Designation: F2529 – 13 (Reapproved 2021)

Standard Guide for
in vivo Evaluation of Osteoinductive Potential for Materials Containing Demineralized Bone (DBM)[1]

1. Scope

1.1 This guide covers general guidelines to evaluate the effectiveness of DBM-containing products intended to cause and/or promote bone formation when implanted or injected *in vivo*. This guide is applicable to products that may be composed of one or more of the following components: natural biomaterials (such as demineralized bone), and synthetic biomaterials (such as calcium sulfate, glycerol, and reverse phase polymeric compounds) that act as additives, fillers, and/or excipients (radioprotective agents, preservatives, and/or handling agents) to make the demineralized bone easier to manipulate. It should not be assumed that products evaluated favorably using this guidance will form bone when used in a clinical setting. The primary purpose of this guide is to facilitate the equitable comparison of unique bone-forming products in *in vivo* heterotopic models of osteoinductivity. The purpose of this guide is not to exclude other established methods.

1.2 The values stated in SI units are to be regarded as standard. No other units of measurement are included in this standard.

1.3 *This standard does not purport to address all of the safety concerns, if any, associated with the use of DBM-containing bone-forming/promoting products. It is the responsibility of the user of this standard to establish appropriate safety, health, and environmental practices involved in the development of said products in accordance with applicable regulatory guidance documents and in implementing this guide to evaluate the bone-forming/promoting capabilities of the product.*

1.4 *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]

D1193 Specification for Reagent Water

D5056 Test Method for Trace Metals in Petroleum Coke by Atomic Absorption

E508 Test Method for Determination of Calcium and Magnesium in Iron Ores by Flame Atomic Absorption Spectrometry

F565 Practice for Care and Handling of Orthopedic Implants and Instruments

F895 Test Method for Agar Diffusion Cell Culture Screening for Cytotoxicity

F981 Practice for Assessment of Compatibility of Biomaterials for Surgical Implants with Respect to Effect of Materials on Muscle and Insertion into Bone

F1854 Test Method for Stereological Evaluation of Porous Coatings on Medical Implants

F2131 Test Method for *In Vitro* Biological Activity of Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) Using the W-20 Mouse Stromal Cell Line

F2721 Guide for Pre-clinical *in vivo* Evaluation in Critical Size Segmental Bone Defects

2.2 *Federal Documents:*[3]

21 CFR 58 Good Laboratory Practice for Nonclinical Laboratory Studies

21 CFR 820 Quality System Regulation

21 CFR 1270 Human Tissue Intended for Transplantation

21 CFR 1271 Human Cells, Tissues, and Cellular and Tissue-Based Products

21 CFR 610.12 General Biological Products Standards— General Provisions—Sterility

Q1E Evaluation of Stability Data FDA Guidance for Industry

Container and Closure Integrity Testing in Lieu of Sterility Testing as a Component of the Stability Protocol for Sterile Products FDA Guidance Document

Eligibility Determination for Donors of Human Cells, Tissues, and Cellular and Tissue-Based Products Guidance for Industry

Guidance for the Preparation of a Premarket Notification Application for a Surgical Mesh Guidance for Industry and/or for FDA Reviewers/Staff and/or Compliance

2.3 *AAMI/ISO Documents:*[4]

AAMI TIR 17 Compatibility of Materials Subject to Sterilization

AAMI/ISO 22442-01 Medical Devices Utilizing Animal Tissues and Their Derivatives—Part 1: Application of Risk Management

AAMI/ISO 22442-03 Medical Devices Utilizing Animal Tissues and Their Derivatives—Part 3: Validation of the Elimination and/or Inactivation of Viruses and Transmissible Spongiform Encephalopathy (TSE) Agents

2.4 *ANSI/AAMI/ISO Documents:*[5]

ANSI/AAMI/ISO 11137 Parts 1–3 Sterilization of Health Care Products—Radiation

ANSI/AAMI/ISO 10993 Biological Evaluation of Medical Products

2.5 *ICH Documents:*[6]

ICH Harmonised Tripartite Guideline Q5C Quality of Biotechnological Products—Stability Testing of Biotechnological/Biological Products

ICH Harmonised Tripartite Guideline Q5A (R1) Viral Safety Evaluation of Biotechnology Products Derived from Cell Lines of Human or Animal Origin

2.6 *USP Document:*[7]

United States Pharmacopeia Chapter <71>

3. Terminology

3.1 *Definitions:*

3.1.1 *additive*—ingredients that may be used to preserve the product, provide radioprotection, and/or act as bulk filler and/or binding agent.

3.1.1.1 *Discussion*—It has no intended mode of action, such as causing cells to transform lineage, once implanted.

3.1.2 *biologically active carrier*—a component added to a DBM-containing bone-forming product that results in a physiological and/or biochemical transformation in the implant site independent of other constituents in the bone-forming product.

3.1.2.1 *Discussion*—The transformation may be desirable or untoward. A biologically active carrier may also contribute to the physical or chemical properties/characteristics of that bone-forming product.

3.1.3 *bone*—hard connective tissue of the skeletal system in vertebrates comprised of collagen, growth factors, and an inorganic rigid matrix containing calcium, phosphate and other minerals, and various cellular elements, including osteoblasts, osteocytes, osteoclasts, and hematopoietic cells.

3.1.3.1 *Discussion*—Bone is made up of cortical and cancellous bone tissue. It serves as the point of attachment for muscles and tendons and is load-bearing.

3.1.4 *bone-forming product (containing DBM)*—as used in this guide, a DBM-containing bone-forming product may be comprised of multiple components including, but not restricted to, demineralized bone, growth factors, differentiation factors, osteoprogenitor cells, mesenchymal stem cells, biologically active carrier(s), and/or non-biologically active carrier(s).

3.1.5 *bone marrow*—tissue located in the cancellous portion and cavities (medullary canal) of most bones.

3.1.5.1 *Discussion*—Bone marrow is highly vascular and occurs in two forms: white/yellow marrow, comprised mostly of adipose cells located primarily in the long bones, and red marrow, which primarily produces and contains pluripotent stems cells and red blood cells, platelets, and white blood cells derived from them. In adults, red marrow is located primarily in the flat bones.

3.1.6 *bone tissue*—the tissue component of a bone comprised of a mineralized collagenous matrix formed and maintained through the action of osteoblastic cells and osteocytes remodeled through the action of osteoclasts.

3.1.7 *cartilage*—connective tissue that is a major constituent of the embryonic and young vertebrate skeleton, largely replaced with bone and bone marrow bone with maturation.

3.1.7.1 *Discussion*—It is comprised mostly of Type II collagen and proteoglycans and found in joints, the outer ear, bronchi, and larynx. There are three major types of cartilage: hyaline cartilage, which is adapted for joint surfaces by virtue of its smoothness and ability to withstand compression; fibrocartilage, found in the outer ear, nose, and meniscus; and elastic cartilage, found in the outer ear and epiglottis. Cartilage is also formed by the action of bone morphogenetic protein(s) (BMPs) in concert with other peptide factors on mesenchymal stem cells.

3.1.8 *cortical bone*—thin superficial layer of compact bone tissue that constitutes the primary load-bearing component of a bone.

3.1.9 *demineralized bone*—bone tissue wherein the average mineral content, typically measured as calcium, is less than or equal to 8 % by dry weight.

3.1.9.1 *Discussion*—As used in this guide, dry weight means lacking significant or measurable residual moisture (<5 %).

3.1.10 *differentiation factors*—proteins with the ability to induce or alter cell differentiation and/or proliferation with subsequent tissue morphogenesis.

3.1.10.1 *Discussion*—For example, BMP-2, BMP-4, and BMP-7(OP-1) are differentiation factors with the ability to

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[4] Available from Association for the Advancement of Medical Instrumentation (AAMI), 4301 N. Fairfax Dr., Suite 301, Arlington, VA 22203-1633, http://www.aami.org.

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

[6] Available from International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH), ICH Secretariat, c/o IFPMA, 15 ch. Louis-Dunant, P.O. Box 195, 1211 Geneva 20, Switzerland, http://www.ich.org.

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

induce bone and cartilage formation in tissues or implantation sites that would otherwise not form bone or cartilage by causing mesenchymal progenitor cells to differentiate into chondrocytes and/or osteoblasts.

3.1.11 *endochondral ossification*—process by which bones in embryonic and juvenile animals grow while maintaining functional proportions of the skeleton and joints.

3.1.11.1 *Discussion*—Bone-forming products considered in this guide mimic this process: post-implantation inflammation leads to recruitment of mesenchymal stem cells, and the BMPS/growth factors cause the mesenchymal stems cells to differentiate into chondroblasts, which synthesize cartilage matrix. The chondrocytes undergo hypertrophy and calcify their extracellular matrix. This matrix is then resorbed by osteoclasts, angiogenesis occurs, and osteoprogenitor cells migrate to the calcified cartilage by means of the neovasculature. Osteoblasts synthesize bone on the cartilage scaffold and new bone marrow forms, and is incorporated into the new tissue and ultimately is remodeled.

