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Cover Letter

THE OHIO STATE UNIVERSITY

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August 13, 2018

Dr. Avital Braiman
Director of Editorial
Journal of Visualized Experiments
1 Alewife Center, Suite 200
Cambridge, MA 02140

Dear Dr. Braiman:

I am pleased to submit an original research protocol entitled "Detection of Proteases by Fluorescent Peptide Zymography", by Ameya Deshmukh, Jessica Weist and Jennifer Leight, for consideration for publication in the *Journal of Visualized Experiments.*

In this manuscript, we describe the protocol for a modified zymographic technique in which we covalently link fluorescent peptides to polyacrylamide gel matrices using an azido-PEG3-maleimide crosslinker. This manuscript provides an in-depth protocol as a companion to our recently published article in *Biotechniques*. This technique integrates degradable moieties through a covalent reaction into a zymogram gel. This covalent incorporation is a critical feature of this method, which now enables incorporation of a vast array of degradable substrates. Covalent attachment overcomes the susceptibility of these substrates to diffuse out of the gel during electrophoresis or development. The modified gels were used to measure the activity of various proteases in purified samples as well as in conditioned cell media, enabling detection of proteases not currently observed with gelatin zymography. Fluorescent peptide zymography greatly expands the library of proteases that can be detected and presents a technique for improving the sensitivity of current methods through design of new substrates.

We believe that this manuscript is appropriate for publication by the *Journal of Visualized Experiments* because it outlines an original technique which researchers in various disciplines can use to separate proteases by molecular weight and detect their activity. Fluorescent peptide zymography can complement current efforts aimed at elucidating the roles of proteases in disease, development and wound healing.

We have no conflicts of interest to disclose.

Thank you for your consideration!

Sincerely,

Jennifer Leight

Jennifer L. Leight, Ph.D.
Assistant Professor of Biomedical Engineering
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TITLE:

Detection of Protease Activity by Fluorescent Peptide Zymography

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KEYWORDS:
Zymography, Fluorescent Peptide, Protease, Matrix Metalloproteinase (MMP), Covalent Crosslinking, Cancer, Conditioned Media, Electrophoresis

SUMMARY:
Here, we present a detailed protocol for a modified zymographic technique in which fluorescent peptides are used as the degradable substrate in place of native proteins. Electrophoresis of biological samples in fluorescent peptide zymograms enables detection of a wider range of proteases than previous zymographic techniques.

ABSTRACT:

The purpose of this method is to measure the proteolytic activity of complex biological samples. The samples are separated by molecular weight using electrophoresis through a resolving gel embedded with a degradable substrate. This method differs from traditional gel zymography in that a quenched fluorogenic peptide is covalently incorporated into the resolving gel instead of full length proteins, such as gelatin or casein. Use of the fluorogenic peptides enables direct detection of proteolytic activity without additional staining steps. Enzymes within the biological samples cleave the quenched fluorogenic peptide, resulting in an increase in fluorescence. The fluorescent signal in the gels is then imaged with a standard fluorescent gel scanner and quantified using densitometry. The use of peptides as the degradable substrate greatly expands the possible proteases detectable with zymographic techniques.

INTRODUCTION:

Gel zymography is a biological technique used to measure proteolytic activity within biological

samples, such as body fluids or cell culture media[1–3]. The samples are separated by their molecular weights with electrophoresis through a polyacrylamide gel embedded with a degradable substrate. Common degradable substrates include gelatin, casein, collagen and elastin, which have been used to measure the activity of matrix metalloproteinases (MMPs) -1, -2, -3, -7, -8, -9, and -11, in addition to a variety of cathepsins[1,2,4–8]. After electrophoresis, the enzymes are renatured and allowed to degrade the protein within the gel. In traditional gel zymography, the gel is stained with a protein dye, such as Coomassie Blue, and protease activity is detected as a loss of signal, *i.e.,* white bands (degradation of protein) on a dark blue background.

Here, we describe a protocol for an alternative method of gel zymography, in which the degradable substrate is a short, fluorogenic peptide covalently incorporated into the polyacrylamide gel (**Figure 1**). The substitution of synthetic peptides as the degradable substrates enables detection of a wider range of proteases as compared to traditional gel zymography with native proteins[9]. Covalent linkage of the fluorogenic peptide prevents peptide diffusion and migration during gel electrophoresis observed with previous methods[9,10]. Furthermore, the use of a fluorogenic substrate enables direct detection of protease activity without additional staining and de-staining steps. The overall goal of this method is the detection of protease activity in biological samples via the covalent incorporation of fluorogenic peptides in zymogram gels.

PROTOCOL:

1. Preparation of the Resolving Gel Layer

1.1. Prepare a 10% polyacrylamide resolving gel solution as per **Table 1**. Add the Tetramethylethylenediamine (TEMED) and Ammonium Persulfate (APS) immediately prior to pouring the gel as their addition initiates the polymerization reaction.

1.2. Fill an empty 1.5 mm mini-gel cassette half way (5 mL) with the 10% resolving gel solution.

1.3. Add a thin layer of isopropanol (~500 µL) to the top of the polyacrylamide gel to produce a level gel and prevent bubbles. Use the leftover polyacrylamide solution to track the progress of the polymerization reaction. When the polyacrylamide in the tube has completely solidified, the reaction is complete (~40 min).

2. Preparation of the Azido-PEG3-maleimide Linker Molecule

2.1. While the first resolving gel layer is polymerizing, retrieve the azido-PEG3-maleimide kit from the -20 °C storage and allow the components to reach room temperature. There are two components in each kit. Vial 1 contains a maleimide-NHS ester, an off-white to grey solid. Vial 2 contains azido-PEG3-amine, a slightly yellow oil.

NOTE: The authors suggest using the 25 mg azido-PEG3-maleimide kit as it can only be stored for short periods of time (1-2 hours) at -20 °C after being prepared before it begins to degrade. 25 mg is sufficient to produce 10 peptide gels. Use gels within 3 weeks of preparation.

2.2. Dissolve the components of Vial 2 in the manufacturer recommended volume of dimethyl sulfoxide (DMSO) and vortex for 30 s to ensure the liquids have been mixed well.

2.3. Transfer the contents of Vial 1 into a clean, dry 100 mL round-bottom flask containing a stir bar.

NOTE: Rinse the flask with acetone and dry completely prior to usage to prevent moisture from interfering with the reaction.

2.4. Immediately insert a rubber septum stopper with a diaphragm that can be punctured with a syringe into the mouth of the flask. Work quickly to prevent moisture from entering the flask.

2.5. Insert two 18 gauge syringe needles into the diaphragm and connect one to an inert gas tank (*e.g.* argon gas). Allow the inert gas to fill the flask for 3 min. Mix the components of Vials 1 and 2 under inert gas to prevent undesirable reaction products.

CAUTION: The second syringe needle is to provide a vent, thereby allowing the atmospheric air contained within the flask to flow out of the flask as it fills with inert gas. Do not forget to include a vent needle!

2.6. Shut off the inert gas and detach it from the needle. Using a syringe, inject the full contents of Vial 2 into the flask.

2.7. Remove both needles and syringe and allow the components to mix for 30 min at room temperature while stirring.

2.8. Remove the rubber septum stopper and transfer the contents to a clean 5 mL centrifuge tube. The azido-PEG3-maleimide solution must be used within 1 hour at room temperature.

3. Preparation of the Peptide Resolving Gel Layer

3.1. Once the first resolving gel layer has polymerized, pour off the isopropanol layer. Rinse the top of the gel by pipetting 1 mL of deionized water on the top of the gel and then pour off the water.

