Detailed Scratch Wound Cell Invasion Protocol for Matrigel + HT1080 cells

Detailed Demonstration Protocol

This protocol describes the measurement of HT1080 fibrosarcoma cell invasion and (control) migration in 1 x 96 well plate, without test samples. 50% of wells are used to measure invasion and 50% migration.

Materials

- Growing stocks (T75, 37°C, 5%CO_2) of HT1080 fibrosarcoma cells (ATCC: CCL-121) in:
- 100ml of DMEM cell culture media + 10% FBS + 0.1mM NEAA + 1mM sodium pyruvate
  - DMEM (Life Technologies 32430)
  - FBS Fetal bovine serum, characterized (Thermo Scientific - HyClone SH3007103)
  - 10mM NEAA (Life Technologies 11140)
  - 100mM Sodium pyruvate (Life Technologies 11360)
- 96-well ImageLock™ plate (Essen BioScience: 4379) 1 per assay
- 96-well microplate (Corning: 3595) 1 per assay
- Trypsin/EDTA (Life Technologies 25200)
- D-PBS (w/o Ca^{2+}, Mg^{2+}, pH 7.0 - 7.3)
- Matrigel standard (BD 354234)
- IncuCyte™ 96-Well Scratch Wound Cell Invasion Accessories (Essen: 4444) include:
  - 2 x CoolBox 96F System plus 2 x CoolSink 96F (Essen: 1500-0078-A00)
  - 1 x extra CoolSink 96F (Essen: 1500-0080-A00)
  - CoolBox M30 System with CoolRack (Essen: 1500-0079-A00)

Protocol

IMPORTANT:
In advance of the experiment it is important to have stored the Cool pack accessories at the correct temperatures for at least 4h:
Coolbox x2 (block with gelpack: -20°C), Coolsink 96F x2 (4°C), Coolsink 96F x1 (37°C).
CoolBox M30 System (block with gelpack: -20 °C) with CoolRack (4°C).
The Cool Packs are used to ensure close temperature control of Matrigel in microplate plates. Matrigel stock (standard or GFR) is stored at – 20°C. At 4 - 8 °C, Matrigel is a viscous liquid. Gelling will occur slowly at 4 – 8°C, and more rapidly when warmed to 37 °C. For this reason it is imperative to store Matrigel working solutions at 4-8 °C at all times to avoid unwanted gelling. It is easier to handle low volume (<500µL) Matrigel solutions using pre-cooled (from a fridge), wide bore pipette tips or serological pipettes. Ahead of the experiment, Matrigel (standard and GFR) can be defrosted and stored at -20°C in 1ml aliquots.
Matrigel batches are supplied at a range of concentrations. We recommend sourcing a batch of Matrigel with a concentration of >9mg/ml and an endotoxin level of <3 (EU)/ml. The following protocol assumes a Matrigel stock concentration of 9.6 mg/ml.

Day 1 – Thaw Matrigel

1. Defrost Matrigel by placing the bottle into an ice bucket which is then placed in the fridge (+ 4 °C) overnight. When fully thawed there should be no gel clumps visible. If aggregates are present replace the bottle on ice and thaw at + 4 °C for a longer period of time. After thawing, chill ten 1ml eppendorf tubes in the CoolSink M30 System (10min). Using a pre-cooled serological pipette (10ml) aliquot (1ml) the Matrigel into each tube and store at -20 °C.

Day 2 – Coat the ASSAY PLATE with thin layer of Matrigel (10min, wait 1d)

1. Using the CoolSink M30 System with CoolRack, thaw one 1ml aliquot of 9.6 mg/ml Matrigel (~1 h). In a 15 ml Falcon tube, dilute Matrigel stock to 100 µg/ml in HT1080 culture media by slowly pipetting 63 µl of 9.6 mg/ml Matrigel into 5.937 ml culture media. Pipette five times to mix, ensuring any residual Matrigel in the pipette tip has been washed into the vial.
2. Dispense 50 µl of diluted Matrigel into each well of a 96-well ImageLock™ plate. Gently rock the plate to ensure even coating of each well. Place the plate in a 37 °C incubator, 5% CO_2 overnight.

