

MMP activity detection in zymograms

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Summary

Matrix metalloproteinases (MMP) belong to a distinguished class of zinc-depending 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 co-polymerized 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 of 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 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)**.

TABLE 1

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 non-invasive evaluation of disease activity and mucosal healing in ulcerative colitis (27).

Regarding to 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 Fig. 1-3). Zymography can be performed as a substrate zymography, in which the substrate of the certain MMP is incorporated, co-

polymerized in a sodium dodecyl sulfate (SDS) polyacrylamide gel and MMPs are separated according to their molecular weights. 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 1 + 2

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 **(28)**. 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 **(29)**].

FIG 3

1.3 Types of zymography substrates

MMPs cleave different substrates, therefore, the substrate, which is co-polymerized 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 co-polymerized 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 co-polymerized 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

For pulverized tissue samples

1. Homogenization buffer: 500 mL double distilled water (ddH₂O), 0.335 g (50 mM) Tris base, 1 mL (0.5%) Triton X-100. In 500 mL beaker dissolve compounds in 500 mL ddH₂O. 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.

For organ perfusates

2. Concentrating tubes: Amicon Ultra-4 30 kDa centrifugal filter unit with Ultracel-30 membrane for perfusate concentration.

For cell culture lysates

3. Phosphate Buffered Saline tablets (PBS): In 200 mL beaker dissolve 1 PBS tablet in 100 mL dd H₂O Adjust to pH 7.2 with 1 mM NaOH prepare freshly; Homogenization buffer (see above step 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 at room temperature) dissolved in 4.5 mL ddH₂O.
- 4 10% (w/v) SDS (sodium dodecyl sulfate) solution. Weigh 10 g SDS and dissolve in 100 mL ddH₂O (Storage: at room temperature for 1 year).
- 5 10% (w/v) ammonium persulfate (APS) solution: Dissolve 100 mg of APS in 1 mL ddH₂O. 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 ddH₂O. Bring solution to 1950 mL with ddH₂O. Add SDS. Bring solution to 2000 mL total volume with ddH₂O. 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 solution: Renaturation buffer (Bio-Rad). Store: at +4 °C for 1 year. Add 1x mL Renaturation buffer (Bio-Rad) + 9x mL ddH₂O. Storage: prepare freshly.

- 12 Development solution: Development buffer (Bio-Rad) Storage: at +4 °C for 1 year.
Add 1x mL Development buffer + 9x mL ddH₂O. Storage: prepare freshly.
- 13 For negative control, use 10 mM ethylene glycol tetraacetic acid (EGTA, binds Ca²⁺ ions, which is obligatory for activity of MMPs). Dissolve 381 mg EGTA in 90 mL dH₂O + 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 ddH₂O. 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 ddH₂O. Storage: at room temperature.
- 15 Destaining solution: 40 mL methanol, 80 mL acetic acid, 880 mL ddH₂O. Storage: at room temperature

2.2. 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: at room temperature) dissolved in 4.5 mL phosphate buffer.
- 2 Phosphate buffer (126 mM)

2.3. Elastin zymography

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

Elastin solution: 54 mg soluble elastin from bovine neck ligament dissolved in 4.5 mL ddH₂O.

2.4. Materials for in situ zymography

EnzCheck Gelatinase/Collagenase Assay Kit (Invitrogen)

Assay includes the following reagents:

DQ gelatine from pig skin: five vials (1mg DQ substrate lyophilized from 1 mL of PBS in each vial).

10x Reaction Buffer: 50 mL

1,10 o-phenanthroline monohydrate: 30mg powder in a vial.

Collagenase type IV. from Clostridium Histolyticum: 500U collagenase powder in a vial.

3. Methods

3.1. Sample preparation

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 1**).
3. Add 4x volume homogenization buffer to the sample (e.g. 30 mg sample and 120 µL buffer).
4. Homogenize the mixture by Pellet Pestle Motor for 3x10 sec (**see Note 2**)
5. Centrifuge the homogenate at 4 °C for 10 min at 10000 g, and collect the supernatant, and store at -80 °C for maximum 1 month.
6. Measure protein concentration by a BCA kit. Usually 20x dilution of tissue homogenates is required.

Preparing perfusate samples

1. In case of perfusate sample, use Amicon Ultra 30 kDa concentrating tubes to concentrate 4x3 mL perfusate sample to 50-100 μ L.
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 \times g for 20 min, 4 $^{\circ}$ 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 3x dilution of perfusate concentrates is required.