3.2. Retrieve the thiol-functionalized fluorescent peptide from -80 °C storage and allow it to thaw at room temperature.

NOTE: The thiol-functionalized peptide can be prepared as described previously[11,12]. Commercially available peptides can also be used but require the addition of a terminal cysteine residue to enable the maleimide-thiol click reaction. Dissolve the peptide to a stock concentration of 10 mM and store it at -80 °C in small (30 uL) aliquots to limit repeated freeze-thaw cycles.

3.3. Prepare a 10% resolving gel solution containing the azido-PEG3-maleimide linker molecule and the fluorescent peptide as per **Table 1**. Add the TEMED and APS immediately prior to pouring the gel as their addition initiates the polymerization reaction.

3.4. Fill half of the remaining portion of the gel cassette (3 mL) with the peptide resolving gel solution.

NOTE: A multi-layer resolving gel approach reduces the amount of peptide and linker necessary for each gel. The size of the peptide resolving gel layer can be adjusted to accommodate a larger range of molecular weights as needed.

3.5. Pipette a thin layer of isopropanol (~500 µL) to the top of the polyacrylamide gel to produce a level gel and prevent bubbles. Use the leftover polyacrylamide solution to track the progress of the polymerization reaction. When the polyacrylamide in the tube has completely solidified, the reaction is complete (~40 min).

NOTE: The fluorescent peptide is light sensitive. Keep the gels covered with aluminum foil to prevent photobleaching during gel preparation, electrophoresis, washing and development.

3.6. Pour off the isopropanol layer and rinse the top of the peptide resolving gel with deionized water as in step 3.1.

3.7. If using the gels immediately, proceed to step 4, otherwise, immerse the prepared gels in 100 mL of 1X phosphate buffered saline (PBS) at 4 °C in a plastic box to prevent the gels from drying out. Wrap the box in aluminum foil to prevent photobleaching. Gels can be stored in PBS for up to 3 weeks prior to usage.

4. Preparation of the Stacking Gel

4.1. Prepare a 5% stacking gel solution as per **Table 1**. Add the TEMED and APS immediately prior to pouring the gel as their addition initiates the polymerization reaction.

4.2. Fill the remaining empty portion of the gel cassette (~2 mL) with the stacking gel solution.

4.3. Quickly insert a 1.5 mm gel comb into the stacking gel layer, making sure no bubbles remain trapped under the wells. Use the leftover polyacrylamide solution to track the progress of the polymerization reaction. When the polyacrylamide in the tube has completely solidified, the reaction is complete (~10 min).

4.4. Gently remove the comb and the tape from the back of the gel cassette.

5. Preparation of Biological Samples for Electrophoresis

5.1. Prepare conditioned cell media, cell lysates, tissue homogenates, and MMP standards as described elsewhere under non-reducing conditions[2]. Do not heat samples.

5.2. For example, prepare conditioned cell media as follows:

5.2.1. Plate 40,000 cells/cm^2 cells in a 6-well plate in 10% fetal bovine serum (FBS) culture media. Incubate cells in a humidified chamber (5% CO_2 at 37 °C) for 24 hours and allow them to reach 70-80% confluence.

NOTE: If the cells have not reached the desired confluency after 24 hours, allow them to grow in 10% FBS culture media for an additional 24 hours.

5.2.2. Wash the cells twice with PBS and add 2 mL of serum-free culture media. Incubate the cells in a humidified chamber (5% CO_2 at 37 °C) for an additional 24 hours.

5.2.3. Using a serological pipette, collect the conditioned media from each well. Centrifuge the media at 1200 rpm for 3 minutes to remove any cell debris. Take the supernatant and concentrate using a 15 mL, 10 kDa molecular weight cutoff centrifugal filter unit. Centrifuge the filter units at 4000 x g for 15 min in a swinging bucket rotor or 5000 x g for 15 min in a fixed angle rotor.

NOTE: This step is optional but can enhance the intensity of the proteolytic bands in the peptide zymography gels.

5.2.4. Transfer the concentrated filtrate to a fresh 1.5 mL centrifuge tube. Aliquot and store samples at -80 °C for up to three freeze-thaw cycles.

5.3. Quantify protein content using a standard protein quantification assay (*e.g.* BCA, Bradford Assay, *etc.*).

6. Electrophoresis of Biological Samples in Peptide Zymography Gels

6.1. Dissolve samples in conventional zymography sample buffer (62.5 mM Tris-HCl, pH 6.8, 25% glycerol, 4% SDS, 0.01% bromophenol blue). For cell and tissue samples, ~30 µg of total protein per well is recommended, and 50-100 ng of protein for MMP standards.

6.2. Add 400 mL of 1x Tris-Glycine SDS Running Buffer to the gel apparatus. Load up to 35 µL of sample per well. Run the samples at 120 V at 4 °C for 1.5 hours or until the molecular weight standards (which have a visible orange color) indicate that the proteases of interest are within the peptide resolving gel layer.

NOTE: Most MMPs and their variants fall within the range of 35-100 kDa. When the molecular weight standards indicate that those weights are within the peptide resolving gel layer, electrophoresis can be stopped. The same principle can be applied to other classes of proteases

with known molecular weights. If there is an interest in detecting multiple proteases over a larger range of molecular weights, reduce the size of the resolving gel layer and increase the size of the peptide resolving gel layer.

6.3. Following electrophoresis, remove the gels from the plastic cassette and wash gels three times for 10 min each at room temperature under gentle agitation in renaturing buffer containing 2.5% Triton X-100, 1 µM $ZnCl_2$, and 5 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.5.

6.4. Transfer gels to a developing buffer solution containing 1% Triton X-100, 1 µM $ZnCl_2$ and 5 mM $CaCl_2$ in 50 mM Tris-HCl, pH 7.5 for 15 min. Replace with fresh developing buffer solution and incubate gels at 37 °C under gentle agitation for 24 hours, making sure the gels are fully submersed in the solution.

7. Imaging of Peptide Zymography Gels

7.1. After 24 hours, image gels using a fluorescent gel scanner/imager using the appropriate excitation and emission filters. For example, the peptide gels shown in the representative results are conjugated with Fluorescein and were imaged using an excitation filter of 488 nm and an emission filter of 521 nm. Using the appropriate filters for your fluorophore will maximize the detection of proteolytic activity.

NOTE: Images can also be taken with a gel imager equipped with a UV transilluminator, often used for the imaging of DNA gels stained with ethidium bromide. Image the gels using the UV transilluminator (365 nm) setting and an emission filter of 590 nm.

