Chapter 6

MMP Activity Detection in Zymograms

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Abstract

Matrix metalloproteinases (MMP) belong to a distinguished class of zinc-dependent endopeptidases. Zymography is a semi-quantitative tool for determining the activity of different MMP isoenzymes in a variety of biological samples. In substrate gel zymography, protein samples of different origin (tissue, cell lysates, plasma/serum, perfusates, other liquids) are separated in sodium dodecyl sulfate (SDS) polyacrylamide gels containing copolymerized substrate (gelatin, casein, elastin, etc.), and after incubation-enabling substrate cleavage by MMPs, MMP activities are detected after the gel staining as transparent bands against a dark-blue background. In situ zymography is a histological modification of substrate zymography in frozen sections, allowing detection of the localization of the MMP activities within the tissue. Here, we describe detailed experimental protocols of all abovementioned techniques and provide examples for several sample measurements.

Key words Matrix metalloproteinase activity, Substrate zymography, Gelatin, Casein, In situ zymography

1 Introduction

1.1 Matrix Metalloproteinases in Health and Disease

Matrix metalloproteinases (MMPs) are enzymes that belong to the family of zinc-dependent endopeptidases and are known to play a crucial role in the dynamic processing of the extracellular matrix (ECM) facilitating the degradation of matrix material. On the other hand, MMPs have been also shown to be present intracellularly, thereby influencing physiological as well as pathological intracellular signal transduction processes and the contractile machinery. Therefore, MMPs are interesting drug targets for several pathologies (*see* for a review: [1]). The family of MMPs include close to 30 members (*see* for reviews: [2, 3]); many of them shown to be activated due to different physiological as well as pathological situations in different tissues. Some of the important family members together with their characteristic features are shown in Table 1. MMPs are synthesized as zymogens and can be activated by proteolytic cleavage of an amino-terminal domain, by oxidative/nitrosative-induced conformational

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Table 1
The family of MMPs and their characteristic features

MMP codes	Alternative names	Molecular weight (kDa)		Substrates	Pathologies
		Z[a]	A[a]		
MMP-1	Interstitial collagenase	57	52	Gelatin	Atherosclerosis, melanoma, heart failure
MMP-2	Gelatinase A, type IV collagenase	75/72	64	Gelatin, elastin	Myocardial infarction, heart failure, gastritis, rheumatoid arthritis
MMP-3	Stromelysin-1	57	45	Gelatin, elastin, casein	Brain injury, neurodegeneration
MMP-7	Matrilysin	28	19	Gelatin, elastin, casein	Tumor-induced osteolysis, colon cancer
MMP-8	Neutrophil collagenase	75	57	Gelatin	Coronary artery disease, angina
MMP-9	Gelatinase B	92	86	Gelatin, elastin	Myocarditis and subsequent dilated cardiomyopathy, ulcerative colitis
MMP-10	Stromelysin-2	57	44	Gelatin, elastin, casein	Lung cancer
MMP-11	Stromelysin-3	51	44	Gelatin, elastin, casein	Tumor progression, breast carcinomas
MMP-12	Macrophage metalloelastase	54	22	Gelatin, elastin, casein	Granulomatous skin diseases, inflammatory disorders
MMP-13	Collagenase-3	65	48	Gelatin	Breast carcinomas
MMP-14	MT1-MMP	66	54	Gelatin, casein	Tumor growth by activating MMP-2
MMP-15	MT2-MMP	76	N/A	Fibronectin Laminin	Obesity, preeclampsia, ovarian carcinoma
MMP-16	MT3-MMP	64	52/30	Gelatin, casein	Breast cancer
MMP-17	MT4-MMP	71	67	N/A	Preeclampsia
MMP-19	RASI-1	57	N/A	Gelatin	Rheumatoid arthritis
MMP-20	Enamelysin	54	42.5		Amelogenesis imperfecta

(continued)

Table 1 (continued)

MMP codes	Alternative names	Molecular weight (kDa)		Substrates	Pathologies
		Z[a]	A[a]		
MMP-21	N/A	N/A	N/A	N/A	Melanoma, ovarian and colon carcinomas
MMP-22	N/A	N/A	42	N/A	N/A
MMP-23	N/A	N/A	N/A	N/A	Breast cancer
MMP-24	MT5-MMP	N/A	N/A	N/A	Brain tumors
MMP-25	MT6-MMP, leucolysin	56	38	pro-MMP-2	Inflammatory hyperalgesia
MMP-26	Endometase	29	19	Gelatin	Lung cancer

[a]Z and A indicates the zymogen or active form(s) of MMPs, respectively

change (without a change in molecular weight), or by phosphorylation [2, 4]. Activities of MMPs are tightly regulated by their endogenous tissue inhibitors (TIMPs) [5].

