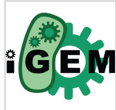

Feb 24, 2020

iGEM Calibration Protocol - Red Fluorescent Proteins in Plate Readers

Forked from Calibration Protocol - Plate Reader Fluorescence Calibration

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1 Works for me dx.doi.org/10.17504/protocols.io.bcdjis4n

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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 fluorescent protein would be an ideal way to standardize the amount of fluorescence in our E. coli cells, the stability of these proteins and the high cost of their purification are problematic. We therefore use the dye Texas Red, which has similar excitation and emission properties to many red fluorescent proteins, but is cost-effective and easy to prepare.

You will prepare a dilution series of Texas Red 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 Texas Red concentration. You will be able to use this to convert your cell based readings to an equivalent Texas Red concentration. Before beginning this protocol, ensure that you are familiar with the flourescence settings and measurement modes of your instrument. You will need to know what filters your instrument has for measuring red fluorescence, making sure it can adequately excite and record emmission. Recommended spectra for Texas Red are excitation at 596 nm and emmission at 620 nm.

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.

EXTERNAL LINK

https://2019.igem.org/Measurement

ATTACHMENTS

iGEM Data Analysis Template - Fluorescence Standard Curve - v1.xlsx

GUIDELINES

For a full set of calibrations, you should run two protocols: this fluorescence calibration curve with Texas Red, 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

NAME	CATALOG #	VENDOR
96 well plate		

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NAME	CATALOG #	VENDOR
PBS		
Texas Red		

STEPS MATERIALS

NAME	CATALOG #	VENDOR
Texas Red		
PBS		

MATERIALS TEXT

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

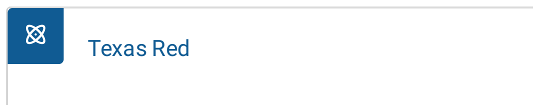
BEFORE STARTING

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

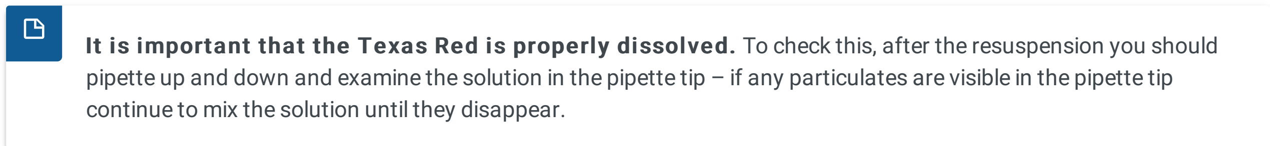
Note: The iGEM Abs600 (OD) calibration protocol with microspheres calibration method is a pre-requisite for carrying out this protocol. You will need data from that calibration to analyse the results of this protocol.

Prepare the Texas Red stock solution

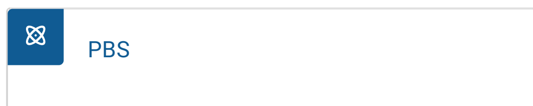
1 Spin down Texas Red kit tube to make sure pellet is at the bottom of tube



2 Make a Texas Red 10X reference stock solution (20 µM) by resuspending the dye powder in 1mL of 1X PBS.



It is important that the Texas Red 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.



3 **[OPTIONAL]** If you have access to a spectrophotometer, you can calculate the concentration of your Texas Red reference stock solution even more accurately using the Beer-Lambert law.

3.1 Measure the stock solution's absorbance at 586 nm and calculate concentration using an extinction coefficient of 82.786 mM^{-1} cm^{-1}.

3.2 Change the starting concentration value in the spreadsheet in step 21 to reflect your more accurate measurement when doing this. This value is defined in cell B1 in the "Texas Red Std curve" sheet. The rest of the document will update automatically after you make this change.

