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Raouf A. Khalil *Editor*

Zymography

Biological and Clinical Applications, Volume 2

Humana Press

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Edited by

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Preface

Zymography is a molecular biology technique to detect enzyme activity in an electrophoretic gel or a tissue section. This simple, easy-to-perform, and cost-effective technique has recently received increasing attention from researchers, scientists, and clinicians. This book on *Zymography: Methods and Protocols* provides up-to-date information on the current knowledge, and latest advances and protocols of the zymography technique. The book includes highly organized chapters with point-by-point description of the steps involved in a successful zymography method, and appropriate data collection, analysis, and interpretation. The step-by-step description of the zymography method is intended to reach a broad spectrum of researchers in the fields of biology, science, and medicine. The book describes how to determine the activity of various isozymes, allozymes, and families of proteinases to advance the fields of enzymology and molecular evolution and provide useful biomarkers for various biological processes, pathological conditions, and clinical disorders. Hence, many scientific and medical fields will benefit from this state-of-the-art bibliographical collection of the zymography technique.

The purpose of this book is to bring together the currently used techniques and the emerging advances in the zymography methods. Hence, the book is of great value to both experts in the field and incoming new scientists aspiring to learn and perform successful zymography technique. All chapters have been written following the guidelines and classic format employed in the *Methods in Molecular Biology* laboratory protocol book series. The chapters start with an Introduction and a brief outline of the basic theory to be analyzed. The Materials Section then lists all chemicals, reagents, buffers, and other ingredients necessary for the experiments. The Methods Section then includes a detailed step-by-step description of the experimental protocol. The chapter then describes the appropriate methods for data collection, analysis, and interpretation. A detailed Notes Section will then describe potential pitfalls and provide useful tips, hints, advice, and troubleshooting for key steps in the experimentation, data collection, analysis, and interpretation, thus complementing the Method section.

Because of the multiple enzymes, substrates, activators, and inhibitors that can be assessed by zymography, and the numerous applications of the technique in science and medicine we divided the special issue into two volumes. Volume I includes four parts (I, II, III, IV) that focus on zymography principles, process, and pitfalls, and Volume II highlights in situ and in vivo zymography, and some of the zymography special applications in biological processes and clinical biomarkers. In Volume II, we present three Parts (I, II, and III). Part I includes chapters that introduces in situ zymography and localization of bright green-fluorescent gelatinase activity in tissue sections, in situ zymography in formalin-fixed paraffin-embedded and mineralized tissues, and in vivo zymography as an essential activity assay for studying the activity of matrix metalloproteinases (MMPs) in a cell-specific manner in the brain. Part II focuses on biological applications of zymography with chapters on fundamentals of zymography and its applications to the study of biological samples, gelatin zymography to quantify MMP-2 and MMP-9 in complex biological specimens, detection of proteolytic enzymes in polyacrylamide gels supplemented with diverse biological substrates, evaluating MMP levels in primary mouse glial cultures, exploring

enzymatic activity in dental research, profiling of digestive proteases in a freshwater invertebrate model, casein zymography for rapid and robust detection of calpain protease activity in meat product, detection of lipases and proteases in crude extracts of microbial proteins, and detection of enzymes from fungal endophytes. Part III focuses on potential clinical applications of zymography with chapters describing assessment of MMP-2 and MMP-9 hydrolytic activity in preclinical and clinical tissue samples, the use of zymography to assess circulating MMP-2 and MMP-9 in plasma and serum, and in pathological conditions, the use of zymography for the detection of bacterial proteases, gelatin zymography of proteases in chronic wound samples, in-gel zymography of amyotrophic lateral sclerosis-associated variants of superoxide dismutase-1, assessing gelatinase activity in normal and disease uterine tissue and cells, gelatin zymography of conditioned media from tumor cell lines, analysis of MMPs secretion in cancer cells, evaluating MMP activity in human hepatocellular carcinoma tissues and cells, and detecting gelatinase activity during invadopodia-mediated invasion by tumor cells.

Importantly, these chapters were written by researchers and scientists from different parts of the world, thus promoting different viewpoints and highlighting different approaches in the preparation of materials, execution of the methods, troubleshooting of potential pitfalls, data analysis, and interpretation of the results. Thanks to the excellent work of the contributing authors, and the careful review of our dedicated reviewers and editors, the book series collated these important topics for our readers in a clear, concise, and informative fashion. I encourage every researcher, clinician, medical, graduate, and undergraduate student with aspiration to learn and utilize zymography in their research to read this state-of-the-art synopsis on the ins and outs of the technique.

