymerization [20]. Monoclonal antibodies and polyclonal antibodies are large multidomain proteins of which the interaction among the domains affects the stability and their tendency to aggregate [23,24]. Antibodies of a certain isotype are highly conserved in the Fc region but with a specific Fab region. This Fab region largely determines the antibodies stability and aggregation as does any post-translational modifications [24]. Formulation in drug development is one strategy to improve drug stability by inclusion of stabilizing sucrose and other carrier proteins. Discussion of formulation strategies can be found elsewhere and is out of scope of this chapter [25].For long-term critical reagent management, reagent variability occurs due to differences in the animal immune responses between individual animals and within an animal as it matures. This presents potential difficulty of ensuring a long-term consistency among batches of antibody supplies. Furthermore difference of immune response between preclinical species and human toward a biological drug may lead to differences between ADA of preclinical species and humans, thus resulting in a difference in immunogenicity assay performance. Although immunoglobulin may be considered more stable than some other nonglobulin proteins [26], oxidation and reduction of the antibody under storage conditions that leads to the presence of heavy and light chains of antibody in a reagent preparation is a known. The stability of the labeled antibodies employed as detection antibodies may also be affected by the chemical conjugation. Unstable conjugation may lead to stability issues of a labeled antibody and consequently the performance of the immunogenicity assay.

Cell line & other critical reagents for cell-based Nab assays

Often the most critical reagent of a cell-based Nab is the availability of a robust and stable cell line that yields a specific and sensitive cellular response to the drug product and is relevant to its biological mechanism of action. Cell culture conditions can impact directly the stability of the cell line throughout the passage of the cell proliferation under *in vitro* culture conditions. Problems such as clonal stability may affect the sensitivity and reproducibility of a potency and Nab assay. Established cell lines relevant with the appropriate phenotypic and cellular response to the mechanism of action of a drug may be used in the potency and Nab assays with the advantage of being readily available and consistent within a number of passages required to support the life cycle of the drug-development program. Primary cells (e.g., peripheral blood monocyte and human umbilical vein endothelia cells) have also been used for potency and Nab assay, largely in part due to the consideration of mechanism of action of the drugs [27]. However, the inherent difference among the sources (donors) of primary cells and the insufficient availability to support long-term clinical development programs presents significant challenges for the development of a robust and stable primary cell-based Nab assay.

Other critical reagents are also important for a stable cell-based Nab assay. For example, serum is commonly used in growth media for cell lines. Batch variation of fetal bovine serum can have a significant impact of the proliferation and differentiation, and hence the stability of a cell line. Growth factors required for cell proliferation and functions are important in establishing a stable cell supply for a Nab assay. Although these may be considered as 'off-the-shelf' reagents, depending on internal operational process, these also may be considered as part of the critical reagent portfolio.

Platform-specific critical reagents

Platform-associated critical reagents are also part of the overall critical reagents for immunogenicity assays. Depending on the design of the assay and platform technologies, these reagents may include reagents ranging from biotinylation to proprietary labeling reagents that couple enzymes for the optical detection of the analyte. Secondary detection antibodies are often used in a LBA. The source and labeling of a secondary antibody may lead to significant changes to the assay signal and thus the analytical performance. Although reagent providers maintain quality control and characterizations of labeled reagents under production process, it is necessary to consider this as part of the overall critical reagent strategy in the management of the immunogenicity assays in support of a biologics

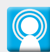
drug life cycle. These ‘off-the-shelf’ platform-specific reagents are not the focus of this chapter.

Strategy, analytical criteria & management of critical reagent stability

Critical reagent stability issues encompass those related to the variation in reagent generations and those related to the stability during the reagent storage or material inventory management throughout the drug life cycle. Although strategy of critical reagent management varies among laboratories and drug-development programs (e.g., early vs late clinical development), some common analytical criteria exist and thus the approaches [22]. The analytical criteria and assays required for assessing the critical reagent stability are summarized in **Table 8.1** and derived based on the intended use of the reagents and the physicochemical nature of the reagents. Some of the common key considerations of reagent stability are discussed further in the following sections.

Critical reagent generation & stability

Changes of the protein expression process and variation in the quality of a drug necessitate the monitoring or revalidation of an immunogenicity assay depending on the nature of the variation of the drug. For example, change of the glycosylation of an antibody may lead to change of affinity of an antibody and immune response to the drug [20]. The extent of reagent recharacterization may include physicochemical analysis (e.g., molecular mass, critical post-translation modification and structural integrity). Functional analysis employing LBA and cell-based potency assays provide reagent quality information not readily available from physicochemical analysis. The requirement and selection of a reagent analysis should be based on the nature of the changes expected and the intended use of the reagent with respect to the assay design. For example, a minor change of glycosylation of a secondary antibody as detection reagent may not necessitate a full physicochemical characterization of the reagent, whereas a change in glycosylation of a drug antibody may result in a change of immunogenicity response and its interaction with ADA, thus requiring a more thorough characterization of the reagent change [20]. For externally provided reagents, a certificate of analysis is often the document provided with minimal data on the material stability and physicochemical analysis. Understanding the tests performed for the certificate of analysis



Critical reagent stability management is supported by analytical assays. The extent of analytical assays may include physicochemical characterization and functional assays. The nature of the biological drug and specific impact stability parameters on immunogenicity assay performance determine the reagent characterization and their stability acceptance criteria.

Table 8.1. Critical reagents stability and analytical monitoring for immunogenicity assays.

Example	Concentration[†]	Degradation analysis[‡]	Binding activity	Functional potency	Neutralizing activity	Cell proliferation	Differentiation & phenotypic markers
Biological drug							
Monoclonal antibodies	Yes	Yes	Yes	Yes	NA	NA	NA
Peptides	Yes	Yes	Yes	Yes	NA	NA	NA
Fusion protein	Yes	Yes	Yes	Yes	NA	NA	NA
Antibody–drug conjugate	Yes	Yes	Yes	Yes	NA	NA	NA
Anti-drug antibody & labeled reagent							
Anti-drug antibody	Yes	Yes	Yes	Yes	Yes	NA	NA
Labeled drug	Yes	Yes	Yes	Yes	NA	NA	NA
Labeled secondary antibody	Yes	Yes	Yes	Yes	NA	NA	NA
Cell lines							
Transfected cell lines for biological drug expression	NA	NA	NA	NA	NA	Yes	Yes
Stable cell lines for cell-based potency assay	NA	NA	NA	Yes	Yes	Yes	Yes

[†]Concentration analysis may include total concentration and purity analysis.
[‡]Degradation analysis may include aggregation, fragmentation, oxidation and reduction, denaturation, and deamination.
NA: Not applicable; Opt: Optional; Yes: Recommended.

and their association with the intended use of the critical reagents is important. Establishing a sustaining reagent quality management system built on critical analytical monitoring **(Table 8.1)** both internally and externally in collaboration with reagent vendors is part of the strategy in ensuring reagent quality and stability.

Maintaining a trending of assay performance is crucial in determining whether the stability of the protein has been preserved or whether the consistency of quality or stability should be under investigation [22,23]. To help addressing the batch-to-batch reagent variation, a batch switch approach entails having a period of overlap between the new and old lots of the critical reagents in order to cross test the two reagent batches, and thus ensuring that there is no significant deviation of assay performance.

Critical reagent storage stability

Similar to the storage conditions of other protein reagents, critical reagents such as monclonal antibodies, polyclonal antibodies, antibody–drug conjugate or fusion protein or peptide drugs are generally stored at -60 to -100°C and may be stable up to 10 years without a freeze–thaw cycle [22]. Consideration should be given to the issues such as size of a single aliquot in storage to align with the expected use of the materials and thus avoid a repeated freeze–thaw cycle. Protein reagent stability may be significantly shortened to <1 week when stored at 4°C during analytical assay run. In addition to physicochemical characterization, LBA or functional assays may also be used to assess the reagent stability **(Table 8.1)**. For example, quality controls or positive controls are used to determine the sensitivity of an assay as part of the assessment of freeze–thaw stability of respective biological drug and ADA reagents.

Buffers that are used to reconstitute bulk critical protein reagents may impact their stability. High pH can speed up the deamination process and it is in these cases that a neutral pH formulation buffer is used. Phosphate-buffered saline pH drops during freeze–thawing, and this could impact the stability [20,22–23]. Additives, such as cryprotectant glycerol, may be added to the formulation buffers to minimize the freeze–thaw effects. Stabilizers, such as carrier proteins (BSA), may need to be added to minimize nonspecific adsorption of reagents. Although these additives may be used to improve the stability of the protein of

The storage stability of critical biological reagents must be closely monitored by physicochemical characterization and functional assays throughout the life cycle of the planned use of the reagents. Considerations on the appropriate aliquot size for storage and stabilizer coupled with a monitoring program may assist the effective management of reagent stability important to the quality of immunogenicity assays.

interest, they can also negatively impact the performance of the assay. For example, sodium azide, which is used as an antimicrobial agent, should not be used with HRP conjugates because azide inhibits peroxidase activity. A change of formulation of a biological drug for storage stability needs to be approached cautiously as this may lead to significant concern on its safety and efficacy profile.

Assignment of the reagent expiration date generally regards the originating date where a critical reagent is ready for the intended use as the commencing date from which a period of expiration is assigned. The period of expiration must be based on acceptable physicochemical characterization and functional assay data within the allowable range of variation. Extension of expiration must be done with supporting data generated following the original characterization methods or by selected partial characterization based on the (bio)chemistry of the drug molecule, institutional practices and agency guidance.

Conclusion

Ensuring critical reagent stability is pivotal to the analytical performance of immunogenicity assays throughout the drug-development life cycle. An effective reagent management strategy may require careful planning with respect to the different phases of biological drug-development program, involving selection, development and maintenance of reagent generation systems (e.g., cell lines, culture conditions, purification and process optimization, and analytical assays required). The recommendation of establishing a knowledge-based institutional database to facilitate information capture and quality management process [22], and in conjunction with purpose and regulatory guidance-driven analytical supports represents a comprehensive approach. Despite different internal institutional practices, the common scientific rationale based on the reagent biochemistry serves as the fundamental consideration in developing individual practice to ensure stability of critical reagents for immunogenicity assay.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

Summary.

- Ligand-binding assay and biochemical or cell-based neutralizing assays are the most common immunogenicity assays for biological therapeutics. These assays are incorporated throughout the drug-development and postapproval life cycle.
- Critical reagents of immunogenicity assays included (but not limited to) biological drugs, their anti-drug antibody, labeled detection antibodies or ligands, reference materials and key platform-specific reagents.
- Ensuring reagent stability sufficient for a biological drug-development starts with managing stability of reagent generation and ends with reagent storage supported by analytical assays to monitor reagent stability.
- Analytical assays for monitoring reagent stability may include physicochemical characterization and functional assays designed based on the nature of biological drug, respective impact of an individual stability change, institutional practices and regulatory guidance.

References

1. Kariolis MS, Kapur S, Beyond antibodies: using biological principles to guide the development of next-generation proteintherapeutics. *Curr. Opin. Biotechnol.* pii, S0958-1669(13)00074-8 (2013).
2. Butterfield AM, Chain JS, Ackermann BL, Konrad RJ. Comparison of assay formats for drug-tolerant immunogenicity testing. *Bioanalysis.* 2(12) 1961–1969 (2010).
3. Menendez AT. Strategic selection and development of immunogenicity binding methods. *Bioanalysis.* 4(12), 1491–1508 (2012).
4. Leng SX, McElhaney JE, Walston JD, Xie D, Fedarko NS, Kuchel GA. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. *J. Gerontol. A. Biol. Sci. Med. Sci.* 63(8) 879–884 (2008).
5. McGiven JA, Thompson IJ, Commander NJ, Stack JA. Time-resolved fluorescent resonance energy transfer assay for simple and rapid detection of anti-*Brucella* antibodies in ruminant serum samples. *J. Clin. Microbiol.* 47(10), 3098–3107 (2009).
6. Choudhury F, Williams A, Johnson P. Validation and comparison of two multiplex technologies, luminex and mesoscale discovery, for human cytokine profiling. *J. Immuno. Methods* 340(1), 55–64 (2009).
7. Mora JR, Obenauer-Kutner L, Patel V. Application of the Gyrolab™ platform to ligand-binding assays: a user's perspective. *Bioanalysis* 2(10), 1711–1717 (2010).
8. Houser B. Bio-Rad's Bio-Plex® suspension array system, xMAP technology overview. *Arch. Physiol. Biochem.* 118(4) 192–196 (2012).
9. Spriggs FP, Zhong ZD, Safavi A *et al.* Ligand binding assays in the 21st century laboratory: platforms. *AAPS J.* 14(1), 13–118 (2012).
10. Lofgren JA, Dhandapani S, Pennucci JJ *et al.* Comparing ELISA and surface plasmon resonance for assessing clinical immunogenicity of panitumumab. *J. Immunol.* 178, 7467–7472 (2007).
11. Nechansky A. HAHA-nothing to laugh about. Measuring the immunogenicity (human anti-human antibody response) induced by humanized monoclonal antibodies applying ELISA and SPR technology. *J. Pharm. Biomed. Anal.* 52(1), 252–254 (2010).
12. Li J, Schantz A, Schwegler M, Shankar G. Detection of low-affinity anti-drug antibodies and improved drug tolerance in immunogenicity testing by Octet® biolayer interferometry. *J. Pharm. Biomed. Anal.* 54(2), 286–294 (2011).
13. Tatarewicz S, Moxness M, Weeraratne D *et al.* A step-wise approach for transfer of immunogenicity assays during clinical drug development. *AAPS J.* 11(3), 526–534 (2009).
14. Shankar G, Devanarayan V, Amaravadi L *et al.* Recommendations for the validation of immunoassays used for detection of host antibodies against biotechnology products. *J. Pharm. Biomed. Anal.* 48(5), 1267–1281 (2008).

