Enabling Consistency in Pluripotent Stem Cell-Derived Products for R&D and Clinical Applications Through Material Standards.

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Running title

Material standards for the clinical application of hPSCs.

Key words

Pluripotent stem cell, human, embryonic stem cell, hESC, human induced pluripotent stem cell, hiPSC, standardization, translation, reference standards, reference materials, regulation, pre-clinical, clinical adoption, scale-up, manufacturing,

Summary

- There is a need for physical standards (reference materials) to ensure both reproducibility and consistency in the production of somatic cell types from human pluripotent stem cell sources.
- We outline the need for reference materials (RM) in relation to the unique properties and concerns surrounding hPSC-derived products and suggest in-house approaches to RM generation relevant to basic research, drug screening and therapeutic applications.

Abstract

Human pluripotent stem cells (hPSCs) have an unparalleled potential as a source of somatic cells for drug screening, disease modelling and therapeutic application. Undefined variation and product variability following differentiation to the lineage or cell type of interest impede efficient translation and may obscure the evaluation of clinical safety and efficacy. Moreover, in the absence of a consistent population, data generated from *in vitro* studies may be unreliable and irreproducible. Efforts to devise approaches and tools that facilitate improved consistency of hPSC-derived products, both as development tools and therapeutics products, will aid translation. Standards exist in both written and physical forms; but because many unknown factors persist in the field, premature written standards may inhibit rather than promote innovation and translation. Here we are focused on the derivation of physical standards-RM. We outline the need for RM and assess approaches to in-house RMgeneration for hPSC-derived products, a critical tool for the analysis and control of product variation that can be applied by researchers and developers. We then explore potential routes for the generation of RM, which include both cellular and non-cellular materials as well as novel methodologies that may provide valuable tools to measure and account for variation. Multiparametric techniques to identify 'signatures' for therapeutically relevant cell types, such as neurons and cardiomyocytes that can be derived from hPSCs, would be of significant utility, although physical RM will be required for clinical purposes.

Introduction

Human pluripotent stem cell (hPSC) technologies have a unique potential to address the increasing burden of unmet clinical need for many intractable diseases. However, a grasp of the fundamental biology, which is necessary to ensure invariant and reproducible safe and effective cellular products from batch-to-batch and patient-to-patient, has eluded our reach. The field generally lacks standards that will enable scalable, automated manufacturing to build regulatory confidence and meet clinical needs.

Written standards exist in a vast range of industries, enabling dialogue between stakeholders via a common set of 'rules' or 'guidelines'. Typically developed through consensus that emerges through an incremental process of discussion and revision amongst experts, standards can establish specifications, set minimum requirements and provide a route by which valid comparisons can be made. Standards can also serve to protect the integrity of manufacturers, stimulate consumer confidence and facilitate the uptake of new technologies into the market [1-3]. Physical standards are materials used for specific comparative purposes to validate and provide a benchmark for assessments.

Outlining the need for routes to generating both developer-specific and, where appropriate, consensus physical (material) standards for stem cell translation has value for two primary reasons. Firstly, physical standards will support and enable improved reproducibility and product consistency in research. Currently the field, and biomedical research in general, suffers from issues of irreproducibility [4, 5], which impedes progress and effective collaboration and could damage the public perception of stem cell research. Secondly, there are commercial and translational benefits of incorporating standardization principles early in R&D, by building a base for quality assessment to prevent undue delays throughout clinical trials because of deficiencies in necessary tools and data to meet regulatory requirements. Therefore, approaches for the derivation of standards, and especially, physical (material) standards may benefit early-stage researchers conducting pre-clinical phase investigation, as well as those engaged further along the translation pathway. However, it is vital to strike the proper balance here to ensure that the benefits of standardization are not achieved at the expense of hindering innovation. Here we are not trying to identify the preferred PSC line to generate clinical products, nor a specification for the optimal cell type. Instead we are focused on the need and possible mechanisms by which physical standards-RM, can be produced to analyse and therefore facilitate the

consistent and reproducible generation of products from hPSCs. As consensus (international) RM may have limited application and will be more challenging to achieve, we focus on in-house developer-specific RMthat can be generated by research laboratories and companies alike that are engaged in pre-clinical research, and that can be applied in clinical trials and beyond to meet with regulatory expectations. These principles and approaches also have application in drug screening and toxicology studies that utilize PSC-derived somatic cells with underlying expectations for reproducibility and consistency.

