Cell Culture

MCF-7, MDA-MB231, MDA-MB468, T47D, and HEK 293T cells were cultured in DMEM medium supplemented with 10% FBS and 1% penicillin/streptomycin. HCC-1806 cells were cultured in RPMI-1640 medium with similar supplements, while MCF10A cells required a specialized commercial medium. All cells were maintained in a CO2 incubator at 37°C with over 90% humidity. Cell experiments were conducted in a sterile, toxin-free biosafety cabinet or ultra-clean bench to ensure cleanliness, dust-free environment, and dryness, thereby preventing contamination.

Cell Recovery

Disinfect the biosafety cabinet for 30 minutes, and turn on the thermostatic sterilized water bath. Preheat the medium to be used.

Take out the frozen cells from the -80°C ultra-low temperature freezer or liquid nitrogen, and immediately place them in a 37°C thermostatic sterilized water bath. Gently shake the cryovial to accelerate the thawing process, ensuring the thawing time does not exceed 1 minute.

Add 500 µL of complete medium to the cryovial, mix thoroughly by pipetting, and centrifuge at room temperature at 800 rpm for 5 minutes.

Discard the supernatant, gently resuspend the cells in 1 mL of complete medium, and transfer the cells to a 6 cm culture dish containing 4 mL of medium. Incubate at 37°C in a CO2 incubator.

After 12 hours, replace the medium, and perform cell passaging on the second day.

Cell Passage

Disinfect the biosafety cabinet for 30 minutes, and turn on the thermostatic sterilized water bath. Preheat the medium to be used.

When the cells in the culture dish reach 85% confluence, completely aspirate the medium and wash once with PBS.

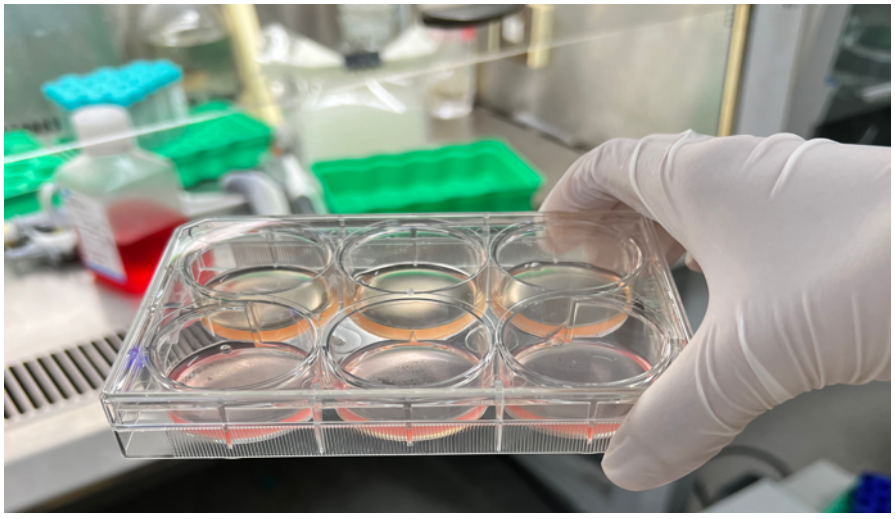
Thoroughly aspirate the remaining PBS, add an appropriate amount of trypsin, and gently shake to cover all the cells. Digest in a 37°C CO2 incubator.

Under the microscope, when the edges of the digested cells appear transparent, the cells become round, and some cells detach, add complete medium to stop the digestion. Gently pipette the cells to detach most of them from the dish and transfer them to a centrifuge tube.

Centrifuge at room temperature at 800 rpm for 3 minutes.

Completely aspirate the supernatant, add an appropriate amount of complete medium, gently pipette to create a single-cell suspension, and transfer the cells to a culture dish with complete medium. Incubate at 37°C in a CO2 incubator.

Replace the medium after 12 hours.

