Plate Reader Calibration & Analysis

Purpose

Plate readers report fluorescence values in arbitrary units that vary widely from instrument to instrument. Thus, absolute fluorescence values cannot be directly compared from one instrument to another. In order to compare fluorescence output of test devices between teams, it is necessary for each team to create a standard fluorescence curve.

Materials

- Fluorescein
- PBS
- 300 uL silica beads
- ddH2O

Equipment

- 96 well plate (preferably black, with clear, flat bottom)
- Pipettes and tips
- Centrifuge
- Plate reader
- Eppendorf tube

Procedure

Prepare the Fluorescein Stock Solution

1. Spin down the fluorescein kit tube to make sure the pellet is at the bottom of the tube.
2. Prepare a 10X fluorescein stock solution (100 μM) by resuspending fluorescein powder in 1mL of 1X PBS.
3. Pipette up and down and examine the solution in the pipette tip. If any particulates are visible in the pipette tip, continue to mix the solution until they disappear.
4. Dilute the 10X reference stock solution with 1X PBS to make a 1X reference working solution with a concentration of 10 μM.
5. Serial dilutions will be performed across columns 1-11. Column 12 must contain PBS buffer only.

a. Set up the plate with the fluorescein stock in column 1 and an equal volume of 1X PBS in columns 2 to 12. Perform a serial dilution by consecutively transferring 100 µl from column to column with good mixing.
    i. Add 100 µl of 1X PBS into wells A2, B2, C2, D2....A12, B12, C12, D12.
    ii. Add 200 µl of fluorescein 1X stock solution into A1, B1, C1, D1.
    iii. Transfer 100 µl of fluorescein stock solution from A1 into A2.
    iv. Mix A2 by pipetting up and down 3x and transfer 100 µl into A3.
    v. Mix A3 by pipetting up and down 3x and transfer 100 µl into A4.
    vi. Mix A4 by pipetting up and down 3x and transfer 100 µl into A5.
    vii. Mix A5 by pipetting up and down 3x and transfer 100 µl into A6.
    viii. Mix A6 by pipetting up and down 3x and transfer 100 µl into A7.
    ix. Mix A7 by pipetting up and down 3x and transfer 100 µl into A8.
    x. Mix A8 by pipetting up and down 3x and transfer 100 µl into A9.
    xi. Mix A9 by pipetting up and down 3x and transfer 100 µl into A10.
    xii. Mix A10 by pipetting up and down 3x and transfer 100 µl into A11.
    xiii. Mix A11 by pipetting up and down 3x and transfer 100 µl into liquid waste. Do not continue the serial dilution into column 12.
    xiv. Repeat dilution series for rows B, C, D.

Measure Fluorescence

1. Measure the fluorescence of all samples in your plate reader. Ensure that any automatic gain setting is off.
    a. If you will be using your data in conjunction with measurements from the **Plate Reader Abs600 (OD) Calibration protocol**, make sure you use the same instrument settings for both protocols.
2. Obtain the tube labeled “Silica Beads” from the Measurement Kit and vortex vigorously for 30 seconds.
3. Immediately pipet 100 µL microspheres into a 1.5 mL eppendorf tube.
4. Add 900 µL of ddH_2O to the microspheres.
    a. Vortex well. This is your Microsphere Stock Solution.
5. Serial dilutions will be performed across columns 1-11. Column 12 must contain ddH2O only.
    a. Set up the plate with the microsphere stock solution in column 1 and an equal volume of 1x ddH2O in columns 2 to 12. You will perform a serial dilution by consecutively transferring 100 µl from column to column with good mixing.

i. Add 100 µl of ddH2O into wells A2, B2, C2, D2....A12, B12, C12, D12.
ii. Vortex the tube containing the stock solution of microspheres vigorously for 10 seconds.
iii. Immediately add 200 µl of microspheres stock solution into A1.
iv. Transfer 100 µl of microsphere stock solution from A1 into A2.
v. Mix A2 by pipetting up and down 3x and transfer 100 µl into A3.
vi. Mix A3 by pipetting up and down 3x and transfer 100 µl into A4.
vii. Mix A4 by pipetting up and down 3x and transfer 100 µl into A5.
viii. Mix A5 by pipetting up and down 3x and transfer 100 µl into A6.
ix. Mix A6 by pipetting up and down 3x and transfer 100 µl into A7.
x. Mix A7 by pipetting up and down 3x and transfer 100 µl into A8.
xi. Mix A8 by pipetting up and down 3x and transfer 100 µl into A9.
xii. Mix A9 by pipetting up and down 3x and transfer 100 µl into A10.
xiii. Mix A10 by pipetting up and down 3x and transfer 100 µl into A11.
xiv. Mix A11 by pipetting up and down 3x and transfer 100 µl into liquid waste. Do not continue the serial dilution into column 12.
xv. Repeat dilution series for rows B, C, D.

6. **IMPORTANT:** Re-Mix (pipette up and down) each row of your plate immediately before putting in the plate reader!
    a. This is important because the beads begin to settle to the bottom of the wells within about 10 minutes, which will affect the measurements. Take care to mix gently and avoid creating bubbles on the surface of the liquid.
7. Measure OD600 of all samples in the instrument. Disable any path length correction setting on your instrument, if it has one.
8. Record the data. Import the data into the provided Excel spreadsheet (iGEM Data Analysis Template - Particle Standard Curve - v1.xlsx).
9. Record all data in your notebook, as well as the gain setting you used in your instrument (if available). Import the data into the provided Excel spreadsheet (iGEM Data Analysis Template - Fluorescence Standard Curve - v1.xlsx).

Measure Sample

1. Suspend E. coli Nissle cells by pipetting suspension up and down.
2. Seed 1000-500 cells/well in 50µL of medium. Centrifuge to ensure cells reach the bottom of the well.

3. Incubate seeded plates overnight.
4. Add 100 µL of 1X PBS into wells E2, E3 ....E12.
5. Add 200 µlL of suspension into E1.
6. Transfer 100 µL of solution from A1 into A2.
7. Mix A2 by pipetting up and down 3x and transfer 100 µl into A3.
8. Mix A3 by pipetting up and down 3x and transfer 100 µl into A4.
9. Mix A4 by pipetting up and down 3x and transfer 100 µl into A5.
10. Mix A5 by pipetting up and down 3x and transfer 100 µl into A6.
11. Mix A6 by pipetting up and down 3x and transfer 100 µl into A7.
12. Mix A7 by pipetting up and down 3x and transfer 100 µl into A8.
13. Mix A8 by pipetting up and down 3x and transfer 100 µl into A9.
14. Mix A9 by pipetting up and down 3x and transfer 100 µl into A10.
15. Mix A10 by pipetting up and down 3x and transfer 100 µl into A11.
16. Mix A1 by pipetting up and down 3x and transfer 100 µl into liquid waste.
17. **IMPORTANT!** Re-Mix (pipette up and down) each row of your plate **immediately before** putting in the plate reader!
    a. *This is important because the beads begin to settle to the bottom of the wells within about 10 minutes, which will affect the measurements.*
18. Measure OD_{600} of all samples in the instrument. Disable any path length correction setting on your instrument, if it has one.
19. Compare with the standard curve to determine GFP concentration.

Safety Precautions

Ensure to wear appropriate protective equipment (including lab coat, gloves, goggles, and close-toed shoes) at all times during experimentation. Tie back long hair and secure any loose items. Follow all laboratory procedures, as outlined in safety training. Never ingest or inhale any laboratory chemicals.

References

https://2019.igem.org/wiki/images/e/ed/Plate_Reader_Fluorescence_v3.pdf

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