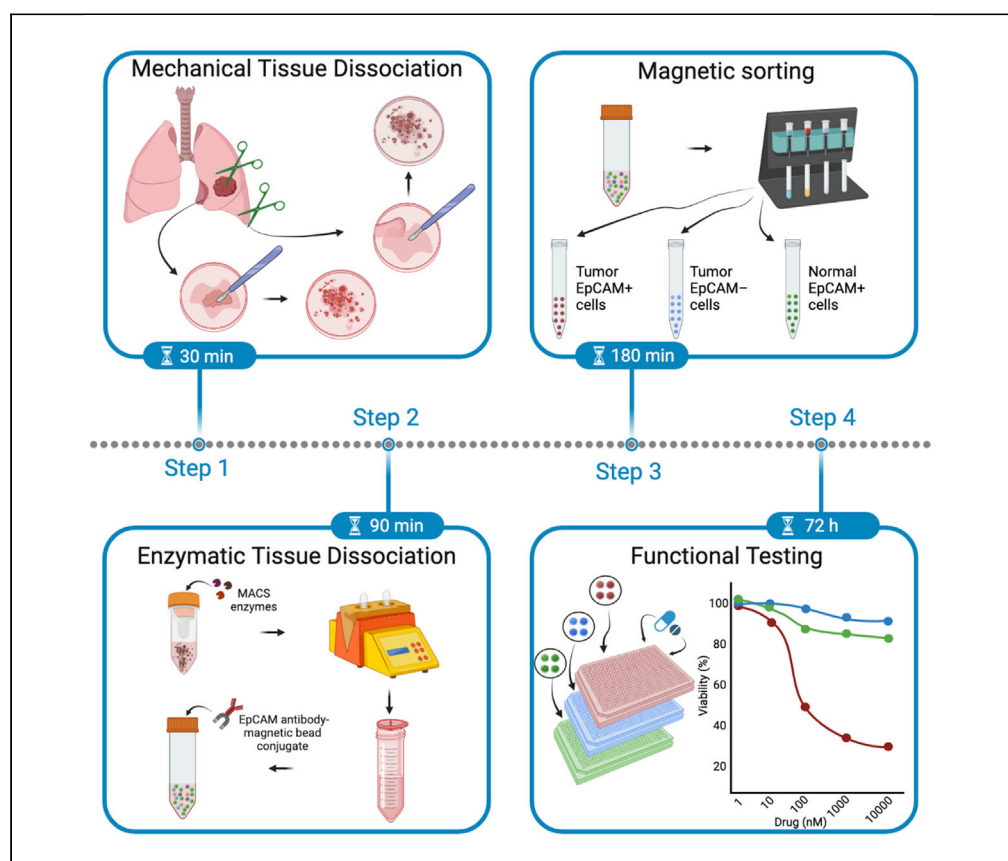


Protocol

Protocol to utilize fresh uncultured human lung tumor cells for personalized functional diagnostics



Drug sensitivity data acquired from solid tumor-derived cultures are often unsuitable for personalized treatment guidance due to the lengthy turnaround time. Here, we present a protocol for determining *ex vivo* drug sensitivities using fresh uncultured human lung tumor-derived EpCAM⁺ epithelial cells (FUTCs). We describe steps for drug testing in FUTCs to identify tumor cell-selective single or combination therapy in 72 h of sample processing. The FUTC-based approach can also be used to predict *in vivo* resistance to known targeted therapies.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Fresh uncultured tumor-derived cells (FUTCs) can be used for *ex vivo* drug testing

FUTC drug profiling provides diagnostic results within three days of sample collection

2,500 cells/per well suffice to provide robust drug response data

Profiling of matched tumor and normal cells pinpoints cancer-selective treatments

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Protocol

Protocol to utilize fresh uncultured human lung tumor cells for personalized functional diagnostics

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SUMMARY

Drug sensitivity data acquired from solid tumor-derived cultures are often unsuitable for personalized treatment guidance due to the lengthy turnaround time. Here, we present a protocol for determining *ex vivo* drug sensitivities using fresh uncultured human lung tumor-derived EpCAM⁺ epithelial cells (FUTCs). We describe steps for drug testing in FUTCs to identify tumor cell-selective single or combination therapy in 72 h of sample processing. The FUTC-based approach can also be used to predict *in vivo* resistance to known targeted therapies. For complete details on the use and execution of this protocol, please refer to Talwelkar et al. (2021).

BEFORE YOU BEGIN

Drug sensitivity testing on live cancer cells collected from patients provides individualized information that can be used to guide treatment decisions. The clinical value of this strategy is determined by the speed with which results are returned to the clinician. Approaches such as patient-derived 2D cultures, organoids, or xenografts are clinically less viable in this scenario due to their poor “take rate” and protracted establishing time (Letai et al., 2022). We propose a simple and direct assay for determining therapy options for individual lung cancer patients by using fresh uncultured tumor-derived EpCAM⁺ epithelial cells (FUTCs) for immediate drug sensitivity testing.

Institutional permissions

The patient’s informed consent must be obtained from the relevant ethical bodies in order to use clinical samples for research. For the present study, ethical permissions were approved by the Helsinki University Central Hospital (HUCH) and all the procedures were conducted in accordance with protocol approved by the Coordinating Ethics Committee of the University of Helsinki (License numbers: 85/13/03/00/2015 and HUS-1204-2019).

General preparations

1. Autoclave non-sterile equipment, e.g., pipettes, pipette tips, glass petri dishes, etc. listed in the [key resources table](#) (KRT).
2. Prepare and warm the required media ahead of time.



3. Check the safety data sheets (SDS) for all chemicals in the procedure and wear the appropriate personal protective equipment (PPE).

Preparation of stock solutions for F-medium

⌚ Timing: 1–2 h

4. Adenine stock solution (2.4 mg/mL).
 - a. Weigh 120 mg of adenine and dissolve it in 45 mL of 0.5 M HCl solution using a magnetic stirrer.
 - b. If the solution does not become clear, add a few drops of concentrated HCl.
 - c. Filter sterilize using a 0.22 μ m filter and aliquot to appropriate (5 mL) volumes.
 - d. Store at -20°C for up to six months.
5. Insulin stock solution (4 mg/mL).
 - a. Dissolve 50 mg of insulin in 2.5 mL of 0.5 mM HCl for 1 h using a magnetic stirrer.
 - b. Add 10 mL of sterile MQ water. If the solution does not clear, reduce the pH of the solution.
 - c. Filter sterilize using a 0.22 μ m filter and aliquot in sterile tubes. Aliquots of 500 μ L are useful as these can be used to prepare 500 mL of F-medium. Avoid repeated freeze-thaw cycles.
 - d. Store at -20°C for up to six months.

⚠ **CRITICAL:** Insulin dissolves when the pH of the solution is around 2.5–3; if the solution does not become clear, reduce the pH with HCl.

6. Rho kinase inhibitor (Y-27632) stock solution (10 mM).
 - a. Dissolve 5 mg of Y-27632 in 1.5 mL of sterile MQ water.
 - b. Aliquot in sterile tubes. Aliquots of 500 μ L are useful as these can be used to prepare 500 mL of F-medium. Avoid repeated freeze-thaw cycles.
 - c. Store at -20°C for up to one month.
7. Human recombinant (hr) EGF stock solution (0.1 mg/mL).
 - a. To dissolve the EGF, add 1 mL of sterile MQ water to the EGF vial.
 - b. Pipette up and down to ensure complete dissolution.
 - c. Aliquot in sterile tubes. Aliquots of 50 μ L are useful as these can be used to prepare 500 mL of F-medium. Avoid repeated freeze-thaw cycles.
 - d. Store at -20°C for up to 3 months.
8. Cholera toxin stock solution (0.5 mg/mL).
 - a. Add 2 mL of sterile MQ water into the tube with 1 mg of cholera toxin.
 - b. Pipette up and down to ensure complete dissolution.
 - c. Aliquot in sterile tubes (250 μ L per tube).
 - d. Store at 4°C .

