Two-fold Broth Microdilution Method for Determination of MIC

Applied in BioNMR Group of Prof. Anne Ulrich (Chair of Biochemistry and IBG2, KIT, Germany) by Dr. Marina Berditsch and students

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Bacterial strains

To characterize antibacterial activity of peptides or conventional antibiotics we use mostly four quality control strains, which are recommended by DSMZ and in [2]. The set contains two Gram-negative strains: *E.coli* DSM 1103 (ATCC 25922) and *Pseudomonas aeruginosa* DSM 1117 (ATCC 27853), as well as two Gram-positive strains: *Staphylococcus aureus* DSM 1104 (ATCC 25923) and *Enterococcus faecalis* DSM 2570 (ATCC 29212).

Preparing of peptide stock solutions

1. Take out the flask with peptide powder from -20°C and equilibrate at room T° (ca. 30 min for a 50 ml "Falcon" tube).
2. Sterilize MilliQ (bi-distilled, deionised) H_2O using membrane filter Filtropur S with pore size 0.2µm (Sarstedt AG&Co., Nümbrecht, Germany) – to be used for dilution of the absolute EtOH.
3. Weight-out about 1 mg of each peptide to prepare stock solutions (2 ml low-binding "Eppendorf" tubes). Stocks are prepared with the final concentration of **1024 µg/ml**, in EtOH:MilliQ H_2O 1:1 (H_2O from step 2).
4. When not used, store stock solutions at -20°C (50% EtOH will not be frozen).
5. Use stock solution directly, if the highest desired concentration is 256 µg/ml (4-fold); If the highest desired concentration is 128 µg/ml, prepare the appropriate volume of **512 µg/ml working solution,** dissolving stock solution 1:1 in 50% EtOH.
6. Use **working solution of 256 µg/ml** in 50% EtOH, if peptide shows tendency to precipitate at 128 µg/ml (for example, Gramicidin S), but MIC value is expected or known to be significantly lower.

We don't employ the method for MIC determination as recommended in [2], which is modified for cationic antimicrobial peptides. In the method [2] the stocks are prepared by addition of 0,01% acetic acid and 0,2% of BSA, reasoning that this avoids precipitation of some peptides [3]. We found these conditions to bring other problems: i) BSA itself binds hydrophobic peptides and reduces effective concentration of them; ii) acetic acid significantly decreases pH of the medium, inhibiting bacterial growth. We recommend instead, to prepare the stock solutions in 50% EtOH. This solvent system, in our hands, never influenced bacterial growth (multiple control experiments). Precipitation problem can even be ignored in our two-fold dilution method, but persists when 10-fold stock solutions are used and multiple-step dilution is employed as recommended in [2,4]. For instance, in chemically defined media we have observed precipitation of such peptides as Gramicidin S or Temporin L being stronger than in complex MH medium.

Adjustment of two-fold microdilution of peptides

Done directly in microtiter plates Nunclon™ (use F, PS, not „tissue-culture treated", Nunc GmbH & Co., Wiesbaden, Germany). The method is based on the protocol of Amsterdam [1]. Our modifications are indicated with asterisks:

1. Dispense 50 µl of 2-fold MH medium into each well in the column 1, 50 µl of 1-fold original MH medium into each well in the columns from 2 to 11 and 100 µl into each well in the column 12. Use 300 µl Electronic Multichannel Pipette (Mettler-Toledo GmbH, Gießen, Germany).
2. Pipette 50 µl of appropriate 4-fold concentrated peptide ("stock" or "working") solution***** in two or four repetitions into the wells in column 1 (antibiotic concentration will be diluted this way 1:1). Use 100 µl Manual Research Pipette (Eppendorf, NY, USA).
3. Adjust two-fold dilution of peptides in the next columns from 1 to 10****** using 100 µl Manual Multichannel Pipette (MMP, Eppendorf, NY, USA):
    a. Mix solutions (use MMP) in column 1 **by steady circular motions of tips*****, release the rests of solution from the tips. Do not splash.
    b. Withdraw (use MMP) 50 µl of the peptide mix and move into the column 2, thus dissolving 1:1.
    c. Repeat dilution procedure (use MMP) up to column 10 and additionally

discard 50 µl of solutions from this last column.

4. Use column 11 as a **positive growth control** (no antibiotic) and column 12 as a **negative control for medium sterility** (no cells).
5. Transfer microtiter plates into the closable (with lid) tray or box until inoculation time (to avoid evaporation, which is most often occurs on the plate edges, when no care is taken).

* Peptides are first diluted 1:1 in the dilution procedure and then additionally 1:1 by the inoculation (totally 4-fold).

