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Standard Guide for Evaluating Extracellular Matrix Decellularization Processes[1]

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1. Scope

1.1 This document provides guidance on the characterization and evaluation of the decellularization processes used to produce decellularized extracellular matrix (dECM) materials which will be used as medical products in direct or indirect contact with the body. The decellularization process may be performed on tissue from human or other mammalian sources or produced *in vitro* from human or other mammalian cells. The dECM may or may not be recellularized prior to use. Decellularized ECM material derived from non-mammalian tissue or cells and decellularized ECM material used for non-medical purposes may follow the framework provided but may require additional considerations outside the scope of this document.

1.2 Biological tissues are composed of a structural extracellular matrix (ECM) and embedded cells. The intent of a decellularization process is to disrupt and/or remove cells and cellular components from an ECM material while maintaining key structural and/or compositional properties of the material. Decellularization comprises process steps intended or expected to result or aid in the disruption of source tissue cells and/or removal of cellular content from the material undergoing decellularization. Actions that are intended to rinse or otherwise remove decellularization reagents or by-products should also be considered in that context as part of the decellularization process. Purifications or other isolations of specific ECM components are not considered decellularization and are outside the scope of this document.

1.3 This document describes relevant parameters of decellularization processes used to prepare extracellular matrix materials as medical products.

1.4 This document provides guidance on the measurement of specific and general properties of dECM. This includes both the analysis of cellular material as well as the assessment of the effects of decellularization on dECM properties such as composition, structure, and material properties.

1.5 This document does not provide guidance on the assessment of the host response subsequent to the implantation or other *in vivo* placement of dECM medical products. Such assessments should instead be conducted as part of biocompatibility studies or other safety and efficacy studies. At a minimum it is recommended that the finished product composed of dECM material shall be assessed in a relevant model that represents the biological responses that the product is expected to experience to ensure that the final material is functioning in accordance with design intentions. An *in vivo* model will generally be used, but cellular or *ex vivo* models may also be satisfactory when appropriate.

1.6 This document provides guidance on determining pertinent quality attributes as well as developing and assessing acceptance criteria related to ensuring the consistent evaluation and use of decellularization in manufacturing medical products. Acceptance criteria should address the adequacy of cellular disruption and removal of cellular remnants. Acceptance criteria should define acceptable levels for retention of extracellular matrix components. Acceptance criteria may place limits on damage to retained components. Acceptance criteria should place limits on the persistence of decellularization reagents. This document also provides recommendations on developing process parameters and associated process controls.

1.6.1 This guide recommends attributes as representative measures of decellularization in the direct function of removing cells and cell components. These attributes can also be used to show process consistency, capability, or equivalency. Recommendation of these attributes does not confer additional significance related to product safety and performance.

1.6.2 No consensus has been established regarding decellularization thresholds or classifications. This guide therefore cannot suggest acceptance criteria and instead recommends commonly measured attributes to develop acceptance criteria specific to the design of each unique material and its intended use.

1.7 Decellularized products will require evidence of safety and/or efficacy beyond that related to evaluating the decellularization process. Commonly referenced standards include the ISO 10993 series (see ISO 10993-1) for biocompatibility of medical devices and the ISO 22442 series for medical devices

[1] This guide is under the jurisdiction of ASTM Committee F04 on Medical and Surgical Materials and Devices and is the direct responsibility of Subcommittee F04.42 on Biomaterials and Biomolecules for TEMPs.

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utilizing animal tissues and their derivatives. These assessments are not in the scope of this document, though they may help to identify relevant functional characteristics and test methods as discussed in 5.2.9.

