*Revised: 22–March–2001*

# *EnzChek® Gelatinase/Collagenase Assay Kit*

**D-12060 DQ™ collagen, type I from bovine skin, fluorescein conjugate**
**D-12052 DQ™ collagen, type IV from human placenta, fluorescein conjugate**
**D-12054 DQ™ gelatin from pig skin, fluorescein conjugate**
**E-12055 EnzChek® Gelatinase/Collagenase Assay Kit**

*Quick Facts*

***Storage upon receipt:***
- –20°C
- Desiccate
- Protect from light

***Ex/Em of digestion product:*** 495/515 nm

***Note:*** Avoid freeze-thaw cycles after reconstituting.

## *Introduction*

The extracellular matrix (ECM) serves not only as a scaffolding to stabilize tissue structure, but also has been observed to influence the development, migration, proliferation, shape and metabolic function of cells that contact it.[1] Matrix metalloproteinases (MMPs; e.g. gelatinases, collagenases and stromelysins), which digest collagen, gelatin (denatured collagen) and other components of the ECM, are important for both normal development and carcinogenesis. When cells from one tissue invade a neighboring tissue, as in angiogenesis, wound healing, fetal tissue development and metastasis of tumors, MMPs are released to facilitate the breakdown of barriers opposing the invading cells.[2] There are a number of different MMPs that are specific for the various ECM components. For instance, gelatinase A (MMP-2, 72 kDa) is primarily responsible for the degradation of the helical domains of type IV collagen, the principal collagen of basement membranes,[3] while interstitial collagenase (MMP-1) is more selective for type I collagen.[4] MMPs are regulated by a number of inhibitor proteins, termed tissue inhibitors of metalloproteinases (TIMPs), and the relationship between MMPs and their respective TIMPs plays a key role in the regulation of growth, invasion and metastasis of neoplastic cells.[2]

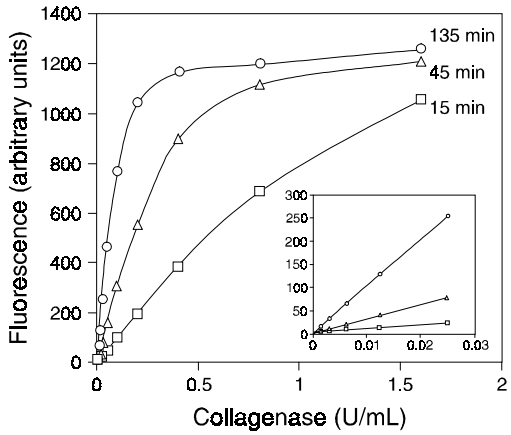


***Figure 1.*** *Assay of collagenase from Clostridium histolyticum using the EnzChek Gelatinase/Collagenase Assay Kit. Reactions contained 100 µg/mL DQ gelatin and the indicated amount of collagenase. After incubation times of 15, 45 and 135 minutes, fluorescence was measured using a microplate reader set for excitation at 485 ± 10 nm and emission detection at 530 ± 15 nm. Background fluorescence (150 arbitrary units) has been subtracted from each value. The inset shows an enlargement of the plot to more clearly depict the values for low collagenase concentrations.*

Molecular Probes' EnzChek® Gelatinase/Collagenase Assay Kit (E-12055) provides the speed, high sensitivity and convenience required for measuring gelatinase or collagenase activity or for screening inhibitors in a high-throughput format.[5] The EnzChek kit contains DQ™ gelatin, fluorescein conjugate — gelatin so heavily labeled with fluorescein that the fluorescence is quenched. This substrate, which is also available as a standalone product (D-12054), is efficiently digested by most, if not all, gelatinases and collagenases to yield highly fluorescent peptides. The increase in fluorescence is proportional to proteolytic activity (Figure 1) and can be monitored with a fluorescence microplate reader, minifluorometer or standard fluorometer.

Collagenase purified from *Clostridium histolyticum* is provided with the EnzChek Gelatinase/Collagenase Assay Kit to serve as a control enzyme. Using 100 µg/mL DQ gelatin and a two-hour incubation period, the assay can detect the activity of this enzyme down to a final concentration of $2 \times 10^{-3}$ U/mL (7 ng protein/mL), where one unit is defined as the amount of enzyme required to liberate 1 µmole of L-leucine equivalents from collagen in 5 hours at 37°C, pH 7.5. We have found that an incubation time of 24 hours can increase the sensitivity approximately tenfold (data not shown). At high enzyme concentrations (e.g. 0.2–0.4 U/mL), incubation times can be as short as 15 minutes (Figure 2). Using human gelatinase A (not provided) and 100 µg/mL DQ gelatin, the EnzChek Gelatinase/Collagenase assay is able to detect concentrations as low as $3 \times 10^{-4}$ U/mL with a 24 hour incubation, where one unit of gelatinase A is defined as the amount that can hydrolyze 1 mg of type IV collagen within one hour at 37°C, pH 7.5.

