QCM™ High Sensitivity Non-cross-linked Collagen Invasion Assay, 24-well (8 µm) Colorimetric

Catalog No. ECM1401
Sufficient for analysis of 24-samples

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

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Introduction

Metastasis, the process by which tumor cells colonize tissues distant from the site of the primary tumor, consists of multiple phases: invasion of cells from the primary tumor into adjacent tissues, passage of the tumor cells through the vascular lining into the bloodstream (intravasation), adhesion to the endothelium at the site of implantation, traversal across the endothelium (extravasation) and proliferation at the distant site. The first step, invasion, requires that the tumor cell traverse one or more extracellular matrix elements, depending on the tumor type. Epithelial tumors must breach a basement membrane, which is a dense sheet of interwoven cross-linked type IV collagen and laminin, in order to extend into neighboring tissues. In addition, many tumors contain a reactive stroma containing fibrillar collagen, another cross-linked matrix element. Finally, the process of intravasation requires passage through the endothelial basement membrane.

The mechanisms by which premetastatic cells breach such barriers are the focus of intense study. In many solid tumors of epithelial origin, a morphological change from a cuboidal epithelial phenotype to a spindle-shaped mesenchymal phenotype (termed the epithelial-mesenchymal transition, or EMT) presages the invasion of the cells across ECM barriers. A key feature of the EMT is the increased expression of proteases, chiefly matrix metalloproteinases, which cleave the components of the ECM. However, alternative forms of cell invasion have been observed and are likely to contribute to the invasive potential of a tumor. Some invasive cells adopt an amoeboid phenotype, and migrate by rear-force propulsion and membrane blebbing; such amoeboid cells invade the ECM by squeezing through pores and appear not to require proteases. Distinctions between amoeboid and mesenchymal invasion are not clear-cut, and some tumor cells will adopt different morphologies in response to environmental conditions.

The most widely used *in vitro* assay to assess invasive potential of a cell type employs a chamber with a porous filter at the bottom, which is coated with a gelled layer of ECM, usually collagen or basement membrane extract. Cells are applied to the top of the gel, and a chemoattractant is provided in the well below the chamber, such that invasive cells traverse the ECM and move through the pores to the other side, where they are then quantified. EMD Millipore provides such assays, coated with either basement membrane extract (Catalogue No. ECM550, ECM554, ECM555) or collagen (Catalogue No. ECM551, ECM552, ECM556).

As our understanding of migration modes expands, more consideration is being given to the properties of the ECM employed in such *in vitro* models of invasion, and the extent to which they reflect the properties of the ECM of the tumor microenvironment. Basement membrane extracts contain the constituents, but lacks the covalent cross-links, of a basement membrane *in vivo*. Two forms of fibrillar collagen are used in invasion assays. Acid-extracted collagen, which is employed in EMD Millipore's Collagen Invasion Assays (Catalogue No. ECM551, ECM552, ECM556), retains intermolecular covelent cross-links, whereas pepsin-solubilized collagen, also known as atelo-collagen, lacks such cross-links. It has been demonstrated that amoeboid invasion only occurs in matrix environments lacking cross-links.

EMD Millipore's QCM™ High Sensitivity Non-cross-linked Collagen Invasion Assay utilizes the same format of matrix-coated porous filters as the existing products, but employs a non-cross-linked, atelo-collagen that provides a more pliable environment that permits higher levels of cell invasion to occur. For moderately invasive cell types, invasion can be detected at earlier time points with the High Sensitivity Collagen Invasion Assay. In less invasive cell types, the High Sensitivity Collagen Invasion Assay is able to detect invasion that is not apparent with the original QCM™ Collagen Invasion Assay.

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Kit Components

1. 24-well Cell Invasion Plate Assembly: (Part No. CS210537) Two 24-well plates (8 µm) with 12 non-cross-linked collagen-coated inserts per plate (24 inserts total/kit).
2. Cell Stain: (Part No. 90144) One 20 mL bottle.
3. Extraction Buffer: (Part No. 90145) One 20 mL bottle.
4. Cotton Swabs: (Part No. 10202) 50 each.
5. Forceps: (Part No. 10203) One each.

