Chapter 9

Gel-Based Gelatin Zymography to Examine Matrix Metalloproteinase Activity in Cell Culture

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Abstract

Gelatin zymography, first described by Heussen and Dowdle in the 1980s, is a widely used valuable tool in research and diagnostics. The technique identifies gelatinases by the degradation of their preferential substrate as well as by their molecular weight (kDa). We here describe detailed methodology for the detection of pro- and active- forms of both MMP-2 (gelatinase A) and MMP-9 (gelatinase B) in cells using norepinephrine-stimulated H9c2 cardiomyoblasts as model. An easy to follow step-by-step protocol has been carefully written for reliable results. We also suggest an acceptable method for quantification of gelatin zymograms.

Key words Cell culture, Extracellular matrix, MMPs, Gelatinases, Gelatin zymography, Proteolytic activity

1 Introduction

Matrix metalloproteinases (MMPs) are a family of at least 26 endopeptidases (23 identified in humans) that act as key players in extracellular matrix (ECM) remodeling in various physiological and pathological conditions [1]. This class of Zn^{2+}- and Ca^{2+}-dependent proteases is intricately involved in physiological processes such as embryogenesis and angiogenesis as well as pathological conditions including tumor metastasis, inflammation, arthritis, chronic obstructive pulmonary disease (COPD), and cardiovascular complications like hypertrophy, stroke, and heart failure [2]. These regulatory proteases have been subdivided into collagenases (MMP-1, MMP-8, MMP-13, and MMP-18), gelatinases (MMP-2 and MMP-9), stromelysins (MMP-3, MMP-10, MMP-11, and MMP-12), matrilysins (MMP-7 and MMP-26), membrane-type MMPs (MMP-14, MMP-15, MMP-16, and MMP-25), and others, based on their substrate specificity [3]. Structurally, MMPs are multi-domain proteins consisting of four distinct domains: an

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amino terminal hydrophobic pro-domain, a Zn^{2+}-containing catalytic domain, a flexible hinge region, and a carboxy terminal hemopexin-like domain which determines its substrate-specific nature [4]. They are produced in latent inactive form and are hence called "zymogens." Activation of MMPs requires cleavage of its inhibitory N-terminal domain (~10 kDa) [5]. MMP activity is closely regulated by tissue inhibitors of matrix metalloproteinases (TIMPs), a group of four endogenous MMP inhibitors which bind to the catalytic site of MMPs in a 1:1 ratio. An imbalance in MMP-TIMP levels consequently disrupts ECM integrity and thus affects associated biological functions [6, 7].

Gelatinases, MMP-2 and MMP-9, are two members of the MMP family that have been extensively studied owing to their consistent association with a number of physiological and pathological processes. These were originally described as type IV collagenases because of their ability to promote hydrolysis of collagen IV, a major component of ECM [8, 9]. However, they were also found to hydrolyze denatured collagen I (gelatin) efficiently and thus began to be referred as "gelatinases" [10]. This ability of MMP-2 and MMP-9 was suitably harnessed to develop "gelatin zymography," a relatively easy yet powerful technique for detection of MMP activity in biological samples [11]. This technique was utilized by our group, and the important role of curcumin in suppressing gelatinase B mediated norepinephrine induced cardiac stress in H9c2 cells was established [12].

Gelatin zymography is an electrophoretic technique which utilizes sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) wherein gelatin is impregnated in the polyacrylamide gel matrix. The samples are prepared under nonreducing conditions, i.e., without boiling, and any reducing agent like β-mercaptoethanol or dithiothreitol. After the electrophoretic run, the gel is washed off SDS by incubation in nonionic detergent, Triton X-100. The gel is digested in calcium-containing buffer for an optimized length of time such that the partially renatured enzymes degrade gelatin leaving a clear zone on the gel. Subsequently, the gel is stained with Coomassie Brilliant Blue to distinguish areas of digestion against a darkly stained background [13]. A number of zymography techniques have been developed over years and are tabulated in Table 1.