MMPs play an important role in many physiological and pathological processes, including embryogenesis, wound healing, inflammation, cardiovascular diseases, and tumor development or progression [2, 6]. Increased activities of different MMPs have been reported to be connected with different pathological situations such as ischemia-reperfusion injury [7], myocardial contractile dysfunction [8], heart failure [9], arthritis [10], neurodegenerative disorders [11], cancer invasion and metastasis [12], liver cirrhosis [13], fibrotic lung disease [14], periodontal disease [15] as well as with responses to some invasive interventions like anthracycline treatment [16, 17] or chest irradiation [18] used in the cancer therapy. On the other hand, inhibition of MMP activities has been shown to be connected with some kinds of tissue protection such as ischemic preconditioning [19, 20] or flavonoid-induced cardioprotection [17]. Moreover, pharmacological inhibition of MMP activities has been shown to be cardioprotective in animal models of acute myocardial infarction [21, 22] and has also been shown to be altered in coronary artery disease patients [23]. Being able to detect MMPs at early stages of the disease is opening a perspective to use MMPs as diagnostic markers. MMPs have been well-investigated in clinical studies of cardiovascular diseases: MMP-2 and -9 in Chagas cardiomyopathy [24] and MMP-9 in ST-segment elevation myocardial infarction [25]. Protein expression and activation of MMP-2 and MMP-9 has clinical relevance and prognostic value in patients with colorectal cancer [26]. Fecal MMP-9 is a useful tool for the differential diagnosis of diarrheic disorders and in the noninvasive evaluation of disease activity and mucosal healing in ulcerative colitis [27].

Regarding the abovementioned facts, determination of MMP activities belongs to very useful methodologies in biomedical research and is of high clinical importance since it seems to be a powerful diagnostic and/or therapeutic tool for the detection or follow-up of the abovementioned pathologies.

1.2 MMP Activity Detection in Biological Samples

MMP activities can be determined by zymography in different kinds of biological samples such as heart, brain, liver, lung tissues, blood vessels, or in isolated or cultured cell lineages (*see* Figs. 1, 2, and 3). Zymography can be performed as a substrate zymography, in which the substrate of the certain MMP is incorporated, copolymerized in a sodium dodecyl sulfate (SDS) polyacrylamide gel, and MMPs are separated according to their molecular weights.

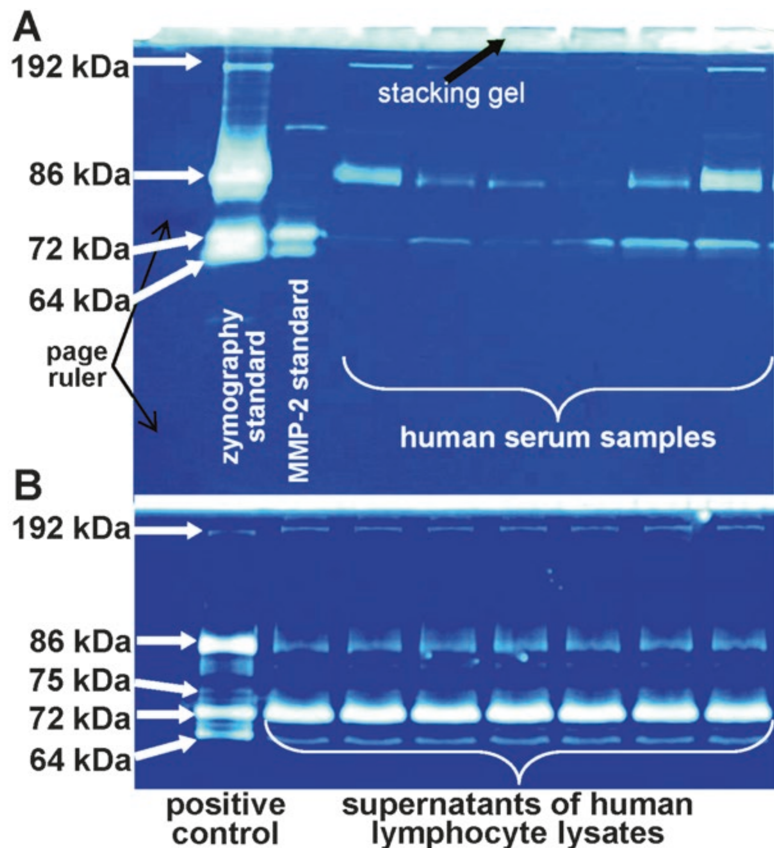

Fig. 1 Representative gelatin zymograms performed from human samples. (*Panel A*) Human serum samples from patients with coronary artery disease. Some of the patients have increased MMP-2 and/or MMP-9 activity as indicated by the zymographic intensity at 72 or 86 kDa, respectively. (*Panel B*) Gelatin zymogram from human isolated lymphocytes. 72 kDa MMP-2 activity is markedly visible; however, other activities at 64 kDa (MMP-2) and at 86 kDa (MMP-9) can also be observed. Weak gelatinolytic activity signals can be seen in both panels at 192 kDa, which may indicate the dimerized form of MMP-9 [28]