The clinical potential of PSC-derived products

The utility of hPSCs in disease modelling is beginning to be demonstrated and some of these models are now finding application as drug screening tools (reviewed in [6-8]). However, it is the application of PSCs as a cell source for therapeutic intervention that still garners the greatest enthusiasm within a healthcare context. To date, ESC-derived products have entered a limited number of clinical trials, pioneered by Geron Corporation (Menlo Park, CA, USA, assets now owned by Asterias Biotherapeutics) and more recently Advanced Cell Technology (ACT) Incorporated (Santa Monica, CA, USA), with a Pfizer/UCL trial scheduled to begin in 2014 [9, 10]. Although Geron did not complete its trial of ESC-derived oligodendrocyte precursors for acute spinal cord injury due to internal competing fund allocations, it pioneered a regulatory path and demonstrated the data requirement for testing PSC-derived products in humans [10]. This exercise proved educational not only for the private sector but also for the regulators themselves, who until that time had no experience evaluating the safety of hPSC-derived products in actual patients.

Standards for hPSCs in their undifferentiated state are important for cell banking, both to demonstrate comparability and to show that cell lines are stable over time [11, 12]. A number of engaging perspectives on standards for hPSCs can be found [11, 13-15]. However, as hPSCs in their undifferentiated state will not be the final product delivered to research subjects or patients, standards should extend to validating early-stage translational research, as well as the manufacture and scale-up process by which large numbers of somatic cells are derived from hPSCs. As with any cell therapy, due to the unique nature of each product, written standards have limited utility for hPSC-derived

products. Instead material standards, generated on a case-by-case basis will be required to validate process, method and product consistency. While such material standards will be the responsibility of the developer and will vary in accordance with the particular developer's technology, product and target indication, a clearer understanding of the need, requirements and potential approaches by which these materials standards could be produced will benefit academic researchers and industry alike.

The challenges of hPSC translation

The application of hPSC-derived products in a clinical setting is challenged by numerous inherent and unique barriers to translation, including scalability and manufacturability, as well as a variety of regulatory challenges (see Box.1 for an overview of challenges to the translation of hPSC-derived products).

hPSCs are highly reactive to their external environment. They may undergo significant changes in response to different culture conditions, to extended time in culture, and following cryopreservation [16-18]. Stability is also a key issue, both genetic stability and physiological stability of hPSC-derived products. In comparison to cells harvested from adult donors, hPSC-derived populations may have an increased propensity for continued proliferation, differentiation, and/or maturation. This was exemplified by the increased frequency of cyst formation from the ESC-derived oligodendrocyte precursor cell population in Geron's pre-clinical animal studies, which resulted in the FDA placing the trial on hold. This issue was ultimately resolved by an additional level of cell selection [19]. Furthermore, hPSCs and their differentiated progeny have been demonstrated to be highly heterogeneous at the population level, with differentiation protocols asynchronously generating a variety of cell types [20]. Researchers are focusing on the generation of homogeneous differentiated populations from hPSCs that would be amenable to clinical demands. However, it has been argued that mixed populations might be preferable to a single cell type in some cases, if survival or efficacy of administered cells is enhanced by the presence of interacting cell types. It will be more challenging to characterize and control the consistency and quality of mixed populations, acknowledging that the level of heterogeneity in cell products will always exceed that of traditional small molecules. These inherent characteristics and current methodologies all create significant challenges for the development of RM for hPSC-derived products.

Box 1. Challenges to the translation of hPSC-derived products.

- Phenotypic and genetic instability
- Capacity to generate adult phenotypes
- Cost of cell culture processes
- IP protection clarity
- Differentiation efficiencies and time scales
- Tumorigenicity risks
- Immunological considerations
- Paucity regulatory and sponsor familiarity
- Limited positive cell therapy outcomes to date
- Each product must be considered by regulatory authorities on a case-by-case basis

Standards and reference materials

Standards fall into two main categories, written and physical (material), which must be clearly distinguished (Figure 1). Written standards include codes of practice, standard operating procedures (SOPs), agreed terminology, guidelines and pharmacopoeia methods. Pharmacopoeia, particularly relevant to this discussion, are a series of monographs and general chapters documentation for active substances that outline the minimum requirements, describing identity, permissible levels of impurities in addition to appropriate methods to define purity and potency with accompanying expected ranges (USP, 2011). Pharmacopoeia may require the use of consensus physical (material) standards which currently only exist for small molecules and a limited number of biologics. The single example of a cell therapy monograph is currently being developed by the U.S Pharmacopoeia Convention (USP) for sipleucel-T (Provenge) (Dendreon, Seattle, WA, USA), a T cell therapy for advanced prostate cancer that has been authorized by the FDA, although approval of the monograph may encounter challenges and application may be limited (USP, 2013). There are a variety of local or regional organisations concerned with the publication of pharmacopoeia and/or production of physical reference standards (see Table 1). In addition, organizations such as the World Health Organisation (WHO), and the Joint Committee for Traceability in Laboratory Medicine (JCTLM)- the latter in the context of laboratory medicine and *in vitro* diagnostics, that lead and co-ordinate in the establishment of higher order international RM [21].