Materials Not Supplied

- Precision pipettes sufficient for aliquoting cells
- Sterile cell culture hood
- CO_2 incubator appropriate for subject cells
- Tissue culture growth medium appropriate for subject cells, such as DMEM containing 10% FBS
- Harvesting buffer: EDTA or trypsin cell detachment buffer. Suggested formulations include a) 2 mM EDTA/PBS, b) 0.05% trypsin in Hanks Balanced Salt Solution (HBSS) containing 25 mM HEPES, or other cell detachment formulations as optimized by individual investigators
- Quenching Medium: serum-free medium containing 5% BSA (for EDTA/PBS) or soybean trypsin inhibitor (for trypsin)
- Sterile PBS or HBSS to wash cells
- 70% ethanol
- Distilled water
- Low speed centrifuge and tubes for cell harvesting
- Hemocytometer or other means of counting cells
- Trypan blue or equivalent viability stain
- Chemoattractants (eg. 10% FBS) or pharmacological agents for addition to culture medium, if screening is desired
- Microplate reader (560 nm)
- 24-well tissue culture plate
- 96-well plate

Storage

Store kit materials at 2-8℃ for up to 4 months fro m date of receipt. **Do not freeze.**

Assay Instructions

Cell Preparation

Prepare subject cells for investigation as desired. The following procedure is suggested for adherent cells only and may be optimized to suit individual cell types.

1. Use cells that have been passaged 2-3 times prior to the assay and are 80% confluent.

   ***Note*:** Use of early passage cells is recommended, as some invasive cell lines are known to show declines in invasion activity with prolonged passaging.

2. Starve cells by incubating 18-24 hours prior to assay in appropriate serum-free medium (DMEM, EMEM, or equivalent).
3. Visually inspect cells before harvest, taking note of relative cell numbers and morphology.
4. Wash cells 2 times with sterile PBS or HBSS.
5. Add 5 mL Harvesting Buffer (see Materials Not Supplied) per 100 mm dish and incubate at 37°C for 5-15 minutes.
6. Gently pipet the cells off the dish and add to 10-20 mL Quenching Medium (see Materials Not Supplied) to inactivate the Harvesting Buffer.
7. Centrifuge cells gently to pellet (1500 RPM, 5-10 minutes).
8. Gently resuspend the pellet in 1-5 mL serum-free medium, depending upon the size of the pellet.
9. Count cells and bring to a volume that gives 0.5–1.0 x 10^6 cells per mL.
10. If desired, add additional compounds (cytokines, pharmacological agents, etc.) to cell suspension.

Assembly of Cell Invasion Assay Wells

Perform the following steps in a tissue culture hood:

1. For optimal results, bring plates and reagents to room temperature (25°C) prior to initiating assay.
2. Sterilize forceps with 70% ethanol and handle inserts with forceps.
3. Add 300 µL of prewarmed serum free media to the interior of the inserts. Allow this to rehydrate the collagen layer for 15-30 minutes at room temperature.
4. After rehydration from step 3, carefully remove 250 µL of media from the inserts without disturbing the collagen–coated membrane.
5. Prepare a cell supension containing 0.5-1.0 x 10^6 cells/mL in chemo-attractant-free media.
6. Add 250 µL of prepared cell suspension from step 5 to each insert.
7. Add 500 µL of serum free media in the presence or absence of chemo-attractant (e.g. 10% fetal bovine serum) to the lower chamber.

   Note: *Ensure the bottom of the insert membrane contacts the media. Air may get trapped at the interface.*

8. Cover plate and incubate for 24 - 72 hours at 37°C in a CO_2 incubator (4-6% CO_2).
9. Carefully remove the cells/media from the top side of the insert by pipetting out the remaining cell suspension, and place the invasion chamber insert into a clean well containing 400 µL of Cell Stain. Incubate for 20 minutes at room temperature.