I would like to take this opportunity to express my deepest gratitude to Professor John Walker, the Series Editor of *Methods in Molecular Biology*, who gave me the opportunity to organize this topical and timely book on zymography. My special thanks to our outstanding and hard-working Associate Editor Ms. Anna Rakovsky, who spared no effort to ensure the highest quality of the chapters. I also would like to acknowledge our contributing authors for their excellent chapters and for sharing their expertise in providing the readers with helpful notes to ensure proper execution of the technique and obtaining meaningful data. I particularly wish to thank our readers for their interest in zymography. I encourage all of you to provide feedback and contact me directly if you have any questions, comments, suggestions, criticism, or ideas that could further enhance our knowledge to help us achieve our goals and meet the highest expectations of our readers.

Boston, MA, USA ***Raouf A. Khalil***

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Chapter 15

Zymography: A Simple and Powerful Tool for the Assessment of MMP-2 and MMP-9 in Pathological Conditions

Joseph George

Abstract

Zymography is an electrophoretic technique to measure the proteolytic activity of native enzymes on a *nonreducing sodium dodecyl sulfate polyacrylamide* gel. It is a simple and powerful tool to assess the amount of various proteases present in both physiological and pathological conditions. The concerned protease degrades the protein substrate that is incorporated with the gel and resolves during the incubation period. Staining with Coomassie brilliant blue (CBB) reveals the sites of proteolysis as clear white bands. The intensity and area of the bands are linearly related to the amount of protease present in the loaded sample. Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9), also known as gelatinases, have the indigenous property to digest gelatin and several other protein molecules present in the extracellular matrix. Here, we describe the detailed protocols and methods of zymography, with a special emphasis on the determination of gelatinases present in conditioned culture media and tissue extracts.

Key words Zymography, *Polyacrylamide* gel electrophoresis, Matrix metalloproteinases, MMP-2, MMP-9, Gelatinases

Abbreviations

APS:	Ammonium persulfate
ATCC:	American type culture collection
CBB:	Coomassie brilliant blue
DTT:	Dithiothreitol
ECM	Extracellular matrix
EDTA:	Ethylenediaminetetraacetic acid
MCT:	Monocrotaline
MMPs:	Matrix metalloproteinases
MMP-2:	Matrix metalloproteinase-2
MMP-9:	Matrix metalloproteinase-9
PBS:	Phosphate-buffered saline
PMA:	Phorbol-12-myristate-13-acetate
PMSF:	Phenylmethylsulfonyl fluoride

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SDS: Sodium dodecyl sulfate
SDS-PAGE: *Sodium dodecyl sulfate polyacrylamide* gel electrophoresis
TBS: Tris-buffered saline
TEMED: *N*,*N*,*N*′,*N*′-tetramethylethylenediamine
β-ME: β-mercaptoethanol

1 Introduction

Matrix metalloproteinases (MMPs) are a large family of calcium-dependent, zinc-containing endopeptidases involved in tissue remodeling, wound healing, and also in cancer progression. MMPs are responsible for cleaving all structural elements of the extracellular matrix (ECM) as well as a variety of non-ECM proteins [1]. Currently, 28 MMPs have been reported, out of which 23 are expressed in human tissues [2]. There are *24 genes* encoding MMPs in humans, including the duplicated MMP-23 gene. MMPs are secreted in inactive pro-MMP form, which undergoes proteolytic cleavage by various proteinases or other MMPs into an active form that can degrade ECM proteins [3]. MMPs play a prominent role in tissue remodeling in both physiological and pathological conditions [4].

Matrix metalloproteinase-2 (MMP-2) and matrix metalloproteinase-9 (MMP-9) are secreted as inactive enzymes, pro-MMP-2 and pro-MMP-9, with a molecular weight of 72 kDa and 92 kDa, respectively [5, 6]. A cysteine residue in the N-terminal pro-domain binds to the zinc atom at the active site of MMP-9, thus maintaining latency [7]. Activation of MMP-9 requires a disruption of the cysteine interaction with the zinc atom, thus exposing the catalytic site [7]. Multiple ways have been demonstrated for the activation of MMP-2, including thrombin cleavage of the propeptide of MMP-2 at specific sites [8]. The active forms of MMP-2 and MMP-9 are around 64 kDa and 82 kDa molecules, respectively, and are called gelatinases [9]. They have the indigenous property to digest gelatin (denatured collagen) and several other protein molecules present in the ECM. Mouse MMP-9 shares 72% identity and 99% homology with human MMP-9 [1]. Mouse MMP-9 contains 23 extra amino acids, mainly between amino acids 486–501 and 705–711. This results in mouse MMP-9 having an apparent molecular weight of 105 kDa for pro-MMP and around 95 kDa for the active molecule [1, 10, 11].