RM are highly characterized physical materials used with analytical methods for a specific comparison purpose, and are a regulatory expectation globally [22-25]. Physical (material) standards can be sub-segmented into certified (consensus) and in-house (developer-specific) materials (Figure 1). While certified RM are available for many biological substances, they have not yet been produced for cell therapies. The NIBSC has produced a RM of untouched (enrichment using negative selection techniques to prevent activation) $CD4^+$ cell certified RM, intended for use in clinical diagnostics. This standard comprises fixed peripheral blood mononucleated cells (PBMCs) pre-labelled with a CD4 antibody conjugated to FITC (NIBSC, SS-222), this approach may have some relevance for cell therapies, as discussed below [26]. Certified (consensus) RM may have application when applied to specific characterization methodologies but are highly unlikely to have broad application, especially when considering the need for case-by-case development of cell therapies such as hPSC-derived products. Therefore, we focus here exclusively on in-house (developer-specific) RM. There are two main categories of in-house, developer-specific RM, 'product' and 'method' (Figure 1).

Potential approaches to developing reference materials for hPSC-derived products

The aim of this discussion is to support the development of approaches and assays that facilitate consistency and comparability in the production of differentiated cell types, such as cardiomyocytes, neurons and T cells from hPSCs, or 'hPSC-derived products'. Batch-to-batch variation, if not assessed and controlled, will affect the quality of clinical hPSC-derived products. Moreover, approaches that enable the consistent production of differentiated cells will also be of significant benefit to drug and toxicity screening. There are numerous potential causes of variation in the production of somatic cells from hPSCs. 'Products', such as cardiomyocytes, are generated from hPSCs by a highly dynamic differentiation process that may utilize a variety of methodologies [27], typically occurring via a number of stages. Even when considering the production of one specific product, from one hPSC line, using a single methodology, it is inevitable that the final product will demonstrate variability between individual batches. This variability will arise in the form of variable levels of heterogeneity and 'purity' of cell types, as well as inconsistencies in the differentiation and/or maturation stage of the product. Additionally, both genomic and phenotypic stability may demonstrate variation. Here we detail approaches to RM generation that may mitigate current inconsistency and irreproducibility concerns.

An overview of the possible approaches to establish RM for hPSC-derived products identifies two main categories: those that use living, cellular RM, either as product or method RM, and those involving non-living, non-cellular materials such as beads, DNA or RNA samples as method RM (Table 2).

Product RM approaches

'Product' RM should be representative of the product and are used to validate comparability assessments throughout the product's lifecycle including process change and optimisation as well as to detect process drift (Figure 2i).

The typical approach, which is common practice in the pharmaceutical industry, is to generate primary and secondary RM that are samples of the product batch generated for pre-clinical and then pivotal studies. Secondary RM are the 'working' samples used as a comparator in the relevant tests. Once samples are depleted, the secondary RM is generated from another batch and, through rigorous characterization, determined to be sufficiently comparable to the primary RM via a direct comparison. Ideally there would be a sufficient quantity of primary RM to last for the lifecycle of the product (over 10 years) however, and as discussed below, there may be limitations that preclude this period of coverage.

A second potential methodology to product RM generation involves a 'pooling' approach, in which cells from a number of different batches are pooled. If RM are generated using a pooling approach then differences due to heterogeneity within the product are averaged out, providing a broad-ranging background against which measurements can be assessed. This may be beneficial in a number of circumstances.

In a limited number of cases biologically equivalent cellular populations may be suitable product RM. For example if $CD4^+$ T lymphocytes are derived from hPSC lines then $CD4^+$ T lymphocytes harvested from healthy donors may be suitable as a product RM, or potentially as a method RM for a limited number of assays. This approach may only be appropriate for a limited number of cell types that can be obtained from healthy donors

without causing a detrimental effect. While this approach may have potential utility, it must be acknowledged that heterogeneity in the 'biologically equivalent' RM would need to be analysed and accounted for.