15 EMA. *Guideline on Immunogenicity Assessment of Monoclonal Antibodies Intended for* In Vivo *Clinical use.* EMA, London, UK, EMA/CHMP/BMWP/86289/2010 (2012).

16 US FDA. *Guidance for Industry Assay Development for Immunogenicity Testing of Therapeutic Proteins.* US FDA, MD, USA (2009).

17 Gupta S, Devanarayan V, Finco D *et al.* Recommendations for the validation of cell-based assays used for the detection of neutralizing antibody immune responses elicited against biological therapeutics. *J. Pharm. Biomed. Anal.* 55(5), 878–888 (2011).

18 Finco D, Baltrukonis D, Clements-Egan A *et al.* Comparison of competitive ligand-binding assay and bioassay formats for the measurement of neutralizing antibodies to protein therapeutics. *J. Pharm. Biomed. Anal.* 54(2), 351–358 (2011).

19 Cludits L, Meager A, Thorpe R, Wadhua M. Detection of neutralizing interleukin-17 antibodies in autoimmune polyendocrinopathy syndrome-1 (APS-1) patients using a novel non-cell based electrochemiluminescence assay. *Cytokine* 50(2), 129–137 (2010).

20 Geist BJ, Egan AC, Yang TY, Dong Y, Shankar G. Characterization of critical reagents in ligand-binding assays: enabling robust bioanalytical methods and lifecycle management. *Bioanalysis* 5(2), 227–244 (2013).

21 Rup B, O'Hara D. Critical ligand binding reagent preparation/selection: when specificity depends on reagents. *AAPS J.* 9(2), E148–E155 (2011).

22 O'Hara DM, Theobald V, Egan AC *et al.* Ligand binding assays in the 21st century laboratory: recommendations for characterization and supply of critical reagents. *AAPS J.* 14(2), 316–328 (2012).

23 Staack RF, Stracke JO, Stubenrauch K, Vogel R, Schleypen J, Papadimitriou A. Quality requirements for critical assay reagents used in bioanalysis of therapeutic proteins: what bioanalysts should know about their reagents. *Bioanalysis* 3(5), 523–534 (2011).

24 Lowe D, Dudgeon K, Rouet R *et al.* Aggregation, stability, and formulation of human antibody therapeutics. In: *Advances in Protein Chemistry and Structural Biology (Volume 84).* Donev R (Ed.). Academic Press, Oxford, UK, 41–61 (2011).

25 Swain S, Mondal D, Beg S *et al.* Stabilization and delivery approaches for protein and peptide pharmaceuticals: an extensive review of patents. *Recent Pat. Biotechnol.* PMID:23441815 (2013) (Epub ahead of print).

26 Randall SA, McKay MJ, Pascovici D *et al.* Remarkable temporal stability of high-abundance human plasma proteins assessed by targeted mass spectrometry. *Proteomics Clin. Appl.* 6(11–12), 626–634 (2012).

27 Yu L, Liang XH, Ferrara N. Comparing protein VEGF inhibitors: *in vitro* biological studies. *Biochem. Biophys. Res. Commun.* 408(2), 276–281 (2011).

Research Article

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Bioanalysis

Critical reagent characterization and re-evaluation to ensure long-term stability: two case studies

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Characterization of critical reagents can mitigate adverse impact to ligand-binding assay performance. We investigated the conjugation conditions of a bispecific protein to SULFO-TAG NHS-Ester™ ruthenium to resolve a steady increase in ligand-binding assay background signal. Functional and biophysical attributes in stability samples revealed low pH (4.0) conjugation and formulation buffers were key to decrease aggregate formation. We also identified pH-specific (3.0) purification conditions to reduce aggregate levels from 37% to <5% of a mouse IgG3 reagent antibody. These case studies support the utility of biophysical and functional characterization of critical reagents as a proactive approach to maintain long-term stability and provide the basis for our recommendations a risk-based approach to establish re-evaluation intervals for traditional and novel reagents.

First draft submitted: 21 August 2020; Accepted for publication: 26 February 2021; Published online: 22 April 2021

Keywords: characterization • critical reagents • LBA • long-term stability • re-evaluation

Background

Large molecule biologicals comprising antibodies, proteins, receptors and target ligands are examples of critical reagents employed in ligand-binding assays (LBAs) to assess pharmacokinetic (PK), biomarker and immunogenicity (e.g., antidrug antibodies [ADAs], neutralizing antibodies) parameters [1–5]. Critical reagents are subjected to various biophysical characterization and stability testing strategies to ensure high quality, specificity, long-term stability and supply [1,4–7]. However, novel proteins and complex modalities for which there are no empirical data for stability or optimal storage conditions can present a higher risk over time [8].

The current generation of new LBA technologies offering greater sensitivity, lower sample volumes and multiplexing abilities compared with ELISA poses unique challenges regarding the generation or procurement of critical reagents. Often, many reagent labeled based platforms require biologicals to be modified with chemical moieties such as small molecule tags (e.g., biotin or digoxigenin), metals (e.g., ruthenium [Ru]), single fluorophores (e.g., alexa fluor 647, fluorescein isothiocyanate), multiple fluorophores (Förster-type energy-transfer) or enzymes (e.g., alkaline phosphatase or horseradish peroxidase) for use as capture or detector reagents depending on the assay format. However, the addition of chemical moieties can introduce changes to the native form of the protein and may alter properties such as charge, conformation or stability [9] resulting in a loss or change in the molecular recognition site [2,3] or self-aggregation [10] affecting LBA performance. Although methods to detect the biophysical changes of conjugated reagents such as LC–MS [3], MALDI-TOF-MS [11], Biotective Green [12] or 4′-hydroxyazobenzene-2-carboxylic acid (HABA) [13,14] have been explored, none can accurately predict the impact they will have when used in an LBA or the long-term stability of the molecule.

Conventionally, there is a biased approach to the characterization of critical reagent conjugates which mainly focuses on physical or structural attributes, for instance, conjugate incorporation ratio, prior to functional testing by LBA. Indeed, the quest to reproduce a critical reagent conjugate or identify the presence of remaining unconjugated protein or label is supported by a concern for decreased LBA specificity, sensitivity and consequently assay performance. Aside from UV/VIS spectrometry which is reliant upon discrete wavelengths of proteins, quantitation and differentiation of conjugated (Ru and fluorophores) and unconjugated species of protein are performed in

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Table 1. Examples of biophysical and functional methods.

Reagent attribute	Characterization method
Biophysical test	
Concentration	A_{280}, BCA
MW and purity	SDS-PAGE, SEC
Aggregation	SEC, DLS, A_{320}
Specificity	Octet, Biacore
Affinity (mAbs)	Octet, Biacore, Gyrolab™
Degree of labeling (conjugates)	LC–MS, HABA
Functional test	
Binding	LBA, nAb, flow, receptor binding, biomarkers

Octet and Biacore are biosensor-based platforms that use BLI and SPR, respectively, as well-established techniques for characterizing biomolecular interactions in real time.
BCA: Bicinchoninic acid assay; BLI: Bio-layer interferometry; DLS: Dynamic light scattering; HABA: 4′-hydroxyazobenzene-2-carboxylic acid; LBA: Ligand-binding assay; mAb: Monoclonal antibody; nAb: Neutralizing antibody; SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel; SEC: Size exclusion chromatography; SPR: Surface plasmon resonance.

an artificial setting including addition to resins, plates, chips, beads, etc. Traditional analytical methods such as mass spectrometry (MS), size exclusion chromatography (SEC) and HABA require manipulation of the native structure including degradation or migration through a resin for analysis. Although chromatographic methods such as SEC [14] and procedures such as HABA are subjected to inaccuracies [12,15], these protocols have utility especially when used to monitor changes in comparison to those determined at the baseline.

Reagent conjugates are a subset in the arsenal of proteins which are ordinarily well studied. Although the workhorses of LBA, innovative and complex proteins such as those offered as the next generation of drug modalities are becoming more mainstream in these applications; therefore, a plan should be formulated to address the distinct traits when used as critical reagents. Traditional reagents such as monoclonal antibodies (mAbs) and polyclonal antibodies (pAbs) are well documented; however, occasional issues such as nonsecretion of the cell line attributed to lack of clonality, lot to lot variability due to diverse antibody response in animals or aggregation during purification can arise which will need to be resolved. Our goal was to devise a risk-based strategy for a proactive approach to mitigate stability issues based upon categories of reagent type. In this article, we will present a case study which will explore the impact of reagent stability of a protein conjugated with Ru in an LBA. The scope of our inquiry included formulation buffers, storage conditions, characterization methods and interval testing. A second case study will describe an optimized protein A purification method of a mouse mAb (IgG3) where considerable aggregation (37%) was observed in the initial purified lot over a protein G column. Elution and neutralization buffers encompassing a range of pHs and characteristics were tested, and the resultant purification products were characterized by SEC and performance by LBA. This article will also provide the authors' recommendations to monitor critical reagent stability through a combination of functional and biophysical testing (Table 1).

Case study 1

A bispecific mAb (drug A) which was prone to aggregation under certain pH conditions was conjugated to biotin and Ru for preclinical sample testing in a bridging ADA LBA. A steady increase in the LBA background signal using drug A-biotin (capture) and -Ru (detector) over a 6-month period was observed resulting in the failure of assay quality controls (Figure 1). The presence of aggregates and unconjugated Ru observed in the drug A-Ru reagent was suspected to impact the LBA background; thus, we initiated an investigation to explore the optimal conjugation and storage conditions to prepare a stable lot of drug A-Ru over a 28-day period.

Case study 2

A mouse antidrug B reagent antibody (IgG_3) (specific to domain A on a multispecific drug) produced in cell culture and purified by protein G using 100 mM glycine, pH 2.8 exhibited elevated levels (37%) of high molecular weight (HMW) species (Figure 2). Attempts to remove the HMW species by preparative SEC consumed 80% of the purified antibody (20 mg recovered from 100 mg of starting material) and still contained 15% aggregate. Aside from the concern of a reagent containing HMW species well above the acceptance criteria of 5%, exploration of purification conditions precludes the practice of aggregate removal by preparative SEC which can be inefficient and expensive [16]. These challenges were the impetus for this investigation.

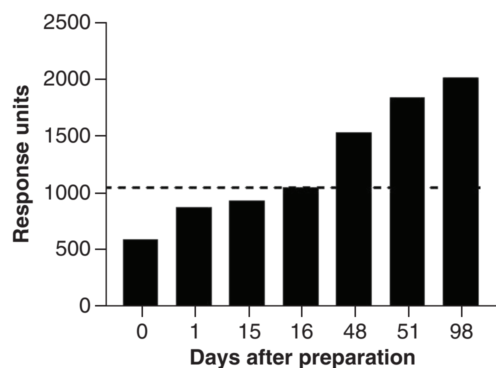

Figure 1. Negative control response units for antidrug antibody assay over time. The x-axis denotes the date of ligand-binding assay testing, and the y-axis depicts response units. Negative controls were stored at -70°C and thawed the day of use. Closed bar: negative control response unit; dotted line: negative control acceptance limit.

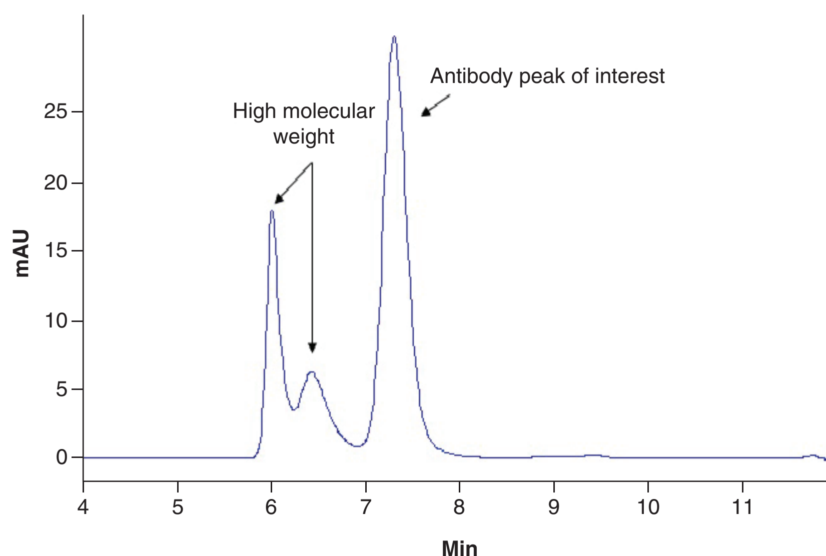

Figure 2. Size exclusion chromatography of mouse antidrug B after protein G purification. The x-axis denotes time in minutes, and the y-axis depicts mAU.
mAU: Milli absorbance unit.