⚠ **CRITICAL:** Cholera toxin is highly toxic following inhalation, oral consumption, or dermal exposure and should be handled with extreme caution.

9. Heat inactivated FBS.
 - a. Heat inactivate the FBS at 56°C for 30 min.
 - b. Aliquot and store at -20°C . Aliquots of 25 mL are useful as these can be used to prepare 500 mL of F-medium. Avoid repeated freeze-thaw cycles.
10. EDTA-BSA Buffer

Reagent	Final concentration	Amount
BSA	0.5%	2.5 g
EDTA	2 mM	292 mg
PBS	1 x	500 mL

Dissolve the solution using a magnetic stirrer. Pass the solution through a 0.22 μm filter and store at 4°C–8°C for up to 2 weeks.

Alternatives: Other supplements can be used instead of EDTA, such as anticoagulant citrate dextrose formula-A (ACD-A) or citrate phosphate dextrose (CPD). In place of BSA, other proteins such as human serum albumin, human serum, or fetal bovine serum can be used (FBS). Also, commercial buffers such as MACS BSA Stock Solution (#130-091-376) and autoMACS™ Rinsing Solution can be used instead of EDTA-BSA buffer.

⚠ **CRITICAL:** Do not use PBS containing Ca^{2+} or Mg^{2+} .

⚠ **CRITICAL:** All media stocks and solutions should be sterile or sterile-filtered before use.

Preparation of F-medium

⌚ **Timing:** 1–2 h

11. Prepare the F-medium in a sterile bottle by adding the medium components listed in the [materials and equipment](#) section below.

⚠ **CRITICAL:** Reagent stocks, media, solutions, and samples should be processed in a biosafety cabinet class II and precautions should be used when handling human tissue and chemicals.

Preparation of MACS tumor tissue dissociation reagents

⌚ **Timing:** 15 min

12. Enzyme H.
 - a. Reconstitute the Enzyme H lyophilized powder in each vial with 3 mL of DMEM.
 - b. To avoid repeated freeze-thaw cycles, prepare aliquots of appropriate volume.
 - c. Aliquots can be stored at –20°C for up to six months.
13. Enzyme R.
 - a. Reconstitute the Enzyme R lyophilized powder in the vial with 2.7 mL of DMEM.
 - b. To avoid repeated freeze-thaw cycles, prepare aliquots of appropriate volume.
 - c. Aliquots can be stored at –20°C for up to six months.

Note: Make sure to thoroughly mix the suspension before use.

14. Enzyme A.
 - a. Reconstitute the Enzyme A lyophilized powder in the vial with 1 mL of DMEM.
 - b. To avoid repeated freeze-thaw cycles, prepare aliquots of appropriate volume.
 - c. Aliquots can be stored at –20°C for up to six months.

Alternatives: To reconstitute the Enzymes R, H, or A, RPMI 1640 medium could be used instead of DMEM.

Note: Medium used for reconstitution of enzymes should not contain FBS or antibiotic-antimycotic.

Preparation of drug plates and equipment

⌚ **Timing:** 30 min

15. To screen cancer-selective drugs of interest, prepare drug sensitivity and resistance testing (DSRT) plates in advance by dispensing compounds into 384-well plates using an Echo 550 liquid handler, at five concentrations covering a 10,000-fold concentration range.
 - a. Compounds dissolved in DMSO have a final volume of 2.5 nL or 25 nL per well, whereas compounds dissolved in aqueous have a final volume of 25 nL or 250 nL per well of a 384-well plate. The strategy presented in the following table could be used to transfer drug volumes from stock plate to DSRT plates.

Final values in 384 well DSRT plate		Transfer condition for DMSO drugs		Transfer condition for aqueous drugs	
Final concentration (nM)	Total volume (μl)	Stock plate (μM)	Transfer volume (nL)	Stock plate (μM)	Transfer volume (nL)
10,000	25	10,000	25	1,000	250
1,000	25	1,000	25	1,000	25
100	25	1,000	2.5	100	25
10	25	10	25	10	25
1	25	10	2.5	1	25

- b. Use 0.1% DMSO and 100 μM benzethonium chloride as negative and positive controls, respectively.

Note: To get a better understanding of data uniformity, positive and negative controls should be scattered across the plate. Pressurized storage pods mentioned in the [key resources table](#) can be used to store drug plates for up to one month at room temperature (20°C).

Preparation before sample collection and processing

⌚ Timing: 2 h

16. Fill an ice bucket with ice for sample transportation.
17. Add 1% penicillin and streptomycin to DMEM or RPMI 1640.
18. Prepare complete F-medium according to the list in the [key resources table](#).
19. Prepare 1 × PBS solution.
20. Prepare fixative to fix tissue samples. To make 300 mL of fixative, measure 270 mL of 1 × PBS and 30 mL of 37%–41% formaldehyde.

⚠ **CRITICAL:** Chemical fume hoods should be used for all procedures involving formaldehyde processing.

21. Prepare 1.5 mL reaction tubes containing 1 mL of RNAlater solution, for storage of tissue samples for RNA isolation.
22. Collect liquid nitrogen into a safety tank for the snap freezing of DNA and protein samples.

⚠ **CRITICAL:** Only trained individuals should handle liquid nitrogen. To avoid asphyxiation risks, liquid nitrogen should only be handled in well-ventilated spaces with the necessary safety gear. Liquid nitrogen exposure can result in frostbite.

23. Before you begin, ensure that the following items are readily available.
 - a. Ice-cold HBSS.
 - b. Sterile 1.5 mL tubes.
 - c. MACS-C tubes.
 - d. Razor blades/scalpel.

- e. Falcon tubes, both 15 mL and 50 mL.
- f. Histology cassettes and a suitable marker pen or pencil.
- g. 70 μ m cell strainer.
- h. CryoTube vials, 2 mL.
- i. Incubator (37°C) with a rotator.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Patient-derived lung cancer tissue and tumor-adjacent normal healthy tissue (age range: 25–82, median age: 66, male: 7, female: 10)	Helsinki University Central Hospital	NA
Chemicals, peptides, and recombinant proteins		
DMEM medium	Gibco	11965084
RPMI 1640 medium	Life Technologies	21875034
F12 medium	Life Technologies	21765029
Fetal Bovine Serum (FBS)	Gibco	10270106
Penicillin-Streptomycin (10,000 U/mL)	Gibco	15140122
HBSS	Sigma	H6648
Phosphate buffered saline (PBS)	Lonza	BE17-517Q
Ethanol absolute \geq 99.8%	VWR Chemicals	20821.365
DMSO	Thermo Fisher Scientific	327182500
EDTA	Sigma	EDS-500G
BSA	Sigma	A2153
Adenine	Sigma	A8626
Insulin	Sigma	I2643
Y-27632 (Rho kinase inhibitor)	MedChemExpress	HY-10583
hrEGF	Corning	354052
Hydrocortisone	Sigma	H4001
Cholera Toxin	List Biological laboratory	100B
Red Blood Cell lysis buffer	Sigma	11814389001
EpCAM (CD326) MicroBeads	Miltenyi	130-061-101
RNAlater solution	Thermo Fisher Scientific	AM7021
Formaldehyde (37%–41%)	Fisher Chemical	F/1501/PB15
Critical commercial assays		
MACS Human Tumor Dissociation Kit (contains enzymes H, R and A)	Miltenyi	130-095-929
CellTiter-Glo Cell Viability Assay	Promega	G9681
Software and algorithms		
BREEZE	FIMM / University of Helsinki	http://dsrt.fimm.fi
GraphPad Prism	GraphPad Software Inc.	N/A
Microsoft Excel	Microsoft Corporation	N/A
Other		
GentleMACS C Tube	Miltenyi	130-093-237
MACS LS column	Miltenyi	130-042-401
MACS dissociator	Miltenyi	130-093-235
MACS magnet	Miltenyi	130-042-501
OctoMACS separator	Miltenyi	130-042-109
SuperMACS II separator	Miltenyi	130-044-104
MACSMix Tube Rotator	Miltenyi	N/A
Echo 550 Liquid Handler	Labcyte	N/A