Detailed Scratch Wound Cell Invasion Protocol: Matrigel + HT1080 cells

Day 3 – Seed cells onto the ASSAY PLATE (30min, wait 4h)

1. Remove a freshly confluent T75 flask of HT1080 cells from the cell incubator and place within a sterile cell culture hood.
2. Remove media, and wash cells with D-PBS (10ml).
3. Add 1ml Trypsin/EDTA to detach cells, leave for 2-3min.
4. Add 9ml of culture media, and re-suspend cells using a 10ml stripette.
5. Perform a cell count (e.g. trypan blue staining + haemocytometer).
6. Centrifuge the cell suspension (185 ×g, 4 min) and re-suspend the cell pellet in culture media at $2.5x10^5$ cells/ml.
7. Remove ASSAY PLATE from the incubator and aspirate and discard the Matrigel from each well and seed cells (100 µl per well, i.e. 25K cells per well) into every well. Do not leave any empty (dry) wells - these will damage the WoundMaker™.
8. Allow the cell plate to sit at room temperature for 5min to allow cells to evenly disperse across the bottom of the plate. Place the lidded plated into a 37 °C incubator, 5% CO_2 **for 4h**.

Day 3 – Prepare the REAGENT plate (30min)

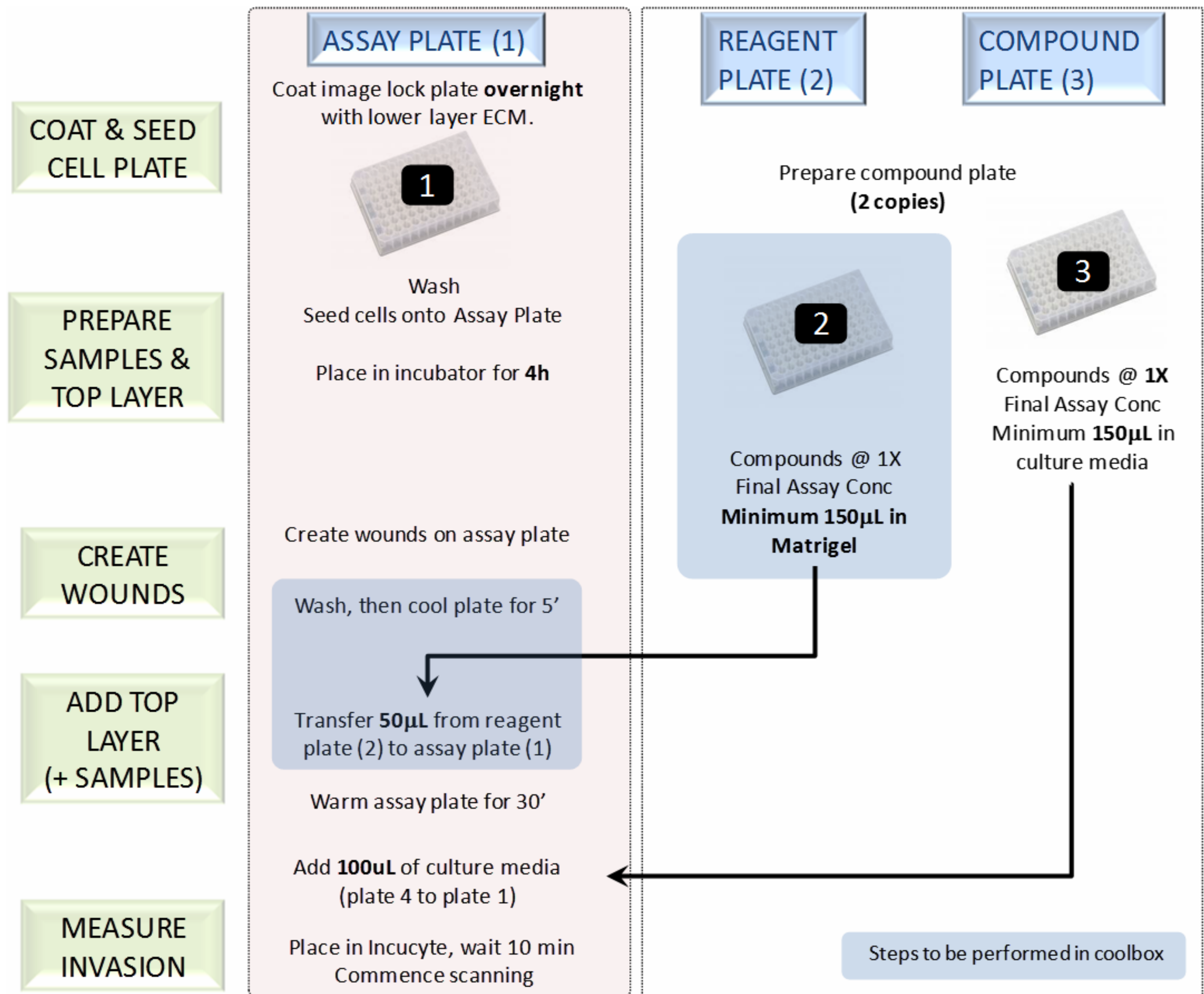
1. Place a standard 96-well plate in the pre-chilled CoolBox 96F at 4 - 8 °C for 5 min. Keep this plate on the CoolBox throughout the protocol.
2. Prepare a working stock (5ml) of 8 mg/ml Matrigel in cold culture media. In a 15ml falcon tube add 4.17ml of 9.6 mg/ ml Matrigel to 0.83ml of cold culture media (+4°C). Pipette five times to mix, ensuring any residual Matrigel in the pipette tip has been washed into the vial. Keep on ice.
3. Add 200µL Matrigel working stock to each well of columns 1 & 4 (for Invasion wells).
4. Add 200µL of culture media to each well of columns 7 & 10 (for Migration control wells).
5. Remove bubbles from by gently squeezing a wash bottle (containing 100% ethanol with the inner straw removed) to blow vapor over the surface of each well. Try to keep the tip of the wash bottle approximately 5cm from the media surface.
6. **NOTE:** in sample testing experiments, the REAGENT PLATE should contain samples prepared (diluted) in Matrigel/ culture media, at the final assay concentration. A second plate containing compounds (the COMPOUND PLATE, prepared in media) also needs to be made.