7.2. Conduct densitometric evaluation of band intensities using ImageJ as described elsewhere[13].

REPRESENTATIVE RESULTS:

Using the method described here, two fluorescent protease-degradable peptides were incorporated into polyacrylamide gels: GGPQG↓IWGQK$(PEG)_2$C (abbreviated as QGIW throughout the text and figures) and GPLA↓C^{pMeOBzl}WARK$(PEG)_2$C (abbreviated as LACW throughout the text and figures). ↓ indicates the site of cleavage. QGIW is a collagen-I derived sequence designed to detect cellular collagenases[14]. LACW is a sequence that has been optimized for the detection of MMP-14 and MMP-11[15]. The peptides are labeled with dabcyl (quencher) and fluorescein (fluorophore) using N-hydroxysuccinimide (NHS)-ester-amine chemistry[11]. It can be difficult to develop new fluorogenic peptides that have adequate fluorescence quenching and are soluble in standard buffers. Therefore, adapting peptide sequences from commercially available fluorescent protease substrates to include a C terminal cysteine is often a successful strategy to develop new fluorogenic sensors. To demonstrate the ability of peptide zymography to separate complex protease mixtures, conditioned media was collected from two different cancer cell lines. HT1080 fibrosarcoma cells and MDA-MB-231 breast adenocarcinoma cells were plated in 10% FBS media for 24 hours, after which the media was replaced with serum-free media for an additional 24 hours. Conditioned media samples were collected and concentrated using

10 kDa molecular weight cutoff centrifugal filter units. The protein content of the media was measured using a standard μBCA assay. 30 μg of protein from conditioned media were electrophoresed. As positive controls, wells with type I bacterial collagenase (100 μg) or purified, activated MMP-9 (125 ng) were also included. The gels were incubated for 24 h in developing buffer to allow MMP cleavage of the degradable substrates within the gels (**Figure 2**) and then imaged. Fluorescent imaging revealed numerous bands were visible within the LACW peptide gels (**Figure 2A**[14]), while only a single band was apparent within QGIW gels (**Figure 2D**[14]). In comparison to gelatin zymography (**Figure 2G**[14]), LACW gels were able to detect more proteolytic bands, demonstrating the ability of peptide zymography to detect a wider range of proteases present within biological samples than traditional methods using native substrates.

To verify the identity of the visualized bands as MMPs, peptide zymography gels were incubated in development buffer containing either 20 μM GM6001, a broad-spectrum MMP inhibitor, or 10 μM E-64, a general cathepsin inhibitor. Treatment of the LACW peptide gels with GM6001 (**Figure 2B**[14]) decreased the intensity of the bands, while treatment with E-64 (**Figure 2C**[14]) had no discernable effect. Treatment of the QGIW peptide gels with GM6001 resulted in complete ablation of the previously seen bands (**Figure 2E**[14]). As expected, E-64 did not have any effect (**Figure 2F**[14]). In both peptide gels, GM6001 inhibited purified MMP-9 activity but did not affect bacterial collagenase activity, further verifying that the visualized increase in fluorescence was a result of proteolytic activity by MMPs present within the tested biological samples.

To compare the sensitivity of peptide zymography to the current gold standard, gelatin zymography, a sensitivity analysis was conducted using purified, activated MMP-9. Serial dilutions of MMP-9 (1-250 ng) were electrophoresed in LACW, QGIW and gelatin zymography gels (**Figure 3A**[14]). Following development and fluorescent imaging, band intensities were quantified with ImageJ and plots of normalized band intensity were generated to calculate EC_{50} values—the concentration that produces 50% of the maximum signal (**Figure 3B**[14]). LACW peptide gels were able to detect the smallest concentrations of MMP-9, with an EC_{50} value of 17.28 ng, as compared to QGIW and gelatin zymograms with values of 59.50 ng and 31.85 ng, respectively. These data indicate that the use of peptide zymography can match or exceed the sensitivity limits of native substrates like gelatin.

FIGURE & TABLE LEGENDS:

Figure 1: Schematic of the fluorescent peptide zymography process. (A) Preparation of the multi-layer polyacrylamide resolving gel. A standard 10% resolving gel solution is used to form the first layer of the peptide zymography gel. A second 10% resolving gel layer containing a quenched, fluorescent peptide and an azido-PEG3-maleimide linker molecule is then polymerized on top of the first layer. The final top layer is a 5% stacking gel. **(B)** Standard electrophoresis under non-reducing conditions is used to separate protease-containing samples in the functionalized polyacrylamide gels. The gels are washed to remove SDS and to allow the proteins to renature. The gels are then incubated in a development buffer for 24 h at 37 °C, allowing the proteases to cleave the fluorogenic peptides, resulting in increased fluorescence. This fluorescence, corresponding with protease activity, is then captured using a fluorescent gel

imager at an excitation of 488 nm and emission of 521 nm (Adapted with permission from Biotechniques and Future Science[14]).

Figure 2: **Detection of cell-secreted proteases in human cancer cell lines.** Analysis of collagenase enzyme (100 µg), MMP-9 (125 ng) and conditioned cell media from HT1080 fibrosarcoma (30 µg) and MDA-MB-231 adenocarcinoma breast cancer (30 µg) cell lines in LACW and QGIW peptide gels. Gels were treated with DMSO (vehicle control) **(A & D)**, treated with GM6001 **(B & E)** or treated with E-64 **(C & F)**. **(G)** Gelatin zymogram of HT1080 and MDA-MB-231 conditioned cell media (Adapted with permission from Biotechniques and Future Science[14]).

Figure 3: **Comparison of MMP-9 Sensitivity of Peptide and Gelatin Zymograms. (A)** QGIW (top), LACW (middle) and gelatin (bottom) zymography gels were subjected to serial dilutions of MMP-9. **(B)** Normalized band intensities were plotted against MMP-9 concentration and fit to a four parameter variable slope curve. EC_{50} values indicate concentration at half the maximum signal. Results are represented as n=3, mean $\pm$ SD (Adapted with permission from Biotechniques and Future Science[14]).

Table 1: **Reagent table for preparing fluorescent peptide zymography gels.** Concentrations and volumes for the preparation of the multi-layer peptide zymography gel.

DISCUSSION:

Current zymographic techniques rely on the incorporation of native substrates into polyacrylamide gels for the detection of proteolysis. While these techniques have garnered widespread use, they are still limited in the number of proteases they can detect. Here, a protocol was described in which fluorescent, protease-degradable peptides are incorporated into the polyacrylamide resolving gel. Covalent coupling using an azido-PEG3-maleimide linker molecule enables the separation and detection of a wider variety of proteases than is currently attainable with native substrates. The highly tunable nature of fluorescent peptides affords researchers the ability to design substrates that can target their proteases of interest. Numerous peptide substrates have been identified for a wide variety of proteases using peptide libraries, and there are a growing number of commercial sources manufacturing custom peptides. It can be difficult to develop new fluorogenic peptides that have adequate fluorescence quenching and are soluble in standard buffers. Therefore, adapting peptide sequences from commercially available fluorescent protease substrates to include a C-terminal cysteine is often a successful strategy to develop new fluorogenic sensors.

While completing this protocol, care should be taken while handling fluorescent peptide gels to prevent excessive exposure to light as this can significantly reduce the detected fluorescent signal. Additionally, the current concentration of peptide used in each gel is 75 µM. This can be adjusted to lower concentrations to conserve peptide, keeping in mind that the azido-PEG3-maleimide solution must be added to the solution in at least a 20 molar excess to the peptide. The azido-PEG3-maleimide kit can be purchased in 3 sizes (25, 100 and 1000 mg). The authors recommend purchasing the 25 mg kit as the prepared solution can only be stored at short periods

of time at -20 °C. Furthermore, a 25 mg kit is sufficient to prepare 10 peptide gels, which must be used within 3 weeks of preparation.

One of the limitations of zymography is the difficulty in discerning the exact identity of visualized protease bands due to significant overlap in molecular weights. In future studies, it will be critical to conduct secondary analysis to determine their identity using techniques such as mass spectrometry[16,17]. Another limitation of zymography is the refolding of proteins to their active conformation following partial denaturing by SDS and electrophoresis. These processes can cause a change in the active conformation of the protease, rendering proteolytically inactive proteins, active. For example, pro-MMP-2 can be detected in gelatin zymograms despite having the inhibitory pro-domain intact due to its renaturation to an intermediate active form. Supplementary methods like enzyme-linked immunosorbent assay (ELISA) or Western Blots can be used to determine the identity and total presence of a protease of interest.

