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1 **TITLE:**
2 Gelatin Zymography to Detect Gelatinase Activity in Melanoma Cells
3
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17 **SUMMARY:**
18 Metalloproteases (MMPs) are secreted by many cells, including malignant melanoma. MMP-
19 mediated cleavage of extracellular matrix components leads to the increased invasive potential
20 of these cells. Gelatin zymography, presented here, is a quantifying method for studying
21 gelatinase activity manifested as a digested gelatin area on a polyacrylamide gel.
22

23 **ABSTRACT:**
24 Melanoma cells, having highly invasive properties, exhibit the formation of invadopodia—
25 structures formed by tumor cells and responsible for the digestion of the surrounding
26 extracellular matrix (ECM). Several metalloproteases (MMPs) are secreted by cells to hydrolyze
27 ECM proteins. They are mainly secreted through structures known as invadopodia. ECM
28 degradation is crucial for tumor cells while forming metastases as the cells heading towards blood
29 vessels must loosen dense tissue.
30

31 One group of metalloproteases secreted by melanoma cells comprises the gelatinases, i.e.,
32 metalloproteases 2 and 9. Gelatinases cleave gelatin (denatured collagen), a few types of
33 collagen (including type IV), and fibronectin, all structural components of ECM. This paper
34 describes a gelatin zymography assay to analyze the gelatinase activity of melanoma cells. This
35 approach is based on analyzing the extent of digestion of a substrate (gelatin) added to a
36 polyacrylamide gel. Several advantages, such as simplicity, sensitivity, low cost, and
37 semiquantitative analysis by densitometry, as well as the detection of both active and inactive
38 forms of MMPs, make this assay valuable and widely used.
39

40 This protocol describes how to concentrate medium devoid of intact floating cells, cell debris,
41 and apoptotic bodies. Next, it focuses on preparing polyacrylamide gel with gelatin addition,
42 performing sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), removing
43 SDS, and staining of the gel to detect gelatin-free bands corresponding to the activity of
44 gelatinases secreted by melanoma cells. Finally, the paper describes how to quantitatively

45 analyze data from this assay. This method is a good alternative for estimating the gelatinase
46 activity of melanoma cells to a fluorescent gelatin degradation assay, western blot, or enzyme-
47 linked immunosorbent assays (ELISAs).
48
49 **INTRODUCTION:**
50 Matrix metalloproteinases (MMPs) are a family of Zn^{2+}-containing endopeptidases that cleave
51 various ECM proteins and nonECM proteins, such as growth factors, cell receptors, proteinases,
52 and their inhibitors[1–4]. ECM-substrate specificity of MMPs is dependent on the peptide domains
53 and motifs, as well as similarities in their sequences, thus defining their subgroups. There are, for
54 example, collagenases digesting various types of collagens, as well as gelatin and aggrecan;
55 gelatinases cleaving gelatins and collagens; and matrilysins or MT-MMPs that digest various ECM
56 proteins[5].
57
58 This paper focuses on two gelatinases: MMP-2 and MMP-9, as they can digest denatured collagen
59 (gelatin) proteolytically, allowing the detection of their activity using a gelatin zymography
60 assay[3,6]. Although MMP-2 and MMP-9 bear a strong structural resemblance, they do not have
61 identical substrate specificity[7]. A C-terminal hemopexin-like domain of MMPs is responsible for
62 recognizing substrate sequence[8]. Slight differences in their catalytic domains are responsible for
63 the differences in the substrate selectivity of MMP-2 and MMP-9, e.g., MMP-2, unlike MMP-9,
64 can cleave native type I collagen[9]. Nevertheless, their proteolytic activities can be unquestionably
65 determined with gelatin zymography as they both can cleave gelatin[3,6].
66
67 MMPs are already known to be involved in both physiological and pathological conditions. They
68 were found to impact cell migration, invasion, spreading, and adhesion, thus impairing
69 angiogenesis, inflammation, tumor progression, and metastasis[10–13]. As they take part in various
70 important processes, they are extensively studied due to their high therapeutic or diagnostic
71 potential[14–16]. MMP-2 (gelatinase A) occurs as a 72 kDa proenzyme whose prodomain binds Zn^{2+}
72 in the catalytic site, leading to the inhibition of enzymatic activity[9]. MMP-2 can be activated
73 through the cleavage of its zymogen's prodomain by MMP-14 (MT1-MMP), thrombin, and
74 activated protein C[17–20]. Therefore, the mass of active MMP-2 is lower (~64 kDa). In contrast,
75 MMP-9 (gelatinase B) is expressed as a ~92 kDa proenzyme and is activated by the cleavage of
76 the N-terminal domain to obtain the 83 kDa protein. MMP-9 maturation results from a
77 prodomain cleavage by serine proteases, other MMPs, and as a response to the oxidative stress[21].
78
79 The progression and malignancy of melanoma are highly dependent on the ability of the tumor
80 cells to digest ECM, as it is "a barrier" limiting the cells from progression and metastasis
81 formation[22]. Cells need to first penetrate the basal membrane (BM) to enter the dermis, migrate
82 toward blood vessels, adhere to vascular endothelium, and reach the blood. It has been shown
83 that gelatinase expression was increased in different cancers and was correlated with increased
84 invasion and migration and a worse prognosis for patients[23,24]. MMP-2 was highly expressed in
85 melanoma cells, with its activation state correlated with progression[25,26]. MMP-9 was also found
86 to accumulate in human skin tumors and melanoma cell lines[27,28].
87
88 Due to the high correlation between the properties of MMPs with the invasiveness of melanoma

89 cells, the availability of a simple, sensitive, low-cost, functional assay to determine their presence
90 and activity is crucial for better understanding the biology of melanoma and designing new
91 diagnostic techniques for their detection. This paper describes the gelatin zymography technique
92 in detail, as it can be considered the best candidate for this purpose. This approach is based on
93 SDS-electrophoresis under denaturing but nonreducing conditions, using polyacrylamide gels
94 prepared with the addition of gelatin[29,30]. Although proteins, including MMP-2 and MMP-9, are
95 denatured in the presence of SDS during electrophoresis, washing in buffer containing Triton X-
96 100 causes their renaturation as the result of an SDS:Triton X-100 exchange[31].
97
98 These renatured MMPs digest gelatin during gel incubation in an incubation buffer, which can be
99 finally observed as clear zymolytic bands in the Coomassie Blue-stained gel[5]. The amount and the
100 area of digested gelatin presented as transparent bands corresponding to the gelatinolytic
101 activity of MMPs can be determined using both commonly used and open-source applications—
102 ImageLab and ImageJ[29,32]. Although this method has many advantages, it also possesses some
103 limitations mentioned in the discussion. This "step by step" protocol with notes and comments
104 performed on the different melanoma cell lines should be sufficient for reproducibility and
105 optimization to obtain representative results. **Figure 1** presents the steps of the described
106 procedure.
107
108 **PROTOCOL:**
109
110 **1.   Cell culture medium collection and concentration**
111
112 **1.1.**   Seed the melanoma cells (here A375, SK-MEL-28, Hs 294T, WM9, WM1341D cell lines)
113 into tissue-culture 75 cm^2 flasks in complete medium [Dulbecco's modified Eagle's medium-high
114 glucose with reduced concentration (1.5 g/L) of NaHCO$_3$, supplemented with 10% (v/v) Fetal
115 Bovine Serum (FBS), 1% (v/v) L-Glutamine, and 1% (v/v) Antibiotic-Antimycotic].
116
117 **1.2.**   Culture the cells under standard conditions (5% CO$_2$, 37 °C).
118
119 **1.3.**   After the cells reach 80–90% confluence, aspirate and discard the supernatant and wash
120 the flasks 3 times with serum-free medium or PBS warmed to 37 °C to remove residual medium
121 in the culture flask.
122
123 NOTE: The supernatant (complete medium) should be removed completely before adding the
124 serum-free medium. FBS contains various MMPs, which may lead to false-positive results and
125 wrong data analysis and interpretation[29].
126
127 **1.4.**   Add 10 mL of warm serum-free medium and incubate the cells at 37 °C in a humidified
128 atmosphere of 5% CO$_2$ for 48–72 h.
129
130 NOTE: The duration of cell incubation in the serum-free medium may affect cell viability, as well
131 as the amount of secreted proteins. Therefore, the duration of cell starvation must be optimized
132 before performing zymography depending on the type of cell line. The trick is to choose when

133 cells are most viable and secrete the highest amount of MMPs. All melanoma cell lines used here
134 were cultured in a serum-free medium for 48 h without affecting viability. The condition of the
135 cells was verified using a phase-contrast microscope, although an XTT or MTT cell viability assay
136 can be performed.

