



Designation: F2131 – 21

Standard Test Method for *In Vitro* Biological Activity of Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) Using the W-20 Mouse Stromal Cell Line¹

This standard is issued under the fixed designation F2131; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ϵ) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This test method describes the method used and the calculation of results for the determination of the *in-vitro* biological activity of rhBMP-2 using the mouse stromal cell line W-20 clone 17 (W-20-17). This clone was derived from bone marrow stromal cells of the W++ mouse strain.²

1.2 This test method (assay) has been qualified and validated based upon the International Committee on Harmonization assay validation guidelines³ (with the exception of inter-laboratory precision) for the assessment of the biological activity of rhBMP-2. The relevance of this *in-vitro* test method to *in-vivo* bone formation has also been studied. The measured response in the W-20 bioassay, alkaline phosphatase induction, has been correlated with the ectopic bone-forming capacity of rhBMP-2 in the *in-vivo* Use Test (UT). rhBMP-2 that was partially or fully inactivated by targeted peracetic acid oxidation of the two methionines was used as a tool to compare the activities. Oxidation of rhBMP-2 with peracetic acid was shown to be specifically targeted to the methionines by peptide mapping and mass spectrometry. These methionines reside in a hydrophobic receptor binding pocket on rhBMP-2. Oxidized samples were compared alongside an incubation control and a native control. The 62, 87, 98, and 100 % oxidized samples had W-20 activity levels of 62, 20, 7, and 5 %, respectively. The incubation and native control samples maintained 100 % activity. Samples were evaluated in the UT and showed a similar effect of inactivation on bone-forming activity. The samples with 62 % and 20 % activity in the W-20 assay demonstrated reduced levels of bone formation, similar in level with the

reduction in W-20 specific activity, relative to the incubation control. Little or no ectopic bone was formed in the 7 and 5 % active rhBMP-2 implants.

1.3 Thus, modifications to the rhBMP-2 molecule in the receptor binding site decrease the activity in both the W-20 and UT assays. These data suggest that a single receptor binding domain on rhBMP-2 is responsible for both *in-vitro* and *in-vivo* activity and that the W-20 bioassay is a relevant predictor of the bone-forming activity of rhBMP-2.

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

1.5 *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.*

1.6 *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. Terminology

2.1 *rhBMP*—recombinant human bone morphogenetic protein.

2.2 *GDF*—growth and differentiation factor.

3. Summary of Test Method

3.1 In this test method, the mouse stromal cell line W-20-17 is used as a target cell line for rhBMP-2. The W-20-17 cells exhibit increased alkaline phosphatase activity in response to rhBMP-2. Optical density at 405 nm of the p-nitrophenol generated from the alkaline phosphatase substrate is used as a measure of alkaline phosphatase enzyme level. The test method is performed in a 96-well plate format. A similar test

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² Thies, R. S., Bauduy, M., Ashton, B. A., Kurtzberg, L., Wozney, J. M., and Rosen, V., "Recombinant Human Bone Morphogenetic Protein-2 Induces Osteoblastic Differentiation in W-20-17 Stromal Cells," *Endocrinology*, Vol 130, 1992, pp. 1318–1324.

³ Guideline for Industry, ICH-Q2A Text on Validation of Analytical Procedures, November 1996, International Committee on Harmonization, March 1995, <http://www.fda.gov/cder/guidance/index/htm>.

method based upon the same cell line has been developed using chemiluminescent detection of alkaline phosphatase.⁴

4. Significance and Use

4.1 Although the test method can be used for assessment of the bioactivity of crude preparations of rhBMP-2, it has only been validated for use with highly pure (>98 % by weight protein purity) preparations of rhBMP-2.

5. Interferences

5.1 There have been no systematic studies of interfering substances for this test method. There is anecdotal evidence that trypsin and some rhBMP-2 formulation buffers can interfere with the assay. Additionally, the source of fetal bovine serum is an important variable. Each lot should be tested in all parts of the assay where it is required to determine the appropriateness of the lot. This is particularly important if the fetal bovine serum vendor is changed.