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
0.22 μ m filter	Fisherbrand	15206869
70 μ m strainer	Falcon	4708389
33 mm syringe filter	Fisherbrand	15206869
Scalpel	Fisher	O501/11798343
384-well plate	Corning	3712
1.5 mL eppendorf tubes	Sarstedt	72.706
50 mL conical tubes	Greiner	227261
15 mL conical tubes	VWR	VWRI525-1068
25 mL serological pipette	VWR	VWRI612-3698
10 mL serological pipette	VWR	VWRI612-3700
5 mL serological pipette	VWR	VWRI612-3702
Cryo tubes	Greiner	V3135-500EA
Glass petri dish	Thermo Fisher Scientific	11750844
Histo cassette	VWR	720-2233
Pressurized Storage Pods	Roylan Developments Ltd	N/A
Biotek MultiFlo FX RAD (MultiFlo)	BioTek	N/A
PheraStar FS plate reader	BMG LABTECH	N/A
Multi-channel pipette	Sartorius	BH-PLP8C
Drugs and benzethonium chloride	FIMM drug library	https://www2.helsinki.fi/en/infrastructures/drug-discovery-chemical-biology-and-screening/infrastructures/fimm-high-throughput-biomedicine-unit

MATERIALS AND EQUIPMENT

Reagents for F-medium preparation

Reagent	Stock concentration	Final concentration	Volume for 500 mL F-medium
Adenine	2.4 mg/mL	24 μ g/mL	5 mL
Insulin	4 mg/mL	5 μ g/mL	625 μ L
Y-27632	10 mM	10 μ M	500 μ L
hrEGF	0.1 mg/mL	10 ng/mL	50 μ L
Hydrocortisone	1 mg/mL	0.4 μ g/mL	200 μ L
Cholera toxin	0.5 mg/mL	10 ng/mL	10 μ L
FBS	n/a	5%	25 mL
Penicillin-Streptomycin	10,000 U/mL	1%	5 mL
F12 medium	N/A	N/A	348 mL
DMEM medium	N/A	N/A	116 mL

△ CRITICAL: Cholera toxin is highly toxic when ingested, inhaled or upon dermal exposure, and should be handled with extra care.

Note: All stock solution reagents required for F-media preparation should be stored at -20°C for up to six months, except cholera toxin, DMEM, F12, and F-media are stored at 4°C for up to two months.

STEP-BY-STEP METHOD DETAILS

This protocol begins with the collection of tumor and normal lung tissue samples, which are then mechanically and enzymatically digested to prepare a single cell suspension. The mixture of cells originating from lung tissue is then further fractionated based on the presence of the epithelial marker epithelial cell adhesion molecule (EpCAM), also known as CD326. Both healthy and cancerous epithelial cells express EpCAM. In non-small cell lung cancer (NSCLC), 40%–85% of adenocarcinomas (ACs) and 85%–98% of squamous cell carcinomas (SCCs) are reported to be EpCAM⁺ (Went et al., 2004; Kim et al., 2009).

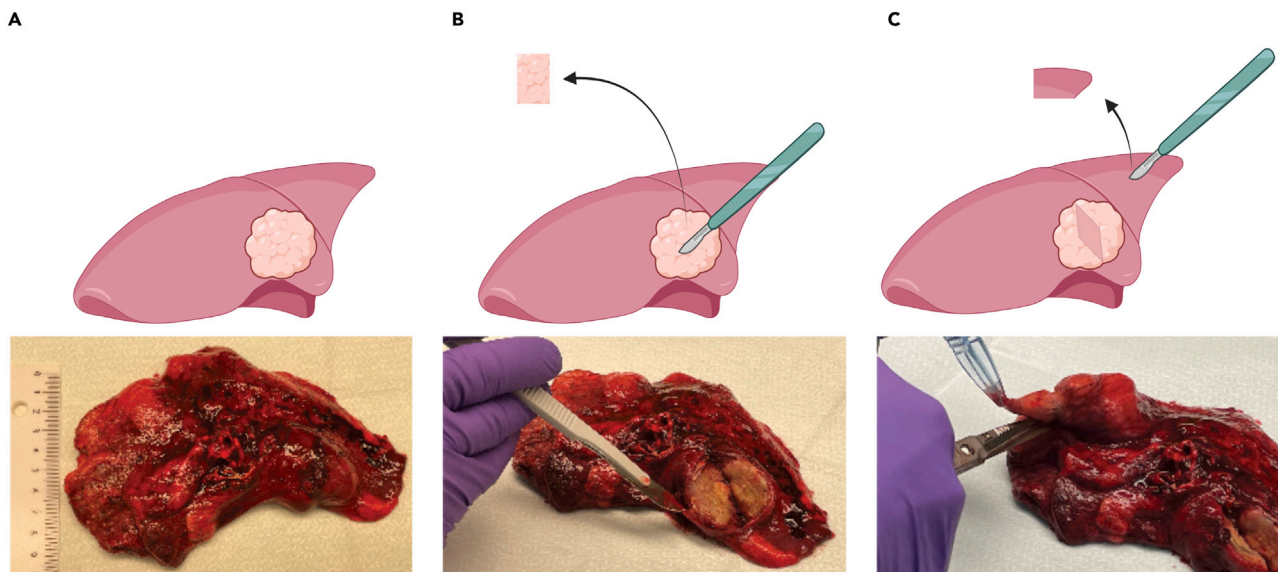


Figure 1. Dissection of tumor and normal tissue

(A) Surgically removed tumor-bearing lung lobe.

(B) The pathologist cuts the tumor site in the middle and removes a 3–4 cm section of the tumor for the FUTC processing. Reserve 1–3 cm³ of tissue pieces for the FUTC assay.

(C) Normal tissue is sampled from the same lung lobe, from a region adjacent to the tumor.

Lung tissue-derived single cells are labeled with EpCAM (CD326) antibody conjugated magnetic MicroBeads for positive selection of epithelial cells from both normal and tumor tissue. Different fractionated cell types (tumor EpCAM⁺, tumor EpCAM[−], and normal EpCAM⁺) are then tested for drug sensitivity and resistance, and cell viability data is analyzed to identify cancer-cell selective drug vulnerabilities.

Collection of normal lung and tumor tissue samples

⌚ Timing: 15 min

Collection of viable (non-necrotic) tissue samples is a critical step to achieve a maximum yield of viable cells. This step of the protocol highlights the factors that need to be considered while collecting tissue samples.

1. Collect tumor and tumor-adjacent normal lung tissue samples from surgically resected tumors lung lobe/s carrying tumor/s (lobectomy) (Figure 1).

Note: A trained thoracic surgeon or pathologist should dissect tumor and normal lung tissue because it is critical to collect fresh and viable tissue samples for efficient cultivation and further analysis. To dissect tumor and normal tissue areas, separate sterile scalpels should be used.