138 **1.5.** Collect the media from the cell cultures after 48–72 h and transfer them to 15 mL tubes.
139 Keep the media constantly on ice to avoid protein degradation.

141 **1.6.** Centrifuge the media for 20 min at 7,000 × g at 4 °C[33].

143 NOTE: Centrifugation is crucial for removing floating intact cells, cell debris, and apoptotic bodies,
144 as they may be sources of active MMPs. Skipping this step could lead to false-positive results and
145 wrong data analysis and interpretation. Optionally, media can be filtered through 0.22-μm filters
146 to remove apoptotic bodies, large microvesicles, and cell debris[34]. It is important to centrifuge
147 the media before transferring to -20 °C for storage, as freezing and thawing of cells may lead to
148 their damage, thus releasing intracellular components[35].

150 **1.7.** Store the media at -20 °C if required before further analyses (STOP POINT 1).

152 **1.8.** Thaw the media on ice and concentrate them to achieve the desired final concentrate
153 volume using ultracentrifugal filter units with a 10-kDa cutoff (see the **Table of Materials**),
154 according to the manufacturer's recommendation.

156 NOTE: Here, the obtained concentration factor was approximately 20 times. As the molecular
157 weight of the detected MMPs is 43–215 kDa, it is recommended to use ultracentrifugal filter units
158 with 10 kDa Nominal Molecular Weight Limit (NMWL). Using higher NMWL (30 kDa) may result
159 in losing the MMP molecules with lower molecular weight (e.g., 43 kDa collagenase MMP-1).

161 **1.9.** Transfer the concentrated medium immediately to -80 °C and store at -80 °C (STOP POINT
162 2).

164 NOTE: Fresh or thawed samples should be kept on ice during the next steps of the protocol to
165 avoid protein degradation.

167 **1.10.** Measure the concentration of proteins in collected and concentrated media using, e.g.,
168 the Bradford or BCA method, according to the manufacturer's recommendation (STOP POINT 3).

170 ==**2. Preparation of SDS-PAGE separating and stacking gels with gelatin (1 mg/mL) for gelatin**==
171 ==**zymography**==

173 **2.1.** Place the spacer plate with a short plate into a casting frame to form a cassette and place
174 the frame on the casting stand.

176 NOTE: The spacer plate with 0.75–1.5 mm integrated spacers can be used (here 1 mm). Ensure

177 that the working surface is stable and perfectly leveled.

178

179 **2.2.**   Prepare 2.65 mg/mL of gelatin by weighing the appropriate amount of gelatin and
180 dissolving it in ultrapure or sterile deionized H_2O by warming the solution to 65 °C. Sterilize the
181 solution using a 0.22-µm syringe filter. Cool the gelatin solution down to room temperature
182 before preparing the separating gel.

183

184 NOTE: The gelatin solution can be stored for 1 week at 4 °C. Long-term storage may cause
185 bacterial contamination and the presence of proteolytic enzymes secreted by them[36].

186

187 **2.3.**   Prepare 10 mL of 10% separating polyacrylamide gel by mixing 3.95 mL of 2.65 mg/mL
188 gelatin, 3.3 mL of 30% acrylamide/bis-acrylamide, 2.5 mL of 1.5 M Tris-HCl (pH 8.8), 48 µL of 10%
189 SDS, 80 µL of 50% glycerol, 70 µL of H_2O, 48 µL of 10% APS, and 4.8 µL of TEMED.

190

191 NOTE: CAUTION! Wear gloves and goggles when handling acrylamide/bis-acrylamide. APS (an
192 activator) and TEMED (catalyst of gel polymerization) must be added at the end of gel
193 preparation, after which it is important to work quickly and efficiently. CAUTION! TEMED must
194 be added under a fume hood. This volume of the separating gel is sufficient to prepare two
195 separating gels with a 1 mm spacer suited to the referenced electrophoresis apparatus (see the
196 **Table of Materials**). If a lower percentage of the gel is required, recalculate the volumes of the
197 components to obtain the desired percentage of acrylamide in the gel.

198

199 **2.4.**   Mix the 10% separating gel solution by inversion six to eight times, avoiding air bubble
200 formation, and pour the solution into the gel cassette sandwich (step 2.1) up to 75% of the height
201 of the short plate.

202

203 NOTE: The thickness of the gel, the amount of loaded protein, and incubation time in the
204 incubation buffer (see step 5.1.3 for composition) should be optimized to obtain areas of the gel
205 with the fully digested gelatin bands separated for individual MMPs. Ultimately, this depends on
206 the amount of MMPs secreted by a given cell type.

207

208 **2.5.**   Layer the top of the gel with 70% ethanol.

209

210 NOTE: It is essential to remove bubbles and prevent the gel from drying out and forming contact
211 with air, which is unsuitable for polymerization.

212

213 **2.6.**   Leave the gel for approximately 30 min at room temperature. When the polymerization
214 is complete and a clear separation line between the gel mix and ethanol layers is visible, remove
215 the ethanol layer carefully.

216

217 **2.7.**   Prepare 4% stacking gel (5 mL) by mixing 3.05 mL of H_2O, 0.67 mL of 30% acrylamide/bis-
218 acrylamide, 1.25 mL of 0.5 M Tris-HCl (pH 6.8), 50 µL of 10% SDS, 50 µL of 10% APS, and 5 µL of
219 TEMED. Mix the 4% stacking gel solution by inversion six to eight times.

220

221   NOTE: This volume is sufficient to prepare two stacking gels with a 1 mm spacer.
222
223   **2.8.**   Insert a comb on the top of the gel sandwich cassette immediately and fill it with the
224   stacking gel solution.
225
226   **2.9.**   Incubate the stacking gel for approximately 30 min at room temperature to allow it to
227   polymerize completely.
228
229   **2.10.**   Wrap the gels with moist paper towels, place them in a plastic bag, and keep the gels at
230   4°C for at least overnight but no longer than 1 week.
231
232   **2.11.**   Load the gel sandwich cassette into an electrode assembly, transfer it to the tank, and fill
233   it with 1x SDS-PAGE buffer (250 mM Tris-HCl, 1.92 M glycine, 1% (w/v) SDS) before running the
234   SDS-PAGE electrophoresis.
235
236   NOTE: SDS-PAGE buffer can be stored at 4 °C for extended periods.
237
238   **2.12.**   Remove the comb from the gel.
239
240   **3.   Preparation of SDS-PAGE separating and stacking gels for total protein content
241   determination**
242
243   NOTE: The steps in section 3 are similar to the steps in section 2 describing gel preparation for
244   gelatin zymography. Note that the composition of the gels is different. A gel for total protein
245   content determination must not contain gelatin, as it will also be stained by Coomassie Brilliant
246   Blue solution.
247
248   **3.1.**   Prepare 10 mL of 10% separating gel by mixing 3.34 mL of 30% acrylamide/bis-acrylamide,
249   2.5 mL of 1.5 M Tris-HCl (pH 8.8), 100 μL of 10% SDS, 3.99 mL of H_2O, 50 μL of 10% APS, and 20
250   μL of TEMED.
251
252   **3.2.**   Mix the solution by inversion, avoiding air bubble formation, and transfer the solution to
253   the gel cassette sandwich up to 75% of the height of the short plate.
254
255   **3.3.**   Layer the top of the gel with ethanol and leave the gel for approximately 30 min at room
256   temperature. Remove the ethanol.
257
258   **3.4.**   Prepare 5 mL of 4% stacking gel by mixing 650 μL of 30% acrylamide/bis-acrylamide, 1.25
259   mL of 1.5 M Tris-HCl (pH 8.8), 50 μL of 10% SDS, 2.99 mL of H_2O, 25 μL of 10% APS, and 12.5 μL
260   of TEMED.
261
262   **3.5.**   Place a comb on the top of the gel sandwich cassette and fill it with stacking gel solution.
263
264   **3.6.**   Leave the gel for approximately 30 min at room temperature.