This article demonstrates the use of fluorescent peptide substrates for enhancing the sensitivity of current zymographic techniques. Using purified MMP-9, a concentration gradient analysis was conducted comparing LACW, QGIW and gelatin zymography gels. Currently, gelatin zymography is the gold standard technique by which the gelatinases (MMP-2 and -9) are detected in biological samples. Comparing the EC_{50} values of the three substrates, LACW peptide gels had the lowest values, indicating the highest sensitivity. Utilizing different peptide sequences designed for detection of specific proteases can potentially enhance these sensitivities even further. Treatment of the gels with an MMP activating agent such as 4-aminophenylmercuric acetate (APMA) or heparin can also be used to boost a weak signal as previously described[18].

In addition to the measurement of protease activity for biological studies, protease-degradable peptides are also often used for crosslinking synthetic hydrogels for tissue engineering and drug delivery applications. Controlled degradation is critical for these applications. Currently, the degradation kinetics of these peptides are characterized using single, purified enzymes. However, determining which enzymes cells actually produce and are responsible for cleavage of these peptides has been difficult to determine. The use of peptide zymography to quantify cell and tissue-specific enzyme release will greatly aid in the rational design of these peptide crosslinking sequences.

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DISCLOSURES:

The authors have nothing to disclose.

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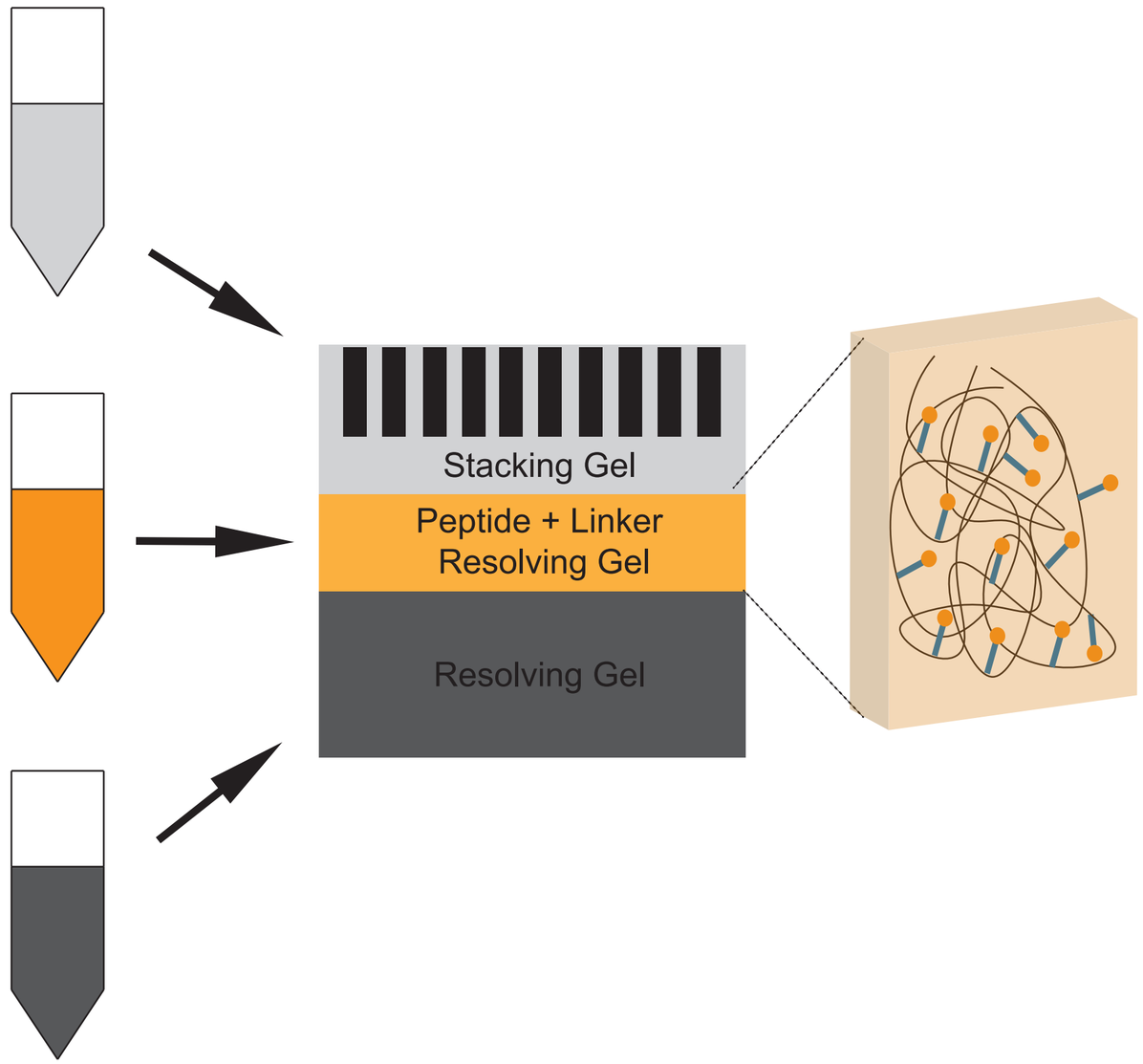
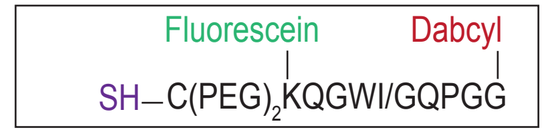
A

5% Polyacrylamide Stacking Gel

10% Polyacrylamide Resolving Gel +
75 µM MMP Degradable Peptide +
1.5 mM Azido-PEG3-Maleimide Linker

10% Polyacrylamide Resolving Gel





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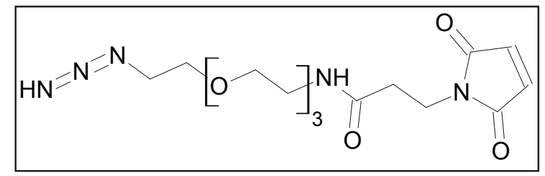
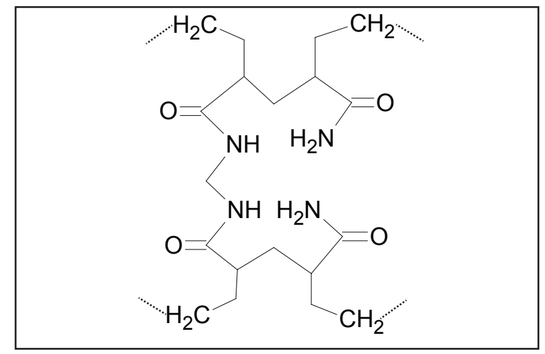