The main considerations of whether these approaches may be suitable, in addition to the critical criteria of relevance in accordance with the product, are those of stability, feasibility and cost (Figure 3). Stability will need to be monitored over time and stability profiles determined so that new RM can be prepared before expiry. As batch sizes for hPSC-derived products are expected to be smaller than is typically seen with small molecules, and even biologics, the amount of product that can be stored as RM will also be limited. The practicalities of RM quantity requirements will vary and will need to be mapped out and planned for by developers. An additional challenge for approaches that rely on cellular RM is that cell banks of differentiated products will need to be cryopreserved which will impact on viability but also potentially functional parameters. This becomes an issue if the product is to be used 'fresh' but is less of a concern if the product will be cryopreserved as part of the production process.

Method RM approaches

'Method' RM are used to qualify, validate and define acceptance criteria for specific assays, to calibrate methods and equipment, and to identify method drift over time (Figure 2ii).

Cell lines may be valuable method RM in some settings. An example of such an approach is the use of embryonal carcinoma line 2102Ep, which has a good stability profile in culture. This cell line demonstrated utility in flow cytometry assays for the characterization of a range of hESCs by the International Stem Cell Initiative (ISCI) [28], in spite of a number of reported biological differences between hESCs and the EC line [15, 29]. This approach may also be relevant for a number of differentiated cell types, if an appropriate cell line is available or can be produced. Immortalization of primary cells to generate stable cell lines impacts upon signalling pathways and some phenotypic characteristics; therefore the suitability of cell lines as method RM will depend on the application. It should also be

acknowledged that the standardization, characterization and qualification of cell lines as method RM would take a considerable amount of work-up and validation.

Non-cellular/(nonviable) method RM could include fixed cells, beads, DNA/RNA samples and reference cytokines. Fixed cells, most suitably from a product sample, can be used as a RM for assays that compare cell surface marker expression, such as the clinical application of the NIBSC $CD4^+$ T cell sample for HIV testing, as well as applications to assess heterogeneity/composition criteria [26]. However, the process of fixation changes the properties of the cell, which may negate use as a method RM when compared to the non-fixed product in some flow cytometry-based assays. Bead-based approaches have been used routinely for flow cytometry purposes, to both calibrate and establish baseline readings for cytometers and to apply compensation settings. However, bead-based approaches also encounter stability issues and would most likely need to be used in conjunction with a cellular method RM.

RM for molecular biology assays should be easily achievable as DNA, and RNA samples in the appropriate conditions, demonstrate good stability profiles, are easily stored and can be generated in large quantities relative to the amount of material required for any given assay. In most cases method RM for molecular biology assays would be produced from a sample of the product batch. One issue to be considered is selection of suitable positive controls in assays such as RT-PCR. (Semi)-quantitative measurements of gene expression are typically made in relation to the expression of 'housekeeping genes'. However, a number of studies have identified changes in the expression levels of these presumably stable genes in concordance with the differentiation status of hPSCs and other stem cell types and, therefore, are an unsuitable baseline for these assays [30, 31]. Similarly, while microarray-based assays detect the relative levels of all transcripts in the genome, they do not identify alternatively spliced transcripts that may be critical for cellular function. RNA sequencing (RNAseq) is growing in popularity and has the potential for identifying splice variants and absolute amounts of transcripts. A challenge for an RNAseq approach, however, is the large variation in results from different sequencing labs, a problem that must be solved by a normalization method before sequencing-based assays can be reliable- another example of the need for physical reference materials to enable comparability testing. Written standards that identify a consensus minimum requirement for qRT-PCR and microarray assays have been described [32, 33].

Reference cytokines, such as those used to calibrate ELISA assays, are available in some instances in the form of certified (international) RM. However, while a reference cytokine can serve to define standard curves and therefore a link to a known concentration of that cytokine for an assay such as an ELISA, the reference cytokine is not a RM for the biological assay that results in cytokine release.