265

NOTE: The gel can be prepared just before SDS-PAGE electrophoresis or stored for a few days at 4 °C. For gel storage, wrap the gel in a wet paper towel and place it in a plastic bag to keep it moist.

3.7. Put the cassettes with the gels in the tank, load the gel sandwich cassette into an electrode assembly, and transfer the assembly to a tank with 1x SDS-PAGE buffer [250 mM Tris-HCl, 1.92 M glycine, 1% (w/v) SDS] before running the SDS-PAGE electrophoresis.

3.8. Remove the comb from the gel.

4. **Sample loading and electrophoresis running**

4.1. Prepare each SDS-PAGE sample for zymography by mixing a 1:1 ratio of 3–20 µg (here 10 µg) of concentrated medium with 2x nonreducing loading buffer [10 mM Tris pH 6.8, 1% (w/v) SDS, 10% (v/v) glycerol, 0.03% (w/v) bromophenol blue] based on the estimated concentration of protein in the medium.

NOTE: Loading buffer should be stored at 4°C. Using β-mercaptoethanol and dithiothreitol (DTT) is not recommended due to their ability to destroy disulfide bonds between cysteine residues, which results in the inability of the MMP to refold after electrophoresis.

4.2. Prepare the gel for the determination of total protein content by mixing samples in a 1:3 ratio with 4x reducing buffer [40% (v/v) glycerol, 240 mM Tris (pH 6.8), 8% (w/v) SDS, 0.04% (w/v) bromophenol blue, 5% (v/v) β-mercaptoethanol].

NOTE: CAUTION! Wear gloves and goggles, and work under the fume hood. Loading buffer should be stored at -20 °C. The same amount of protein should be loaded into each lane. Samples can be optionally stored at -80 °C for a few days if required (STOP POINT 4).

4.3. Incubate the samples for gelatin zymography for 20 min at 37 °C before running the electrophoresis.

NOTE: Do not heat the samples at a temperature higher than 37 °C. Thermal denaturation of proteins may lead to the inactivation of protease activity or prevent the refolding of the enzymes causing false-negative results and wrong data interpretation[31].

4.4. Incubate the samples for total protein content determination for 10 min at 95 °C.

4.5. Spin the samples for a few seconds in a small table centrifuge and load them into the wells of the gels.

4.6. Optionally, load a molecular weight ladder and negative (samples that have been boiled for 10 min at 95 °C) and positive control samples (e.g., recombinant MMP-2 and MMP-9 or a cell

309   line known to secrete MMP-2 and MMP-9[36]) into the wells.

310

311   **4.7.**   Run electrophoresis, keeping it on ice until the dye flows out of the gel to provide good

312   MMP-9 and MMP-2 band separation. Use the following SDS-PAGE electrophoresis conditions:

313   initially, 20 mA per gel, increasing the power to 40 mA per gel when the samples enter the

314   resolving gel.

315

316   NOTE: The maximum electric current should not exceed 130 mA for four gels. Otherwise, the gel

317   will get overheated during electrophoresis. Electrophoresis can be performed in a box containing

318   ice or in a cold room (4 °C), if available.

319

320   **5.**   **Activation, staining, and destaining of the polyacrylamide gel**

321

322   **5.1.**   Gel washing and activation

323

324   5.1.1.   Remove the zymography gel from between the short and spacer plates and transfer it to

325   a plastic container with washing buffer [50 mM Tris pH 7.5, 150 mM NaCl, 10 mM $CaCl_2$, 2.5%

326   (v/v) Triton X-100]. Prepare washing buffer on the day of gelatin zymography.

327

328   NOTE: As Triton X-100 is very viscous, pipet it according to the rules for pipetting viscous liquids

329   to add the same volume of Triton X-100 each time the buffer is prepared[37].

330

331   5.1.2.   Incubate the gel twice in the washing buffer, each time for 30 min with gentle agitation

332   to remove SDS from the gel and replace it with Triton X-100.

333

334   5.1.3.   Transfer the gel to a plastic tank containing incubation buffer (50 mM Tris pH 7.5, 150

335   mM NaCl, 10 mM $CaCl_2$).

336

337   NOTE: For long-term storage, keep the incubation buffer at 4 °C. Prepare fresh buffer if there are

338   any signs of microbial contamination or precipitation.

339

340   5.1.4.   Incubate the gel in the incubation buffer for 12–20 h (here 16 h) at 37 °C.

341

342   NOTE: The thickness of the gel and amount of loaded protein influence the incubation period in

343   the incubation buffer, which should be optimized to obtain the areas of well-digested gelatin in

344   the gel.

345

346   **5.2.**   Gel staining and destaining

347

348   5.2.1.   Prepare the staining solution containing an aqueous solution of 0.5% (w/v) Coomassie

349   Brilliant Blue R-250, 30% (v/v) of ethanol, and 10% (v/v) of acetic acid.

350

351   NOTE: CAUTION! Work under the fume hood and wear gloves when using acetic acid. Dissolve

352   the weighed amount of Coomassie Blue in water, add ethanol, and mix the solution. In the end,

353 slowly add acetic acid to the ethanol-water solution to avoid an exothermic reaction.
354
355 5.2.2. Prepare the destaining solution containing 30% (v/v) of ethanol and 10% (v/v) of the
356 acetic solution in H_2O.
357
358 NOTE: Mix ethanol with H_2O. Add acetic acid slowly to the ethanol-water solution at the end to
359 avoid an exothermic reaction.
360
361 5.2.3. Transfer the gel to a plastic container with Coomassie Brilliant Blue solution and stain the
362 gel for 30 min at room temperature with gentle agitation.
363
364 NOTE: FastGene Q-Stain can also be used for staining the gel. The advantage of this dye is that it
365 is ready to use and that the stained gel does not require any destaining step.
366
367 5.2.4. Incubate the gel in the destaining buffer at room temperature with gentle agitation to
368 destain. Continue the destaining until clear bands representing the gelatinase activity are visible.
369
370 NOTE: If the staining with Coomassie Brilliant Blue solution is very intense, change the destaining
371 buffer a few times during incubation.
372
373 5.2.5. Keep the gel moisturized and do not allow it to dry out until its visualization.
374
375 NOTE: Replace the destaining buffer with water to slow down the destaining process until
376 visualization.
377
378 **6.   Visualization of proteins in polyacrylamide gels**
379
380 NOTE: See the **Table of Materials** for details on the imaging system and software used to visualize
381 proteins in polyacrylamide gels (**Figure 2A–C**). Other readily available gel visualization systems
382 (such as iBright Imaging Systems, UVP PhotoDoc-It Imaging System, E-Gel Imager, and Azure
383 Imagers) can also be used for this purpose.
384
385 **6.1.**   Switch imaging equipment on and open the software.
386
387 **6.2.**   Click **File | New project** to open a new project.
388
389 **6.3.**   Choose the **application type** by clicking **Select… | Protein gels | Coomassie blue.**
390
391 **6.4.**   Transfer the gels, one by one, into the imaging system and check the gel position using
392 the **Position Gel** button.
393
394 **6.5.**   Visualize the gel by clicking **Run protocol.**
395
396 **6.6.**   Save the image as .scn to allow further analysis using the referenced software.

