A fast impedance-based antimicrobial susceptibility test

Daniel C. Spencer
Teagan Paton
Timothy J.J. Inglis
J. Mark Sutton
Hywel Morgan (hm@ecs.soton.ac.uk)

Method Article

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Abstract

There is an urgent need to develop simple and fast antimicrobial susceptibility tests that allow informed prescribing of antibiotics. In this protocol we describe a method for a label-free AST that can deliver results within an hour, using an actively dividing culture as starting material.

Introduction

In this protocol an actively dividing bacterial isolate is incubated in the presence of the antibiotic for 30 minutes, and then approximately 10^5 cells are analysed one-by-one with microfluidic impedance cytometry for 2-3 minutes. The measured electrical characteristics reflect the phenotypic response of the bacteria to the mode of action of a particular antibiotic, in a 30-minute incubation window.

Reagents

Cell media

Tryptic soy broth (TSB) *[Sigma: 22092]*

Cation Adjusted Mueller Hinton Broth (MHB) *[Sigma: 90922]*

Hanks media *[Sigma: H6648]*

Agar plates *[Sigma L5542]*

Antibiotics

Meropenem *[Sigma M2574]*

Colistin *[Sigma 1148001]*

Gentamicin *[Sigma G4918]*

Ciprofloxacin – *[Sigma 17850]*

Amoxicillin/Clavulanic acid *[Sigma A8523 and Sigma 33454]*

Cefoxitin *[Sigma 1098107]*

Reference Particles

Polybead® Microspheres 1.50µm *[Polysciences 17133-15]*

Bacterial isolates

Klebsiella pneumoniae strain 43358

Klebsiella pneumoniae strain ATCC 700603

Klebsiella pneumoniae strain 18397

Klebsiella pneumoniae strain 43292

Klebsiella pneumoniae strain 44271

Klebsiella pneumoniae strain KS1

Klebsiella pneumoniae strain KS11

Klebsiella pneumoniae strain K1

Klebsiella pneumoniae strain 1705

Klebsiella pneumoniae strain K14

Acinetobacter baumannii strain NCTC 13424

Acinetobacter baumannii strain ATCC 17978

Klebsiella pneumoniae strain 51851

Klebsiella pneumoniae strain NCTC 13368

Klebsiella pneumoniae strain CFI-014

Pseudomonas aeruginosa strain NCTC 13437

Pseudomonas aeruginosa strain PA01

Escherichia coli strain CFL 161

Escherichia coli strain NCTC 12923

Klebsiella pneumoniae strain51851

Klebsiella pneumoniae strain NCTC 13438

Klebsiella pneumoniae strain 51851

Klebsiella pneumoniae strain TW3

Klebsiella pneumoniae strain NCTC 13368

Klebsiella pneumoniae strain M6

Klebsiella pneumoniae strain NCTC 13368

Klebsiella pneumoniae strain NCTC 5054

Klebsiella pneumoniae strain NCTC 13438

Klebsiella pneumoniae strain M6

Escherichia coli strain LEC001

Escherichia coli strain NCTC 12923

Staphylococcus aureus strain NCTC 13616

Staphylococcus aureus strain NCTC 6571

Equipment

–Zurich Instruments HF2IS impedance analyser (Zurich Instruments AG, Zurich, Switzerland)

–Shaking incubator set to 36-37^{o}C with an air atmosphere.

–Vortex mixer for falcon and eppendorf tubes

–Single cell impedance cytometer glass chips. Custom - see procedure section for chip fabrication

–Single cell impedance cytometer electronics hardware -see application note here for an example https://www.zhinst.com/sites/default/files/documents/2020-03/zi_hf2is_appnote_microfluidics_2020.pdf

–A syringe pump (set to 30uL/min with a 1mL syringe, e.g. KD Scientific 788101, Harvard Apparatus 70-4500)

–Adjustable pipettes (P10, P20, P300, P1000)

Procedure

Protocol 1

Step 1. Take a single colony of bacteria from a refrigerated or frozen plate, add to 10mL of Tryptic soy broth (TSB) and place in an incubator overnight at 37^{o}C to revive the cells. This is sample A.