Day 3 – Wound, add top layer & schedule scans (40 – 50min)

1. Carefully remove the ASSAY plate from the incubator.
2. Follow the 96-well WoundMaker™ procedure to simultaneously create wounds in all wells. Visually inspect wounds with an inverted microscope, to ensure that you have correctly used the WoundMaker™.
3. Immediately (and carefully) wash the cells twice with cell culture media. After the final wash, add culture media (100 µl) to each well and place plate in a pre-chilled CoolBox 96F to equilibrate for 5 min. Remove and discard (cool) media from the ASSAY PLATE.
4. Transfer the Matrigel solution and culture media from the REAGENT PLATE to the ASSAY PLATE: 50µl per well Matrigel to Col 1 – 6 and 50µl per well culture media to Col 7 – 12. Remove bubbles as before.
5. Place the ASSAY PLATE on to the pre-warmed CoolSink 96F in a 37°C, 5% CO_2 incubator for 30 min.
6. Add 100µl of additional culture media to each well of the ASSAY plate. Do not mix. Remove bubbles. Place the de-bubbled cell plate in the IncuCyte ZOOM®, close the drawer and incubator door.
7. Allow the cell plate to equilibrate in the IncuCyte ZOOM® for 10min prior to scheduling the first scan – this will reduce the likelihood of condensation on the lower side of the plate which can hinder image quality.
8. In the IncuCyte ZOOM® software, schedule repeat scanning for every 3h for 48h, with the first scan to commence immediately. Set the assay plate to scan one image per well (Wide Mode if available) and ensure 'scan type' is set to Scratch Wound.
9. Wash and store the WoundMaker™ according to the established Wash Protocols.