3.1.12 *evidence of endochondral bone formation*—chondroblasts, chondrocytes, cartilage, osteoblasts, osteocytes, new bone, and bone marrow.

3.1.13 *excipient*—constituents of a DBM-containing bone-forming product that are not the active substance.

3.1.14 *fascia*—fibrous connective tissue between two layers of tissues such as muscle, or an internal organ and the abdominal wall.

3.1.15 *fibrous connective tissue*—connective tissue produced by fibroblasts.

3.1.16 *fillers*—an ingredient that may used to expand the volume of the implant material and may possess osteoconductive properties but does not cause cells to transform lineage once implanted.

3.1.17 *gelatin capsule*—capsule made of gelatin used to contain materials for implantation.

3.1.18 *growth factors*—proteins produced by cells that stimulate or inhibit cell proliferation and may affect differentiation.

3.1.19 *heterotopic*—in the context of this guide, an implantation/transplantation site that is not in direct contact with bone, such as skeletal muscle.

3.1.20 *intermuscular*—within that space occurring between anatomically identifiable muscles, frequently associated with little to no bleeding at the time of surgical dissection.

3.1.21 *intramuscular*—within an anatomically identifiable bundle of muscle tissue, frequently associated with occurrence of bleeding at the time of surgical dissection.

3.1.22 *mesenchymal stem cells*—cells derived from mesenchyme that are capable of proliferating and differentiating into connective tissue such as cartilage and bone, blood, lymphatic, and blood vessels.

3.1.23 *non-biologically active carrier*—a component added to a DBM-containing bone-forming product that by itself does not result in the differentiation or proliferation in the cells infiltrating the implant site.

3.1.23.1 *Discussion*—A carrier may also contribute to the physical or chemical properties/characteristics of that bone-forming product.

3.1.24 *nude mouse*—a mouse with a genetically deficient immune system as the result of a dysfunctional rudimentary thymus and thus is deficient in T-cells and incapable of producing cytotoxic effector cells and a graft-versus-host response.

3.1.24.1 *Discussion*—Therefore the T-cell response to xenogenic material is abrogated. However, the animals are able to mount an innate immune response (macrophages, giant cells, and granulocytes) and thus are capable of mounting an immunological response to non-biocompatible products.

3.1.25 *nude rat*—a rat with a genetically deficient immune system as the result of a dysfunctional rudimentary thymus and thus deficient in T-cells and incapable of producing cytotoxic effector cells and a graft-versus-host response.

3.1.25.1 *Discussion*—Therefore the T-cell response to xenogenic material is abrogated. However, the animals are able to mount an innate immune response (macrophages, giant cells, and granulocytes) and thus are capable of mounting an immunological response to non-biocompatible products.

3.1.26 *orthotopic*—in the context of this guide, an implantation/transplantation site within or in direct contact with bone tissue.

3.1.27 *ossicle*—a spherical piece of bone consisting of an outer core of cortical bone and an inner region of trabecular bone and bone marrow.

3.1.28 *osteoblasts*—cells exhibiting the appropriate protein and gene expression characteristics necessary for the formation and maintenance.

3.1.29 *osteoconduction*—the ability of a material/bony implant site to support new bone formation by providing an environment in which pre-existing osteoid cells capable of synthesizing and secreting components essential to the formation lead to growth of new host bone into/onto the material.

3.1.30 *osteogenic*—indicating the presence of cells capable of synthesizing and secreting components essential to the formation of bone at some implant site as well as the osteoconductive capacity necessary for causing/allowing bone formation in a tissue or implantation site.

3.1.30.1 *Discussion*—Additionally, BMPs would also be considered osteogenic as these are some of the components secreted by cells that cause the cells to be osteogenic.

3.1.31 *osteoinduction*—the ability of a substance to stimulate cells to differentiate along some osteoprogenitor pathway resulting in cells capable of synthesizing and secreting components essential to the formation of bone at an implant site.

3.1.32 *osteoprogenitor cells*—cells with the capacity to migrate, proliferate, and/or differentiate into osteoblasts under osteoinductive, osteoconductive, and/or osteogenic conditions.

3.1.33 *subcutaneous*—below the skin and above the fascia covering the underlying tissue.

3.1.34 *trabecular bone*—bone tissue comprised of spicules that form a lattice filled with bone marrow.

3.1.34.1 *Discussion*—It is also commonly referred to as cancellous bone. Trabecular bone constitutes the secondary load-bearing component of a bone.

4. Significance and Use

4.1 This guide covers animal implantation methods and analysis of the explanted DBM-containing material to determine whether a material or substance possesses osteoinductive potential, as defined by its ability to cause bone to form *in vivo* at a site that would otherwise not support bone formation, that is, heterotopically in a skeletal muscle implant site. For *in vitro* evaluation see Test Method F2131 for *in vitro* assessment of rhBMP 2.

4.2 The test methods described here may be suitable for defining product specifications, cGMP lot release testing, research evaluation, regulatory submission, and so forth, but a positive outcome should not be presumed to indicate that the product will be osteoinductive in a human clinical application. At present, the only direct assays to assess new bone formation are *in vivo*, since the property of bone conduction or induction can only be assessed in a heterotopic or orthotopic site in a living animal. When these products are implanted in an orthotopic site, osteogenic factors already present at the implantation site may contribute to and enhance bone formation in conjunction with the osteoconductive nature of the product. Thus, orthotopic implantation of products may result in bone formation by acting on existing bone-forming cells and not by causing mesenchymal stem cells to become osteochondroprogenitor cells. In contrast, when these products are implanted in a heterotopic site, no native osteogenic factors are present to contribute to or enhance bone formation. Thus, heterotopic implantation of products will only result in new bone formation by causing mesenchymal stem cells to become osteochondroprogenitor cells. *In vitro* assays have been described and some believe they may correlate to the results obtained from *in vivo* assays. However such *in vitro* assays measure only some of the biochemical marker(s) associated with *in vivo* bone formation and are therefore only indirect assays for osteoinductive activity or the capacity to promote new bone formation. Many factors or combinations of factors contribute to osteoblast progenitor cells differentiating and/or proliferating into bone-forming cells *in vitro* that are both osteoinductive and osteoconductive when they are implanted *in vivo*. Thus, only an *in vivo* assay method currently directly considers the many potential factors involved in new bone formation induced by DBM-containing biomaterials. The qualification of a DBM or DBM-containing material should also encompass product characterization such as that described in Appendix X1.

5. Animal Models

5.1 *General Note*—Appropriate positive and negative controls shall be used for research purposes and during qualification of new test articles into a validated system, but are not required for routine lot release testing once validated. For example, negative controls could be 4M guanidine hydrochlo-ride inactivated DBM (**1**),[8] mineralized cancellous bone and heat inactivated DBM (**2**) and positive controls could be previously *in vivo* qualified DBM.

5.2 *Athymic (nude) Rodents*—Mice (mu/mu) or rats (nu/nu) with genetically deficient immune systems are the preferred animal models for assessing bone formation for products comprised of human demineralized bone in heterotopic sites. Athymic animals have a dysfunctional rudimentary thymus and thus are T-cell deficient and incapable of producing cytotoxic effector cells and a graft-versus-host response. Therefore, the T-cell response to xenogenic material is absent. However, the animals are able to mount an innate immune response (macrophages, giant cells, and granulocytes) and thus are capable of having an immunological response to non-biocompatible or microbiologically (culture) positive products. Gender is generally not a factor; however, male athymic mice/rats should be considered for implantation unless estrous cycles in female animals have been demonstrated not to negatively impact the desired studies. In addition, adolescent mice/rats (six to nine weeks old at the time of implant) are recommended as studies have demonstrated that older animals may not respond as effectively to osteoinductive materials (**3, 4**). The choice of animal, mouse versus rat, is determined to a large extent by the size (volume) of the material being tested.

5.3 *Implant Mass/Volume*—In general, implant mass/volume (DBM+Excipient/additive/filler) of 15 to 120 mg can be conveniently assessed using the nude mouse, whereas larger samples of 100 to 250 mg are able to be tested in the nude rat model. The amount of DBM implanted into a nude mouse should ideally be 20 ± 5 mg (**3, 4**) and for implantation into nude rats the amount of DBM should be 40 ± 5 mg (**1**). For example, a recommended athymic mouse implant may be comprised of 20 mg of DBM and 60 mg of excipient for a total sample size of 80 mg. It is recommended that the DBM+excipient groups contain the same amount of bone implanted as negative and positive controls so the results are comparable and the potential effects of the excipient on osteoinductive potential can be determined.

