2023 iGEM InterLab study

Derived from: InterLab 2022 - Calibration Protocol v2

Automated multicolor fluorescence per particle calibration protocol

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 biological devices, it is necessary to create a standard fluorescence curve. This variant of the protocol uses two replicates of three colors of dye, plus beads. Adapted from

- https://dx.doi.org/10.17504/protocols.io.bht7j6rn
- https://dx.doi.org/10.17504/protocols.io.6zrhf56.

Protocol in short: You will use the three color calibrants and the silica nanoparticles to perform serial dilutions from a known initial concentration. First, you will put 200uL of each solution in the first columns of the plate, and then you will aspirate 100uL and drop them into the corresponding well of the next column. After that you will mix very well by pipetting up and down three times, and then you will transfer again 100uL into the well in the next column. Repeating this until the 11th column and discarding those last 100uL. Finally you will fill all the wells to a total volume of 200uL with either water or PBS when appropriate. This step ensures that we have the same volume in the wells for calibration and for the experiments.

Important note: For the calibration and your experiments you must use the same type of plates and the same volumes. You must also use the same settings (e.g., filters or excitation and emission wavelengths) for the calibration and for the experiment. If you do not use the same plates, volumes, and settings, the measurements will not be valid.

Protocol Outputs:

- `fluorescein and bead fluorescence measurements of calibration plate`
- `sulforhodamine 101 fluorescence measurements of calibration plate`
- `cascade blue fluorescence measurements of calibration plate`
- `NanoCym 950nm monodisperse silica nanoparticle absorbance measurements of calibration plate`

Protocol Materials (from the Measurement Kit)

- Fluorescein (0.1 umoles dried down)
- Cascade Blue (0.1 umoles dried down)

- Sulforhodamine 101 (0.02 umoles dried down)
- NanoCym 950nm monodisperse silica nanoparticles (3e9 beads/mL)

Protocol Materials (provided by the Team)

- Water, sterile-filtered, BioReagent, suitable for cell culture
- Phosphate Buffered Saline 1X (pH 7.4)
- 96 well plate black with transparent flat bottom
- Foil-covered or amber tubes.
- Pipette for 1 mL (single)
- Plate reader able to measure absorbance at 600nm and fluorescence either with filters or monochromator.
- Opentrons OT-2
- OT-2 Pipette P300 (Gen 2) or OT-2 Pipette P1000 (Gen 2) [Link]
- Tip rack OT-2 compatible
- Tube rack OT-2 compatible (tested labware: aluminum block)

Protocol Steps:

Making the stock and working solutions

Note: Resuspend all the fluorescent calibrants in amber or foil-covered tubes and do not leave them out in the light for too long, as they are light sensitive.

Fluorescein

1. Provision the stock reagent tube with Fluorescein calibrant from the Measurement Kit. This is a powder. Spin down to make sure the pellet is at the bottom.
2. Transfer 1.0mL of Phosphate Buffered Saline (PBS) to stock reagent tube Fluorescein calibrant. Resuspend pipetting up and down a few times, and vortex 30 seconds. This is now the reconstituted Stock Fluorescein Solution 10X with a concentration of 100 uM in PBS.
3. Obtain a tube to make the working concentration solution 1X Fluorescein.
4. Dilute the Stock Fluorescein Solution 10X with 1X PBS to make a 1X reference working solution with a concentration of 10 uM. E.g. dilute 100 uL of 10X fluorescein stock into 900 uL 1X PBS.

Sulforhodamine 101

5. Provision the stock reagent tube containing Sulforhodamine 101 calibrant from the Measurement Kit. This is a powder. Spin down to make sure the pellet is at the bottom.
6. Transfer 1.0mL of PBS to stock reagent tube Sulforhodamine 101 calibrant. Resuspend pipetting up and down a few times, and vortex 30 seconds. The reconstituted Stock Sulforhodamine 101 Solution has a final concentration of 20 uM in PBS.
7. Obtain a tube to make the working concentration solution 1X Sulforhodamine 101.
8. Dilute the Stock Sulforhodamine 101 Solution 10X with 1X PBS to make a 1X reference working solution with a concentration of 2 uM. E.g. dilute 100 uL of 10X Sulforhodamine 101 stock into 900 uL 1X PBS.

Cascade Blue

9. Provision the stock reagent tube containing Cascade blue calibrant from the Measurement Kit. This is a powder. Spin down to make sure the pellet is at the bottom.
10. Transfer 1.0mL of Water to stock reagent tube Cascade Blue calibrant. Resuspend pipetting up and down a few times, and vortex 30 seconds. The reconstituted Stock Cascade Blue Solution 10X has a final concentration of 100 uM in Water.
11. Obtain a tube to make the working concentration solution 1X Cascade Blue.
12. Dilute the Stock Cascade blue Solution 10X with Water to make a 1X reference working solution with a concentration of 10 uM. E.g. dilute 100 uL of 10X Cascade blue stock into 900 uL water.

NanoCym 950nm monodisperse silica nanoparticles

13. Provision the stock reagent container containing NanoCym 950nm microspheres
14. This tube contains 850uL of NanoCym 950nm monodisperse silica nanoparticles with a concentration of 3e9 microspheres/mL in Water.
15. Vortex NanoCym 950 nm microspheres, they are ready to be used.

Calibration Protocol

Set the OT-2 deck

16. Place the tip rack in deck slot 9
17. Place the tube rack in deck slot 1
18. Place the 96 well plate in deck slot 7

Set the OT-2 tube rack

19. Place Fluorescein 1X in tube rack slot A1
20. Place Sulforhodamine 101 1X in tube rack slot A2
21. Place Cascade blue 1X solution in tube rack slot A3
22. Place NanoCym 950nm in tube rack slot A4
23. Place 1.5 mL tubes with 1.3 mL PBS in tube rack slots B1, B2, B3 and B4
24. Place 1.5 mL tubes with 1.3 mL water in tube rack slots C1, C2, C3 and C4
25. Close the OT-2 door

A1:sulforhodamine_1x	A2:fluorescein_1x	A3: cascade_blue_1x	A4: microspheres_1x		
B1: PBS	B2: PBS	B3: PBS	B4: PBS		
C1: Water	C2: Water	C3: Water	C4: Water		

Your tube rack should be like this.

Run the script

26. Open the Opentrons App in the computer connected to the OT-2
27. Load the script (run_iGEM2022_rgb_od_libre.py) in the Opentrons App
28. Run the script/protocol using the Opentrons App

Measurement

29. Measure Fluorescein fluorescence of calibration plate (wells A1:B12) with excitation wavelength of 488.0nm and emission filter of 530.0nm and 30.0nm bandpass.
30. Measure Sulforhodamine 101 fluorescence of calibration plate (wells C1:C12 and D1:D12) with excitation wavelength of 561.0nm and emission filter of 610.0nm and 20.0nm bandpass.
31. Measure Cascade blue fluorescence of calibration plate (wells E1:E12 and F1:F12) with excitation wavelength of 405.0nm and emission filter of 450.0nm and 50.0nm bandpass.
32. Measure NanoCym 950nm monodisperse silica nanoparticles absorbance of calibration plate (wells G1:G12 and H1:H12) at 600.0nm.
33. Import data for fluorescein fluorescence, sulforhodamine 101 fluorescence, cascade blue fluorescence, and absorbance measurements into the provided Excel file.

    Note: If your equipment does not have the exact wavelengths of bandpass wavelengths, choose the nearest one and take note (you will be asked for this information in the data uploading process).

Protocol version: 1.0a