MMP-2 (gelatinase A, EC 3.4.24.24) and MMP-9 (gelatinase B, EC 3.4.24.35) have been extensively studied due to their consistent association with various cancers. MMP-9 plays a prominent role in tumor cell invasion, metastasis, and angiogenesis in several cancers and is considered a cancer biomarker [12]. The

invasion and metastasis of tumor cells require the degradation of the existing ECM, and therefore, all malignant cells upregulate MMPs, especially MMP-9 [13]. Inhibition of MMP-9 activity is considered a potent strategy to arrest tumor cell invasion and angiogenesis in various cancers [14]. Gelatinases are also upregulated in various other pathological conditions, such as pulmonary arterial hypertension (PAH) [15], *chronic obstructive pulmonary disease* (*COPD*) [16], and liver fibrosis [17], and are targeted for therapeutic applications.

Zymography is a powerful technique to assess the proteolytic activity of native enzymes on a polyacrylamide gel loaded with the specific enzyme substrate [18]. Another prominent feature of the technique is the ability to differentiate between the pro-enzyme and active forms semiquantitatively on the gel [19]. It is important to note that the *sodium dodecyl sulfate polyacrylamide* gel electrophoresis (SDS-PAGE) should be prepared without the addition of reducing agents such as β-mercaptoethanol (β-ME) or dithiothreitol (DTT), usually used in Western blots, in order to reduce the disulfide bridges in proteins for a better separation on SDS-PAGE gels. The addition of a reducing agent might destroy the proteolytic activity of gelatinases and therefore should be avoided. In the zymography for gelatinases, the protein mixture present in the sample is separated on a 10% SDS-polyacrylamide gel copolymerized with 1% porcine skin gelatin and incubated with a suitable buffer containing calcium chloride at 37 °C overnight to digest the gelatin substrate. After fixing and staining the remaining protein on the gel with Coomassie Brilliant Blue R-250 or Amido black, clear visible bands can be observed on areas where MMP-2 and MMP-9, as well as pro-MMP-2 and pro-MMP-9, are present. A detailed protocol to assess the MMP-2 and MMP-9 activity in conditioned culture media, cell lysate, and tissue extracts employing SDS-PAGE gel loaded with gelatin is described in detail below.

2 Materials

2.1 Chemicals and Reagents

1. Pierce Bradford protein assay kit.
2. Phorbol-12-myristate-13-acetate (PMA).
3. Ammonium persulfate (APS), 10% w/v. Always prepare fresh. Otherwise, the gel may not polymerize.
4. Gelatin from porcine skin.
5. TEMED (*N*,*N*,*N*′,*N*′-tetramethylethylenediamine). Store the original bottle in dark at 4 °C.
6. Kaleidoscope prestained protein standards.
7. Sodium dodecyl sulfate (SDS).
8. Ethylenediamine tetraacetic acid (EDTA).