This protocol holds the advantage of detecting both latent and active forms of gelatinases (72 and 64 kDa for MMP-2, 92–95 and 82 kDa for MMP-9). The denaturation in the presence of SDS followed by partial renaturation in the presence of Triton X-100 allows the pro-form to activate and exhibit gelatinolytic activity. Difference in molecular weight between the two forms (pro-form migrates lesser than active form) permits activity by both to be seen on gel. SDS also helps to disrupt any non-covalent interactions

Table 1
Types of zymography and its associated MMPs/TIMPs

S. No.	Zymography	MMP/TIMP	Remarks
Substrate zymography			
1.	Gelatin zymography	MMP-2, MMP-9; MMP-1, MMP-8, and MMP-13 to a lesser extent	Simplest and most widely used type of zymography
2.	Casein zymography	MMP-11, also for MMP-1, MMP-3, MMP-7, MMP-12, and MMP-13	Pre-running of gel to avoid casein gradient in the zymogram is important
3.	Collagen zymography	MMP-1, MMP-13; MMP-2 and MMP-9 may also be detected	Incorporation of native collagen fibers in gel is challenging
4.	Carboxymethylated (CM) transferrin zymography	MMP-3, MMP-7	Usually accompanied with addition of heparin to samples
5.	Fibronectin zymography	MMP-3	Vitronectin and laminin zymography may be done along with it
Modified substrate zymography			
1.	Heparin-enhanced substrate zymography	MMP-7, MMP-1, MMP-13	Addition of heparin to samples during or prior to electrophoresis improves the detection limit for MMP
2.	Reverse zymography	TIMP family (TIMP-1, TIMP-2, TIMP-3, and TIMP-4)	Both substrate (gelatin) and MMP (MMP-2) are incorporated in gel
3.	In situ zymography	Usually performed for MMPs whose fluorophore-conjugated substrates are easily available like FITC-gelatin	Assessment of functional activity on site within the histochemical or cytochemical sections
4.	Real-time zymography	Holds advantage over conventional protocol	Utilizes fluorophore-conjugated substrate to monitor proteolytic activity in real time
5.	Real-time reverse zymography	Holds advantage over conventional protocol	Similar to real-time zymography, gel contains specific MMP along with fluorescent-labeled substrate

between gelatinases and TIMPs [14]. Gelatin zymography is very useful as a qualitative tool for the detection and analysis of the level and type of gelatinases expressed in different cell types/tissues at any time point or after a treatment; however, it lacks the ability to be classified as quantitative. Despite this, owing to its ease-of-conduct and the useful information it generates, it remains a method of

choice among researchers involved in the field of matrix biology [15]. Moreover, a few methods have been devised to quantify zymograms utilizing image processing softwares like Image J, and thus, the technique may be considered as semiquantitative [16].

Here, we provide a detailed protocol to set up gel-based gelatin zymography and guide on sample preparation from cells. We also discuss the detailed procedure for quantification of gelatin zymogram as accepted and used widely.

2 Materials

Make all solutions in ultrapure water by purifying deionized water to attain a sensitivity of 18 MΩ cm at 25 °C (dH_2O). Prepare and store all reagents at room temperature (unless indicated otherwise). Ensure adherence to good laboratory practices (GLPs) while conducting the experiment.

2.1 Cell Culture Components

1. Cell line: Rat heart-derived H9c2 cardiomyoblasts obtained from National Centre for Cell Science (NCCS), Pune (*see* **Note 1**).
2. Growth media (Dulbecco's modified eagle medium (DMEM, pH 7.4)): 25 mM glucose, 4 mM L-glutamine, 1 mM sodium pyruvate, 44 mM sodium bicarbonate, and 11 mM HEPES, supplemented with antibiotic (100 units/mL penicillin and 100 μg/mL streptomycin) and 10% fetal bovine serum (FBS). Prepare, filter, and sterilize the media with a 0.22 μm filter. Store at 4 °C (*see* **Note 2**).
3. Trypsin-EDTA solution: 0.25%, sterile-filtered, suitable for cell culture, 2.5 g porcine trypsin, and 0.2 g EDTA-4NA per liter of Hanks' balanced salt solution (HBSS), with phenol red, pH 7.0–7.6.
4. 1× Phosphate-buffered saline (PBS): 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, and 1.47 mM KH_2PO_4. Adjust to a final pH of 7.4, autoclave, and store at 4 °C for long-term use. Warm to 37 °C before usage.
5. Cell culture flask: Surface area 75 cm^2, canted neck, vented cap, sterile, rectangular bottom, surface treated, polystyrene.
6. Culture dish: Surface area 55 cm^2, inside *D* (diameter) × *H* (height) 100 mm × 20 mm, sterile, round bottom, surface treated, polystyrene.
7. Humidified CO_2 incubator: 5% CO_2, 37 °C.
8. Inverted microscope.