2. Immediately after dissection, collect the tissue pieces in 30 mL of ice cold HBSS containing antibiotics (100 U/mL of penicillin and 100 mg/mL of streptomycin).
 - a. Use separate tubes for collecting normal or tumor tissue pieces.
 - b. Transport the samples to the cell culture laboratory using a styrofoam ice box with a closed lid.
3. After reaching the cell culture lab, transfer tissue pieces to sterile petri plates and wash gently three times with ice-cold HBSS containing antibiotics.

Note: Give a gentle wash to tumor tissue pieces. Washing fragile tumor tissue pieces vigorously can lead to the detachment of small tissue fragments in the solution, resulting in the loss of material.

4. After the last wash with HBSS, add 3.7 mL of ice-cold DMEM or RPMI 1640 medium.
 - a. Using a scalpel, cut the tissue pieces into smaller pieces for analysis of DNA, RNA, and protein, as well as for preparation of formalin-fixed, paraffin-embedded (FFPE) tissue blocks.
 - i. Immediately after HBSS wash, snap freeze $\sim 0.5 \text{ cm}^3$ samples of the tumor and normal tissue for DNA and protein extraction and store at -80°C .
 - ii. For RNA extraction, submerge a piece of $<0.5 \text{ cm}^3$ tissue samples in approximately 5 \times volumes of RNeasy lysis solution at room temperature (20°C). Store at -80°C .
 - iii. To prepare FFPE blocks, place $>0.5 \text{ cm}^3$ tissue samples in histo cassettes and mark the cassettes with a histology marking pen that is resistant to formalin, ethanol, isopropanol, and xylene.
 - b. Reserve 1–3 cm^3 of tissue pieces for the FUTC assay.

Mechanical dissociation of normal lung and tumor tissue samples

⌚ Timing: 15 min

Mechanical digestion of the sample will break down the larger tissue pieces into smaller fragments, allowing effective further dissociation using an enzymatic procedure. This part of the protocol demonstrates how to dissociate normal and tumor tissue samples using a Miltenyi Biotec's gentleMACS Dissociator (Figure 2).

⚠ **CRITICAL:** This procedure should begin immediately following the surgical resection.

5. Move the tissue pieces into sterile glass petri plates at room temperature and add ice-cold DMEM or RPMI 1640 medium without FBS.
 - a. Remove all excess fat, fibrotic, and necrotic areas from the sample.
 - b. Cut the tissue into small 1–2 mm pieces with a sterile scalpel (Figure 2).

Note: It is important to work quickly and keep the samples cold on ice until initiating mechanical dissociation. This will minimize the loss of cell viability.

Enzymatic dissociation of normal lung and tumor tissue samples

⌚ Timing: 90 min

6. Prepare the enzyme mix by adding 1 mL of DMEM (or RPMI 1640) medium, 200 μL of enzyme H, 100 μL of enzyme R and 25 μL of enzyme A to a gentleMACS C tube.

Alternatives: A mix of collagenase and dispase can be used instead of MACS enzymes, and RPMI 1640 can be used instead of DMEM.

Note: Lower volumes of DMEM and enzymes are recommended for processing smaller tissue samples. For tissue samples smaller than 1 cm^3 , use half of the volume of the enzyme mix (2.5 mL in total).

7. Transfer the tissue samples minced using a scalpel into a gentleMACS C tube containing the DMEM and enzyme mix.
8. Close the C tube tightly and attach it upside down to a gentleMACS dissociator.

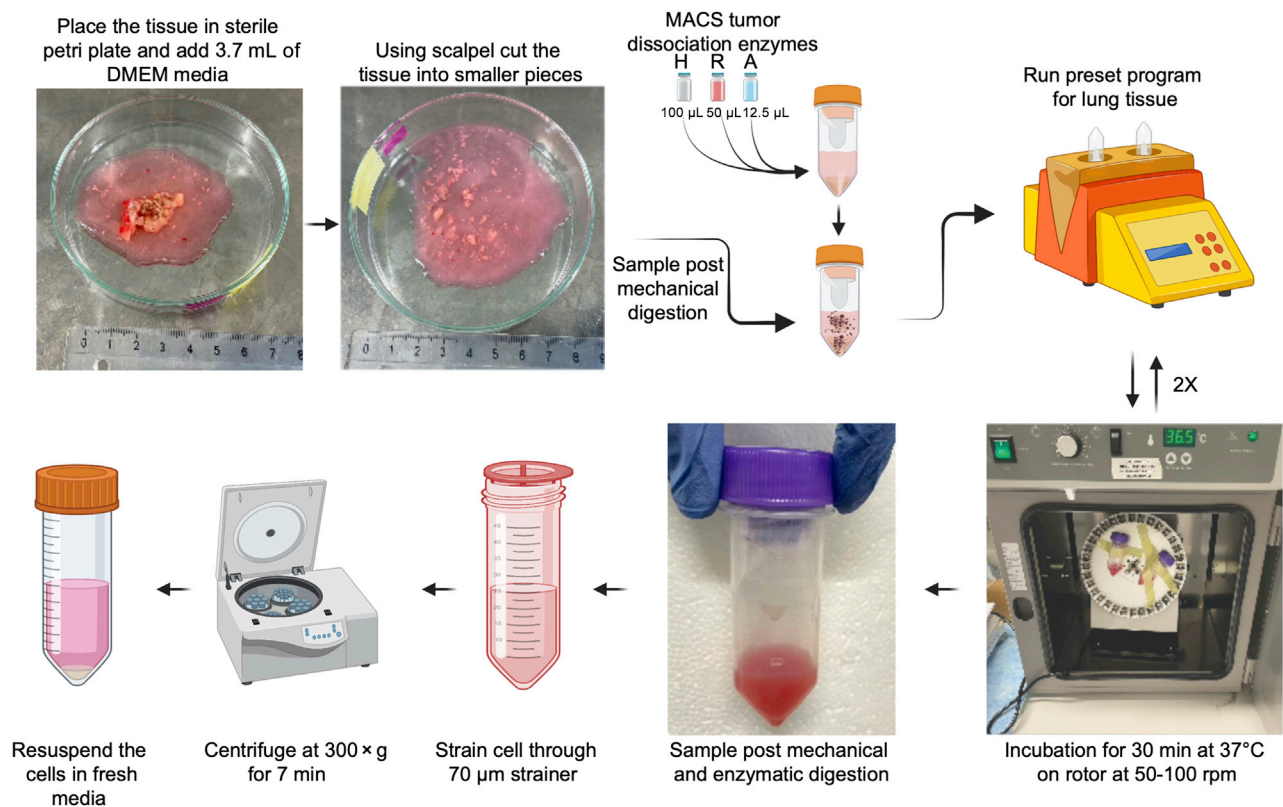


Figure 2. Mechanical and digestion of the lung tissue pieces

Place the tissue material in a sterile petri dish and add 3.7 mL of ice-cold DMEM medium. Using a scalpel, chop the tissue pieces as small as possible. Transfer the chopped tissue pieces to the MACS C tube and run the preset program on the MACS dissociator designed for lung tissue dissociation. To ensure proper tissue dissociation, incubate the tissue pieces at 37°C on rotation at 50–100 rpm. Collect the single cell suspension by straining the dissociated tissue pieces through a 70 µm cell strainer. Centrifuge the cells to pellet, discard the supernatant and resuspend them in fresh DMEM medium.

Note: Ensure that the tissue pieces are in the enzymatic dissociation mix and not attached on the walls of the tube.

9. Run the gentleMACS preset 'program h_tumor_01' recommended for lung tissue.
10. Detach the gentleMACS C tube from the dissociator when the spin is over.
11. Incubate the sample in the incubator for 30 min at 37°C under continuous rotation at 50–100 rpm (Figure 2).

△ **CRITICAL:** After step 11, cool the centrifuge to 4°C.