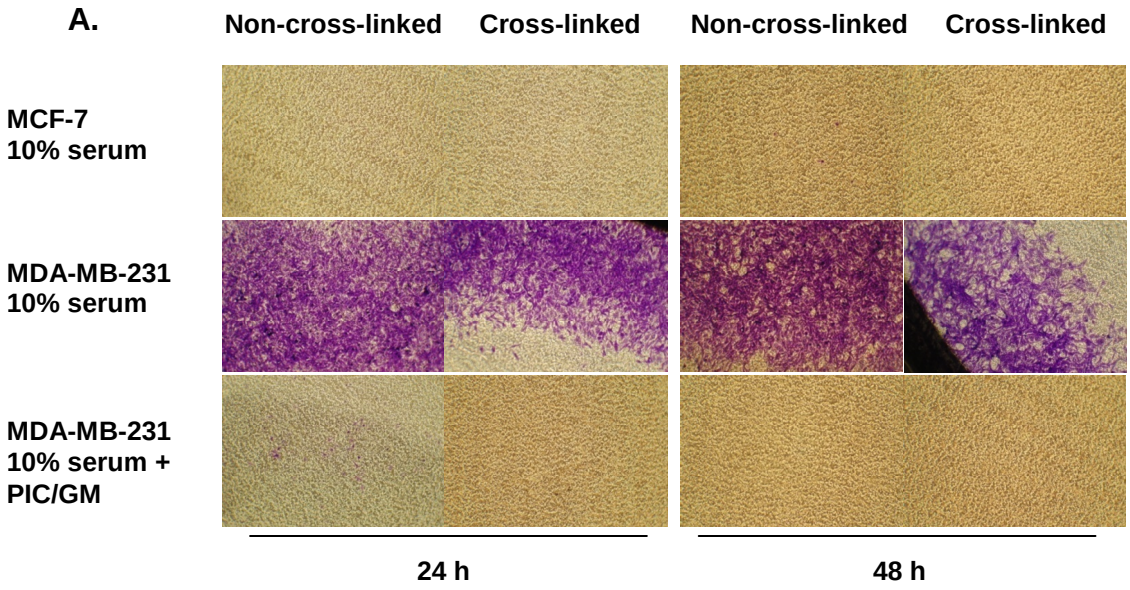
10. Dip insert into a beaker of water several times to rinse.
11. While the insert is still moist, use a cotton-tipped swab to gently remove non-invading cells/collagen layer from the interior of the insert. Take care not to puncture the polycarbonate membrane. Be sure to remove all cells on the inside perimeter, as any remaining cells inside the insert will contribute to background staining. Repeat procedure with a second, clean cotton-tipped swab.
12. Allow insert to air dry.
13. (Optional) View the underside of the membrane under a microscope and photograph. Cells may be counted manually or with the aid of an imaging application.
14. Transfer the stained insert to a clean well containing 200 µL of Extraction Buffer for 15 minutes at room temperature. Extract the stain from the underside by gently tilting the insert back and forth several times during incubation. Remove the insert from the well.
15. Transfer 100 µL of the dye mixture to a 96-well microtiter plate suitable for colorimetric measurement.
16. Measure the Optical Density at 560 nm.

Data Analysis

Results of the QCM™ High Sensitivity Non-cross-linked Collagen Invasion Assay may be illustrated graphically by the use of a "bar" chart. Inserts without cells, but treated with Cell Stain and Extraction Buffer are typically used as "blanks" for interpretation of data. A typical cell invasion experiment will include control chamber migration without chemoattractant. Cell invasion may be induced or inhibited in test wells through the addition of cytokines or other pharmacological agents.

The following figures demonstrate typical invasion results. **One should use the data in the next pages for reference only. This data should not be used to interpret actual assay results.**

