EROD assay protocol

Revision 1.3; 2010-09-14

Materials

1. 60mM Tris, pH 7.1-7.4@37C; store at RT or 4C
2. 50mM Tris, pH 7.1-7.4@37C; store at RT or 4C
3. 100uM Ethoxyresorufin (7-ER) stock **[20x]:** 1mg/41.45mL (24.3ug/mL); FW = 241.24
   Dissolve 1mg of 7-ER in 41.32mL of 15% MeOH, 85% 60mM Tris (pH 7.1-7.4); store at -20C in 1mL aliquots. Keep away from light and minimize repeated freeze/thawing. Note: Light should not be a factor for up to 2hrs at RT, and a few freeze/thaw cycles are acceptable.
4. 100ng/uL Resorufin std: 1mg/10mL (100ng/uL); FW = 213.19
   Dissolve 1mg of Resorufin (NOT the sodium salt) in 85% 60mM Tris (pH 7.8)/15% MeOH; store at -20C
5. BSA, 5.32mg/mL in 50mM Tris, pH 7.1-7.4 **[5x]**: 80.6uM; FW = 66000
   Dissolve 5.32mg of BSA in 50mM Tris, pH 7.1-7.4; sterile filter and/or freeze aliquots for long-term use. Store at 4C or -20C. Minimize freeze/thawing.
6. NADPH, 6.7mM in 50mM Tris, pH 7.1-7.4 **[6.67x]**: 1mg/130.7uL (7.65mg/mL); FW = 833.35
   Dissolve 25mg in 4.4mL 50mM Tris, pH 7.1-7.4; store at -20C
7. 85% 60mM Tris/15% MeOH (abbreviated as Tris/MeOH in this protocol); store at RT or 4C
8. 2M Glycine, pH 10.3-10.4@RT: 15g/100mL H20 (FW = 75.1); pH with 10N NaOH; store at RT or 4C
9. Fluorescence plate reader @535-550nm excitation, 570-590nm emission (preferred- much more sensitive) OR spectrophotometer @572nm (much less sensitive, by at least 1000x)

EROD rxn mix: quick reference

Reagent	Volume (uL) per 100uL rxn mix	Volume (uL) per 250uL rxn mix
Tris, 50mM, pH 7.4	67.5	168.75
BSA, 5.32mg/mL in 50mM Tris	20.0	50.0
7-ER, 100uM, in MeOH/50mM Tris 15%/85%	5.0	12.5
NADPH, 6.7mM in 50mM Tris	7.5	18.75

EROD rxn mix: final concentrations

A. BSA = 1.0mg/mL
B. 7-ER = 5uM
C. NADPH = 0.5mM

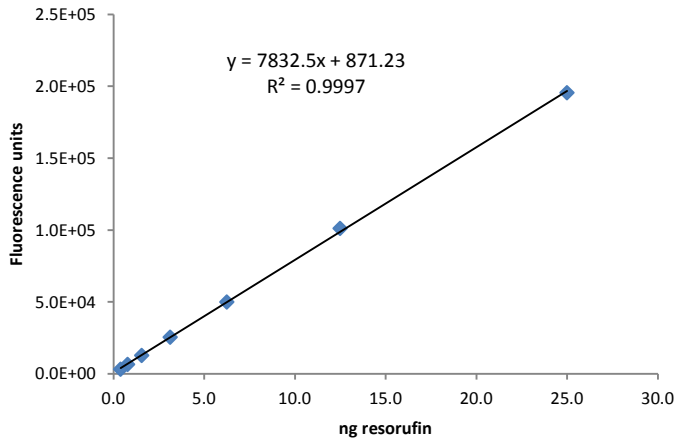

Figure 1- Typical (fluorescence) standard curve for resorufin in 1.25:1.00 ratio of Rxn mix:2M Tris

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Resorufin standard—for spectrophotometer

In an aqueous solvent, when fully dissolved, resorufin has a brilliant fuschia color. Before full dissolution, resorufin exists as a dull-red, opaque to semi-opaque solution.

1. In a 1.7mL microfuge tube, weigh out a minimum of 1mg of resorufin on an analytical scale.
2. Add 1mL of Tris/MeOH and transfer as much solution as possible from the microfuge tube into a 15-50mL conical tube.
3. Add 1mL of Tris/MeOH and transfer as much solution as possible from the microfuge tube into a 15-50mL conical tube. Repeat 3-4 more times to ensure that as much actual resorufin was transferred from the microfuge tube into the conical.
4. Confirm the total volume in the conical tube, and add additional Tris/MeOH to reach 100ng of resorufin/mL of Tris/MeOH (1mg resorufin = 10mL final volume).
5. Dilute the 100ng/uL resorufin solution (will still be cloudy/dull red at this stage) 1:1 with Tris/MeOH to make a 50ng/uL solution (this should be much clearer, as well as a more brilliant red/fuschia color).
6. Your top standard will be 50ng/uL. Make 2x serial dilutions in Tris/MeOH until you have seven total standards, from 50ng/uL→0.78125ng/uL.
7. When making your standard curve, add 10uL of your standards into your diluents. At 572nm, you should have near-perfect linearity within the middle five standards. Blank with Tris/MeOH.