Methods

Standard conjugation protocol

The conjugation chemistry to prepare the drug A-Ru reagent targeted positively charged primary amines, more specifically, epsilon amines, which are mainly found on lysine residues. Optimal reaction of (II) tris-bipyridine-(4-methylsulfonate) NHS ester Ru with these primary amines occurs at pH 7–9 to form stable amide bonds. At lower pH, the labeling efficiency decreases due to hydrolysis and inactivation of the NHS ester groups as well as a reduction in primary amine protonation. A standard molar coupling ratio (MCR) of 12:1 with MSD SULFO-TAG NHS-Ester™ Ru (Meso Scale Discovery®, Meso Scale Diagnostics, LLC, MD, USA) is performed unless a more optimal MCR is determined for a specific protein. Free conjugate which has not bound to the drug is removed by passive diffusion by Slide-A-Lyzer™ (slide-a-lyzer, Thermo Fisher Scientific, IL, USA; 10K MW cutoff). Calculation of actual MCR protein is determined by OD readings per manufacturer's instructions.

Experimental conjugation protocol

Previous experiments determined that an MCR of 6:1 (phosphate-buffered saline [PBS], pH 7.2 pre- and post-conjugation) for drug A-Ru was optimal for LBA performance. Multiple factors were evaluated including the conjugation reaction buffer (PBS, pH 7.2 versus formulation buffer, pH 4.0) during modification. One bulk reagent was prepared in each respective reaction buffer and then processed for the removal of unconjugated Ru by passive diffusion (slide-a-lyzer) or centrifugation over a resin (Zeba™ Spin Desalting Columns [zeba spin column, Thermo Fisher Scientific], 40K MW cutoff) per manufacturer's instructions into drug A formulation buffer, pH

Table 2. Summary of experimental conjugation and stability conditions.

Step	Default conditions	Experimental conditions
Conjugation reaction buffer	PBS, pH 7.2	• PBS, pH 7.2 • Low pH, 4.0
Removal of free ruthenium	Dialysis cassette, 10K MWCO	• Dialysis cassette, 10K MWCO • Zeba spin column, 40K MWCO
Formulation buffer post-conjugation	PBS, pH 7.2	Low pH, 4.0
Storage temperature	-70°C	• 4°C • -70°C (freeze–thaw) • 37°C

MWCO: Molecular weight cutoff; PBS: Phosphate-buffered saline.

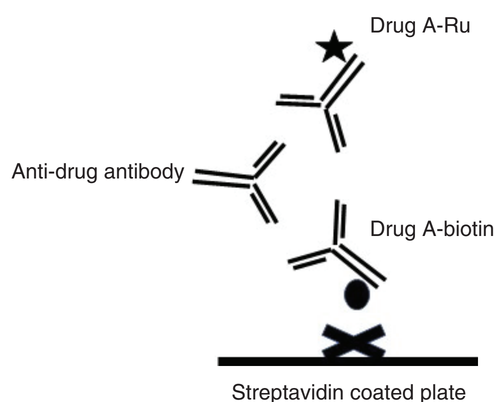

Figure 3. Schematic of antidrug antibody assay format.

4.0 and stored at 4°C, 37°C, -70°C (3 freeze–thaw cycles) until analyzed. Each experimental drug A-Ru lot was subjected to accelerated stability conditions to test the stability of the protein (Table 2). All reagents were assessed by LBA ($\log_{10}$ titer, background response units and positive control [PC] S/N ratio) and HPLC–SEC for HWM species.

Ligand-binding assay for drug A-Ru

The LBA bridge format utilizes drug A-biotin and drug A-Ru (SULFO TAG) to capture ADA in study samples on blocked streptavidin-coated Multi-Array® plates (Figure 3). The negative control and study samples were diluted in assay buffer containing 4% cynomolgus monkey serum. When read on the Meso Scale Discovery SECTOR™ Imager 6000, bound complexes containing drug A-Ru produce an electrochemiluminescent signal which is reported as response units. Data are analyzed using a 4-parameter nonlinear fit.

PK ligand-binding assay for mouse antidrug B

Multidomain drug B was diluted in 2.5% mouse serum and added to a blocked Meso Scale Discovery Multi-Array plate coated with mouse antidrug B (specific to domain A) followed by addition of anti-domain B reagent labeled with Ru to detect the bound drug. Data were acquired as previously described (LBA for drug-A Ru section) and plotted on a $\log_{10}$ scale.

High-performance liquid chromatography size exclusion chromatography (HPLC–SEC)

HPLC–SEC analysis was performed using a YMC-Pack Diol-200, 300 × 8.0 mml. D. S-5 μm, 20 nm (Waters, Milford, MA, USA) resin using PBS (drug A-Ru, case study 1) or 20 mM sodium phosphate, 400 mM sodium chloride, pH 7.2 (mouse antidrug B, case study 2) in the mobile phase in at a flow rate of 0.75 ml/min. MW standards were used to monitor the performance of the run and estimate the MWs of peaks in samples.

Purification for case study 2

Recombinant protein A Fast Flow 1 ml column (GE Healthcare, IL, USA) was used to purify 20 ml of conditioned media containing the mAb by ÄKTA™ fast protein liquid chromatography (FPLC). New columns were used for experimental purifications to avoid protein A leaching and reagent cross contamination. Columns were equilibrated

Table 3. Summary of percent high molecular weight in test purification elution and neutralization buffer lots.

Resin	Purification elution buffer	Neutralization buffer	% of HMW content
Protein G	100 mM Glycine-HCl, pH 2.8	1 M Tris, pH 9.0	37
Protein A	100 mM Glycine-HCl, pH 2.5	1 M Tris, pH 9.0	10
Protein A	100 mM Glycine-HCl, pH 2.5	1 M Hepes, pH 7.3	14
Protein A	100 mM Glycine-HCl, pH 3.0	1 M Tris, pH 9.0	<0.1
Protein A	100 mM Glycine-HCl, pH 3.0	1 M Hepes, pH 7.3	<0.1
Protein A	2.0 M arginine, pH 3.0	1 M Tris, pH 9.0	12
Protein A	2.0 M arginine, pH 3.0	1 M Hepes, pH 7.3	15
Protein A	3.0 M sodium thiocyanate, pH 8.8	1 M Tris, pH 9.0	16
Protein A	3.0 M sodium thiocyanate, pH 8.8	1 M Hepes, pH 7.3	16

HMW: High molecular weight.

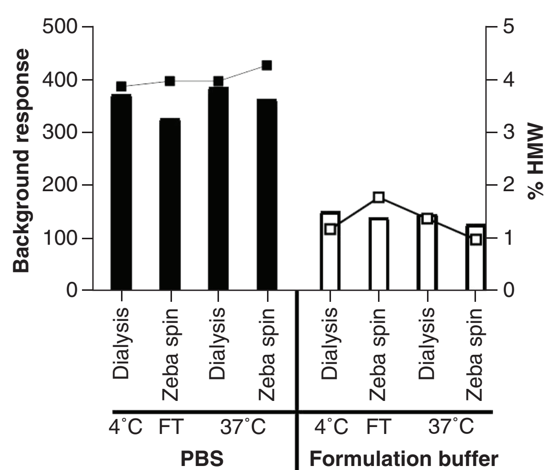

Figure 4. Summary of Ru background and high molecular weight species on drug A-Ru preps collected on day 28. The x-axis denotes conjugation reaction conditions (PBS, pH 7.2 [closed bars], formulation buffer, pH 4.0 [open bars]); storage temperature (4°C, -70°C F/T, 37°C); removal of unconjugated ruthenium (dialysis, zeba spin column) and the left y-axis denotes background in response units. The solid line represents the %HMW species (denoted on right y-axis) (PBS, pH 7.2 [closed squares]; formulation buffer, pH 4.0 [open squares]).
%HMW: Percent high molecular weight; FT: Freeze–thaw; PBS: Phosphate-buffered saline.

with PBS, and samples were eluted at a flow rate of 1 ml/min using the elution agents in Table 3. Absorbance was monitored at A_{280}. Eluted fractions were collected into neutralizing buffers, a nucleophilic buffer, 1 M Tris, pH 9.0 or a zwitterion, 1 M Hepes, pH 7.3 where applicable, and fractions containing antibody were pooled and dialyzed against PBS using a slide-a-lyzer. Samples were stored at -70°C until analysis.

Preparative SEC for case study 2

Gravity flow purification to isolate monomeric species from the mouse anti-drug B reagent antibody containing 37% aggregate was performed using a 490 ml Sephacryl S-200 high resolution column (GE Healthcare). Antibody was concentrated using ammonium sulfate precipitation to a final volume of 5 ml in PBS and loaded on the column equilibrated in PBS. Fractions were collected, and absorbance at A_{280} was measured. Fractions from the monomer peak were pooled, dialyzed into PBS and filtered through a 0.2 µm filter (Nalgene, NY, USA). The antibody was stored at -70°C until analysis.

Results

In case study 1, the significant factor to reduce the LBA background signal while maintaining consistent assay performance was the conjugation reaction buffer. All drug A-Ru preparations conjugated in formulation buffer, pH 4.0 exhibited a decrease in HMW species and LBA background signal while retaining acceptable performance ($\log_{10}$ titer, S/N ratio of PC) when compared with reactions conducted in PBS, pH 7.2. Figure 4 depicts the background signal of the negative control and the %HMW species in drug A-Ru preps on day 28 of the stability study at all conditions. The storage temperature (4°C, -70°C freeze–thaw, and 37°C) or the removal of unconjugated Ru had minimal impact on these LBA parameters.

Predictably, the reaction conducted in PBS, pH 7.2 provided a more efficient labeling environment producing drug A-Ru with a calculated MCR of 3.5:1 as compared with the pH 4.0 reaction which resulted in a calculated MCR of 2:1. The increased incorporation rate offers a higher S/N ratio (41–47 in PBS, pH 7.2 vs 28–29 in

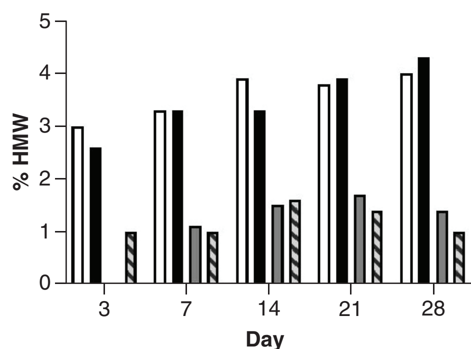

Figure 5. Summary of percent high molecular weight species in 37°C stability samples. %HMW species in drug A-Ru formulated in phosphate-buffered saline and processed by dialysis (clear bar); or zeba spin column (black bar); low pH formulation buffer and processed by dialysis (gray bar); or zeba spin column (striped bar). The x-axis denotes the day of testing and the y-axis denotes the %HMW.
%HMW: Percent high molecular weight.

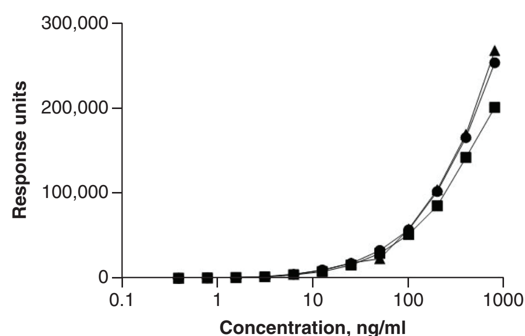

Figure 6. Ligand-binding assay analysis of purified mouse antidrug B antibody lots. Lot 1 containing 37% HMW species (closed circles); Lot 2, preparative size exclusion chromatography of Lot 1 containing 15% high molecular weight species (closed squares); and Lot 3, experimental lot purified with <0.1% HWM species (closed triangles). The x-axis denotes the concentration (ng/ml), and the y-axis denotes response units.

formulation buffer, pH 4.0; data not shown), but it does not affect the PC $\log_{10}$ titer (3.2–3.4 for all samples; data not shown).

All samples exhibited <5% HMW species throughout the stability study which is our desired metric; however, the drug A-Ru samples prepared in PBS, pH 7.2 appear to be trending higher over time which was not observed in the lots prepared in formulation buffer, pH 4.0 (Figure 5). Incubation of the conjugated reagent post-dialysis at 37°C provided an environment to accelerate any potential stability issues which may arise later which has been observed with this protein in just 2 months.

For the second case study, the combination of pH and the property of the elution buffer were critical in the prevention of aggregate formation in the mouse IgG3 mAb during purification. Neutralization buffer, Tris versus Hepes, was not a factor. Elution from a protein A resin with 100 mM glycine-HCl, pH 3.0 was the only condition that produced HMW levels below the limit of detection (<0.1%) agnostic of neutralization agents (Table 3) which is a marked difference from the original lot containing 37% HMW aggregate that was generated by elution with 100 mM glycine-HCl, pH 2.8 over a protein G column and neutralized with Tris.