2.2 Total Protein Extraction and Estimation Components

1. Protein extraction buffer: 20 mM HEPES-NaOH (pH 7.9), 20% glycerol, 500 mM NaCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100, and protease inhibitor cocktail (without metalloprotease inhibitors).
2. Protease inhibitor cocktail: For use with mammalian cell and tissue extracts, does not contain EDTA, inhibits serine, cysteine, and acid proteases, and aminopeptidases but not metalloproteinases. Contains individual components, including 104 mM AEBSF (serine protease inhibitor), 80 μM aprotinin (serine protease inhibitor), 4 mM bestatin (aminopeptidase inhibitor), 1.4 mM E-64 (cysteine protease inhibitor), 2 mM leupeptin (serine and cysteine protease inhibitor), and 1.5 mM pepstatin A (acid protease inhibitor). Store at −20 °C.
3. Bradford reagent: Ready to use or prepared as described. For Bradford reagent (1×, 1000 mL), dissolve 100 mg Coomassie Brilliant Blue G250 in 50 mL of 95% ethanol (C_2H_5OH). Add 100 mL of 85% (w/v) *o*-phosphoric acid (H_3PO_4). Now, add 500 mL H_2O to this solution and mix well. Filter the solution using Whatman filter paper and make up the volume to 1000 mL. Store the reagent in amber-colored bottle at 4 °C (*see* **Note 3**).
4. BSA (bovine serum albumin): To be used as standard. Dissolve BSA in ultrapure water to prepare a stock solution of 2000 μg/mL. Serially dilute the stock in water to make BSA dilutions (used as standards)—1500, 1000, 750, 500, 250, 125, and 0 μg/mL (blank). The dilutions can be stored at 4 °C for long (*see* **Note 4**).
5. Refrigerated benchtop microcentrifuge.
6. Ice.
7. 96-well microplate: With/without lid, sterile/non-sterile, flat bottom, polystyrene, capacity ≥300 μL/well.

2.3 Gelatin Zymography Components

1. SDS-PAGE gel: 30% acrylamide, dH_2O, 1.5 M Tris–HCl (pH 8.8)/1 M Tris–HCl (pH 6.8), 10% ammonium persulfate (APS), TEMED, 10% sodium dodecyl sulfate (SDS). For 500 mL acrylamide solution, add 145 g acrylamide (29 parts) and 5 g bis-acrylamide (1 part) to 350 mL dH_2O in the dark and stir the solution until the components dissolve completely. Filter and store at 4 °C in amber-colored bottle. For 100 mL of 1.5 M Tris–HCl (pH 8.8), weigh 18.17 g Trizma base and dissolve in 50 mL water. Set pH to 8.8 with HCl and make up the volume to 100 mL. Similarly, for 100 mL of 1 M Tris–HCl (pH 6.8), weigh 12.114 g Trizma base and adjust pH to 6.8 with HCl. For 10% APS, dissolve 0.5 g APS in 5 mL of dH_2O and store at 4 °C in amber-colored tube. For 10% SDS, add 10 g SDS to 50 mL dH_2O and make up the volume to 100 mL

once the detergent dissolves completely. Composition for 10% resolving gel (20 mL)—6.6 mL of 30% acrylamide, 8 mL dH_2O, 5 mL of 1.5 M Tris–HCl pH 8.8, 200 µL of 10% SDS, 160 µL of 10% APS, and 16 µL TEMED. Composition for 4% stacking gel (5 mL)—650 µL of 30% acrylamide, 3.7 mL dH_2O, 625 µL of 1 M Tris–HCl pH 6.8, 50 µL of 10% SDS, 50 µL of 10% APS, and 5 µL TEMED.