Note: Your second standard, which should be a total of 250ng of resorufin, corresponds to the theoretical upper limit of resorufin that can be generated in the assay, assuming 5uM of 7-ER in 200uL of final reaction volume (241.24ng 7-ER total). The upper limit of linearity for this assay using absorbance is ~300ng, while the lower limit is ~30ng.

Resorufin standard- for fluorimetry—THIS IS PREFERRED

Prepare standards as outlined for spectrophotometer protocol, but start top standard at 5ng/uL and make serial dilutions down to 0.078125ng/uL. You should have perfect linearity throughout the entire standard curve, with the possible exception of your top standard. Again, add 5-10uL of each standard for your standard curve. Using fluorescence, the lower (linear) limit of sensitivity is ≤ 30pg of resorufin.

Plate preparation- frozen

A. For plates that you wish to freeze down for later analysis, remove plate from incubator and place on ice.
B. Wash with cold 1x PBS twice and aspirate off all liquid.
C. Keeping plate on ice, transfer to -80C
D. Store for up to 3-4 weeks

Plate preparation- live

A. Prepare and warm EROD reaction mix- does not need to be sterile, but make sure that the individual components are at least clean (i.e. no visible growth—this is mainly an issue with the BSA).
B. In the hood, wash plates with warm cell culture grade, sterile 1x PBS, twice.
C. Aspirate all media and pipette the desired volume of EROD reaction mix into cells- you must add sufficient volume to completely cover the cells, as you will not be shaking for a live protocol
D. Place cells back into incubator and start your timer.
E. See steps

Pre-assay preparation (steps relevant to frozen plate protocol only are labeled with "[F]")

A. Tray with ice, with sufficient space to accommodate the number of plates you wish to run at the same time **[F]**
B. Aluminum foil for each plate, if your shaker/incubator is not in a dark location **[F]**
C. Tape to attach plates to shaker/incubator **[F]**
D. Timer
E. EROD reaction mix
F. Labeled microfuge tubes for transferring completed EROD assay buffer
G. Black –walled solid or clear-bottom 96-well plate

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Running the assay

For live assays in 12-well plates, the rxn volume should be 300uL, which will be sufficient to cover the entire surface area even without shaking. With frozen cells, you may use as little as 200uL, but will need to shake (>200RPM). 250uL is the recommended volume for frozen cells in a 12-well plate; for a 24-well plate, 125-150uL is sufficient with shaking.

1. If using a frozen plate, remove plate from -80C and thaw rapidly in a 37C waterbath. Make sure you do not get any water into your wells. Place thawed plate on ice.
2. (On ice) Pipette the desired volume of EROD reaction mix into your plates. Make sure you dispense the liquid down the sides of the plate, and not directly onto the cells (for frozen, you obviously do not have to worry about damaging the cells, as they are already dead, but you still do not want to lift the cells off of the plate).
3. (On ice) Wrap plate in foil.
4. Incubate for 15-20 minutes at 37C with shaking (according to the literature, the assay is linear for only the first 30 minutes, but you must determine empirically whether this holds true for your conditions).
5. Terminate the assay by placing your plate(s) on ice; using a repeater pipette, add 2M glycine (pH 10.3-10.4 @RT) in a ~1:1.5 ratio of 2M glycine : Total EROD rxn volume (e.g. 200uL 2M glycine : 250-300uL EROD rxn mix). An exact ratio is not important, but if you must deviate, err on the side of more, rather than less glycine. The addition of glycine will permanently terminate the reaction.
6. (You may now proceed at room temperature) Pipette all or a fixed fraction of the now-terminated reaction mixture into microfuge tubes.
7. Spin supernatent for 1-2 minutes at top speed to pellet any cellular debris. 4C is preferred, although RT will suffice.
8. Pipette a fixed fraction of the supernatent into a microplate suitable for fluorescence. If you wish to add liquid to the wells to rinse your pipette, use 50mM tris.
9. For your standards, pipette the same volume of the same ratio of reaction mix : 2M glycine, plus your standards. Pipette the same volume of your standard blanking solution into all non-standard wells to ensure that the volume and composition of each well is identical.
10. Read the plate at specified excitation/emission wavelengths. A 0.5-1.0s integration time should be sufficient.