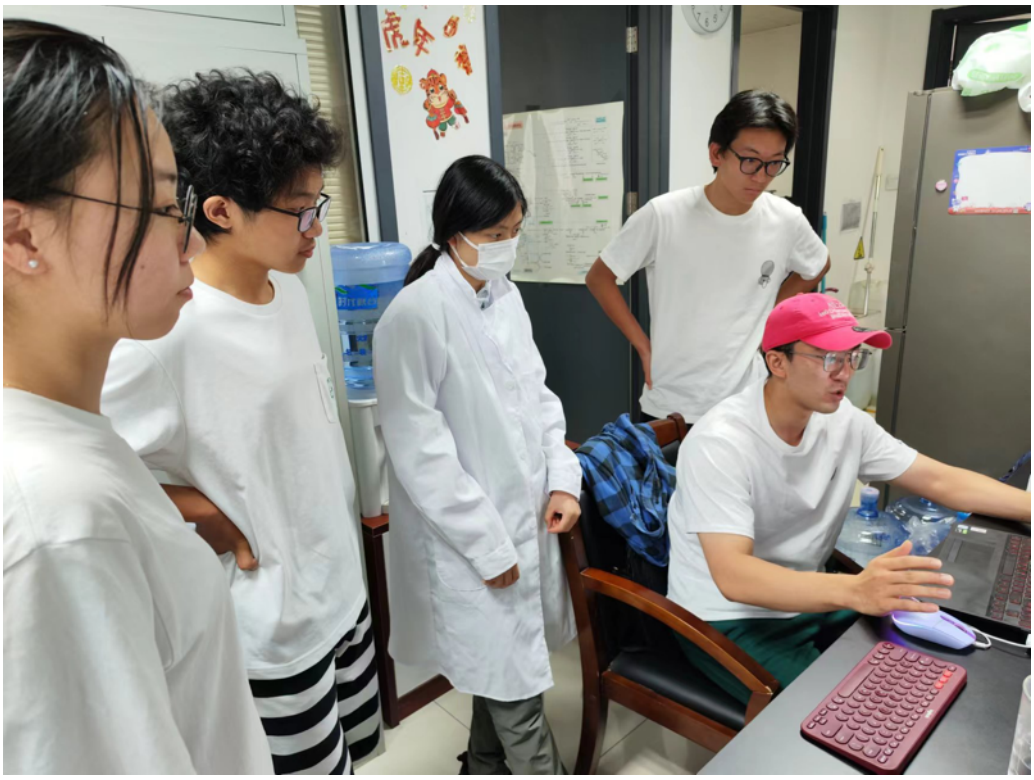


Cryopreservation of Cells

Disinfect the biosafety cabinet for 30 minutes, and turn on the thermostatic sterilized water bath. Preheat the medium to be used.

Repeat cell passaging steps (2) to (5).

Thoroughly aspirate the supernatant, add 1 mL of commercial serum-free cell freezing solution or laboratory-prepared cell freezing solution. Gently pipette to create a single-cell suspension. Transfer the cell suspension to cryovials and place them in a gradient cooling freezing box filled with isopropanol. Store overnight in a -80°C ultra-low temperature freezer, then transfer to a liquid nitrogen tank for long-term storage the next day.