9. Acrylamide-bis-acrylamide: 30% solution [37.5:1 (2.7% cross-linker)].
10. Alternatively, prepare a 30% acrylamide-bis-acrylamide stock solution using 30 g acrylamide and 0.8 g N,N′--methylenebisacrylamide (bis-acrylamide) powder dissolving in distilled water and made up to 100 mL. Store at 4 °C in a dark bottle, which will be stable for up to 6 months (*see* **Note 1**).
11. Separating (lower) gel buffer (4×): (1.5 M Tris–HCl, 0.4% SDS, pH 8.8). Dissolve 182 g of Tris (hydroxymethyl)-aminomethane (Trizma base, Mol Wt. 121.14) in 900 mL of distilled H_2O, adjust pH to 8.8 with concentrated HCl, make up to 1000 mL with H_2O, and then add 4 g of SDS and mix well. It is stable at room temperature.
12. Stacking (upper) gel buffer: (0.5 M Tris–HCl, 0.4% SDS, pH 6.8). Dissolve 30.3 g Tris base in 400 mL of distilled water, adjust pH 6.8 with HCl, add 2 g SDS, and make up to 500 mL.
13. Sample buffer (Laemmli loading buffer)-6×: Mix 7 mL of 4× lower buffer, 3 mL of glycerol, 1 g of SDS, and 1.2 mg of bromophenol blue dye. Store it at –70 °C in 0.5 mL aliquots.
14. Running buffer stock (10×). Dissolve 30.3 g Tris, 144 g glycine, and 10 g SDS in 1 L of distilled water. There is no need to adjust the pH. Add the SDS powder after dissolving Tris base and glycine. Store it at room temperature.
15. Running buffer (1×). Mix 100 mL of 10× running buffer with 900 mL of distilled water to prepare 1× running buffer. It can be stored at room temperature.
16. Triton X-100 (2.5%). Mix 12.5 mL of Triton X-100 with 487.5 mL of distilled water and warm at 45 °C for 15 min to attain equilibrium. It is stable at room temperature for several months.
17. Tris-calcium chloride buffer (developing buffer): Dissolve 8 g of Trizma base (Mol Wt. 121.14) in about 800 mL of distilled water, adjust pH to 7.6 with concentrated HCl, add 1.1 g of $CaCl_2$ and 0.12 g of sodium azide (NaN_3), and make up to 1000 mL with distilled water. It is stable at room temperature.
18. Staining solution: Dissolve 1.25 g of Coomassie Brilliant Blue R-250 in 227 mL methanol, add 46 mL acetic acid, and make up to 500 mL with distilled water. Filter through Whatman No. 1 qualitative filter paper. It is stable at room temperature.
19. Destaining solution: Mix 50 mL methanol, 75 mL acetic acid, and 875 mL distilled water. It is stable at room temperature indefinitely.

20. Renaturing solution (2.5% Triton X-100): Mix 25 ml of Triton X-100 with 975 mL of distilled water, warm at 45 °C for 15 min to attain the equilibrium. It is stable for months at room temperature.
21. Phosphate-buffered saline (PBS): Dissolve 8 g (137 mM) sodium chloride (NaCl), 201 mg (2.7 mM) potassium chloride (KCl), 1.42 g (10 mM) disodium hydrogen phosphate Na_2HPO_4, and 245 mg (1.8 mM) potassium dihydrogen phosphate (KH_2PO_4) in about 950 mL of distilled water. Adjust the pH to 7.4 using concentrated HCl and make up to 1 L. Autoclave the solution and store at room temperature.
22. Tris-buffered saline (TBS): Dissolve 6.057 g Trizma base (50 mM) and 8.766 g (150 mM) sodium chloride in about 800 mL of distilled water, adjust pH to 7.5 with 1 M HCl, and make up to 1 L with distilled water. It is stable at 4 °C for several months.
23. Cell lysis buffer: Dissolve 303 mg Trizma base (25 mM) and 584 mg (100 mM) NaCl in about 80 mL of distilled water, adjust pH to 8.00 with concentrated HCl, add 1 mL of Triton X-100, and make up to 100 mL with distilled water. It is stable at 4 °C for 6 months (*see* **Note 2**).
24. Homogenization buffer: Dissolve 606 mg Trizma base (50 mM) and 876 mg NaCl (150 mM) in about 80 mL of distilled water, adjust pH to 8.00 with concentrated HCl, add 1 mL of Triton X-100, and make up to 100 mL with distilled water. It is stable at 4 °C for several months.

2.2 Apparatus

1. PowerPac Basic Power Supply or equivalent one.
2. Mini-PROTEAN Tetra Vertical Electrophoresis Cell, two-gel, for 1.0 mM-thick handcast gels. This unit includes two-gel vertical electrophoresis system, electrode assembly, tank, lid with power cables, and mini cell buffer dam.
3. Orbital shaker (Digital Mini Rotator) or equivalent.
4. Vortexer, Model BR-2000 or equivalent.
5. Polystyrene petri dishes (cell culture dishes), 150 × 21 mM or equivalent.

3 Methods

3.1 Preparation of SDS-Polyacrylamide Gel

1. Prepare the gelatin solution by dissolving 30 mg of porcine skin gelatin in 7.5 mL of 4× lower gel buffer and 12.5 mL of distilled H_2O (total volume: 20 mL). Incubate at 45 °C for 2 h with intermittent shaking. Make sure that the gelatin has

been dissolved completely. Allow to cool down to room temperature before use. Always prepare fresh (*see* **Note 3**).

