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ENDOCRINE UNIT

Center for Skeletal Research

Histology & Histomorphometry Core

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Protocol for IHC of phosphoERK in growth plate (with tyramide amplification, Perkin Elmer, Cat. No. NEL700)

Controls: 1. no 1st Ab, or 1:100 nonspecific IgG
2. no amplification

Preparation of reagents:

1. 90% methanol, 10% DMSO (for 200ml need 180ml methanol, 20ml DMSO)
2. 1l 1x PBS
3. 0.3% H2O2 (for 200ml need 2ml 30%H2O2 in 200ml methanol)
4. Biotinyl Tyramide: add DMSO (0.3 ml [NEL700A] or 1.2 ml [NEL700]), store at 4C (stable 6 months)
5. Biotinyl Tyramide Working Solution: dilute stock 1:50 with 1x Amplification Diluent, 100ul/slide
6. Wash Buffer (TNT; 0.1M Tris, 0.15M NaCl, 0.05% Tween, pH 7.5): for 3l need 300ml 1M Tris, pH 7.5, 90ml 5M NaCl, 1.5ml Tween, 2610ml water
7. Blocking Buffer (TNB; 0.1M Tris, 0.15M NaCl, 0.5% Blocking Reagent, pH 7.5): for 10ml need 1ml 1M Tris, 0.3ml 5M NaCl, 0.05g Blocking Reagent, heat to 60C to completely dissolve Blocking Reagent, can be stored at -20C in aliquots, at RT no longer than 24h
8. SA-HRP (1:100 in TNB buffer)
9. DAB kit for visualization

Procedural notes:

1. need humidified chamber (slide box with damp paper towels) for ALL incubation steps
2. staining tubs for incubations need volumes of 200ml

Protocol:

1. heat paraffin slides for 30min at 55C to melt wax
2. deparaffinize slides in 2 changes of xylene x 5min
3. rehydrate slides in series of graded alcohol 100%, 80%, 70% x 5 min
4. incubate slides in 90% methanol, 10% DMSO for 20min at RT
5. wash with PBS
6. incubate with 0.3% H2O2 for 20min in methanol
7. wash with PBS
8. block slides with 100ul TNB for 30min in humidified chamber at RT, drain off
9. apply 100ul of 1st Ab (1:500 anti-phosphoERK in TNB buffer, Cell Signaling, Cat.No. 4377S) for overnight at 4°C
10. wash 3x5min with TNT buffer at RT with agitation
11. apply 100ul 2nd biotinylated Ab (1:300 anti-rabbit biotinylated IgG in TNB, Vector, Cat.No. BA1000) for 1h at RT
12. wash 3x5min with TNT buffer
13. apply 100ul SA-HRP (diluted 1:100 in TNB) for 30min at RT
14. wash 3x5min with TNT buffer
15. apply 100ul Biotinyl Tyramide Working Solution for 5 min at RT

16. wash 3x5min with TNT buffer
17. add 100ul of SA-HRP and incubate 30min at RT
18. wash 3x5min with TNT buffer
19. visualize with chromogenic substrate 5min in the DARK
    - 5ml H2O
    - 4 drops DAB
    - 2 drops buffer
    - 2 drops H2O2
    - 2 drops NiCl (optional: will give grey/black stain as compared to brown with DAB only)

look for signal while developing

YES

20. wash with distilled water until desired signal intensity achieved (1-3min)
21. counterstain with hematoxylin (5sec; Histo-core sequence) or toluidine blue (see separate protocol)

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