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Calibration Protocol - Plate Reader Fluorescence Calibration V.3

Version 1 is forked from Calibration Protocol - Fluorescence Standard Curve with Fluorescein

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Protocol status: In development
Calibration for red fluorescent proteins is still a work in progress

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Abstract

Plate readers report fluorescence values in arbitrary units that vary widely from instrument to instrument. Therefore 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. Although distribution of a known concentration of GFP protein would be an ideal way to standardize the amount of GFP fluorescence in our E. coli cells, the stability of the protein and the high cost of its purification are problematic. We therefore use the small molecule fluorescein, which has similar excitation and emission properties to GFP, but is cost-effective and easy to prepare. (The version of GFP used in the devices, GFP mut3b, has an excitation maximum at 501 nm and an emission maximum at 511 nm; fluorescein has an excitation maximum at 494 nm and an emission maximum at 525nm).

You will prepare a dilution series of fluorescein in four replicates and measure the fluorescence in a 96 well plate in your plate reader. By measuring these in your plate reader, you will generate a standard curve of fluorescence for fluorescein concentration. You will be able to use this to convert your cell based readings to an equivalent fluorescein concentration. Before beginning this protocol, ensure that you are familiar with the GFP settings and measurement modes of your instrument. You will need to know what filters your instrument has for measuring GFP, including information about the bandpass width (530 nm / 30 nm bandpass, 25-30 nm width is recommended), excitation (485 nm is recommended) and emission (520-530 nm is recommended) of this filter.

Note: The iGEM Abs600 (OD) calibration protocol with microspheres calibration method is a pre-requisite for carrying out this protocol. You will need data from that calibration to analyse the results of this protocol.

RFP users: This protocol now includes instructions for the use of dyes compatible with some red fluorescent proteins (RFPs). Please note the Measurement committee is still investigating these so this part of the protocol should be considered a work in progress.

Attachments

XLSX

iGEM Data Analysis T...

37KB

Guidelines

For a full set of calibrations, you should run two protocols: this fluorescence calibration curve with fluorescein, and the Abs600 (OD) calibration with microspheres.

Before beginning these protocols, please ensure that you are familiar with the measurement modes and settings of your instrument. For all of these calibration measurements, you must use the same plates and volumes that you will use in your cell-based assays. You must also use the same settings (e.g., filters or excitation and emission wavelengths) that you will use in your cell-based assays. If you do not use the same plates, volumes, and settings, the calibration will not be valid.

Make sure to record all information about your instrument to document your experiment. If your instrument has variable temperature settings, the instrument temperature should be set to room temperature (approximately 20-25 C) for all measurements.

Materials

MATERIALS

96 well plate

PBS

Fluorescein

STEP MATERIALS

Fluorescein

PBS

Fluorescein is provided in the iGEM Measurement Kit. The 96-well plate should preferably be black with a clear flat bottom.

Protocol materials

PBS

96 well plate

PBS

Fluorescein

Fluorescein

Fluorescein

PBS

Before start

Read through this entire protocol carefully before you start your experiment and prepare any materials you may need.

Note: The iGEM Abs600 (OD) calibration protocol with microspheres calibration method is a pre-requisite for carrying out this protocol. You will need data from that calibration to analyse the results of this protocol.

Prepare the fluorescein stock solution

1 Spin down fluorescein kit tube to make sure pellet is at the bottom of tube

Fluorescein

2 Prepare the stock solution of your reference dye. See sub-step 2.1 for Fluorescein and 2.2 for dyes compatible with red fluorescent proteins (note recommendations for these are a work in progress).

2.1 Make a 10X fluorescein stock solution (100 µM) by resuspending fluorescein powder in 1mL of 1X PBS. If you are performing an RFP calibration, skip this and to go 2.2 below instead.

Note

It is important that the fluorescein is properly dissolved. To check this, after the resuspension you should 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.

PBS

2.2 [Work in progress]: Skip this step if you are using fluorescein and continue to step 3.

Two dyes are in testing by the Measurement committee for RFP calibrations: Texas Red and Rhodamine B.

- If you are using Texas Red, we recommend you follow the same protocol as fluorescein; make a 100 µM stock solution in PBS and then continue from step 4 below.

- If you are using Rhodamine B, we recommend you follow this protocol from step 4, but instead of using a 10 µM starting concentration, start with a 50 µM concentration of Rhodamine B and then follow the protocol normally.

3 Dilute the 10X reference stock solution with 1X PBS to make a 1X reference working solution with a concentration of 10 µM. E.g. dilute 100 µL of 10X fluorescein stock into 900 µL 1X PBS.

