

AccuWound 96

User Manual

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Upgrading the Traditional Scratch Assay

Cell migration is critical to many facets of biology, including processes such as wound healing, angiogenesis, and the intravasation and extravasation of cancer cells during metastasis. Although scratch assays/wound healing assays have long been a staple for studying cell migration, they suffer from three drawbacks:

- 1 They are endpoint assays, making it difficult to accurately monitor migration kinetics.
- 2 Manually scratching individual wells limits throughput.
- 3 Inconsistencies in the manually-produced scratches reduce precision/reproducibility.

It was recognized many years ago that xCELLigence real-time cell analysis (RTCA) provides a solution to the first issue with scratch assays. Because it can continuously track wound closure, RTCA yields a quantitative/kinetic readout. However, on its own, xCELLigence RTCA cannot address the problems of low efficiency and poor reproducibility in the scratch production step.

The Agilent AccuWound 96 scratch tool addresses both of these issues. Using 96 spring-loaded pins that make contact with the bottom of Agilent xCELLigence E-Plate wells, combined with a mechanism for precisely dragging these pins laterally across the bottom of the wells, the AccuWound 96 makes it possible to produce 96 identical scratches in roughly 10 seconds. Combining xCELLigence RTCA with the AccuWound 96 tool has revolutionized the scratch assay, providing unparalleled efficiency and data quality (with coefficients of variation that are ~4% versus ~20% for traditional scratch assays).

xCELLigence and AccuWound 96 Scratch Assay Overview

The xCELLigence RTCA instruments use electronic microplates (Agilent E-Plates) that contain gold biosensors embedded in the bottom of each well (**Figure 1**). These biosensors continuously and noninvasively monitor how much of their surface area is covered by cells. After the AccuWound 96 tool is used to simultaneously generate a scratch wound in all 96 wells of the E-Plate, closure of the wound by cell migration and proliferation is recorded in real time.

Step 1 – Cell seeding and proliferation: After seeding adherent cells in the wells, the Agilent E-Plate WOUND 96 is placed inside the xCELLigence instrument, which is housed in a standard tissue culture incubator. The xCELLigence biosensor signal, also known as “Cell Index”, increases as cells attach and proliferate, and then plateaus as cells approach 100% confluence (**Figure 1A** and **A** in **Figure 2**).

Step 2 – Scratch wound production: The E-Plate is removed from the xCELLigence instrument and placed inside the AccuWound 96 scratch tool. Turning a knob slides a spring-loaded PTFE pin across the bottom of each well in the E-Plate. This will selectively remove cells from the biosensors (**Figure 1B**). The E-Plate is then placed back into the xCELLigence instrument, which immediately detects the scratch wound through a drop in the Cell Index (**Figure 2**).

Step 3 – Monitoring cell migration/wound healing: As cells migrate and proliferate to close the wound, they progressively recolonize the gold biosensors (**Figures 1C to 1E**), causing the Cell Index signal to increase (**Figure 2**). The kinetics of this process are easily compared for different conditions, such as $\pm$ drug and gene knockdown.

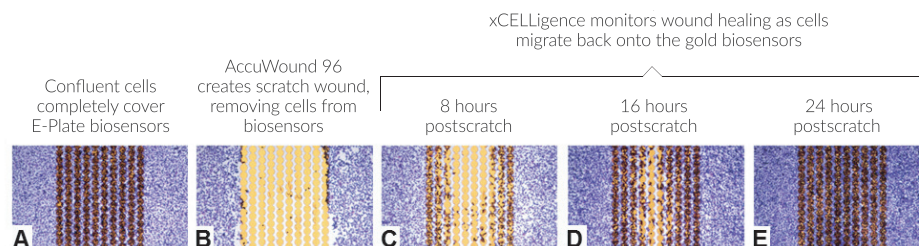


Figure 1. Representative images from a migration/wound healing assay with HT1080 cells on the Agilent E-Plate WOUND 96.

Although xCELLigence data can be acquired without staining, here cells were stained and visualized at different time points (**Figure 1**) to demonstrate the correlation between biosensor coverage and the magnitude of the Cell Index signal (**Figure 2**). Key features of the Cell Index plot, including the specific time points that correspond to images A to D in **Figure 1**, are noted.

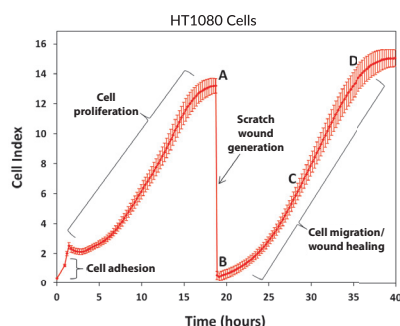


Figure 2. Impedance based real-time monitoring of HT1080 cells adhesion, proliferation, wound generation, and migration/wound healing on an Agilent E-Plate WOUND 96.

AccuWound 96 Scratch Tool

The different parts of the AccuWound 96 scratch tool and wash reservoir are shown in **Figure 3**.