1.8 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices and determine the applicability of regulatory limitations prior to use.*

1.9 *This international standard was developed in accordance with internationally recognized principles on standardization established in the Decision on Principles for the Development of International Standards, Guides and Recommendations issued by the World Trade Organization Technical Barriers to Trade (TBT) Committee.*

2. Referenced Documents

2.1 *ASTM Standards:*[2]

D6797 Test Method for Bursting Strength of Fabrics Constant-Rate-of-Extension (CRE) Ball Burst Test

F2150 Guide for Characterization and Testing of Biomaterial Scaffolds Used in Tissue-Engineered Medical Products

F2212 Guide for Characterization of Type I Collagen as Starting Material for Surgical Implants and Substrates for Tissue Engineered Medical Products (TEMPs)

F2903 Guide for Tissue Engineered Medical Products (TEMPs) for Reinforcement of Tendon and Ligament Surgical Repair

F3142 Guide for Evaluation of ***in vitro*** Release of Biomolecules from Biomaterials Scaffolds for TEMPs

2.2 *ISO Standards:*[3]

ISO 5840-1 Cardiovascular implants -- Cardiac valve prostheses -- Part 1: General requirements

ISO 7198 Cardiovascular implants and extracorporeal systems — Vascular prostheses — Tubular vascular grafts and vascular patches

ISO 10993-1 Biological evaluation of medical devices -- Part 1: Evaluation and testing within a risk management process

ISO/TR 10993-33 Biological evaluation of medical devices — Part 33: Guidance on tests to evaluate genotoxicity — Supplement to ISO 10993-3

ISO 14971 Medical devices – Application of risk management to medical devices

ISO 22442-1 Medical devices utilizing animal tissues and their derivatives -- Part 1: Application of risk management

ISO 22442-2 Medical devices utilizing animal tissues and their derivatives -- Part 2: Controls on sourcing, collection and handling

2.3 *Harmonized Guidance Documents:*[4]

GHTF Study Group 3 – Quality Management Systems, Process Validation Guidance– January 2004 (GHTF/SG3/N99-10:2004 (Edition 2))

ICH Q2(R1) – Validation of Analytical Procedures: Text and Methodology, Step 4

2.4 *USP Documents:*[5]

USP <1285> Preparation of Biological Specimens for Histologic and Immunohistochemical Analysis

USP <1285.1> Hematoxylin and Eosin Staining of Sectioned Tissue for Microscopic Examination

USP Reference Standard Bovine Acellular Dermal Matrix Reference Photomicrographs, Catalog # 1535824

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 *decellularization, n*—a process used to disrupt and/or remove cells and cellular components from a biological material, while maintaining key structural and/or compositional properties of the extracellular matrix material.

3.1.2 *decellularized, adj*—referring to a material, previously containing cells, which has been subjected to decellularization.

3.1.3 *detergent, n*—an amphiphilic compound, containing both a hydrophobic group and hydrophilic group, which enables the solubilization of hydrophobic, hydrophilic, and amphiphilic materials such as the lipid bilayers of cellular membranes. Detergents are surfactants and therefore reduce surface tension in aqueous solutions.

3.1.4 *enzyme linked immunosorbent assay (ELISA), n*—a group of procedures for quantification of specific molecular antigens in a (biological) sample. ELISA methods use antibodies that bind specifically to the molecule of interest. ELISA method variations include Direct, Indirect, Sandwich, and Competitive.

3.1.5 *immunohistochemistry (IHC), n* and *immunofluorescence (IF), n*—the process of detection and visualization of specific antigens/components within a tissue section (IHC) or other sample using labeled antibodies. Antibodies are labeled with chromogenic or fluorescent (IF) markers for visualization.

3.1.6 *intended use, n*—also, intended purpose. Use for which a product, process or service is intended according to the specifications, instructions and information provided by the manufacturer (ISO 14971).

3.1.7 *recellularization, n*—the introduction of viable cells onto or into a decellularized material either *ex vivo* (in culture) or *in vivo*.

4. Significance and Use

4.1 Decellularization is used in the preparation of medical products that make use of the native structure and/or composition of the extracellular matrix derived from a specific tissue

[2] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

[3] Available from American National Standards Institute (ANSI), 25 W. 43rd St., 4th Floor, New York, NY 10036, http://www.ansi.org.