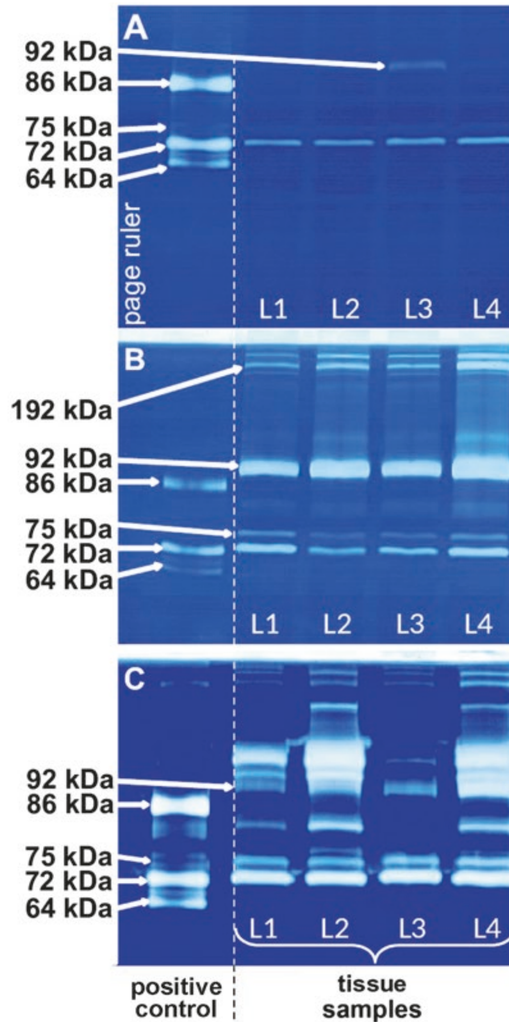

Fig. 2 Representative gelatin zymograms performed from homogenates of murine tissues (*L1–4*: *lanes 1–4*; 15 μg protein was loaded into each wells). *Panel A* represents mouse heart homogenates, which expresses mainly the 72 kDa isoform of MMP-2. Weak signal for MMP-9 can be observed; however, it indicates inappropriate removal of blood from heart samples. (*Panel B*) Lung samples from mice subjected to chronic tobacco smoking (*L2* and *4*) and their controls (*L1* and *3*). A markedly increased intensity can be observed at 92 kDa (MMP-9). (*Panel C*) Samples derived from the aorta of transgenic mice. *L1* and *2*: ApoB100LDL−/− mice, control and Chlamydia pneumoniae (Cpn) infected; *L3* and *4*: ApoE−/−, control and Cpn infected

The activity of MMPs is detected by the absence of gelatin in the gel, which can be visualized by transilluminating the gel. The activity of the certain MMP is proportional with the intensity and the thickness of the corresponding band on zymogram, which can be evaluated electronically by using different software after scanning the gels.