We further characterized the immunoreactivity of three of the purified reagents in LBA: the original lot containing 37% HWM species (lot 1), re-purification of lot 1 by preparative SEC (resulting in 15% HMW species, lot 2) and the experimental lot that was eluted and neutralized with 100 mM glycine-HCl, pH 3.0, 1 M Hepes (<0.1% HMW, lot 3), and minimal impact of signal was observed (Figure 6).

Discussion & summary

LBA employed to analyze PK and immunogenicity parameters are firstly dependent upon high quality critical reagents used as capture and detector reagents. The recommendations that exist to assign re-evaluation dates [8] are based upon the date of generation, for example, purification, production or conjugation, and data are accumulated to provide scientific rationale for decisions [17]. However, as noted in our first case study, historical information on a protein may forecast potential instability issues and provide insight on the best analytical methods to corroborate biophysical attributes such as the formation of protein aggregates and functional performance in an LBA. As we have demonstrated, the successful generation of drug A-Ru critical reagent was achieved through the optimization and refinement of default conjugation conditions that was conducive to the stability of the protein. These changes reduced the presence of HMW species from 10% to <2% which resulted in improved and consistent LBA performance and the successful execution of sample analysis.

Table 4. Critical reagent risk-level categories.

Risk level	Examples	Stability re-evaluation time period	Analytical test
1	Traditional mAbs and pAbs (conjugated or unconjugated)	Follow O'Hara *et al.* • 2–10 years at -60°C to -100°C for unconjugated • 2–5 years at -60°C to -100°C for conjugated	Functional† and biophysical† [28]
2	Conjugated biotherapeutics (other than traditional mAbs; e.g., engineered mAbs, ADCs, Fc fusion proteins, multispecifics, etc.)	Re-evaluation at 1 year and extend as appropriate	Functional and biophysical
3	Reagents with unknown modality-based stability (e.g., conjugated beads, soluble ligands and receptors)	Re-evaluation at a shorter time period (e.g., 3–6 months) and extend as appropriate	Functional and biophysical

†Outlined in Table 1.
ADC: Antibody-drug conjugate; Fc: Fragment crystallizable region; mAb: Monoclonal antibody; pAb: Polyclonal antibody.

For mAbs, unique attributes can influence purification methods since structure, size and isoelectric point can vary for each IgG subclass [18] as well as across species, affecting stability of the molecular structure [19]. The reagent antibody in our study, a mouse IgG3, has the distinct property of protein self-association due to the presence of an additional glycosylation site at amino acid residues 471–473 in the C_H3 domain which may promote fragment crystallizable (Fc) region interaction (Fc–Fc) and complexes and further compound the risk of aggregation [20]. The typical purification method of mammalian antibodies utilizes binding to the C_H2–C_H3 domain of the Fc component (protein A and protein G) [21,22] or to the $F(ab')_2$ region of protein G [23]. To minimize aggregation during the purification process of our mouse antidrug B mAb, we assessed chromatography resins, the parameters of pH and the properties of buffers during both the elution and neutralization steps. Biophysical characterization by SEC was key in the disclosure of the substandard quality of this critical reagent. Although the aggregation of this capture reagent had minimal impact on the performance of the PK LBA, lot to lot consistency of the reagent preps and further assurance of long-term stability necessitated the removal of the aggregated species. In addition, newer bead and labeled-based LBA platforms may prove more sensitive to changes in reagents particularly those that are aggregated. Larger in size, an aggregate may present a new epitope multiple times thus providing a higher avidity over the monomer or, in some cases, epitopes may be masked [24].

In summary, the data presented in these case studies are representative of our experiences with conjugated and purified reagents, and our lessons learned have been applied to resolve other issues that we have encountered. Here, we present two case studies that addressed issues with critical reagents detected by functional testing (case study 1) and biophysical examination (case study 2). Assembly of critical reagents by attributes of similar nature can provide direction to those which are susceptible to molecular instability. Here, we propose a three-tiered risk-level approach that categorizes reagents and recommendations of testing intervals to re-evaluate by selected methods (Table 4). Although accelerated stability studies using kinetic models such as the Arrhenius equation may not provide a direct correlation for extended stability, valuable information can be obtained to portend future issues [25]. Decisions based upon scientific data are imperative, thus analytical tools discerning both the functional and biophysical changes to establish *a priori* acceptance criteria of purified critical reagents and conjugates [8,17,26,27] thereof will set the foundation of attributes to monitor over time. The business decision to implement a testing plan in parallel with best practices and standardized procedures executed by trained scientists will safeguard the critical reagent supply and minimize investigations.

Future perspective

It is crucial to understand fully what factors can influence the integrity of the supply of critical reagents to avoid inherent performance consequences to the LBA. Optimization of conjugation methods, formulation and storage conditions can impact critical reagents and thus merit further monitoring and investigation. The extent of reagent stability characterization is a business decision which should consider reagent type, historical experience, and intended use. New drug and reagent modalities may need more frequent stability testing and characterization than historical reagent types.

Executive summary

- Critical reagents are precious assets and a long-term, quality resupply should be available for assay use and study support.
- Stability of bispecific drug A-Ru was achieved when low pH formulation buffer (4.0) was used for the conjugation reaction and final formulation (case study 1).
- Mitigation of high molecular weight species during purification of murine antidrug B was accomplished using 100 mM glycine-HCl, pH 3.0 for elution; however, it had minimal effect on the performance of the ligand-binding assay (case study 2), demonstrating ligand-binding assay impact is on a case by case basis.
- We propose to characterize critical reagents in functional assays in tandem with the assessment of biophysical properties to identify quality attributes that are predictive of stability and performance in the assay.

Acknowledgments

The authors acknowledge Glenn Miller, Qiang Qu, Mania Kavosi, Sheldon Leung, Kaitlyn Bushey and Denise O'Hara for experimental data.

Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

References

1. Rup B, O'Hara D. Critical ligand binding reagent preparation/selection: when specificity depends on reagents. *AAPS J.* 9(2), E148–E155 (2007).
2. O'Hara DM, Theobald V. Life cycle management of critical ligand-binding reagents. *Bioanalysis* 5(21), 2679–2696 (2013).
3. Geist BJ, Egan AC, Yang T-Y, Dong Y, Shankar G. Characterization of critical reagents in ligand-binding assays: enabling robust bioanalytical methods and lifecycle management. *Bioanalysis* 5(2), 227–244 (2013).
4. King LE, Farley E, Imazato M *et al.* Ligand binding assay critical reagents and their stability: recommendations and best practices from the Global Bioanalysis Consortium Harmonization Team. *AAPS J.* 16(3), 504–515 (2014).
5. O'Hara DM, Theobald V, Egan AC *et al.* Ligand binding assays in the 21st century laboratory: recommendations for characterization and supply of critical reagents. *AAPS J.* 14(2), 316–328 (2012).
6. Pihl S, van der Strate BW, Golob M *et al.* EBF recommendation on practical management of critical reagents for PK ligand-binding assays. *Bioanalysis* 10(19), 1557–1565 (2018).
7. Staack RF, Stracke JO, Stubenrauch K, Vogel R, Schleypen J, Papadimitriou A. Quality requirements for critical assay reagents used in bioanalysis of therapeutic proteins: what bioanalysts should know about their reagents. *Bioanalysis* 3(5), 523–534 (2011).
8. Piccoli S, Mehta D, Vitaliti A *et al.* 2019 white paper on recent issues in bioanalysis: FDA immunogenicity guidance, gene therapy, critical reagents, biomarkers and flow cytometry validation (Part 3 - recommendations on 2019 FDA immunogenicity guidance, gene therapy bioanalytical challenges, strategies for critical reagent management, biomarker assay validation, flow cytometry validation & CLSI H62). *Bioanalysis* 11(24), 2207–2244 (2019).
9. Xu AS, Weant J. Critical reagent stability for immunogenicity assays. In: *Immunogenicity Assay Development, Validation and Implementation.* Future Medicine, London, UK, 92–104 (2013).
10. Tatarewicz S, Miller JM, Swanson SJ, Moxness MS. Rheumatoid factor interference in immunogenicity assays for human monoclonal antibody therapeutics. *J Immunol. Methods* 357(1–2), 10–16 (2010).
11. Haulenbeek J, Piccoli SP. Conjugated critical reagent characterization for ligand-binding assays: using MALDI-TOF-MS as an orthogonal tool to assess assay performance. *Bioanalysis* 6(7), 983–992 (2014).
12. Batchelor RH, Sarkez A, Cox WG, Johnson I. Fluorometric assay for quantitation of biotin covalently attached to proteins and nucleic acids. *BioTechniques* 43(4), 503–507 (2007).
13. Green NM. A Spectrophotometric assay for avidin and biotin based on binding of dyes by avidin. *Biochem. J.* 94(3), 23C–24C (1965).
14. Burgess RR. A brief practical review of size exclusion chromatography: rules of thumb, limitations, and troubleshooting. *Protein Expr. Purif.* 150, 81–85 (2018).
15. Kanagy B. An accurate and simple spectrophotometric assay system for quantitation of biotin: the Quant*Tag™ Biotin Kit. *BioTechniques* 55(4), 210–211 (2013).
16. Cromwell ME, Hilario E, Jacobson F. Protein aggregation and bioprocessing. *AAPS J.* 8(3), E572–E579 (2006).

17. Garofolo W, Savoie N. The decennial index of the white papers in bioanalysis: 'A Decade of Recommendations (2007–2016)'. *Bioanalysis* 9(21), 1681–1702 (2017).

18. Skamris T, Tian X, Thorolfsson M *et al.* Monoclonal antibodies follow distinct aggregation pathways during production-relevant acidic incubation and neutralization. *Pharm. Res.* 33(3), 716–728 (2016).

19. Shimizu M, Nagashima H, Sano K *et al.* Molecular stability of chicken and rabbit immunoglobulin G. *Biosci. Biotechnol. Biochem.* 56(2), 270–274 (1992).

20. Panka DJ. Glycosylation is influential in murine IgG3 self-association. *Mol. Immunol.* 34(8–9), 593–598 (1997).

21. Kronvall G, Seal US, Finstad J, Williams RC Jr. Phylogenetic insight into evolution of mammalian Fc fragment of gamma G globulin using staphylococcal protein A. *J. Immunol.* 104(1), 140–147 (1970).

22. Rispens T, Vidarsson G. *Chapter 9 – Human IgG Subclasses* Academic Press, MA, USA (2014).

23. Stone GC, Sjobring U, Bjorck L, Sjoquist J, Barber CV, Nardella FA. The Fc binding site for streptococcal protein G is in the C gamma 2-C gamma 3 interface region of IgG and is related to the sites that bind staphylococcal protein A and human rheumatoid factors. *J. Immunol.* 143(2), 565–570 (1989).

24. Pan T, Chang B, Wong P *et al.* An aggregation-specific enzyme-linked immunosorbent assay: detection of conformational differences between recombinant PrP protein dimers and PrP(Sc) aggregates. *J. Virol.* 79(19), 12355–12364 (2005).

25. Clancy D, Hodnett N, Orr R, Owen M, Peterson J. Kinetic model development for accelerated stability studies. *AAPS PharmSciTech* 18(4), 1158–1176 (2017).

26. Amaravadi L, Song A, Myler H *et al.* 2015 White Paper on recent issues in bioanalysis: focus on new technologies and biomarkers (Part 3 – LBA, biomarkers and immunogenicity). *Bioanalysis* 7(24), 3107–3124 (2015).

27. Stevenson L, Richards S, Pillutla R *et al.* 2018 White Paper on recent issues in bioanalysis: focus on flow cytometry, gene therapy, cut points and key clarifications on BAV (Part 3 - LBA/cell-based assays: immunogenicity, biomarkers and PK assays). *Bioanalysis* 10(24), 1973–2001 (2018).

28. O'Hara DM, Theobald V, Egan AC *et al.* Ligand binding assays in the 21st century laboratory: recommendations for characterization and supply of critical reagents. *AAPS J.* 14(2), 316–328 (2012).

Review

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Regenerative Medicine

Manufacturing, characterization and control of cell-based medicinal products: challenging paradigms toward commercial use

During the past decade, a large number of cell-based medicinal products have been tested in clinical trials for the treatment of various diseases and tissue defects. However, licensed products and those approaching marketing authorization are still few. One major area of challenge is the manufacturing and quality development of these complex products, for which significant manipulation of cells might be required. While the paradigms of quality, safety and efficacy must apply also to these innovative products, their demonstration may be demanding. Demonstration of comparability between production processes and batches may be difficult for cell-based medicinal products. Thus, the development should be built around a well-controlled manufacturing process and a qualified product to guarantee reproducible data from nonclinical and clinical studies.