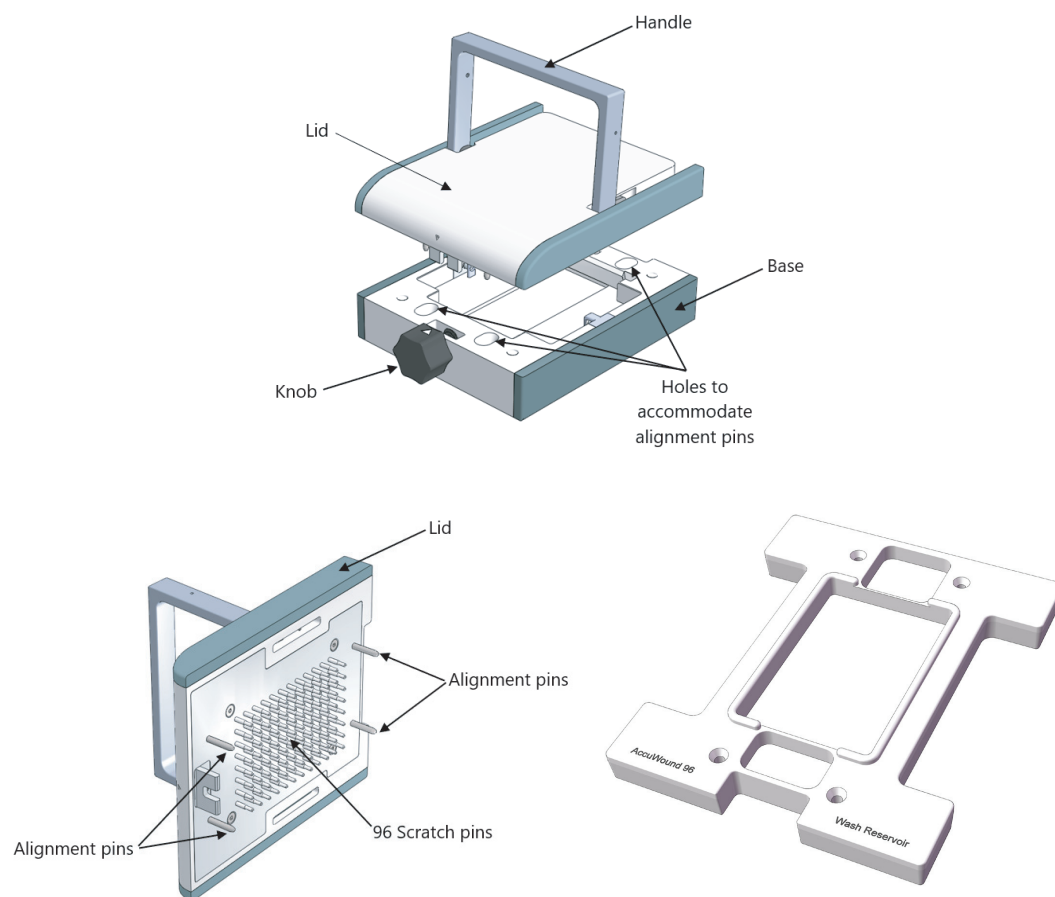


Figure 3. AccuWound 96 scratch tool components and AccuWound 96 wash reservoir.

E-Plate WOUND 96

Agilent produces many 96-well E-Plates. Although all of these are compatible with the AccuWound 96 scratch tool, only the E-Plate WOUND 96 has been optimized for scratch assays. The biosensors in these plates are located exclusively in the central region of the well, instead of spanning the entire well bottom (as is the case for standard E-Plates, compare images in **Figures 4A** and **4B**). The AccuWound 96 scratch tool uses PTFE-tipped scratching pins that have the same width as the gold biosensor strip in an E-Plate WOUND 96. Consequently, the scratching step removes cells from >99% of the biosensor surface area. This causes the sensor's signal to drop to nearly zero (**Figure 4C**), which maximizes the assay window and increases the robustness and reproducibility of the data. While the AccuWound 96 scratch tool will produce identically sized wounds in standard E-Plates, the wound spans only ~40% of the biosensor surface area, resulting in just a ~40% drop in signal (**Figure 4D**).

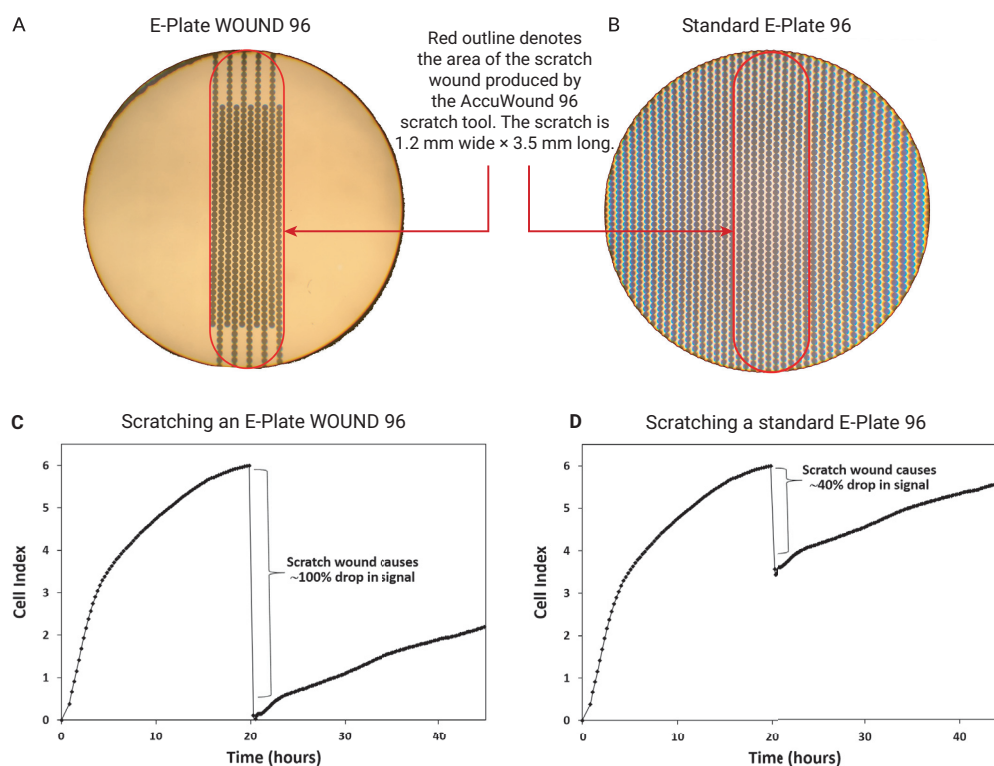


Figure 4. The Agilent E-Plate WOUND 96 is optimized for scratch assays. (A) View of a single well in an E-Plate WOUND 96. The gold biosensors, silhouetted in dark gray, are confined to the central region of the well bottom. (B) View of a single well in a standard E-Plate 96. The gold biosensors span the entire well bottom. (C and D) xCELLigence impedance data for a scratch assay conducted in an E-Plate WOUND 96 (C) and a standard E-Plate 96 (D).

The AccuWound 96 scratch tool is optimized and validated for use with the Agilent E-Plate WOUND 96. However, the AccuWound 96 scratch tool may be compatible with other types of 96-well microplates. But, validation of the scratch quality and consistency will be needed for each particular plate.

For the AccuWound tool to generate scratches of consistent size, the microplate must fit snugly within the AccuWound base. If not, and the plate can be moved laterally within the base, the scratch pins will not consistently remove cells from the exact same location of the wells. If a plate type fits loosely within the AccuWound base, use shims to lock the plate in place before scratching.

Also important in generating scratch wounds of consistent size and quality is the height of the well bottoms in a plate. The spring-loaded pins of the AccuWound 96 are inherently able to accommodate a plate whose bottom is higher than the bottom of the E-Plate WOUND 96. However, some plate types have a bottom that is lower than the bottom in the E-Plate WOUND 96. For these types of plates, the AccuWound 96 scratch pins will not contact the well bottoms or the contact is weak, creating inconsistent scratch wounds. The height of the microplate can be elevated by placing a spacer in the bottom of the AccuWound 96 base.

AccuWound-Compatible xCELLigence Instruments

The AccuWound 96 scratch tool has been optimized for use with the Agilent E-Plate WOUND 96 plates. These 96-well plates can be used with both the xCELLigence single plate (SP, **Figure 5A**) and multiple plates (MP, **Figure 5B**) instruments. Although these instruments can be housed on a benchtop, to control temperature, humidity, and atmospheric composition, they are most often used within a tissue culture incubator (**Figure 5C**) or hypoxia chamber.