Azido-PEG3-Maleimide Linker



Polyacrylamide

B

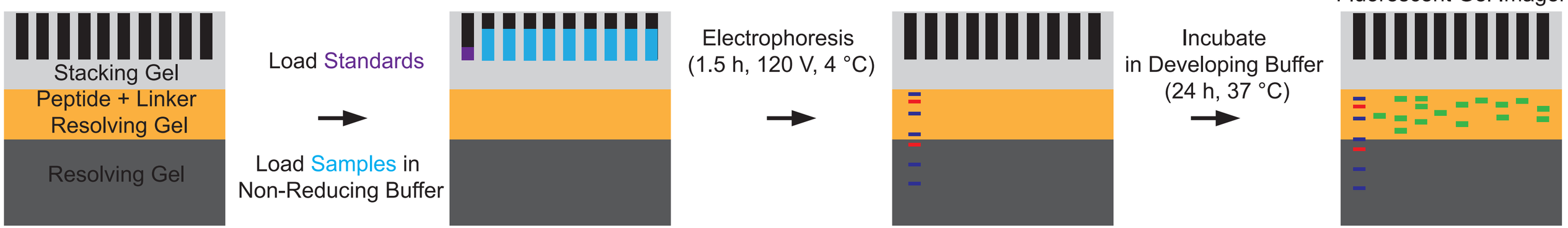


Figure 2

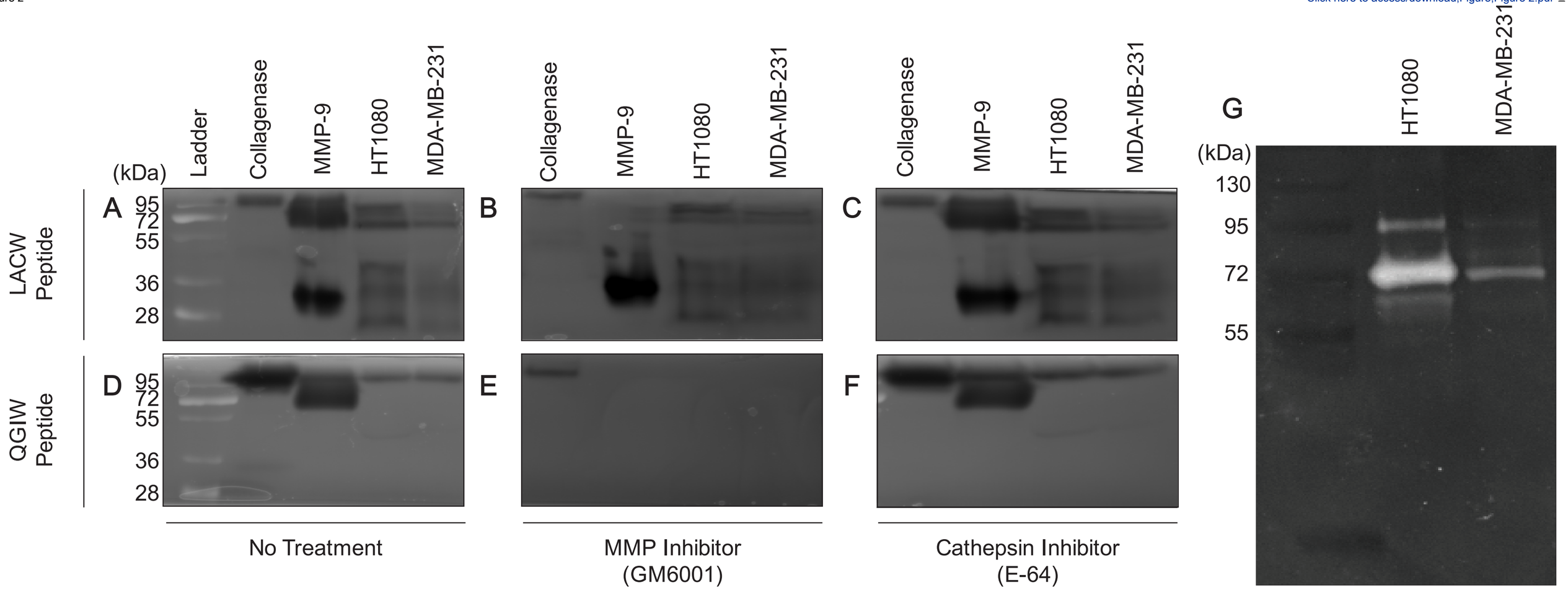
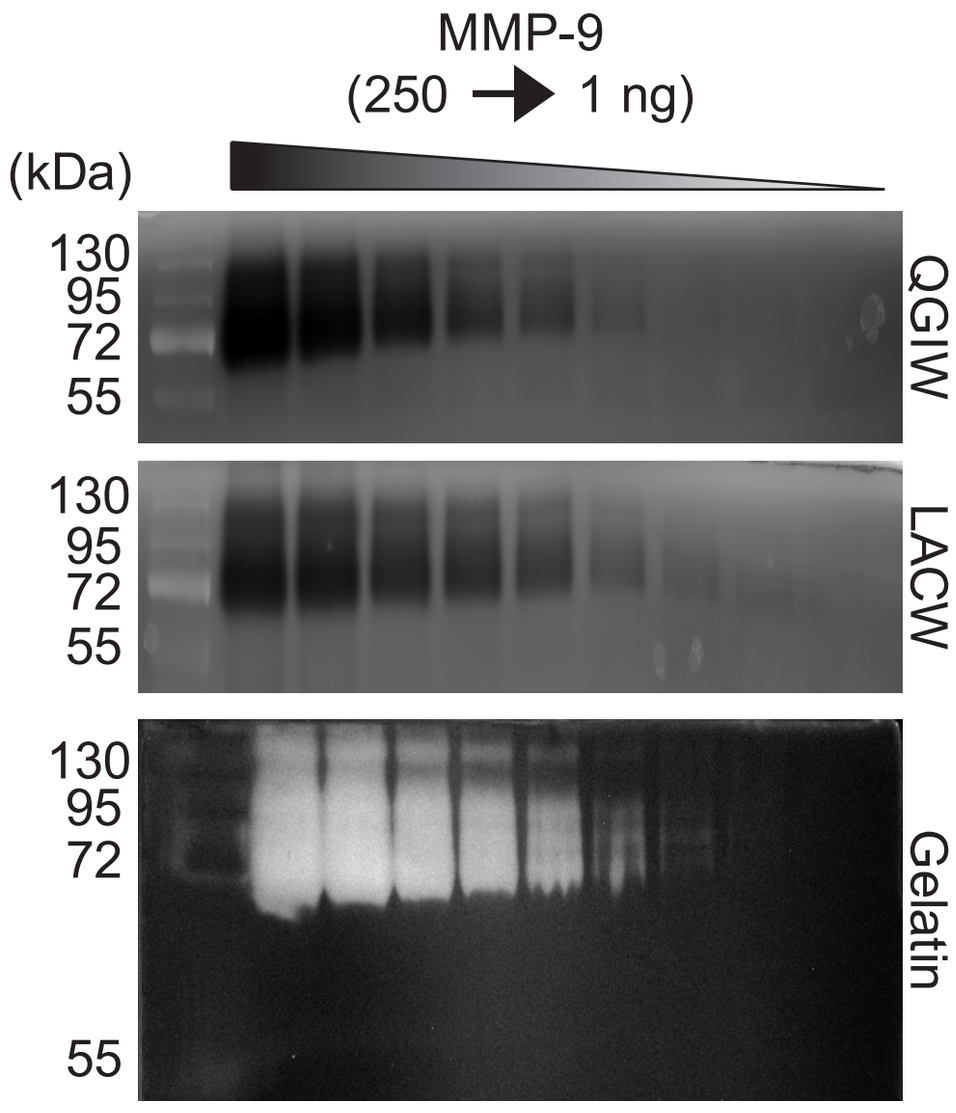