Detailed Scratch Wound Cell Invasion Protocol: Matrigel + HT1080 cells

Schematic diagram of Matrigel Invasion Assay Workflow with test samples



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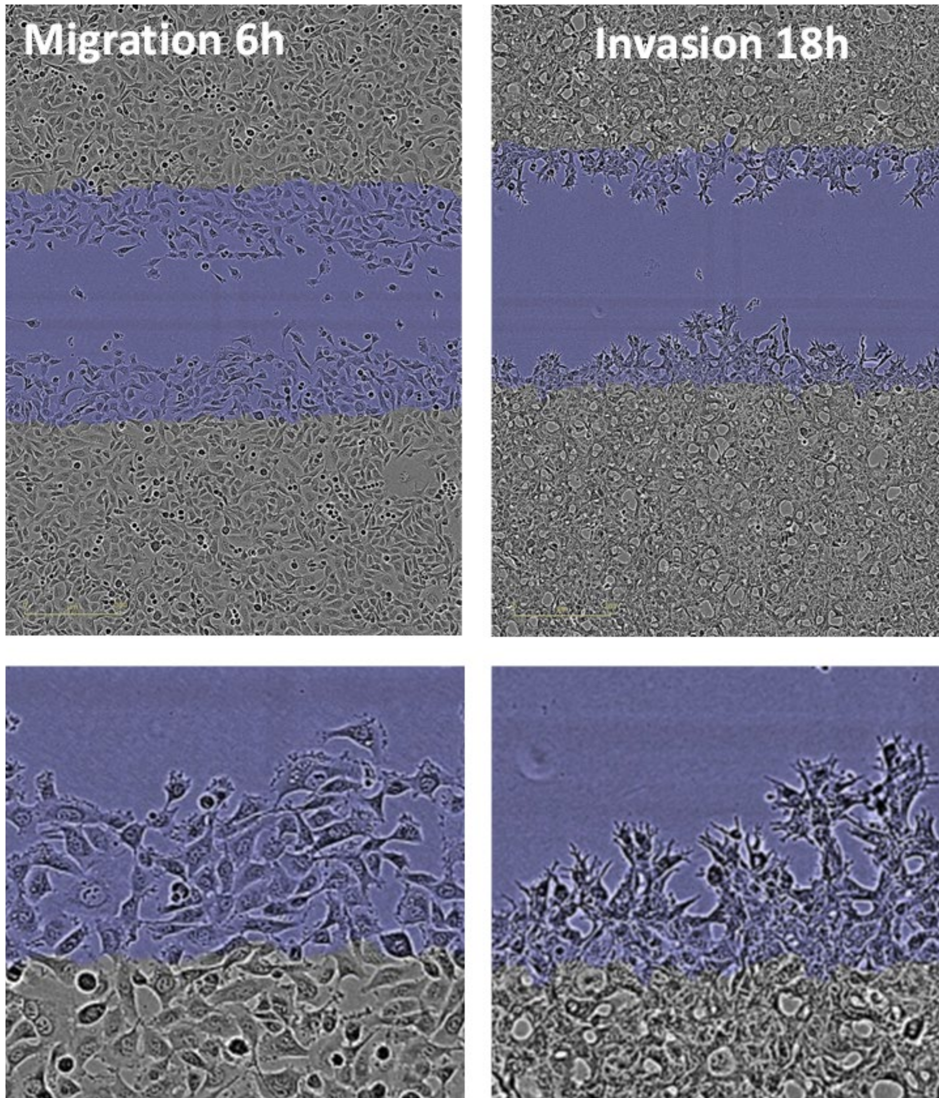
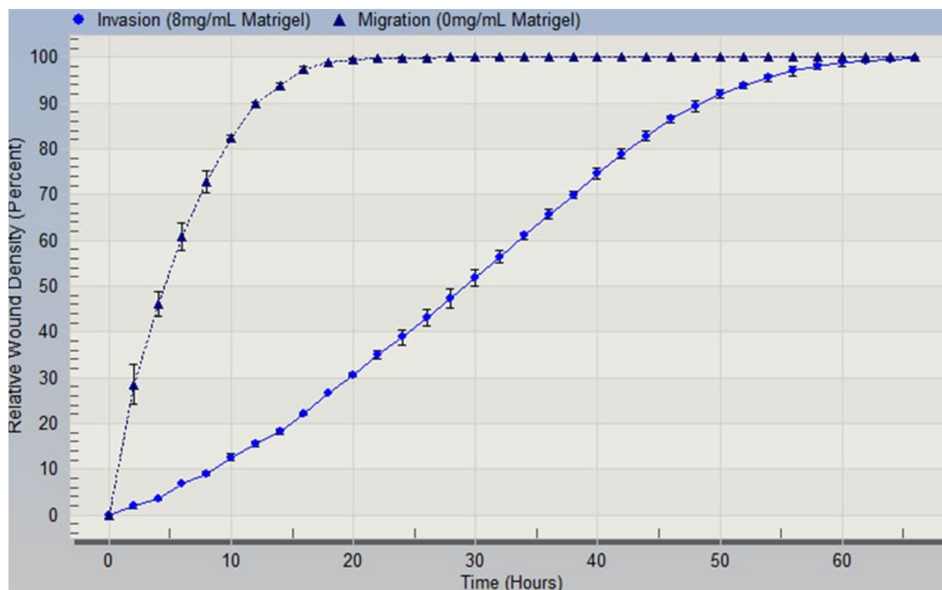


Representative phase contrast images of HT1080 cells (upper; 10X magnification, lower; digitally zoomed) taken at 6h post wounding for migration (left) and 18h post wounding for invasion (right).
Note clean wound boundaries and symmetrical migration/invasion. Also note the characteristic differences in cell morphology.



Summary data time courses of migration and invasion (% relative wound density; points are mean values and vertical bars show standard deviation).

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