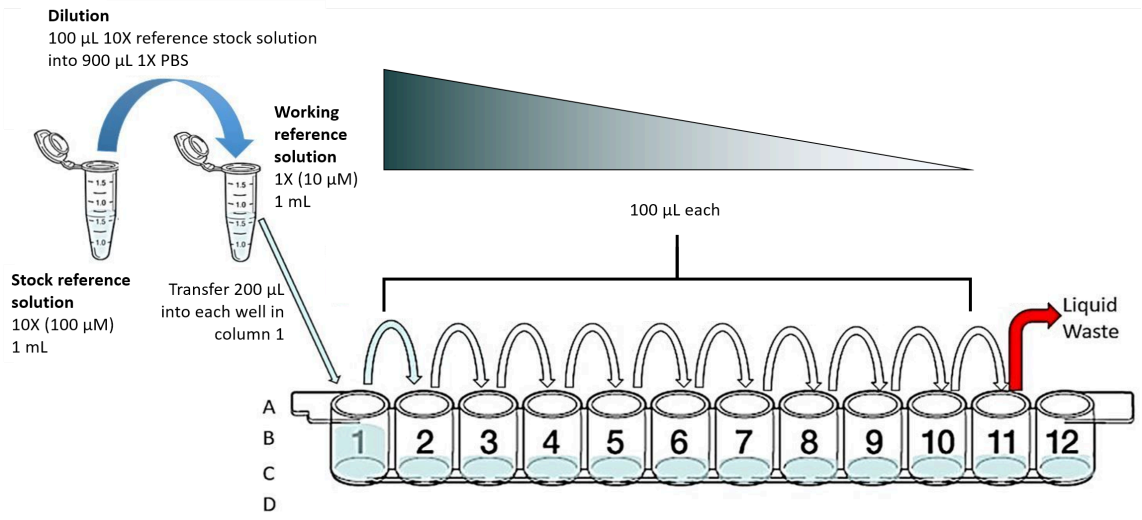
Note

If you are using Rhodamine B, dilute your reference solution to 50 µM instead of 10 µM then continue this protocol normally.

Prepare the serial dilutions of fluorescein

4 Accurate pipetting is essential. Serial dilutions will be performed across columns 1-11. **Column 12 must contain PBS buffer only.** Initially you will setup the plate with the reference working solution in column 1 and an equal volume of 1X PBS in columns 2 to 12.

You will perform a serial dilution by consecutively transferring 100 µL from column to column with good mixing.



5 Add 100 µL of 1X PBS into wells A2, B2, C2, D2....A12, B12, C12, D12

6 Add 200 µlL of fluorescein 1X stock solution into A1, B1, C1, D1

7 Transfer 100 µL of fluorescein stock solution from A1 into A2

8 Mix A2 by pipetting up and down 3x and transfer 100 µL into A3

9 Mix A3 by pipetting up and down 3x and transfer 100 µL into A4

10 Mix A4 by pipetting up and down 3x and transfer 100 µL into A5

11 Mix A5 by pipetting up and down 3x and transfer 100 µL into A6

12 Mix A6 by pipetting up and down 3x and transfer 100 µL into A7

13 Mix A7 by pipetting up and down 3x and transfer 100 µL into A8

14 Mix A8 by pipetting up and down 3x and transfer 100 µL into A9

15 Mix A9 by pipetting up and down 3x and transfer 100 µL into A10

16 Mix A10 by pipetting up and down 3x and transfer 100 µL into A11

17 Mix A11 by pipetting up and down 3x and transfer 100 µLl into liquid waste

Note

Take care not to continue serial dilution into column 12

18 Repeat dilution series for rows B, C, D

Measure fluorescence

19 Measure the fluorescence of all samples in your plate reader . Ensure that any automatic gain setting is off (if your instrument has one).

If you will be using your data in conjuction with measurements from the Plate Reader Abs600 (OD) Calibration protocol, make sure you use the same instrument settings for both protocols.

Protocol

NAME

Calibration Protocol - Plate Reader Abs600 (OD) Calibration with Microsphere Particles

CREATED BY

Paul Rutten

PREVIEW

[RFP calibration, work in progress]: Ignore this if you are using fluorescein. If you are not using fluorescein, remember to adjust your plate reader excitation and emissions settings as appropriate for your red fluorescent protein and the alternative dye you will be using.

Please also be aware you may need to change the wavelength at which you are taking your OD reading. If your fluorescent protein is absorbing at 600 nm, this will interfere with your Abs600 reading. We recommend measuring your OD at 660 nm instead, if this is beyond the excitation spectrum of your protein. You can check the excitation of many commonly use fluorescent proteins at **fpbase.org**. Please note OD readings at 600 and 660 nm cannot be directly compared.

19.1 Obtain the tube labeled “Silica Beads” from the Measurement Kit and vortex vigorously for 30 seconds.

300µl Silica beads

Note

Microspheres should NOT be stored at 0°C or below, as freezing affects the properties of the microspheres. If you believe your microspheres may have been frozen, please contact the iGEM Measurement Committee for a replacement (measurement@igem.org).