Figure 3



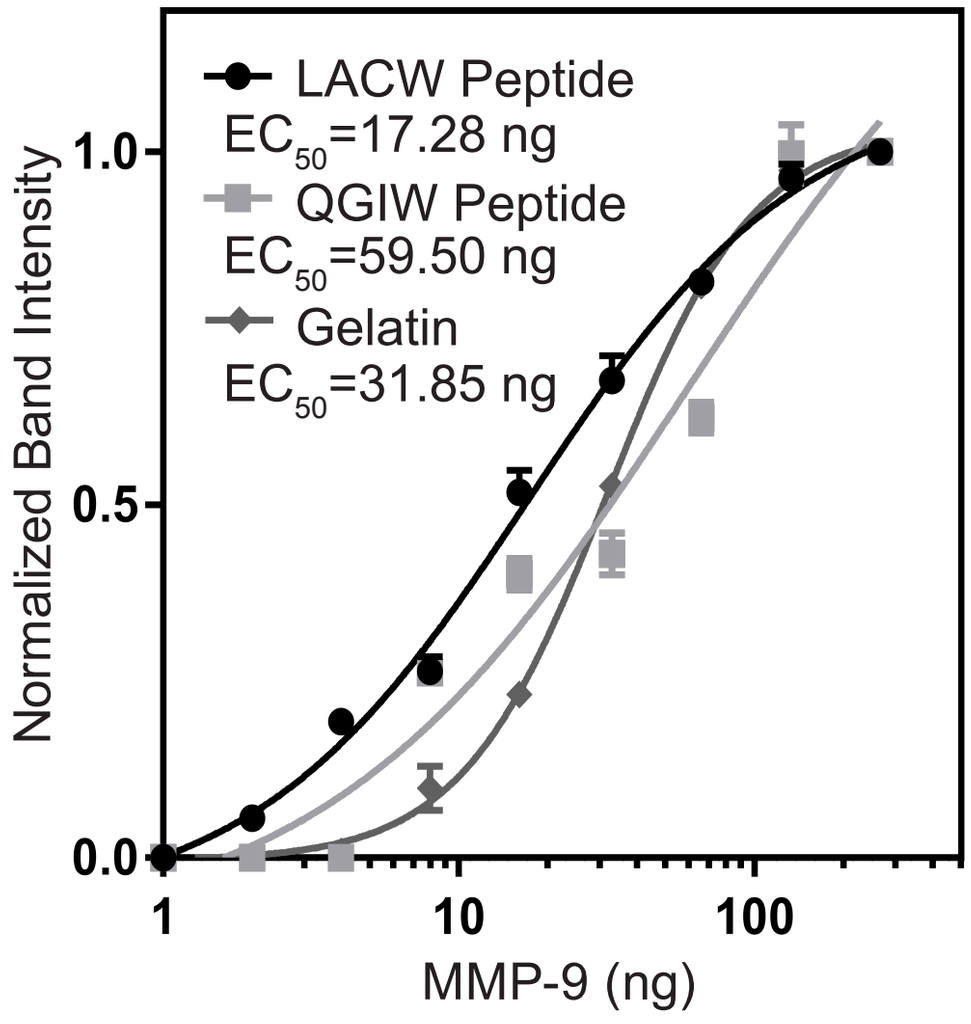


Table 1

Resolving Gel				
	Stock Conc.	Final Conc.	5 Gels	10 Gels
Acrylamide/Bis-Acrylamide (19:1)	40%	10%	10 mL	20 mL
Tris-HCl pH 8.7	1 M	0.375 M	15 mL	30 mL
Sodium Dodecyl Sulfate (SDS)	20%	0.10%	200 µL	400 µL
Deionized H_2O	--	--	14.4 mL	28.7 mL
TEMED	--	--	40 µL	80 µL
APS	10%	0.10%	400 µL	800 µL
		Total Volume	**40 mL**	**80 mL**

Peptide Resolving Gel				
	Stock Conc.	Final Conc.	5 Gels	10 Gels
Acrylamide/Bis-Acrylamide (19:1)	40%	10%	5 mL	10 mL
Tris-HCl pH 8.7	1 M	0.375 M	7.5 mL	15 mL
Sodium Dodecyl Sulfate (SDS)	20%	0.10%	100 µL	200 µL
Deionized H_2O	--	--	6.5 µL	13 mL
Fluorescent Peptide	10 mM	75 µM	150 µL	300 µL
Azido-PEG3-Maleimide CrossLinker	75 mM	1.5 mM	400 µL	800 µL
TEMED	--	--	20 µL	40 uL
APS	10	0.10%	200 µL	400 µL
		Total Volume	**20 mL**	**40 mL**

Stacking Gel				
	Stock Conc.	Final Conc.	5 Gels	10 Gels
Acrylamide/Bis-Acrylamide (19:1)	40%	5%	2.5 mL	5 mL
Tris-HCl pH 6.9	1 M	0.125 M	2.5 mL	5 mL
Sodium Dodecyl Sulfate (SDS)	20%	0.10%	100 µL	200 µL
Deionized H_2O	--	--	14.75 mL	29.5 mL
TEMED	--	--	50 µL	100 µL
APS	10%	0.10%	100 µL	200 µL
		Total Volume	**20 mL**	**40 mL**

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1.5 mm Empty Gel Cassettes	ThermoFisher Scientific	NC2015	
1.5 mm, 10 well Empty Gel Cassette Combs	ThermoFisher Scientific	NC3510	
1x Phosphate Buffered Saline	Fisher Scientific	10-010-049	
20% SDS Solution	Ambion	AM9820	
3x Zymography Sample Buffer	Bio-Rad	1610764	
40% (w/v) Acrylamide/Bis (19:1)	Ambion	AM9022	
6 Well Tissue Culture Plates	ThermoFisher Scientific	087721B	
Amicon Ultra-2 Centrifugal Filter Unit (10 kDa MWCO)	Sigma-Aldrich	UFC201024	
Ammounium Persulfate	Sigma-Aldrich	A3678	
Azido-PEG3-Maleimide Kit	Click Chemistry Tools	AZ107	
Calcium Chloride	ThermoFisher Scientific	BP510100	
Dimethyl Sulfoxide	Fisher Scientific	BP231	
Isopropanol	Scientific	A416P	

Micro BCA Protein Assay Kit	ThermoFisher Scientific	23235
N N N' N'-Tetramethylethylenediamine (TEMED)	Sigma-Aldrich	T9281
PowerPac Basic Power Supply	Bio-Rad	1645050
Precision Plus Protein Dual Color Standard	Bio-Rad	161-0374
PrecisionGlide Hypodermic Needles	Fisher Scientific	14-826
Round Bottom Flask (100 mL)	Fisher Scientific	50-873-144
Septum Rubber Stopper	Fisher Scientific	50-872-546
Sterile Slip Tip Syringe (1 mL)	Fisher Scientific	14-823-434
Triton X-100	Sigma-Aldrich	X100
Trizma hydrochlroide	Sigma-Aldrich	T5941
Typhoon 9410 Molecular Imager	GE Amersham	8149-30-9410
Zinc Chloride	Sigma-Aldrich	208086

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Author(s):	Ameya A. Deshmukh, Jessica L. Weist, Jennifer L. Leight

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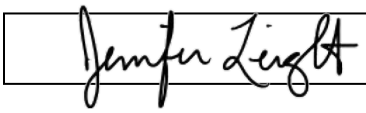
Signature:	
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The authors thank the editors and reviewers for their thoughtful comments. We have carefully considered the contents of the review and have taken steps to improve the manuscript based on these insights. Included below are detailed responses to each comment, in addition to the location of the changes. All changes have been underlined in the revised copy of the manuscript.

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have reviewed the manuscript and made any necessary corrections.

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3. Please provide an email address for each author.

These have now been included on the title page.

4. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"

The authors thank the editor for their suggestion. We have rewritten the short abstract to more clearly emphasize the protocol and its applications. Underlined revisions can be found on page 1 of the manuscript.

5. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

We have amended the introduction to clearly state the goal of this method. Underlined revisions can be found on page 2 of the manuscript.

6. Please spell out each abbreviation the first time it is used.

All abbreviations have been defined at their first use.

7. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns have been removed from the protocol.