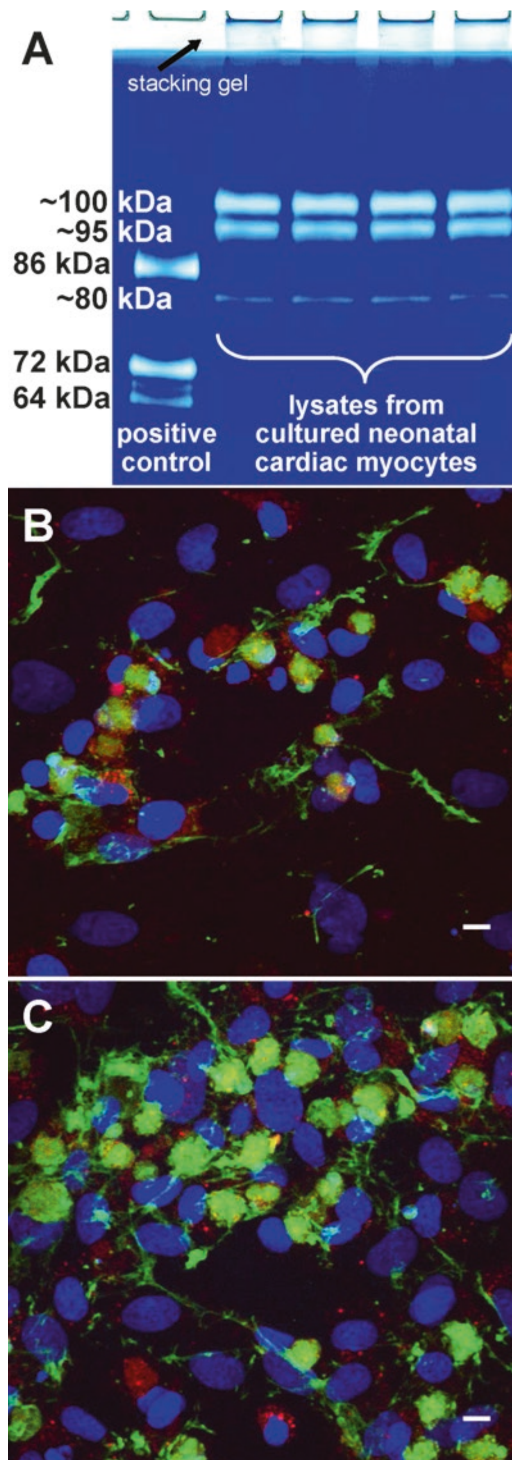

Fig. 3 Examples of MMP zymography in isolated and/or cultured cells. (*Panel A*) Gelatin zymogram from cultured neonatal cardiac myocytes after resuspension. Gelatinolytic activities can be detected at completely different molecular weights than that of heart homogenates (for comparison *see* Fig. 2a). (*Panel B, C*) In situ gelatin zymography in fixed neonatal cardiac myocyte culture in normoxic conditions (*Panel B*) and subjected to simulated ischemia/reoxygeation (*Panel C*). *Panel C* shows an increased gelatinolytic activity (represented as *green fluorescence*—DQ™ fluorescent gelatin; *red fluorescence*: MMP-2 immunostaining by rhodamine-labeled goat anti-mouse antibody, *blue fluorescence*: cell nuclei by Hoechst 33342 staining) in cardiac myocytes, which indicates the presence and activation of MMP-2 during simulated ischemic stress. Scale bars = 20 μm

For the detection of MMP activity in situ in different cell or tissue cultures, in situ zymography is a suitable method (Fig. 3). In in vivo systems, natural inhibitors of MMPs (tissue inhibitors of MMPs, TIMPs) are presented [29]. Therefore, when a study is designed for detecting MMPs activity in a certain physiological or pathological condition, one should calculate not only with the activation, but the inhibition of MMPs by TIMPs as well. For this reason, reverse zymography has been developed, which allows detection of TIMPs activities in gel zymograms. However, this chapter is limited for showing the opportunities to detect MMP activities in biological samples, thus here we do not provide detailed description on the available techniques for detecting TIMPs activity (for TIMP measurements, *see* for review [30]).

1.3 Types of Zymography Substrates

MMPs cleave different substrates; therefore, the substrate, which is copolymerized in the gel, should be determined according to the MMP isoform.

Gelatin zymography is predominantly used for measurements of activities of MMP-2 and MMP-9 as these two MMPs exert strong ability to cleave gelatin as a substrate and are commonly called "gelatinases."

In casein zymography, casein is copolymerized into the polyacrylamide gel as a substrate for MMP cleavage. Casein zymography is used for estimation of proteolytic activity of MMP-7 due to its ability to cleave casein.

The most rarely applied type of zymography is elastin zymography, in which soluble elastin is copolymerized in the gel and, beside the activity of elastases, it can suitably show elastinolytic activity for such MMPs, which cleave basically other substrates like gelatin (e.g., MMP-2).

2 Materials

2.1 Sample Preparation

1. *For pulverized tissue samples*: Homogenization buffer: 500 mL double distilled water (ddH_2O), 0.335 g (50 mM) Tris base, 1 mL (0.5%) Triton X-100. In 500 mL beaker dissolve compounds in 500 mL ddH_2O. Adjust to pH 7.4 with 1 M HCl. Aliquot into 15 mL Falcon tubes. Store at −20 °C for 1 year. Homogenator: Pellet Pestle Motor, Centrifuge (e.g., Hettich Universal 320R), BCA Protein Assay Kit for protein measurement.
2. *For organ perfusates*: Concentrating tubes: Amicon Ultra-4 30 kDa centrifugal filter unit with Ultracel-30 membrane for perfusate concentration.
3. *For cell culture lysates*: Phosphate Buffered Saline tablets (PBS): In 200 mL beaker dissolve 1 PBS tablet in 100 mL ddH_2O. Adjust

to pH 7.2 with 1 mM NaOH prepared freshly; Homogenization buffer (*see* above **item 1**); Amicon Ultra 10 kDa concentrating tubes. Centrifuge (e.g., Hettich Universal 320R)