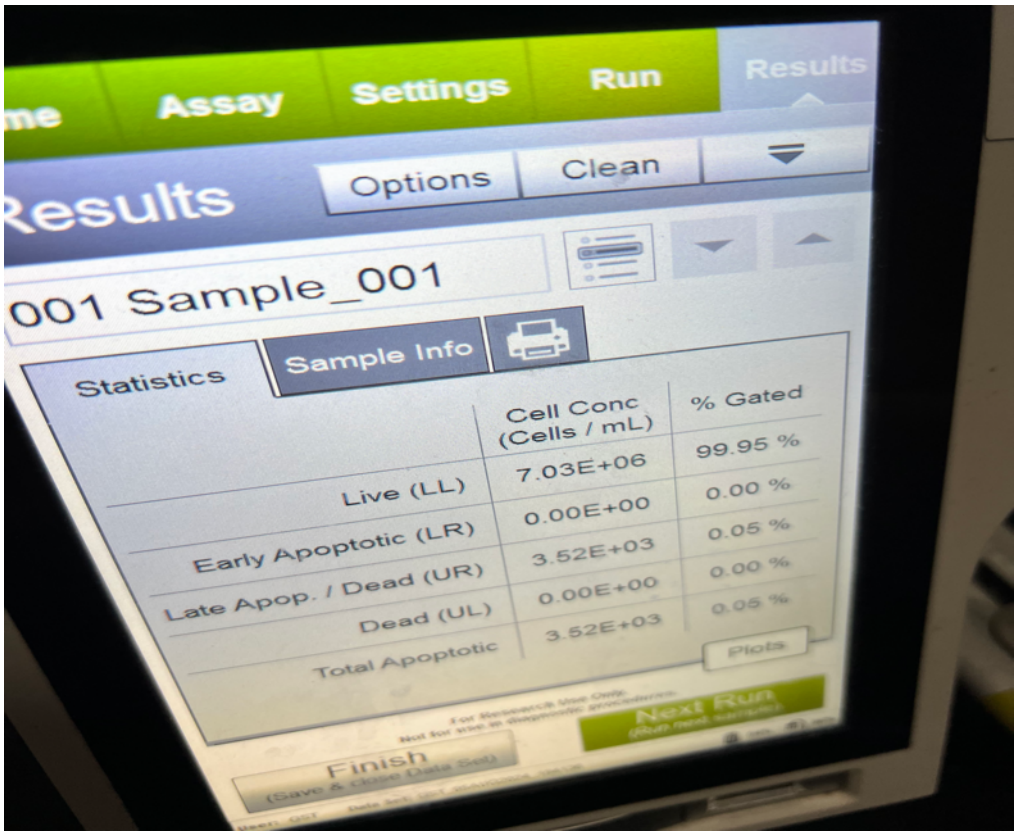
Cell Counting

Disinfect the biosafety cabinet for 30 minutes, and turn on the thermostatic sterilized water bath. Preheat the medium to be used.

Repeat cell passaging steps (2) to (5).

Resuspend the digested cells in 1 mL of complete medium, gently pipette to mix and create a single-cell suspension. Take 50 µL of the cell suspension and add it to a 1.5 mL EP tube containing 950 µL of PBS, then gently pipette to mix.

Vortex to mix thoroughly, then use a small flow cytometer (Muse Cell Analyzer, Luminex Cooperation) to perform cell counting, with a dilution factor of 20x.



Protein Extraction

Use an ice box to pre-cool the NP-40 lysis buffer and PBS needed for the experiment, as well as the protease inhibitor at room temperature.

Aspirate the culture medium from the cultured cells and wash three times with pre-cooled PBS.

Prepare the lysis buffer containing proteinase inhibitors, and add it to the culture dish according to the cell quantity. Lyse the cells on ice for 15 minutes.

Use a cell scraper to detach the cells, transfer the cell lysate to a 1.5 mL EP tube, and sonicate at 30% power for 5 seconds, then pause for 5 seconds, repeating for a total of 5 cycles to achieve complete lysis.

Pre-cool the centrifuge to 4°C and centrifuge at 12,000 g for 15 minutes. Collect the supernatant and measure the protein concentration.

BCA Method for Determining Protein Concentration

1.Using the BCA protein quantification reagent, calculate the number of samples and the volume of working solution needed. Mix BCA Reagent A and Reagent B in a 50:1 ratio, which means 50 volumes of Reagent A to 1 volume of Reagent B. For example, mix 10 mL of Reagent A with 200 µL of Reagent B to prepare 10.2 mL of BCA working solution. The BCA working solution should be prepared fresh and not stored overnight.

2.Add standard samples of 0, 1, 2, 4, 8, 12, 16, and 20 µL into the wells of a 96-well plate to prepare a standard curve for protein. Add the corresponding protein lysate to each well to make the total volume 20 µL in each well. At this point, the protein concentrations in each well will be 0, 0.025, 0.05, 0.1, 0.2, 0.3, 0.4, and 0.5 mg/mL, respectively.

3.Add an appropriate amount of the protein sample to be tested into the wells of the 96-well plate, with each protein having three replicates. Add the corresponding protein lysate to make the total volume 20 µL in each well.

4.Add 200 µL of BCA working solution to the standard curve wells and the sample wells, and incubate at 37°C for 30 minutes (if the color is too light, the incubation can be extended to 60

minutes; conversely, if the color is too dark, reduce the incubation time to 20 minutes).

5.Measure the absorbance at 562 nm using an enzyme marker. Plot the standard curve of absorbance versus protein concentration, calculate the goodness of fit (R^2 value). An R^2 value greater than 0.99 indicates that the curve is acceptable. Compare the absorbance of the sample to the standard curve to determine the protein concentration of the sample.

Preparing SDS-PAGE Gel

Wash the glass plates (both long and short) with distilled water, then rinse with double-deionized water, and place them in a 55°C drying oven. Assemble the glass plates, secure them in the gel casting frame, and after confirming proper alignment, prepare the gel solution

Calculate the total volume based on 8 mL of gel solution for each plate. Prepare the lower separating gel and pour the gel solution into the glass plates. Gently and evenly add anhydrous ethanol sealing gel, removing any bubbles generated during the pouring process while leveling the liquid surface.

After allowing the lower gel to set at room temperature for 30 minutes and observing a clear interface between the separating gel and ethanol, pour out the excess anhydrous ethanol and thoroughly absorb the remaining ethanol with absorbent paper.

