Western Blot/Anti-pCREB

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1. Treat cells. For PC12 or NIH 3T3 cells a moderately confluent 60 mm plate is used.

2. Aspirate media. Rinse cells with PBS, aspirate.

3. Lyse cells in 200 uL of SDS sample buffer (100ºC). Immediately scrape the cells off the plate with a rubber policeman.

4. Put the extract into an eppendorf and boil for 5 min., cool on ice.

5. Load onto 10% SDS-PAGE. I usually load about 1/5 of the lysate from a 60 mm dish. This is about 100 ug protein for PC12 cells.

6. After electrophoresis, electrotransfer to nitrocellulose membrane. I include in the SDS-PAGE prestained molecular weight markers to check the efficiency of the transfer and for M.W. determination.

7. After transfer, rinse the blot 2 times with TBST (10 mM Tris (pH 7.4), 150 mM NaCl, 0.05% Tween-20).

8. Block with 4% BSA in TBST for 45–60 minutes. I use RIA grade BSA from USB (United States Biochemical; Catalog number 10868). I also include 0.02% NaN_3 in this block and reuse it at least 5 times (keep in 4ºC between uses). We have had problems obtaining good signals when we've used poor quality BSA. Others have told me that 10% nonfat dry milk works well for anti-pCREB, but in my hands milk is not as good as BSA from USB (it seems to mask the epitope).

9. Remove block solution and add primary antibody. For anti-pCREB (IgG, 0.7 mg/ml), I use 1:5,000–1:20,000 dilution in 4% BSA in TBST. This should be titrated for optimal results. I incubate with primary Ab 3 hours - overnight at room temp with shaking.

10. Remove the antibody solution and wash the blot 3 times, 5–7 minutes each, with 0.5% BSA in TBST.

11. Incubate with Goat anti-Rabbit/alkaline phosphatase (from Promega; 1:7500 in TBST with 5% BSA) for 1 hour at room temp with shaking. I have also used successfully Amersham ECL detection, which employs goat anti-Rabbit HRP.

12. Wash filter 3 times 7 minutes with 0.5% BSA in TBST.

13. Perform alkaline phosphatase reaction using NBT and BCIP substrates. I find that CREB usually comes up within several minutes of adding the AP substrates, however a one-hour reaction time may be necessary.