5.4 *Implant Numbers/Sample Size:*
5.4.1 In order to achieve a statistically valid outcome for histomorphometry, a minimum of two animals (nude mouse/rat) each with two implants per lot are necessary. This achieves 83 % power at the 95 % confidence interval. This allows for the detection of a 12 % difference in new bone formation. The following is assumed: The percent new bone is 40 %; the standard deviation is 5 %; and the alpha is 0.05.

5.4.2 A minimum of four implants in four different nude rats, for a total of 16 implants per test group, or a minimum of two implants in six different nude mice, for a total of twelve implants per test group, may be required to achieve statistical significance where product variability and performance are uncertain (**1, 3, 4**). However, each investigator will need to perform and report his/her own statistical assessment to account for the variability or product/process change under investigation.

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

5.5 *Heterotopic Implant Locations:*

5.5.1 The preferred heterotopic test site is in the animal's skeletal muscle. Intermuscular pouches in the gluteal muscle or biceps femoris are some appropriate implant sites for the athymic mouse model (**3**, **4**). Intermuscular pouches in the gluteal, biceps femoris, pectoralis, abdominal, or dorsal muscles are some appropriate implant sites for the athymic rat model (**1**). Other sites can be qualified using this guide. Care should be taken that the implant does not touch or rest against a bone as this will provide osteogenic factors to the implant and will bias the data towards a false positive. As a general rule, intermuscular implantation is one in which minimal bleeding occurs at the site of implantation because the implant goes between the fascia. An intramuscular implant is one in which bleeding occurs because the implant goes between the muscle fibers within a muscle bundle such that the blood of the animal mixes directly with the implanted material at the time of implantation if care is not taken to allow the bleeding to stop and the blood reasonably cleared from the implant site. Implantation of DBM intra- versus intermuscularly significantly affects the amounts of new bone formed from the same implant DBM (**3**). Therefore, to obtain the most objective result from this intentionally ectopic model, the intermuscular implantation is the recommended implant methodology described in 7.8.4, thus the amount of blood in the implant site should be minimized so as not to significantly bias the results. Minimizing blood in the implant site allows for a more accurate assessment of the osteoinductive potential of the product; however during clinical use, it is recognized that there will be blood in the implant site as well as bone and osteogenic components. Implants may also be placed subcutaneously over the pectoralis muscles of the chest in the athymic rat; however, subcutaneous sites have proven to be less favorable in their ability to support osteoinduction in the nude mouse (**3**). Furthermore, a false negative result is more probable with a subcutaneous implant model than with the muscle implantation model due to the different embryonic origin of these two tissues.

5.5.2 For cGMP lot release testing and testing to support regulatory submissions, validated implantation methodologies, test and assessment systems shall be employed. In addition, the test article shall be qualified into the validated testing system. The qualification should encompass product characterization as described in Appendix X1 and the successful implantation of three separate lots of product into a minimum of two athymic mice/rats, each receiving a minimum of two implants (as detailed above).

6. Method Validation and New Product Qualification into a Validated Model System

6.1 The validation shall be performed in accordance with cGLP requirements 21 CFR Part 58. The test system (athymic mouse/rat) and procedures shall be validated prior to specific bone-forming product testing.

6.2 Validation should involve the implantation of a minimum of three different lots of DBM, a negative control (for example, inactivated DBM or mineralized cancellous bone), and a positive control (for example, DBM previously demon-strated to possess osteoinductive potential *in vivo*), to demonstrate that the test system is unaffected by the implant methodologies, anesthetizing agents and analgesics.

6.3 It is recommended that non-steroidal anti-inflammatory drugs be avoided because of potential confounding effects.

6.4 A minimum of two animals, each receiving two implants per lot of test article are recommended.

6.5 The in-life portion of the validation shall be 28 to 36 days. Going longer than this time frame makes it difficult to compare results between laboratories, even with appropriate controls. A longer in-life duration should be well justified.

6.6 Recommended explant preparation is described in 7.7.

6.7 DBM to be used a negative control may be inactivated using 4M guanidine hydrochloride or heat (**2**).

6.8 Assessments of osteoinductive potential for the validation may be carried out using quantitative histological analysis such as described in 8.3.

6.9 *Acceptance Criteria:*

6.9.1 Elements of new bone formation include: chrondroblasts/cytes, osteoblasts/cytes, cartilage, osteoid, new bone, and bone marrow. Percent new bone: 75 % of the explants per lot of DBM must demonstrate ≥10 % average new bone formation. By requiring 75 % of the explants to form new bone it demonstrates the intra-lot consistency of each batch/lot tested.

6.9.2 The percent is arrived at by the calculation in 8.3.16.

6.9.3 All negative controls should demonstrate <10 % new bone formation.

6.10 *Recommended Minimum Protocol/Report Contents:*

6.10.1 Title, date, purpose, cGLP statement, sponsor name and address, test facility name and address, test article characterization statement/disclaimer.

6.10.2 Justification for the test system, description of test system (species, gender, age, source, weight range, number of animals involved in the protocol, animal identification, Institutional Animal Care and use Committee (IACUC) protocol number, and approval date), husbandry (receipt, acclimation, housing, environment, diet, water, and USDA animal welfare act statement).

6.10.3 Study design, study test method, test article preparation, test article administration, health observations, termination, histopathology analysis, interpretation of results, control of bias, data analysis, statistical methods, assay validity, acceptance criteria, protocol changes, record retention, references, compliance statements (GLP and AAALAC), test article identification, and test articles disposition.

6.10.4 Report should contain any protocol deviations.

6.10.5 Signature and date of responsible parties performing and requesting the validation.

7. Recommended Implant Methodologies

7.1 *Sample Preparation*—All implant volumes should be normalized to contain equal amounts of DBM as measured by freeze-dried weight. Implants for athymic mice should contain 20 ± 5 mg of DBM, and 40 ± 5 mg for athymic rats (**3**, **4**). Other implant volumes can be utilized but positive and

negative controls should be of equivalent weight and the report/publication should clearly state the volume/weight implanted for test and control articles to allow for appropriate inter-laboratory comparisons.

7.1.1 For DBM powder, paste, or putty products, it is suggested that the sample intended for implantation be placed in a blunt cut 0.5-cc or 1-cc syringe for ease of implantation. Additionally, amalgam carriers and/or pre-sterilized gelatin capsules will aid in implantation.

7.2 *Athymic Mouse Recommended Implant Technique:*

7.2.1 Aseptic technique should be employed during the implantation procedure.

7.2.2 Animals should be housed in sterile microisolator cages and fed with sterile rodent food and water.

7.2.3 For implantation, healthy, young adult (for example, six to nine weeks of age at the time of implant) male athymic mice that have been quarantined for seven (7) days are recommended. Each animal is recommended to receive one injection of pre-operative buprenorphine (0.1 mg/kg IP) or other analgesic approved by the IACUC. Identify each animal with a unique ear punch number and weigh. Record the individual animal identification numbers along with the body weights.

7.2.4 Anesthetize the animal with Isoflurane or other anesthetic approved by the IACUC. The depth of anesthesia should be sufficient to prevent muscular movement. This can be checked by pinching the toe (between the digits) of the animal's hind limbs. If there is a reflex reaction, the animal is not sufficiently anaesthetized to continue with the implantation. A technician shall monitor the animal's respiration during surgery.

7.2.5 Place the anesthetized animal in a prone position on a clean, flat surface in a procedure room or surgical suite.

7.2.6 Shave the lower back of the animal with clippers, if necessary. Scrub the animal's lower dorsal region with surgical scrub. Start from the center and work to the edge of the surgical area. Wipe off the surgical scrub with 70 % isopropyl alcohol (IPA). The surgeon will complete final preparation for aseptic surgery.

7.2.7 Transfer the anesthetized mouse to the surgeon's nose cone.

7.2.8 An example procedure for intermuscular implantation in the gluteal muscle pouch is detailed as follows:

7.2.8.1 Scrub the animal's lower dorsal region with iodine solution. Start from the center and work to the edge of the surgical area. Wipe off the iodine with a clean, sterile gauze pad. Carefully lift the skin located over the spine of the lower dorsal region and open the skin with a single incision that is approximately 0.5 to 1 cm long. Look for any blood vessels that need to be avoided. Pull the skin opening over so that it is aligned above the gluteal muscle. Create a small pocket in the gluteal muscle tissue (Fig. 1), aligned parallel to the muscle fibers, using both blunt and sharp dissection. There should be little to no bleeding during this process. Hemostasis methods such as application of gauze shall be used, as necessary. Take appropriate measures to blot away as much blood as possible as the presence of blood may bias the outcome by providing potentially osteogenic components that are not part of the test article.

7.2.8.2 Insert the tip of the coordinating 3/10 cc syringe and implant the test article into the gluteal muscle pocket. Use of a steel syringe plunger may be necessary in order to push the entire test article through the syringe. Ensure that the entire test article is implanted.