Example Data



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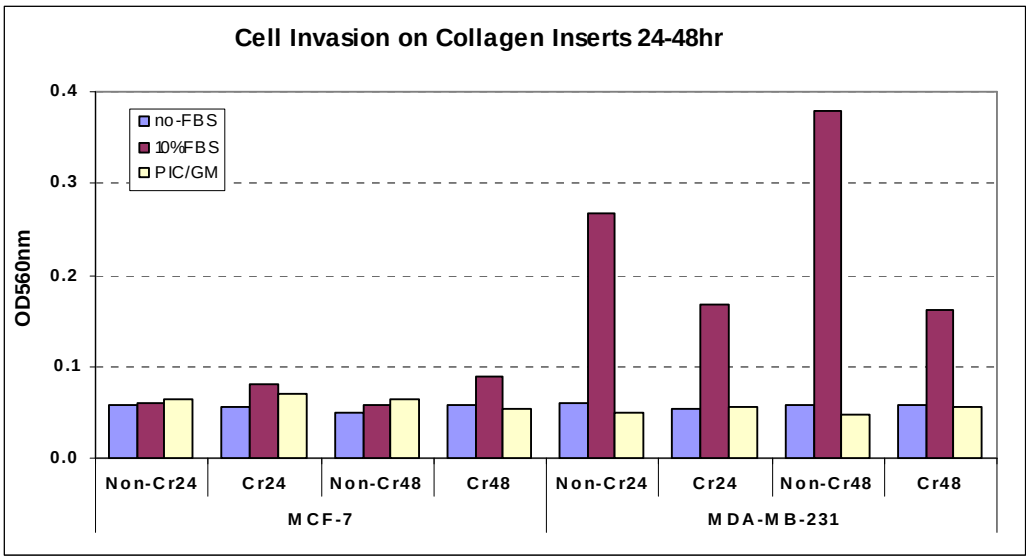
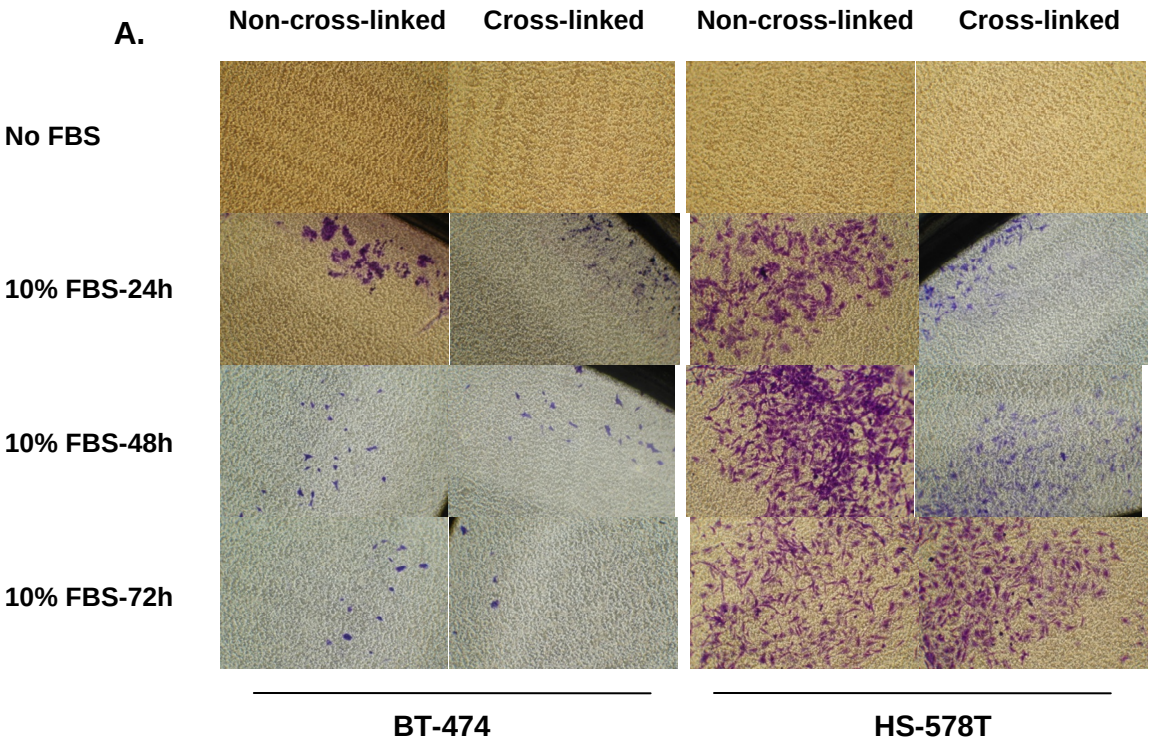


Figure 1: Invasion through cross-linked and non-cross-linked collagen gels by breast cancer cell lines with different invasiveness.