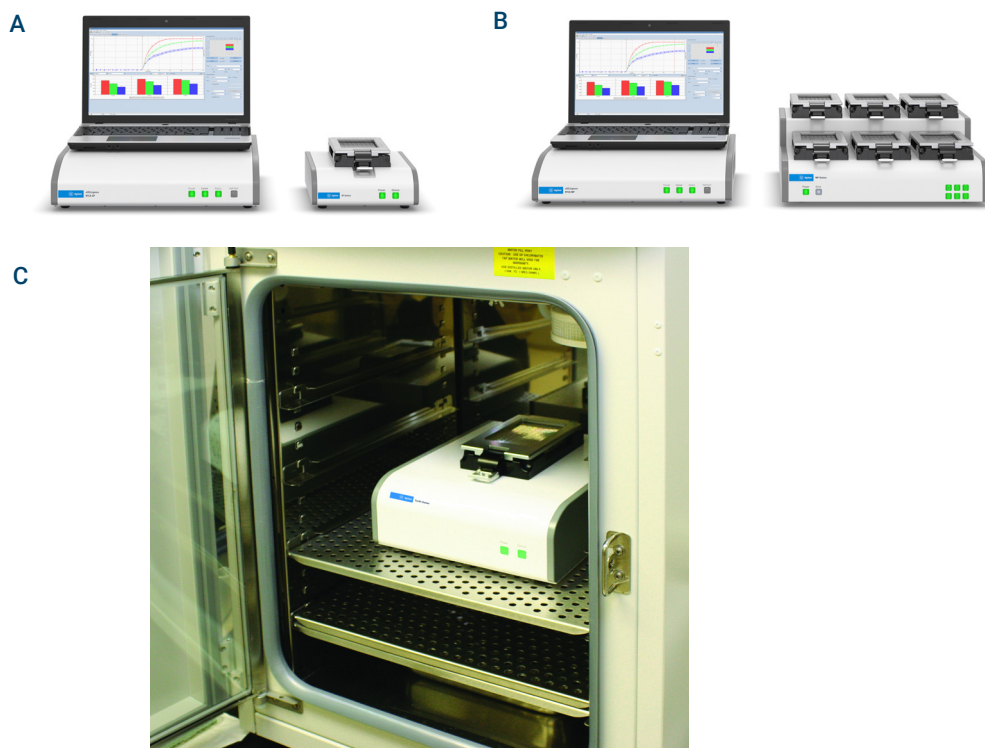


Figure 5. xCELLigence instruments that are compatible with Agilent E-Plate WOUND 96, Agilent AccuWound 96, and conducting scratch wound healing assays. (A) The Agilent xCELLigence SP instrument accommodates a single 96-well plate. (B) The Agilent xCELLigence MP instrument accommodates six 96-well plates. (C) Example of an xCELLigence instrument housed inside a standard tissue culture incubator.

Protocol - Condensed

Initial preparation

Before using the AccuWound 96 tool for the first time, work inside a sterile tissue culture hood and use the provided wash reservoirs for rigorous initial cleaning and sterilization.

- 1 Soak the scratch pins for 5 minutes in each of the following solutions (in order):
 - 0.5% (w/v) Alconox (5 minutes)
 - 1% (w/v) Virkon S (5 minutes)
 - Sterile distilled water (5 minutes)
 - 70% ethanol (5 minutes)
- 2 Air dry the pins inside the hood.
- 3 If the tool will not be used immediately, place the AccuWound lid into the AccuWound base, lock it into position, and store it in a cool, dry, dust-free location.

Day 1

- 1 Seed cells into the wells of an E-Plate WOUND 96. Seeding density should be such that cells will have just reached confluence when the scratch wounds are generated on day 2.
- 2 Allow seeded cells to settle for 30 minutes at room temperature inside the tissue culture hood.
- 3 Place the E-Plate WOUND 96 into the xCELLigence instrument and immediately begin data acquisition to monitor cell adhesion and proliferation.

Day 2

- 1 Immediately before using the AccuWound 96 scratch tool, clean, and sterilize the scratch pins. Working inside a sterile tissue culture hood and using the provided wash reservoirs, soak the scratch pins for 5 minutes in 70% ethanol. Air dry the pins inside the hood.
- 2 Once cells reach confluence (displaying a plateaued impedance signal), pause the xCELLigence data acquisition and transfer the E-Plate WOUND 96 to the sterile tissue culture hood that contains the cleaned, sterilized, and dry AccuWound 96 scratch tool.
- 3 Generate scratch wounds using the AccuWound 96 tool.
- 4 Remove debris-containing media from the scratched wells, gently wash the wells twice with 200 μ L of fresh media, and add fresh media to achieve a final volume of 100 to 200 μ L (which can include drug treatments if desired).
- 5 Place the E-Plate WOUND 96 back into the xCELLigence instrument, but do not immediately start acquiring data. Allow the temperature of the plate and media to equilibrate for 10 minutes, and then begin data acquisition to monitor cell migration and wound closure.
- 6 Data analysis: Quantify wound healing using the slope of the impedance curve, the area under the curve, or the time required for the impedance signal to reach a particular value postscratching.

Protocol - In Depth

NOTE

Never use the AccuWound 96 scratch tool with a “dry” microplate (one that does not contain media within its wells). Dragging the scratch pins across a dry well bottom can potentially damage the pins.

Initial cleaning and sterilization

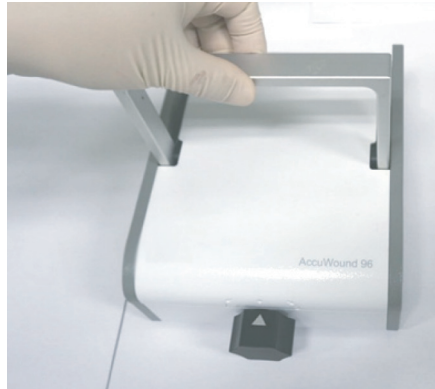
(Alconox → Virkon S → distilled water → 70% ethanol)

When the AccuWound 96 scratch tool is first received, or after having been in storage for a long period, the scratching pins should be subjected to the following washing steps to ensure they are biologically and chemically sterile (free of chemical residues or cellular debris).