7.2.8.3 Close the muscle layer with suture.

7.2.8.4 Repeat for the other gluteal pouch.

7.2.8.5 After both muscle pockets are sutured, place one drop of bupivacaine or IACUC-approved analgesic that will not negatively impact new bone formation, over each pocket.

7.2.8.6 Then close the skin layer with suture.

7.3 Return the animals to a cage lined with a clean, dry, sterile towel drape. Place the cage on the water heating pad
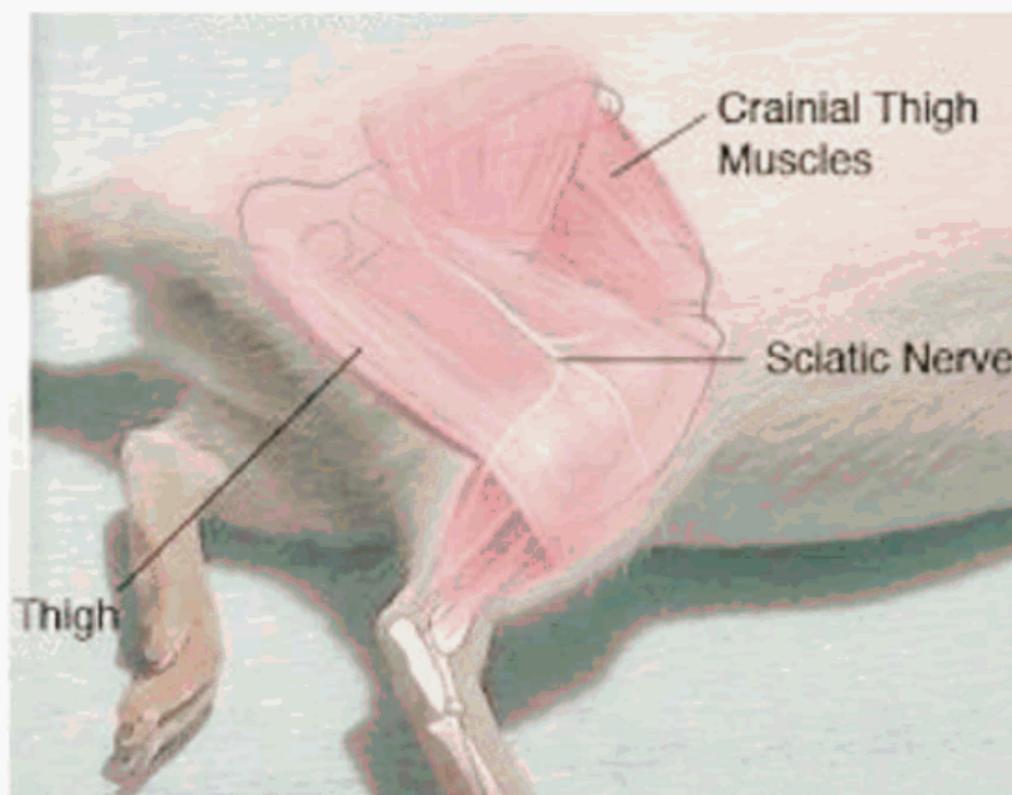

FIG. 1 Intermuscular Gluteal Muscle Implant

(with pump) until the animals are alert and active (moving). Once alert and active, the animals may be moved to clean cages. Segregation of animals who are still sedated and animals that are awake is recommended to reduce risk of cannibalism.

7.4 On the day following surgery, administer one injection of buprenorphine (0.1 mg/kg intraperitoneal (IP)) or IACUC-approved analgesic that will not negatively impact new bone formation and document the dosing. The dose should be injected away from the implant sites.

7.5 *Recommended Observations:*

7.5.1 *General Health*—Observe the animals once daily and record the observations. Observations can occur through direct, physical manipulation of the animals or close cage-side obser-vations. If any abnormal clinical signs or signs of inflammation and/or infection are observed, inform a staff veterinarian and/or designated personnel.

7.5.2 *Clinical Signs*—If a mouse should die during the study, perform a necropsy on the animal. If the animal died on or after Day 14 of the study, harvest and fix the implant sites until a decision is made about the potential utility of processing for histopathology. If the animal dies before Day 14, perform a necropsy and contact the staff veterinarian and appropriate personnel. A terminal weight should always be recorded.

7.6 *Recommended Euthanasia*—At the end of the 28-day implantation period, record the terminal weight of each animal. Record any abnormalities, with respect to the animals' health, on the macroscopic observations section of the worksheet. Euthanize the animals using carbon dioxide (CO_2) asphyxia-tion or another IACUC-approved method.

7.7 *Recommended Explant Methodologies:*

7.7.1 *Gently* palpate the implant area to determine the approximate implant location. Make a longitudinal incision with a scalpel down the gluteal muscle. Allow sufficient time to elapse for the tissue to be cut without bleeding.

7.7.2 Dissect the fascia away, carefully exposing the muscle. Remove the muscle by clipping any attached ligaments and tendons. Occasionally the implanted material may have migrated out of the tissue. In this case, make careful documen-tation of this observation, as sample migration may affect the osteoinductive processes. It is important to also note if the implant migrated to a bone, such as the femur, in the test animal as this may bias the data to the positive.

7.7.3 Carefully trim all extraneous tissue away from the explant. This will facilitate bisection of the implant along the long plane of the bone for histological analysis.

7.7.4 Record any abnormal color, shape, and/or consistency of the muscle and associated implant. Do not allow the tissue to dry out. Place the sample in a tissue cassette or specimen jar containing an 8 to 10× volume of 10 % neutral buffered formalin and allow to fix for at least 72 h. Alternatively, after placement in a cassette, flash-freeze the specimen for fixation.

7.8 *Athymic Rat Recommended Implantation Technique—Biceps Femoris:*

7.8.1 Most details from the gluteal implantation apply and thus only athymic rat-specific and biceps femoris-specific information is detailed below.

7.8.2 For implantation, select healthy, young adult (six to nine weeks of age/50 to 75 g) male athymic rats that have been quarantined for seven (7) days.

7.8.3 Anesthetize the animal with intraperitoneal ketamine (250 mg), xylazine (11 mg), and physiological saline (10 mL) using a dosage of 3.6 mL/kg body weight, or other anesthetic approved by the IACUC.

7.8.4 *Intermuscular Implantation (Biceps femoris):*

7.8.4.1 For hind limb intermuscular implantations, shave the lateral portion of the leg with a sterile disposable shaver if fur is apparent.

7.8.4.2 Locate the femur between the animal's knee and hip.

7.8.4.3 Grasp the skin upward along the femur with tissue forceps and snip the skin with scissors, creating a skin incision approximately 1 cm in length parallel to the femur.

7.8.4.4 Locate the fine line between the two leg muscles near the femur. Approximately midway between the hip and the knee, puncture the membrane between the muscle groups (gluteus superficialis and the biceps femoris) with the tips of the iris scissors, and then open the scissors to stretch the puncture opening.

7.8.4.5 Take care not to open too close to the knee to avoid excessive bleeding. Insert forceps and open in the opposite direction of the stretch to form a pocket between the muscle groups.

7.8.4.6 Take care not to disturb the sciatic nerve, which appears as a white cord located in the plane of the underlying muscle group.

7.8.4.7 While keeping the pocket open with forceps, insert the implant into the muscle just below the femur. Be sure the implant does not separate or come out of the pocket towards the skin. Reproducible placement can be achieved in close proximity to the femur. After both muscle pockets are sutured, place one drop of bupivacaine over each pocket. Close the muscle at the implant site by placing a single suture in the muscle. Close the skin incision with a suture or autoclip staple. Close the muscle layer with suture. Repeat for the other biceps femoris. Then close the skin layer with suture.

8. Techniques to Analyze New Bone Formation

8.1 *Histologic Preparation of Explants:*

8.1.1 Upon explant, the samples can be either flash frozen for cryosectioning or fixed per routine histological procedure. They are then decalcified if desired, dehydrated, infiltrated with paraffin or medium of choice, and cleared. They are typically cut in half along the long axis of the explant, and embedded (cut edge down) in the histology block. Slides are prepared with sections, typically 4 to 7 microns thick, at a minimum of three different levels within the block, and slides are stained as desired to best visualize the new bone-forming elements. Some of these stains are: Hematoxylin and Eosin (H&E), Toluidine blue, Alcian blue, Massons's trichrome, Goldner's trichrome, and Villanueva's stain. If Von Kossa stain is used, special consideration should be taken to not mistake dystrophic calcification for new bone formation.

8.1.2 Of the three slides per specimen, the one that has the fewest wrinkles, most uniform staining, and the largest amount of explanted material shall be used for the osteoinductive evaluation.