6. Apparatus

- 6.1 Polypropylene conical tubes, 15 mL and 50 mL.
- 6.2 Cryovials (Corning or equivalent), sterile, 2 mL.
- 6.3 Eppendorf vials, sterilized.
- 6.4 Variable pipets, (range 20 to 1000 µL) and Multichannel pipets (range 50 to 300 µL).
- 6.5 Biosafety cabinet.
- 6.6 96-Well flat bottom sterile tissue culture microtiter plates, Falcon 3072 or equivalent.
- 6.7 IEC Centra-7R Centrifuge, or equivalent.
- 6.8 CO₂ humidified tissue culture incubator.
- 6.9 Spectrophotometric microplate reader, VMAX/ Spectramax, Molecular Devices, or equivalent.
- 6.10 Hemacytometer, or automatic cell counter.
- 6.11 Inverted microscope.
- 6.12 Tissue culture flasks, Falcon T175 or equivalent.
- 6.13 Sterilized paper towels, or equivalent.
- 6.14 Sterile filter units, 0.2 µm.
- 6.15 Sterile pipets, 1 mL, 5 mL, 10 mL, 25 mL, 50 mL.
- 6.16 9 in. Pasteur pipets, sterilized.
- 6.17 Sterilized pipet tips, (1 to 300 µL and 200 to 1000 µL).
- 6.18 Sterile reagent reservoirs.
- 6.19 –80 °C freezer.
- 6.20 96-Well U-bottom polypropylene sterile tissue culture microtiter plates, Costar 3790 or equivalent.
- 6.21 Water bath.
- 6.22 Orbital shaker.

⁴ Blum, R. S., Li, R. H., Mikos, A. G., and Barry, M. A., "An Optimized Method for the Chemiluminescent Detection of Alkaline Phosphatase Levels During Osteodifferentiation by Bone Morphogenetic Protein 2," *Jour. Cellular Biochem*, Vol 80, 2001, pp. 532–537.

7. Reagents and Materials

- 7.1 W-20-17 mouse stromal cells.⁵
- 7.2 Dulbecco's modified Eagle's medium with 4500 mg/L glucose and 4.0 mM L-glutamine, without sodium bicarbonate (DME/High, JRH Biosciences, 56439 or equivalent).
- 7.3 Sodium bicarbonate (Sigma-Aldrich S4019 or equivalent).
- 7.4 5 M hydrochloric acid.
- 7.5 Heat inactivated (Hi) fetal bovine serum (FBS).
NOTE 1—Each new lot of fetal bovine serum must be evaluated in the assay before use.
- 7.6 200 mM L-Glutamine (Invitrogen Life Technologies, 25030081 or equivalent).
- 7.7 Gentamicin Gibco sterile filtered: 10 mg/mL or equivalent.
- 7.8 Penicillin Streptomycin (PS), contains 10 000 units of penicillin (base)/mL and 10 000 µg of streptomycin (base)/mL, utilizing penicillin G (sodium salt) and streptomycin sulfate in 0.85 % saline (Invitrogen Life Technologies, #15140122 or equivalent).
- 7.9 Phosphate buffered saline, calcium and magnesium free, 1x (PBS-CMF), (Invitrogen Life Technologies (cat. #20012050 or equivalent).
- 7.10 Dimethyl sulfoxide (DMSO), cell culture grade (Sigma-Aldrich or equivalent).
- 7.11 Trypsin-EDTA (0.05 % trypsin, 0.53 mM EDTA · 4Na) (1X), liquid (Invitrogen Life Technologies 25300054 or equivalent).
- 7.12 Glycine (Sigma-Aldrich or equivalent).
- 7.13 Sodium hydroxide (NaOH) 0.2 N and 10 N.
- 7.14 Triton X-100 (J.T. Baker Cat. No. X198-05 or equivalent).
- 7.15 Magnesium chloride, crystalline (MgCl₂ · 6 H₂O).
- 7.16 p-Nitrophenol phosphate (PNPP, Sigma-Aldrich 104(R) phosphatase substrate, product # 1040 or equivalent).
- 7.17 NaCl.
- 7.18 Purified water.
- 7.19 rhBMP-2, 1st WHO Reference Reagent 1997 (5000 units per ampoule, cat. # 93/574, National Institute for Biological Standards and Control).⁶

⁵ This cell line has been deposited in mid-2001. The sole source of supply of the apparatus known to the committee at this time is American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, U.S., <http://www.atcc.org>. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,¹ which you may attend.

⁶ The sole source of supply of the material known to the committee at this time is National Institute for Biological Standards and Control (NIBSC), Blanche Ln., South Mimms, Potters Bar, Herts, EN6 3QG, U.K., <http://www.nibsc.ac.uk>. If you are aware of alternative suppliers, please provide this information to ASTM International Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,¹ which you may attend.