While not a material standard, approaches that create product 'signatures' from complex data sets and applied algorithms might have application in demonstrating comparability. Examples of such an approach that has been developed to assess (undifferentiated) iPSC populations by gene expression analysis include the hPSC Scorecard and PluriTest, either by genome-wide microarray (PluriTest [34]) or PCR analysis of a set of selected genes (Scorecard [35]), establishing a typical gene expression profile. Users analyze their samples via the same method (array or PCR) and compare their samples to the established standard generated, in this case, from previous product batches. Such a comparison informs users as to how similar their sample profile is to the expected profile. This approach might be suitable for the assessment of hPSC-derived products, where a developer would perform gene array (or other) analysis using multiple samples from different product batches, and then, using a similar approach to the PluriTest, an expression signature for the product would be generated with the identification of 'acceptable' levels of variation. This data set could then be used as a 'virtual' method RM when assessing comparability of future product batches using the same methodology, in the context of a specific product. This approach would facilitate a move away from the inaccurate use of a limited set of cell surface markers that are typically used to characterize and identify cell populations. Clearly this approach would require validation and the application of a suitable RM.

Method RM described here will typically display greater stability profiles than product RM due to the inherent plasticity of living cells and their responsiveness to the environment (Figure 3). Feasibility factors, including the ability to generate sufficient batch sizes, are also more amenable for method RM approaches which should, in general, also carry lower costs. A rational mix of method RM for different assays within the overall characterization process will be required, as will robust product RM.

A final consideration is how data will be made available and managed once method/product RM are established. Given that discussion has focussed on development

of in-house RM, there may be no requirement for external publication and management of data. However, in the interests of eventually establishing consensus RM, rapid dissemination of open access and peer-reviewed publications for community wide access would be beneficial as a 'formal' record and to catalyze multi-stakeholder dialogue. Eventually, it may be possible to develop online data repositories managed by experts in both research and regulation that will act as a centralised resource in which in-house RM standards can be pooled and potentially inform development of consensus RM guidelines.

The approaches outlined here are by no means exhaustive. Innovative thinking is required to envisage novel routes to RM that would be appropriate to the unique characteristics of hPSC-derived products and cell-based therapies. It is clear that different approaches will have varying levels of application, depending upon the specific product and clinical application.

Conclusion

Uncontrolled variability and irreproducibility are key considerations for the translation of PSC-derived therapeutics and for the cell therapy field more broadly. Here we have outlined the need for RM, discussed the potential challenges faced by hPSC-derived products, and identified possible approaches to alleviate consistency and reproducibility concerns in the production of hPSC-derived products. A range of cellular and non-cellular approaches to product and method RM generation can be envisioned and have been described here, including relevant considerations. The ambition behind this work is for the research community and tool providers to engage around these requirements and existing industry and regulatory models and terminology, so that potential approaches will be assessed and incorporated into practice where appropriate, and that novel thinking will lead to approaches that more satisfactorily fulfil the needs outlined.

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Tables

Table 1. Organizations concerned with the generation and/or oversight of reference materials. List of major organisations that play a role in the production, guidance and/or directives concerning reference materials for small molecule drugs and biologics.

Organization	Region	Description	Hyperlink
National Institute for Biological Standards and Control (NIBSC)	UK	The leading World Health Organisation (WHO) International Laboratory for Standards, is responsible for >90% of global WHO Standards.	(http://www.nibsc.org)
U.S Pharmacopeia Convention (USP)	US	The official organization that sets standards and generates reference materials implemented by the FDA as law in the United States, used globally in	(http://www.usp.org/about-usp)
World Health Organization (WHO)	International	Publishes the International Pharmacopoeia (Ph. Int.) which aims to harmonize global pharmaceutical standards and administers the establishment of	(http://www.who.int/medicines/publications/pharmacopoeia/overview/en/).
European Directorate for the Quality of Medicines & Healthcare (EDQM)	Europe	Responsible for the European Pharmacopoeia (Ph. Eur.) commission, the evaluation of manufacturer's quality dossiers for certification, and market	(http://www.edqm.eu/en/edqm-homepage-628.html)
Pharmaceutical and Medical Device Regulatory Science Society of Japan (PMRJ)	Japan	Produces and distributes Japanese Pharmacopoeia Reference Standards as prescribed in the Japanese Pharmacopoeia (published by Pharmaceuticals and	http://www.pmrj.jp/hyojun/html/frm031.php?lang=e

Table 2. Potential approaches to generating reference materials for PSC-derived products.

In-house reference material (RM) for hPSC-derived products will enable the analysis and qualification of consistency and promote reproducibility. Product RM are used to ensure that a product batch is representative of an intended product and to identify process drift. Method RM validate data derived from specific assays, define assay acceptance criteria and are a tool to detect method drift.