8. Please revise the protocol to contain only action items that direct the reader to do something (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.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

The protocol has been amended to reflect the changes described above.

9. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below:

2.5: Please specify the size of the syringe needles used in this step.

The size of the needle has been added to the protocol step on page 3.

2.6: Which needle is removed, the one connected to the inert gas tank?

The authors have specified which needle to remove on page 3.

5.2.2: Are the incubation conditions the same as in 5.2.1?

Details on the humidified chamber used in 5.2.2 were added on page 5.

5.2.3: Please mention how to collect the samples and how to concentrate using a centrifugal filter. What are the centrifugation parameters?

Details on the centrifugation parameters have been added to step 5.2.3 on page 5.

5.4: What type of plate is used here?

This step in the protocol is now 6.2. The plate referenced here is the peptide zymography gel.

5.8: Please give an example of the filters and fluorophore used in this step. Please combine 5.8 and 5.8.1.

These two steps in the protocol have been combined into step 7.1. An example of the filters used in this step has been added on page 6.

10. Please include single-line spaces between all paragraphs, headings, steps, etc.

The authors have confirmed that all spaces are single line spaces.

11. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Please see response to #13.

12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Please see response to #13.

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

The authors have highlighted all the steps in the protocol that they wish to be included in the filming process.

14. Figure 1: Please change “Hour/Hours” to “h” and include a space between numbers and their temperature units (4 °C, 37 °C).

The units have been adjusted accordingly to the editor’s suggestions.

15. Figure 2: Please label panels A and B. What is the unit for the numbers in the left panel? Please change “n=3±SD” to “n=3, mean ± SD” or remove it from the figure since this has been defined in the figure legend.

The labels for panels A and B have been added to figure 2. The units for the molecular weight labels have also been added. "n=3±SD" has been removed from the figure.

16. Figure 3: Please provide the unit for the numbers in the left two panels.

The units for the molecular weight labels have been added.

17. Table 1: Please make the number 2 in H2O a subscript.

The number 2 has been made a subscript throughout Table 1.

18. Discussion: Please describe any limitations of the technique.

The authors thank the editors for their suggestion. We have added to the discussion section in order to describe some of the limitations of the technique on page 8 of the manuscript.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
The authors developed a fluorescent peptide zymography method to detect a wider range of proteases in complex biological samples. Although the application of zymography has been widely reported for sensing of various proteases, the present method with several tunable peptides demonstrated enhanced sensitivity and expanded the library of detectable targets. Furthermore, in terms of proteases detection, the work is technically important.

Major Concerns:
1. In the introduction part, the author said "Furthermore, the high tunability of our method allows for the simultaneous detection of numerous proteases, a result that was previously unattainable by traditional, native substrate zymography". Then how to design the specific substrate peptide which covalently couple to the polyacrylamide polymer network in multiplex detection?

We recognize this was not clear in the original manuscript and have clarified how we develop peptide substrates in the introduction on page 2 based on commercially available MMP substrates.

2. In protocol 5.5, "until the molecular weight standard indicates that the proteases of interest are within the peptide resolving gel layer", how to judge whether the proteases of interests are within the peptide resolving gel layer?

Please see our reply to question 3 below.

3. How to ensure that numerous proteases of interest are all within the peptide resolving gel layer when multiple proteases with relatively large molecular weight differences are detected simultaneously?

Given known protease molecular weights (35-100 kDa for most MMPs), the molecular weight standards can be used to estimate when the proteases of interest are within the peptide resolving gel layer – which has a visible orange color. While we describe a two-layer resolving gel approach in this manuscript to save on reagent use, the entire resolving gel could be made with the fluorogenic peptide substrates or the size of the peptide resolving gel layer can be adjusted to accommodate all proteases of interest. We have clarified these points in the protocol on page 5, step 6.2.

Minor Concerns:
1. In protocol 5.7, "Incubate gels at 37 °C under gentle agitation for 24 hours", how to control the area of the enzyme reaction? How to reduce the dispersion of enzyme bands in 24 hours?

The authors thank the reviewer for their question. All of the steps following electrophoresis of the peptide zymography gels are similar to those in traditional gelatin zymography. As such, the gel should be fully submersed within the developing buffer solution during the overnight incubation. Any proteases that become activated under the development conditions and are capable of cleaving the incorporated substrate can then be detected by fluorescent imaging. The authors have clarified this on page 5.

2. In figure 3A and 3C, there are two bands of MMP-9. In figure 3A, B, C, there are more than two bands of HT 1080 and MDA-MB-231, yet there is only one band in D, E and F. Please explain the reason for this result.

Figure 3A, B, C correspond to gels made with the LACW peptide and Figure D, E, and F correspond to gels made with the QGIW peptide. Because of the difference in peptide sequence, each peptide will have a different sensitivity to each protease, as demonstrated in Figure 2. In this figure it is possible that the QGIW is either not cleaved by both forms of MMP-9 or not detected due to the lower sensitivity of the QGIW peptide to MMP-9 (Figure 2). We have added text clarifying this issue.

Reviewer #2:

Manuscript Summary:
Zymography is a useful technique that is inexpensive and does not require antibodies as the authors mention. Technique described here of using crosslinked fluorogenic peptides could be a helpful addition to the field.

Major Concerns:

None

Minor Concerns:
Authors state that zymography has suffered due to a limited number of degradable substrates available for proteases/enzymes to work on, but it might be a bigger limitation for renaturing and refolding proteins into their native/active conformation after the electrophoresis and partial denaturation by SDS.

The authors thank the reviewer for their recommendations. We recognize this limitation in zymographic techniques in general and have now acknowledged it in the discussion on page 8.

Reviewer #3:

Manuscript Summary:
This manuscript describes detail methodology of results previously published in BioTechniques (2018). Except for the abstracts (see below), manuscript is well-written and all essential information (materials, equipment, and methods) are included. Below are concerns (mostly minor) that authors should take into consideration when revising.

Minor Concerns:

1. I have some concerns about the title of the manuscript. Clearly, the method detects the protease activity not the proteases themselves. I suggest that title be changed to "Detection of protease activity…"

The authors believe that due to the limitations on zymography for renaturing and refolding proteins as well as separating MMP-inhibitor complexes, "protease activity" can, in some sense, be misleading. However, the authors also recognize that we do not directly measure protease expression and therefore, we have amended the title of the manuscript to reflect that.

2. Both the short and long versions of the abstracts are poorly written. Both of them need to be re-written.

Abstract: The main texts are easy to read, but for some reason (written by different person?), the abstracts are written in cluttered sentences and even wrong grammar. For example, the last sentence "Fluorescent peptide zymography with several tunable peptides demonstrated enhanced the sensitivity and expanded the library of detectable proteases by zymographic techniques" needs to be corrected. Also, "tunable peptide" is not very clearly defined. What is "highly transient nature of physical entrapment?" Why not just say that the small molecules diffuse out of the gel easily? Instead of "This technique was applied to the detection of proteolysis by purified enzymes and conditioned media… by electrophoresis" authors can just say "This new type of gel was used to run purified enzymes and conditioned media, and the

protease activity was detected by direct fluorescence of the protein band without staining." Authors should strive to write clear sentences.

"Copolymerization" typically represents two monomers making a co-polymer, and it suggests that the multiple components are covalently incorporated into the polymer chain. Therefore, readers will likely mis-interpret "copolymerization of short, fluorescent peptide" as peptide (which has polymerizable moiety) covalently conjugated to polymer during the polymerization process. Therefore, I suggest replacing "copolymerization of peptide…" with "incorporation of peptide during the polymerization (gelation) process."