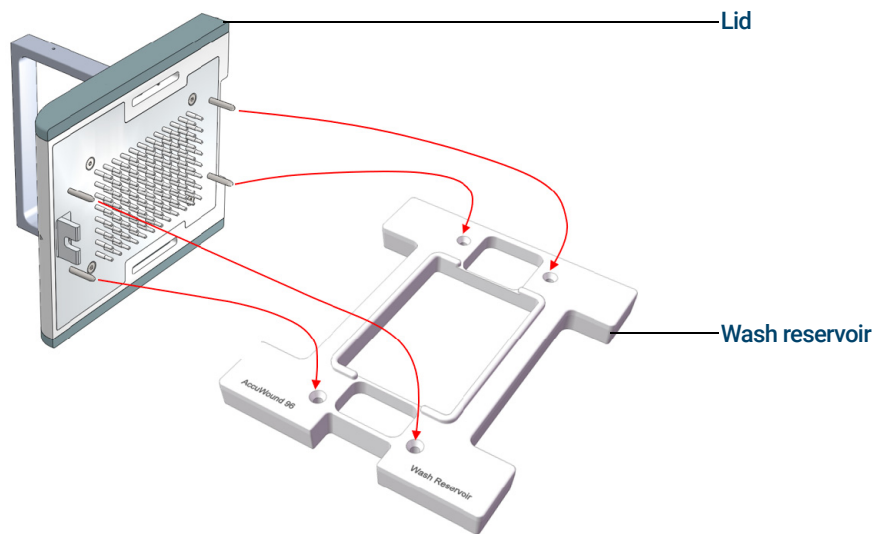
- 1 Produce solutions of Alconox and Virkon S:
 - Add 0.25 g of powdered Alconox (an anionic detergent) to a 50 mL tube. Add sterile distilled water to achieve a final volume of 50 mL. Agitate until all powder has dissolved to produce a 0.5% (weight/volume) solution.
 - Add 0.5 g of powdered Virkon S (a broad-spectrum disinfectant and virucide) to a 50 mL tube. Add sterile distilled water to achieve a final volume of 50 mL. Agitate until all powder has dissolved to produce a 1% (weight/volume) solution.
- 2 Bring the following items into a sterile tissue culture hood:
 - 50 mL of 0.5% Alconox solution (described in [step 1](#))
 - 50 mL of 1% Virkon S solution (described [step 1](#))
 - 500 mL of sterile distilled water
 - 50 mL of 70% ethanol
 - AccuWound 96 scratch tool
 - Both AccuWound wash reservoirs
- 3 Fill one reservoir with the Alconox solution. Using the entire 50 mL is necessary to ensure the AccuWound 96 tool scratch pin tips are submerged during the cleaning and sterilization steps.
- 4 Use the same method as in [step 3](#) to fill the other wash reservoir with the Virkon S solution.
- 5 To remove the pin-containing AccuWound lid from the AccuWound base:
 - a Ensure that the arrow on the large black knob of the base is aligned with the arrow on the lid. If the knob is not rotated to this “centered” position, the AccuWound lid cannot be removed from the base.



- b** Rotate the metal handle from the locked horizontal position to the unlocked vertical position.



- c** Using this handle, lift the pin-containing AccuWound lid straight up.
- 6** Place the pin-containing AccuWound lid into the Alconox-containing wash reservoir so that the scratch pins are submerged. Secure the four large alignment pins of the AccuWound lid in the four alignment holes of the wash reservoir to ensure proper positioning.



NOTE

For all washing and soaking steps, do not agitate the tool or wash reservoir as this can splash the wash solution into the spring-loaded pin mechanism, which can cause corrosion over time.

- 7** Transfer the tool directly from the Alconox solution into the second wash reservoir that contains the Virkon S solution. Let it soak for 5 minutes. While the tool is soaking in Virkon S, aspirate off the Alconox cleaning solution from the other reservoir, rinse the reservoir three times with sterile distilled water, and then fill the reservoir with 50 mL of sterile distilled water.
- 8** After soaking for 5 minutes in Virkon S, transfer the tool into the reservoir that contains sterile distilled water. Let it soak for 5 minutes. While the tool is soaking in water, aspirate off the Virkon S cleaning solution from the other reservoir, rinse the reservoir three times with sterile distilled water, and then fill the reservoir with 50 mL of 70% ethanol.
- 9** After soaking the tool for 5 minutes in sterile distilled water, transfer it into the reservoir that contains 70% ethanol. Let it soak for 5 minutes there.

- 10** Remove the AccuWound lid from the 70% ethanol solution and let it air dry by resting it directly on the floor of the tissue culture hood. The AccuWound lid rests on the four metal alignment pins, preventing the scratch pins from contacting the floor of the hood. Depending on the air flow in the hood, 5 minutes is typically sufficient for all the ethanol to evaporate.
- 11** The tool is now biologically and chemically sterile, and is ready for use. If the tool is not going to be used immediately, place the AccuWound lid into the AccuWound base and lock it into position. Store the tool in a cool, dry, dust-free location.

Seeding cells and monitoring adhesion and proliferation

- 1 Before seeding cells into the E-Plate WOUND 96, a background reading must first be taken. Add 50 μ L of media to each well and then insert the plate into an xCELLigence instrument. Allow the plate and media to equilibrate to 37 °C for 10 minutes and then acquire the background reading. Cells can now be added.
- 2 Seeding cells into the E-Plate WOUND 96 on day 1 and generating the scratch wounds on day 2 is recommended. The goal is to generate the scratch wounds when cells have recently reached confluence. **Table 1** shows some of the commonly used cell seeding densities. Note that these are only suggestions. Users need to optimize cell seeding density based on the cell type they are working with, the growth media being used, and other factors.

Table 1. Commonly used cell seeding densities

Cell type	Number of cells to seed in each well of an E-Plate WOUND 96
HT1080	20,000
3T3	20,000
A549	40,000
MDA-MB-231	20,000

- 3 At the time that a scratch wound is being generated by the AccuWound 96 tool, the volume of media that is present in each well of the E-Plate WOUND 96 microplate should be 100 μ L or less. If more than 100 μ L of media is present, inserting the AccuWound scratching pins into the wells will cause media to spill out. The most straightforward way to satisfy this volume requirement is to simply add 50 μ L of a cell suspension to the 50 μ L of media that is already present in the wells (left over from the background reading).
- 4 After seeding cells into the wells of the E-Plate WOUND 96, leave the plate inside the hood at room temperature for 30 minutes to allow the cells to settle evenly onto the well bottom. Failure to perform this step can result in large well-to-well variation. This is because immediate warming to 37 °C can cause convection currents to form within the well. These currents can push settling cells to the well perimeter, resulting in an uneven distribution of cells on the gold biosensors.
- 5 After letting the cells settle for 30 minutes at room temperature, place the plate into the xCELLigence instrument and monitor adhesion and growth by taking a reading once every 10 minutes. Collecting data over this early time regime helps the user know when to induce the scratch wound (when cells reach confluence the impedance signal plateaus). The rate and magnitude of the adhesion and proliferation phases of the impedance signal, and any other nuances of the curve shape, are all unique to the cell type being studied (and the assay conditions). As a result, they should be consistent from one assay to the next. Monitoring the adhesion and proliferation phases of the impedance signal after cell seeding can serve as an internal quality control measure, helping to ensure that cells are healthy and behaving “normally” before generating the scratch wound. The impedance readings immediately before the scratching step also establish the “baseline” reference point that the scratch and recovery steps are analyzed against.