2.2 Gelatin Zymography

1. 30% Acrylamide/0.8% Bisacrylamide, Store: at +4 °C for 1 year.
2. Separating Gel Solution—1.5 M Tris–HCl, pH 8.8, Store at +4 °C for 1 year.
3. 2% Gelatin Solution: 100 mg gelatin (type A, from porcine skin; stored between 20–25 °C) dissolved in 4.5 mL ddH_2O.
4. 10% (w/v) SDS (sodium dodecyl sulfate) solution. Weigh 10 g SDS and dissolve in 100 mL ddH_2O (Storage between 20–25 °C for 1 year).
5. 10% (w/v) ammonium persulfate (APS) solution: Dissolve 100 mg of APS in 1 mL ddH_2O. Storage: at +4 °C for 1 month
6. TEMED. Storage: at +4 °C for 1 year.
7. Stacking Gel Solution: 0.5 M Tris–HCl/SDS, pH 6.8. Storage: at +4 °C for 1 year.
8. ELFO Buffer (25 mM Tris–HCl, 192 mM glycine, 0.1% SDS, pH 8.3). Storage: at +4 °C for 1 year. Or self-prepared: 28.83 g glycine, 6.0 g Tris base, 2.0 g SDS. Dissolve Tris base and glycine in 1000 mL of ddH_2O. Bring solution to 1950 mL with ddH_2O. Add SDS. Bring solution to 2000 mL total volume with ddH_2O. Storage: at 4 °C for 1 year.
9. Non-reducing loading (sample) buffers: use commercial "Blue" Zymogram Sample Buffer or commercial "Pink" non-reducing lane marker.
10. For positive control, use "zymography standard," containing a mixture of purified and activated MMP-2 and MMP-9; or MMP-2 standard for gelatin and elastin zymography (Fig. 1a). For casein zymography, human, recombinant active MMP-7 can be used.
11. Renaturation buffer: Renaturation buffer (Bio-Rad). Store: at +4 °C for 1 year. Add 1× mL Renaturation buffer (Bio-Rad) + 9× mL ddH_2O. Storage: prepare freshly.
12. Development buffer: Development buffer (Bio-Rad) Storage: at +4 °C for 1 year. Add 1× mL Development buffer + 9× mL ddH_2O. Storage: prepare freshly.
13. For negative control, use 10 mM ethylene glycol tetraacetic acid (EGTA, binds Ca^{2+} ions, which is obligatory for activity of MMPs). Dissolve 381 mg EGTA in 90 mL ddH_2O + 10 mL development buffer.
14. Coomassie Brilliant Blue (0.05%): 250 mg Coomassie Brilliant Blue G-250, 125 mL methanol, 50 mL glacial acetic acid,

325 mL ddH_2O. Dissolve 250 mg Coomassie Brilliant Blue in the mixture of 125 mL methanol and 50 mL glacial acetic acid and dilute it by adding 325 mL ddH_2O. Storage: between 20–25 °C.

15. Destaining solution: 40 mL methanol, 80 mL acetic acid, 880 mL ddH_2O. Storage: between 20–25 °C

2.3 Casein Zymography

All materials and procedures are identical with gelatin zymography except gelatin solution, which is substituted with casein.

1. Casein solution: 100 mg casein (storage: between 20–25 °C) dissolved in 4.5 mL phosphate buffer.
2. Phosphate buffer (126 mM).

2.4 Elastin Zymography

All materials and procedures are identical with gelatin zymography except gelatin solution, which is substituted with elastin.

1. Elastin solution: 54 mg soluble elastin from bovine neck ligament dissolved in 4.5 mL ddH_2O.

2.5 Preparation of Substrate for Electrophoresis

1. *Gelatin solution*: Add 100 mg gelatin to 4.5 mL ddH_2O. Gently heat and mix solution until it dissolves (beaker will be warm to touch, max. 40 °C). If it has cooled down, add 0.5 mL 10% (w/v) SDS aqueous solution to reach final desired volume and concentration. Prepare freshly (*see* **Note 1**).
2. *Casein solution*: Dissolve 100 mg casein in 4.5 mL phosphate buffer. Stir until casein dissolves. Preparation of phosphate buffer: mix 7.12 g Na_2HPO_4 dissolved in 400 mL ddH_2O and 1.56 g NaH_2PO_4 dissolved in 100 mL ddH_2O, set at pH 7.4. Add 0.5 mL 10% w/v SDS aqueous solution to reach final desired volume. Alternatively, dissolve 30 mg casein in 2 mL of 75 mM Tris–HCl, pH 8.8.
3. *Elastin solution*: Add 54 mg κ-elastin to 4.5 mL ddH_2O. Stir until elastin dissolves. Add 0.5 mL 10% w/v SDS aqueous solution to reach final desired volume.

2.6 In Situ Zymography

The EnzCheck Gelatinase/Collagenase Assay Kit from Invitrogen was used. This assay includes the following reagents:

1. DQ gelatine from pig skin: five vials (1 mg DQ substrate lyophilized from 1 mL of PBS in each vial).
2. 10× Reaction buffer: 50 mL.
3. 1,10-phenanthroline monohydrate: 30 mg powder in a vial.
4. Collagenase type IV from *Clostridium histolyticum*: 500 U collagenase powder in a vial.