The metal chelator and general inhibitor of metalloproteinases, 1,10-phenanthroline, is also provided with the EnzChek Gelatinase/Collagenase Assay Kit. This inhibitor can serve as a control to allow researchers to optimize conditions for screening potential gelatinase or collagenase inhibitors. We have found that 1,10-phenanthroline at 0.4 mM effectively inhibits the activity of *Clostridium* collagenase (Figure 3).

As an alternate to the DQ gelatin substrate provided in the EnzChek Gelatinase/Collagenase Assay Kit, Molecular Probes offers DQ collagen, type I and type IV, fluorescein conjugates (D-12060, D-12052). Like DQ gelatin, these quenched substrates are heavily labeled with fluorescein and release fluorescent peptides when enzymatically cleaved. DQ collagens may prove particularly useful in the development of assays for specific gelatinases and their inhibitors. DQ collagens can be used with the EnzChek Gelatinase/Collagenase Assay Kit, and a sample protocol is included below. DQ collagens generally require a longer incubation period than does DQ gelatin. Please note that DQ collagens and DQ gelatin can be digested by proteases other than gelatinases and collagenases.

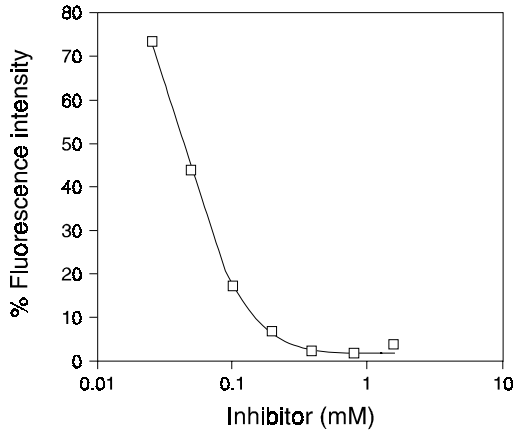


***Figure 3.*** *Inhibition of Clostridium collagenase by 1,10-phenanthroline. DQ gelatin substrate at 100 µg/mL, collagenase at 0.2 U/mL and increasing amounts of 1,10-phenanthroline were incubated together for 25 minutes. Fluorescence was measured as described in Figure 1. All values were corrected for background fluorescence and expressed relative to the fluorescence obtained in the absence of inhibitor.*

## Materials

### Kit Contents

The EnzChek Gelatinase/Collagenase Assay Kit provides the following components:

- **DQ gelatin from pig skin, fluorescein conjugate** (Component A), five vials each containing 1 mg substrate lyophilized from 1 mL of phosphate-buffered saline (PBS), pH 7.2
- **10X Reaction Buffer** (Component B), 50 mL of 0.5 M Tris-HCl, 1.5 M NaCl, 50 mM $CaCl_2$, 2 mM sodium azide, pH 7.6
- **1,10-Phenanthroline, monohydrate** (Component C), ~30 mg (MW = 198.2), a general metalloproteinase inhibitor
- **Collagenase, Type IV from *Clostridium histolyticum*** (Component D), 500 U. One unit is defined as the amount of enzyme required to liberate 1 µmole of L-leucine equivalents from collagen in 5 hours at 37°C, pH 7.5.

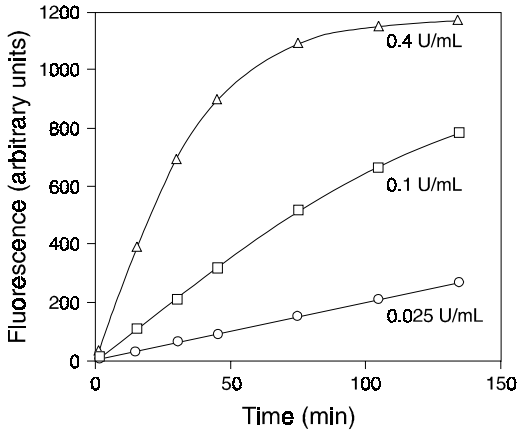


***Figure 2.*** *Kinetics of the EnzChek Gelatinase/Collagenase reaction. Clostridium collagenase at 0.025, 0.1 and 0.4 U/mL was incubated with 100 µg/mL DQ gelatin substrate in 1X Reaction Buffer for the indicated time periods, and fluorescence was measured as in Figure 1. Background fluorescence, determined for a no-enzyme control reaction, has been subtracted from each value.*

Each kit provides sufficient reagents for 250–2000 assays using a fluorescence microplate reader. The actual number of assays depends on the substrate concentration used in each reaction.