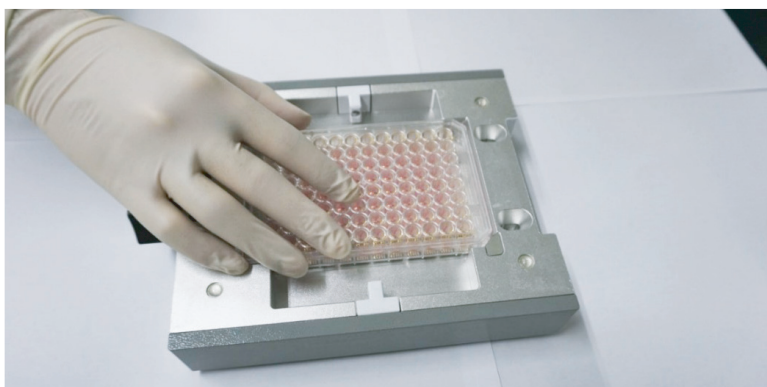
Rapid sterilization of pins in 70% ethanol immediately before scratching

If the AccuWound 96 tool has never been used, or has not been used in a long time, it should be subjected to the rigorous cleaning and sterilization protocol that was described in **“Initial cleaning and sterilization”** on Page 12 (involving Alconox, Virkon S, distilled water, and 70% ethanol). If the tool has been used within the past week and has been stored in a dust-free environment, it can

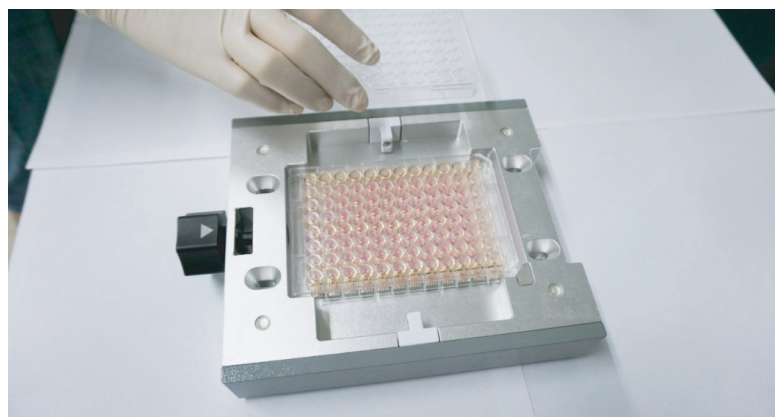
simply be subjected to rapid sterilization by soaking the scratch pins in sterile distilled water for 5 minutes, followed by 70% ethanol for 5 minutes (using the AccuWound wash reservoirs). After air drying the scratch pins in a sterile hood for 5 minutes, the tool is ready for use.

Generate scratch wounds

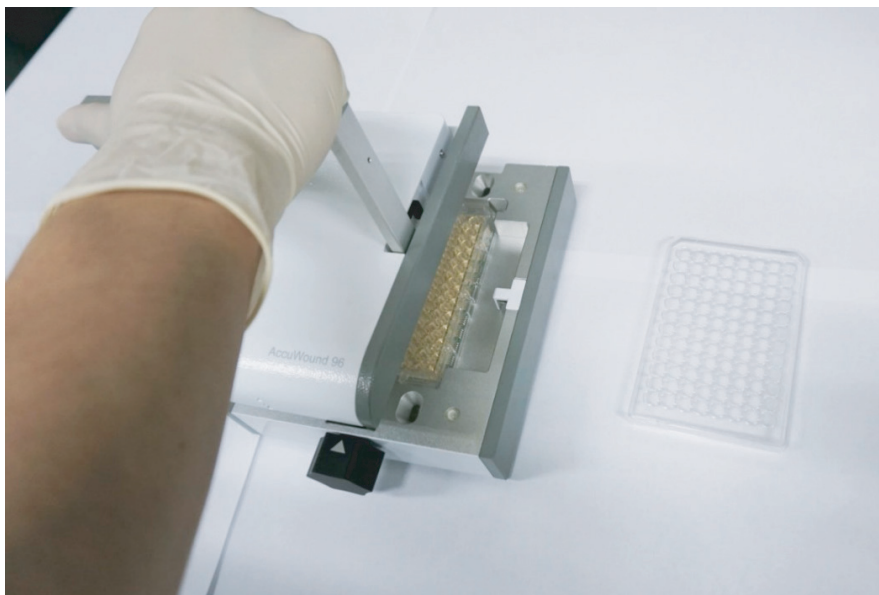
- 1 The rate at which a scratch wound heals can vary depending on how dense or confluent the cells are at the time the wound is generated. To get consistent results from one assay to the next, wounds should always be generated at a similar cell density. For best results, Agilent recommended using the AccuWound to generate scratch wounds just as cells reach confluence.
- 2 Pause data acquisition on the xCELLigence instrument, remove the E-Plate WOUND from the instrument, and transfer the plate into the sterile tissue culture hood where the clean and sterilized AccuWound tool is ready for use. All steps after that should be performed in the hood.
- 3 Remove the lid of the AccuWound tool (using [step 5](#) on Page 12).
- 4 Place the E-Plate WOUND into the base of the AccuWound tool. There is only one orientation for the plate and base; line up the notched corner of the plate with the notched corner of the AccuWound base.



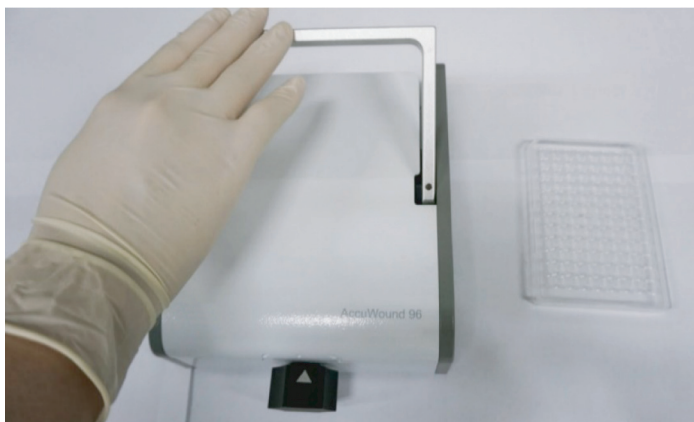
- 5 Remove the lid from the E-Plate WOUND.



- 6 Place the pin-containing AccuWound lid into the E-Plate-containing AccuWound base. The four large alignment pins on the AccuWound lid facilitate alignment with the AccuWound base.



- 7 Lock the AccuWound lid to the AccuWound base by rotating the metal handle from the vertical position down into the horizontal position. When done properly, this results in a clicking noise.