Step 2. Add a 1uL aliquot of sample A to an eppendorf containing 1mL Hanks media and vortex for 1 minute to ensure the sample is thoroughly mixed.

Step 3. Dilute sample A 1000-fold (1uL in 1mL Hanks) and measure the concentration of cells using the impedance cytometer (see impedance cytometer measurements section later for further details).

Step 4. Take an aliquot of sample A and dilute such that the final concentration (called sample B) is 5x10^{5} cells/mL; From step 3, if the concentration of sample A is *N* cells/mL, add a volume of sample A given by *V*= *N* / 5x10^{6} (uL) to 10mL MHB, such that the final concentration (called sample B) is 5x10^{5} cells/mL. Typically sample A will be between 10^{8} and 10^{9} cells/mL, and therefore the volume *V* will be between 10-100uL.

Step 5. Incubate sample B in a shaking incubator for 30 minutes at 37^{o}C.

Step 6. Add aliquots (950uL) to 7 pre-warmed test tubes each containing 50uL MHB and Meropenem at a concentration of 0, 5, 10, 20, 40, 80 and 160 mg/L of meropenem (final antibiotic concentration is 20x lower; i.e. 0, 0.25, 0.5, 1, 2, 4 and 8 mg/L.)

Step 7. Incubate the tubes for 30 minutes at 37^{o}C.

Step 8. Wash the cells and resuspend in Hanks media (spin at 10,000*g for 5 minutes, remove the supernatant and top up to 1mL)

Step 9. Dilute each sample 1:10 (add 100uL from each of the 7 eppendorfs to 7 new eppendorfs containing 900uL Hanks).

Step 10. Add a volume of concentrated bead suspension that contains approximately 10^{4} polystyrene beads (1.5um diameter) to each eppendorf.

Step 11. Load the contents of each tube into a 1mL syringe and measure with the impedance cytometer (see later).

Protocol 2.

Step 1. Streak out a 10uL loop from a frozen glycerol stock onto an agar plate. Place the plate into an incubator overnight at 37^{o}C.

Step 2. Pick three typical colonies from the plate and add to 3mL of MHB - this is sample A.

Step 3. Vortex sample A for 1 minute to ensure that it is well mixed.

Step 4. Dilute sample A 400-fold (2.5uL in 1mL Hanks) and measure using the impedance cytometer to determine the concentration of the bacteria in sample A (see impedance cytometer measurements section later for further details).

Step 5. Assuming the concentration of sample A is N cells/mL, add a volume of sample A given by $V = N / 15 \times 10^5$ uL to 3mL MHB, such that the final concentration (called sample B) is 5×10^5 cells/mL. Typically sample A will be approximately 10^8 cells/mL, and therefore the volume V will be approximately 15uL.

Step 5. Incubate sample B in a shaking incubator for 30 minutes at 37^{o}C.

Step 6. Add aliquots (500uL) to pre-warmed test tubes each containing 500uL MHB and an antibiotic. Concentrations of antibiotics are 4mg/L and 32mg/L for Meropenem , 2 mg/L for ciprofloxacin, 16 mg/L for gentamicin, 8 mg/L for Colistin, 16 mg/L for ceftazidime, along with a control (no antibiotic). Final concentrations of antibiotics will be half the above concentrations.

Step 7. Incubate the tubes for 30 minutes at 37^{o}C.

Step 9. Dilute each sample 1:10 (add 100uL to an new eppendorfs containing 900uL Hanks media).

Step 10. Add a volume of concentrated bead suspension that contains approximately 10^4 polystyrene beads (1.5um diameter) to each eppendorf.

Step 11. Load the contents of each tube into a 1mL syringe and measure with the impedance cytometer (see

Single cell impedance cytometry measurements

Step 1. Load the sample into a 1mL syringe.

Step 2. Connect the syringe to the impedance cytometer tubing.

Step 3. Insert the syringe into a syringe pump.

Step 4. Start the syringe running at 30uL/min.