8.2 *Assessment of Explanted Tissue*—In addition to assessing the explants for new bone-forming elements, any signs of infection or foreign body reaction should also be identified. The presence of either condition would provide a rationale to repeat the implantation if the sample is determined to not be osteoinductive. The infection could have resulted from the implant material, the surgical technique, or trauma to the implant site during the in-life portion of the study.

8.2.1 The individuals performing the assessments shall be trained so they are able to execute the grading methodology with a low degree of inter-evaluator variability. The way this will be achieved is through the use of standard slides appended to this guide or facility established proficiency assessment slides.

8.3 *Example of Quantitative Measurements Using Histomorphometry:*

NOTE 1—This section gives one method by which new bone formation can be quantitiatively assessed. Other methods are available in the literature (1, 2, 5). Other methods can be validated against the method provided herein.

8.3.1 A minimum of one slide shall be prepared from each of four explants per donor lot.

8.3.2 Insert a five by five (24 mm square) grid into the ocular. Grids for many types of microscopes are available from www.reticles.com or contact the microscope manufacturer for their list of applicable reticles. For example the Olympus BX41, model BX41TF uses the following reticle: U-OCMSQ, ZA3264, 24 mm sq, 5/5x5.

8.3.3 Use the 10× objective for a total magnification of 100× to perform the grading for explants from athymic mice and the 4× (40×) objective for athymic rat explants.

8.3.4 If the presence of bone-forming elements in a particular square is uncertain, the objective may be changed to higher power to make the determination and then returned to the 10/4× objective for continuation of grading of the remaining squares within the grid.

8.3.5 Place the objective over the explant in one of the four corners of the explant.

8.3.6 Each field of view will be examined until the entire explant has been observed and graded as detailed below.

8.3.7 A field of view is identified as the area visualized at 100/40× (defined above), which is contained within the confines of the 5×5 grid.

8.3.8 Each field of view has the possibility of generating a total 25 readings from each of the 25 squares contained within the grid.

8.3.9 All squares within the grid containing ≥50 % implant and/or new bone-forming elements will be examined. The average number of fields of view in an athymic mouse/rat explants are 17 to 20 and take approximately 8 to 10 min, in total, to evaluate. For a square within the grid to be considered eligible for grading, the following must be true:

8.3.9.1 Each square must contain ≥50 % implant bone and/or new bone-forming element(s).

8.3.9.2 A new bone-forming element is defined as: chondroblast/cyte, osteoblast/cyte, cartilage, osteoid, new bone, woven bone, and bone marrow.

8.3.9.3 Examples of grids with analysis are included in Appendix X2.

8.3.10 Squares within the grid containing >50 % of empty space, fibrous connective tissue, skeletal muscle, excipient, additive and/or filler, and so forth, shall not be counted even if they contain new bone. Because a square with >50 % bone may only contain "one chondrocyte," eliminating squares with <50 % bone that may contain bone is scientifically balanced and conservative.

8.3.11 A square shall be considered positive for the presence of new bone-forming elements if it meets the criteria listed in 8.3.9 and it contains at least one bone-forming element.

8.3.12 This balances the possibility of excluding a square that contains new bone-forming elements.

8.3.13 If there are multiple new bone-forming elements observed inside one square, the square is still assigned a value of one (1) for the purposes of summing the total number of squares in all the grids/fields of view examined that contain new bone-forming elements.

8.3.13.1 If a new bone-forming element is found in two squares, that is, the chondrocyte is in both squares, it may only be counted one time.

8.3.14 For each grid, the number of squares containing implant bone and/or bone-forming elements is recorded, summed, and used as the denominator to calculate percent new bone.

8.3.15 For each grid, the number of squares containing bone-forming elements is recorded, summed, and used as the numerator to calculate percent new bone.

8.3.16 Percent new bone formation is calculated as follows:

$$\frac{A}{B} \times 100 \qquad (1)$$

where:

A = total number of squares containing new bone-forming elements, and

B = total number of squares containing implant bone and/or new bone-forming elements.

8.3.17 The average percent new bone calculated for 75 % the lot under examination shall be ≥10 % for the lot to be considered to possess osteoinductive potential. For lot release and shelf life testing, having reproducible amounts of bone in most fields of view is indicative of a product with good osteoinductive potential, reproducible manufacturing, and provides the user with a reasonable assurance that the potential for osteoinduction exists in an athymic rodent model.

8.3.18 The acceptance criterion of a minimum of 10 % new bone was arrived upon based upon the following:

8.3.18.1 At least one to two positive squares per field of view is representative of a product with uniform distribution of biologically active demineralized bone. Mathematically, one positive square per field of view will yield a passing score.

8.3.18.2 A result of 9.9 % shall not be rounded to 10.0 %.

8.3.18.3 For example, if there are 220 squares, a score of ≥10 % can be achieved with 22 squares (usually 17 to 20 fields of view, each comprised of 25 squares per explant) demonstrating new bone-forming elements.

8.3.18.4 (22 / 220) × 100 = 10.0 %.

9. Ranking System

9.1 The use of the ranking system allows for a consistent and normalized reporting method between laboratories to minimize inter-laboratory disparities.

9.2 0 = no implant detected upon explant—fails test.

9.3 1 = <10 % new bone-forming elements—fails test.

9.4 2 = 10 to 20 % new bone-forming elements—passes test.

9.5 3 = 21 to 30 % new bone-forming elements—passes test.

9.6 4 = >30 % new bone-forming elements—passes test.

10. Quantitative Assessment—Validity and Maintenance of Validity for Lot Release Testing (Recommended)

10.1 The histopathologists' assessment shall be verified every four to six months by one of two methods: *(1)* visualization and judging of the percent new bone-forming elements on a minimum of 20 slides evaluated by another histopathologist, encompassing the full range of bone formation not seen before, or *(2)* randomly reevaluating 1 % of the slides they assessed over the preceding four to six months and comparing both sets of results. The histopathologist should score an 85 % or better, by either assessment modality, to continue to perform lot release assessment for bone-forming products. If the histopathologist fails the assessment, s/he shall be retrained and retested on a new set of slides that have been previously evaluated. Within a project the histopathologist shall be blind as to the test articles, any applicable controls, and the requesting institution/company.

11. Examples of New Bone-Forming Elements

11.1 Bone induction is defined as the presence of newly formed bone at the implant site. Newly formed bone shall be defined as the presence of one or more of the following bone-forming elements: chondroblasts, chondrocytes, osteoblasts, osteocytes, cartilage, new bone, and bone marrow. Figs. 2-4 illustrate a typical histological preparation of an explant from the implantation of DBM in a nude mouse. In 2, new bone is clearly visible as pockets of chondrocytes that have the potential to form bone at some later time. In addition, there is newly formed bone, bone marrow, and implant DBM that has been "recellularized."