- 8 Generate the scratch wounds within all 96 wells of the plate. The scratch wounds of identical length (3.5 mm) and width (1.2 mm).
- Rotate the black knob on the AccuWound base counterclockwise until it reaches a hard stop.
 - Rotate the knob clockwise back to its original starting position (with the arrow on the black knob aligned with the arrow on the AccuWound lid).
 - Continue to rotate the black knob clockwise until it reaches a hard stop.
 - Rotate the black knob counterclockwise until the arrow on the knob aligns with the arrow on the AccuWound lid.
- 9 Unlock the handle (by lifting it into a vertical position), remove the pin-containing AccuWound lid by lifting straight up, and remove the E-Plate WOUND from the AccuWound base.
- 10 The media in the wells will now contain cellular debris and intact cells that were dislodged during the scratching step. For most applications, this debris-laden media should be removed, followed by two washing steps using 200 μ L of fresh media. Add 100 to 200 μ L of fresh media

that has been prewarmed to the temperature the assay is being run at (typically 37 °C). At the time that fresh media is added back into the wells, different treatments (such as wound healing stimulators or inhibitors) can also be included. As media is removed or added to the wells after scratching, care must be taken to not dislodge or disrupt the remaining adherent cellular monolayer.

- 11 Once media has been replaced, put the lid back onto the E-Plate WOUND and insert the plate into the xCELLigence instrument, but do not immediately start acquiring data. Allow the temperature of the plate and media to equilibrate for 10 minutes, and then begin data acquisition to monitor cell migration and wound closure. During this wound closure phase, data should be acquired every 10 minutes.

Scratching multiple plates

- 1 If multiple plates are to be scratched sequentially and these plates contain the same type of cells, the pins of the AccuWound can be cleaned using the following protocol:
 - Scratch plate 1
 - Soak pins in sterile distilled water for 5 minutes
 - Scratch plate 2
 - Soak pins in sterile distilled water for 5 minutes
- 2 If multiple plates are to be scratched sequentially and these plates contain different types of cells, an extra measure of caution is warranted. Before scratching each new cell type, the pins of the AccuWound tool should be sterilized by going back through the Alconox, Virkon S, distilled water, and 70% ethanol steps that were described in **“Initial cleaning and sterilization”** on Page 12.

Cleaning before storing the AccuWound

- 1 Once all scratch assays have been completed and the AccuWound tool is going to be stored, the pins should be sterilized by going back through the Alconox, Virkon S, distilled water, and 70% ethanol steps that were described in **“Initial cleaning and sterilization”** on Page 12.
- 2 The clean and dry AccuWound lid should always be stored within the AccuWound base.

Example Data

Figure 6 shows a representation of typical scratch assay data for HT1080 cells (seeded at 20,000 cells for each well) and A549 cells (seeded at 40,000 cells for each well) conducted with an xCELLigence instrument and the AccuWound 96 scratch tool. Key features of the data are:

Adhesion phase: Note the rapid increase in signal, due to cells adhering to the well bottom, that is observed for the HT1080 cells over the first ~two hours after cell seeding. Whether or not this distinct adhesion phase is observable varies depending on the cell type and the number of cells that have been seeded (this phase is absent for the A549 cells).

Proliferation phase: When seeded at a subconfluent density, cells display a steady increase in impedance signal as they proliferate and occupy an increasing percentage of the surface area on the well bottom. When cells reach confluence, the impedance signal plateaus. As seen in **Figure 6**, the magnitude of the impedance signal when cells reach confluence varies from one cell type to the next. Scratch wounds should be generated shortly after cells have reached confluence.

Scratch phase: Scratching the E-Plate WOUND with the AccuWound scratch tool removes >99% of the cells from the gold biosensors in the well bottom. Immediately after scratching, the impedance signal drops to ~0. Failure to drop all the way to zero is because a small number of cells are still attaching to the biosensors, typically along the periphery of the wound.

Cell migration/wound healing phase: As cells migrate to close the scratch wound, they reoccupy the gold biosensors and the impedance signal steadily increases. The rate at which this occurs depends on the cell type and the assay conditions (medium composition, temperature, migration inhibiting and stimulating drugs). Note the dramatic difference in migration rate for HT1080 and A549 cells (requiring ~20 hours and ~60 hours for wound closure, respectively).

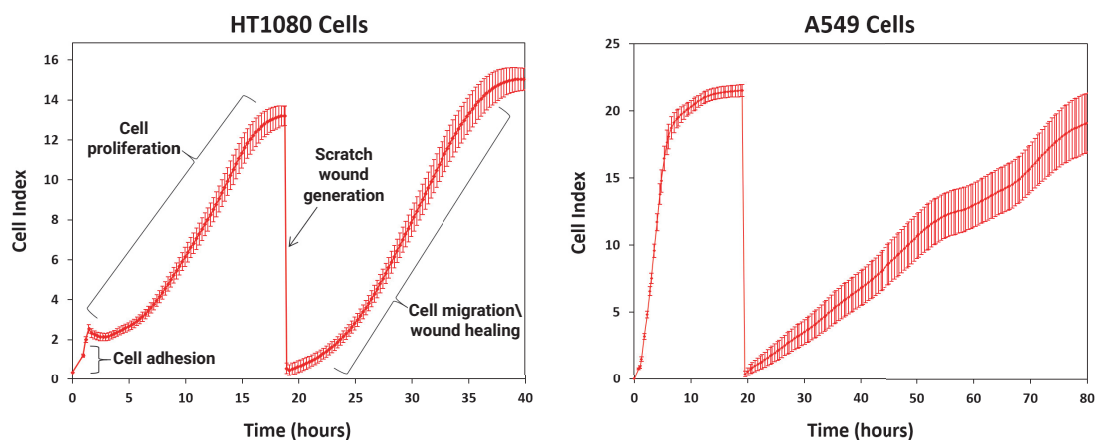


Figure 6. Typical scratch assay in HT1080 (A) and A549 (B) cells on an Agilent E-Plate WOUND 96.

Data Analysis

When monitored by xCELLigence, the rate of scratch wound closure can be quantified using one of the following methods:

- **“Slope”** of the migration/healing phase
- **“Area under the curve”** on Page 22
- **“Time required to reach a particular Cell Index value”** on Page 23

Slope

The slope of the impedance data over a particular time range can be calculated directly within the xCELLigence RTCA Software Pro. Alternatively, the impedance data can be exported from RTCA Software Pro and analyzed in other software, such as Excel. For instructions on how to calculate slope or export impedance data, see the RTCA Software Pro software manual.

The data is zoomed in to the first 10 hours postscratching in **Figure 7** to focus on migration/healing data for four different cell lines. The A549 and MDA-MB-231 cells migration/healing phase is linear, making calculation of slope straightforward. In contrast, the migration/healing phase for NIH-3T3 cells is mostly linear, but displays a small nonlinear “burst” in signal over the first hour postscratching. As a more extreme example of nonlinearity, the signal for HT1080 cells displays significant curvature over the first 10 hours after scratching. In these cases, the calculated slope will be highly dependent upon the time range analyzed, as shown in **Table 2**.

Table 2. Migration/healing of HT 1080 cells.