3 Methods

3.1 Sample Preparation

3.1.1 Pulverized Tissue Sample

1. Weigh out 30–50 mg heart (lung, pancreas, aorta, spleen) tissue powder into a liquid-nitrogen-frozen 1.5 mL Eppendorf tube. Avoid thawing. It can be stored at −80 °C for 2 years.
2. Thaw an appropriate amount of homogenization buffer (*see* **Note 2**).
3. Add 4× volume homogenization buffer to the sample (e.g., 30 mg sample and 120 μL buffer).
4. Homogenize the mixture by Pellet Pestle Motor for 3 × 10 s (*see* **Note 3**).
5. Centrifuge the homogenate at 4 °C for 10 min at 10,000 × *g*, and collect the supernatant, and store at −80 °C for maximum 1 month.
6. Measure protein concentration by a BCA kit. Usually 20× dilution of tissue homogenates is required.

3.1.2 Preparing Perfusate Samples

1. In case of perfusate sample, use Amicon Ultra 30 kDa concentrating tubes to concentrate 4 × 3 mL perfusate sample to 50–100 μL.
2. Pour 3 mL perfusate in the concentrating insert of an Amicon tube.
3. Put concentrating insert into the tube and close it. Spin samples at 7500 × *g* for 20 min, 4 °C.
4. Remove the concentrating insert and discard the flow-through from the tube. Reinsert the concentrating insert.
5. Pour 3 mL perfusate in the insert, recap, and spin it for 20 min. Repeat **steps 3–4** twice more.
6. Pipette out the concentrated sample from the insert into an Eppendorf tube.
7. Measure protein concentration by BCA kit. Usually 3× dilution of perfusate concentrates is required.

3.1.3 Cell Culture Lysates

1. For cell culturing *see* Ref. 31 (culturing neonatal cardiac myocytes).
2. Remove treating solutions and wash cells in 2 mL PBS two times and then remove PBS.
3. Scrape cells from two wells of a 6-well plate in 200 μL zymography homogenization buffer (two wells together are 400 μL and they are collected into one tube after washing both wells). In case of 25 cm^2 flask use 400 μL buffer; in case of 75 cm^2 flask, use 1 mL buffer.
4. Keep Eppendorf tubes with the suspensions on ice and take into −80 °C freezer or concentrate them freshly.

3.2 Concentration of Samples

1. Keep samples on ice.
2. Homogenize the mixture by ultrasonic homogenizer 2× 5 s on ice.
3. Centrifuge cell homogenates at 5000 × *g* for 10 min at 4 °C.
4. Collect supernatant.
5. Centrifuge the supernatant in Amicon Ultra 10 kDa concentrating tubes to increase the sample protein concentration (4000 × *g* for 30–50 min at −4 °C).
6. Put 50–100 μL samples into −80 °C freezer in two aliquots (one aliquot for determination of protein concentration).
7. Measure protein concentration by BCA kit. Usually 3× dilution of cell concentrates is required.

3.3 Preparation of Separating Gel

1. Assemble electrophoresis unit (*see* **Note 4**).
2. Insert a comb between the gel-casting glasses and mark desired level of separating gel on the front glass (at the bottom of the teeth of the comb).
3. Mix 30% acrylamide/0.8% bisacrylamide solution with Tris–HCl pH 8.8, gelatin solution, and ddH_2O (Table 2; *see* **Notes 5** and **6**).
4. Add 10% APS solution and TEMED to the mix quickly (Table 2).

Table 2
Preparation of separating gel between 0.75–1.5 mm thickness

	Final acrylamide concentration in the separating gel (%)					
			8.0			
Stock solutions[a]	**7.0**	**7.5**	**Small gel (15 mL)**	**Large gel (22.5 mL)**	**9.0**	**10.0**
30% acrylamide/0.8% bisacrylamide (mL)	3.5	3.75	4.0	6	4.5	5.0
1.5 M Tris–HCl, pH 8.8 (mL)	3.75	3.75	3.75	5.62	3.75	3.75
ddH_2O (mL)	6.25	6.0	5.75	8.62	5.25	4.75
Gelatin/casein/elastin solution (20/20/12 mg/mL, 1% w/v SDS; mL)	1.5	1.5	1.5	2.25	1.5	1.5
10% w/v Ammonium Persulfate Solution (APS; μL)	50	50	10	75	50	50
TEMED (μL)	10	10	10	15	10	10

[a] *See* **Notes 8** and **9**

5. Swirl to get homogenous gel. Avoid bubbling. Use immediately as polymerization process has begun (*see* **Note 7**).
6. Using a pipette, pour a small amount into sandwich plates and watch for leakage. In the absence of leakage, continue filling up to 1 mm above line.
7. Gently add butanol (with a 27G-needle connected to a 10-mL syringe) along the surface of the gel to remove bubbles (*see* **Note 8**).
8. Allow gel to polymerize (approximately 20 min at 25 °C; *see* **Note 9**). Use this time to prepare stacking gel (without adding TEMED and 10% APS).
9. A layer of H_2O on the surface of the gel will be visible when polymerization is completed. Drain this layer from the unit with a small stripe of blotting/filter paper.