12. Keywords

12.1 animal model; athymic; bioassay; demineralized bone; osteoconductive; osteoinductive
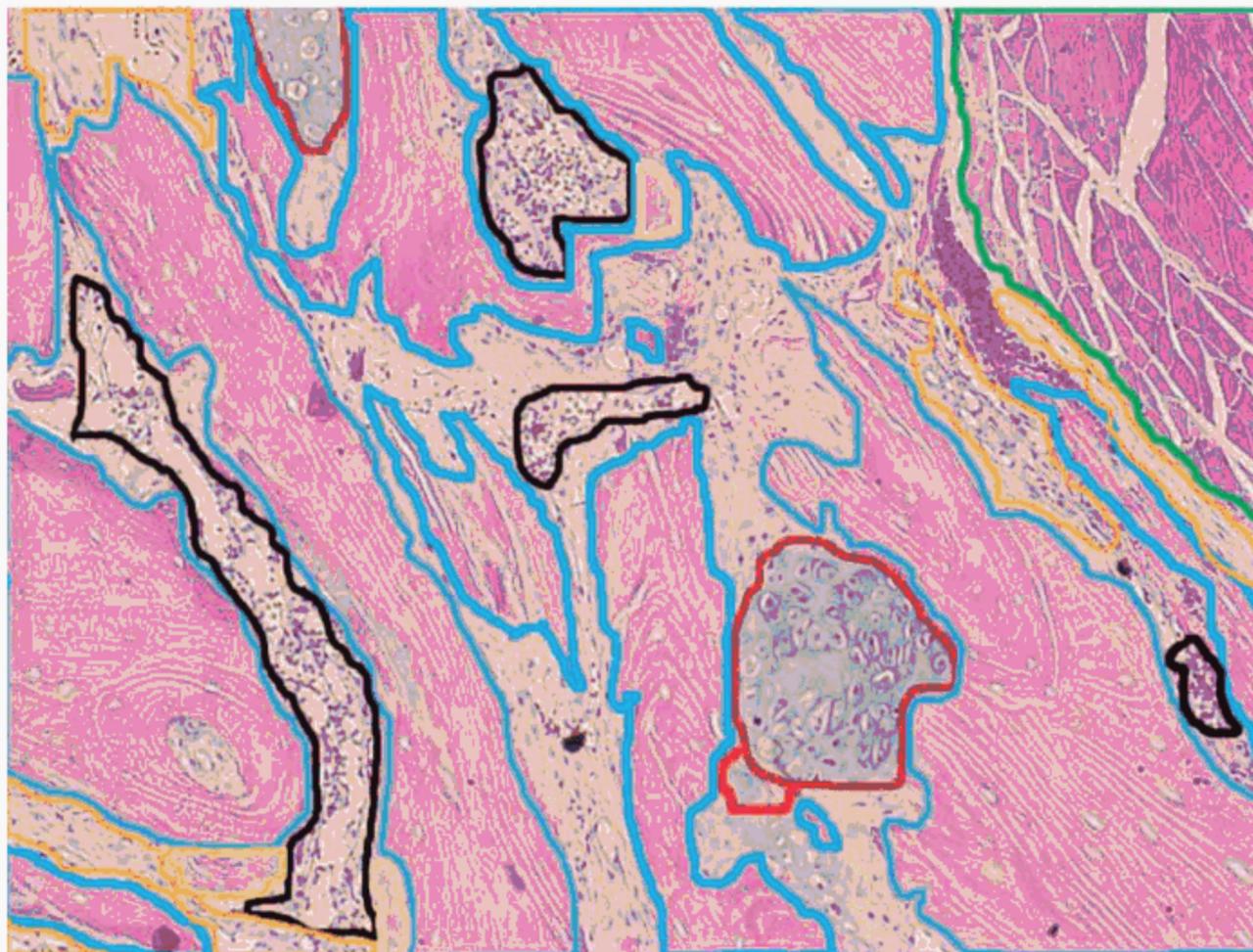
FIG. 2 H&E Stain of Explanted DBM Demonstrating Pockets of Newly Formed Chondrocytes and Bone Marrow (Black – progenitor cells; Blue – implant DBM; Yellow – new bone; Orange – connective tissue; Red – chondrocytes; Green – skeletal muscle)
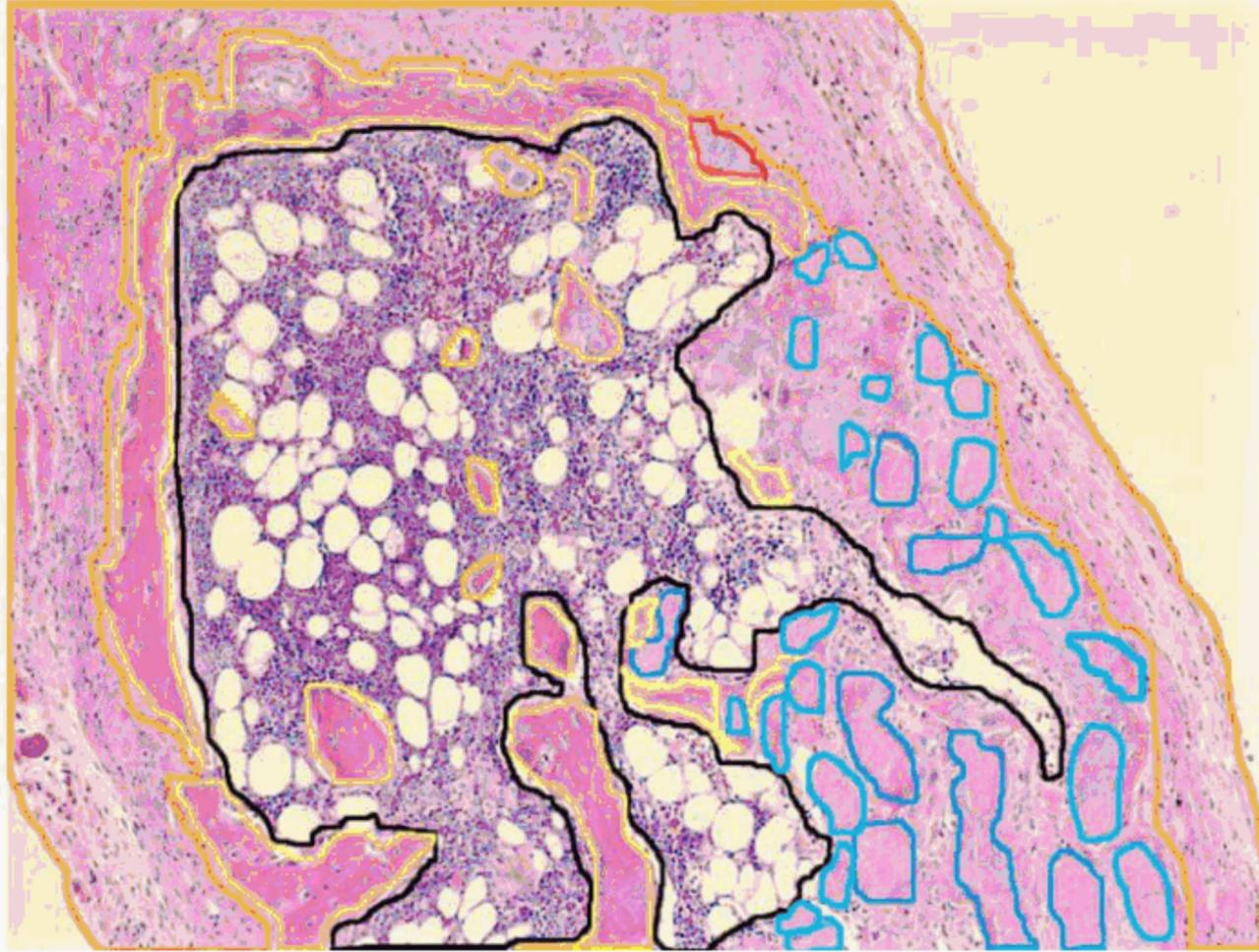
FIG. 3 Histomorphometry of Newly Formed Bone and Implant Bone (Black – progenitor cells and the adipocytes are clear vacuoles contained inside the progenitor cell region; Blue – implant DBM; Yellow – new bone; Orange – connective tissue; Red – chondrocytes)
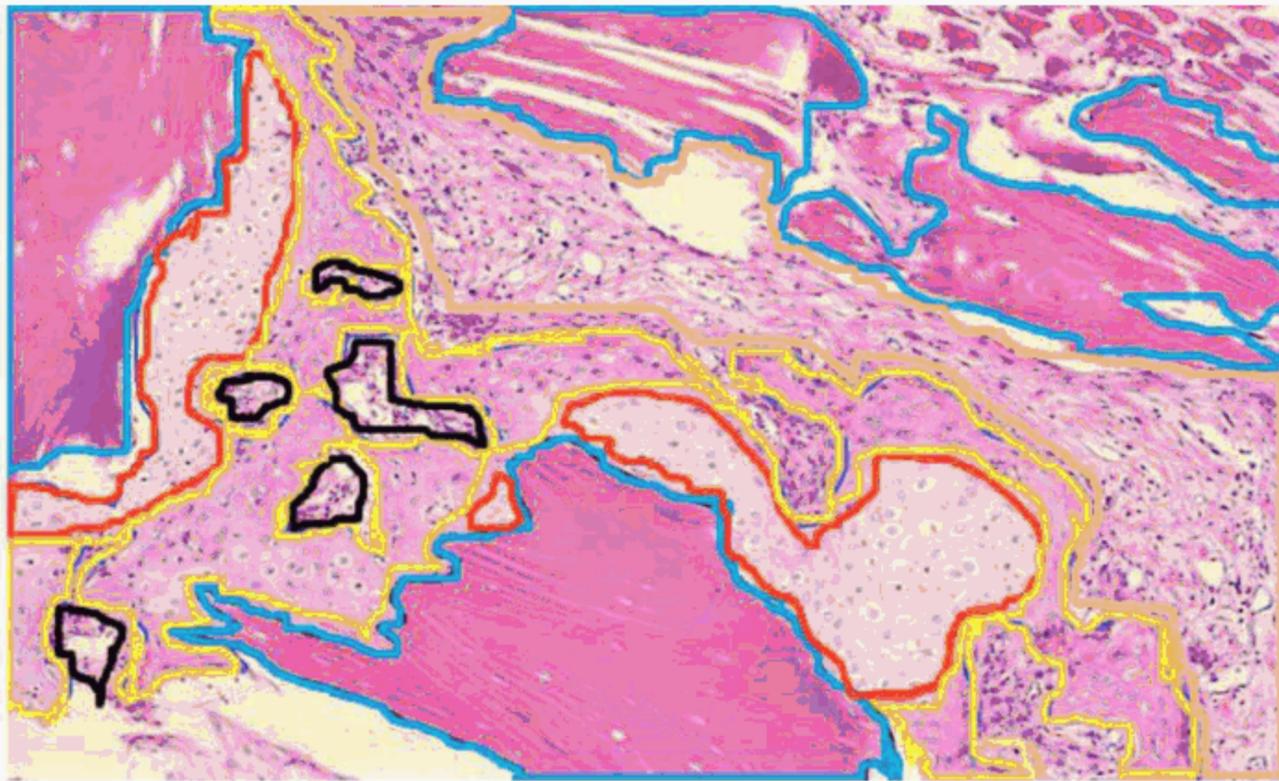

FIG. 4 Example of an Ossicle or Newly Formed Bone Surrounding Bone Marrow (Black – progenitor cells; Blue – implant DBM; Yellow – new bone; Orange – connective tissue; Red – chondrocytes)

APPENDIXES

(Nonmandatory Information)

X1. CHARACTERIZATION

INTRODUCTION

This appendix is provided to provide example guidance on how to control some potential sources of inter-lot and intra-lot variability.