Keywords: cell-based • manufacturing • quality • regenerative • regulatory • therapy

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Through the advent of biotechnology, therapeutic options have been expanded by large biological molecules, antibodies and recombinant proteins. More recently, with increasing knowledge of cell and tissue architecture of health and disease, therapies became targeted, not only to the metabolic, pharmacologic and/or immunological interaction but also to the more complex regeneration, repair and replacement of human tissues. Gene therapy, somatic cell therapy and tissue-engineered medicinal products have been consolidated in the EU legislation as advanced therapy medicinal products (ATMPs) [1]. The present paper outlines particular challenges related to the development of cell-based medicinal products (CBMPs). The term CBMP is used specifically to illustrate common requirements for products, which contain engineered cells or tissues as active substance and encompass medicinal products for somatic cell therapy, tissue-engineered products and combined ATMPs, that is, cells in combination with medical devices.

CBMPs hold high expectations for the treatment of diseases and tissue/organ defects for which traditional therapies and medicinal products have not provided satisfactory outcome [2]. Presently, two products for cartilage repair (ChondroCelect [3] and MACI [4]) have received a marketing authorization (MA) in the EU and a third one for treatment of metastatic, castrate-resistant prostate cancer (Provenge [5]). According to the industry, the clinical translation and commercialization of CBMPs are in strong progress worldwide [6,7], although most developers in EU at this stage come from academia, hospitals or small and medium enterprises (SMEs), often spin-off of those institutions [8].

In contrast to cell/organ transplantation, the developers of new CBMPs are making use of significant manipulation of the cells in an attempt to modulate the differentiation potential and plasticity of cell populations. Cultured cells, however, are complex as pharmaceuticals and require further knowledge and manufacturing experience to support commercial production. The CBMP field is currently struggling with similar problems as the first recombinant proteins 20 years ago – appropriate frameworks for these types

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of products need to be established by both developers and regulators while specific knowledge and control of the cell-based technologies are steadily growing.

What are the relevant legal/regulatory documents to be followed in EU?

The regulatory authorization of ATMPs in the EU is defined by legislation [1] and is concisely described by Kassim and Somerville [9]. Requirements for the MA of medicinal products are described in the pharmaceutical legislation (EudraLex Volume 1). Mandatory requirements are included in Regulations and Directives, while those included in guidelines or guidance documents, reflection papers or similar documents are recommendations based on the current scientific knowledge on a particular subject. Monographs from the European Pharmacopoeia (Ph.Eur.) constitute mandatory requirements while general chapters in the Ph.Eur. include recommendations that are not mandatory unless otherwise stated in a given text. In general, all these requirements apply at the time of EU marketing authorization application (MAA). The control of their gradual application during clinical development is under the remit of national competent authorities for clinical trial approval.

The new regulatory environment (Regulation [EC] No 1394/2007 [1], revised Annex I of Directive 2001/83/EC [10] and EMA guidelines [11–13]) is gradually supporting industry in issues related to CBMPs, but experience is lacking on appropriate analytical methods and novel strategies to build up standardization and quality control. Also the upscale of the manufacturing processes to commercially viable batch sizes of CBMPs generates in itself additional limits and challenges [14]. Tailored approaches provided in recent EU legislation and guidance (Directive 2009/120/EC [10], including EMA Guideline on the Risk-Based Approach for ATMPs [15]), are discussed in depth later.

Cells are much more complex entities than small molecules & therapeutic proteins

Cells are fragile, complex, living systems, capable of responding to external signals. Whenever their environment changes, the cells tend to respond, which makes the quality control of CBMPs a challenging undertaking [2]. Most crucial in any CBMP production, like during *in vivo* tissue morphogenesis, is the spatiotemporal organization of a heterogeneous population of cells into a controlled entity with intended properties and functionality. *In vivo* the mechanisms underlying this organization are governed by co-ordinated and interconnected intracellular and extracellular events that involve, for example, gene expression and imprinting and activation of a variety of signal transduction pathways. There are examples suggesting that *in vitro* processes not based on critical developmental phenomena and used without understanding of the limitations of the cells are unlikely to be successful [16,17]. For example, the factors impacting cell functionality in tissue renewal [18], the location and characterization of the cells [19] and plasticity [20,21] have been shown important for clinical efficacy of the grafts. Therefore, the *in vitro* process should be designed based on crucial parameters controlling cell biological events and following the knowledge on developmental cell biology. Furthermore, appropriate quality control is vital, since a poorly controlled product and production process may have impact on safety and efficacy, which therefore has a direct consequence for the treated patient.

A well-controlled product is one that is characterized in terms of identity, purity and potency, and function, ideally reflecting the intended mode of action. In order to ensure consistent manufacture, product stability and comparability after manufacturing changes, functional testing is important and may provide invaluable information. Some developers are utilizing multiple assays to cover different aspects of characterization, process validation, release, stability and comparability testing.

Major challenges related to a cell-based product are microbiological contamination, arising from either the cells/tissues used as starting material or the production process, and possible dedifferentiation/loss of cell function due to the manipulation. Further quality challenges for CBMPs are related to the consistency of the product and the production process, which may be difficult to validate due to, for example, autologous nature of the products and small sample sizes limiting the number of analytical tests, among others.

Your product is as good as the quality of the starting & raw materials

The cells/tissues used as starting materials, be they of autologous or allogeneic origin, can be very heterogeneous due to interindividual variability of donors, the variable content of cells other than the intended ones and because the isolated cells are not in a synchronized cell cycle. Furthermore, the origin of the cells may have significant impact on the phenotype and functionality of the cells after manipulation, as described, for example, for mesenchymal stem/stroma cells (MSCs) [22]. The age and health status of the donors may also determine the cells' response in culture [23]. Living cells are complex entities and only limited control over cell propagation and manipulation *in vitro* is possible. To further add to the complexity, there are limited possibilities to remove impurities, purification steps might affect the cells themselves and terminal sterilization of these products is impossible. Appropriate counter-measures are to

rigorously control the starting materials with appropriate predefined acceptance criteria that go beyond the requirements for donor testing, and the establishment of standard operating procedures for sourcing and transport of the cells. The sourcing of cells is to be conducted in compliance with the blood or tissues and cells legislations [24–28]; the information needs to be available to the manufacturer of the CBMP in order to comply with the GMP requirements and need to be reflected in the quality dossier. A good panel of suitable, validated/qualified analytical tools also enhances control of variability and acceptable heterogeneity, as described by Davie *et al.* [29].

Detailed documentation of cell cloning or cell banking/storage procedures performed according to regulatory requirements will ensure control over the quality of the starting materials and permit traceability should issues arise.

During past decades, high-risk reagents have been used in research (e.g., animal brain extracts, cholera toxin, and so on). In production of CBMPs for human use, such materials are problematic due to limited possibilities to control the source, purity and safety of these reagents. Thus, alternative raw materials with better safety profile should be considered already in early development of the products.

Adequate acceptance criteria for any other materials used in the manufacturing process, defined as raw materials in the legislation, are essential, particularly for biological materials such as cytokines and growth factors. This serves to ensure manufacturing consistency, comparability and traceability. The acceptance criteria have further relevance for safety, since those reagents may remain in the final product. Specific attention should be given to risks related to the biological source and pooling of the substances used in the raw material as well as testing for and removal of adventitious agents. Depending on the source of the raw material and the substances used in its production, the safety risks to be considered may be different and a risk assessment should be performed to justify their use. A general text regarding the expected quality of these biological raw materials, that is, reagents, is being prepared by the Ph.Eur. and has been released for public consultation in fall 2014. To comply with GMP requirements, the information needs to be available to the manufacturer of the CBMP and reflected in the quality dossier.

Sourcing of complex cells for production of CBMPs: the potentials of embryonic stem cells & induced pluripotent stem cells for the development of cell-based therapies

The discovery and culture of embryonic stem cells (ESCs) in 1998 [30] stirred a worldwide interest for stem cell research, but brought up serious bioethical issues [31]. Along with these concerns, the fact that ESCs form teratomas slowed down the start of first clinical trials and increased the challenges concerning development of human ESC-derived cellular therapeutics [32,33]. Earlier observations showing that transcriptional factors regulate the cell phenotypes [34] led Yamanaka in 2006 to produce induced pluripotent stem cells (iPSCs) similar to ESCs [35]. Immediately after the discovery, iPSCs attracted worldwide attention and became the epicenter of pluripotent stem cell research for their functional properties and capability to give rise to all types of tissues and even whole animals [36].

Nevertheless, iPSCs also form teratomas and may pose the same risk of tumorigenicity as hES cells [37]. iPSCs, when produced by retroviral transfection, carry oncogenic virus and can be considered problematic as starting material to develop CBMPs. The major drawback in current use of pluripotent stem cell-derived therapies is the need to impose a differentiation program toward the intended cell population/function that inevitably will lead to the possible presence of undifferentiated stem cells at the time of administration [38]. Minimizing undifferentiated pluripotent stem cells in CBMPs is crucial because of the risk of malignancy. At present, only few clinical trials were or are conducted with ESCs and only one with iPSs [39], because in-depth knowledge of the differentiation process and characterization of human ESCs and iPSCs is needed prior to their clinical use, in particular of their genetic stability and possible teratogenicity.

The fully established manufacturing process has to be controlled & validated at the commercial scale

For most CBMPs, the manufacturing process from the starting materials to the finished product is a continuous process. A target product profile, based on a sound scientific rationale, aids the establishment of the manufacturing process. This information should be gained through characterization studies and may provide basis for setting the initial specification limits for production and of the final product. A rigid and standardized process is advisable as early as possible [14], although some flexibility might be introduced at later development stages based on knowledge gained. Variability in the manufacturing process should be kept at minimum where possible and sufficient controls need to be in place to verify consistency at intermediate stages after relevant safety and clinical data have been gathered. This basic approach of minimizing change and variability is also applicable for scale-up and/or automation, which should be carefully planned if conducted during later stages of development [40].

Each step of the manufacturing process needs to be adequately defined and validated. For example, cell density, population doubling times and frequency of media changes can all have substantial impact on the growth kinetics of the cells, proliferation/differentiation and signaling, among others, and ultimately on the product and thus should be defined by the process and monitored by in-process controls (IPCs) and tests [41]. Cell storage and transport conditions need to be defined through testing and limits for temperatures, holding/transportation times, and so on, have to be established. If the cells are to be stored frozen, the impact of freezing and thawing steps, including materials used to protect the cells (e.g., DMSO) should be evaluated. Fully validated processes, storage and transport conditions are expected at the time of the MAA.

It is advisable to invest effort in defining the limits of the manufacturing process from very early stages of development. A prudent approach is, for instance, to set the maximum number of cell divisions in culture needed to obtain cell numbers well above the required dose and characterizing the resulting cells to assure they retain the intended biological activity and genetic stability.

Process validation gains a prominent role also in cases where very limited amount of material is available for release testing such as in certain autologous preparations. Where feasible, the introduction of a freezing step could be useful to ensure completion of the release testing prior to patient exposure.

Many of the products are intended for immediate use after production with very short shelf-lives. In cases where the nature of the product limits the possibilities for batch release testing of the finished product, the missing information could be complemented through more extensive product characterization, process validation data, as well as IPCs performed at critical steps [10].

The analytical methods matter

Although new analytical methods undoubtedly will be developed, proper tools are already currently available to characterize and control cell preparations. When a mechanism of action can be associated with a particular protein (marker) or function of the cells, in principle, an assay making use of these characteristics should be available as applied for analyzing biotechnologically manufactured proteins. One example is flow cytometry, which with new, innovative detection and labeling systems can even be used to fingerprint cells. Furthermore, specific bioassays, immunochemical assays, assays for proliferation, apoptosis and enzymatic activity are available for cell characterization and quality control. The key to successful quality management for CBMPs is to find the critical parameters to be monitored at the various steps of the manufacturing process and the corresponding assays that can be used. Appropriate methods are needed as IPCs and/or for batch release testing, process validation and for comparability and stability testing. One of concerns in CBMPs is the effect of procedures on genetic stability of the cells. Physiological stress or *in vitro* culture conditions may induce noticeable damage to the DNA and contribute to the occurrence of cell or chromosomal aberrations; the latter could be spontaneous or recurrent [42,43]. At least two groups of analytical techniques are currently available to assess cytogenetic abnormalities: conventional karyotyping, which is considered a valuable and useful technique to analyze chromosomal stability during nonclinical studies, and molecular cytogenetic techniques, comparative genomic hybridization array and single nucleotide polymorphism array, which could be used to look for recurrent aberrations due to their better sensitivity to detect a low proportion of abnormal cells [42].

