Defluorination Assay

This protocol is to quantify enzymatic defluorination by measuring released fluoride with the alizarin–lanthanum complex. This protocol covers calibration of a media, enzyme screening, measuring and data analysis

Materials

- 5 mM Alizarin-3-methyliminodiacetic acid (Ali)
- 5 mM Lanthanum(III) nitrate hexahydrate (Lan)
- 1.68 M acetate buffer (pH 5.2)
- Acetone (>99.9%)
- 1 M and 1 mM NaF in Milli-Q
- Assay medium: 20 mM HEPES, tap water or demi water
- Strain of interest and its negative control
- M9 medium, fluorochemical of interest

Equipment

- Plate reader (400–700 nm spectral scan)
- Black 96-well microplate
- Multichannel pipette (P200) and reservoirs
- Micropipettes (P200, P1000)
- Centrifuge, shaking incubator
- Analytical balance, pH meter
- Aluminium foil, 1.5 mL tubes, 40 mL falcon tubes

Mix the reagents fresh before every measurement and store the stocks in the dark.

Well Composition

Component	Volume (µL)
Sample or standard	100
Alizarin dye (5 mM)	6
Lanthanum (5 mM)	6
Acetate buffer (1.68 M, pH 5.2)	15
Milli-Q water	98
Acetone, after incubation	75

Per well composition; multiply by the number of wells that will be used. Acetone is added only after the incubation, so keep it out of the pre-mix.

Calibration Curve

Run a calibration in every medium you measure in; the optimal wavelengths are a property of the medium, not of the assay.

1. Prepare standards of 0, 1, 2, 5, 10, 20, 30, 40, 60, 80 and 100 µM NaF in medium.
2. Pipette 100 µL of each standard into the plate in a predetermined layout (replicated >4).
3. Add 125 µL of master mix per well.
4. Incubate for 1 h, with the plate covered in aluminium foil.
5. Immediately add 75 µL acetone and scan each well from 400 to 700 nm.

This fixes the wavelength pair, the calibration line and the detection limits of one medium. Everything measured in that medium is then read against them.

6. Build a table of absorbance with wavelength as the index and one column per well, taking the well names from the first row of the sheet. Convert comma decimals to points, set blank cells to missing and keep only 400–700 nm.
7. Average the replicate wells of each standard into one mean spectrum per concentration, $\bar{A}_c(\lambda)$.
8. Find the isosbestic point: at every wavelength, take how far the spectra sit from the blank,

$$S(\lambda) = \mathrm{mean}_c \left| \bar{A}_c(\lambda) - \bar{A}_0(\lambda) \right| \tag{1}$$

   and take λ_{iso} as the wavelength between 480 and 640 nm where $S(\lambda)$ is smallest, which is where the spectra cross. This needs at least three concentrations, and is determined again for every medium.
9. Pin the denominator to λ_{iso}. There the absorbance does not change with fluoride, so the ratio $R = A_{\mathrm{num}}/A_{\mathrm{den}}$ moves only because the numerator moves.
10. Scan candidate numerators on a 5 nm grid between 620 and 680 nm, and discard a pair unless all of the following hold: the two wavelengths are at least 40 nm apart; the denominator carries real dye absorbance rather than a near-zero baseline; the ratio moves by at least 20% across the ladder; and the slope is positive.
11. Fit every surviving pair over the calibration range and keep its slope, R^2 and LOD. Accept the pairs with $R^2 \geq 0.95$, take the one with the lowest LOD, and lock it for that medium.
12. Fit $\hat{y}_i = b x_i + a$ by least squares, with x the nominal fluoride concentration and y the locked ratio, over at least four points covering three concentrations:

$$s_y = \sqrt{\frac{SS_{\mathrm{res}}}{n-2}}, \qquad R^2 = 1 - \frac{SS_{\mathrm{res}}}{SS_{\mathrm{tot}}} \tag{2}$$

   where $SS_{\mathrm{res}} = \sum (y_i - \hat{y}_i)^2$ is the scatter about the line and $SS_{\mathrm{tot}} = \sum (y_i - \bar{y})^2$ the total scatter about the mean. Read R^2 as descriptive only, since it rises with the width of the concentration range.
13. Calculate the detection limits from the slope b and a standard deviation σ:

$$\mathrm{LOD} = \frac{3.3\,\sigma}{b}, \qquad \mathrm{LOQ} = \frac{10\,\sigma}{b} \tag{3}$$

   Take σ as the standard deviation of the 0 µM wells when there are more than two blanks, and as the residual standard deviation s_y of the fit when there are no replicates.
14. Find the linear ceiling: sort the concentrations from low to high, fit the lowest three, then the lowest four, and so on. The highest concentration at which the fit still holds $R^2 \geq 0.98$ is the ceiling, and above it the response saturates.

Reference values in demi water: 545 nm isosbestic, 630 nm numerator, linear range 0–40 µM, $R^2 = 0.996$, LOD 3.8 µM and LOQ 11.6 µM. Signal saturates above 40 µM

Growing, Washing and Incubating the Cells

15. Grow the strains overnight in M9 with the appropriate antibiotic and inducer.
16. Spin down at 3000 × g for 10 min, resuspend the pellet in 3 mL 20 mM HEPES, and repeat this wash once. Washing removes the growth medium, which otherwise interferes with the assay.
17. If possible, normalise all the samples to the same OD.
18. Resuspend the washed pellet in 3 mL of 20 mM HEPES containing 5 mM of the fluorochemical, and incubate.
19. Take 250 µL samples at timepoints of interest. Spin each at 3000 × g for 10 min, harvest the supernatant and store it at −20 °C.

Running the Measurement

20. Load 100 µL of each supernatant into the plate, at least in duplicate, alongside the blanks and the negative-control strain.
21. Add 125 µL master mix, incubate for 1 h under foil, add 75 µL acetone and scan from 400 to 700 nm.
22. Dilute and re-measure any sample that falls outside the linear range.

Data Analysis Enzyme Defluorination

23. Build the table of absorbance as for the calibration, keeping only 400–700 nm.
24. Divide the two locked wavelengths of the medium's calibration to get each well's ratio $y = A_{\mathrm{num}}/A_{\mathrm{den}}$.
25. Re-zero the plate on its own negative-control strain, so that the general loss of signal caused by incubation is taken out.
26. Convert each well to fluoride with the calibration line and multiply by the dilution factor:

$$x = \frac{y - a}{b} \tag{4}$$

27. Report values below the LOD as not detected, and re-dilute and re-measure anything above the linear ceiling.