X1.1 Excipient/Additive/Filler Characterization

X1.1.1 Excipients, additives, and fillers can be added to aid, for example, handling, radioprotection, and bulking. However, they are not intended to cause cells to transform lineage but they may possess osteoconductive properties. Every effort should be made to use materials that meet at least one of the criteria listed below. Items on the FDA list of items "Generally Regarded as Safe (GRAS)" should not be presumed to be safe for use in bone-forming products, and their safety and efficacy must be determined within the specific product and place(s) of intended use.

X1.1.1.1 United States Pharmacopeia (USP).

X1.1.1.2 American Chemical Society (ACS).

X1.1.1.3 Ultra pure, >99 %.

X1.1.1.4 FDA Master File.

X1.1.1.5 Water – Type II of Specification D1193 – 1199.

X1.1.2 Additionally, excipients, additive,s and fillers should be characterized for their intended purpose, the quality of the source material, and function in the product by means such as:

X1.1.2.1 pH – physiological, if applicable.

X1.1.2.2 Characterization of set times, if applicable.

X1.1.2.3 Radioprotective capabilities.

X1.1.2.4 Preservation capabilities.

X1.1.2.5 Bulking agent characteristics such as viscosity.

X1.2 Demineralized Bone Characterization

X1.2.1 The various attributes described below have been demonstrated in the literature (see Bibliography) to affect the performance of DBM:

X1.2.2 *Particle/Fiber Size*—A specification that details the acceptable parameters for particle size distribution or fiber dimensions, or both, is recommended. The method used to generate and/or partition particles and/or fibers should be validated to meet the written specification reproducibly. One reason for this validation is that the particle/fiber size distribution may impact the reproducibility of the demineralization process and its ability to meet the manufacturer's residual weight percent calcium specification at the least. If it is not possible to validate the sizing process, then a written rationale should be constructed that addresses why the inability to validate this process does not negatively affect the form, fit, or function of the bone-forming product.

X1.2.3 *Weight Percent Residual Calcium*—The average weight percent residual calcium in each lot of DBM post-demineralization should be determined prior to being mixed with an excipient. The weight percent residual calcium must be less than or equal to 8 % per the American Association of Tissue Banks standards for DBM. The method used to determine the weight percent calcium shall be validated to be accurate and precise such that potential interfering substances present in the DBM have been demonstrated not to interfere with the accurate determination of the calcium content in the DBM. For instance, the phosphate component of DBM is a known interfering substance in atomic absorption spectroscopy; this can be overcome with the use of lanthanum (Test Method E508 and Test Method D5056). Additionally, there should be a validated sampling plan that addresses how many samples, from how many locations, must be obtained and assessed to statistically ensure that the entire lot of DBM meets the manufacturer's calcium specification.

X1.2.4 *Residual Moisture*—The American Association of Tissue Banks has established the 6 % criterion for freeze-dried products. For products in which the DBM is freeze-dried and provided separately from the excipient or as a freeze-dried product, the residual moisture shall be maintained at or below 6 % for the shelf life of the product. To that end, a validated accurate and precise method should be employed to ascertain the residual moisture for each lot of DBM and proof that acceptable residual moisture is maintained for the shelf life of the product. Several methods are available including, but not limited to, Karl Fischer, gravimetric loss upon drying, or thermogravimetric (6) methods. Additionally, there should be a validated sampling plan that addresses how many samples, from how many locations, must be obtained and assessed to statistically ensure that the entire lot of DBM meets the manufacturer's residual moisture specification over time.

X1.2.5 *Product Characterization Rationale*—While some of these assessments may seem removed from osteoinductive activity, ensuring that each of these elements listed below is addressed will aid in ensuring the form, fit, and function of the bone-forming product and that the design and manufacturing specifications are met by the final bone-forming product on a consistent basis as outlined in 21 CFR Parts 820 and 1271.

X1.2.5.1 *Donor Eligibility.*

X1.2.5.2 *Viral Inactivation.*

X1.2.5.3 *Sterility.*

NOTE X1.1—It is not possible to obtain meaningful data from USP <71> or 21 CFR 610.12 sterility testing performed on products terminally sterilized to meet a sterility assurance level of ten to the minus six. This is because the rate of false positives/negatives for USP sterility testing is 1:1000 and therefore does not provide a sensitive enough test for these types of products short of testing in excess of a million products. However, end point sterility testing is a regulatory requirement for licensed biologics.

X1.2.5.4 *Endotoxin.*

X1.2.5.5 *Biocompatibility.*

X1.2.5.6 *Shelf Life*—At a minimum, the shelf life of the product is typically assessed by confirming the ability to form bone in a heterotopic implant site for the duration of the marketed shelf life. If accelerated aging testing is used, it shall always be validated with real time data.