3.4 Preparation of Stacking Gel

1. Mix 30% acrylamide/0.8% bisacrylamide solution with Tris–HCl, pH 6.8 and ddH_2O (Table 3).
2. Add 10% SDS, 10% APS, and TEMED quickly (Table 3).
3. Swirl to mix. Avoid bubbling. Use immediately as polymerization process has begun.
4. Place comb in units and then use pipettes to pour stacking gel.
5. Allow gels to polymerize (approximately 15 min at 25 °C). Use this time to make sample calculation.

3.5 Sample Calculation

1. According to the results of protein measurement, sample loading mass and volume should be calculated.
2. From a tissue homogenate, 50 μg protein per lane should be loaded. Since we load 15 μL per lane, this means that the final protein concentration of sample needs to be 50 μg/15 μL.
3. In case you want to load a sample only once, it is enough to prepare 2× volume of one load (30 μL), which means that we

Table 3
Preparation of 5 mL stacking gel

Stock solutions	Volume
30% acrylamide/0.8% bisacrylamide	1 mL
0.5 M Tris–HCl pH 6.8	1.25 mL
ddH_2O	3.05 mL
10% SDS	50 μL
10% w/v Ammonium Persulfate Solution (APS)	25 μL
TEMED	8 μL

should add 6 μL (1/5 part) "pink" or 20 μL (2/3 part) "blue" loading buffer.

4. The remaining 24 or 10 μL, respectively, should contain 2 × 50 μg = 100 μg protein. Therefore, volume of the sample will be: V_1 = 100 μg/protein concentration of your sample.
5. Then we should add ddH_2O to dilute samples. The required volume of ddH_2O is: $V_2 = 30 - V_1$.

Taken together:	
Loading volume:	15 μL
Loaded protein:	50 μg
Prepared volume:	30 μL (2× loading volume)
Prepared (sample) mass:	2× loaded protein (100 μg)
"Pink" loading buffer:	1/5 of prepared volume (6 μL) OR
"Blue" loading buffer:	2/3 of prepared volume (20 μL)
Sample volume:	V_1 = 2× loaded protein (2× 50 μg)/ sample protein concentration (μg/μL)
ddH_2O:	V_2 = Prepared volume (30 μL) − V_1

3.6 Sample Loading and Running Gels

1. Prepare and cool down ELFO buffer: 50 mL ELFO + 450 mL ddH_2O (avoid bubbles, mix it gently) (*see* **Note 10**).
2. Mark gel lanes for sample loading. Do not use the two outside lanes.
3. When gel is polymerized, remove combs by pulling it straight up.
4. Remove gel plates and snap onto electrode assembly (*see* **Note 11**).
5. Fill up the lower and the upper buffer container with tank buffer.
6. In case of casein zymography: pre-run electrophoresis at 40 mV for 15 min at 4 °C before the samples are loaded into the wells. Another possibility is to load sample buffer into one well and pre-run electrophoresis at 4 °C until it reaches the bottom of the gel. Afterwards continue with **step 7**, but keep gels at 4 °C (*see* **Note 12**).
7. Load samples

   For identification of different isoforms of detected MMPs, positive controls (e.g., zymography standard containing

human MMP-2 and -9 or MMP-2 standard) should be used. Page ruler is also useful to detect the different size of bands. Leave at least one lane loaded with sample(s) for negative control (*see* in Subheading 3.6).

8. Connect electrodes properly (red to red, black to black) and set to 90 V.
9. Let the samples run until loading buffer (running front) reaches the bottom of the gel (*see* **Note 13**).
10. Use this time to prepare renaturation and development buffers.

3.7 Washing and Incubating Gels

1. Set dry incubator at 37 °C.
2. Disassemble the casting apparatus.
3. Cut down lanes for negative control (*see* **Note 14**).
4. Wash gels for 40 min in 200 mL renaturation buffer between 20–25 °C (*see* **Notes 15** and **16**).
5. Place gels into 200 mL freshly prepared development buffer (*see* **Note 16**). For negative control, incubate lane(s) separately in development buffer substituted with 10 mM EGTA solution.
6. Incubate gels in dry incubator at 37 °C for 20–40 h (*see* **Note 17**).

3.8 Staining Gels and Preparation for Evaluation of MMP Activity

1. Immerse gels into 0.05–0.1% Coomassie Brilliant Blue solution. Place on a shaker for 1 h (*see* **Note 18**).
2. Incubate gels in destaining solution and place on a shaker (at least for 60 min; *see* **Notes 19** and **20**).
3. Gelatinolytic activities should be detected as transparent bands against the blue background of Coomassie Brilliant Blue-stained gelatin (*see* Figs. 1–3).
4. Scan the gel in transilluminator mode with a special gel scanner or gel-documentation system.
5. Evaluate MMPs activity by using a gel/film evaluation software (e.g., Quantity one, Bio-Rad).

3.9 In Situ Zymography

The present description provides details for performing in situ zymography for MMP-2 in cardiac myocytes (Fig. 3); however, techniques for showing other MMP activities in different tissue sections or cell cultures in situ also exist and a description for general in situ zymography is available in Ref. 32.