### DQ Gelatin Fluorescein Conjugate (D-12054)

DQ gelatin is supplied as a set of five vials, each containing 1 mg of substrate lyophilized from 1 mL of PBS, pH 7.2. Each vial of substrate is sufficient for 50–400 assays, depending on the substrate concentration desired, using the protocol described below in *Assays for Gelatinase/Collagenase Activity* and a fluorescence microplate reader.

### DQ Collagen, Fluorescein Conjugates (D-12052, D-12060)

DQ collagen, fluorescein conjugates (sold separately from the EnzChek Gelatinase/Collagenase Assay Kit) are supplied as 1 mg of substrate lyophilized from 1 mL of PBS. This amount is sufficient for 50–400 assays using the protocol described below in *Adaptations for Using DQ Collagen Fluorescein Conjugates* and a fluorescence microplate reader.

### Storage and Handling

Upon receipt, each kit or DQ substrate should be stored at -20°C, protected from light. Allow reagents to warm to room temperature before opening vials. When stored properly, these reagents are stable for at least six months.

## Experimental Protocol

The following procedures are designed for use with a fluorescence multi-well microplate reader. For use with a standard fluorometer, volumes must be increased accordingly.

### Reagent Preparation

**1.1** Prepare a 1.0 mg/mL stock solution of the DQ gelatin by adding 1.0 mL of deionized water ($dH_2O$) directly to *one* of the five vials containing the lyophilized substrate. It may be necessary to agitate the sample in an ultrasonic water bath for ~5 minutes and heat to 50°C to facilitate dissolution. A 2.5–20 µL volume will be used for each 200 µL volume reaction; thus this stock solution provides substrate sufficient for approximately

50–400 assays using a fluorescence microplate reader and assay volumes of 200 µL per microplate well. Proportionally fewer assays will be possible if the reaction is scaled up to accommodate fluorometer cuvettes. Reconstituted DQ gelatin may be stored in the dark at 4°C with the addition of sodium azide to a final concentration of 2 mM. DO NOT FREEZE the DQ gelatin stock solution; background fluorescence of the substrates may increase upon freezing and thawing.

**1.2** Prepare 1X Reaction Buffer. Dilute 2 mL of the 10X Reaction Buffer in 18 mL $dH_2O$. This 20 mL volume is enough working 1X Reaction Buffer for at least fifty 200 µL assays with ~10 mL excess for performing dilutions and preparing working solutions.

**1.3** If using the *Clostridium* collagenase, prepare a 1000 U/mL stock solution by dissolving the contents of the vial (Component D) in 0.5 mL $dH_2O$. Reconstituted *Clostridium* collagenase can be frozen in aliquots and stored at -20°C for at least six months without significant loss of activity.

**1.4** If using 1,10-phenanthroline as an inhibitor, weigh out 9.9 mg 1,10-phenanthroline from the ~30 mg provided (Component C) and dissolve in 25 µL ethanol or *N,N*-dimethylformamide (not provided). Prepare a 10 mM working solution by adding 10 µL of this solution to 2 mL of 1X Reaction Buffer prepared in step 1.2.

### *Assays for Gelatinase/Collagenase Activity*

**2.1** Add 80 µL of 1X Reaction Buffer (prepared in step 1.2) to each assay well. If an inhibitor is to be used, see *Assays of Gelatinase/Collagenase Inhibitors*.

**2.2** Next, add 20 µL of DQ gelatin solution to the wells: We have found that DQ gelatin concentrations of 12.5–100 µg/mL are appropriate for most experiments. To yield a final substrate concentration of 100 µg/mL, use 20 µL of the 1.0 mg/mL stock solution (prepared in step 1.1). If a lower substrate concentration is desired, dilute the DQ gelatin stock solution as required prior to dispensing. For example, to yield a final substrate concentration of 25 µg/mL, dilute the 1.0 mg/mL DQ gelatin stock solution fourfold in 1X Reaction Buffer and then add 20 µL of this diluted solution to each microplate well. Pipet up and down to mix.

**2.3** Dilute the enzyme of interest in 1X Reaction Buffer. We recommend trying a number of different enzyme dilutions. Add 100 µL of the diluted enzyme, or 100 µL of 1X Reaction Buffer as a negative control, to the substrate samples and mix to begin the reactions. If a positive control is desired, use 100 µL of diluted *Clostridium* collagenase. The *Clostridium* collagenase stock solution prepared in step 1.3 should be diluted to an appropriate concentration in 1X Reaction Buffer. We have found that *Clostridium* collagenase at a final concentration of 0.05–0.2 U/mL is reasonable for a two-hour incubation period. For shorter incubation periods, use more enzyme; for longer incubation periods, use less (see Figures 1 and 2 for guidance). For maximum sensitivity, the incubation period can be as long as 24 hours.