12. Attach the gentleMACS C tube upside down into a gentleMACS dissociator and run the preset 'program h_tumor_02'.
13. Repeat steps 11 and 12.
14. To collect the sample material at the bottom of the C tube, perform a brief centrifugation step: 200 × g for 4 min at room temperature (20°C).
15. Resuspend the sample in the medium contained in the C tube.
16. Place a 70 µm cell strainer on a new 50 mL falcon tube and moisten the strainer surface with 5 mL DMEM to enable efficient cell straining later.
17. To obtain a single cell suspension, strain the cell suspension in the C tube through a 70 µm cell strainer.

18. Wash the cell strainer with 10 mL of fresh medium.
19. Centrifuge cell suspension at $300 \times g$ for 7 min at room temperature (20°C) and aspirate supernatant completely.
20. Resuspend the cells in a 5 mL of F-medium.
21. Count the number of cells.

Note: It is recommended that cells are stained with trypan blue before cell counting to accurately count only the viable cells.

22. Centrifuge the cell suspension at $300 \times g$ at 4°C for 10 min. Aspirate the supernatant completely.

Enrichment of epithelial cells using EpCAM purification

⌚ Timing: 2 h

EpCAM-based enrichment is a widely used strategy to isolate patient-derived epithelial tumor cells from various tissue types, including solid tumors, peripheral blood, bone marrow, lymphoid tissue, and serous effusions. The enrichment of epithelial cells using MACS EpCAM monoclonal antibody-conjugated magnetic MicroBeads, involves the labeling of epithelial cells in cell suspension and the positive separation of these cells by loading the samples onto a MACS Column placed in a strong magnetic field. Cells labeled with magnetic MicroBeads are retained while unlabeled cells pass through the column. Labeled cells can then be collected by removing the column from the magnetic field.

⚠ **CRITICAL:** Before beginning the EpCAM⁺ cell enrichment, make sure that all reagents have been pre-cooled. Also, keep the cells on ice and work fast during the next few steps. Taking these precautions will prevent antibody capping on the cell surface and will avoid non-specific cell labeling. A few recommendations for speeding up the operations are mentioned below.

Note: MACS columns are available in three sizes: MS, LS, and XS. MS columns can separate maximum 2×10^8 total cells or maximum 10^7 EpCAM⁺ cells. LS columns can separate maximum 2×10^9 total cells or 10^8 EpCAM⁺ cells. XS columns can separate maximum 2×10^{10} total cells or maximum 10^9 EpCAM⁺ cells. The column size should be determined by the total number of cells or the quantity of tissue samples available. In the step-by-step protocol below, instructions are provided for magnetic separation using the LS column. Use the same volumes as specified when working with less than 10^7 cells. To process large cell volumes, several columns can be used with OctoMACS and SuperMACS II separators. Follow the manufacturer's protocol to process a total cell numbers greater than 2×10^{10} www.miltenyibiotec.com/FI-en/products/octomacs-separator-and-starting-kits.html - gref.

⚠ **CRITICAL:** In case clumps are formed see [troubleshooting problem 2](#) for solutions.

⚠ **CRITICAL:** Sodium azide is present in reagents. When exposed to acidic conditions, sodium azide produces hydrazoic acid, which is toxic. Before discarding azide chemicals, they should be diluted with running water.

23. Resuspend the cell pellet using the cold EDTA-BSA buffer. For 5×10^7 cells, add 300 μ L of EDTA-BSA buffer.
24. Per 5×10^7 total cells, add 100 μ L of EpCAM (CD326) MicroBeads ([Figure 3](#)).
25. Gently pipette the suspension up and down to mix well and break up cell clumps.
26. Incubate the cell suspension for 30 min in the refrigerator (2°C–8°C).

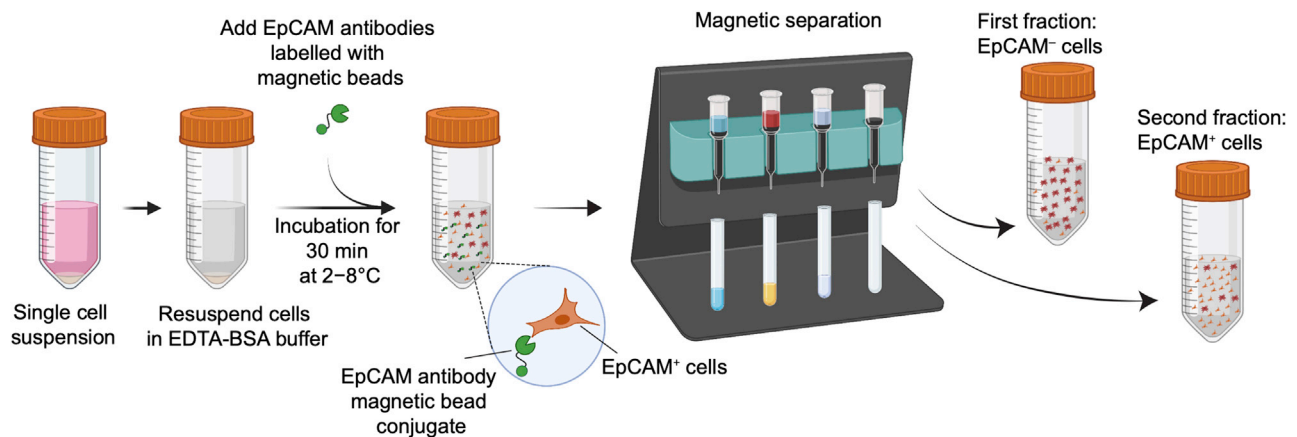


Figure 3. Isolation of epithelial cells using anti-EpCAM antibodies labeled with magnetic beads

27. After incubation is over, wash the cells by adding 10 mL of cold EDTA-BSA buffer per 5×10^7 cells and centrifuge at $300 \times g$ at 4°C for 10 min.

Note: During the centrifugation step (step 27), assemble the column and magnetic separator. Attach the MACS Separator to the MACS MultiStand. Insert the MACS column into a MACS Separator, making sure that the column's wings fit into the Separator's notch provided to insert the column. Put a collection tube below the column. Lastly, prepare the column by adding 3 mL of cold EDTA-BSA buffer to it. Discard the flow-through and place a fresh collection tube under the column.

28. Aspirate the supernatant completely and resuspend the cell pellet using the cold EDTA-BSA buffer. Add $500 \mu\text{L}$ of EDTA-BSA buffer per 10^8 cells.
29. Apply the cell suspension onto the MACS LS column and collect the flow-through, which mostly contains EpCAM⁻ cells. This fraction can be used as an EpCAM⁻ control for drug sensitivity testing (Figure 3).
30. Remove any remaining unlabeled cells in the column by washing the column four times with 3 mL of EDTA-BSA buffer.

Note: As soon as the column reservoir is empty, start the next round of washing by adding EDTA-BSA buffer. Do not allow the column to dry.

31. Immediately after all the washing steps are over, remove the column from the separator and place it on a fresh 15 mL collection tube.
32. Apply 5 mL of cold EDTA-BSA buffer onto the column and push the plunger firmly into the column to flush out the magnetically tagged EpCAM⁺ cells.

Note: To attain a higher purity of EpCAM⁺ cells, the positively selected cell fraction from step 32 must be separated over a second fresh MACS column by repeating steps 29–32.

33. Centrifuge both the EpCAM⁻ and EpCAM⁺ flow-throughs at $300 \times g$ at 4°C for 10 min. Aspirate the supernatant completely and discard it.
34. Resuspend each of the cell pellets in 3–5 mL of F-medium and count the total number of cells.
35. Calculate the volume and cell number required for drug sensitivity testing. We recommend usage of 384-well plates, 2,500 cells per well, robotic dispensing of cells, and testing each condition at least in duplicates.