MCF-7, a non-invasive cell line and MB-MDA-231, a highly invasive cell line, were seeded at 250,000 cells per well in the upper chamber of inserts containing non-cross-linked collagen gels (Non-cross-linked; Catalog No. ECM1401) and cross-linked collagen gels (Cross-linked; Catalog No. ECM551). The wells beneath the chambers contained serum-free media, or media containing 10% FBS alone or with 25 µM MMP Inhibitor GM6001 (GM) and 1x Protease Inhibitor Cocktail (PIC; Sigma). **A:** Invaded cells on the bottom side of the membrane were stained according to *Assay Instructions*. The MB-MDA-231 cells displayed invasion to the bottom of the insert membrane after 24 and 48 hrs, and more cells invaded through the non-cross-linked collagen inserts than through the cross-linked collagen inserts. The MCF-7 cells displayed minimal invasion. The protease inhibitor cocktail along with MMP inhibitor GM6001 blocked MDA-MB-231 cell invasion completely. **B:** Colorimetric measurements were taken according to *Assay Instructions*. The absorbance readout matched with the invaded cells staining data. (***Non-Cr:*** *Non-cross-linked,* ***Cr:*** *Cross-linked)*



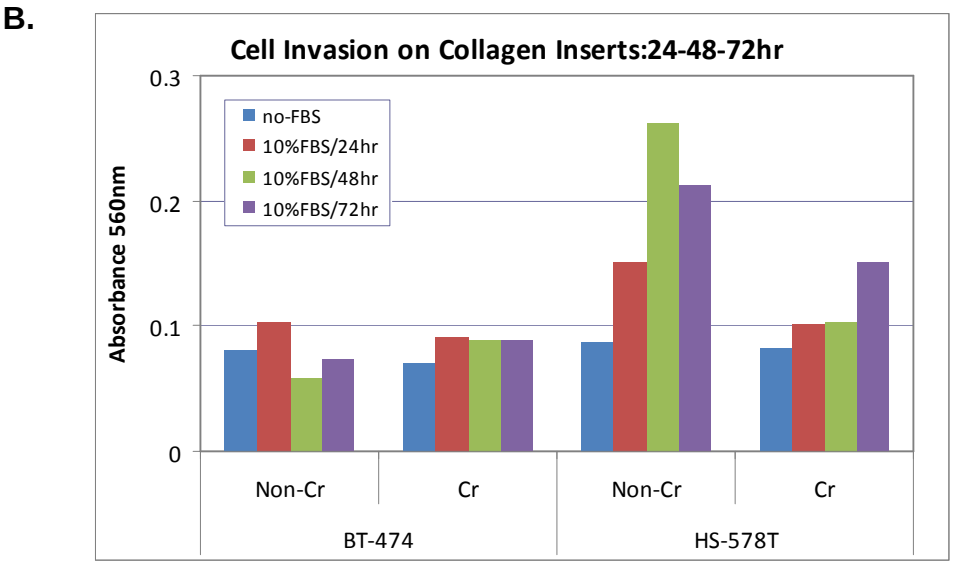


Figure 2: Time course of invasion through cross-linked and non-cross-linked collagen by BT-474 and HS-578T breast cancer cell lines.
BT-474, a minimally invasive cell line, and HS-578T, a highly invasive cell line were seeded at 250,000 cells per well in the upper chamber of inserts containing non-cross-linked collagen gels (Non-cross-linked; Catalog No. ECM1401) and cross-linked collagen gels (Cross-linked; Catalog No. ECM551). The wells beneath the chambers contained serum-free media or media containing 10% FBS. Plates were incubated for 24, 48 and 72 hrs. **A:** Invaded cells on the bottom side of the membrane were stained as described in *Assay Instructions*. HS-578T displayed significant invasion as early as 24 hrs on the non-cross-linked collagen gels, but displayed invasion through the cross-linked collagen gels only after 72 h incubation. Invasion by BT-474 was not observed at any time point with either gel. **B:** Colorimetric measurements were were performed as recommended in *Assay Instructions*. Data were consistent with that observed by microscopic analysis of the stained insert bottoms. (***Non-Cr:*** *Non-cross-linked,* ***Cr:*** *Cross-linked)*