[4] To obtain the referenced GHTF Study Group 3 guidance, visit the website of the International Medical Device Regulators Forum, www.imdrf.org. To obtain the referenced ICH guidance, visit the website of the International Council for Harmonisation (ICH), www.ich.org.

[5] Available from U.S. Pharmacopeial Convention (USP), 12601 Twinbrook Pkwy., Rockville, MD 20852-1790, http://www.usp.org.

source. Upon implantation or placement, the decellullarized product is commonly intended to undergo and/or induce constructive remodeling and incorporation into the native host tissue instead of being recognized as foreign material. Typically, immune system recognition of foreign material leads to encapsulation of the material and an aggressive inflammatory response, causing the ultimate rejection or other failure of the product.

4.2 As described above, decellularization is a recognized technique which allows the use of ECM-derived products in medical treatments with a reduced risk of an adverse host immune response and immune rejection by disrupting and removing cells and/or cell contents while aiming to preserve significant features of the ECM structure and/or composition. More complete decellularization is often associated with a beneficial response (**1**, **2**)[6] but can also be associated with the loss of important ECM components and the loss of structural or biomechanical integrity from the tissue during the decellularization process (**3**, **4**, **5**, **6**). Therefore, given the typical objective of producing a product that does not elicit an adverse immune response while maintaining the integrity of the tissue for its intended surgical application, this guide presents a standard approach to the evaluation of decellularization processes, including assessment of adequate decellularization to achieve this end.

4.3 An ideal decellularization process would completely remove source tissue cells and associated cellular content from a tissue or organ, while minimizing unwanted effects on the remaining ECM. However, a more widely encountered and practical representation of an optimized decellularization process exhibits partial removal and/or disruption of resident cells and cellular material to levels within a set of product-specific ranges (acceptance criteria). This guide is intended to aid in evaluating a decellularization process through the mechanisms and extent of decellularization and any potential impacts on the remaining dECM.

4.4 This standard provides a guide to the following steps in evaluating an extracellular matrix decellularization process:

4.4.1 Selecting attributes and test methods for characterization (Section 5)

4.4.2 Developing decellularization acceptance criteria for selected attributes (Section 6)

4.4.3 Documenting and analyzing the decellularization process flow (Section 7)

4.4.4 Performing a characterization of the decellularization process by testing decellularized ECM materials using the selected attributes, methods, and acceptance criteria (Section 8)

4.5 Decellularization processes vary widely in practice, utilizing a variety of reagents, temperatures, pressures, and/or mechanical forces in parallel and/or in sequence. While any one factor may act through consistent mechanisms, its effect will vary according to the decellularization process in its entirety as well as the particular tissue structure. As such, each part of a decellularization process should be understood and analyzed within the context of the complete process sequence and its action upon the type of tissue. For example, a process developed for dermis will likely not translate directly to a heart valve and the doubling of process time will affect each process differently, so the decellularization process will have to be adjusted to account for the difference in tissue properties and desired attributes at the conclusion of the process. Within the context of this guide, analysis of a processing step should not suggest material testing. Analysis is meant to demonstrate an understanding of the relevant mechanisms of decellularization and the relevant mechanisms of adverse effects on the ECM material.

4.6 Decellularization acceptance criteria and ECM integrity acceptance criteria should be developed based on the intended use of the dECM material. This guide suggests some considerations that should be used to develop and justify acceptance criteria.

4.6.1 Decellularization acceptance criteria already established for a source ECM and decellularization process allow for controlled changes to the decellularization process. Significant changes include changes to the processing mechanisms, reagents/materials, reagent concentrations, and controls as well as changes in source ECM materials. Prior to any significant change to a decellularization process, a decellularization process analysis should be conducted on the process steps which are subject to change. In addition, testing against the established decellularization acceptance criteria should be conducted on dECM material produced with the proposed process changes. A risk management process may then be utilized to ensure that any risks associated with the proposed changes are acceptable.