397

6.7. Additionally, export file as .tif by clicking **File | Export | Export for Analysis**, which will be useful for analysis using Fiji (STOP POINT 5).

7. **Data analysis**

7.1. Gel analysis using the imaging software

7.1.1. Open the imaging software and images of the zymogram and the gel representing the total protein content.

7.1.2. Invert the image to obtain black bands on a white background. Under **Go to the Image transform setting**, click on the **Invert image display**.

7.1.3. Select lines and bands representing MMP-2 and MMP-9 manually or automatically using the **Analysis Tool Box | Lane and Bands section**. Create one band per line and adjust its size to include the whole line area for total protein content determination.

7.1.4. Adjust the lines and bands using the tools available in the **Lane and Band section** (**Figure 2A'–C'**).

7.1.5. Go to the **Analysis Table** and copy the Volume (intensity) and molecular weight of each lane and band (**Figure 2A"–C"**).

7.1.6. Divide the **Volume** of each lane per **Volume** of the selected control lane (here A375) to compare the total protein content between lanes (**Figure 2A"**).

7.1.7. Divide the **Volume** of each studied lane per **Volume** of the total protein lane corresponding to it (Coomassie Blue-stained) to normalize the **Volume** of MMP activity to the total protein content (**Figure 2B",C"**).

7.1.8. To compare the activity of gelatinases between lines, divide the **Volume** of the studied line/studied condition per **Volume** of the control line/standard condition (here A375) (**Figure 2B",C"**).

7.2. Alternative gel analysis using Fiji software

7.2.1. Open the Fiji software and open the inverted gel image (black bands on a white background).

7.2.2. Outline the first lane with gelatin-digested bands using the **rectangle** tool.

7.2.3. Choose **Analyze | Gels | Select First Lane** in the Fiji toolbar to select and mark the first lane.

7.2.4.   Transfer the first outlined selection (yellow rectangle) into the next lane and click **Analyze | Gels | Select Next Lane** to select and mark the next lane.

7.2.5.   Select all lanes presented on the gel in the same way (**Figure 3A–C**).

7.2.6.   Go to **Select Analyze | Gels | Plot Lanes** to make the lane profile plots (Figure 3A'–C').

7.2.7.   Using the **Straight** tool, separate bands with vertical lines at the edges of each peak and then draw horizontal lines to "close" their area (**Figure 3A'–C'**; yellow lines).

7.2.8.   Measure the size of each peak using the **Tracking** tool. Click in the area of each peak to select it and wait for the peaks to be outlined in yellow.

7.2.9.   Look for the size of each selected peak in the **Results** table (**Figure 3A"–C"**).

7.2.10. Divide the "area" of the studied line/condition per "area" of the control line/standard condition (**Figure 3A"–C"**).

7.3.   Data presentation

7.3.1.   Show the results obtained with the imaging software as the MMP activity normalized to total protein content and control line (**Figure 4A,B**).

7.3.2.   Present the results obtained with Fiji as the MMP activity normalized to total protein content and control line (**Figure 4C,D**).

REPRESENTATIVE RESULTS:
In this protocol, we describe the procedure for gelatin zymography (**Figure 1**) using media obtained from five melanoma cell lines (A375, SK-MEL-28, Hs-294T, WM9, WM1341D) as samples. The zymography approach shown here includes two separate SDS-PAGE electrophoreses. One SDS-PAGE electrophoresis results in the Coomassie Blue-stained gel, representing the total protein content loaded in each line (**Figure 2A**). It is used for the normalization of gelatinase activity data. The second SDS-PAGE electrophoresis results in the gel with loaded MMPs that digest gelatin added to the gel. The result of gelatin zymography is the zymogram which, when stained with Coomassie Blue solution, should exhibit transparent bands at the proper molecular weight position, depending on the types of secreted MMPs (**Figure 2B,C**). These gelatin-digested areas are mostly identified as the gelatinolytic activity of MMP-9 and MMP-2.

The zymograms in this paper show the presence of both latent (pro) and active forms of MMP-9 and MMP-2 in all tested melanoma cell lines (**Figure 2B,C**). Two forms of both MMPs are observed owing to the different activation mechanisms of pro-MMPs and active MMPs. Active MMPs are activated proteolytically *in vitro*, whereby their N-terminal domains are cleaved, decreasing their

485 molecular mass[7]. Pro-MMPs are denatured during SDS-PAGE electrophoresis, leading to the
486 opening of their active sites and their autocatalytic activation but without proteolytic cleavage[38].
487 Subsequent renaturation causes protein refolding to make MMP-mediated gelatin digestion
488 possible. Hence, pro-MMPs also exhibit gelatinolytic activity in gelatin zymography; however,
489 they can be distinguished from the active MMPs because of their higher, zymogenic molecular
490 mass[39].
491
492 In the case of MMP-9, these data show that the molecular weight for pro- and active forms are
493 approximately 93 and 80 kDa, respectively (**Figure 2B',B''**). Pro-MMP-2 is represented as a 68-
494 kDa band and the active form of MMP-2 as 60 kDa (**Figure 2C',C''**). The molecular weight of MMP-
495 2 as a proenzyme is 72 kDa in the literature. Nevertheless, the cleavage of MMP-2's prodomain
496 results in its activation and changes the mass to 64 kDa[9]. MMP-9 is present in cells in the 92 kDa
497 proenzyme form, and its activation by cleavage leads to the decrease in its protein mass to 83
498 kDa. The reason for these differences in the molecular masses of MMPs presented here
499 compared to the literature is that the samples and the protein ladder migrate differently because
500 the protein ladder, unlike the samples, is reduced[31]. Although gelatin zymography is commonly
501 used to detect MMP-2 and MMP-9 activity, the activity of other MMPs, such as MMP-1
502 (collagenase 1, 43 kDa), may also be visible after gelatin zymography[40]. These bands, if visible,
503 have much less intensity because gelatin is not their main substrate[5]. MMP-9 complexes with α_2-
504 microglobulin-related protein (125, 215 kDa) can also be detected after gelatin zymography, as
505 they are based on disulfide bridges that are not dissociated in gelatin zymography[31, 41].
506
507 Densitometric analysis using ImageLab or ImageJ applications helps determine the total protein
508 content in each lane (**Figure 2A', 3A'**) and the level of gelatinase activity in melanoma cell lines
509 (**Figure 2B',C'** and **Figure 3B',C'**). The result of densitometric analyses using ImageLab is the
510 **volume** (the sum of all the intensities within the band boundaries) of the band as well as
511 molecular weight (**Figure 2A''–C''**). ImageJ gives information about the peak area representing
512 each band (**Figure 3A''–C''**). The more intense and the bigger the digested areas are, the higher
513 the MMP activity that the test cell lines exhibit.
514
515 Here, we observed the highest pro-MMP-9 activity in Hs 294T cells and the highest activity of
516 active MMP-9 in SK-MEL-28 cell line (**Figure 2B''**, **Figure 3B''**, **Figure 4A**, and **Figure 4C**). Although
517 data analysis using both applications showed that MMP-9 activity is higher in SK-MEL-28 cells,
518 there were differences in the observed values. In the case of the ImageJ data, the MMP-9 activity
519 is more than 5 times higher than that of A375 cells but only approximately 3 times higher in the
520 ImageLab data (**Figure 2B''**, **Figure 3B''**, **Figure 4A**, and **Figure 4C**). In the case of MMP-2
521 gelatinase, we observed a dramatically lower level of pro-MMP2 activity in the SK-MEL-28 cell
522 line (**Figure 4B** and **Figure 4D**). However, the level of active MMP-2 was comparable in its activity
523 among different melanoma cell lines when the ImageLab software was used for analysis (**Figure**
524 **2C''** and **Figure 4B**). Nevertheless, the data obtained from the ImageJ application indicated
525 approximately 1.5 times higher MMP-2 activity in Hs 294T and 1.7 times in WM9 and WM1341D
526 cells compared to A375 cells (**Figure 3C''** and **Figure 4D**). The slight differences in the results
527 between the ImageLab and ImageJ analyses arise from the different calculations used by these
528 applications.