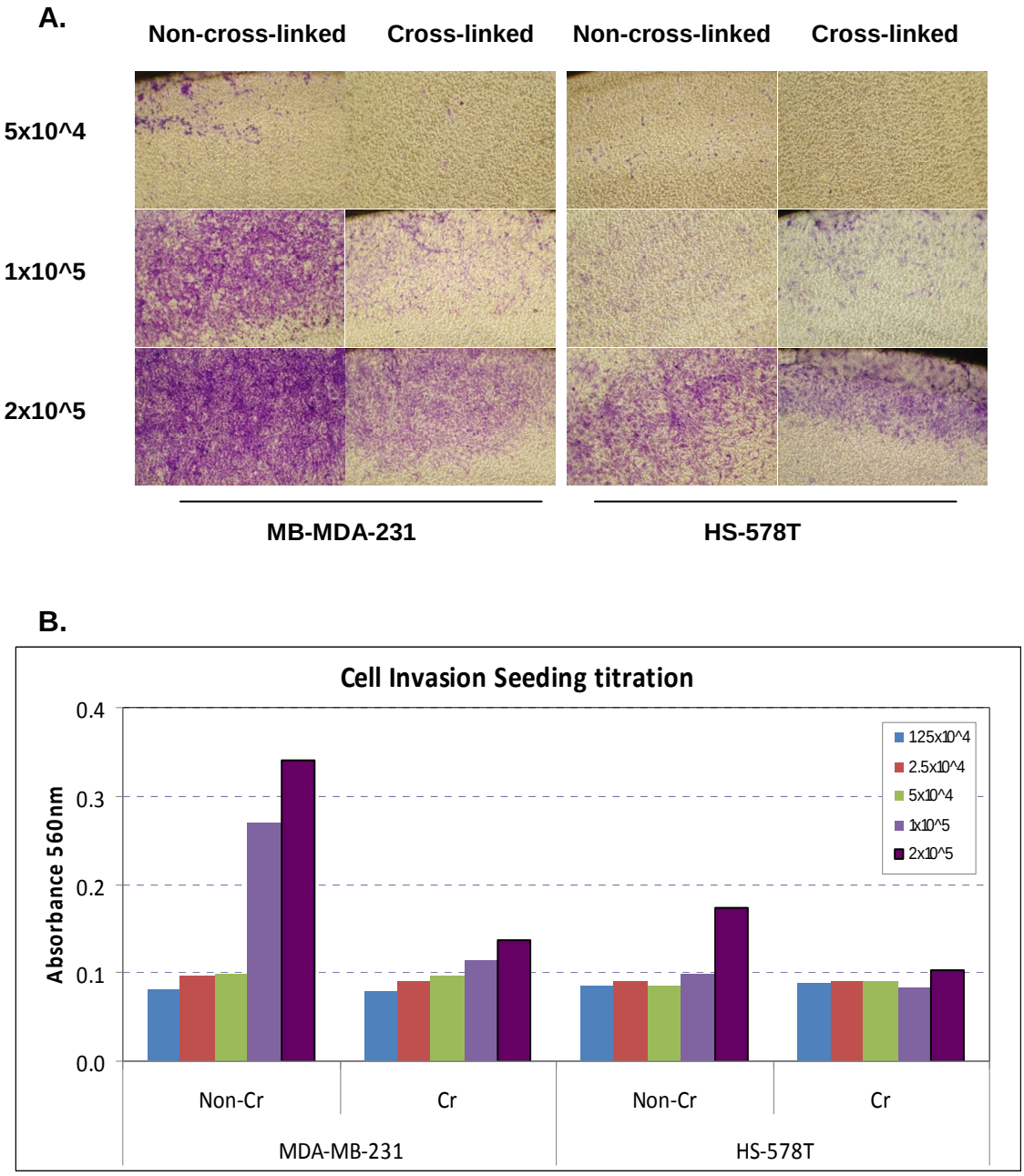


Figure 3: Effect of cell number on assay performance.
MB-MDA-231 and HS-578T cells were seeded with the indicated number of cells per well in the upper chamber of inserts containing non-cross-linked collagen gels (Non-cross-linked; Catalog No. ECM1401) and cross-linked collagen gels (Cross-linked; Catalog No. ECM551). The wells beneath the chambers contained 10% FBS, and plates were incubated for 48 h. **A:** Invaded cells on the bottom side of the membrane were stained and analyzed by light microscopy as described in *Assay Instructions*. Both MB-MDA-231 and HS-578T invaded non-cross-linked collagen with as few as 5x10^4/insert, whereas invasion through cross-linked collagen was only observed with 1x10^5 or more cells per insert. **B:** Colorimetric measurements were performed as recommended in *Assay Instructions*. Although colorimetric detection requires more cells than