4.7 Measurements of decellularization attributes using the source extracellular matrix material as a reference can provide a valuable frame of reference and determination of percent change for exploratory and informational purposes. However, acceptance criteria based on percent change from the source material are more prone to variability in the final product due to variability in the source material. Acceptance criteria based on measurements of the dECM alone are more stable and simpler to implement.

4.7.1 The preparation of decellularized medical products involves variability originating in the source material as well as the processing; both types of variability can affect the consistency of the end product (dECM) and its performance in meeting predetermined acceptance criteria. A complete characterization of a decellularization process will include statistical ranges for each measured attribute. Statistical correlations may be explored to connect variation in source material and processing to end product attributes. These correlations can help prioritize source material and process controls to address uncontrolled variability.

5. Decellularized Material Attributes and Testing Methods

5.1 *Attributes Related to Cellularity and Cellular Remnants*—The following attributes can be used to measure the efficacy of disruption and removal of cellular contents. to develop a holistic understanding of the decellularization

[6] The boldface numbers in parentheses refer to a list of references at the end of this standard.

process, its effects on ECM properties, and the consistency of those effects. The effect of cell remnants on host response is not attributable to a single mechanism; instead there is evidence for a variety of possible relevant mechanisms, with implications for the end product and its eventual performance *in vivo*. There is a common practice within the tissue engineering community in which characterization of decellularization includes, at a minimum, DNA quantification and nuclear localization (staining). This document recommends setting acceptance criteria for those measures as well as quantification of a representative cellular membrane component, and a representative intracellular molecule, as shown in Table 1. These attributes are recommended to evaluate the removal of cells and cell components; this recommendation does not directly confer relevance to product safety or performance beyond measuring the extent of decellularization. Producers are encouraged to use their discretion to implement additional acceptance criteria; examples are provided in Table 2. The descriptions below provide context on why an attribute may be relevant to a product's intended use and how to measure it. These descriptions do not attempt to include all limitations of the referenced methods or all possible attributes and methods. All decellularization methods should be appropriately validated; for quantitative measures, this should include spike recovery studies to measure the sensitivity of the sample preparation and testing methods. In particular, inefficient extraction methods may give falsely low values especially for low analyte concentrations. Because material properties may be altered during processing, test method validations should include source ECM to the extent that measurements of source ECM are used in evaluation. Further guidance on validation of test methods can be found in ICH Q2(R1).

5.1.1 *Phospholipids*—Phospholipids comprise the cell membrane, nuclear membrane, and the membranes of all organelles and vacuoles. As such, the presence of phospholipids in decellularized materials is representative of the presence of membrane molecules in general. Immunoglobulins IgG and IgM have consistently been shown to bind to membrane-bound antigens identifying donor cells found in non-decellularized xenografts, activating the immune complement system (**7**) and resulting in an adverse immune response. For standard decellularization techniques the presence of these antigens as a group can be approximated as remaining in proportion to the quantity of remaining phospholipids.

5.1.1.1 *Phospholipid Quantification Methods*—Several assay kits which quantify phospholipid content after extraction from tissue are available. Most methods either measure the phosphorus bound to lipids or the choline groups bound to lipids. Choline groups are found on a consistent proportion of human serum phospholipids (**8**) and should be assumed to be consistent within any species and organ. When reporting measurements using a choline-specific reaction, the results should be labeled as "choline-containing phospholipids." Extraction techniques for quantification of phospholipids vary, but generally depend on cell lysis and membrane solubilization, involving the use of detergents, alcohols, chloroform, tissue homogenization, and/or sonication. As with many tissue components, remaining phospholipids may only partially come into solution, especially if the extraction buffer is similar to a decellularization reagent. In these cases, an orthogonal extraction technique would be preferred.

5.1.2 *Membrane Proteins and Other Membrane Molecules*—Similar to the detection of phospholipids, ubiquitous or otherwise representative membrane molecules may be considered as a stand-in for membrane-bound antigens. For example, Major Histocompatibility Complex (MHC) molecules may be of interest; they mediate the T cell response in vertebrates against intracellular and extracellular pathogens as well as discrimination between self and non-self molecules. In humans, MHC class I proteins are found on all nucleated cells and MHC class II proteins are found on antigen-presenting cells.

