Proximity ligation assay on coverslips Entered by Kevin Janes
Janes Lab Protocols 7/18/22

1. Plate cells at the desired density on 12 mm round No.1.5 coverslips (Thomas #1217N79) at the base of a 24-well plate.
   - *The DuoLink PLA Fluorescence protocol uses reactions of 40 µl total volume on a surface of 1 cm^2*
   - *Our protocol uses 25 µl total volume per coverslip to preserve reagents*
   - *No. 1.5 coverslips provide the proper working distance for microscope objectives*
2. Aspirate the culture medium and wash cells with 500 µl PBS.
3. Fix coverslips in freshly prepared 3.7% PFA and incubate for 15 min at room temperature.
   - *Stock solutions of 37% PFA should be prepared according to Wang et al.* Meth. Enzymol*. 85:514 (1982) and stored in single-use aliquots at –20°C*
   - *Fix slides in a fume hood and dispose of PFA as hazardous waste*
4. Wash coverslips 3 × 5 min in PBS.
5. Block slides in a humid chamber for 1 hr at room temperature in blocking solution: 1× Western Blocking Reagent (Roche #11921673001) diluted in PBS + 0.3% Triton X-100.
   - *Humid chambers can be constructed with an inverted pipette-tip box containing a paper towel saturated with water*
   - *Coverslips should be handled with fine foreceps and lifted off the bottom of the 24-well plate with a curled hypodermic needle while the PBS is still in the well*
   - *25 µl of blocking solution is sufficient for each coverslip*
   - *Place each coverslip face-down on a piece of Parafilm in the humid chamber*
6. Add primary antibody at the appropriate dilution in blocking solution overnight at room temperature.
   - *25 µl of primary-antibody solution is sufficient for each coverslip*
   - *Antibody dilutions must be determined empirically, although 1:100 or 1:200 dilutions are common*
   - *Place each coverslip face-down on a piece of Parafilm*
7. Wash coverslips 2 × 5 min in 1x Wash Buffer A at room temperature.
   - *Washes can be done in a clean 24-well plate*
8. Vortex PLUS and MINUS PLA probes and dilute each 1:5 in the DuoLink Antibody Diluent
   - *5 µl PLUS probe + 5 µl MINUS probe + 15 µl Antibody Diluent per coverslip*
   - *Prepare as a master mix for all samples*
9. Add PLA probes and incubate in a humid chamber for 1 hr at 37ºC.
10. Wash coverslips 2 × 5 min in 1x Wash Buffer A at room temperature.
    - *Washes can be done in the same 24-well plate as before*
11. Dilute 5x DuoLink Ligation Buffer to 1x in ddH_2O and dilute Ligase 1:40 in 1x Ligation Buffer.
    - *0.625 µl Ligase in 25 µl 1x Ligation Buffer per coverslip*
    - *Prepare as a master mix for all samples*
12. Add Ligase and incubate in a humid chamber for 30 min at 37ºC.
13. Wash coverslips 2 × 5 min in 1x Wash Buffer A at room temperature.
    - *Washes can be done in the same 24-well plate as before*
14. Dilute 5x Amplification Buffer to 1x in ddH_2O and dilute Polymerase 1:80 in 1x Amplification Buffer.
    - *0.3125 µl Polymerase in 25 ml 1x Amplification Buffer per coverslip*
    - *Prepare as a master mix for all samples*
15. Add Polymerase and incubate in a humid chamber for 100 min at 37ºC.
16. Wash coverslips 2 × 10 min in 1x Wash Buffer B at room temperature.
    - *Washes can be done in the same 24-well plate as before but different wells on the plate*
17. Wash coverslips in 0.01x Wash Buffer B for 1 min.
    - *Dilute 1x Wash Buffer B to 0.01x in ddH_2O immediately before use*
    - *Washes can be done in the same 24-well plate as before*
18. Mount coverslips with 10 µl DuoLink InSitu Mounting Media with DAPI.
19. Seal the edge of the coverslip with nail polish and allow to air dry.
20. Remove residual salts with a Kimwipe and air dry.
21. Store slides at 4ºC until imaging.

Buffer recipes

- 1x Wash Buffer A (for 1 L total volume):
  One Wash Buffer A pouch
  1000 ml ddH_2O

  Store a 4ºC long term and bring working aliquot to room temperature before use

- 1x Wash Buffer B (for 1 L total volume):
  One Wash Buffer B pouch
  1000 ml ddH_2O

  Store a 4ºC long term, bring working aliquot to room temperature before use, and dilute to 0.01x at room temperature immediately before use