2. Gelatin: From porcine skin, for electrophoresis, Type A (*see* **Note 5**).
3. Lab hot plate: With uniform heating and maintenance of temperature.
4. 1-D protein electrophoresis unit (casting stand, electrophoresis tank along with suitable power pack).
5. 1× SDS-PAGE running buffer (pH 8.3): 192 mM glycine, 25 mM Tris base, 0.1% SDS. Store the buffer at room temperature.
6. Pre-stained protein ladder: 10–250 kDa, for use in SDS-PAGE (*see* **Note 6**).
7. Zymogram sample buffer (2×): 62.5 mM Tris–HCl, pH 6.8, 25% glycerol, 4% SDS, and 0.005% bromophenol blue. Contains no reducing agent. Use one part sample with one part of this dye.
8. Triton X-100: Add 2.5 mL of detergent to 97.5 mL dH_2O to make a final volume of 100 mL (2.5% v/v). Vortex thoroughly to dissolve the detergent (*see* **Note 7**).
9. Zymography digestion buffer: 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM $CaCl_2$, and 0.1% Brij-35. Weigh the components and dissolve in dH_2O. Make up the volume of buffer once all salts dissolve completely. Add 0.1% Brij-35 (nonionic detergent) with a cut tip in the final solution. Use freshly prepared buffer (*see* **Note 8**).
10. Gel rocker/incubator shaker.
11. Coomassie Brilliant Blue staining solution (500 mL): Dissolve 1.25 g Coomassie Brilliant Blue R250 in 225 mL methanol. Add 50 mL of glacial acetic acid and 225 mL of water to the solution. Stir the solution for 2–3 h and filter through a Whatman filter paper. Store the solution at room temperature.
12. Destaining solution (500 mL): Mix methanol (150 mL), glacial acetic acid (50 mL), and water (300 mL) in a ratio of 3/1/6 (v/v/v). Store the solution at room temperature.
13. White light transilluminator.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Cell Culture

Follow GLP guidelines for cell culture techniques (*see* **Note 9**).

1. Seed the suspension of H9c2 cells covering the entire surface of 100 mm culture dish containing 12 mL of growth media (*see* **Note 10**).
2. Gently swirl the plate to allow the cell suspension to settle uniformly in the dish with growth media.
3. Allow the cells to adhere to the surface of culture dish and grow to a confluency of about 60–70% in a humidified CO_2 incubator maintained at 37 °C and 5% CO_2.
4. Give suitable treatment to the cells as per the plan of experiment to be conducted.
5. Harvest the cells for total protein extraction after the treatment. Aspirate the media and add 5 mL of pre-warmed 1× PBS per dish to wash the cells.
6. Remove PBS and add 600 µL pre-warmed trypsin-EDTA solution to each dish. Incubate the dish in the CO_2 incubator at 37 °C for 3–4 min until the attached cells begin to round off and fall from the surface of the dish.
7. Add 5 mL of complete growth media (DMEM with 10% FBS) to stop trypsin activity and flush all the cells to clear the surface.
8. Transfer the cell suspension in a 15 mL centrifuge tube and spin at 200 × *g* for 10 min (*see* **Note 11**).
9. Decant the supernatant and break the pellet before resuspending it in 1 mL of ice-cold PBS. Transfer the contents in a 1.5 mL microfuge tube and spin in a refrigerated centrifuge at 400 × *g* for 10 min to remove traces of any media.

3.2 Total Protein Extraction and Estimation

1. Carefully decant supernatant from the tube and break the pellet before adding prechilled protein extraction buffer (with protease inhibitor cocktail) (*see* **Note 12**).
2. Incubate in ice for 30 min and tap the tube intermittently after every 10 min.
3. Centrifuge at 9600 × *g* for 15 min at 4 °C and collect the supernatant in fresh tube.
4. Aliquot the extracted protein in ice and store at −80 °C for long-term use (*see* **Note 13**).

5. Estimate the protein concentration using the standard protocol followed for Bradford assay using BSA as standard (*see* **Notes 14–16**).