Once the anhydrous ethanol is completely air-dried, add the upper concentrated gel. Simultaneously, slowly insert the comb along one side to avoid generating bubbles.

After allowing the upper gel to set at room temperature for 30 minutes, the gel should be fully solidified. The prepared gel can be stored in the electrophoresis buffer at 4°C or used immediately.

SDS-PAGE Gel Electrophoresis

Prepare the electrophoresis tank, electrodes, buffer, and gel.

Assemble the gel and electrodes, then pour the buffer into the inner and outer tanks up to the marked line.

Slowly remove the comb and gently flush out any debris from the wells using a pipette tip.

Mix the protein sample with loading buffer, heat at 96°C for 10 minutes, and cool before loading the sample.

Run electrophoresis at 80V constant voltage for 30 minutes, ensuring the bromophenol blue bands fully enter the separating gel and the protein marker separates. Then switch to 120V constant voltage for 1 hour until the bromophenol blue bands reach the bottom of the gel.

After electrophoresis, proceed to either transfer or directly stain the gel with Coomassie Brilliant Blue or silver staining.

Semi-Dry/Wet Transfer Membrane Development

Prepare PVDF membrane, methanol, transfer buffer, ice box, and ice in advance.

Activate the PVDF membrane with methanol, then soak it in the transfer buffer.

Transfer conditions:

Wet transfer: constant voltage of 100 V for 90 minutes.

Semi-dry transfer: constant voltage of 25 V, limited to 1 A for 17 minutes.

After transfer, block the membrane with 5% BSA or 5% non-fat dry milk at room temperature for 1 hour.

Dilute the primary antibody and add it to the membrane after blocking. Incubate at room temperature for 2 hours or overnight at 4°C.

After primary antibody incubation, place the membrane on a shaker and wash it with TBS-T for 5 minutes, repeating the wash 4 times.

Coomassie Brilliant Blue Staining

Prepare Coomassie Brilliant Blue staining solution and destaining solution. Place the SDS-PAGE gel on a shaker and stain for 20 minutes.

After staining, recover the staining solution and destain using the Coomassie Brilliant Blue destaining solution. Change the destaining solution every hour until the bands are clear and the background is clean.

After destaining, take a photograph of the gel for analysis.

Agarose Gel Electrophoresis

Prepare agarose gel: Clean the agarose gel casting frame and a comb of appropriate dimensions. Assemble the casting frame. Take a clean 250 mL triangular glass bottle, weigh 1 g of agarose powder, pour it into the bottle, and add 1 × TAE buffer to a final volume of 100 mL, preparing a 1% agarose gel (the gel concentration can be adjusted between 0.8% and 1.2% based on the size of the DNA molecules).

Place the triangular bottle in a microwave and heat on high for 2 minutes. After removing it, add Goldview nucleic acid dye, gently shake the bottle to ensure even mixing, and pour the solution into the assembled casting frame.

Allow the agarose gel to sit in the dark at room temperature for 30 minutes until it solidifies. Remove the comb and prepare for electrophoresis.

Agarose gel electrophoresis: Prepare 2 L of 1 × TAE buffer, pour it into the horizontal electrophoresis tank, and place the gel into the tank. Gently tap the gel to expel any air bubbles from the sample wells. Mix the DNA samples with the loading buffer evenly, and slowly add it to the sample wells, avoiding turbulence that could cause the samples to overflow. After

loading, add a DNA marker to the wells on both sides of the samples.

Electrophorese at a constant voltage of 120 V for 30 minutes, or at 150 V for 20 minutes. Once electrophoresis is complete, place the gel into an ultraviolet imaging system for photography and analysis.

Plasmid Construction

To construct a plasmid, begin by disinfecting the biosafety cabinet for 30 minutes and preparing the PCR reaction system for target gene amplification.
This involves combining 1 μg of target gene fragment, 1 μL of forward primer (10 μM), 1 μL of reverse primer (10 μM), 25 μL of 2× Pfu PCR mix, and filling with double deionized water to 50 μL in a PCR tube. The PCR amplification follows this cycle repeated 35 times: 95°C for 10 minutes (pre-denaturation), 95°C for 30 seconds (denaturation), 58°C for 30 seconds (annealing), and 72°C for extension at 1000 bp/minute, with a final extension of 72°C for 5 minutes and indefinite storage at 4°C. For point mutation plasmids, additional primers (FM and RM) are required. Separate F-end and R-end fragment systems are prepared using the same PCR components, followed by fragment amplification and a bridging reaction repeated 8 times using similar PCR conditions.
After this, 1 μL of forward and reverse primers (10 μM) is added, and another PCR round is conducted. The resulting product, an amplified target gene fragment, can be analyzed by agarose gel electrophoresis or stored at -40°C.