2. Fix the electrophoresis glass plates with 1 mM-thick spacers on the casting frame and casting stand. All items are available with the Mini-PROTEAN Tetra Vertical Electrophoresis System.
3. Prepare the 10% separating (lower) gel by mixing 10 mL of 30% acrylamide-*bis*-acrylamide solution, 7.5 mL of 4× lower gel buffer, 12.5 mL of distilled water (use the above prepared 20 mL gelatin solution), 150 μL of 10% freshly prepared APS, and 20 μL TEMED. The total volume would be 30.170 mL. This is enough for four numbers of 1 mm-thick mini gels.
4. Pipette out about 6.5 mL of the prepared separating gel solution into gel cassettes through the sides of the glass plate, avoiding the formation of air bubbles.
5. After loading the separating gel, gently add 1–2 drops of isopropyl alcohol on top of the gel to prevent air contact and allow smooth and uniform gelation at the top.
6. Allow the gel to polymerize for 1–2 h at room temperature. In order to check the polymerization of the gel poured into the cassettes, save the balance of the gel solution in the glass beaker. If the gel has polymerized in the beaker, then it has also polymerized inside the gel plates.
7. Prepare the 4.8% upper stacking gel by mixing 1.6 mL of 30% acrylamide, 2.5 mL of upper gel buffer, 5.9 mL of distilled water, 50 μL of 10% APS (100 mg/mL), and 5 μL of TEMED. This will be enough for four mini gels of 1 mm thickness.
8. Tilt the polymerized gel and gently pipette out the isopropyl alcohol.
9. Place the 1 mm thick 10-well comb between the glass plates at the top. Make sure that it is level.
10. Pour the upper stacking gel at the top of the polymerized separating gel without trapping bubbles on the comb. If an air bubble is noticed, lift up the comb and insert it again, avoiding air bubbles.
11. Allow the stacking gel to polymerize at room temperature for 30–60 min.
12. It is better to use the prepared gels on the same day. The gels can also be stored (with the comb on top) after being wrapped well with cellophane sheet at 4 °C for a few days (*see* **Note 4**).

3.2 Sample Preparation

Serum-Free Conditioned Cell Culture Media

Since serum might contain gelatinases, serum-free conditioned media is necessary for gelatin zymography (*see* **Note 5**).

1. Culture two sets of any tumor cell line of interest in 6-well culture dishes to approximately 80% confluence in regular growth media (*see* **Note 6**).
2. Culture HT-1080 human fibrosarcoma cells in one well as a positive control, which express large quantities of MMP-2 and MMP-9 (*see* **Note 7**).
3. Remove the regular culture media and wash the cells twice with sterile PBS or serum-free media. Add a fixed amount of serum-free media to all the wells.
4. Treat the second set of cells with serum-free media containing PMA at a concentration of 200 ng/mL. Mix 10 μL of PMA (concentration 20 μg/mL) with 990 μL of serum-free media to obtain a concentration of 200 ng/mL. There is no need to treat HT-1080 cells with PMA (*see* **Note 8**).
5. Incubate the cells with a minimum amount of serum-free media at 37 °C in a CO_2 incubator for about 12 h. Certain cell lines may require longer incubation periods (*see* **Note 9**).
6. Collect the media and centrifuge at 500 × *g* for 5 min at 4 °C to remove the debris and floating cells.
7. Collect the supernatant and store at −20 °C for up to 2 days prior to the assay. After thawing, should not freeze the samples again for the next assay.
8. It is advisable to carry out the zymography for gelatinases as early as possible because the enzyme activity could reduce over time, even at −20 °C (Fig. 1).

Figure 2 demonstrates zymography for MMP-9 and MMP-2 (both pro- and active MMPs) in conditioned cell culture media of eight different tumor cell lines. All the cell cultures, including the HT-1080, were treated with 200 nmoles of PMA per ml of serum-free medium.

Preparation of Cell Lysates

Measurement of MMP-2 and MMP-9 activities in cell lysate is useful to study the rate of synthesis and to screen therapeutic agents (*see* **Note 10**).

1. Remove the culture media and wash the cells twice with sterile PBS.
2. Add 0.5 mL of cold lysis buffer to 100 mm culture dishes and 250 μL to each 6-well culture dish.
3. Using a cell scraper, collect the cells completely into the lysis buffer and transfer them to 1.5 mL of Eppendorf tubes.
4. Incubate the lysate in an ice bath for 15 min.