529

530 **FIGURE AND TABLE LEGENDS:**

531

532 **Figure 1: Schematic presentation of gelatin zymography protocol**. Zymography can be divided
533 into three main parts: i) Sample preparation, ii) SDS-PAGE electrophoresis, and iii) gel staining
534 and analysis. The scheme shows the main steps of each part of this method with an extra
535 annotation, which is important for the proper performance of the gelatin zymography. Stop
536 points are indicated in red. The figure was assembled with the Servier Medical Art by Servier,
537 licensed under a Creative Commons Attribution 3.0 Unported License[42]. Abbreviations: SDS-PAGE
538 = sodium dodecyl sulfate-polyacrylamide gel electrophoresis; RT = room temperature.

539

540 **Figure 2: Representative results of gelatin zymography with bioinformatic analysis of gelatinase**
541 **activity of MMPs in different melanoma cell lines using the ImageLab application.** (**A**) After SDS-
542 PAGE electrophoresis under denaturing but nonreducing conditions, the gel was stained with
543 Coomassie Blue R-250 solution and visualized. (**A', A"**) Analysis of total protein content in each
544 lane. Every lane at its full height was selected and adjusted as one band (**A'**). The intensity of the
545 signal of the total protein content for every lane was then automatically measured and presented
546 in a table (**A"**). Additionally, the total protein content was normalized to the second lane (here,
547 A375 cells). (**B,C**) Zymograms were obtained in gelatin zymography, representing the gelatin-free
548 area digested by pro- and active-MMP-9 (**B**) and pro- and active-MMP-2 (**C**). Before analysis, the
549 images representing white bands on the dark background were reversed to obtain the image
550 showing black bands on a white background (shown as lower panels of **B** and **C**). (**B',C'**)
551 Densitometric analysis of the gelatinolytic activity of MMPs. Bands representative for pro- and
552 active forms of (**B'**) MMP-9 and (**C'**) MMP-2 were selected and adjusted. (**B",C"**) Tables showing
553 densitometric data for gelatinolytic activity of (**B"**) MMP-9 and (**C"**) MMP-2 were obtained. The
554 molecular weight and volume (intensity) of bands were measured automatically. The tables
555 present the intensity of specific bands, normalized to the total protein content and then to the
556 volume of the band from the second lane (A375).

557

558 **Figure 3: Representative results of gelatin zymography and bioinformatic analysis of the**
559 **gelatinolytic activity of MMPs in different melanoma cell lines using the ImageJ application.**
560 Gels were visualized with ImageLab, but MMP activity was analyzed using the ImageJ application.
561 (**A–A"**) Analysis of the total protein content. (**A**) Every lane at its full height was selected and then
562 adjusted as one band (**A'**), and the line profile plots for each lane were created. (**A"**) Next, the
563 area of peaks was automatically measured and presented in a table. The peak area values
564 normalized to the second lane (A375 cell line) are also shown. (**B,C**) Zymograms with selected
565 lines were obtained after the gelatin zymography. (**B',C'**) Plot profiles showing gelatin-free areas
566 digested by pro- and active forms of (**B'**) MMP-9 and (**C'**) MMP-2. (**B",C"**) Densitometric analysis
567 of the gelatinolytic activity of MMP-2 and MMP-9. Tables showing peak areas determined using
568 ImageJ data for (**B"**) MMP-9 and (**C"**) MMP-2 activity. The peak areas were measured
569 automatically. Tables also show the peak areas for specific bands normalized to the total protein
570 content and the volume of the band from the second lane (here, A375 cells).

571

572 **Figure 4: Final results showing the gelatinolytic activity of MMP-2 and MMP-9, determined**

573 **using gelatin zymography, presented as graphs.** The values presented in **Figure 3A",B",C"** and
574 **Figure 4A",B",C"** were used to create graphs representing the gelatinase activity of the MMPs.
575 Data are presented as MMP activity, normalized to the total protein content and A375 cell line.
576 (**A, B**) Results obtained using ImageLab software, showing the activity of pro- and active forms of
577 (**A**) MMP-9 and (**B**) MMP-2 gelatinases. (**C, D**) The ImageJ software results show the activity of
578 pro- and active forms of (**C**) MMP-9 and (**D**) MMP-2 gelatinases.
579
580 **DISCUSSION:**
581 Despite the "step by step" protocol elaborated here, gelatin zymography requires optimization
582 depending on the samples/cell lines being analyzed. Different cell types and cell lines (melanoma
583 cell lines shown here) may secrete both forms (pro- and active) of MMP-2 and MMP-9 but with
584 different gelatinase activity. The optimization of the procedure includes mainly the duration of
585 cell starvation, the thickness of the polyacrylamide gel, the amount of loaded protein, and the
586 duration of gel incubation in the incubation buffer. Extended cell culture times may result in
587 higher amounts of secreted MMPs, requiring less protein to be loaded on the gel. Furthermore,
588 the gelatin copolymerized with a thinner gel will be digested more efficiently and rapidly by
589 MMPs, leading to a reduction in gel incubation time to obtain sufficiently destained bands and
590 reliable results. Despite optimization, repeating the protocol may still yield different-looking
591 zymograms because of incompletely mixing gelatin within the gel during gel preparation,
592 variation in temperature during SDS-PAGE electrophoresis or gel incubation in the incubation
593 buffer, and possible partial degradation of samples during freeze-thaw cycles.
594
595 It is important to optimize the percentage of a gel. Here, we presented zymograms performed
596 with 8% gels for MMP-2 and 10% gels for MMP-9. However, the percentage of the gel depends
597 on the form of the MMP to be detected and the distribution pattern of the activity of the pro-
598 and active forms of MMPs. For example, if the studied cells secrete a high level of MMP-2 with
599 high gelatinase activity, this may obscure latent MMP-2 activity. In such a situation, the gel
600 percentage must be decreased to separate the bands or reduce the incubation time in the
601 incubation buffer to visualize less intense smaller bands.
602
603 The next critical step during zymography is maintaining a constant temperature of 4°C during
604 SDS-PAGE. This will help prevent the active forms of the MMPs from digesting the gelatin during
605 SDS-PAGE. The digestion of gelatin at high temperatures will be manifested as blurred bands and
606 smears. It is recommended to show the gelatin-digested areas due to all forms of MMPs secreted
607 by the test cells on one zymogram. However, as presented here, it is necessary to run more than
608 one SDS-PAGE experiment with different gel percentages to distinguish between different forms
609 of MMPs or calculate their ratios. Alternatively, a gradient gel can be prepared. Its advantages
610 include an increased range of molecular weights that can be separated in a single gel and better
611 separation of proteins with close molecular weight[43].
612
613 Additionally, to detect the weak activity of MMPs, the duration of incubation in the incubation
614 buffer should be prolonged, as this period affects the size of the gelatin-digested area. However,
615 prolonged gelatin digestion may reduce the reliability of the densitometric analysis of the more
616 intense bands as all the gelatin will be over-digested. Positive controls will help estimate the

molecular mass of MMPs, e.g., recombinant MMP-2 and MMP-9 or a cell line with known MMP activity. Negative controls can be reduced samples (boiled for 10 min at 95°C) or samples without gelatinase activity of MMPs. The negative control can also be the medium used for cell starvation to check whether the buffers used in the experiment are free from contamination, e.g., bacterial proteases[36]. Treatment of cells with tissue inhibitors of metalloproteinases (TIMPs) cannot be considered a negative control for gelatin zymography as they dissociate from MMPs during gelatin zymography[5]. It is crucial to culture the cells in a serum-free medium before sample collection and remove cell debris by centrifugation as FBS and cells contain various MMPs[44]. This could lead to false-positive results.