Note: If the number of cells is not sufficient to test several drugs and a robotic dispensing facility is not available, a few drugs can be manually tested using the following steps (46–49).

Drug sensitivity and resistance testing (DSRT): Robotic reagent dispensing

⌚ Timing: 1–2 h

Robotic dispensing of cells and reagents allows improved accuracy and speed, which are both important factors in optimizing the protocol for diagnostic test development. However, the use of a robotic dispenser increases the required number of cells since the priming step (done to wet the tubing and tips with the same solution that will be dispensed later; doing this step prior to dispensing improves accuracy) requires an extra number of cells. Furthermore, robotic dispensing can also be used for precise dispensing of CellTiter-Glo, a reagent used in this assay to measure cell viability at 0 h.

Note: When exposed to cellular ATP, luciferase in the CellTiter-Glo produces a luminescent signal; thus, the luminescent signal produced by CellTiter-Glo is proportional to the number of metabolically active cells in the well.

36. Remove the drug plates from the storage pod.
37. In addition to drug plates, seed tumor-derived EpCAM⁺ and EpCAM[−] cells as well as EpCAM⁺ cells derived from normal healthy tissue into two empty 384-well plates for cell viability assessment at 0 h and 72 h.
38. Using Biotek MultiFlo FX RAD (MultiFlo), drug sensitivity testing can be performed using the following procedures (Table 1, Figure 4).

Note: While preparing MultiFlo, make sure that the flow of liquid from the tip is steady and fully vertical. To troubleshoot dispensing errors, backflush is recommended with sterile Milli-Q and 70% ethanol.

⚠ **CRITICAL:** Before cell seeding, ensure that the cell suspension is homogeneous and break any cell clumps by pipetting up and down.

Note: To dispense multiple cell types, repeat the ‘preparing MultiFlo’ step and priming with F-medium before cell seeding. Also, prepare the appropriate volume of medium and reagents for two or more plates.

Optional: To avoid edge effects in viability and drug sensitivity testing plates, do not use wells at the edge of the plate. For robust assessment, measure viability in at least ten replicate wells.

39. Immediately after cell seeding, place the plate on a plate shaker at 300 rpm for 5 min to dissolve drugs and to achieve uniform distribution of cells.
40. Incubate DSRT plates after cell seeding for 72 h at 37°C.
41. To measure cell viability at 0 h, 25 μ L CellTiter-Glo can be dispensed using MultiFlo (see details of the dispensing program in Table 1).

Note: Warm up the CellTiter-Glo ahead of time so it may be utilized as soon as the 0 h plates are ready for viability readout measurement.

Note: After dispensing CellTiter-Glo, wash and empty the MultiFlo cassette according to Table 1.

Table 1. Step-by-step instructions for robotic reagent dispensing

Step	Action#	Action	Reagent	Volume	Time
Preparing the MultiFlo cassette	38.a	Prime-purge	sterile Milli-Q	4 mL	2 min
	38.b	Prime-purge	70% ethanol	4 mL	2 min
	38.c	Prime-purge	sterile Milli-Q	4 mL	2 min
	38.d	Prime	sterile PBS	3 mL	2 min
Dissolving compounds	38.e	Prime	F-medium	3 mL	2 min
	38.f	Dispense	F-medium	5 μ L per well	3 min
Cell seeding ^a	38.g	Prime	cell suspension	3 mL	2 min
	38.h	Dispense	cell suspension	20 μ L per well	5 min
CellTiter-Glo dispensing ^b	38.i	Prime-purge	sterile Milli-Q	4 mL	2 min
	38.j	Prime-purge	70% ethanol	4 mL	2 min
	38.k	Prime-purge	sterile Milli-Q	4 mL	2 min
	38.l	Prime	CellTiter-Glo	3 mL	2 min
	38.m	Dispense	CellTiter-Glo	25 μ L per well	5 min
Washing MultiFlo cassette	38.n	Prime	sterile PBS	3 mL	2 min
	38.o	Prime-purge	sterile Milli-Q	4 mL	2 min
	38.p	Prime-purge	sterile Milli-Q	4 mL	2 min
	38.q	Prime-purge	70% ethanol	4 mL	2 min
Empty MultiFlo cassette ^c	38.r	Purge	air	–	20 s

^aIncubate the DSRT and (72 h) viability plates for 72 h after cell seeding.

^bCellTiter-Glo dispensing is only done for the 0 h viability plates.

^cEvery time the equipment is operated, maintenance tasks involving washing and emptying MultiFlo cassettes are carried out. After each usage, the cassette should be immediately cleaned to prevent clogging.

42. To induce cell lysis, place the 0 h cell viability plate on the plate shaker at room temperature (20°C) for 5 min.
43. To remove the bubbles, spin the plate for 5 min at 200 \times g.
44. Record the CellTiter-Glo luminescence signal using a plate reader compatible with the CellTiter-Glo-based assay.
45. After the 72 h incubation (step 40), add CellTiter-Glo and proceed as in steps 41–44.

Drug sensitivity and resistance testing (DSRT): Manual reagent dispensing

⌚ Timing: 2 h

Manual dispensing of cells or drugs is an option in case the robotic facility is unavailable or the number of cells is insufficient for robotic dispensing. Instead of adding cells to the drug plates, in the manual reagent dispensing approach, cells are first added to the empty wells of the 384-well culture plate on the day of sample processing. The, next day, after overnight (20 ± 4 h) incubation, drugs are manually dispensed on top of the cells, and the plates are subsequently incubated for 72 h. CellTiter-Glo is added on the cells and its luminescence signal is recorded at the end of incubation. The steps outlined below describe how to manually execute FUTC-based drug sensitivity testing.

46. In a 384-well plate, manually seed the EpCAM⁺ cells from step 36 (2,500 cells per well in 15 μ L of medium). Place the plate on a plate shaker at 300 rpm for 5 min to achieve uniform distribution of cells (Figure 4).
47. After overnight (20 ± 4 h) incubation, add the different drug concentrations on top of the cells in 10 μ L of medium.
48. Place the plate on a plate shaker at 300 rpm for 5 min to dissolve drugs.
49. Incubate for 72 h and analyze viability with CellTiter-Glo as described in steps 41–44.