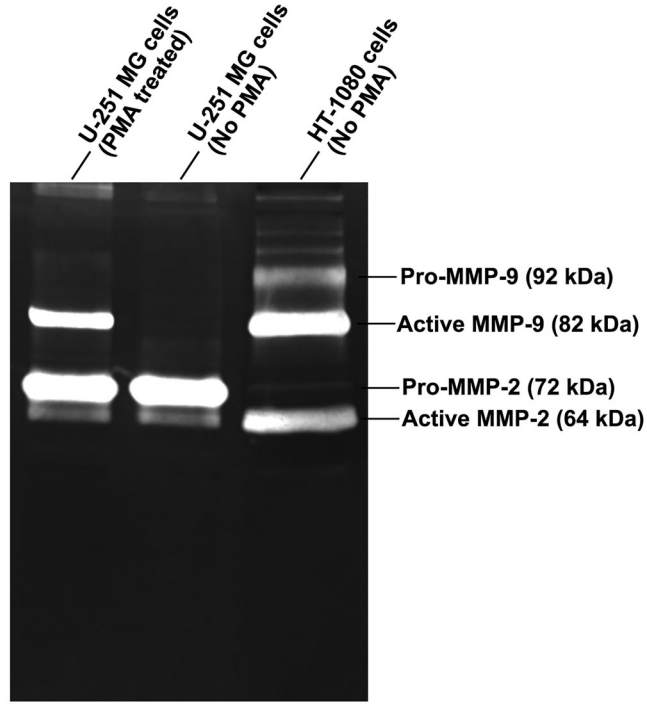

Fig. 1 Gelatin zymography for matrix metalloproteinase (MMP)-9 and MMP-2 activities in conditioned cell culture media of U-251 MG human glioblastoma cell line (ATCC) to demonstrate the stimulatory effect of phorbol-12-myristate-13-acetate (PMA) in the secretion of MMP-9. The U-251 sample depicting active MMP-9 was treated with 200 nmoles of PMA per ml of serum free media for 12 h. The conditioned culture media from the HT-1080 (human fibrosarcoma) cell line without PMA treatment was used as a positive control for MMP-9 and MMP-2 activity (*see* **Note 14**)

5. Vortex each tube for at least 15 sec and centrifuge at 13,000 × *g* for 5 min at 4 °C in a microcentrifuge.
6. Collect the supernatant into new Eppendorf tubes.
7. Measure the protein concentration in the supernatant using the Bradford protein assay or any other appropriate method, and adjust the sample volume accordingly in order to obtain a uniform concentration of protein in each sample.
8. Keep the lysate on ice for immediate assay or store at −20 °C.

Preparation of Tissue Extracts

Measurement of gelatinases in different tissue samples, such as liver or lung, is important to evaluate the upregulation of MMPs in various pathological conditions. Gelatinase activity may be absent or weak in most healthy tissues. HT-1080 cell culture media can be used as a positive control to assess the gelatinase activity in various tissue samples.