** The titration could also be started from the raw A and gone to the raw H, if the MIC value is expected at the middle of the concentration range with 8 sampling points (A-H).

*** To avoid air bubbles during dilution, don't mix the peptide solutions by up/down suctions (6-8 times) as recommended in [1].

Standard procedure for the preparation of inoculation material

Bacterial susceptibility depends on the bacterial state, which is defined by growth phase and growth rate. The aim of this procedure is to refresh bacterial cells from the Cryocultures, which are stored at -80°C and to obtain a standard inoculation material.

1. Inoculate 10 ml BBL™ Mueller Hinton (MH) medium (Becton, Dickinson and Co, Sparks, MD, USA) by single glass beads from the individual vials of bacterial Cryobank™ System (Mast Diagnostica, Reinfeld, Germany).
2. Incubate the culture overnight at 37°C and 200 rpm in the shaker Unimax 1010 (Heidolph Instruments, Schwabach, Germany).
3. Perform three-phase streack-plating of the overnight culture onto MH agar to obtain single colonies [5]. Store MH plates with colonies at 4°C before use (not longer than 2 weeks).
4. Collect colonies and prepare cell suspensions in 500 µl of MH medium (use 2 ml "Eppendorf" tubes).
5. Calculate required volume of suspension to inoculate the overnight pre-culture in 10 ml MH medium. Initial optical density OD_{550} = **0.02** should be reached. For instance: OD_{550} = 4 is determined in the suspension, 4/**0.02** = 200 is the dilution coefficient. Inoculation volume for 10 ml is 10.000/200 = 50 µl.

6. Grow overnight pre-cultures not longer than 18 hours and use for inoculation of the **test cultures** in 10 ml MH medium by adjustment of initial OD_{550} to **0.2**.
7. Incubate **test cultures** for 3-4 hours at 37°C and 200 rpm to reach the earlier exponential growth phase (i.e. OD_{550} = 1-2).
8. Dilute test cultures to OD_{550} = **0.2**, which is used for the cell count. According to **McFarland standard**, bacterial suspension with OD_{550} = 0.125-0.250 contains 1-2x10^8 CFU/ml. However, our results of cell count confirmed this value only for Gram-positive bacteria. Gram-negative bacteria (*E.coli* DSM 1103 (ATCC 25922) and *Pseudomonas aeruginosa* DSM 1117 (ATCC 27853), counted 1-2x10^9 CFU/ml at OD_{550} =0.2. Therefore, use 1000-fold dilution for Gram-negative and 100-fold dilution for Gram-positive bacteria.
9. Inoculate microtiter plate by addition of 50 µl of diluted bacterial suspension to each well (with the exception of negative control, *i.e.* column 12). Use Electronic Multichannel Pipette and follow column direction 11→1.
10. Incubate microtiter plates at 37°C for 20-22 hours.

Evaluation of growth results by respiration activity of bacteria

1. Add 20 µl of the redox indicator Resazurin in concentration 0.2 mg/ml to each well. Use Electronic Multichannel Pipette.
2. Incubate for the next 2 hours at 37°C.
3. Determine bacterial respiration (indicate the growth), as a difference in absorption of the pink-coloured reduced form (Resafurin) at 570 nm and the blue-coloured oxidized form of Resazurin at 600 nm. Use Microtiter Plates Reader „FLASH Scan 550“ (Analytik Jena, Jena, Germany).
4. Alternatively, inspect plates visually. Assume, that the cultures in the pink wells contain mostly living cells, while blue color is demonstrated by the wells, where growth is inhibited.

References:

[1] Daniel Amsterdam (1996). Susceptibility testing of antimicrobials in liquid media. In: *Antibiotics in laboratory medicine*, Loman, V., ed., 4th ed. Williams and Wilkins, Baltimore, MD, p. 52-111.

[2] Irith Wiegand, Kai Hilpert & Robert E. W. Hancock (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances, *Nature Protocols*, 3(2): 163-175.

[3] Deborah A. Steinberg et al. (1997). Protegrin-1: a Broad-Spectrum, Rapidly Microbicidal Peptide with in Vivo Activity, *Antimicrobial Agents and Chemotherapy*, 41(8): 1738-1742.

[4] European Committee for Antimicrobial Susceptibility Testing (EUCAST) of the European Society of Clinical Microbiology and Infectious Diseases (ESCMID) (2003). Determination of minimum inhibitory concentrations (MICs) of antibacterial agents by broth dilution. *Clinical Microbiology and Infection*, 9(8): 1-7.

[5] http://www.jlindquist.net/generalmicro/102streakplate.html
http://www.youtube.com/watch?v=_1KP9zOtjXk&feature=related