19.2 Immediately pipet 100 µL microspheres into a 1.5 mL eppendorf tube

19.3 Add 900 µL of ddH_2O to the microspheres

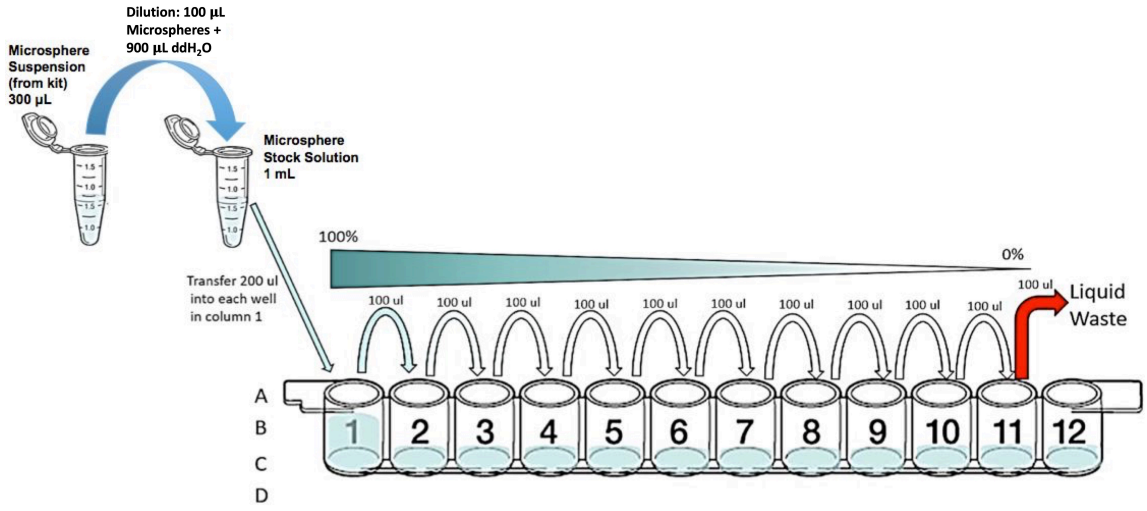
ddH20

19.4 Vortex well. This is your Microsphere Stock Solution

19.5 Accurate pipetting is essential. Serial dilutions will be performed across columns 1-11. **Column 12 must contain ddH_2O only**.

Initially you will setup the plate with the microsphere stock solution in column 1 and an equal volume of 1x ddH_2O in columns 2 to 12.

You will perform a serial dilution by consecutively transferring 100 µl from column to column with good mixing.



19.6 Add 100 µl of ddH_2O into wells A2, B2, C2, D2....A12, B12, C12, D12

19.7 Vortex the tube containing the stock solution of microspheres vigorously for 10 seconds

19.8 Immediately add 200 µl of microspheres stock solution into A1

19.9 Transfer 100 µl of microsphere stock solution from A1 into A2

19.10 Mix A2 by pipetting up and down 3x and transfer 100 µl into A3

19.11 Mix A3 by pipetting up and down 3x and transfer 100 µl into A4

19.12 Mix A4 by pipetting up and down 3x and transfer 100 µl into A5

19.13 Mix A5 by pipetting up and down 3x and transfer 100 µl into A6

19.14 Mix A6 by pipetting up and down 3x and transfer 100 µl into A7

19.15 Mix A7 by pipetting up and down 3x and transfer 100 µl into A8

19.16 Mix A8 by pipetting up and down 3x and transfer 100 µl into A9

19.17 Mix A9 by pipetting up and down 3x and transfer 100 µl into A10

19.18 Mix A10 by pipetting up and down 3x and transfer 100 µl into A11

19.19 Mix A11 by pipetting up and down 3x and transfer 100 µl into liquid waste

Note

Take care not to continue serial dilution into column 12

19.20 Repeat dilution series for rows B, C, D

19.21 **IMPORTANT!**

Re-Mix (pipette up and down) each row of your plate **immediately before** putting in the plate reader! (This is important because the beads begin to settle to the bottom of the wells within about 10 minutes, which will affect the measurements.)

Note

Take care to mix gently and avoid creating bubbles on the surface of the liquid

19.22 Measure OD_{600} of all samples in instrument. Disable any path length correction setting on your instrument, if it has one.

If you will be using your data in conjuction with measurements from the **Fluorescence standard curve** protocol, make sure you use the same instrument settings for both protocols.

19.23 Record the data in your notebook. Please note your standard curve should still work well even if a few of your measurements are saturing the instrument

19.24 Import data into this Excel sheet:

iGEM Data Analysis Template - Part...

19.25 You have now completed this calibration protocol

20 Record the data in your notebook. Also record the gain setting that you used in your instrument, if available. Please note your standard curve should still work well even if a few of your measurements are saturing the instrument

21 Import data into this Excel sheet provided (fluorescein standard curve tab):

iGEM Data Analysis Template - Flu...

Congratulations!

22 You have now completed this calibration protocol