Designation: F2131 – 02(Reapproved 2007)$^{\varepsilon 1}$

Standard Test Method for *In Vitro* Biological Activity of Recombinant Human Bone Morphogenetic Protein-2 (rhBMP-2) Using the W-20 Mouse Stromal Cell Line[1]

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.

ε^1 NOTE—Formatting and grammar were corrected editorially throughout in April 2007.

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 guidelines[3] (with the exception of interlaboratory 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.

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 and health practices and determine the applicability of regulatory limitations prior to use.*

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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[1] This test method 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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[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*, 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 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 *CO_2 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,* (1-300 µL and 200-1000 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.*

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).[6]

7.3 Sodium bicarbonate (Sigma—Aldrich S4019 or equivalent).[7]

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).[8]

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).[8]

7.9 Phosphate Buffered Saline, Calcium and Magnesium Free, 1x (PBS-CMF), (Invitrogen Life Technologies (cat. #20012050 or equivalent).[8]

7.10 Dimethyl sulfoxide (DMSO), cell culture grade (Sigma-Aldrich or equivalent).[7]

7.11 Trypsin-EDTA (0.05 % trypsin, 0.53 mM EDTA · 4Na) (1X), liquid (Invitrogen Life Technologies 25300054 or equivalent).[8]

7.12 Glycine (Sigma —Aldrich or equivalent).[7]

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).[9]

7.15 Magnesium Chloride, Crystalline ($MgCl_2 \cdot 6\ H_2O$).

7.16 p-Nitrophenol phosphate (PNPP, Sigma—Aldrich 104(R) phosphatase substrate, product # 1040 or equivalent).[7]

7.17 NaCl.

7.18 Purified water.

[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.*, 80, 2001, pp. 532-537.

[5] This cell line has been deposited in mid-2001 at the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, U.S., http://www.atcc.org.

[6] This medium has been found satisfactory for this purpose and is available from JRH Biosciences, P.O. Box 14848, Lenexa, KS 66215, U.S., http://www.jrhbio.com.

[7] This material has been found satisfactory for this purpose and is available from Sigma-Aldrich Corp., 3050 Spruce St., St. Louis, MO 63103, U.S., http://www.sigmaaldrich.com.

[8] This material has been found satisfactory for this purpose and is available from Invitrogen Life Technologies, 1600 Faraday Ave., P.O. Box 6482, Carlsbad, CA 92008, U.S., http://www.invitrogen.com.

[9] This material has been found satisfactory for this purpose and is available from J. T. Baker, (Mallinckrodt Baker, Inc.), 222 Red School Ln., Phillipsburg, NJ 08865, U.S., http://www.jtbaker.com.

7.19 rhBMP-2, 1st WHO Reference Reagent 1997 (5000 Units per ampoule, cat. # 93/574, National Institute for Biological Standards and Control).[10]

7.20 rhBMP-2 internal control, >1 mg/mL (stored at −80°C).

8. Procedure

8.1 *Solution Preparation:*

8.1.1 *DME Low Bicarb:*

8.1.1.1 Dissolve 66.87 g DME/High and 11.13 g sodium bicarbonate in 4.5 L of purified water.

8.1.1.2 Adjust the pH to 7.3 ± 0.10 with 5 M HCl and bring solution to 5 L with purified water.

8.1.1.3 Filter through a 0.2 µm filter into sterile bottles.

8.1.1.4 Store at 2 to 8°C. The solution expires in 8 weeks.

8.1.2 *Hi FBS:*

8.1.2.1 Thaw the desired amount of FBS at ambient temperature, or 2 to 8°C.

8.1.2.2 Adjust the water bath to a temperature of 56 ± 2°C.

8.1.2.3 Place the bottle of FBS into the water bath so that the entire contents of the bottle are immersed in water.

8.1.2.4 Heat the bottle for 45 min, swirling periodically.

8.1.2.5 Remove the bottle from the water bath and allow to cool to room temperature. Aliquot 50 mL of the FBS in sterile 50-mL conical tubes.

8.1.2.6 Label each container with name, lot number, expiration date, and the heat inactivation date. Store at −20 ± 10°C or 2 to 8°C.