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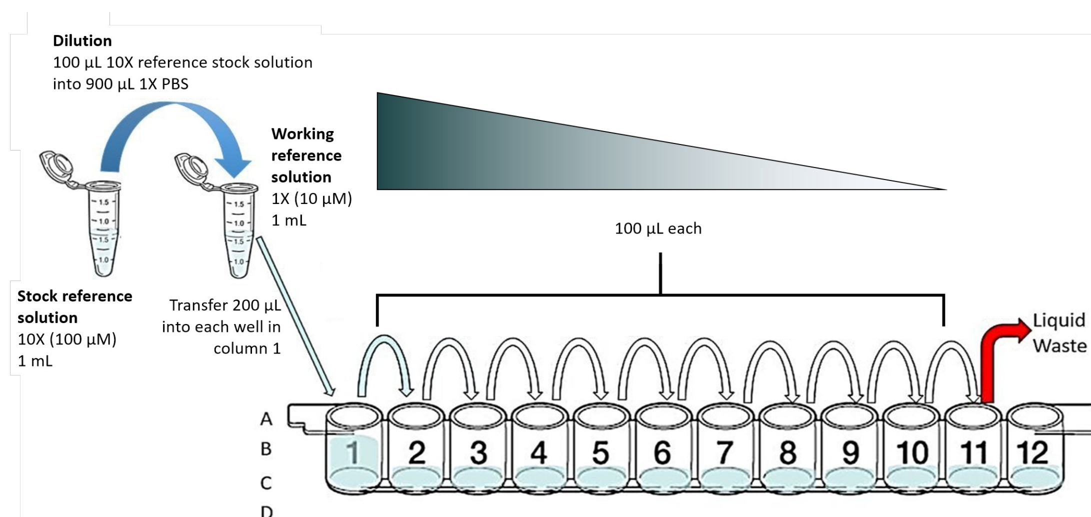
4 Dilute the 10X reference stock solution with 1X PBS to make a 1X reference working solution with a concentration of 10 µM. E.g. dilute 100 µL of 10X Texas Red reference stock into 900 µL of 1X PBS.

Prepare the serial dilutions of Texas Red in a 96-well plate

5 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.



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

7 Add 200 µL of Texas Red reference working solution (1X) into A1, B1, C1, D1

8 Transfer 100 µL of Texas Red reference working solution from A1 into A2

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

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

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

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

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

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

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

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

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

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

Take care not to continue serial dilution into column 12

19 Repeat dilution series for rows B, C, D

Measure fluorescence

20 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.

Please also be aware you may need to change the wavelength at which you are taking your OD reading when using red fluorescence. If your red 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.

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

by Paul Rutten,

The University of Oxford

PREVIEW RUN

Remember to set your plate reader's excitation and emission settings correctly for red fluorescence.

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

300µl Silica beads

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).

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

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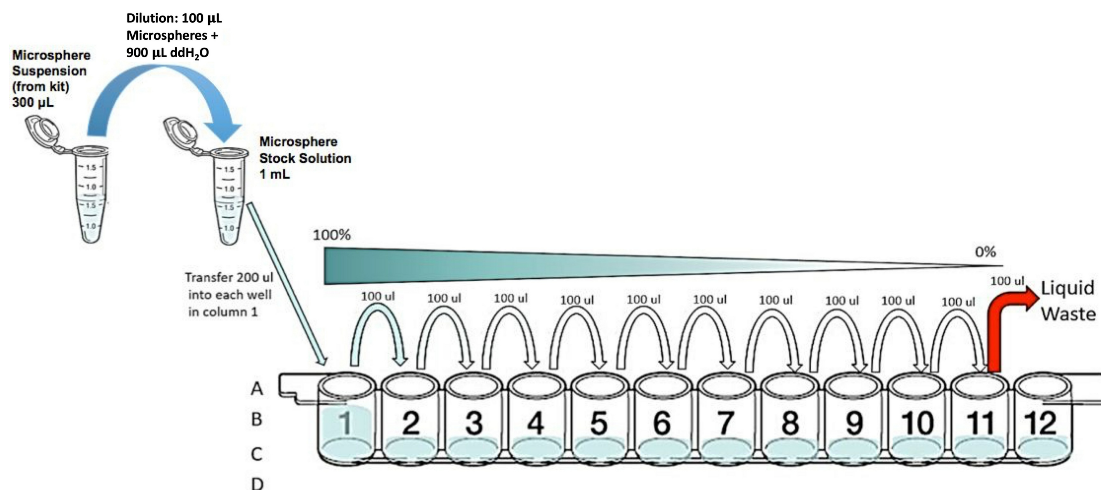
20.3 Add 900 µL of ddH_2O to the microspheres

20.4 Vortex well. This is your Microsphere Stock Solution

20.5 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.



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

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

20.8 Immediately add 200 µl of microspheres stock solution into A1

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

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

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

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

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

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

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

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

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

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

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

Take care not to continue serial dilution into column 12

20.20 Repeat dilution series for rows B, C, D

20.21 **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.)

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

20.22 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.

20.23 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

20.24 Import data into this Excel sheet:

20.25 You have now completed this calibration protocol

21 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

22 Import data into this Excel sheet provided (Texas Red standard curve tab):

Congratulations!

23 You have now completed this calibration protocol

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