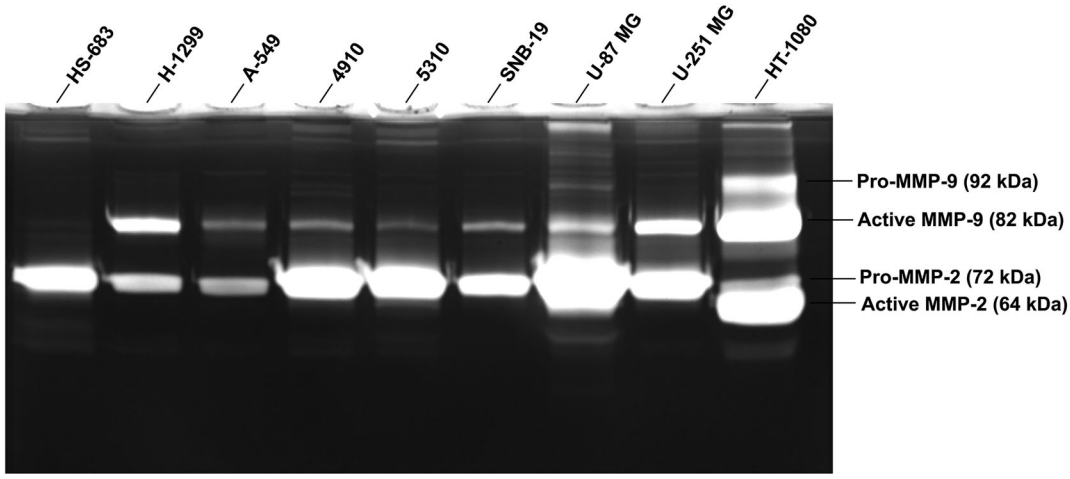

Fig. 2 Gelatin zymography for MMP-9 and MMP-2 in conditioned cell culture media for eight different tumor cell lines in confluent culture (HS-683 human glioma (ATCC); 4910 and 5310 are human glioma xenograft cell lines; SNB-19, U-87 MG, and U-251 MG are human glioblastoma (ATCC); H-1299 and A-549 are human lung carcinoma (ATCC). All the cell cultures were treated with 200 nmoles of PMA per ml of serum-free media for 12 h. HT-1080 (human fibrosarcoma) cell line (ATCC) culture media was used as a positive control for MMP-9 and MMP-2 activity, where both MMP-9 and MMP-2 are highly expressed

1. Weigh 100 mg of fresh lung or liver tissue from mice or rats and add 900 µL of ice-cold homogenization buffer (listed above) and homogenize well with a handheld homogenizer (*see* **Note 11**).
2. Centrifuge the homogenate at 13,000 × *g* for 10 min at 4 °C and collect the supernatant.
3. Determine the protein concentration using the Bradford protein assay or any other suitable method, and adjust the volume of the supernatant accordingly to have a uniform protein concentration in all the samples.
4. Keep the tissue supernatant on ice for immediate gelatinase activity assay or store at −20 °C.

Figure 3 depicts gelatin zymography for MMP-9 and MMP-2 in the lung tissue extracts of C57BL/6 J control and monocrotaline-treated mice. Due to the presence of 23 extra amino acids in the mouse MMP-9 molecule, the apparent molecular weight of pro-MMP-9 is 105 kDa and active MMP-9 is 97 kDa in mice [10, 20]. There is no such difference in the molecular weight of MMP-2 in mice compared to other species.

3.3 Electrophoresis and Staining

1. Mix 25 µL of clear conditioned media or 25 µL of cell lysate (about 20 µg protein) or 25 µL of lung tissue extracts (about 30 µg protein) with 5 µL of 6x sample buffer to obtain a final

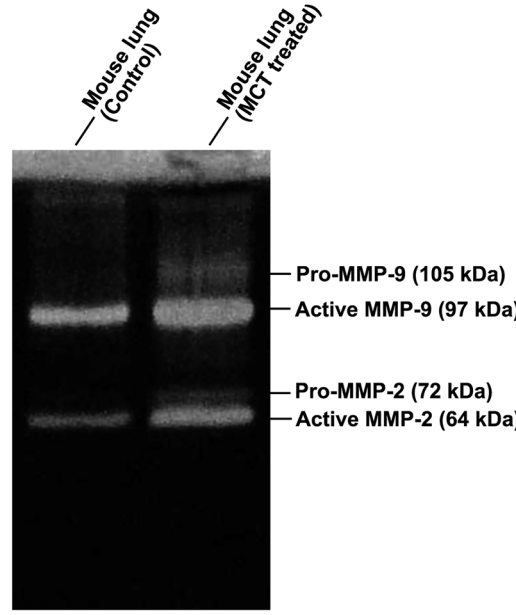

Fig. 3 Gelatin zymography for MMP-9 and MMP-2 in the lung tissue of C57BL/6 J control mice and after the treatment with monocrotaline at a concentration of 60 mg/100 g body weight once a week for eight consecutive weeks to induce pulmonary arterial hypertension. Treatment with monocrotaline increased both MMP-9 and MMP-2 activity

concentration of 1×. Mix thoroughly with a gentle vortex. Do not heat the samples (*see* **Note 12**)

2. Transfer the gel with the glass plates to the vertical electrophoresis chamber and fix it on the stand facing the gel inside. Pour 1× running buffer into the chamber up to the level of the spacing plates.
3. Load 25 μL of sample containing 1× sample buffer in each lane.
4. Run 5–10 μL of Kaleidoscope prestained protein ladder to identify the pro-MMPs and active MMPs based on molecular weights.
5. Close the lid and connect the electrophoresis chamber to the negative and positive poles of the power pack. Run the system for 1–2 h at 100 volts until the bromophenol blue marker dye present in the sample buffer reaches the bottom.
6. After the electrophoresis, carefully remove the spacer plate, and transfer the gel to a 150 mm cell culture plate containing the renaturing solution (2.5% Triton X-100).
7. Remove SDS from the gels with two washes in the renaturing solution for 20 min each at room temperature on an orbital shaker.