The absence of visible bands could result from reduced samples, no or very low MMP activity in samples, too short incubation of a gel in the activation buffer, too high gelatin concentration in the gel, or insufficient loaded protein. First, the amount of protein can be standardized by loading a broad range of protein amounts into the gel[45]. The absence of latent forms of the MMPs could be due to problems with the SDS:Triton X-100 exchange not leading to renaturation of pro-MMPs and subsequent gelatinase activation. As mentioned earlier, smears with blurred bands may be caused by gelatin digestion by MMPs activated during electrophoresis. A zymogram should exhibit sharp bands (areas with digested gelatin) against a dark background. Suppose the digested areas of the gelatin in the gel are not completely transparent compared to the dark blue background. In that case, it indicates insufficient gel destaining or incomplete gel digestion by MMPs, probably due to excessive gel thickness. Prolonged decolorization of the gel can result in less contrast between the background and the gelatin-digested areas. This may lead to problems with the analysis and unreliable results.

Although gelatin zymography is considered a quantitative assay because of densitometry used for data analysis, data interpretation must be done carefully. Additionally, data analysis is strongly dependent on the quality of zymograms, e.g., separation of bands and their size and shape. As gelatin-digested areas are manually selected and adjusted during analysis, the results may vary between data obtained from ImageJ and ImageLab. Moreover, although pro-MMPs — activated by SDS during SDS-PAGE (opening of active sites without proteolytic cleavage) and refolded — exhibit activity in gelatin zymography, they may not be physiologically active inside cells[46]. Further, if the active form of an MMP exhibits high gelatinolytic activity on a gelatin zymogram, its activity may not be high inside a cell. MMPs are bound to their inhibitors, which block their activity *in vitro*[47] but dissociate from the MMPs during gelatin zymography, allowing them to be active and digest gelatin[5]. Thus, the results of gelatin zymography may not reflect the state of MMP activity inside a cell. Hence, we recommend normalizing MMP activity to the total protein content detected for every cell line. This will avoid misinterpretation of data in the case of unequal amounts of the loaded protein.

The main advantage of gelatin zymography is its sensitivity, which is much higher than that of western blot analysis. Even picograms of MMPs can be detected with gelatin zymography[39]. Additionally, the MMP activity measured by gelatin zymography may not strictly correlate with the amounts of MMPs secreted by the cells. This assay allows the detection of two types of MMPs that share gelatin specificity. An alternative or supplement for gelatin zymography is a

661 fluorescent gelatin degradation assay[48]. This may help confirm the presence of gelatinase activity
662 in melanoma cell lines. The basis of the gelatin degradation assay is an estimation of the activity
663 of invadopodia based on their ability to digest a fluorescent gelatin-coated surface. The final data
664 are usually presented as the size of the gelatin-fluorescein layer digested per cell or per area of a
665 single cell. Although fluorescent gelatin degradation assay facilitates the correlation of gelatin
666 degradation with the number and activity of invadopodia, it cannot, unlike gelatin zymography,
667 distinguish the types of MMPs and estimate their ratios[49–51]. Additionally, the invadopodia
668 formed by cells may not always colocalize with the gelatin-degradation area, and their number
669 may not correlate with the size of the gelatin-digested area in melanoma cells[52]. As invadopodia
670 may contain proteases other than MMPs, such as seprase[53], the fluorescent gelatin degradation
671 assay is not specific for MMPs. The gelatin zymography assay identifies and determines MMP
672 activity based exclusively on their molecular weight. Additionally, MMP activity can be
673 determined in concentration units in gelatin zymography. Another method that detects
674 gelatinase activity is the ELISA targeting specific MMPs[54]. Although the method is highly sensitive
675 and quantitative, it is definitely more expensive than gelatin zymography and cannot detect pro-
676 and active forms of MMPs.
677
678 Gelatin zymography may be useful as a diagnostic technique for detecting MMP activity in
679 biopsies from patients with melanoma, as it has been found that melanoma cells are rich in
680 MMPs, which correlates with a poor prognosis for patients[55]. Thus, MMPs could be promising
681 biomarkers for estimating melanoma occurrence, invasiveness, or progression. Gelatin
682 zymography has been described as a method for detecting disease progression and metastasis
683 of breast, pancreatic, bladder, ovarian, prostate, and brain tumors[56]. By correlating the activity
684 of MMPs in the sera of breast cancer patients with their clinicopathological parameters, Minafra
685 and colleagues showed that the determination of MMP activity may be a putative diagnostic tool
686 not only to confirm the presence of breast cancer in patients but also to distinguish breast cancer
687 subgroups[57].
688
689 The literature shows that gelatin zymography has been successfully used to determine MMP
690 activity in tumor cell lines, such as breast cancer with different metastatic potential (e.g., MCF-7,
691 ZR-75-1, MDA-MB-435, MDA-MB-231, Hs578T)[58, 59], ovarian cancer (SKOV3 and COV504)[60], and
692 other different gynecological cancers[61]. Moreover, this technique is also used for tissue samples
693 obtained from patients with colon[61], rectal[62], breast[58, 63], oral[64], prostate[65], and gastrointestinal[66]
694 cancers or in clinical research on MMP activity in the sera of normal individuals and breast cancer
695 patients[57]. The use of gelatin zymography is also noted in noncancer cells crossing through or
696 remodeling tissue barriers such as monocytes, macrophages, or osteoclasts[67, 68]. The activity of
697 MMPs in these cells arises from podosomes — structures similar to invadopodia[69]. Altogether,
698 these reports demonstrate that a wide range of samples can be analyzed using gelatin
699 zymography.
700
704

705   **DISCLOSURES:**
706   The authors declare no competing financial interests.
707
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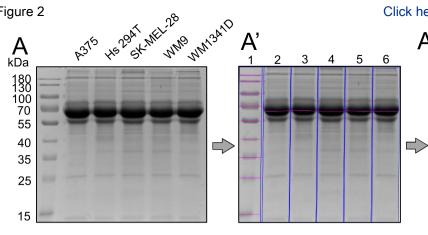
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875   and adhesion of monocytes/macrophages via CD147 signalling pathway in rheumatoid
876   arthritis. *Rheumatology*. **47** (9), 1299–1310 (2008).