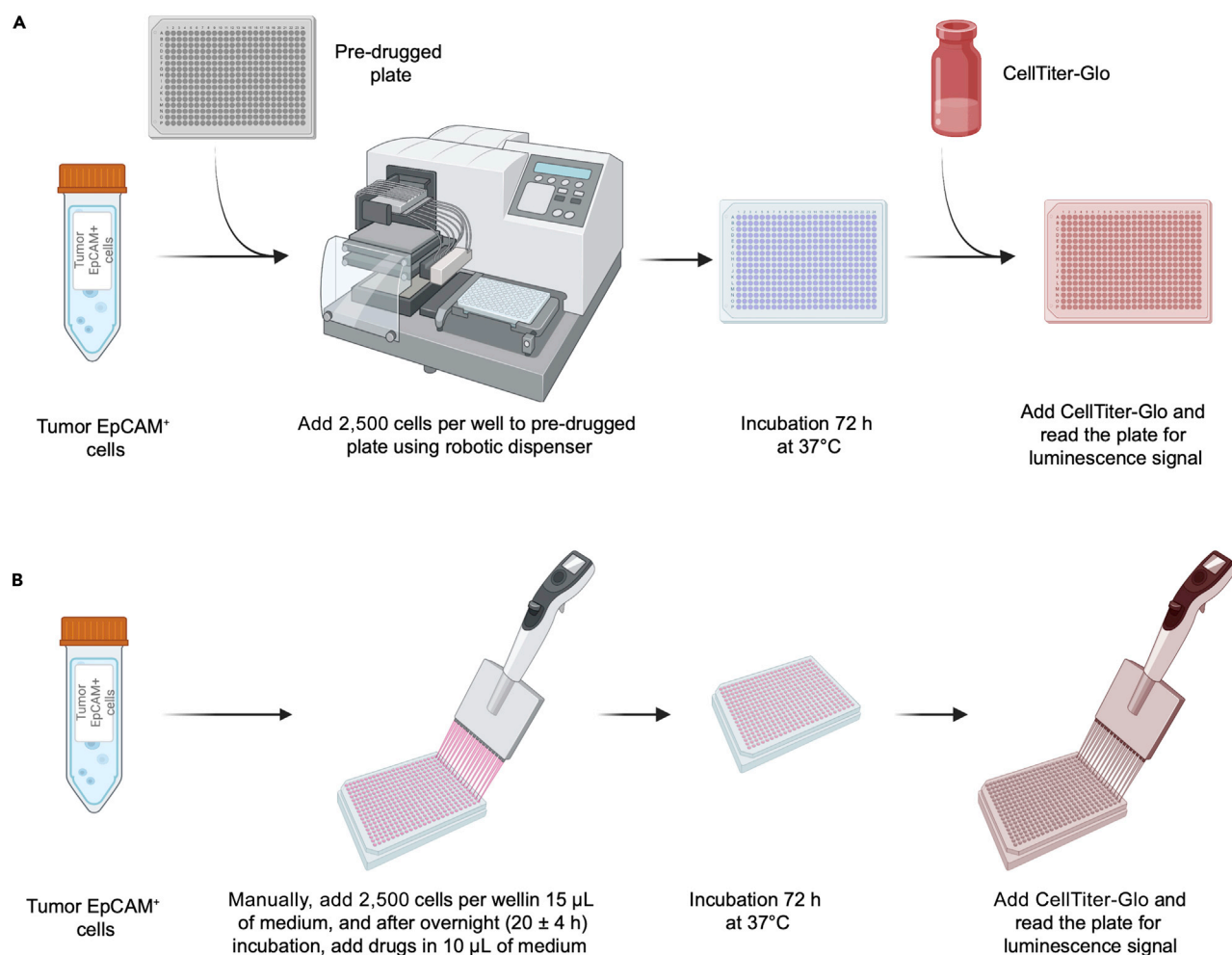


Figure 4. FUTC-based pharmacological screening

(A and B) Drug sensitivity and resistance testing with (A) robotic and (B) manual reagent dispensing methods.

Drug sensitivity and resistance testing (DSRT): Data analysis

⌚ Timing: 2 h

To analyze drug sensitivity data, an interactive web-based interactive portal called BREEZE can be employed (Potdar et al., 2020). BREEZE is a one-stop solution for extracting various data features from drug sensitivity testing, such as EC50, IC50, area under the curve (AUC), and drug sensitivity score (DSS). BREEZE's dynamic data visualization tools enable systematic data analysis and exploration at many levels. BREEZE can be found at <http://dsrt.fimm.fi> or <http://breeze.fimm.fi>.

Note: The video tutorial available on the BREEZE website is an excellent starting point for getting acquainted with the DSRT data analysis protocol. This video tutorial can be found at the BREEZE website <http://breeze.fimm.fi>.

Alternatives: Although the BREEZE portal is recommended for evaluating the DSRT data because it accelerates the process and provides visual insights into data quality and analysis, basic data analysis may also be done with MS-excel and GraphPad Prism. Excel may be used to calculate percent viability for each data point, and GraphPad Prism can be used to plot

dose response curves. To compare drug responses, IC50 values generated in GraphPad Prism can be used instead of the DSS values.

Data submission

The Breeze data analysis portal accepts a variety of data input formats, but drug names, their concentration ranges, and CellTiter-Glo measurement raw reads are essential for DSRT data analysis. The raw CellTiter-Glo readout values are used to calculate the percentage inhibition for each data point based on the negative (DMSO) and positive (benzethonium chloride) control values on the corresponding plate. To support the data submission step, a template of the data input structure is available for download (https://breeze.fimm.fi/DSRT_documentation/docs.html).

50. Visit the BREEZE online portal at <http://dsrt.fimm.fi/> or <http://breeze.fimm.fi/>.
51. Click the browse button under the 'Data analysis process' tab and upload the annotation file with the CellTiter-Glo raw data from the drug screen. The annotation file should include positive and negative controls, which are represented as 'POS' or 'BzCl' and 'NEG' or 'DMSO' under the 'Drug name' columns. The example annotation file is depicted in [Figure 5](#).
52. Select viability as a readout.
53. Select the 4PL curve-fitting algorithm.
54. Select DSS2 as a DSS method.
55. Depending on the requirements, different clustering algorithms can be used.
56. When the uploading of the input data file is complete, press the Start button, and a progress bar will appear.
57. When the data is processed, the "Check Results" button becomes active, allowing you to view the results. The link to the "Final report" can be emailed to the user.

Data analysis: Quality control

Technical errors during drug screening processes like spatial plate variability, errors in dispensing, edge effects due to uneven evaporation of the plate edges, in addition to poor cell viability/metabolic activity or elevated cell death of FUTCs can significantly compromise the overall data quality. Therefore, keeping track of the potential errors is crucial before performing the actual data analysis. Data analysis using the BREEZE portal provides information on the multiple quality control parameters like Z' factor, strictly standardized mean difference (SSMD), signal/background ratio, SD, coefficient of variation, and central tendency of controls. Z' factor, a screening window coefficient that indicates data robustness, is primarily used to assess data quality before any further analysis. Screens with a Z' factor value less than 0.2 were not considered for further analysis.

Data analysis: Drug response

Breeze provides several options for combining dose response data into a single metric, including IC50, EC50, AUC, and DSS. DSSs were generated to compare drug responses across samples using dose response curve metrics such as the IC50, slope, top, and lower asymptotes ([Yadav et al., 2014](#)).

EXPECTED OUTCOMES

Mechanical and enzymatic dissociation procedures used in tumor tissue processing for the FUTC assay are stressful for the primary cells and may result in cell death. Anoikis may also occur because of cellular detachment during dissociation or culture. The loss of cell viability could have a significant impact on the utility of FUTCs for assessing response to pharmacological agents. Therefore, the change in cellular viability must be carefully examined during the assay. So far, our results show that FUTCs either maintain or increase cellular viability over the course of a three day ex vivo culture period. See [Figures 1B and 3B](#) in our original report ([Talwelkar et al., 2021](#)). The inclusion of the ROCK inhibitor Y-27632 in the F-medium used for growing FUTCs potentially helps in the survival and recovery of the primary cells. In most cases, cell viability ex vivo is directly related to the percentage of Ki-67+ cancer cells in tumor tissue. See [Figures 1B and 3B](#) in our original report ([Talwelkar et al., 2021](#)).