Postscratching time range analyzed (Hours)	Slope
0-2	0.19
2-4	0.39
4-6	0.66
6-8	0.97
8-10	1.1
0-10	0.67

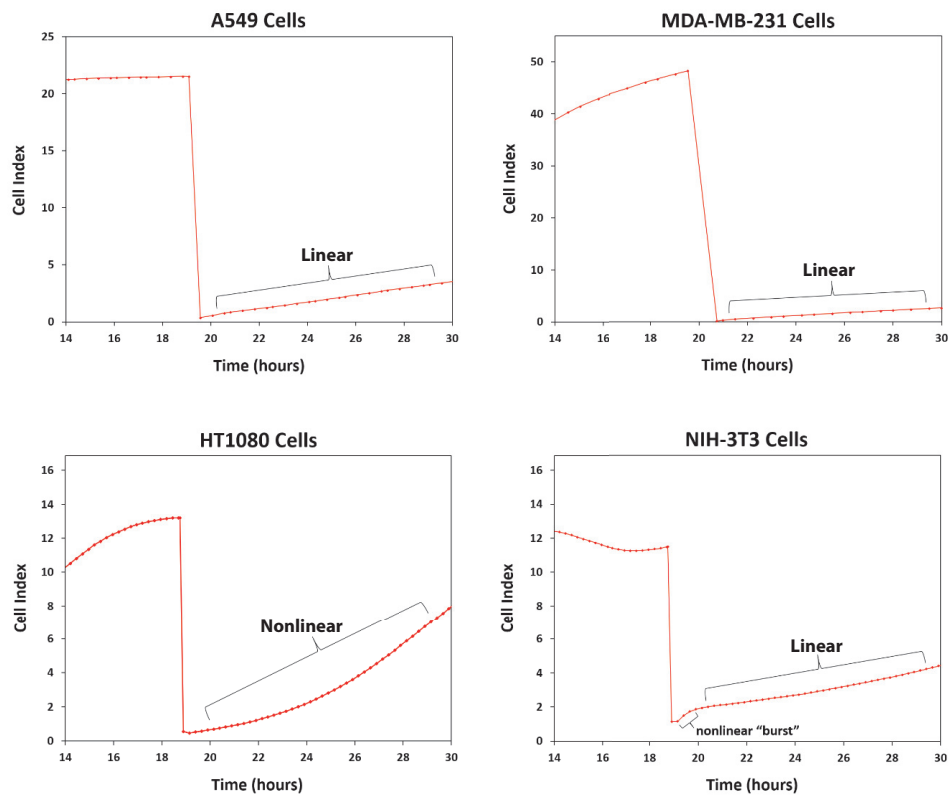


Figure 7. Zoomed in view of the first 10 hours of migration/healing for four different cell lines.

Slope-based analyses might still be possible for nonlinear migration/healing data, but take care to select appropriate regions of the curves for analysis. For example, when analyzing the slope for samples that have been titrated with a drug that inhibits migration, it is possible that the linearity of the migration/healing phase could vary as a function of drug concentration. In this instance, carefully select the time range for slope analysis so all the samples display curve structures that are qualitatively similar. In other words, it would not be appropriate to choose a time range where some samples are perfectly linear and other samples are nonlinear. If it is not possible to satisfy this requirement, then an alternative means of quantification should be employed, see **"Area under the curve"** on Page 22.

Area under the curve

Determine the area under the curve (AUC) for a specific time range within the migration/healing phase. For specific instructions on how to calculate AUC directly within the xCELLigence software, please see the RTCA Software Pro manual. **Figure 8** is an example using HT1080 cells, where the actin polymerization inhibitor cytochalasin D inhibits the rate of wound closure in a dose-dependent manner. The extent of this inhibition has been quantified by calculating AUC for a time range that spans from 21 hours (immediately after the scratch was generated) to 32 hours. The time range that is used for AUC calculations is arbitrary, but typically spans from the moment the scratch wound has been generated until a time where the fastest healing sample has recovered to a Cell Index value that is <50% of what the Cell Index value was immediately before scratching. In **Figure 8** the AUC values are listed, but these can be plotted as a function of drug concentration to produce a dose response curve (see **“Time required to reach a particular Cell Index value”** on Page 23).

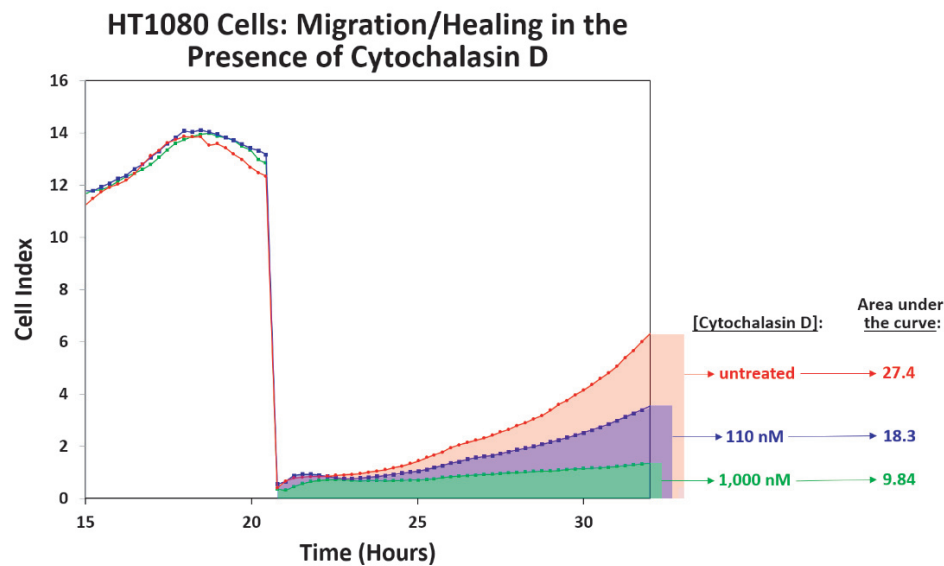


Figure 8. Quantifying migration/healing using area under the curve.

Time required to reach a particular Cell Index value

Users can determine how long it takes for the impedance signal to increase back to a particular value postscratching. The actual chosen value is arbitrary, but is typically selected as <50% of the Cell Index value immediately before scratching. In some cases, for all samples to be analyzed, a Cell Index much lower than the <50% of the Cell Index value must be chosen before scratching.

Figure 9 highlights where migration/healing of HT1080 cells is inhibited in a dose-dependent manner by the actin polymerization inhibitor cytochalasin D.

Immediately before scratching, the impedance signal has a value of approximately 13. The red horizontal dashed line in **Figure 9** denotes how long it takes for each scratched sample to recover to half of this value, Cell Index = 6.5. The 330 nM (pink), 1,000 nM (lime green), and 3,000 nM (yellow) samples would not be evaluable (they do not reach a Cell Index of 6.5 within this time window). By lowering the threshold to Cell Index = 2 (denoted by the black horizontal dashed line in **Figure 9**), the 330 and 1,000 nM samples can be included in the analysis, but the 3,000 nM sample is still excluded. While the threshold could be lowered even further to include the 3,000 nM sample, this would confine the analysis to a short time window after scratching. The disadvantage of a short time window is that at early time points after scratching, the samples are still tightly clustered and have not yet demonstrated their dose-dependent differences in migration/healing.