8.1.3 *Growth Medium:*

8.1.3.1 Combine the following components in the corresponding proportions (v/v):

Component	Proportion (% v/v)	Example: 500 mL (mL)
DME Low Bicarb	85.5	427.5
Hi FBS	10.0	50.0
L-Glutamine (200 mM)	4.0	20.0
Gentamicin	0.5	2.5

8.1.3.2 Filter through a 0.2 µm filter and store at 2 to 8°C in a sterile container.

8.1.4 *Assay Medium:*

8.1.4.1 Combine the following components in the corresponding proportions (v/v):

Component	Proportion (% v/v)	Example: 1000 mL (mL)
DME Low Bicarb	87.0	870.0
Hi FBS	10.0	100.0
L-Glutamine (200 mM)	2.0	20.0
Penicillin/streptomycin	1.0	10.0

8.1.4.2 Filter through a 0.2 µm filter and store at 2 to 8°C in a sterile container.

8.1.5 *NaCl, 0.9 % w/v:*

8.1.5.1 Dissolve 9 g NaCl in approximately 800 mL of purified water and bring to a final volume of 1 L with purified water.

8.1.5.2 Filter through a 0.2 µm filter and store in a sterile container at room temperature.

8.1.6 *12.5 % Triton X-100:*

8.1.6.1 Mix 12.5 mL Triton X-100 with 87.5 mL of 0.9 % NaCl.

8.1.6.2 Filter through a 0.2 µm filter and store in a sterilized container at room temperature.

8.1.7 *Freezing Medium:*

8.1.7.1 Prepare freezing medium immediately before the freezing procedure by adding DMSO to growth medium (see 9.1.3) to 20 % v/v.

Component	Proportion (% v/v)	Example: 100 mL
Growth Medium	80	80 mL
DMSO	20	20 mL

8.1.8 *Glycine Buffer:*

8.1.8.1 Dissolve 0.75 % (w/v) glycine in required volume of purified water. Adjust the pH of the solution to 10.3 ± 0.1 with 10 *N* NaOH.

8.1.8.2 Add 0.8 % (v/v) of 12.5 % Triton X-100.

8.1.8.3 Add 0.13 % (w/v) $MgCl_2 \cdot 6H_2O$ and mix well.

Component	Example: 1000 mL
Glycine	7.5 g
$MgCl_2 \cdot 6H_2O$	1.3 g
12.5 % Triton X-100	8.0 mL
Water	To 1000 mL

8.1.8.4 Filter through a 0.2 µm filter and store in a sterile container at room temperature. The solution has a one month expiration.

8.1.9 *Assay Mix:*

8.1.9.1 Take a sufficient volume of the glycine buffer to cover developing needs (that is, 5 mL glycine buffer per plate).

8.1.9.2 Add 0.34 % (w/v) p-nitrophenol phosphate within one (1) h of use and mix well.

NOTE 2—The assay mix must be made on day of use.

Component	Example: 50 mL for 10 plates
Glycine buffer	50 mL
PNPP substrate	170 mg

8.2 *Cell Line Storage and Cell Banking Procedure:*

8.2.1 Store the cells in 1 mL aliquots in 2 mL cryovials at 5 × 10^5 cells/mL in freezing medium (see 8.1.7).

8.2.2 Prepare cells to make a working cell bank (100+ vials).

8.2.3 Thaw the vial of W-20-17 cells obtained from ATCC or other source following the procedure described in 8.3.

8.2.4 In order to obtain the expected cell number, subculture the cells by expanding them through one or two additional passages (repeat steps in 8.3).

NOTE 3—The viability should be in the range ≥80 %.

8.2.5 Determine the number of vials to be made based on total cell number obtained following procedure 8.2.2. Label the appropriate number of cryovials as follows:

Cell Line Name WCB
Passage Number
Freezing Date
Preparation Reference Number
Initials

8.2.6 Decap the cryovials in the biosafety cabinet.

8.2.7 Dilute the cell suspension to one half the appropriate volume with 2 to 8°C cold freezing medium without DMSO. Volume should be one half of the appropriate volume for the desired cell suspension for freezing. The second half of the

[10] This material has been found satisfactory for this purpose and is available from the National Institute for Biological Standards and Control (NIBSC), Blanche Ln., South Mimms, Potters Bar, Herts, EN6 3QG, U.K., http://www.nibsc.ac.uk.