Short abstract: First sentence should be how fluorescent peptide zymography was produced.

The authors thank the reviewer for their suggestions. The long and short abstracts have been re-written to provide a clearer summary of the method and key findings. Furthermore, copolymerization has been replaced with "incorporation" in the long abstract and throughout the manuscript. These revisions can be found underlined on page 1.

3. Azido-PEG3-maleimide is not a cross-linker. Traditionally, the term "crosslink" is used for describing connection between polymer chains. The utility of azido-PEG3-maleimide is conjugating peptide to the polymer backbone. Therefore, grafting molecule or linker molecule is more appropriate.

The authors thank the reviewer for this observation. We have replaced any references to azido-PEG3-maleimide as a "crosslinker" to a "linker molecule" throughout the manuscript.

4. Vial 2 is introduced abruptly without introducing vial 1. Make sure to describe both solutions (or both vials). Also, it would be helpful to clearly list what is in the two vials.

A description of the contents of both vials has been introduced on page 4.

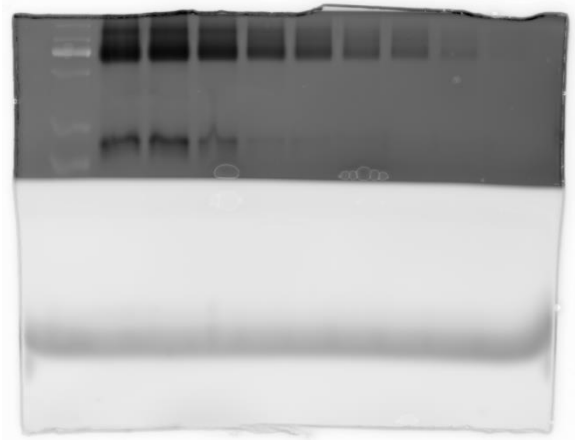
5. It is unclear how azido group adds to the polymer chain during the polymerization reaction. Authors mentioned this in the original BioTechnique paper. I wonder if authors still believe in their original conjecture that azido group adds to the amino side chain. I think it is much more likely that a radical reaction to the azido group results in incorporation of the peptide directly on the polymer backbone (not side chain), since azido can partake in radical reactions. Amino group, on the other hand, is known to be inert toward clink chemistry (hence click reaction is used a lot in bioconjugation in the presence of amino groups). Authors can take this opportunity to re-think and perhaps provide more likely scenario for covalent link between peptide and polymer backbone (which is the main idea behind this work).

We agree that it remains unclear within the literature and the field how the azido group reacts with the acrylamide/poly-acrylamide. However, data from our previous work (Deshmukh et al.

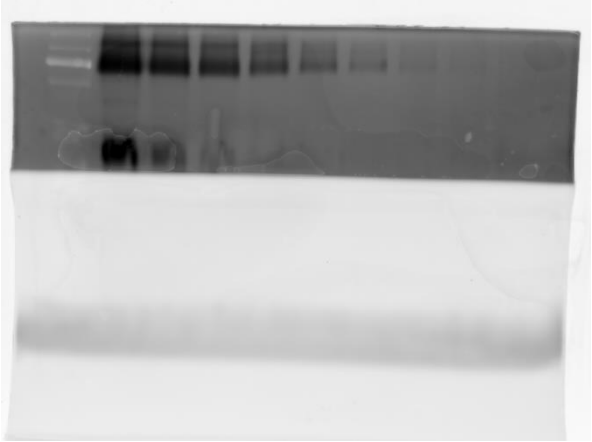
Biotechniques 2018), indicates stable incorporation of the fluorogenic peptide only upon inclusion of the linker molecule. Upon further thought and review of the literature, the mechanism may be what the reviewer suggests or possibly a mechanism in which 1) hydrolysis of the amide occurs followed by 2) the Schmidt reaction. Amide groups within the polyacrylamide structure can be hydrolyzed to carboxylic acids under the basic conditions of the polyacrylamide solution. This is followed by a reaction between the azide functional group in the linker and the carboxylic acid. However, because both these mechanisms remain speculative without literature support specifically about the azido/acrylamide reaction, we have taken out all mentions of the chemical mechanism beyond that the fluorogenic peptide is stably incorporated.

6. For the polymerization reaction of the peptide resolving gel, I wonder if there is any effect on whether peptide was added first and allowed to react to the linker molecule before polymerization or it was added directly to the polymerization reaction. This is because the thiol group of cysteine is known to be a good chain transfer agent and that it can reduce the MW of the polymer.

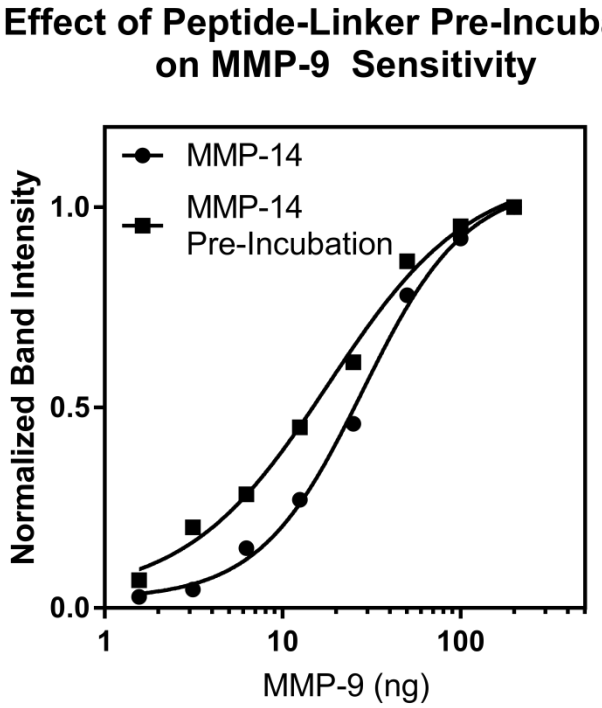
This is an intriguing possibility. We have generated zymogram gels in which 1) we pre-incubate the peptide with the linker and then add this to the acrylamide solution prior to polymerization or 2) the linker and peptide are added directly to the acrylamide solution prior to polymerization. We have observed no differences in how far the samples or ladder migrate during electrophoresis with either approach – which would be one indication of changes in the polyacrylamide network. We did, however, observe increased sensitivity to a concentration gradient of MMP-9 with approach 1 (see below). However, since we have carefully optimized approach 2, found this protocol to empirically work with many different kinds of samples, and either approach does not significantly change the experimental results, we would prefer to describe the original approach (#2) throughout the protocol and results. We are including the results here for appraisal by the reviewers and editors, but we believe these results may be distracting to the reader if included in the protocol and not provide additional benefit. If advised by the editor, we can include this data in the manuscript.



Approach 1: Pre-Incubation of LACW Peptide w/ azido-PEG3-maleimide linker prior to addition to the polyacrylamide solution



Approach 2: Direct addition of LACW Peptide and azido-PEG3-maleimide linker to the polyacrylamide solution



Approach	**EC_{50}**
Approach 1- Pre-incubation	18.02
Approach 2- Direct addition	28.06

7. There is no information about the sample buffer and running buffer. If it is conventional buffers, it needs to be explicitly said so in the manuscript.

Details about the contents of the sample and running buffers have been added to the protocol on page 5.

8. In the representative results section, the peptides should be written from N terminus to C terminus (Cystein on the left side).

The authors have reviewed the orientation of the peptide and would like to note that the cysteine is in fact in the C-terminal position. Therefore, the orientation of the notation is correct.

9. Line 291: "in to" should be "into"

These changes have been made as per the reviewers request