

Appendix B1

Protocol for CV1 + hAR + Luciferase Assay

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6 cm dish CV1 LUCIFERASE ASSAY (hAR)

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Monday

1. recount cells: best to count 50-100 cells per 5x5 in hemocytometer, count 2 5x5 grids and average, count $\times 10^4 =$ cells/ml
2. plate 0.42×10^6 CV1 cells/6 cm dish by preparing large mix of cells and media so 4 ml media/plate containing 5% bovine calf serum, DMEM-H/20 mM Hepes (2 M Hepes stock, pH 7.2, filter), penicillin and streptomycin, 2 mM L-glutamine, spread cells evenly. Cells usually added from master mix; swirl often while adding cells to the plates.

Tuesday

prepare CaPO_4 precipitates for groups of up to 6 plates using freshly made solutions: for large assays of same DNA, pool the precipitates before adding to the plates.

(a) make 2 M CaCl_2 : 2.94 g $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ bring to 10 ml with sterile autoclaved dH_2O , filter sterilize

(b) make 2X HBS: 500 ml 8.2 g NaCl
 12.5 ml 2 M Hepes
 0.2 g $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$

bring to 500 ml with sterile autoclaved dH_2O from TC room, pH with 5 N NaOH (takes 150-200 μl), pH to 7.11-7.14, sterile filter, make 27.5 ml aliquots (25 ml needed for 100 6 cm plates), store frozen at -20°C

[for 50 ml 2XHBS: 14 ml 1 M NaCl, 0.25 ml 2 M Hepes Na salt, 750 μl 0.1 M Na_2HPO_4 , bring to 45 ml with sterile ddH_2O (use sterile autoclaved water), add about 45 μl 5 M NaOH, pH to 7.11-7.14, filter sterilize, store pH electrode in pH 7 buffer, NOT H_2O]

(c) prepare DNA one or more days before assay

make dilutions of DNA stocks so additions are ~ 3 -10 μl

add expression and reporter DNA to bottom of 14 ml polystyrene round-bottom (17x100 mm) Falcon tubes, store frozen -20°C

50 ng pCMVhAR (or 10 ng pCMVhAR1-660 (ABC))

5 μg MMTV-luciferase

[For PSA-luciferase use 5 μg reporter/dish, 100 ng pCMVhAR/dish]

(d) per 6 cm dish, add to tubes containing DNA:

210 μl sterile H_2O

30 μl 2 M CaCl_2 (final 0.125 M CaCl_2)

240 μl 2X HBS, vortex briefly, let sit 30 min at RT

vortex briefly, add 475 μl of mix per well, return plates to incubator, incubate 4 h

example for 6 dishes:

DNA	H_2O	2 M CaCl_2	2XHBS
0.3 μg pCMVhAR	1.26 ml (2 x 630 μl)	180 μl	1.44 ml (2 x 720 μl)
30 μg MMTV-Luc			

- (e) aspirate plates, add 1.5 ml glycerol shock medium, incubate 3 min RT, aspirate, wash 4 ml PBS, aspirate, add 4 ml serum free, phenol red free DMEM-H, Hepes, P/S, glutamine \pm hormone, return to incubator for overnight

Glycerol Shock Medium: use 5% DMEM-H red

dishes \times 1.5 ml/dish = total volume (make extra)

total volume \times 15% = amount of glycerol

total volume – amount of glycerol = amount of media to add with glycerol

Wednesday aspirate media, add 4 ml fresh phenol red-free, serum-free DMEM-H, P/S, Hepes, glutamine ± hormone, add DHT to stock media as needed and add to plates, incubate overnight 37°C

Thursday remove media, wash with 4 ml PBS, aspirate to dry; add 0.5 ml/plate lysis buffer; rock plates 20-30 min, not much longer at RT. Transfer 100 µl from each well to 96 well Nunc flat bottom standard nontreated white plate. Read on LumiStar automated luminometer that injects 100 µl luciferin stock and 100 µl reading buffer

Lysis buffer: 2 mM EDTA, 1% Triton X-100, 25 mM Trizma (Tris base) phosphate, pH 7.8

D-Luciferin: prepare 1 mM D-luciferin (K⁺ salt, MW 318.41) in dH₂O store in 10 ml aliquots at -20°C covered with foil (D-luciferin is light sensitive) use 100 µl/sample, save extra at -20°C, Na⁺ salt sometimes turns yellow but is probably still good, pH of H₂O might be off, better to use K⁺ salt) (from Analytical Luminescence)

Reading buffer: The optimal pH for the reaction is pH 7.8 cold; if glycylglycine and ATP are carefully pH cold, then the final will be pH 7.8

Stock	Amount to <u>20 ml final</u>	Amount to <u>100 ml final</u>	<u>Final conc</u>
0.5 M glycylglycine, pH 7.8 cold	1 ml	5 ml	25 mM
1 M MgCl ₂	300 µl	1.5 ml	15 mM
100 mM ATP in dH ₂ O (bring to pH 7.8 cold with 1 M NaOH, CRITICAL, store -80°C 1 ml aliquots) Sigma - tissue culture grade	1 ml	5 ml	5 mM
50 mg/ml BSA dH ₂ O	200 µl	1 ml	0.5 mg/ml
dH ₂ O	17.5 ml	87.5 ml	
need 40 ml for 100 plates			