Designation: F2131 − 02 (Reapproved 2012)

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

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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*, 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.

[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.

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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).

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.

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[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.

7.19 rhBMP-2, 1st WHO Reference Reagent 1997 (5000 Units per ampoule, cat. # 93/574, National Institute for Biological Standards and Control).[6]

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 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 ≥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. The volume should be one half of the appropriate volume for

[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.

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 Two 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 2 dilution in trypan blue (that is, 50 µL cells + 50 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:

$$\frac{233\ (\text{number of live cells counted}) \times 2\ (\text{dilution factor})}{8\ (\text{number of outer squares counted})} = 58.25$$

$$58.25 \times 10^4\ \text{cells/mL}\ (\text{accounts for dimensions of hemacytometer}) = 5.8 \times 10^5\ \text{cells/mL}$$

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.

$$5.8 \times 10^5\ \text{cells/mL} \times 2.5\ \text{mL cells}\ (\text{volume undiluted cells from s00109}) = 1.4 \times 10^6\ \text{cells recovered}$$

$$(1.4 \times 10^6\ \text{cells})/(2 \times 10^5\ \text{cells/flask}) = 7\ \text{flasks}$$

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.

$$12.5\ \text{mL cells}\ (\text{diluted volume of cells from s00110})/7\ \text{flasks}\ (\text{from s00112}) = 1.8\ \text{mL cells/flask}$$

Add 28.2 mL growth medium

+ 1.8 mL cell suspension to each of 7 flasks

8.3.6 Place the flasks in a 37 ± 2°C, 5 ± 0.5 % CO_2 humidified incubator for 4 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 4 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:

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

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 cells/mL in a volume of 20 mL/plate, is calculated using the following equation:

$$1.3 \times 10^6\ \text{cells/mL}\ (\text{conc. of cells}) \times 11.2\ \text{mL cells}\ (\text{volume of cells}) = 1.5 \times 10^7\ \text{cells}$$

$$1.5 \times 10^7\ \text{cells}\ (\text{total \# of cells})/5 \times 10^4\ \text{cells/mL}\ (\text{desired conc.}) = 300\ \text{mL}\ (\text{volume of cells at } 5 \times 10^4\ \text{cells/mL})\ (300\ \text{mL})/(20\ \text{mL/plate}) = 15\ \text{plates}$$

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 ± 0.5 % CO_2 humidified incubator overnight.

8.5 *Preparation of an* In-Vitro *Assay Standard:*

8.5.1 The current in-house reference batch of rhBMP-2 is aliquoted into sterile vials at a concentration of >1 mg/mL and stored at −80 ± 8°C.

8.5.2 The activity of the working bioassay standard is monitored through the evaluation of the standard curve parameters. If the analysis indicates that the material has changed, the working standard may be requalified or, if appropriate, a new standard obtained.

8.6 *Assay Plate Preparation:*

8.6.1 Each assay plate includes a standard curve, an internal control, test samples, and media blanks (background).

NOTE 9—A log of the internal control activity result is maintained to monitor trends in the assay and in activity of the material as a measure of consistency.

8.6.2 Thaw a vial of rhBMP-2 internal control (a representative in-house reference material) and international reference reagent and samples.

8.6.3 All test samples are diluted off-plate in assay medium to 2-fold above the final concentration (highest point of standard curve).

8.6.4 Add 165 µL assay medium to all the wells of the polypropylene dilution plate, except the first wells of the dilution series (row B, columns 2 through 11, Fig. 1).

8.6.5 Add 215 µL of reference material, internal control, or sample in duplicate (all at 1369 ng/mL) to the first well of the dilution series (row B).

8.6.6 Transfer 50 µL from the first well into the second well of the dilution series containing 165 µL assay medium and mix well. Continue through the remaining wells of the dilution series, discarding 50 µL from the final volume of the last well in the series. The final volume in all wells will be 165 µL.

8.6.7 At time of use, gently invert the assay plates containing the adhered cells to discard the medium. The assay plates are then blotted on sterilized paper towels to remove any excess medium.

8.6.8 100 µL of assay medium is added to each well of the assay plate (which results in a final 1 in 2 dilution).

8.6.9 100 µL per well is transferred from the polypropylene dilution plate to the assay plate containing the adhered cells using a multichannel pipet. Transfer is accomplished by transferring one row at a time from the dilution plate to the corresponding row of the assay plate.