RM category	RM description	Type	Explanation
Product	Primary/ secondary	Cellular	Generated as per product. Primary and secondary RM. Secondary RM is used as the working material which, when depleted is replace with product from a new batch and qualified against the primary RM.
Product	Pooled	Cellular	Generated as per product. RM are produced from a pooled bank of cells, a potential benefit is that variability is averaged across the population.
Product/ Method	Biological equivalent	Cellular	For a limited number of cell types that can be harvested from donors non-invasively e.g. from blood, biological equivalent cell populations can be used as a RM e.g. expression of CD4 levels on peripheral blood T cells and on PSC-derived T cells.
Method	Cell lines	Cellular	Cells lines may have application in a number of characterization assays.
Method	Non-cellular	Non-cellular	Samples such as fixed cells for cell surface marker staining, DNA samples for sequencing or genotyping and RNA for expression profiling.
Not a physical RM	'Virtual'	Non-cellular	Use of transcriptome, proteome, phosphoproteome, or epigenetic mapping to generate a complex data set that when computational algorithms are applied identifies a product 'signature' e.g. concept from PluriTest, PSC scorecard (Mueller et al., 2011, Bock et al., 2011).

Figures

Figure 1. Overview of standards.

Standards are either written, such as guidance, standard operating procedures (SOPs) and pharmacopoeia, or physical (material). Physical (material) standards in turn are typically either generated and used as part of an certified (consensus/international) effort or developed in-house (local). In-house RM are the focus of the discussion here, both product RM and method RM.

Figure 2. Reference materials are required to demonstrate product comparability over time (product RM) and to validate assay results (method RM).

Product and method RM have multiple applications, two of which are depicted a) Product RM are required to demonstrate that product drift does not occur and that a particular batch is representative of the pre-clinical or pivotal trial data demonstrating identity, purity, safety and potentially mechanism of action/potency. RM generated with Batch B will be retained to use as a comparator with Batch D. b) Method RM are needed to validate assay results alongside the product sample.

Figure 3. Assessing the suitability of alternative reference materials (RM) approaches. Alternative routes to generating product and method RM include cellular (PSC-derived; primary/secondary and pooling, non-PSC-derived; cell lines and biological equivalent cells) as well as non-cellular approaches. A third approach, while not a physical RM is to use large data sets e.g. gene array, generated for a specific product that can then be stored as a 'virtual' signature to facilitate comparability. When alternative RM are assessed for characteristics of suitable reference materials it is clear that biological relevance correlates inversely with many desirable criteria such as stability, cost, feasibility and potential for global comparisons. (blue= desirable/positive features e.g. high biological relevance, low cost; grey= neutral feature e.g. limited biological relevance, mid-range cost; red= undesirable/negative features e.g. low biological relevance, high cost).