5.1.2.1 *Membrane Molecule Quantification and Localization Methods*—As with other specific molecules, immune-based techniques are recommended due to their high specificity and precision: ELISA for quantification and IHC or IF for localization.

5.1.3 *αGal*—Galα 1,3Galβ1,4GlcNAc (αGal) is a carbohydrate residue found on cell-surface and secreted glycoproteins and glycolipids in all mammals aside from humans, apes, and Old World monkeys. Humans naturally develop antibodies against αGal. The degree of relevance of αGal to the host immune response is debated. Reduction of αGal may be accomplished through decellularization processes with or without αGal-specific enzymatic treatments (**9**, **10**). Testing for αGal is not recommended if the source material does not contain αGal.

TABLE 1 Recommended Attributes and Testing Methods – Cellularity and Cell Remnants

Recommended Attribute for Characterization	Typical Molecule(s) of Interest	Common Test Methods	Section Reference
DNA quantification	DNA	Hoechst, draq5, Quantifluor, Picogreen	5.1.4
Nuclear localization (staining)	Cell nuclei	Hematoxylin and Eosin, Feulgen (Schiff's), 4',6-diamidino-2-phenylindole (DAPI)	5.1.6.1
Quantification of a representative cellular membrane component	Phospholipids	Phosphorus detection, Choline detection	5.1.1
	Membrane Proteins (for example, Major Histocompatibility Molecules (MHC))	ELISA	5.1.2
Quantification of a representative intracellular molecule	α-Smooth Muscle Actin (αSMA), β-Actin, Vimentin	ELISA	5.1.5

TABLE 2 Example Discretionary Attributes and Testing Methods – Cellularity and Cell Remnants

Discretionary Attribute for Characterization	Common Characterization Modes	Common Test Methods	Section Reference
αGal (quantification recommended when αGal reduction is intended)	Quantification Localization	ELISA (for example, M86, IB4 isolectin) IHC or IF (for example, M86, IB4 isolectin)	5.1.3
DNA	Localization	Feulgen (Schiff's), Hoechst, draq5, DAPI, Cyanine	5.1.6
	Fragment length analysis	Agarose gel electrophoresis	
Membrane components	Localization	IHC or IF (for example, MHC I)	
Damage Associated Molecular Pattern Molecules (DAMPs)	Low molecular weight Hyaluronan or Heparan Sulfate quantification	Fluorophore assisted carbohydrate electrophoresis (FACE)	5.1.5.1
	Proteins (for example, High Mobility Group Box 1 (HMGB1), S100) quantification	ELISA	
	Proteins (for example, HMGB1, S100) localization	IHC/IF	

5.1.3.1 *αGal Quantification Methods*—Quantification may be performed through standard Enzyme Linked Immunosorbent Assay (ELISA) methods. The IB4 isolectin, with affinity for αGal, may be used as a substitute for typical anti-αGal antibodies such as the monoclonal antibody M86 (**11**, **12**). A complete approach using the M86 antibody and appropriate αGal positive and negative controls has been developed as an industry standard in China and published (**13**). Alternatively, IHC or IF staining may be used as a semi-quantitative measurement.

5.1.4 *DNA*—Commercially available decellularized products have been shown to exhibit a wide range of DNA content (**14**) and evidence suggests a correlation of the quantity or localization of remaining DNA in decellularized ECM products with the severity of adverse host inflammatory response (**1**, **2**). DNA complexes can elicit innate immunity through activation of Toll Like Receptors (TLRs) or TLR-independent mechanisms (**15**). DNA quantification and localization have historically been used as measures of cellularity. Therefore, they, along with DNA fragment-length analysis, can be measures of decellularization by serving as a representative of all nuclear material, even if there is a non-specific link to the immune response. In measuring DNA, the analysis may exclude single-stranded nucleotide chains (for example, ssDNA) and very short fragments of double-stranded nucleotide chains.