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882

Figure 1

Preparation of samples

Growing the cells in full medium
Until 80–90 % of confluence

Growing the cells in serum-free medium
for 48-72 h

Cell medium collection
at 4 °C

Cell debris removal
centrifugation 20 min, 7000 x *g*, 4 °C

STOP POINT 1 (store at -20 °C)

Medium concentration using ultracentrifugal Filter
with 10 kDa molecular weight cutoff

STOP POINT 2 (store at -80 °C)

Protein concentration measurement
Bradford or BCA method

STOP POINT 3 (store at -80 °C)

SDS-PAGE

SDS-PAGE samples preparation
+ 2x non-reducing loading buffer,
20 min incubation at 37 °C

STOP POINT 4 (store at -80 °C)

Preparation of the gel with gelatin
+ at least 12 h incubation at 4 °C

Running electrophoresis
20 – 40 mA per gel at 4 °C

Gel washing – removing of SDS
2 x 30 min at RT in washing buffer

Gel activation
12 h incubation at 4 °C in incubation buffer

Gel staining and analysis

Staining of the gel
30 min at RT in Coomassie
Brilliant Blue solution

Destaining of the gel
at RT in destaining buffer

Gel visualization
with dedicated equipment

STOP POINT 5

Analysis of the gelatin digested area
ImageJ (Fiji) software

Analysis of band intensity
ImageLab software

Figure 2

A''

The total protein content

Lane	Volume (Intensity)	normalization (to the 2nd lane)
2	2408296320	1.000
3	2473947046	1.027
4	2581455594	1.072
5	2559288018	1.063
6	2521873408	1.047

B''

pro-MMP-9

Lane	Mol. Wt. (kDa)	Volume (Intensity)	normalization (to the total protein content)	normalization (to the 2nd lane)
2	92.2	104013432	104013432	1
3	93.7	197530025	192288203	1.849
4	95.3	121698005	113534728	1.092
5	93.7	105316596	99103176	0.953
6	91.5	124100507	118511418	1.139

active MMP-9

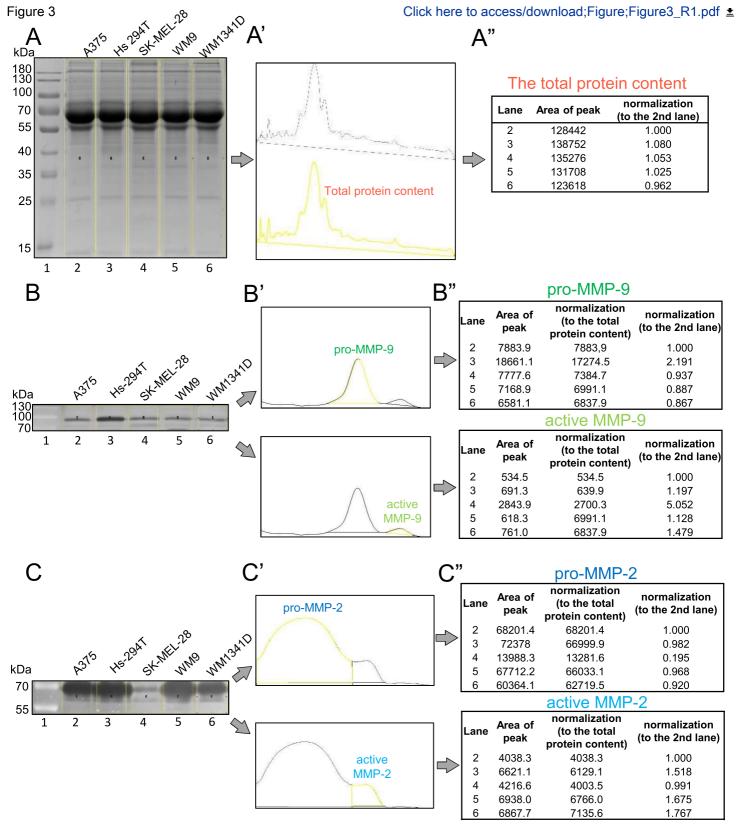| Lane	Mol. Wt. (kDa)	Volume (Intensity)	normalization (to the total protein content)	normalization (to the 2nd lane)
2	80.3	57230340	57230340	1
3	81.0	55122224	53659455	0.938
4	79.1	187481056	174905173	3.056
5	79.1	68265648	64238143	1.122
6	79.1	42791936	40864724	0.714

C''

pro-MMP-2

Lane	Mol. Wt. (kDa)	Volume (Intensity)	normalization (to the total protein content)	normalization (to the 2nd lane)
2	68.2	153409494	153409494	1
3	68.5	152469615	148423553.9	0.967
4	68.7	57791755	53915190.8	0.351
5	68.7	193573704	182153331.6	1.187
6	68.2	207489205	198144556.8	1.292

active MMP-2

Lane	Mol. Wt. (kDa)	Volume (Intensity)	Normalization (to the total protein content)	normalization (to the 2nd lane)
2	59.9	97482315	97482315	1
3	59.8	82044645	79867439.8	0.819
4	59.9	78319945	73066387.7	0.750
5	59.9	101254188	95280440	0.977
6	59.9	96986484	92618524	0.950

Figure 3

A — The total protein content

Lane	Area of peak	normalization (to the 2nd lane)
2	128442	1.000
3	138752	1.080
4	135276	1.053
5	131708	1.025
6	123618	0.962

pro-MMP-9

Lane	Area of peak	normalization (to the total protein content)	normalization (to the 2nd lane)
2	7883.9	7883,9	1.000
3	18661.1	17274.5	2.191
4	7777.6	7384.7	0.937
5	7168.9	6991.1	0.887
6	6581.1	6837.9	0.867

active MMP-9

Lane	Area of peak	normalization (to the total protein content)	normalization (to the 2nd lane)
2	534.5	534.5	1.000
3	691.3	639.9	1.197
4	2843.9	2700.3	5.052
5	618.3	6991.1	1.128
6	761.0	6837.9	1.479

pro-MMP-2

Lane	Area of peak	normalization (to the total protein content)	normalization (to the 2nd lane)
2	68201.4	68201.4	1.000
3	72378	66999.9	0.982
4	13988.3	13281.6	0.195
5	67712.2	66033.1	0.968
6	60364.1	62719.5	0.920

active MMP-2

Lane	Area of peak	normalization (to the total protein content)	normalization (to the 2nd lane)
2	4038.3	4038.3	1.000
3	6621.1	6129.1	1.518
4	4216.6	4003.5	0.991
5	6938.0	6766.0	1.675
6	6867.7	7135.6	1.767

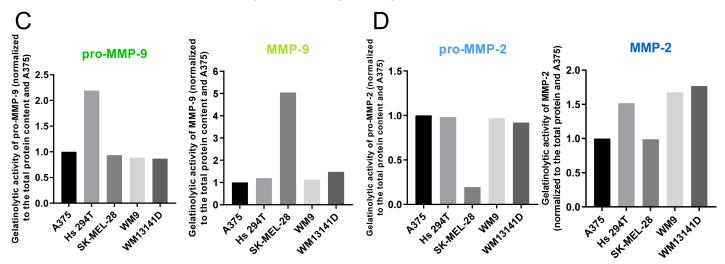Figure 4

Analysis using ImageLab software

Analysis using ImageJ software

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Table of Materials
Table of Materials (3).xls

Please note that we have a hybrid filming/production option where after the manuscript is accepted, the authors would film themselves based on a script that we have provided. The authors would then send us the raw footage so that we can produce and edit the final video. There would be no additional cost for this service. The pricing is only dependent on whether the article is published under standard or open access.

Please let us know if you want to convert to this hybrid filming/production model.

Response: Yes, we would like to convert to hybrid filming/production model.

Editorial and production comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Response: The protocol has been proofread by all the authors. We improved the protocol so that it no longer contains any spelling, grammar issues, or typos in the text. We hope, we caught all the mistakes.
2. Please do not include references in the abstract section.
Response: References have been removed from the abstract.
3. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note."
Response: All sentences in each step of the protocol have been verified for the imperative tense. A few non-imperative sentences have been transferred to the "notes"). We used phrases such as "could be, should be…" only in the notes.
4. Please include a single line space between each step, substep and note in the protocol section.
Response: That has been done.
5. Please ensure you answer the "how" question, i.e., how is the step performed?
Response: We have ensured that each step includes information about how the step was performed.
6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step.
Response: We have checked every single step of the protocol and we confirm that none of them contain more than 2-3 actions.
7. Please ensure the results are described in the context of the presented technique. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc.
Response: We confirm that. In the "result chapter" the information that the results of gelatin zymography are zymograms, obtained by Coomasie Blue staining, what we can observe in zymograms and how to analyze, quantify and normalize this data (densitometric analysis).
8. Please obtain explicit copyright permission to reuse any figures from a previous

publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

Response: In this protocol, we did not reuse any figures from our previous publications.