Cell culture lysates

1. For cell culturing see ref. **(30)** (culturing neonatal cardiac myocytes)
2. Remove treating solutions and wash cells in 2 mL PBS two times, then remove PBS.
3. Scrape cells from 2 wells of a 6-well plate in 200 μ L zymography homogenization buffer (2 wells together are 400 μ L and they are collected into 1 tube after washing both wells). In case of 25 cm^2 flask use 400 μ L 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 $^{\circ}$ C freezer or concentrate them freshly.

3.2 Concentration of samples

1. Keep samples on ice.
2. Homogenize the mixture by ultrasonic homogenizer 2x 5 sec 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 2 aliquots (1 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. Preparation of substrate for electrophoresis

a) gelatin solution

Add 100 mg gelatin to 4.5 mL ddH₂O.

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.

(Note 3)

b) casein solution

Dissolve 100 mg casein in 4.5 mL phosphate buffer.

Stir until casein dissolves.

Preparation of phosphate buffer: mix 7.12 g Na₂HPO₄ dissolved in 400 mL ddH₂O and 1.56 g NaH₂PO₄ dissolved in 100 mL ddH₂O, set at pH 7.4.

Add 0.5 mL 10% w/v SDS aqueous solution to reach final desired volume.

OR

Dissolve 30 mg casein in 2 mL of 75 mM Tris HCl, pH = 8.8

c) elastin solution

Add 54 mg κ -elastin to 4.5 mL H₂O

Stir until elastin dissolves.

Add 0.5 mL 10% w/v SDS aqueous solution to reach final desired volume.

2. Assemble electrophoresis unit. (Note 4)
3. Mark desired level of separating gel on unit (use comb).
4. Mix 30% acrylamide/0.8% bisacrylamide solution with Tris HCl, pH 8.8, gelatin solution and ddH₂O (Table 2).
5. Add 10% APS solution and TEMED to the mix quickly (Table 2)
6. Swirl to get homogenous gel. Avoid bubbling. Use immediately as polymerization process has begun. (Note 5)
7. 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.
8. Gently add butanol (with a 27G-needle connected to a 10-mL syringe) along top to remove bubbles. (Note 6)
9. Allow gels to polymerize (approximately 20 min at 25 °C). Use this time to prepare stacking gel (without adding TEMED and 10% APS) (Note 7)
10. A layer of H₂O on top of the gel will be visible when polymerization is complete. Drain this layer from the unit with a small stripe of blotting paper.

3.3. Preparation of stacking gel

1. Mix 30% acrylamide/0.8% bisacrylamide solution with Tris HCl, pH 6.8 and ddH₂O (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.

3.4. 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 should add 6 µl (1/5 part) “pink” or 20 µl (2/3 part) “blue” loading buffer.
4. The remaining 24 µl or 10 µl, respectively, should contain 2×50 µg = 100 µg protein. Therefore, volume of the sample will be: $V_1 = 100 \mu\text{g} / \text{protein concentration of your sample}$.
5. Then we should add ddH₂O to dilute samples. The required volume of ddH₂O 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 μ l) OR
“Blue” loading buffer:	2/3 of prepared volume (20 μ l)
Sample volume:	$V_1 = 2 \times$ loaded protein (2 \times 50 μ g)/sample protein concentration (μ g/ μ L)
ddH ₂ O:	$V_2 =$ Prepared volume (30 μ l)- V_1

3.5. Sample loading and running gels

1. Prepare and cool down ELFO buffer: 50 mL ELFO + 450 mL ddH₂O (avoid bubbles, mix gently, cool down). (**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 straight up.
4. Remove gel plates and snap onto electrode assembly. (**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 40mV 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. (**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 one lane loaded with sample for negative control (see in section 3.6).
8. Connect electrodes properly (red to red, black to black) and set voltage at 90 V.

9. For 8% gel run till dye gets until the bottom of gel. (**Note 13**)
10. Use this time to prepare renaturation solution and development buffer.

3.6. Washing and incubating gels

1. Set dry incubator at 37 °C.
2. Disassemble gel apparatus.
3. Cut down lane for negative control. (**Note 14**)
4. Wash gels for 40 min in 200 mL Renaturation solution at room temperature.
(**Note 15, 16**)
5. Wash gels for 20 min 200 mL Development buffer. (**Note 15, 16**)
6. Place gels in fresh 200 mL development buffer. (**Note 16**) For negative control, incubate lane separately in 10 mM EGTA solution.
7. Incubate gels into dry incubator at 37 °C for 20-40 h. (Even before staining, gelatinolytic activity should be visible, if not, longer incubation should be applied). (**Note 17**)

3.7. Staining gels and preparation for evaluation of MMP activity

1. Even before staining, gelatinolytic activity should be visible (hold the gel up against a dark background to visualize).
2. Put gels in Coomassie Brilliant Blue solution. Place on a shaker for 1 h. (**Note 18**)
3. Put gels in destaining solution. Place on shaker (at least 40 min). (**Note 19, 20**)
4. Gelatinolytic activities should be detected as transparent bands against the blue background of Coomassie Brilliant Blue stained gelatin (see Figures 1-3).
5. Scan the gel in transparent mode with a special gel scanner.