5.1.4.1 *DNA Quantification Methods*—Several dye reagents, such as the Hoechst, draq5, Quantifluor, and Picogreen dyes, exhibit fluorescent properties upon binding with DNA molecules. Assay procedures using these dyes are commonly used and should be qualified for the intended use. As with other quantification methods, DNA must be extracted or liberated from ECM using qualified techniques. In most cases extraction techniques are generally applied utilizing one or more of the following: cell lysis, tissue homogenization, enzymatic digestion, and sonication.

5.1.4.2 *DNA Staining Methods*—Localization of DNA through histologic staining methods can also be used to assess decellularization. Aside from providing a semi-quantitative measure of DNA content, localization also allows insight into the mechanisms of DNA persistence or removal. These methods allow for the localization of both DNA concentrated as intact nuclei, nuclear remnants and stray DNA from disrupted nuclei. Regions with increased concentrations of stray DNA indicate effective cellular disruption but reduced efficacy of cellular remnant removal. Common DNA nucleotide stains include the Feulgen stain (Schiff's reagent) and the Hoechst, draq5, DAPI, and Cyanine dyes.

5.1.4.3 *DNA Fragment Length Analysis*—The lengths of DNA fragments are commonly measured through agarose gel electrophoresis. This method results in bands or smears that can be compared to DNA fragment standards to determine the distribution of DNA fragment lengths. The DNA fragment length distribution provides context for the total DNA quantity in a tissue and also indicates the action of DNA disruption and removal. Different decellularization mechanisms may produce the same measured quantity of DNA but with substantially different fragment distributions.

5.1.5 *Intracellular Molecules*—Intracellular molecules may be measured or localized as another measure of decellularization. Some, such as αSMA (α-Smooth Muscle Actin) or β-Actin, are ubiquitous in eukaryotic cells and may serve as representative markers of overall intracellular remnants. Others may be indicative of remnants from cell subsets, such as the use of Vimentin, which is generally specific to the cytoskeleton of mesenchymal cells (**16**).

5.1.5.1 *Damage Associated Molecular Pattern Molecules (DAMPs)*—Evidence suggests that particular molecules associated with cell and/or tissue damage can initiate immune responses when released from a cell or displayed on the cell membrane, recreating the signals of cellular necrosis (**17**, **18**). Suspected DAMPs include DNA, RNA, HMGB1, ATP, adenosine, low-molecular-weight Hyaluronan, Heparan Sulfate, and the S100 family of proteins. These molecules may remain in ECM after decellularization. Levels of HMGB1 in decellularized materials in particular have been shown to differ following different decellularization processes (**19**).

5.1.5.2 *Intracellular Molecule Quantification and Localization Methods*—As for other specific molecules, immune-based techniques are recommended due to their high specificity and precision: ELISA for protein quantification and IHC or IF for protein localization.

5.1.6 *Other Methods:*

5.1.6.1 *Nuclear Staining*—Histological nuclear staining allows for visualization and localization of intact cellular nuclei.

Regions of intact nuclei indicate reduced efficacy of cellular disruption. Common nuclear staining methods include various forms of Hematoxylin. USP General Chapter <1285.1> provides guidance on performing Hematoxylin and Eosin staining. As an example, the USP Bovine Acellular Dermis Reference Micrographs provide examples of stained sections with acceptable and unacceptable levels of intact nuclei, according to that particular tissue and use. DAPI and the Feulgen stain are commonly used *in vitro* to identify nuclei, but, as mentioned in 5.1.4.2, their visualization is based on binding to and reactions with DNA molecules, respectively. Intact nuclei and localized DNA are not the same, so users of these techniques should consider how DNA degradation, for example due to DNase treatment, may affect results.