3.3 Gelatin Zymography

1. Prepare zymography sample to be loaded in gel (20–40 μL). Calculate the volume of protein sample in μL required to load at least 30 μg protein in gel based on the estimated concentration. Mix it with suitable amount of zymogram sample buffer so as to bring the dye at 1× working concentration (with respect to the total volume of zymography sample being loaded on gel). Make up the remaining volume with protein extraction buffer. Do not boil the samples. Keep them in ice until loaded (*see* **Note 17**).
2. For gel preparation, weigh 20 mg gelatin and allow it to swell for 15 min in 8 mL of dH_2O being used to prepare 20 mL of 10% resolving gel. Warm it on hot plate at 40–50 °C for 2–3 min or until it dissolves completely. Allow it to cool down before adding rest of the components to prepare the resolving gel (10%). Pour the gel between the plates for casting and allow it to polymerize. Overlay the gel with water-saturated butanol (*see* **Note 18**).
3. Decant the top layer and immediately pour the stacking gel (4%) in the space between the casting plates. Insert a comb so as to make deep wells and allow the gel to polymerize. Ensure there are no air bubbles and at least 1 cm of space for sample to stack properly in the stacking gel.
4. Put the casting unit in the electrophoresis tank filled with SDS-PAGE running buffer and remove the comb carefully. Flush the wells with a syringe before loading the protein sample.
5. Load the samples and pre-stained protein ladder. Run the gel at desirable voltage and current specifications recommended for the electrophoresis unit.
6. Transfer the gel to a box (12 × 10 cm) without cutting the stacking and wash it in 2.5% Triton X-100 (around 50–75 mL for one gel of 8 × 8 cm) for 1 h at room temperature by gently rocking the gel on a rocker (*see* **Note 19**).
7. Transfer the gel to a fresh box (12 × 10 cm) containing zymography digestion buffer (50–75 mL) with/without inhibitor and incubate it at 37 °C for 24 h with constant gentle rocking. Cover the gel box with aluminum foil during incubation (*see* **Notes 20** and **21**).
8. Stain the gel with Coomassie Brilliant Blue staining solution followed by destaining until distinct white bands of MMP activity on gelatin are observed against a blue background of the gel when placed over a white light transilluminator.

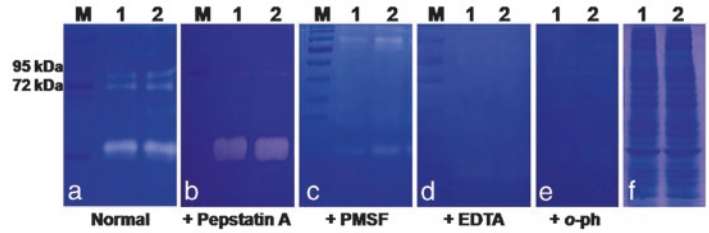

Fig. 1 Gelatin substrate zymography: Total protein samples were subjected to electrophoresis on 10% SDS-PAGE gel containing gelatin (1 mg/mL). Gelatinolytic activity was observed as distinct white bands against a blue background on the gel (M, pre-stained protein ladder; 1, control; 2, norepinephrine treated). (**a**) Gelatin zymogram showing MMP-2 and MMP-9 activity at 72 kDa and 92 kDa, respectively. (**b**) Gelatin zymogram with pepstatin A (aspartyl protease inhibitor) in sample and gel. (**c**) Gelatin zymogram containing PMSF (serine protease inhibitor) in sample and gel. (**d**) Gelatin zymogram with EDTA (divalent cation chelator) in sample and gel. (**e**) Gelatin zymogram with *o*-phenanthroline (zinc chelator) in sample and gel. (**f**) 25 μg of total protein was run on 10% SDS PAGE gel and used as a reference for equal loading

9. Identify the MMP (pro- and active form) based on the molecular weight close to which the activity is observed on the gelatin zymogram (use pre-stained protein ladder as the reference). Also, analyze the zymogram for any difference in MMP activity between the experimental groups taking equal loading SDS-PAGE gel for the same set of samples as a reference/loading control (Fig. 1).

3.4 Gel Scan and Quantification

1. Carefully remove the zymogram from water and place it in a plastic sheet protector.
2. Scan the gel at a resolution of 300 dpi or higher and save the image in ".jpg" format.
3. Open Image J (software can be freely downloaded from http://rsbweb.nih.gov/ij/) and go to "File," then "Open" and select the image to be analyzed. Zoom in or out to an appropriate size.
4. Visualize the image in black and white by setting it to 8-bit format. Go to "Image," then "Type" and select "8-bit."
5. Select the "Rectangular tool" from the tool bar and draw a rectangle along Lane 1 starting from the top of the gel to the bottom.
6. Go to "Analyze," then "Gels" and "Select first lane."
7. Click in the middle of the box drawn over the first lane and move it over to the next lane. Go to "Analyze," then "Gels" and "Select next lane." Repeat this process and make a box along all lanes. All boxes will be at same level vertically.