In some cases, the exact test methods are difficult to define as mandatory for different CBMPs and thus the applicant may choose and justify the selection of a particular approach for the quality control of a CBMP. Sterility testing, for example, is a requirement for CBMPs and for many other medicines (injectables). While the assay for sterility is described in an Ph.Eur. monograph (i.e., mandatory requirement), it cannot be applied as a release test for most CBMPs as described (e.g., volume of sample needed, time to completion, and so on). Therefore, new monographs and general texts for alternative sterility testing have been included in the Ph.Eur [44,45]. They may be easier to conduct, but still are limited by the time needed to get results (reduction from 14 to 7 days) and therefore they cannot be applied as a decisive release test in cases where the final cell product has a very limited shelf-life. Alternative faster methods are being developed and are welcome, but there is still little experience about their robustness as compared with the traditional Ph.Eur. method. Therefore, while it is possible to conduct sterility testing using an innovative approach, most probably, justification of its use will have to include validation against the Ph.Eur. method at the time of MAA. Experience gained by the applicant or others (e.g., scientific literature) can be supportive for this justification and the information be included as part of the risk assessment. In any case, all analytical tools need to be robust, sensitive, specific and reliable. For the release of commercial batches and stability assays, method validation according to International Conference on Harmonisation requirements [46] is expected. For other assays, less stringent requirements may apply, yet they also need to be qualified for the intended use.

Potency assays are essential

A CBMP final product and especially its active substance need to be characterized to a level that ensures that only a safe and efficacious product will be administered to a patient. Where mixed cell populations are administered, characterization studies need to provide the scientific support that the result of potency assay(s) applied to the whole product is mediated by the cell population(s) responsible for the therapeutic effect rather than other populations present.

Characterization studies should also support development of suitable assays for in-process, release and stability testing. Physicochemical methods are mainly needed to establish identity, cell number and viability, purity and genetic stability of the product. However, as described by Bravery *et al.* [47] physicochemical parameters alone, including cell surface marker tests, cannot be considered sufficient for testing of engineered viable cells, which by definition are biological medicinal products [10]. Biological activity/potency is one of the key parameters for biological products and an ultimate link between quality, safety and efficacy of the product. Functional assays are used for the development of the products and they are relevant for characterization and validation of the manufacturing process, as well as for characterization of the active substance. Valid, functional potency tests are also imperative for comparability testing, as well as for safeguarding process and product consistency [47–49].

Often surrogate potency assays have been designed and developed based on the identification/quantification of cell surface markers, of an RNA sequence and/or morphological characteristics that are not directly associated to the pharmacological, metabolic or immunological function of the product. In other cases, the potency assay has been based on cell viability or self-renewal activity data. In most of these cases, such proposed potency assays do not represent the function of the product in itself and cannot be considered a quantitative measure of the biological activity. Assessment of general functional properties like cell growth, survival and migration is useful but not sufficiently specific to measure potency of a product.

A functional potency assay must be able to assess the relevant biological effect, that is, bone-forming capacity, paracrine effects, activation of a metabolic pathway, expression of specific receptors in response to external stimuli in the tissue microenvironment, potential for differentiation, bone marrow homing capacity, stimulation of the immune function or secretion of cytokines to mention a few functional properties. Ideally, one or more properties that reflect directly the intended therapeutic action of the product can be used in a multiple approach to establish the potency of the product.

The potency assay(s) could be *in vitro* (cell-based assays) or *in vivo* (in relevant animal models) assays, although the *in vivo* assays are better suited for product characterization and validation of other assays than for quality control. Furthermore, functional tests may be too complex and time consuming for release testing, especially if the products are intended for immediate use after production with very short shelf-lives. In such case, the 'multiple' approach for potency testing can be used to validate a surrogate assay for release. Potency assays used for batch release should be sensitive, reliable, robust and easy to conduct.

Can we measure comparability of two similar cell batches?

In the life cycle of a CBMP, changes of the starting materials, reagents or the manufacturing processes are inevitable. Upscale of allogeneic CBMP manufacturing process required for Phase III clinical trials or registered product release into market is likely to include substantial modifications. Having gathered relevant safety and efficacy data with a given product, there should be tools available to establish the comparability of the cell-based product before and after the introduced change. This comparability exercise may not be a simple feat and, in addition to the analytical approaches, may require further nonclinical and clinical testing. In such situations, functional *in vitro*/*in vivo* assays are invaluable. As already mentioned for potency, it is recommended to establish multiple assays during the development to cover the needs of characterization and process validation and to overcome the limitations related to batch release testing.

Several scenarios are described below to illustrate that the extent of the comparability studies will vary according to the stage of development of the product or the affected manufacturing step. The role of the comparability exercise is to demonstrate that pre- and post-change product perform equally, thereby confirming the relevance of previously obtained results. As an example, changes of the supplier of a biologically active raw material might trigger the need for comparability data. If clear quality criteria for the reagent were in place, data on the performance of the reagent and characteristics/functionality of the cells at the specific step could suffice. On the other side of the spectrum, substantial changes to the manufacturing process of a product, from which clinical data are already gathered, inevitably lead to differences between the 'old' and the 'new' product requesting more extensive comparability data to justify the differences and address the possible impact on product safety and/or efficacy. In this case, functional testing plays a key role and absence of such assays might result in the need for bridging nonclinical

studies and/or additional clinical data. Examples for possible substantial changes to the manufacturing process include changes to the cell culture medium, the culturing process, the isolation procedures, analytical procedures and the formulation to name a few.

MSCs isolated from a variety of human tissues are possibly the most commonly developed allogeneic cell therapies. MSC characterization is often reduced to a set of three criteria defined for bone marrow-derived MSCs. Those are cell adherence to the plastic, expression of CD73, CD90 and CD105 markers (and absence of hematopoietic lineage markers), and differentiation into mesenchymal cell types under modified culture conditions, as summarized by International Society for Cellular Therapy [50]. More recent studies suggest that within this broad definition of MSCs, there are in fact several specific types of cells with differing characteristics and functionality [51]. Furthermore, recent publications indicate that cell markers vary with the microenvironment of the tissues from where they are collected as well as with the culturing *in vitro* [52]. While molecular mechanisms for MSC therapeutic activity for particular indication may not yet be clear and fully characterized, using preset criteria not reflecting the mode of action remains a poor approach to demonstrate batch-to-batch comparability. Keeping in mind that it is not uncommon that CBMP development may take more than a decade, it is advisable to define comparability criteria and release assays based on state-of-the-art technology platforms. The International Society for Cellular Therapy criteria or examples of limited marker sets for characterization of previously developed therapy candidates may not represent the optimal choice and possibly will be considered outdated by the time a product reaches the evaluation stage. In any case, phenotypic profile combined with functional testing remains the best supportive demonstration of comparability. Therefore, even during the early stage of CBMP development, a strategy for comparability testing should be developed to manage change and assure a 'filiation' of pre- and post-change product and data gathered for MAA.

Manipulation & changes in cell characteristics may have an impact on cell fate, persistence, engraftment & overall efficacy of a CBMP

Questions that often come up when manipulated cells are administered to patients are whether the biodistribution of transplanted cells is unchanged, what proportion of cells remained structurally and viably intact, whether they adapt to the heterologous tissue environment and remain capable of exerting the expected mechanism of action (e.g., to repair, replace and regenerate a damage tissue, to secrete growth factors, to express a metabolic function or any other).

These questions are not easily answered, since the initial dose of cells, their administration (infusion or local placement and insertion), their migration capacity, state of differentiation as well as the formulation used (cells being encapsulated or attached on the membrane, with and without biocompatible scaffolds or other substances) all have an impact. Therefore, close monitoring of the functional activity and assessment of structural integrity/sustainability of a given CBMP is important to correlate with pharmacology. In cases, where surrogate assays are needed for batch release testing (e.g., due to short shelf-lives), *in vivo* nonclinical assays and noninvasive technologies may provide valuable information for the product and allow the surrogate assays to be correlated with functionality/biological activity of the product.

Increasing complexity to the third dimension: combined products

In the area of regenerative medicine, cell-based products may include also other materials (e.g., structural components, biomolecules and feeder cells, among others), which add an additional level of complexity. For combined ATMPs, Directive 2009/120/EC [10] considers that the medical device component can be either an integral part of the active substance – where the cells are cultured together with the structural component, or of the finished product – when the substantially manipulated cells are combined with the device at the time of manufacture of the finished product or at the time of administration of the finished product. In CBMPs combined with additional substances such as scaffolds, matrices, biomaterials or biomolecules, the cells alone are rarely responsible for the entire mechanism of action (be it tissue regeneration, repair or replacement or treatment, prevention or diagnosis of a disease): the medical device or additional substances will contribute to the intended therapeutic effect.

For combined products, the components (cells and other materials) need to be characterized separately and in the combination, thus extending the characterization and functional testing requirements of the final product. It has become clear that manufacturing steps to create scaffolds from human tissues can impact the final combination product. One recent example has shown that decellularization of human tracheas may compromise mechanical integrity of the resulting scaffold and hence that of the combined ATMP [53]. Therefore, it is imperative to also validate the manufacturing procedures of the noncellular components, and to extend the characterization studies to *in vivo*

follow-up of the combinations to ensure integrity and sustainability in clinical use.

A further level of complexity arises with the presence of living cells derived from different animal/human (autologous or allogenic) sources used as feeder layers of the CBMPs. Although the feeder cells do not directly contribute to the CBMPs mode of action, they often remain in the final product, and may impact culture reproducibility and overall product safety and efficacy. A rigorous production and characterization of master and working cell banks, for example, can greatly decrease the feeder cell culture variability generally found in autologous feeder cell cultures established without master cell banking. Furthermore, cell banking allows an extensive characterization of the cells and manufacturing under GMP conditions, thus decreasing also the risk of transmissible diseases. Moreover, validation of the methods used to mitotically arrest the feeder cells is also key to decrease possible tumorigenicity risks. Finally, possible immune reactions against animal/allogeneic material are also representing a risk that should be mitigated. Therefore, a thorough analysis of the feeder cell source, production process, quality attributes and controls, homogeneity, lack of proliferation and potential risks is paramount for utilization of feeder cells for CBMP clinical use.

Are patient cells suitable for therapeutic use?

As explained previously, CBMPs are complex entities that will respond to external signals, including the health status and effects of concomitant medication in both the donor and the recipient. This should be taken into consideration when cells are sourced and subsequently used for cell therapy. Especially important is the reflection on the possible introduction of tumorigenic or genetically altered cells into the product when sourcing the starting material from cancer patients after standard treatments (e.g., irradiation and chemotherapy).

Chemical and biological molecules are also able to manipulate cells phenotypically and genetically, including cell activation, expansion and differentiation. While much is known about how transcription factors and growth factors regulate differentiation programs of stem/pluripotent cell, there are very limited data available about effects of patient medication on cells when concomitantly used during the cell therapy. Especially, possible effects on dedifferentiation/loss of function/apoptosis of cells should be considered. Risk mitigation through selection of appropriate donors and adequate exclusion criteria for the clinical use should be clearly delineated before embarking into development of new CBMPs.

How can the risk-based approach help through the mission?

CBMPs contain a wide variety of cell-based products with different specific risks and risk factor profiles and, as discussed above, they may face limitations and challenges not foreseen for other medicinal products. Thus, a flexible, case-by-case regulatory approach is necessary. Consequently, the option of a risk-based approach has been implemented in the legislation for ATMPs [10]. The risk-based approach provides a strategy to determine the extent of quality, nonclinical and clinical data to be included in the MAA. The intention and the details of the methodological application are outlined in the recently published guideline on the risk-based approach [15]. It is important to note that the process starts at the beginning of the CBMP development and matures as scientific knowledge on the product increases. Even if this flexible approach is used, it is important to highlight that the collected data to be presented at the time of the MA application should be in accordance with the scientific guidelines relating to quality, safety and efficacy of a CBMP [11,12]. Moreover, the strategy of the risk-based approach may be also to use it as a scientifically sound justification for any deviation from the technical requirements for a cell-based product as defined in Directive 2009/120/EC [10].

The principles and the methodology of the risk-based approach are based on the identification of specific risks associated with the clinical use of a CBMP and risks inherent to the CBMP that are linked to quality, manufacturing and administration of the product. Their identification may be supported by published adequate scientific data. It should be noted that the risks of a CBMP are often not different from those of other classes of ATMPs or even other classes of medicinal products. They may include, for example, unwanted immunogenicity, tumor formation, unintended biodistribution/cell homeostasis and (ectopic) tissue formation, contaminants from the production process, as well as toxicity due to toxic degradation products from structural components.

Risks can be mitigated to some extent through the quality management system for manufacture using starting materials of appropriate quality and validated processes. It should be noted that frequently used risk management and process tools are not directly linked to the risk-based approach for CBMPs as outlined in the respective guideline [54].

To conclude on a specific risk associated with the CBMP under development, the contribution of specific risk factors should be evaluated by a systematic approach of individual risk/risk-factor combinations. It is important to highlight that the different risk factors associated with an individual risk are mainly

CBMP product specific, but nevertheless multifactorial. Risk factors may be related for instance to the nature and composition of the CBMP, the manufacturing process as well as nonclinical and clinical aspects.

Examples of risk factors associated with a cell-based product are: the origin of cells or tissues, the level of cell manipulation, the presence of feeder cells of different sources, noncellular components, microbiological contamination arising from the cells or tissues used as starting material or from the production process, the ability of CBMP to proliferate and/or differentiate, possibility for cell transformation to malignancies, the ability of the CBMP to initiate an immune response, possible ectopic engraftment of the cells in nontarget tissues, the specific mode of administration and the duration of exposure of the CBMP. In the MAA, the developer will have to justify that the provided scientific quality, nonclinical and clinical data and/or published information are sufficient to address the overall risks and risk factors for which a reasonable relationship has been identified.