X2. EXAMPLE OF GRIDS

X2.1 See Figs. X2.1-X2.3.
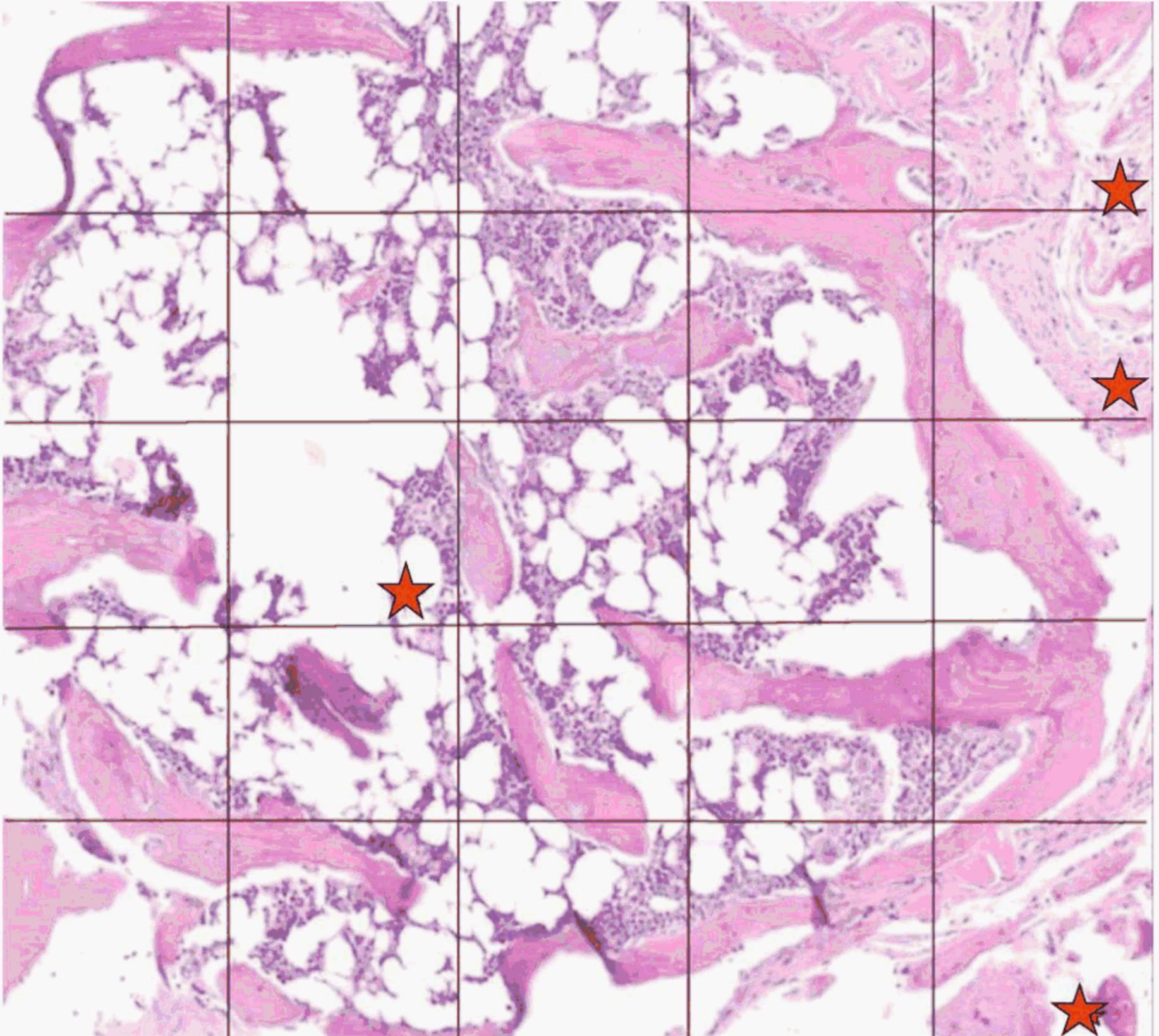
FIG. X2.1 DBM Fibers, H&E, 100×. Red stars = not counted squares. Total squares = 21. Squares with new bone formation = 21.
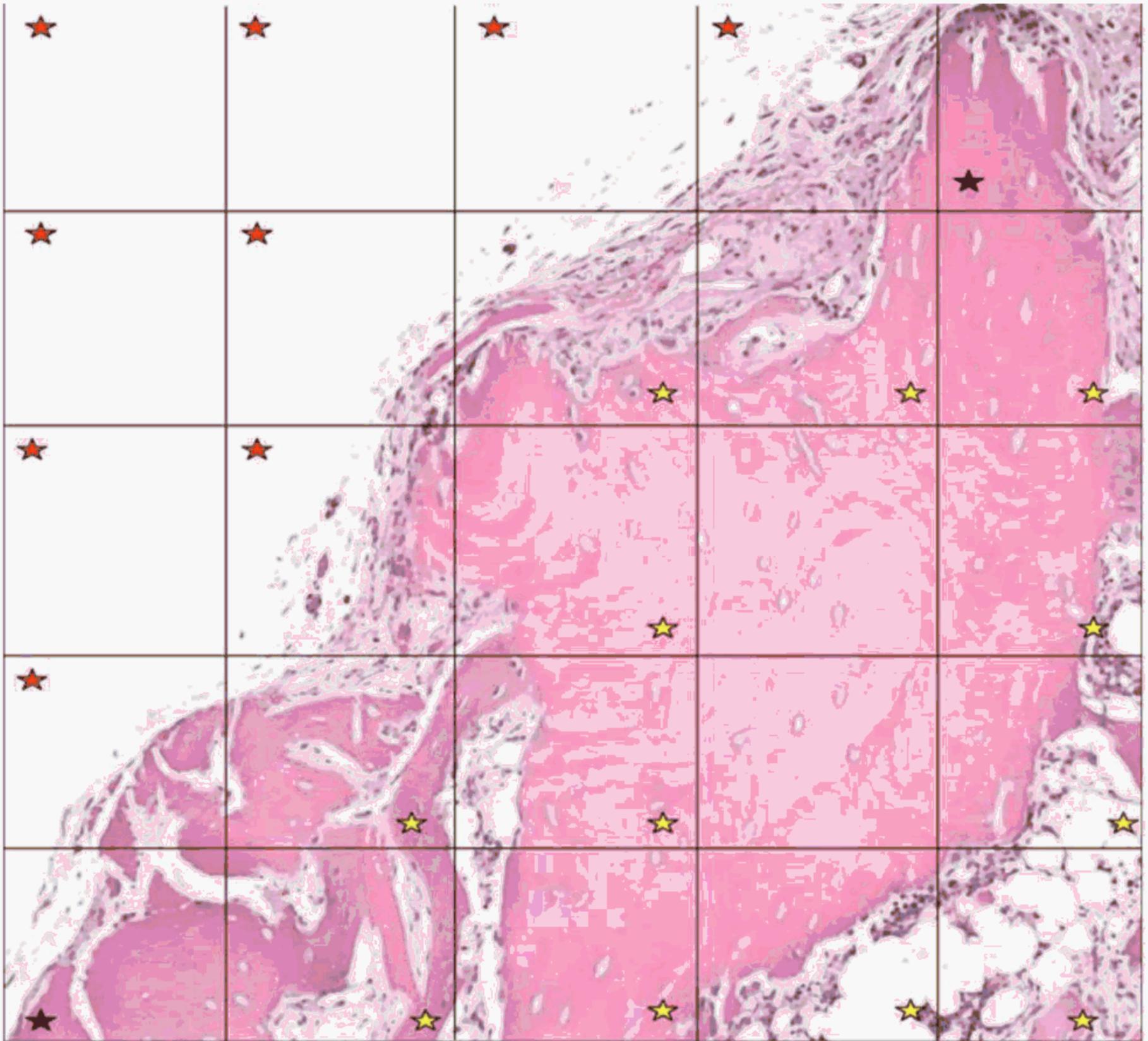
FIG. X2.2 DBM Explant, H&E, 100×. Red stars = not counted squares. Black stars = squares that defined beginning of grading. Total squares = 16. Squares with new bone formation (yellow stars) = 12.
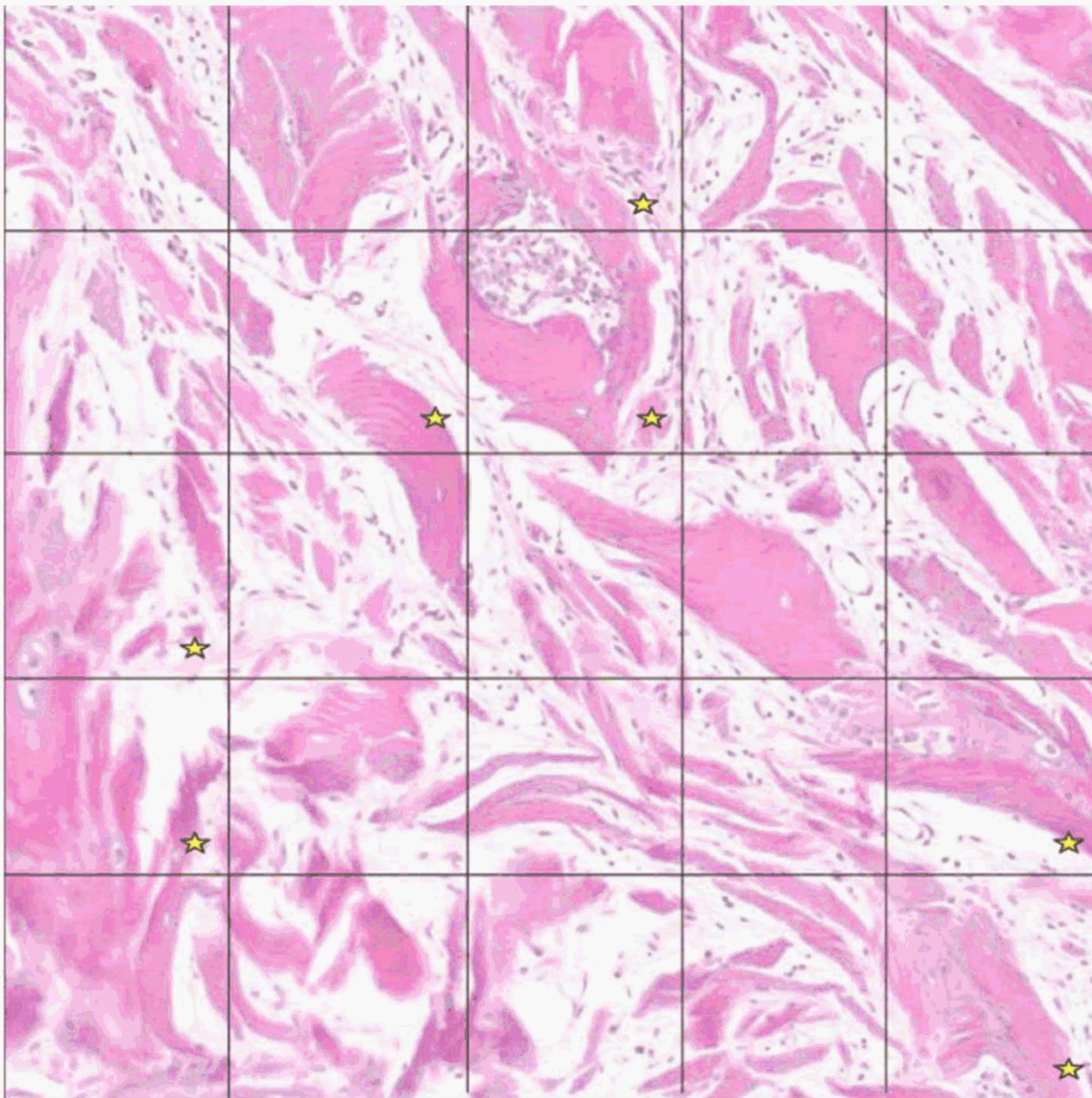
FIG. X2.3 DBM Fibers, H&E, 100×. Total squares = 25. Squares with NB (yellow stars) = 7.

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