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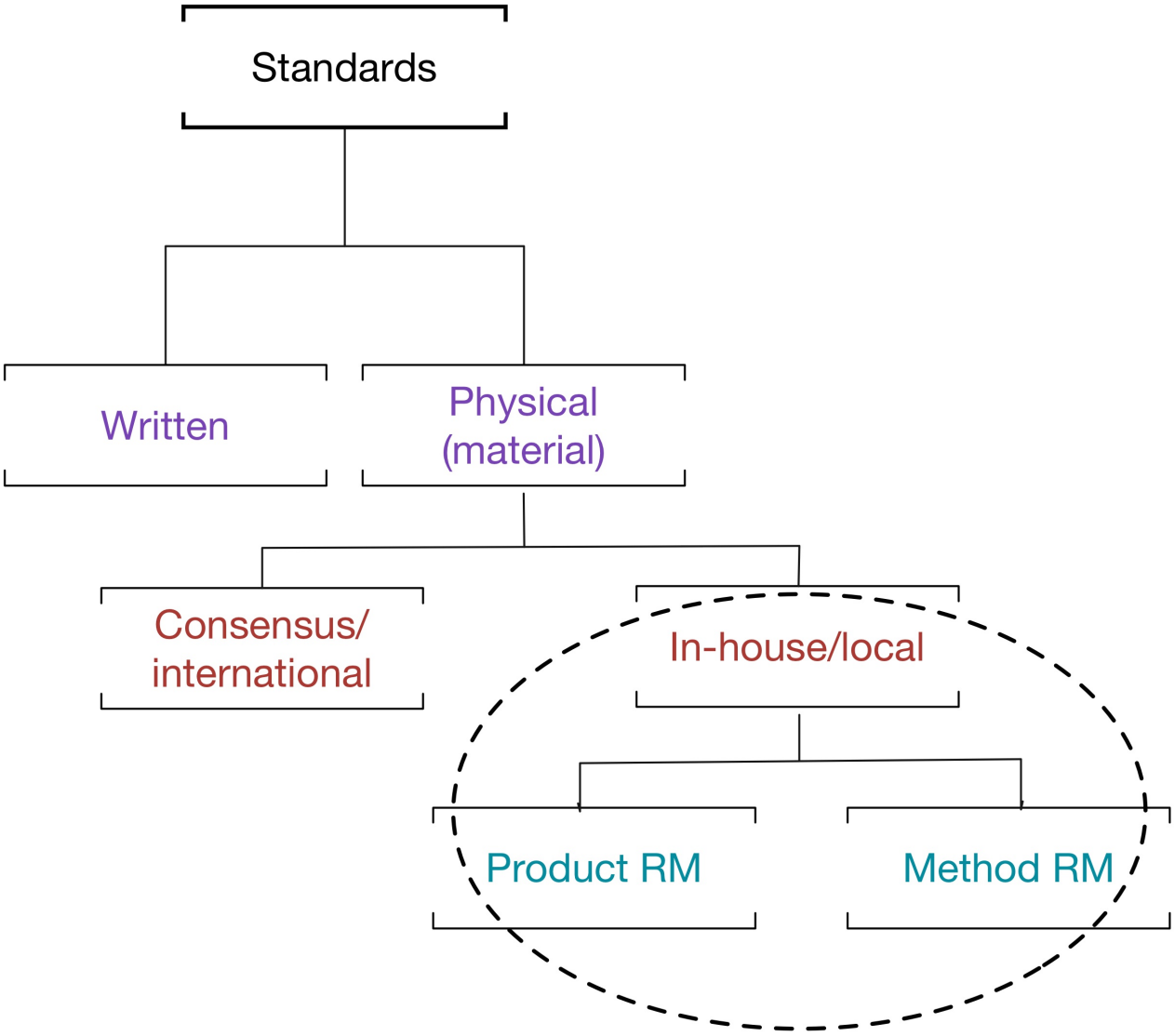