5.2 *Attributes Related to ECM Integrity*—Assessing ECM integrity is primarily conducted through compositional measurements that may be supported by structural analyses. The compositional portion of the assessment should include the relative composition of major ECM components as well as any minor components or non-compositional properties with important functional roles. Major ECM components include any class or specific type of ECM molecules that is known or expected to comprise at least 5 % (50 mg of each 1 g) of the tissue dry weight. The ECM classes of collagens and glycosaminoglycans should always be considered major ECM components even when they do not meet this criterion. Composition is generally reported in proportion to sample dry weight, though in some circumstances sample wet weight or sample semi-dry weight serves as a more appropriate denominator. Depending on the intended use of the decellularized product, assessment of ECM integrity should include additional properties such as structure, cell-ECM interactions, biomolecule release profiles, and testing of physical material properties. There are very many potential biomolecules that may be considered functionally important to a specific intended use and included in such assessments. This document does not attempt to provide a list of such biomolecules. Assessed properties may also relate to the state of modifications or reactions such as cross-linking, glycosylation, sulfation, or oxidation. This document recommends setting compositional acceptance criteria for major ECM components, including total collagens and glycosaminoglycans, as shown in Table 3. Producers may use their discretion to implement additional ECM integrity acceptance criteria; examples are provided in Table 4. The attribute descriptions below provide context on why they may be relevant to decellularization and how to measure them. However, these descriptions do not attempt to include all limitations of the referenced methods.

5.2.1 *ECM Composition Methods: General Notes*—Typical methods of measuring ECM components are destructive and require digestion of the ECM material through enzymatic and/or non-enzymatic means. Many ECM component classes, such as collagens, elastin, glycosaminoglycans, and soluble proteins, can be measured through partially specific biochemical spectrophotometric assays. Liquid chromatography, when available, can similarly be employed for partially specific quantification assays. Specific quantification is generally achieved through use of ELISA methods, though mass spectrometry and other proteomic techniques are increasingly common. For all techniques, sample preparation is critical in maintaining the sensitivity of the measurement. Further, it is important to test for and minimize interference from reagents used in the decellularization process and/or sample preparation through method validations involving spiking studies. All ECM measurements should be appropriately validated for use on both source ECM and decellularized ECM.

5.2.2 *ECM Structural Methods: General Notes*—Histology is the most common technique used for structural analysis of ECM materials. The structure of multiple major ECM components may be visualized through any of several single-color or multi-color stains such as Alcian Blue, which stains glycosaminoglycans (GAGs); Masson's Trichrome, which stains muscle/keratin, collagen/bone, and cell nuclei, or Movat's Pentachrome, which stains collagen, GAGs, fibrin, muscle, and elastin/cell nuclei. In addition, specific ECM components should be visualized when they are indicative of or integral to specific structures which provide functionality in the intended use. Such visualization can be achieved through IHC, IF, or analogous techniques. MicroCT and electron microscopy (SEM and TEM) are valuable for assessing micro- and nanostructures (for example, collagen fiber alignment and periodicity), as well as topographical details, but lack the molecular specificity of histological techniques. Surface imaging with molecular specificity is possible through various forms of Raman spectroscopy and second harmonic generation (SHG) can provide collagen structural images at depths up to several hundred microns (**21**). USP General Chapter <1285> provides guidance on the preparation of samples for both histological staining and immunohistochemistry.

TABLE 3 Recommended Attributes and Testing Methods – ECM Integrity

Recommended Attribute for Characterization	Typical Molecule(s) of Interest	Common Test Methods	Section Reference
Collagen quantification	Total collagen	Hydroxyproline quantification with Chloramine T/dimethylaminobenzaldehyde (**20**)	5.2.4
Glycosaminoglycan quantification	Total glycosaminoglycans	Uronic acid quantification with meta-hydroxydiphenyl (**20**)	5.2.6.1
	Sulfated glycosaminoglycans	1,9-dimethylmethylene blue (DMMB), Blyscan	
Quantification of any other Major ECM Components (≥50 mg/1 g dry weight)	Dependent on product composition	ELISA, Mass Spectrometry	5.2.1