1. Culture neonatal rat cardiac myocytes [31] or other cell types in 24-well tissue culture plate at the density of 10^5 cells/well for 3 days.
2. Replace the growth medium (DMEM—Dulbecco's Modified Eagle Medium—supplemented with Glu, AB/AM, 1% FBS)

with a “stress” solution containing DQ substrate at 40 μg/mL concentration. In case of control group, replace the medium of the cells with a control solution containing DQ substrate at the abovementioned concentration.

3. Subject cells to circumstances according to your aim/project, which may induce intracellular MMP-2 activation.
4. For negative control samples, use ilomastat (at 0.5 μM final concentration), or other non-specific MMP inhibitor (e.g., 1,10-phenanthroline, between 1 and 5 mM final concentration, SB-3CT between 1 and 10 nM final concentration).
5. Subsequently, replace “stress” solution with growth medium containing DQ substrate at 40 μg/mL concentration (250 μL volume must be applied onto the cells).
6. Replace the medium, and wash cells twice with Dulbecco’s-PBS.
7. Rinse cells in 3.7% paraformaldehyde in PBS between 20–25 °C for 15 min.
8. Wash cells twice with PBS.
9. Rinse coverslips with mounting medium and view fluorescent signal under fluorescent microscope.
10. If it is necessary, you can combine in situ zymography with immunocytochemistry. In this case, after the fixation you should continue with an appropriate immunostaining protocol.

4 Notes

1. Gelatin is very sensible. Make sure that gelatin dissolves completely (clear, transparent solution without any opalescent particle). Avoid gelatinization: prolonged cooling leads to gel formation. When SDS is added, avoid precipitation. If gelatin precipitates, try to heat again. If precipitates do not disappear from the solution, prepare a new gelatin solution. Precipitated gelatin does not polymerize homogenously in the gel, which may lead to equivocal results.
2. Avoid reducing agents (e.g., dithiothreitol; DTT) or protease inhibitors (e.g., phenylmethanesulfonyl fluoride; PMSF) in the homogenization buffer. They may reduce or inhibit MMP activities, thereby may lead to false results.
3. Avoid ultrasonic homogenizer. It can destroy the native structure of MMPs; therefore, enzymes may lose their activities.
4. 7.5–10% polyacrylamide gels are recommended for gelatin zymography. For casein zymography, we recommend to use

10–15% gels according to the MW of active MMP-7 (19–21 kDa; 28–30 kDa pro-MMP-7).

5. Mix 15.0 mL of separating gel. This volume is sufficient for 1 small unit (containing ten lanes; i.e., two gels), or mix 22.5 mL for a triple-wide unit (containing 30–34 lanes).
6. In order to avoid leakage, ensure that spacers, comb, and glass plates are aligned properly.
7. Make sure that gel is horizontal and there is no difference in the levels of the two edges.
8. Avoid "shooting" of butanol. Butanol should cover the whole surface of the gel uniformly.
9. TIP: leave pipette tip in the remnant of separating gel; when it is polymerized, you will be able to lift it with the pipette.
10. Calculate the necessary volume of ELFO buffer according to the buffer tank.
11. Eliminate bubbles under the gel, they may disturb gel running.
12. Casein migrates in gel during electrophoresis. Due to its low molecular weight (23 kDa), the zone containing casein can obscure MMP-7 (latent form: 29 kDa, active form: 20 kDa) after staining. Therefore, pre-run of casein-embedded gel is recommended before classical zymogram procedure in order to get excess of casein out of the gel. The amount of remaining casein is sufficient for detection of MMP activities [33].
13. Average time for gel running: 1.5–2 h.
14. Avoid gel rupture. Cut the different corner of the gels to be able to identify the gels later (e.g., cut the bottom left corner of gel #1, and both the top and bottom left corners for gel #2). (Ensure that the gel is oriented correctly so that you don't accidentally cut the right side corner.)
15. Before preparation of renaturation and development buffers, make sure that the buffers do not contain any visible contamination (e.g., fungal particles). Buffers should be clear and transparent.
16. Make sure that gels immerse in the buffers and are not attached to the wall of the dish.
17. Even before staining, gelatinolytic activity can be visible. Hold the gel up against a dark background to visualize it, if not, longer incubation should be applied. A pilot zymography is recommended to run for setting up the optimal incubation time. For example, 20-h incubation is recommended in case of human plasma, rat heart, and rodent lung samples, while 40-h incubation is required for mouse heart samples.

18. Staining can be longer, if it is necessary. The gel should be dark blue, protein ladder should be invisible.
19. Use clear dish and change destaining solution after 5–10 min, if it becomes bluish.
20. Leave the gel in destaining solution until the stacking gel becomes completely destained (transparent) again (*see* Figs. 1 and 3). It can last even for 12 h.

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