However, do ensure that there is no spilling over of the box into the next lane horizontally and each box must cover the lane completely.

8. Now, go to "Analyze," then "Gels" and "Plot lanes." A new window showing a plot with intensity profile of the entire lane for each box selected appears on the screen. The area of drop in intensity (light pixels correspond to digested area on gel) is represented as a peak. Identify the peak corresponding to the band of interest and draw a straight line across its base to enclose the area completely. Repeat this process for all lanes.
9. Select "Wand tool" on the tool bar and determine area of each closed peak by clicking on the same. If a lane shows no band at the desired position (absence of MMP activity in the sample), consider the area of peak to be zero.
10. Copy the data in excel, write the lane number and group label across the value and plot it as a bar graph. The data can be normalized to an MMP standard to avoid any gel-gel variation especially when values are being pooled from replicate gels to determine statistical significance of the result.

4 Notes

1. This protocol has been described utilizing embryonic rat heart-derived H9c2 cardiomyoblasts. However, any other adherent/suspension cell type may be used for extraction of total cell protein and conduct of gelatin zymography.
2. The standard guidelines for maintenance of each cell line are detailed by the concerned cell culture repository like American Type Culture Collection (ATCC). DMEM growth medium with the formulation as described in Subheading 2 has been prescribed for H9c2 cells.
3. Bradford solution must be prepared and stored in the dark. The reagent has a reddish brown color at acidic pH (when prepared) which changes to blue on binding to protein. The presence of detergent or alkali in glassware can lead to the reagent turning blue at the time of preparation itself. Such a solution must not be used.
4. BSA standards: Apart from being abundant, affordable, and relatively stable, this protein offers desirable biochemical reactivity with protein estimation methods including BCA, Bradford, and Lowry. It is an intermediately sized protein (66 kDa) containing 607 amino acids which represent all amino acids in a balanced form.
5. Gelatin is a heterogeneous mixture of water-soluble proteins of high average molecular masses, present in collagen. These

proteins are extracted by boiling the relevant skin, tendons, ligaments, bones, or tissue in water. Dry gelatin must be stored in air-tight container at room temperature to maintain its shelf life for years. It is soluble in warm-to-hot water than cold.

6. Pre-stained protein ladder is a mixture of color-stained proteins which are used as size standards in protein electrophoresis (SDS-PAGE). The ready-to-use ladder is supplied in a loading buffer and is directly loaded on gel without boiling. A pre-stained protein marker helps to monitor the progress of gel during electrophoresis and is also visible after Coomassie staining of zymogram which stains the entire gel blue due to the presence of gelatin in the gel. Ensure that the ladder has at least 2–3 red/orange/green bands rather than blue for clarity in gel visualization. The ladder will help estimating the approximate size of separated proteins (MMPs) which have digested gelatin present in the gel and made it visible in the form of white band against blue background.
7. Triton X-100: It is a nonionic mild surfactant that breaks protein-lipid, lipid-lipid associations but not protein-protein interactions. It does not denature protein and thus help obtain it in its native and active form. The detergent is viscous, and hence, a cut tip must be used to add the detergent.
8. Brij-35 is a nonionic light detergent that ensures enzyme stabilization and minimizes the risk of nonspecific interactions.
9. The key for good laboratory practice in cell culture is to ensure that all procedures are carried out to a standard that precludes microbial contamination as well as cross-contamination with other cell lines. Some of the basic precautions are listed as follows: (a) Surface sterilize the work space, materials, and equipments with 70% ethanol and UV before beginning work. (b) Ensure that the growth media and other reagents are sterile and used inside the biosafety cabinet only. Screw the cap of reagent bottle tightly and seal the neck after use. (c) Disinfect all materials before removing them from the hood. Also, surface sterilize the work area with 70% ethanol and UV, after use. (d) Routinely examine the cultures and growth media for evidence of any gross microbial contamination. (e) Clean the cell culture facility regularly. (f) Follow appropriate guidelines for disposal of biological waste (infectious and noninfectious) that include sharps contaminated with biological waste, liquid as well as solid waste.
10. Use ≥3 culture dishes (100 mm) per group in an experiment to get sufficient amount of protein after extraction.
11. The speed of centrifuge must not exceed 400 × *g* for mammalian cells.