Figure 1 Overview of standards

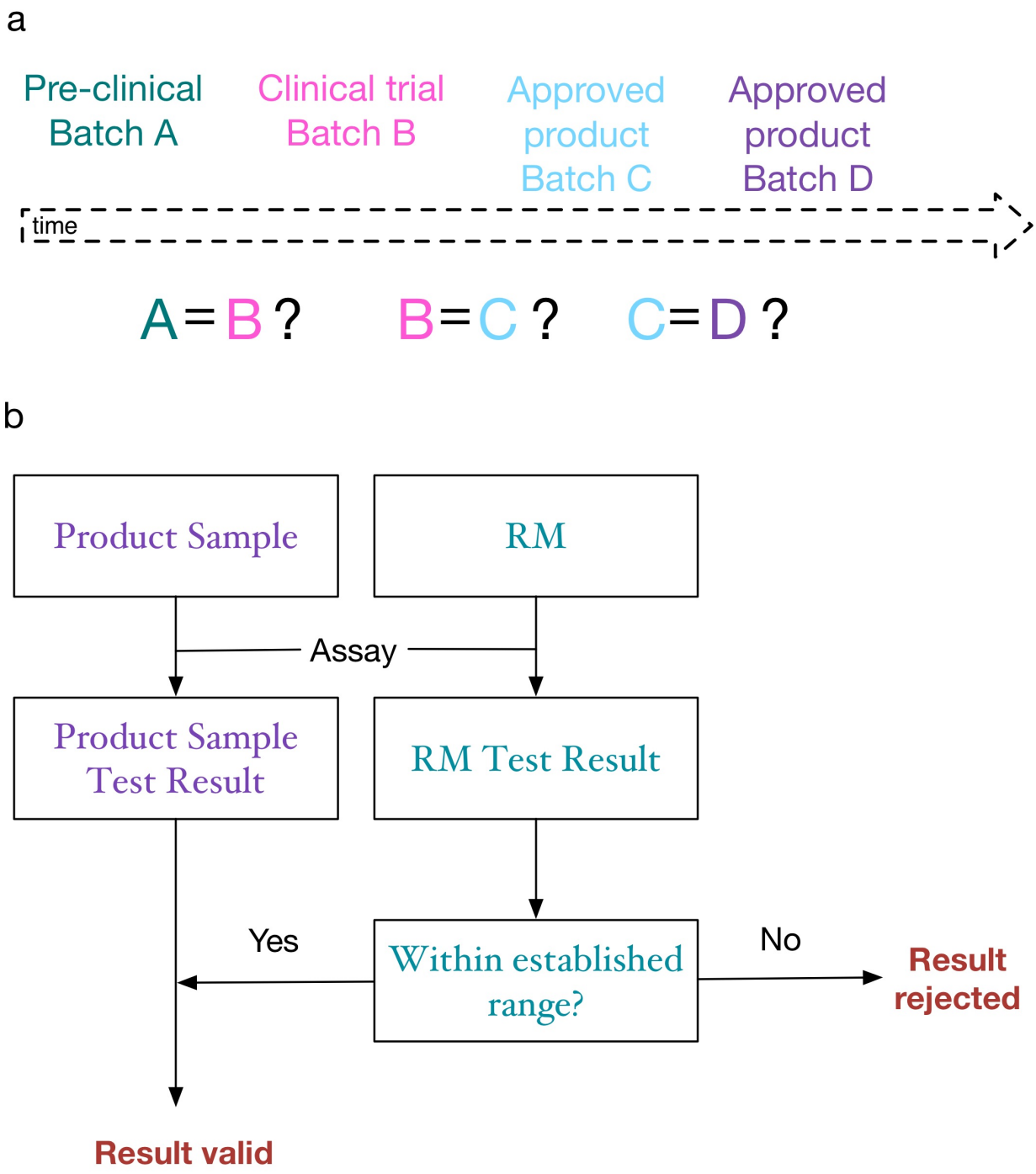

Figure. 2 Reference materials are required to demonstrate product comparability over time (product RM) and to validate assay results (method RM).

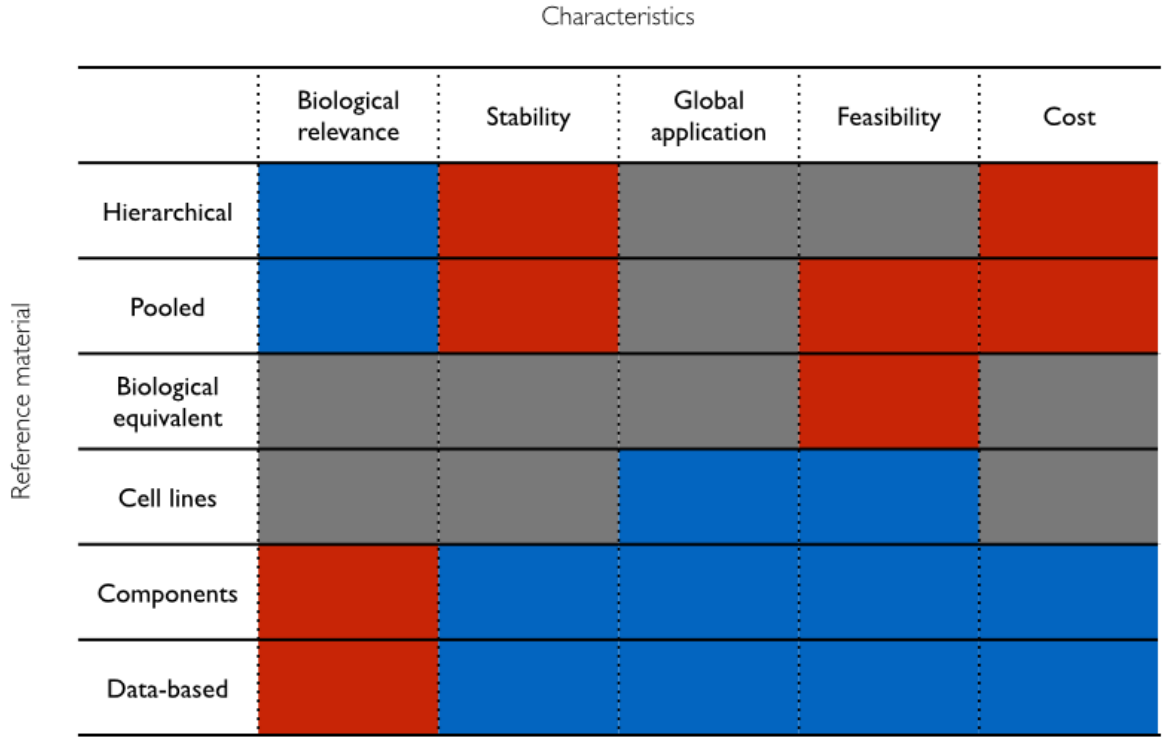

Figure 3. Assessing the suitability of alternative reference materials (RM) approaches.