Analyzing the time required for the Cell Index signal to recover to a particular value can be very useful in some contexts, **Figure 9** highlights a potential challenge associated with this method.

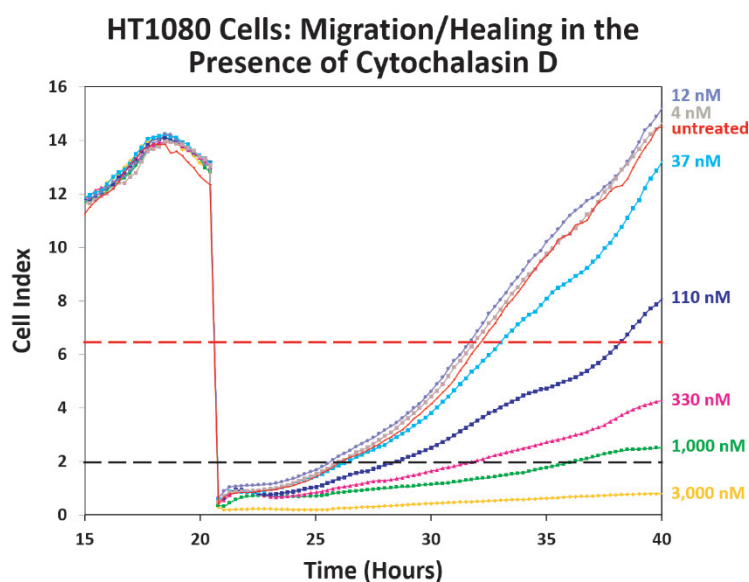


Figure 9. Analyzing how long it takes for HT1080 cells to migrate/heal in the presence of the inhibitor cytochalasin D.

Comparing the three data analysis methods

Although one approach might be easier to apply in some contexts, the three data analysis methods described will provide similar results. To illustrate this, the same data set from HT1080 cells treated with different concentrations of cytochalasin D, was analyzed using all three methods. The plots in **Figure 10** show the concentration-dependent effect that cytochalasin D has on slope (A), area under the curve (B), and the time required to reach a Cell Index of 2 (C). There are two points of interest in plot C. First, since the sample treated with 3,000 nM cytochalasin D never reached a Cell Index of 2, it could not be included in this analysis (see **“Time required to reach a particular Cell Index value”** on Page 23). Second, to facilitate visual comparison of the three different analysis methods, plot D is the reciprocal of plot C. When the data for all three analysis methods are plotted together (E), it is clear that they are qualitatively similar.

When data from the analysis methods is fitted to the four-parameter logistic dose-response equation, it yields IC₅₀ values of 102 nM (slope), 185 nM (area under curve), and 372 nM (time to reach Cell Index = 2). These values compare to the range of 130 to 380 nM that has been reported in the literature.

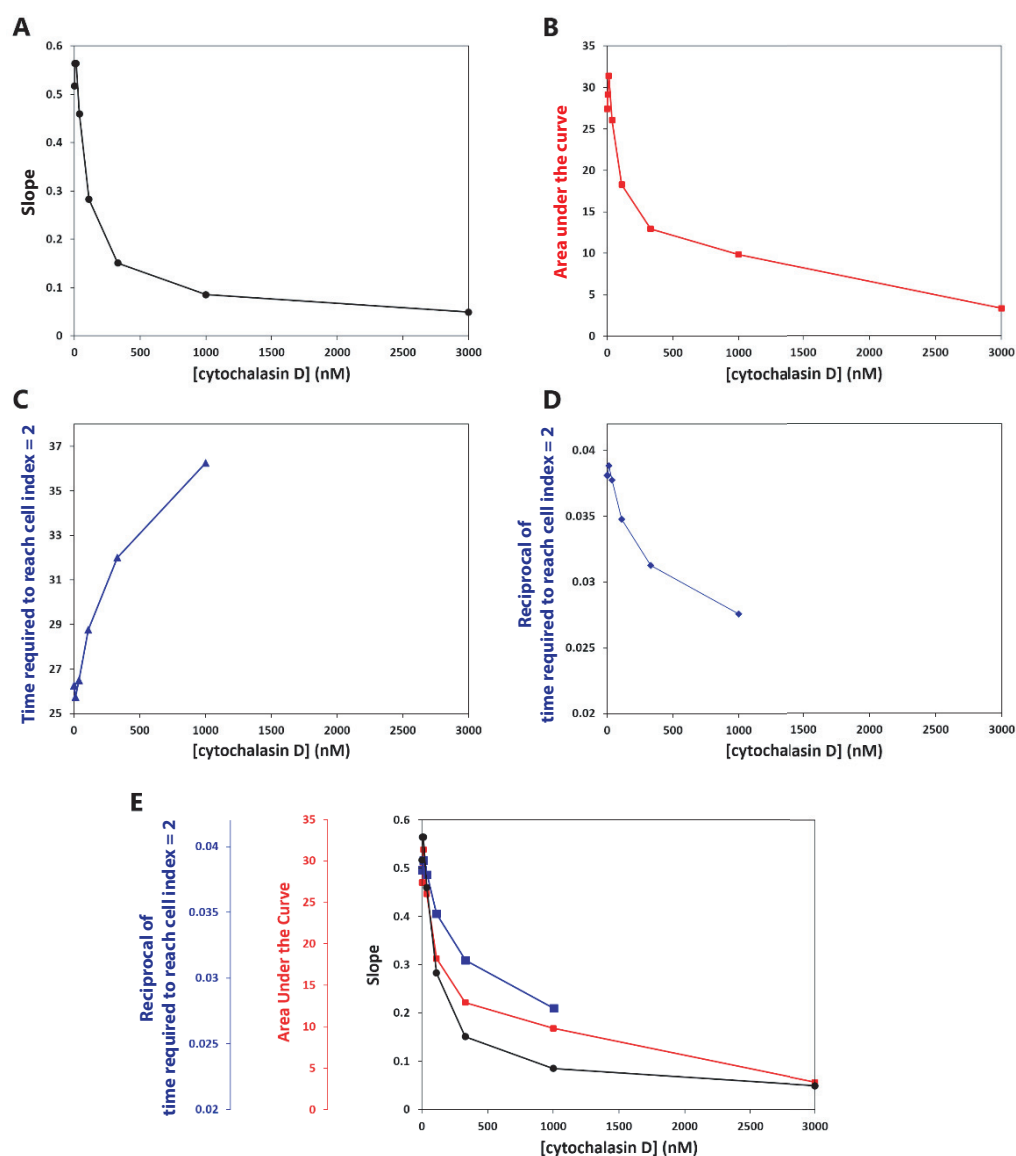


Figure 10. Comparing data from all three analysis methods. Dose-response curves are shown for cytochalasin D inhibition of HT1080 cell migration/healing.

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