9. As we are a methods journal, please ensure that the Discussion explicitly cover the following in detail in 3-6 paragraphs with citations:

a) Critical steps within the protocol.

b) Any modifications and troubleshooting of the technique.

c) Any limitations of the technique

d) The significance with respect to existing methods

e) Any future applications of the technique

Response: We confirm that the discussion covers all these information with citations. As the discussion included 8 paragraphs, according to this requirement we change the organization of paragraphs to obtain 6 ones.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Mrowczynska et al. describes the gelatin zymography assay using melanoma cell lines as example. The method is based on loading sups of cancer cells on SDS-PAGE containing gelatin, in denatured but non-reducing conditions, followed by renaturation of proteins in the gel (MMPs). The MMPs are then allowed to degrade the gelatin which is included in the gel, the gel is stained with Coomassie blue, and degradation can be observed as white bands in places where the gelatin was degraded and in the size of the specific MMP that was active. The level of degradation can then be quantified using image analysis software.

Major Concerns:

Overall, the manuscript is well written and the protocol is detailed and explained, with attention (in notes) to details that may be critical for the success of the experiment. I have a few non-technical comments which could be addressed in the introduction and/or discussion parts of the manuscript:

We would like to thank the Reviewer for the revision of our protocol and valuable comments. Below, the Reviewer will find our responses to the comments and suggestions. All changes in the protocol done by us are presented as tracked changes. The lines numbers with the changes done are indicated according to the "Simple Markup" view in the "Review" option.

1. Please mention other cell lines (of other types of cancer) that could be successfully used in the gelatin zymography assay, and corresponding references.

Response: According to the Reviewer's request, we have added the information (with references) about other cell lines used in gelatin zymography at the end of the discussion part (lines 578-584). In addition to other cancer cell lines (such as breast or gynecological cancer cell lines), we decided to mention as well about the tissue samples obtained from patients with different types of cancers (colon, rectal, breast, oral, prostate, gastrointestinal), as we think it will be a great supplement for this part of the topic.

2. Can the method be used or has it been used for non-cancer cells/cell lines? for primary cancer cells?

Response: We have found papers showing the usage of zymography in non-cancer cells such as monocytes, macrophages, and osteoclasts (lines 584-588). They exhibit MMPs' activity thanks to the structures called podosomes. This allows them to cross and remodel the tissue barriers, therefore, manifesting their activity.

3. In the discussion part, please explain more why this method is preferable on ELISA, and especially why is it better than the fluorescent degradation assay in which MMPs activity is observed locally in cells and can be correlated with invadopodia activity.

Response: According to the Reviewer's suggestion, we have added this fragment in the discussion part (lines 556-565):" . Although fluorescent gelatin degradation assay gives an opportunity to correlate the degradation of gelatin with the amount and activity of invadopodia, it cannot, opposite to the gelatin zymography, distinguish the types of MMPs and estimate their ratio. Additionally, the formed by cells invadopodia may not always co-localize with the gelatin-degradation area and their numbers may not correlate with the size of the gelatin-digested area in melanoma cells. As the invadopodia may contain other than MMPs proteases such as seprase, the fluorescent gelatin degradation assay is not specific for MMPs. Gelatin zymography assay identifies and determines exclusively MMPs' a activity based on their molecular weight. Additionally, in the gelatin zymography, the activity of MMPs can be determined in concentration units.".

The main two reasons for gelatin zymography to be preferable on ELISA is that it is cheaper and allows to distinguish latent and active forms of individual MMPs (lines 565-568).

4. Lines 539-543: While the application of the method to diagnostics of melanoma patients is very relevant and important, the sentence is not so clear to me. Could you please elaborate and describe an example for such usage in more details (from the reference mentioned).

Response: As the Reviewer wished, we elaborated this fragment of discussion about the diagnostic usage of gelatin zymography and the determination of MMPs activity. We added a few confirmations with references that this technique can be as well used for diagnostic of different types of cancer even with differentiation of their subtypes. Changes have been done in lines 572-578.

Minor Concerns:

1. Lines 127-128: could you please specify the cuttoff of the Amicon filter and explain why this size is chosen?

Response: According to the Reviewer's request, the cutoff of Amicon filters with the explanation why we chose this size has been pointed out in 1.8 step with its notes. (lines 130-137).

2. Line 512: please remove an extra comma.

Response: The comma has been removed.

Reviewer #2:

Manuscript Summary:

The manuscript describe in detail the gelatin zymography assay to analyze the gelatinase activity of melanoma cells. The authors were very clear and exaustive; the protocol is well described as well as the annotation for the proper execution of the gelatin zymography.

We thank the Reviewer for the revision of this protocol. Below, the Reviewer will find our responses to the comments and suggestions. We would like to inform you that any changes in the protocol done by us after the revision are presented as tracked changes. The lines numbers with the changes done are indicated according to the "Simple Markup" view in the "Review" option.

Minor Concerns:

I have a doubt about the volumes of APS and Temed to use to prepare the gels. Even if the volume is 10 ml i both cases, the authors use different volumes.

Response: We have corrected the volumes. We have noted that we did mistakes in the case of one gel (step 3.4, lines 210-212), instead of 10 ml there should have been 5 ml. Additionally, the volumes were calculated not for 10 ml and for 5 ml, but for 3,9 ml . We would like to apologize for that. We have corrected that. These amounts now are optimal for this type of experiment.

There are several typos in the text.
Response: The protocol has been proofread by all the authors. We hope that we have corrected the protocol so that it no longer contains any typos in the text.

Reviewer #3:

We thank the Reviewer for the revision of this protocol. Below, the Reviewer will find our responses to the comments and suggestions. We would like to inform you that any changes in the protocol done by us after the revision are presented as tracked changes. The lines numbers with the changes done are indicated according to the "Simple Markup" view in the "Review" option.
Major concerns:
1. Line 24, the mentioning of invadopodia and invadopodia assay in the abstract is not appropriate for this manuscript, since the method was not related to invadopodia. Notably MMPs are also secreted by cancer cells without invaodpodia.
Response: The Reviewer rightly pointed out that invadopodia activity assay should not be included in the abstract. We decided to mention about this assay, because invadopodia are important structures for MMPs secretion and activity. We removed this fragment. The assays for the determination of invadopodia activity have been mentioned by us in the discussion part, where we compared the different techniques used for MMPs activity (lines 551-563). According to the Reviewer's #1 request, we added as well the information about the usage of gelatin zymography in non-cancer cells which do not form invadopodia, but podosomes responsible for the presence of MMPs activity in these cells (lines 584-587).
2. Line 149, here weighing acrylamide for every single experiment is cumbersome and unsafe, since acrylamide has toxicity. The stock solutions of acrylamide/Para acrylamide are quite stable and most people use stock solutions for the preparation of PAGE gels. There were also commercial stock solution available. The authors should modify this.
Response: There is a misunderstanding. We have written in line 149 (new line 152) that gelatin is weighted by us every time before performing the gelatin zymography. Acrylamide for preparation of the gels was used in 30% solution form (line 158).
3. Line 253, most zymography assays include Zn2+ in the incubation buffer, since MMPs are Zn metalloproteases and Zinc is essential for their activities. It is unclear why the authors didn't have ZnCl2 in their solution.
Response: We aware of the fact that MMPs need zinc ion to function. However, because our protocol works, we assume that processing of the samples does not cause dissasiocation of Zn^{2+} from MMPs as they can digest gelatin under experiment conditions. One can buy gel and buffers for gelatin zymography from Thermo Fisher Scientific/Novex (#ZY00100BOX, #LC2670 and #LC2671) or Bio-Rad (#1610765 and #1610766), and these reagents do not contain zinc salt neither.
4. Please indicate that other instrument/software could also be used to image the gel.

Response: According to the Reviewer's request, other image systems have been mentioned in this protocol to use for gel visualization (lines 303-305).

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