

**IHC protocol for mice Anti-phosphoSMAD3  
(rabbit polyclonal ab-51177 Abcam)**

**Day1**

Paraffin Sections

1. Heat paraffin slides for at least 1hr at 55C to melt wax
2. Deparaffinize slides in 2 changes of xylene x 5min each
3. Rehydrate in graded alcohol 100% → 95% → 70%, 3min each
4. PBS wash 5min
5. **Antigen Retrieval 1:** Incubate with 90% methanol+10%DMSO 20min RT. Wash PBS 3min
6. **Endogenous peroxidase blocking** slides in **0.3%** H<sub>2</sub>O<sub>2</sub> in methanol(200mL methanol+2mL H<sub>2</sub>O<sub>2</sub>): 20min RT
7. **Antigen Retrieval 2:** Citrate buffer pH=6.0 →20 min water bath 95-97C. Wait 30 min
6. PBS Wash 5 min
8. **Endogenous unspecific protein blocking** in TNB 30 min RT
- Do Not Wash
9. **Primary AB-** 1:100 anti-pSMAD3 in TNB 4C overnight

**Day 2**

10. TBST wash 3x 5min each
11. **Secondary AB-1:300** anti-rabbit Biotinylated in TNB 1HR RT
12. TBST wash 3x 5min each
13. **SA-HRP** (diluted 1:100 in TNB ) 30min RT
14. TBST wash 3x 5min each
15. **Biotinyl Tyramide** working solution 10min RT
16. TBST wash 3x 5min each
17. SA-HRP (diluted 1:100 in TNB) 1hr RT
18. TBST wash 3x 5min each
19. DAB Detection
  1. 5ml ddH<sub>2</sub>O +2 drops buffer + 4 drops DAB + 2 drops H<sub>2</sub>O<sub>2</sub>
20. Stop reaction in ddH<sub>2</sub>O
21. Counterstain with Fastgreen 1 min → Acetic Acid 30 sec → ddH<sub>2</sub>O
22. Dehydrate in graded alcohol 70% → 95% → 100% → Xylene
23. Coverslip