**2.4** Incubate the samples at room temperature, protected from light, for an appropriate time. Because the reaction is continuous (not terminated), fluorescence may be measured at multiple time points.

**2.5** Measure the fluorescence intensity in a fluorescence microplate reader equipped with standard fluorescein filters. Digestion products from the DQ gelatin (or DQ collagen) substrate have absorption maxima at ~495 nm and fluorescence emission maxima at ~515 nm.

**2.6** For each time point, correct for background fluorescence by subtracting the value derived from the no-enzyme control.

### *Assays of Gelatinase/Collagenase Inhibitors*

This is a sample protocol illustrating the utility of the EnzChek Gelatinase/Collagenase Assay Kit for the detection of enzyme inhibitors. In actual practice, incubation times, enzyme concentrations, substrate concentrations and inhibitor concentrations will have to be optimized for the particular experimental conditions. If a preincubation of the inhibitor and enzyme is desired, the protocol should be altered accordingly.

**3.1** Dilute the inhibitor of interest in 1X Reaction Buffer (prepared in step 1.2). An 80 µL volume of inhibitor will be used for each 200 µL reaction. The provided inhibitor, 1,10-phenanthroline, can serve as a control inhibitor. Make dilutions of 1,10-phenanthroline from the 10 mM working solution prepared in step 1.4. Figure 3 shows that 0.1–0.5 mM 1,10-phenanthroline (final concentration) is a suitable concentration for use with *Clostridium* collagenase at 0.2 U/mL; the appropriate concentration range may be different for the other enzymes. Include a no-inhibitor control for all enzymes being assayed.

**3.2** Add 80 µL of the diluted inhibitor (or no-inhibitor control) to each assay well.

**3.3** Next, add 20 µL of DQ gelatin stock solution to each assay well (see step 2.2).

**3.4** Dilute the enzyme of interest, and *Clostridium* collagenase if desired (see step 2.3), in 1X Reaction Buffer. Add 100 µL of the diluted enzyme, or 100 µL of 1X Reaction Buffer as a blank, to the sample wells preloaded with substrate and inhibitor.

**3.5** Incubate the samples at room temperature, protected from light, for an appropriate time, e.g. 1–2 hours. Because the reaction is continuous (not terminated), fluorescence may be measured at multiple time points.

**3.6** Measure the fluorescence intensity in a fluorescence microplate reader equipped with standard fluorescein filters. Digested products from the DQ gelatin and DQ collagen substrates have absorption maxima at ~495 nm and fluorescence emission maxima at ~515 nm.

**3.7** For each time point, correct for background fluorescence by subtracting the values derived from the no-enzyme control.

### *Adaptations for Using DQ Collagen, Fluorescein Conjugates*

For researchers desiring a more specific substrate than DQ gelatin, DQ collagen type I or type IV conjugates, can be used with the EnzChek Gelatinase/Collagenase Assay Kit.

**4.1** The DQ collagen substrates should be reconstituted in $dH_2O$ as described in step 1.1 for the DQ gelatin substrate.

**4.2** Follow the procedures described in *Assays for Gelatinase/Collagenase Activity* or *Assays for Gelatinase/Collagenase Inhibitors* using the DQ collagen substrate in place of the DQ gelatin. Due to the more complex structure of collagen relative to gelatin, longer enzyme incubation times are generally required for digestion of the DQ collagens than for digestion of the DQ gelatin substrate.

## References

**1.** Alberts, B., Bray, D., Lewis, J., Raff, M., Roberts, K and Watson, J., *Molecular Biology of the Cell, 2nd Edition*, Garland Publishing, Inc. (1989) pp. 802–824; **2.** Chem Biol 3, 895 (1996); **3.** J Biol Chem 270, 5872 (1995); **4.** Lab Invest 78, 687 (1998); **5.** Anticancer Res 19, 3809 (1999).

## Product List

*Current prices may be obtained from our Web site or from our Customer Service Department.*

| Cat # | Product Name | Unit Size |
|---|---|---|
| D-12060 | DQ™ collagen, type I from bovine skin, fluorescein conjugate | 1 mg |
| D-12052 | DQ™ collagen, type IV from human placenta, fluorescein conjugate | 1 mg |
| D-12054 | DQ™ gelatin from pig skin, fluorescein conjugate *special packaging* | 5 x 1 mg |
| E-12055 | EnzChek® Gelatinase/Collagenase Assay Kit *250-2000 assays* | 1 kit |

## Contact Information

Further information on Molecular Probes' products, including product bibliographies, is available from your local distributor or directly from Molecular Probes. Customers in Europe, Africa and the Middle East should contact our office in Leiden, the Netherlands. All others should contact our Technical Assistance Department in Eugene, Oregon.

Please visit our Web site — **www.probes.com** — for the most up-to-date information

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