visualization by microscopy, colorimetric detection of invasion through non-cross-linked collagen gels requires fewer cells than through cross-linked collagen gels. (***Non-Cr:*** *Non-cross-linked,* ***Cr:*** *Cross-linked)*

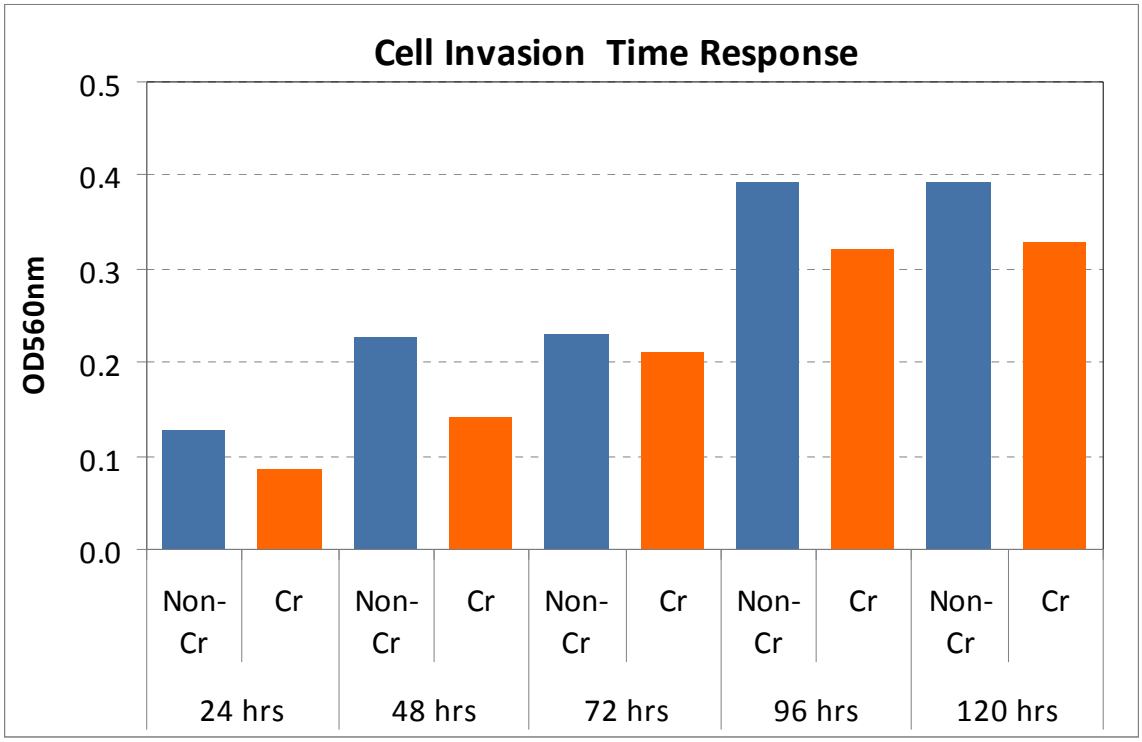


Figure 4: Longer term time course of cell invasion.
HS-578T cells were seeded at 2x10^5 cells/insert into the upper chamber of inserts containing non-cross-linked (Catalogue No. ECM1401) and cross-linked (Catalogue No. ECM551) collagen gels. Lower chambers of the wells contained media with 10% FBS. After incubation of the plates for the indicated intervals, colorimetric quantitation was performed as described in *Assay Instructions.* Invasion through non-cross-linked collagen inserts was detected as early as 24 hrs, whereas invasion through cross-linked collagen was detected at 48 h and beyond.

References

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