6. Evaluate MMPs activity by using a gel/film evaluation software (e.g. Quantity one, Bio-Rad)

3.8 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 are also existing and a description for general in situ zymographies is available in ref. **(31)**.

1. Culture neonatal rat cardiac myocytes **(30)** 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 supplemented with Glu, AB/AM, 1%FBS) with a “stress” solution containing DQ substrate at 40 $\mu\text{g}/\text{mL}$ 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-o-phenanthroline at 1-5 mM final concentration, SB-3CT at 1-10 nM final concentration).
5. Subsequently, replace “stress” solution with growth medium containing DQ substrate at 40 $\mu\text{g}/\text{mL}$ concentration (250ul volume must be applied onto the cells).
6. Replace the medium, and wash cells twice with D-PBS.

7. Rinse cells in 3.7% PFA in PBS at room temperature 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 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.
- 2 Avoid ultrasonic homogenator. It can destroy the native structure of MMPs, therefore enzymes lost their activities.
- 3 Gelatin is very sensible. Make sure that gelatin dissolves completely (clear, transparent solution without any opalescent particle). By cooling avoid gel formation (too long cooling). When SDS is added, avoid precipitation. If gelatin precipitates, try to heat again. If precipitates will not disappear from the solution, make a new gelatin solution. Precipitated gelatin does not polymerize homogenously in the gel and leads irrelevant results in the enzyme activity.
- 4 In order to avoid leakage, ensure that spacers, comb and glass plates are aligned properly.
- 5 Make sure that gel is horizontal and there is no difference in the levels of the two edges.
- 6 Avoid "shooting" the butanol, and butanol covers the hole top surface of the gel uniformly.
- 7 TIP: leave pipette tip in the left over separating gel, when it is polymerized you will be able to lift it with the pipette.
- 8 Mix 15.0 mL of separating gel. This volume is sufficient for 1 small unit (contains 10 lanes) (i.e. 2 gels), or for 1 triple-wide unit (contains 30-33 lanes) mix 22.5 mL.
- 9 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 (19-21kDa; 28-30 kDa pro-MMP-7).

- 10 Calculate the necessary volume of ELFO buffer according the used 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 **(32)**.
- 13 Average time for gel running: 1.5-2 hours.
- 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 buffer, 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 20-hour incubation is recommended for lung samples, 40-hour for heart samples. Individual differences may occur, therefore the authors recommend to run a pilot zymography for set up incubation time.
- 18 Staining can be longer, if it is necessary. The gel should be dark blue.
- 19 Use clear dish and change destaining solution after a couple of minutes (5-10 min), if it becomes bluish.

20 Leave the gel in destaining solution until the stacking gel become completely destained (transparent) again (Fig .1 and 3). It can last even for 12 hours.

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Figure legends

Figure 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 (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 **(33)** (Roomi et al., 2014).

Figure 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. L1-2: ApoB100LDL^{-/-} mice, control and *Chlamydia pneumoniae* (Cpn) infected; L3-4: ApoE^{-/-}, control and Cpn infected.

Figure 3: Examples of MMP zymography y 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 and C: In situ gelatin

zymography in fixed neonatal cardiac myocyte culture in normoxic conditions (Panel B) and subjected to simulated ischemia/reoxygenation (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.

Table 1: The family of MMPs and their characteristic features.

MMP codes	Alternative names	Molecular weight (kDa)		Substrates	Pathologies
		Z*	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, pre-eclampsia, ovarian carcinoma
MMP-16	MT3-MMP	64	52/30	gelatin, casein	breast cancer
MMP-17	MT4-MMP	71	67	N/A	pre-eclampsia
MMP-19	RASI-1	57	N/A	gelatin	rheumatoid arthritis
MMP-20	enamelysin	54	42.5		amelogenesis imperfecta
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

*Z and A indicates the zymogen or active form(s) of MMPs, respectively.

Table 2: Preparation of separating gel between 0.75-1.5 mm thickness (**Note 8, 9**)

STOCK SOLUTIONS	Final acrylamide concentration in the separating gel (%)					
	7.0	7.5	8.0		9.0	10.0
			small gel (15 mL)	large gel (22.5 mL)		
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 ₂ O (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; μ L)	50	50	10	75	50	50
TEMED (μ L)	10	10	10	15	10	10

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 ₂ O	3.05 mL
10% SDS	50 μ L
10% w/v Ammonium Persulfate Solution (APS)	25 μ L
TEMED	8 μ L