What should be in place for early clinical studies: in terms of control of the product

While the requirements for a MAA in the EU are clearly outlined in the ATMP Regulation and in the EMA guidelines, pinpointing the exact requirements for early clinical studies is difficult. This is because the scientific background knowledge differs widely for different ATMPs. Furthermore, it is acknowledged that being on the right track toward a MA is not necessarily the same as getting a clinical trial approved. This divergence arises from the fact that the clinical trial is approved as a self-standing undertaking considering mainly safety aspect for the patients, but generally not with the view of whether the resulting data are the ones needed to complete a MAA. During the clinical trial stage, quality data are summarized in the Investigational Medicinal Product Dossier, which expands in volume as more data and experience become available. At the MAA stage, assessment has a wider scope of ensuring that the applicant is capable of reproducibly and consistently manufacturing a high quality product. Thus, the entire manufacturing history and particularly manufacturing control and validation form a core part of the assessment. In addition, full validation according to International Conference on Harmonisation guidelines [46] only becomes compulsory at the MAA stage.

The approval of clinical trials is in the remit of EU Member States, not the EMA. To harmonize the approach to clinical trial assessment and approvals, a voluntary harmonization approach to share the assessment of multinational trials (VHP procedure) is already available and used by the Member States.

A complete revision of the EU clinical trials framework into a fully integrated European system has been adopted in 2014. The new Clinical Trial Regulation 536/2014/EC [55] is meant to facilitate in particular the conduct of multinational clinical trials with single points for submission and reporting Member States for approval. It will apply no earlier than May 2016.

As previously outlined, the first step should be the definition of a target product profile based on state-of-the-art scientific knowledge. The two main outcomes of this exercise need to be the definition of the product and a proposed mechanism of action. It is essential that the specifications proposed reflect this mechanism of action and that characterization studies and later clinical trial data serve to verify the validity of the hypothesis. Although not directly applicable to ATMPs, some reflections provided in the Guideline on the requirements for quality documentation concerning biological investigational medicinal products in clinical trials [56] namely on the requirements toward increasingly validated processes and assays are of relevance.

The already mentioned characterization studies need to link the product with state-of the-art knowledge, that is, provide product-specific data to support scientific claims. A further important point for products that consist of cell mixtures is to make an effort to identify the cell population responsible for the biological effect and to experimentally address the question whether additional cell populations present have a positive or negative effect or no contribution to the mechanism of action.

Conclusion

Consistent production, appropriate quality control and comparability testing are demanding for novel CBMPs. Therefore, the developers are advised to be prepared from early on to foresee possible challenges and limitations in the manufacturing and quality development. Special attention should be paid to starting and raw materials, characterization of the active substance/final product, analytical tools and special issues/components of the product to ensure consistent, well-qualified product. For CBMPs the quality is directly linked to safety and efficacy/functionality of the product. Thus, only with standardized and well-controlled products can the results from nonclinical and clinical studies be robust. In case there are significant challenges foreseen, the developers should engage with regulators to seek for possible solutions. The available guidelines should guide and support developers and the risk-based approach

is warmly recommended as a tool to get flexibility to standard regulatory requirements.

Future perspectives of regulatory requirements

Many of the novel cell-based therapies are developed by small academic groups, hospitals, nonprofit organizations and SMEs [8], which might not have as their immediate goal to translate their scientific development into authorized medicines. Furthermore, they might lack expertise, experience and the financial resources to move an ATMP from the research phase into clinical trials and finally into a MAA. There are hundreds of papers published on cell therapy or tissue engineering every year, but only few therapies have been reviewed by the Regulatory Authorities for market access. Additionally, there is a big discrepancy between the number of patients treated so far in Europe with ATMPs and the amount of available good quality clinical data, which can be used for regulatory purposes: some of these products were legally available in some member states (e.g., as transplants or medical devices) without (extensive) pre-approval clinical trials or a systematic collection of safety and efficacy data during clinical use. As a result, there are treatments with long clinical history, but with serious difficulties in proving their effectiveness in a scientific manner.

The growth of the ATMP market in Europe therefore is still far from that predicted by the Commission in 2003 [57]. There are multiple elements contributing to this situation. Among them are: changes in manufacturing protocols, changes of the manufacturing sites, problems with proper design of clinical trials and also with gathering funds for long-term clinical trials [58]. Also, the regulatory framework for ATMPs came only in operation in the beginning of 2009. Whereas the regulatory framework for gene and somatic cell therapy medicinal products was set in 2003, the ATMP Regulation defined tissue-engineered products as medicinal products and included ATMPs in the mandatory scope of the centralized procedure.

The ATMP Regulation has been specifically developed for and adapted to ATMPs, aiming to provide a clear regulatory route for these products to gain access to the European market. It provides for specific adaptations for ATMPs, such as the risk-based approach, GMP, GCP and technical (dossier) requirements, as well as two new pre-authorization procedures: ATMP classification and ATMP certification. However, as this legislation builds on the general legislation for medicinal products, it has been reported [59,60] that it is onerous for ATMP developers to comply with all the regulatory requirements for medicines (such as manufacture and quality control under GMP, the need to set up and maintain an EU wide pharmacovigilance system) and that the dossier requirement is not sufficiently adequate or adapted to ATMP under development. For the latter, it cannot be stressed enough that the provision of the risk-based approach, as explained in detail above, provides a unique legal possibility for ATMPs to tailor the quality, nonclinical and clinical dossier content as specified in Directive 2009/120/EC [10], based on specific risks (or lack thereof). This regulatory flexibility, which is not available to other medicines, is at present not used to its full extent. Further refinement can be expected as the ATMP Regulation 1394/2007/EC may be further revised in the near future, considering the stakeholders' recommendations present in the EC report on its 5 years' application [60].

Regulatory challenges are linked to the specificities of the products, to the profile of the ATMP developers or, likely to a combination of both. Regulatory challenges with ATMPs, linked to the product specificities, encountered by Committee for Advanced Therapies (CAT) include (note that this is not a restricted list): how to regulate CBMP composed of nonmanipulated cells for indications that are considered 'nonhomologous'; how to manage the inherent difficulties to conduct clinical trials involving a surgical intervention, or how to employ a surgical comparator, with the expectation for randomized controlled trials in the MAA; how to authorize novel treatment schemes whereby other (non-ATMP) products such as immunomodulators are required to be administered prior, after or together with the ATMP to obtain the required clinical effect. It may also be challenging to justify the 'new active substance' (NAS) status for ATMPs based on the same cell type that is part of another previously licensed CBMP. As part of the evaluation of the MAA, the Rapporteurs will also review the NAS claim by the applicant and this will be adopted by the CAT and Committee for Human Medicinal Products. In order to substantiate the NAS claim, the applicant will have to identify the active substance in the finished product. For most CBMPs, the manufacturing process from the starting materials to the finished product is a continuous process, so the applicant will have to set the demarcation between the active substance and the finished product. Both the ATMP legislation and the applicable guidelines are not descriptive in this respect. For combined ATMPs, Directive 2009/120/EC [10] provides a legal interpretation of the status of the medical device component. However, the applicant needs also to demonstrate whether the medical device or additional substances will contribute to the intended therapeutic effect, thus being integral part of the product. These aspects should be included in the justification of the NAS status of the CBMP, for example, to justify the

NAS status of a chondrocyte-scaffold product after the approval of a CBMP containing a chondrocyte suspension.

CBMPs are not yet 'mainstream medicines', and as mentioned previously, they are a very diverse group of products, ranging from relatively simple progenitor cell products (e.g., chondrocytes) up to very complex CBMPs like 3D scaffolds harboring *in vitro* differentiated and genetically modified cell types. Consequently, the scientific guidelines for these products might not always be fully applicable to a given CBMP under development. This, taken together with the fast evolution in science (e.g., first iPSC-based product entering in clinical development in Japan [39]), puts considerable challenges, both scientific and regulatory, to developers and regulators.

In order to address the regulatory hurdles and the novel scientific challenges, the advice to developers of CBMPs is to get in contact with EU national regulatory authorities and/or the EMA as early and as frequently as possible.

Scientific issues not addressed in the guidelines can be addressed in a National or EMA scientific advice (SA) procedure. The latter includes routine involvement of the CAT in order to ensure that the best available expertise in Europe is used, and also to provide a link with the Committee that later, should the development program be successful, will be responsible for scientific assessment of the dossier. The CBMP developers should make most use of the SA presubmission activities offered by EMA to ensure that the questions asked to CAT/Committee for Human Medicinal

Executive summary

Cells are much more complex entities than small molecules & therapeutic proteins

- Cell-based medicinal products (CBMPs) are complex pharmaceuticals, for which the development should be built around a defined manufacturing process and a qualified product to ensure robust and meaningful nonclinical and clinical results.

Your product is as good as the quality of the starting & raw materials

- All starting and raw materials should be controlled and tested according to predefined acceptance criteria with special focus on biologically active materials and their quality and consistency.

Sourcing of complex cells for production of CBMPs: the potentials of embryonic stem cells & induced pluripotent stem cells for the development of cell-based therapies

- The use of pluripotent stem cells as starting material requires in-depth knowledge of the differentiation process and characterization of the stem cells prior to their clinical use, in particular of their genetic stability and possible teratogenicity.

The fully established manufacturing process has to be controlled & validated at the commercial scale

- A rigid and standardized manufacturing process is advisable as early as possible and variability should be kept at minimum.

The analytical methods matter

- Key to successful quality management for CBMPs is to find the critical parameters of the product and the corresponding assays for testing.

Potency assays are essential

- Potency test(s) are essential for CBMPs as a link between quality, safety and efficacy of the product. Functional potency assays are critical for comparability testing and should be considered as early as possible.

Can we measure comparability of two similar cell batches?

- A strategy for comparability testing should be developed for every product to manage changes. Defined quality criteria for starting and raw materials, for all intermediates and for the final product are needed for the comparability strategy.

Increasing complexity to the third dimension: combined products

- For combined products, the components (cells and other materials) need to be characterized processes validated both separately and in the combination.

How can the risk-based approach help through the mission?

- The risk-based approach provides a unique legal possibility for ATMPs to tailor the quality, nonclinical and clinical dossier content, based on specific risks (or lack thereof).

What should be in place for early clinical studies: in terms of control of the product

- For clinical trials, specifications proposed for the product should reflect the targeted mechanism of action. The characterization studies and later clinical trial data should serve to verify the validity of the hypothesis.

Future perspectives of regulatory requirements

- Developers of CBMPs should get in contact with national regulatory authorities and/or the European Medicines Agency as early and as frequently as possible, when support is needed to overcome challenges and limitations in the development.

Products are addressing all parts of the development (quality, nonclinical and clinical) in an optimal way, and are formulated in such a way that the most adequate feedback can be obtained. Another procedure available to SMEs developing ATMPs registered at the EMA's SME office is the ATMP certification procedure [61,62]. In this procedure, the CAT provides a scientific evaluation of early quality and nonclinical data. In other words, the CAT certification procedure is the only procedure before a MAA evaluation that provides assessment of data; SA provides feedback on Applicant's questions, but does not include assessment.

Several entry doors are therefore available from early stages of development. These include interactions with the SA offices at national level or at the EMA Innovation Task Force informal meetings, such as Innovation Task Force briefing meetings, where regulatory queries can be addressed directly to the regulators. Another important tool for CBMP developers is to make use of the ATMP classification procedure [63]: not only it will give regulatory clarity on whether a particular product will be fulfilling the definition of an ATMP, but it is also a useful tool to establish first contacts with the CAT.

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Financial & competing interests disclosure

The authors have no relevant affiliations or financial involvement with any organization or entity with a financial interest in or financial conflict with the subject matter or materials discussed in the manuscript. This includes employment, consultancies, honoraria, stock ownership or options, expert testimony, grants or patents received or pending, or royalties.

No writing assistance was utilized in the production of this manuscript.

References

Papers of special note have been highlighted as:
• of interest; •• of considerable interest

1 Regulation (EC) No 1394/2007 of the European Parliament and of the Council on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004. Official Journal of the European Union, L324, 121–137 (2007).

•• **Comprises the main legal framework for cell-based medicinal products in EU and is thus considered as most relevant for this publication.**

2 Fischbach, MA, Bluestone, JA, Lim, WA. Cell-based therapeutics: the next pillar of medicine. *Sci. Transl. Med.* 5(179), 1–6 (2013).

3 EMA. European Public Assessment Report: ChondroCelect (2009). www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/000878/human_med_000698.jsp&mid=WC0b01ac058001d124

4 EMA. European Public Assessment Report: MACI (2013). www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002522/human_med_001660.jsp&mid=WC0b01ac058001d124

5 EMA. European Public Assessment Report: Provenge (2013). www.ema.europa.eu/ema/index.jsp?curl=pages/medicines/human/medicines/002513/human_med_001680.jsp&mid=WC0b01ac058001d124

6 Mason C, Mason J, Culme-Seymour E, Bonfiglio GA, Reeve, BC. Cell therapy companies make strong progress from October 2012 to March 2013 amid mixed stock market sentiment. *Cell Stem Cell* 12(6), 644–647 (2013).