12. Add ~150 μL of protein extraction buffer to cell pellet obtained by pooling cells from 3 × 100 mm dishes (60–70% confluent) of each group. Use 1 μL of protease inhibitor cocktail for every 100 μL of protein extraction buffer used or as recommended in the reagent usage instructions.
13. The samples may be stored for long, if aliquoted and stored properly at −80 °C. Avoid freeze thaw cycles. However, it is better to use the protein as fresh as possible. Long-term storage of samples may affect MMP activity.
14. Bradford assay: The basic principle of this colorimetric test lies in complexing of proteins present in the sample with Coomassie Brilliant Blue G under acidic conditions resulting in a color change of the reagent from reddish brown to blue, marked as a shift in the absorption spectrum maximum from 470 nm (cationic/unbound form) to 595 nm (anionic/bound form). The increase in absorption at 595 nm is proportional to the amount of bound dye and thus, to the amount of protein in the sample. Reducing agents such as DTT and β-mercaptoethanol do not cause interference with this reagent. However, the presence of SDS in extraction buffer may affect the assay.
15. Bradford assay protocol: Add 5 μL of BSA standard/sample to each well of 96-well microplate followed by quick addition of 200 μL of 1× Bradford reagent per well in dark. Wrap the plate with aluminum foil and incubate it at 37 °C for 15 min. Read the plate on a spectrophotometer and record A_{595} (absorbance at 595 nm) values. Plot the scatter diagram of the absorbance values obtained for BSA dilutions and use linear regression to determine the standard curve for Bradford assay. This curve will show the relation between protein concentration and A_{595} values in $y = mx + c$ form ($y = A_{595}$; m = slope of the line; x = protein concentration in μg/mL; c = intercept on y-axis). Use this equation to calculate the protein concentration for all unknown samples. Multiply the calculated concentration with appropriate dilution factor, if used.
16. Adjust the initial seeding concentration of cells/number of dishes to be plated per group and protein extraction buffer volume in such a way that the total protein obtained is at a concentration of 6–7 μg/μL. This will ensure that ≥30 μg protein can be conveniently loaded on gel.
17. Also, prepare protein samples using Laemmli sample buffer to run an SDS-PAGE gel in parallel to the zymogram. This buffer contains β-mercaptoethanol and theses samples are boiled before loading. The gel serves as a reference for loading equal amount of protein in all the lanes.

18. Water-saturated butanol (or any organic solvent) helps cut contact of resolving gel with air and thus prevents oxidation. It avoids mixing of trace water with gel, its evaporation leading to shrinkage of gel. This produces a smooth and completely level surface on the top of resolving gel so that bands are straight and uniform.
19. It is advised not to cut the stacking gel after the electrophoresis and retain it till the end of experiment. This portion of gel will help indicate if the gel has been completely destained since it does not contain gelatin and will get clear upon destaining. On the other hand, the resolving gel (containing gelatin) shall be entirely blue even after destaining.
20. *o*-Phenanthroline (1,10-phenanthroline monohydrate) is a metalloproteinase inhibitor that chelates divalent metals like zinc and iron. It is soluble in methanol and can be prepared at a stock concentration of 200 mM. The stock remains stable at −20 °C for months. Effective concentration: 10 mM in sample and 50 mM in digestion buffer. EDTA is also a metalloproteinase inhibitor that chelates divalent ions like Ca^{2+}. It is used at a concentration of 10 mM in sample and 50 mM in digestion buffer. Zinc- and calcium-dependent MMPs show no activity in the presence of these inhibitors.
21. Enzyme (MMP) activity is best observed at 37 °C. Keep the gels in an incubator shaker with controlled temperature and speed if the room temperature is not suitable. Increasing the length of incubation in digestion buffer may also be of help.

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