DNA Purification / Gel Recovery

Start by sterilizing the ultraclean workstation for 30 minutes. Then, equilibrate the spin column by adding 500 µL of Buffer BL, allowing it to sit for 1 minute, followed by centrifugation at 12,000 rpm for 1 minute.

Discard the waste liquid and reuse the spin column the same day. Run agarose gel electrophoresis at 150 V for 20 minutes, cut out the target band under UV light, and minimize exposure. Place the gel piece (no more than 700 mg) into a 1.5 mL EP tube and add 1 µL of Buffer MB per 1 mg of gel. Shake at 55°C and 300 rpm for 10 minutes, flipping every 3 minutes to aid dissolution.

Once dissolved and cooled to room temperature, transfer the solution to the equilibrated spin column, centrifuge at 12,000 rpm for 1 minute, discard the waste liquid, and repeat for higher recovery efficiency. If the solution volume exceeds 900 µL, transfer it in batches. Wash the column with 600 µL of Buffer MW (ethanol added), centrifuge for 30 seconds, discard the waste, and repeat the wash step before air-drying for 4 minutes. Add 35 µL of pre-heated (55°C) Endo-Free Buffer EB to the column membrane, let it sit for 5 minutes, and centrifuge at 13,000 rpm for 2 minutes. Repeat the elution process, then measure DNA concentration using a NanoDrop spectrophotometer or store the sample at -40°C.

Digested Vector and Target Gene Fragment

To digest the amplified target gene fragments and vectors, prepare the digestion reaction mixture in a PCR tube by combining 1 µL of upstream primer F, 1 µL of downstream primer R, 5 µL of 10× CutSmart Buffer, 3 µg of vector, all recovered amplified fragments, and double deionized water up to 50 µL. Incubate the mixture in a 37°C water bath for 3 hours (or overnight if required by the restriction endonucleases).

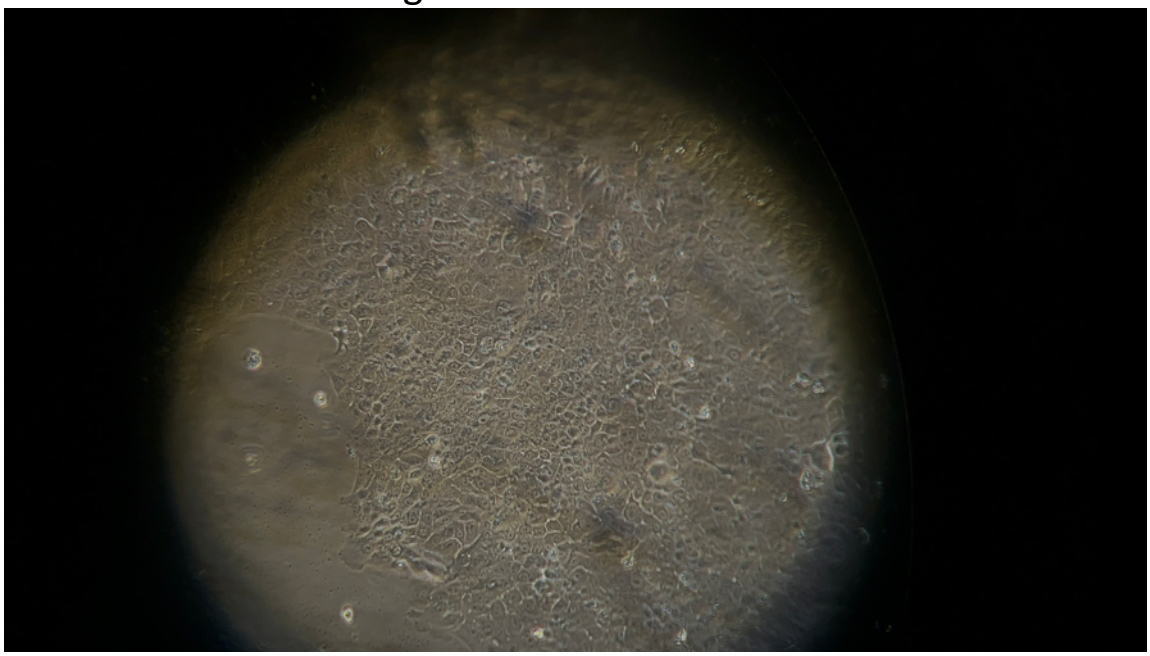
After digestion, perform agarose gel electrophoresis to recover the digested vector and target gene fragments.