8.6.10 The plates are placed in a humidified incubator at 5 ± 0.5 % CO_2, 37 ± 2°C for 24 ± 4 h.

8.7 *Takedown of Plates:*

8.7.1 Invert the plates to gently discard the medium from the plates and blot on paper towels to remove any excess medium. Add 200 µL of PBS-CMF to all wells. Invert the plates to discard the PBS-CMF from the plates and blot again.

8.7.2 Add 50 µL of purified water to all wells of the plates. Place the plates at −80 ± 8°C to freeze.

8.7.3 Remove the plates from the freezer and allow to thaw at room temperature. Once thawed, return the plates to −80 ± 8°C and freeze.

NOTE 10—The plates can remain in the freezer overnight after either the first or second freeze, until ready to develop.

8.8 *Developing of Plates:*

8.8.1 The plates are thawed at room temperature after the second freeze.

8.8.2 Once the plates have thawed completely and reached room temperature, add 50 µL of assay mix to each well of the assay plate and place on an orbital shaker.

8.8.3 Take periodic outside diameter (OD) readings of the plates at 405 nm on the VMAX microplate reader and return to the orbital shaker.

8.8.4 When the highest reading of the standard curve is at least 1.00 OD units above background, add 100 µL of 0.2 *N* NaOH to each well of the assay plate.

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<tr><td>A</td><td colspan="12">EMPTY</td></tr>
<tr><td>B</td><td rowspan="7">B
K
G</td><td colspan="2" rowspan="7">Reference Material
Start at 1369 ng/mL, serial dilute 4.3-fold thru 7 wells</td><td colspan="2" rowspan="7">Internal Control
Start at 1369 ng/mL, serial dilute 4.3-fold thru 7 wells</td><td colspan="2" rowspan="7">Sample 1
Start at 1369 ng/mL, serial dilute 4.3-fold thru 7 wells</td><td colspan="2" rowspan="7">Sample 2
Start at 1369 ng/mL, serial dilute 4.3-fold thru 7 wells</td><td colspan="2" rowspan="7">Sample 3
Start at 1369 ng/mL, serial dilute 4.3-fold thru 7 wells</td><td rowspan="7">E
M
P
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Y</td></tr>
<tr><td>C</td></tr>
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<tr><td>F</td></tr>
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NOTE 1—Empty wells contain assay medium.

FIG. 1 96-Well Plate Assay Template

8.8.5 Immediately read each plate at 405 nm, exporting the raw data to a data disc.

9. Calculation of Results

9.1 *Activity Determination*—The activity of the sample is determined using the "four parameter logistic equation" model for regression analysis (see below). Non-linear regression should be performed on the data using suitable data analysis software. The program should initially generate the four parametric fit for the reference material as a standard curve alone, then run a parallelism test comparing the standard curve with each sample curve by fitting all available data points for each sample (full curve). Four parameter logistic equation:

$$Y = ([A - D]/[1 + (X/C)^B]) + D \tag{1}$$

where:

A = maximum optical density,
B = slope of the curve,
C = rhBMP-2 concentration that corresponds to the ED50 point, and
D = minimum optical density.

9.2 The activity of each sample relative to the reference material is calculated.

9.3 Assays that do not meet the acceptance criteria are considered invalid and shall be repeated.

10. Precision and Bias

10.1 *W-20 Acceptance Criteria*—The W-20 acceptance criteria should be established for each laboratory based on intralaboratory precision for specific reference material and internal control. Tolerance intervals should be calculated for the relevant parameters. The essential parameters that are required to demonstrate consistency of the assay performance are activity values of internal control; slope and asymptotes of standard, internal control and sample curves. A test of parallelism (equidistant) of the sample and internal control curve and reference standard curve also needs to be included in assay acceptance criteria.

10.2 *Statement of Precision*—The precision of the W-20 assay was calculated based on historic database and expressed as cumulative assay variability. Precision was calculated using activity value of internal controls from 1032 assays performed at Genetics Institute in Bioassay laboratory during period of two years.

10.3 *Intermediate Precision*—The cumulative variability was expressed as the coefficient of variation (% CV) of the mean activity value of internal control, % CV = 22.

10.4 *Interlaboratory Precision*—Interlaboratory variation has not yet been determined for this test method. An interlaboratory study may be useful in the future, depending upon the need for widespread implementation of this test method.

10.5 *Statement on Bias*—There is no estimate of bias. The results are relative to a reference material.

11. Keywords

11.1 *in vitro* bioactivity assay; recombinant human bone morphogenetic protein; W-20 cell line

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