7 Buckler, L. Signposts along the way to cell therapy success. *BioProcess Int.* 1(3), S6–S11 (2013).

8 Maciulaitis R, D'Apote L, Buchanan A, Pioppo L, Schneider CK. Clinical development of advanced therapy medicinal products in Europe: evidence that regulators must be proactive. *Mol. Ther.* 20(3), 479–482 (2012).

9 Kassim, SH, Somerville RPT. From lipoproteins to chondrocytes: a brief summary of the European Medicines Agency´s Regulatory Guidelines for Advanced Therapy Medicinal Products. *Hum. Gene Ther.* 24(6), 568–570 (2013).

10 Commission Directive 2009/120/EC of amending Directive 2001/83/EC of the European Parliament and of the Council on the Community code relating to medicinal products for human use as regards advanced therapy medicinal products. Official Journal of the European Union, L243, 3–12 (2009).

•• **Comprises the main legal framework for cell-based medicinal products in EU and is thus considered as most relevant for this publication.**

11 EMA. Guideline on human cell-based medicinal products. www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500003894.pdf

• **Includes available guidelines for CBMPs, as well as outstanding articles in this area, published by product developers, International Society for Cellular Therapy (ISCT) and other relevant parties.**

12 EMA. Guideline on xenogeneic cell-based medicinal products. www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/12/WC500016936.pdf

13 Scientific guidelines applicable to ATMPs. www.ema.europa.eu/ema/index.jsp?curl=pages/regulation/general/general_content_000298.jsp&mid=WC0b01ac05800862bd

• **Includes available guidelines for CBMPs, as well as outstanding articles in this area, published by product developers, ISCT and other relevant parties.**

14 Pattasseril J, Varadaraju H, Lock LT, Rowley JA. Downstream technology landscape for large-scale therapeutic cell processing. *BioProcess Int.* 11(3), S38–S47 (2013).

15 EMA. Guideline on the risk-based approach according to annex I, part IV of Directive 2001/83/EC applied to Advanced therapy medicinal products (2013). www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2013/03/WC500139748.pdf

- **Includes available guidelines for CBMPs, as well as outstanding articles in this area, published by product developers, ISCT and other relevant parties.**

16 Sipione S, Eshpeter A, Lyon JG, Korbutt GS, Bleackley RC. Insulin expressing cells from differentiated embryonic stem cells are not beta cells. *Diabetologia* 47(3), 499–508 (2004).

17 Kim H, Han JW, Lee JY *et al.* Diabetic mesenchymal stem cells are ineffective for improving limb ischemia due to their impaired angiogenic capability. *Cell Transplant.* doi:10.3727/096368914X682792 (2014) (Epub ahead of print).

18 Park CH, Minn YK, Lee JY *et al. In vitro* and *in vivo* analyses of human embryonic stem cell-derived dopamine neurons. *J. Neurochem.* 92(5), 1265–1276 (2005).

19 Pellegrini G, Golisano O, Paterna P *et al.* Location and clonal analysis of stem cells and their differentiated progeny in the human ocular surface. *J. Cell Biol.* 145(4), 769–782 (1999).

20 Bouacida A, Rosset P, Trichet V *et al.* Pericyte-like progenitors show high immaturity and engraftment potential as compared with mesenchymal stem cells. *PLoS ONE* 7(11), e48648 (2012).

21 López-Ruiz E, Perán M, Picón-Ruiz M *et al.* Cardiomyogenic differentiation potential of human endothelial progenitor cells isolated from patients with myocardial infarction. *Cytotherapy* doi:10.1016/j.jcyt.2014.05.012 (2014) (Epub ahead of print).

22 Wegmeyer H, Bröske AM, Leddin M *et al.* Mesenchymal stromal cell characteristics vary depending on their origin. *Stem Cells Dev.* 22(19), 2606–2618 (2013).

23 Stolzing A, Jones E, McGonagle D, Scutt A. Age-related changes in human bone marrow-derived mesenchymal stem cells: consequences for cell therapies. *Mech. Ageing Dev.* 129(3), 163–173 (2008).

24 Directive 2002/98/EC of the European Parliament and of the Council setting standards of quality and safety for the collection, testing, processing, storage and distribution of human blood and blood components. Official Journal of the European Union, L33, 30–40 (2003).

25 Commission Directive 2005/61/EC implementing Directive 2002/98/EC of the European Parliament and of the Council as regards traceability requirements and notification of serious adverse reactions and events. Official Journal of the European Union, L256, 32–40 (2005).

26 Directive 2004/23/EC of the European Parliament and of the Council on setting standards of quality and safety for the donation, procurement, testing, processing, preservation, storage and distribution of human tissues and cells. Official Journal of the European Union, L102, 48–58 (2004).

27 Commission Directive 2006/17/EC implementing Directive 2004/23/EC of the European Parliament and of the Council as regards certain technical requirements for the donation, procurement and testing of human tissues and cells. Official Journal of the European Union, L38, 40–52 (2006).

28 Commission Directive 2006/86/EC implementing Directive 2004/23/EC of the European Parliament and of the Council as regards traceability requirements, notification of serious adverse reactions and events and certain technical requirements for the coding, processing preservation, storage and distribution of human tissues and cells. Official Journal of the European Union, L294, 32–50 (2006).

29 Davie NL, Brindley DA, Culme-Seymour EJ, Mason C. Streamlining cell therapy manufacture: from clinical to commercial scale. *BioProcess Int.* 10(3), S24–S29 (2012).

30 Thomson JA, Itskovitz-Elder J, Shapiro SS *et al.* Embryonic stem cells lines derived from human blastocysts. *Science* 282(5391), 1145–1147 (1998).

31 Baker M. Court lifts cloud over embryonic stem cells. *Nature* 493(7432), 282–283 (2013).

32 Blum B, Benvenishy N. The tumorigenicity of human ECS. *Adv. Cancer Res.* 100, 133–158 (2008).

33 Cunningham JJ, Ulbright TM, Pera MF, Looijenga LH. Lessons from human teratomas to guide development of safe stem cell therapies. *Nat. Biotechnol.* 30(9), 849–857 (2012).

34 Graf T. Historical origins of transdifferentiation and reprogramming. *Cell Stem Cell* 9(6), 504–516 (2011).

35 Takahashi K, Yamanaka S. Induction of pluripotent stem cells from mouse embryonic and adult fibroblasts by defined factors. *Cell* 126(4), 663–676 (2006).

36 Saha K, Jaenisch R. Technical challenges in using human induced pluripotent stem cells to model disease. *Cell Stem Cell* 5(6), 584–595 (2009).

37 Abad M, Mosteiro L, Pantoja C *et al.* Reprogrammimg *in vivo* produces teratomas and iPSC cells with totipotency features. *Nature* 502(7471), 340–345 (2013).

38 Tohyama S, Hattori F, Sane M *et al.* Dinstinct metabolic flow enables large-scale purification of mouse and human pluripotent stem cell derived cardiomyocytes. *Cell Stem Cell* 12(1), 127–137 (2013).

39 Cyranoski D. iPS cells in humans. *Nat. Biotech.* 31(9), 775 (2013).

40 Brindley D, Moorthy K, Lee J-H, Mason C, Kim H-W, Wall I. Bioprocess forces and their impact on cell behavior: implications for bone regeneration therapy. *J. Tissue Eng.* 2011, 620247 (2011).

41 Kirouac DC, Zandstra PW. The systematic production of cells for cell therapies. *Cell Stem Cell* 3(4), 369–381(2008).

- **Includes available guidelines for CBMPs, as well as outstanding articles in this area, published by product developers, ISCT and other relevant parties.**

42 Barkholt L, Flory E, Jekerle V *et al.* Risk of tumorigenicity in mesenchymal stromal cell-based therapies – bridging scientific observations and regulatory viewpoints. *Cytotherapy* 15(7), 753–759 (2013).

43 Lorenzo Y, Haug Berg K, Ustgaard-Andersen K *et al.* Trypsin for dissociation of limbal cells for engineering of grafts may induce DNA strand breaks in the harvested cells. *J. Ocular Biol.* 1(2), 1–6 (2013).

44 Council of Europe. European Pharmacopoeia Text 5.1.6: alternative methods for control of microbiological quality (01/2008:50106). www.google.co.uk/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&cad=rja&uact=8&ved=0CCYQFjAA&url=https%3A%2F%2Fwww.edqm.eu%2Fsite%2FSession_II_Part_1_Slides-en-798–2.html&ei=FAQ1VPr_CIiMsQS164HIBA&usg=AFQjCNG_cx-5Z6kErCH17FS6v4iRHD5FpQ&bvm=bv.76943099,d.cWc

45 Council of Europe. European PharmacopoeiaGeneral Method 2.6.27: microbiological control of cellular products. www.google.co.uk/url?sa=t&rct=j&q=&esrc=s&source=web&cd=2&ved=0CCYQFjAB&url=https%3A%2F%2Fwww.edqm.eu%2Fsite%2FIndex_6th_Edition_64_English-en-8025–2.html&ei=aAQ1VIePBrb-sASu1ICoCg&usg=AFQjCNGAS_mFk0–4hVIdVwanTsJl57RrZg&bvm=bv.76943099,d.cWc

46 EMA. ICH guideline Q2(R1) Validation of Analytical Procedures: text and methodology (1995). www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2009/09/WC500002662.pdf

47 Bravery, C, Carmen, J, Fong, T *et al.* Potency assay development for cellular therapy products: an ISCT review of the requirements and experiences in the industry. *Cytotherapy* 15(1), 9–19 (2013).

- **Includes available guidelines for CBMPs, as well as outstanding articles in this area, published by product developers, ISCT and other relevant parties.**

48 Rayment EA, Williams DJ. Mind the gap: challenges in characterizing and quantifying cell- and tissue-based therapies for clinical translation. *Stem Cells* 28(5), 996–1004 (2010).

49 Guthrie K, Bruce A, Sangha N, Rivera E, Basu J. Potency evaluation of tissue engineered and regenerative medicine products. *Trends Biotechnol.* 31(9), 505–514 (2013).

50 Dominici M, Le Blanc K, Mueller I *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells: the International Society for Cellular Therapy position statement. *Cytotherapy* 8(4), 315–317 (2006).

51 Hoogduijn MJ, Betjes MG, Baan CC. Mesenchymal stromal cells for organ transplantation: different sources and unique characteristics? *Curr. Opin. Organ Transplant.* 19(1), 41–46 (2014).

52 Murphy MB, Moncivais K, Caplan AI. Review: mesenchymal stem cells: environmentally responsive therapeutics for regenerative medicine. *Exp. Mol. Med.* 45, 1–16 (2013).

53 Partington, L, Mordan, NJ, Mason, C *et al.* Biochemical changes caused by decellularization may compromise mechanical integrity of tracheal scaffolds. *Acta Biomater.* 9(2), 5251–5261 (2013).

54 EMA. Guideline on good pharmacovigilance practices (GVP), Module V – risk management systems (Rev 1) (2014). www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/06/WC500129134.pdf

55 Regulation (EU) No 536/2014 of the European Parliament and of the Council on clinical trials on medicinal products for human use, and repealing Directive 2001/20/EC. Official Journal of the European Union, L158, 1–76 (2014).

56 EMA. Guideline on the requirements for quality documentation concerning biological investigational medicinal products in clinical trials (2012). www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/05/WC500127370.pdf

57 European Commission, Joint Research Centre. Human tissue-engineered products: today's markets and future prospects (2003). http://ec.europa.eu/health/files/advtherapies/docs/ipts21000en_en.pdf

58 Rosenblatt B. Meeting regulatory challenges for cell-based therapies. Contrasting novel and familiar biologics. *BioProcess Int.* 10(3), S8–S11 (2013).

59 European Commission, Health Consumers Directorate General. Summary of the responses to the public consultation on the Regulation on Advanced Therapies (2013). http://ec.europa.eu/health/files/advtherapies/2013_05_pc_atmp/2013_04_03_pc_summary.pdf

60 European Commission. Report from the Commission to the European Parliament and the Council in accordance with Article 25 of Regulation (EC) No 1394/2007 of the European Parliament and of the Council on advanced therapy medicinal products and amending Directive 2001/83/EC and Regulation (EC) No 726/2004 (2014). http://ec.europa.eu/health/files/advtherapies/2014_atmp/atmp_en.pdf

61 EMA. Procedural advice on the certification of quality and nonclinical data for small and medium sized enterprises developing advanced therapy medicinal products (2010). www.ema.europa.eu/docs/en_GB/document_library/Regulatory_and_procedural_guideline/2010/01/WC500070030.pdf

62 EMAy. Guideline on the minimum quality and non-clinical data for certification of advanced therapy medicinal products (2010). www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2010/01/WC500070031.pdf

63 EMA. Reflection paper on classification of Advanced Therapy Medicinal Products (2012). www.ema.europa.eu/docs/en_GB/document_library/Scientific_guideline/2012/12/WC500136422.pdf

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