Connection of Vector and Target Gene Fragment

After digestion, the vector and target gene fragments are ligated using T4 ligase, with incubation at room temperature for 2 hours or overnight at 16°C.
For sticky ends, a molar ratio of vector to target gene fragments of 1:7 is used to improve ligation efficiency. The ligation mixture is prepared by combining 80 ng of target gene fragments, 35 ng of vector, 1 µL of 10× T4 ligase buffer, 1 µL of T4 ligase, and double deionized water to a total volume of 10 µL.

Plasmid Transformation

Disinfect the ultra-clean bench for 30 minutes and prepare a foam box filled with ice.
Thaw competent cells from the -80°C freezer by placing them on ice for 5 minutes. For newly ligated plasmids, combine 15 µL of the ligation product with 35 µL of competent cells, gently mix by pipetting, and incubate on ice for 30 minutes. For mature plasmids, add 100 ng to 15 µL of competent cells and incubate on ice for the same duration.
Preheat a water bath to 42°C and perform a heat shock on the competent cells for 90 seconds, then return them to ice for 2 minutes. Add 500 µL of antibiotic-free LB medium to the heat-shocked cells, and for newly synthesized plasmids, incubate the mixture in a shaker at 220 rpm and 37°C for 1 hour. Choose the appropriate LB agar plates based on the plasmid resistance, plating 250 µL for newly synthesized plasmids and 100 µL for mature plasmids. Finally, incubate the plates at 37°C for 14 hours and observe the growth of bacterial colonies.



Protein Purification

To construct the protein expression plasmid for purification, transform the plasmid into BL21(DE3) competent cells, following the outlined plasmid transformation steps.
Select a single colony and grow the bacteria at 37°C for 8 hours, saving 500 µL of the culture while collecting 500 µL for sampling. Induce 1 mL of the culture with 0.5 mM IPTG (or an optimized gradient concentration) at 18°C for 20 hours, then run the collected bacterial protein on SDS-PAGE and stain with Coomassie Brilliant Blue to check for successful induction.
If successful, grow the selected colonies overnight at 37°C in two 50 mL centrifuge tubes to expand the culture to 80 mL, then add this to 1 L of medium and grow at 37°C for 6 hours. Induce with IPTG at a final concentration of 0.5 mM when the culture cools to 18°C, and incubate for 20 hours. Centrifuge at 12,000 rpm for 2 minutes to collect the cells, then resuspend the bacterial culture in Protein Purification Buffer A (final volume under 50 mL) with protease inhibitors, using an ultrasonic disruptor (5 seconds on, 3 seconds off, power at 55%, for 99 cycles) to lyse the cells. Centrifuge at 18,000 rpm at 4°C for 20 minutes and pour the supernatant into a new centrifuge tube, repeating the centrifugation step once more. Filter the supernatant through a 0.22 µM filter, then use an AKTA avant system with a GST column for protein purification, washing and eluting with Protein Purification Buffer B. Concentrate the eluted protein using an Amicon® Ultra filter (10 kDa MWCO) by centrifuging at 4,000 rpm at 4°C until the desired volume is achieved.
Measure the protein concentration with a NanoDrop spectrophotometer, mix with 30% glycerol, and store at -80°C in an ultra-low temperature freezer.

Medicine Screening

We conducted high-throughput drug screening in our lab using a PRCXI SC9210 fully automated liquid handling workstation, with a technician facilitating the process.
The compounds screened were sourced from the MCE novel known active compound library, which contains 1220 compounds, all dissolved in either 100% DMSO or H2O to prepare a stock solution at a concentration of 1 mM. The cell lines used included MCF-10A normal breast epithelial cells and the MDA-MB-231 triple-negative breast cancer cell line, seeded in 96-well culture plates at a concentration of approximately 5000 cells per well, totaling 100 µL per well. After allowing 24 hours for cell adherence, 1 µL of each compound was added to the culture medium using the automated platform, resulting in a final concentration of 10 µM. The experimental controls included a negative control of pure DMSO at 10 µM and a positive control of cisplatin at 10 µM. After 48 hours of drug treatment, the technician removed the culture medium, added ATP detection reagents, and performed luminance detection with a microplate reader or ATP probe effect detection using a Revvity high-content imaging system.