Step 5. Start the ziControl software which controls the Zurich Instruments impedance analyser, and apply a measurement voltage (see measurement settings below).

Step 6. Record data for 2-3 minutes (depending on the required number of bacteria to measure).

Step 7. Turn off the measurement voltage.

Step 8. Flush through with a suitable cleaning reagent (e.g. buffer, NaOH, NaClO).

Repeat steps 1-8 for each remaining sample.

Measurement settings (ziControl)

Sample rate: 230k samples/second

Applied frequencies: 5MHz and 40MHz

Applied voltage: 2v pk-pk

Data analysis

Step 1. Determine the impedance of each particle by extracting the peak signal amplitude using convolution for each applied frequency.

Step 2. Determine the mean signal of the 1.5um beads

Step 3. Normalise the opacity-cell size scatterplot by a single linear multiplier for each axis to ensure the mean of the beads is at Opacity =1 and diameter = 1.5um.

Step 4. Define a contour is defined around the population of cells not exposed to antibiotic. This is termed the unexposed contour should include 50% of the cell population.

Step 5. For each antibiotic exposed sample, normalise the data (as per step 3) and determine the number of cells that fall within the unexposed contour.

Single cell impedance cytometer chip fabrication

Step 1. Deposit 10nm titanium and the 200nm platinum onto two 6 inch glass substrates

Step 2. Spin-coat a suitable photoresist (e.g. S1813) and pattern by exposure to UV light through a 7 inch photomask

Step 3. Develop the resist by washing with a suitable resist developer.

Step 4. Pattern the meta layers using Ion beam milling.

Step 5. Strip the photoresist.

Step 6. Spin-coat SU-8 to the desired channel thickness (e.g. 30um) on to one of the two patterned wafers

Step 7. Expose to UV light through a 7 inch photomask and develop.

Step 8. Align and bond the second wafer to the first wafer by vacuum bonding at 180°C, 10kN for 6 hours.

Step 9. Carefully scribe and dice the wafer to release the individual cytometer chips.

Troubleshooting

If the microcytometer chip gets blocked, try flushing with buffer from outlet back to the inlet. If this is unsuccessful, replace with a new chip. If the cytometer is frequently clogging, filter all reagents with a 0.22um (sterile) filter.

Time Taken

Anticipated Results

A populations of beta-lactam exposed cells from an isolate that is **sensitive** to the antibiotic are expected to **increase** in low-frequency impedance and **decrease** in electrical opacity relative to an unexposed sample of the same isolate. A populations of beta-lactam exposed cells from an isolate that is **resistant** to the antibiotic are expected to not change significantly in low-frequency impedance or electrical opacity relative to an unexposed sample of the same isolate.

A populations of aminoglycoside exposed cells from an isolate that is **sensitive** to the antibiotic are expected to **decrease** in low-frequency impedance and **increase** in electrical opacity relative to an unexposed sample of the same isolate. The bacterial concentration is expected to decrease relative to the unexposed aliquot. Populations of aminoglycoside exposed isolates that are **resistant** to the antibiotic are expected to not change or decrease slightly in low-frequency impedance and not change significantly in electrical opacity relative to an unexposed sample.

A populations of Colisitin exposed cells from an isolate that is **sensitive** to the antibiotic are expected to **decrease** in low-frequency impedance and **increase** in electrical opacity relative to an unexposed sample of the same isolate. The bacterial concentration is expected to decrease relative to the unexposed aliquot. Populations of Colistin exposed isolates that are **resistant** to the antibiotic are expected to not change or decrease slightly in low-frequency impedance and not change significantly in electrical opacity relative to an unexposed sample.

A populations of fluoroquinolone exposed cells from an isolate that is **sensitive** to the antibiotic are expected to **increase** in low-frequency impedance and not change significantly in electrical opacity relative to an unexposed sample of the same isolate. Populations of fluoroquinolone exposed isolates that are **resistant** to the antibiotic are expected to not change significantly in low-frequency impedance or in electrical opacity relative to an unexposed sample.

References

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