WELL	PLATE	DRUG_NAME	CONCENTRATION	SCREEN_NAME	WELL_SIGNAL
A3	1	Trametinib	1	PLT87_Tumor_EpCAMpositive	10168
A4	1	Trametinib	10	PLT87_Tumor_EpCAMpositive	8614
A5	1	Trametinib	100	PLT87_Tumor_EpCAMpositive	6395
A6	1	Trametinib	1000	PLT87_Tumor_EpCAMpositive	4358
A7	1	Trametinib	10000	PLT87_Tumor_EpCAMpositive	1328
L18	1	Gefitinib	1	PLT87_Tumor_EpCAMpositive	13086
L19	1	Gefitinib	10	PLT87_Tumor_EpCAMpositive	13224
L20	1	Gefitinib	100	PLT87_Tumor_EpCAMpositive	11216
L21	1	Gefitinib	1000	PLT87_Tumor_EpCAMpositive	10968
L22	1	Gefitinib	10000	PLT87_Tumor_EpCAMpositive	1509
A9	1	dms0		PLT87_Tumor_EpCAMpositive	12531
L15	1	dms0		PLT87_Tumor_EpCAMpositive	14104
M22	1	dms0		PLT87_Tumor_EpCAMpositive	11819
P8	1	dms0		PLT87_Tumor_EpCAMpositive	13544
A16	1	BzCl		PLT87_Tumor_EpCAMpositive	1461
D12	1	BzCl		PLT87_Tumor_EpCAMpositive	1455
H5	1	BzCl		PLT87_Tumor_EpCAMpositive	1590
L20	1	BzCl		PLT87_Tumor_EpCAMpositive	1578

Figure 5. Example annotation file for drug screen data analysis using BREEZE

Personalizing treatment for cancer patients based on their *ex vivo* response to biopsy-derived cancer cells is a five-decade-old concept. Since the 1970s, researchers have developed assays to evaluate the *ex vivo* responses of chemotherapy drugs using cancer cells derived from patients, which are known as chemotherapy sensitivity and resistance assays (CSRA). However, due to their lack of utility to accurately predict clinical responses to chemotherapy drugs, these CSRA never became part of the cancer diagnostic tool kit (Letai, 2017). One of the primary reasons could be the use of a pool of patient-derived cancer and stromal cell mixtures for assessing chemotherapy responses, as well as a lack of understanding of how these chemotherapy agents affect normal healthy cells. To address this, we used EpCAM-based sorting to enrich cancer cells from tumor and normal epithelial cells from lung tissue, allowing us to determine cancer cell-selective pharmacological responses. First, 13 of 19 clinical tissue samples (68%) were found to have high EpCAM expression. Second, genomic analysis of EpCAM⁺ fractions collected after separation revealed that cancer cell enrichment (a high percentage of cells with a genomic aberration compared to reference tumor tissue) could be achieved even in samples with low EpCAM expression on cells from tissue samples. Third, in samples with moderate or low EpCAM expression levels, genotype-matched drug sensitivities were observed. Finally, a comparison of drug responses of FUTCs with and without EpCAM enrichment revealed that only enriched epithelial cancer cells had genotype-matched cancer-cell selective drug sensitivities (see Table S2 and Figures S2D, S3A, and 4A in our original published study, Talwelkar et al., 2021). Overall, these findings showed that EpCAM-based sorting can be used to reliably identify cancer cell-selective therapeutic vulnerabilities for lung cancer patients.

Analysis of FUTC-based drug responses revealed genotype-matched therapeutic vulnerabilities in samples with known oncogenic dependencies, and more crucially, such responses were observed only in cancer cells, not in normal epithelial cells (see Figures 4 and 5 in our original manuscript) (Talwelkar et al., 2021). Patient samples with *EGFR*, *MET*, *KRAS*, and *ALK*-rearrangement were sensitive to *EGFR*, *MET*, *MEK*, and *ALK* inhibitors, respectively. Treatment-adaptive activation of signaling pathways were biochemically measurable in a *KRAS* mutant sample. Furthermore, a *KRAS* mutant sample showed known combinatorial sensitivities to the *MEK* inhibitor trametinib and the *BCL-2*/*BCL-xL* inhibitor navitoclax. FUTC profiling was also used to guide the compassionate treatment of a lung cancer patient with an *EGFR* mutation (see Figure 6 in our original report) (Talwelkar

[et al., 2021](#)). This analysis exposed treatment responses that cannot be predicted by tumor genetic testing. Following clinical application of FUTC-guided combination therapy, the tumor burden and level of tumor markers were significantly reduced. Additionally, retrospective analysis showed that FUTC profiling successfully predicted clinical non-responses to the treatments that were administered to the patient based on genetic testing.

In conclusion, the FUTC assay can be used to screen anti-cancer drugs *ex vivo* and identify treatments with promise for clinical application. Given that many solid tumor types contain EpCAM⁺ epithelial cancer cells, the FUTC assay can likely be adapted to additional solid tumor pathology types, with particular translational promise for patients with advanced malignancies that lack therapeutic options.

LIMITATIONS

There are a few aspects that need to be considered when developing the FUTC assay for diagnostic analysis. In this protocol, the molecular profile of the solid tumor tissue sample influences the quality of the drug screen data. Furthermore, samples with a low number of EpCAM⁺ epithelial cancer cells may not provide enough cells for high-throughput screening, while tumor tissues with large necrotic regions may compromise tumor cell viability *ex vivo*. While our preliminary tests with smaller biopsy samples indicated promise, further investigation is required to develop the FUTC assay for routine use on small biopsy samples available in the clinic. Last, the translational reliability of the FUTC assay must be thoroughly investigated in clinical trial settings.

TROUBLESHOOTING

Problem 1

Inefficient digestion of the tissue (steps 5–22).

Potential solution

Inefficient tissue digestion may occur if a larger piece of tissue is used than recommended. To circumvent this challenge, a smaller piece of tumor tissue could be used to achieve effective tissue digestion. Prolonging the time of the mechanical or enzymatic digestion may also aid in digestion.

Problem 2

Over-digestion of the tissue (steps 15–20).

Potential solution

If the cell suspension becomes viscous after dissociation, this could be due to DNA release. In this case, centrifuge the cell suspension at 300 × *g* for 5 min, then remove the supernatant and replace it with a fresh 5 mL of DMEM. Incubate for 5 min at room temperature (20°C) with 200 U/mL DNase. Centrifuge the cell suspension at 300 × *g* for 5 min, then remove the supernatant and replace it with 5 mL of fresh DMEM.

Problem 3

Clumps occur following EpCAM labeling (step 27).

Potential solution

To obtain a single-cell solution before the magnetic separation, pass cells through a 30 μm nylon mesh (e.g., Pre-Separation Filter, Miltenyi, #130-041-407). Another way is to add DNase to the sample to avoid the formation of clumps. Shaking the tube during the incubation of cells with EpCAM-labeled magnetic beads also prevents the formation of clumps. Mix the solution before loading it onto MACS columns.

Problem 4

Clogged column during EpCAM magnetic separation (steps 29–32).

Potential solution

Mix the cell suspension inside the column with a 1 mL pipette tip to break up cell clumps and allow the flow-through to pass through the column. If this does not work, terminate the magnetic separation and push the liquid using a plunger.

Problem 5

Low yield of EpCAM⁺ cells (steps 24–34).

Potential solution

The poor yield of EpCAM⁺ cells following magnetic separation could be attributed to a low number of EpCAM⁺ cells in tumor tissue. In this situation, EpCAM⁺ cancer cells can be isolated by using magnetic microbeads labeled with other antibodies that recognize epithelial cells, such as cytokeratin 8 and cytokeratin 18.

Problem 6

Low QC values due to technical issues (step 57).

Potential solution

BREEZE's quality control data visualization aids in the interpretation and detection of technical issues such as edge effects, striping, patterning, related to dispensing cells, medium, and drugs. BREEZE is also excellent for observing signal windows, compound performance and distribution, variation among plates, and outliers. Using BREEZE to identify an issue will help in identifying a solution.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Emmy Verschuren (emmy.verschuren@helsinki.fi).

Materials availability

This study did not generate new materials.

Data and code availability

This study did not generate/analyze new data or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, S.S.T., K.W., and E.W.V.; investigation, S.S.T., M.I.M., A.H., N.L., J.R., and A.K.; writing – original draft, S.S.T., I.A.K.L., K.W., and E.W.V.; writing – review & editing, S.S.T., I.A.K.L.,

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DECLARATION OF INTERESTS

The authors declare no competing interests.

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