8. Incubate the gel for another 30 min in about 50 mL of Tris-calcium chloride buffer (developing buffer) with gentle agitation.
9. Decant the developing buffer and replace the gel with fresh developing buffer. Incubate the gel at 37 °C overnight in a closed tray or culture dish with a lid.
10. Decant the developing buffer and stain the gel with Coomassie Brilliant Blue R-250 for 30–60 min or until uniform staining of the gel. The staining solution could be reused, but it is better to use fresh stain each time (*see* **Note 13**).
11. Destain the gel in the destaining solution until the MMP activity appears as clear, sharp bands.
12. Identify the MMP bands (pro-MMPs and active MMPs) from their ability to digest gelatin and also from their respective molecular weights compared to the protein ladder.

4 Notes

1. Unpolymerized acrylamide is a neurotoxin. Use gloves and mask while preparing and handling the solution.
2. If the cell lysate is going to be used for protein assays (e.g., Western blotting), protease inhibitors such as aprotinin, leupeptin, phenylmethylsulfonyl fluoride (PMSF), and pepstatin may be added to the lysis buffer just before use; otherwise, they are not required.
3. It is important to use high-quality gelatin powder suitable for electrophoresis; otherwise, clear sharp bands may not appear on the gel.
4. If the gels are not wrapped properly in cellophane sheet or stored for longer periods at 4 °C, they might get dried and detach from the glass plate. This may not be visible to the naked eye through the glass. However, while running the gel, this may result in uneven movement of the protein molecules present in the loaded sample. Never store the gels in the freezer compartment.
5. Since gelatinases (MMP-2 and MMP-9) are extracellular enzymes, a prominent amount of the enzyme is secreted into the cell culture medium [21]. Therefore, it is recommended to assay gelatinases in the culture medium rather than the cells.
6. Since many tumor cell lines require activation with phorbol-12-myristate-13-acetate (PMA) to stimulate the secretion of MMPs, especially MMP-9, it is recommended to set up two sets of cultures, one treated with PMA and the other without PMA treatment.

7. HT-1080 cells are highly invasive epithelial cells derived from the connective tissue of a patient with fibrosarcoma. HT-1080 cells can express large quantities of the active form of both MMP-2 and MMP-9 without the mitogenic stimulation of PMA (Fig. 1).
8. Treatment of HT-1080 cells with PMA could produce large amounts of active MMP-2 and MMP-2. Figure 2 demonstrates the production of large quantities of both the active form of MMP-2 and MMP-9 after the treatment with PMA in culture.
9. Most normal cell lines and some tumor cell lines have no effect on PMA treatment with regard to the stimulation and secretion of MMPs in culture. Mostly, it coincides with the invasive properties of the tumor cells. Figure 1 demonstrates the effect of PMA on the secretion of MMP-9 in U-251 human glioblastoma cells maintained in serum-free media for 12 h. The secretion of MMP-9 was completely absent in samples without PMA treatment. However, treatment with PMA has no effect with regard to the synthesis and secretion of MMP-2 (Fig. 1).
10. The determination of MMP-2 and MMP-9 activities in the cell lysate provides valuable information regarding their expression, which plays a pivotal role in tumor cell invasion, metastasis, and cancer progression. In addition, the assessment of gelatinases in the cell lysate is useful to evaluate the effects of MMP inhibitors that would be helpful to arrest tumor cell invasion and angiogenesis [22].
11. Since homogenization can generate heat, hold the samples in an ice bath. Use a powerful homogenizer to reduce the homogenization time.
12. When you study the effect of a compound or drug screening on the activity of MMPs in a tumor cell line, it is not necessary to adjust the protein concentration in the conditioned media, which may affect the results.
13. The staining solution, Coomassie Brilliant Blue R-250, can be reused multiple times. However, the strength of the stain will decrease with every reuse. Since the staining solution is not expensive, it is advisable to use fresh stain every time in order to have reproducible results.
14. The U-251 MG human glioblastoma cell line is currently not available from ATCC. However, it is available from other sources, including Sigma-Aldrich. Since the genetic background of both U-251 MG and U-373 MG are the same, ATCC stopped the distribution of both cell lines.

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