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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 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.

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. 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.

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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 *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 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.*

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 ($MgCl_2 \cdot 6\ H_2O$).

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]

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[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.

[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.

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 eight 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 ± 5 °C or 2 to 8 °C.

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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

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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 8.1.3) 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 the 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 1.0×10^6 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 American Type Culture Collection (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 ≥85 %.

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. The volume should be one half of the appropriate volume for the desired cell suspension for freezing. The second half of the cold freezing medium should be made with culture medium (see 8.1.7, 20 % DMSO). The final DMSO concentration shall be 10 %.

8.2.8 Slowly add the half-volume of culture medium with 20 % DMSO to the other half of the volume of the cell suspension.

8.2.9 Using a sterile pipet, transfer 1 mL of cell suspension to each of the labeled cryovials on ice. Repeat until all vials are filled. Gently mix the cell suspension during the filling process to prevent settling of the cells.

NOTE 4—The period of time from the addition of the DMSO-containing medium to the start of the freezing process should not exceed 45 to 60 min.

8.2.10 Transfer the cryovials to an insulated box or rack. Store the box or rack at −80 °C for 20 to 24 h.

8.2.11 After 20 to 24 h, transfer the vials to a liquid nitrogen dewar or freezer.

8.2.12 Perform test thaws to check the viability and assay performance of cells.

NOTE 5—It is recommended to perform mycoplasma and sterility testing on the new bank.

8.3 *Preparation of Cells for the Assay:*

8.3.1 Vials of cells (W-20-17, stored in liquid nitrogen) are quick thawed in a 37 ± 2 °C waterbath. The contents of the vials are combined in a 15-mL conical tube and mixed thoroughly. The total volume is recorded.

8.3.2 An aliquot of the undiluted cell suspension is taken and used to make a 1 in 3 dilution in trypan blue (that is, 50 µL cells + 150 µL trypan blue). Add 10 mL growth medium to the remaining cells. Live cells (that is, cells not stained by trypan blue) are counted in all four outer squares on both sides of the hemacytometer. To calculate the number of cells per mL of cell suspension, use the following example:

Live cells: 233; Dead cells: 20

$$(233 \text{ live} \div 253 \text{ total}) \times 100 = 92\ \% \text{ viable cells}$$

$$\frac{233\ (\text{number of live cells counted}) \times 3\ (\text{dilution factor})}{8\ (\text{number of outer squares counted})} = 87.375$$

$$87.375 \times 10^4 \text{ cells/mL (accounts for dimensions of hemacytometer)} = 8.7 \times 10^5 \text{ cells/mL}$$

$$8.7 \times 10^5 \text{ cells/mL} \times 2.5 \text{ mL (volume of undiluted cells)} = 2.2 \times 10^6 \text{ total number of cells}$$

8.3.3 A final concentration of 2×10^5 cells per flask is required. The concentration is achieved by appropriate dilutions of the cell suspension using growth medium.

8.3.4 To calculate the number of flasks and the appropriate volume of cells to be used, use the following example with cell numbers from 8.3.2.

$$2.2 \times 10^6 \text{ total cells} \div 12.5 \text{ mL (volume of diluted cell suspension)} = 1.8 \times 10^5 \text{ cells/mL}$$

$$(\text{volume of diluted cell suspension} = \text{volume of undiluted cells} + 10 \text{ mL growth medium})$$

8.3.5 To determine the volume of cell suspension to be added to each of the calculated number of flasks, use the following example with the numbers from 8.3.4.

$$2 \times 10^5 \text{ cells per flask} \div 1.8 \times 10^5 \text{ cells/mL} = 1.1 \text{ mL per flask}$$

$$30 \text{ mL per flask} - 1.1 \text{ mL cells} = 28.9 \text{ mL growth medium per flask}$$

8.3.6 Place the flasks in a 37 ± 2 °C, 5 ± 1.0 % CO_2 humidified incubator for four days.

NOTE 6—A final volume of 30 mL per T175 flask is required.

NOTE 7—Do not prepare partial flasks, that is, less than 2×10^5 cells/flask.

8.4 *Plating of Cells:*

8.4.1 After four days, aspirate the medium from the flasks.

8.4.2 Add 10 mL PBS-CMF to each flask; swirl and lay the flasks flat to cover the monolayer. Remove the PBS-CMF.

8.4.3 Add 10 mL trypsin to each flask; swirl and lay the flasks flat, making sure the monolayer is coated. Allow the flasks to sit for approximately 5 min to detach the cells from the surface.

8.4.4 Add 20 mL of growth or assay medium to each flask. Mix by swirling the flasks. Transfer the contents of the flasks to sterile 50-mL conical tubes. Rinse the flasks with 10 mL of either medium and add to the 50-mL conical tubes containing the cells. Centrifuge at 1200 rpm for 10 min.

8.4.5 Aspirate the supernatant off the pellets.

8.4.6 Loosen the pellets by tapping on the bottom of each tube. Resuspend the cells in the first 50-mL conical tube with the appropriate volume of assay medium calculated from the following equation:

$$\text{Volume of media} = \frac{(\text{number of tubes} \times 2) + 2 \text{ mL}}{2}$$

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8.4.7 Transfer the contents of the first tube to the second tube, resuspending the cells. Continue to transfer the cell suspension from tube to tube until all pellets are combined.

8.4.8 Add the same volume of assay medium calculated in 8.4.6 to the first tube to rinse the tube. Transfer this medium from tube to tube to rinse.

8.4.9 An aliquot of the cells is taken from the cell suspension and diluted 1 in 2 in trypan blue (that is, 50 µL cells + 50 µL trypan blue) and the live cells are counted on a hemacytometer using trypan blue exclusion. The cells/mL of the cell suspension is calculated using the method described in 8.3.2.

8.4.10 The appropriate number of plates, with a cell concentration of 5×10^4 to 10×10^4 cells/mL in a volume of 20 mL/plate, is calculated using the following equation:

$$2.0 \times 10^6 \text{ cells/mL (conc. of cells)} \times 11.2 \text{ mL cells (volume of cells)} = 2.2 \times 10^7 \text{ cells}$$

$$2.2 \times 10^7 \text{ cells (total \# of cells)} \div 10 \times 10^4 \text{ cells/mL (desired conc.)} = 220 \text{ mL (volume of cells at } 10 \times 10^4 \text{ cells/mL) (220 mL)/(20 mL/plate)} = 11 \text{ plates}$$

8.4.11 The appropriate volume of assay medium is added to the cells to achieve the final volume of diluted cell suspension calculated in 8.4.10.

8.4.12 Add 200 µL of diluted cell suspension to each well of the appropriate number of flat-bottomed tissue culture microtiter plates calculated in 8.4.10. Let the plates sit on a non-vibrating surface at room temperature for 20 min prior to incubation.

NOTE 8—Do not prepare partial plates.

8.4.13 Place the plates in a 37 ± 2 °C, 5 ± 1.0 % CO_2 humidified incubator overnight.