Western Blot Analysis: Detection of Proteins using Alkaline Phosphatase

Section of Cancer Genomics, Genetics Branch, NCI
National Institutes of Health

Reagents

Alkaline Phosphatase Substrate Kit
Vector, Cat. SK-5400
Glycine
BioRad, Cat. 161-0716
Methanol
Non-fat Dry Milk (NFDM)
Carnation Brand, Grocery Store of Choice
PVDF Membrane
BioRad, Cat. 162-0181
Sodium chloride (NaCl)
Mallinckrodt, Cat. 7581
Tween 20
Sigma, Cat. P1379
Tris, 1 M, pH 7.5
Quality Biological, Inc., Cat.351-006-100
Tris, 1 M, pH 9.5
Tris Base
BioRad, Cat. 161-0717
Vectastain ABC-AP Kit
Vector, Cat. AK-5001 (Rabbit Ig)
Vector, Cat. AK-5002 (Mouse Ig)
Water, Milli-Q

Preparation

10X Running Buffer

Tris Base	30.3 g	f.c. [250 mM, pH 8.3]
Glycine	144.2 g	f.c. [1.92 M]
Milli-Q Water	1.0 L	

Store at 4˚C

1X Running Buffer

10X Running Buffer	100 ml	
Milli-Q Water	890 ml	
10% SDS	10 ml	f.c. [0.1%]

Transfer Buffer

Tris Base	3.03 g	f.c. [25 mM, pH 8.3]
Glycine	14.4 g	f.c. [192 mM]
Milli-Q Water	1.1 L	

10X TTBS

1M Tris (pH 7.5)	500 ml	f.c. [0.5 M]
NaCl	87.66 g	f.c. [1.5 M]
Tween 20	10 ml	f.c. [1%]

*Bring volume to 1 L with sterile distilled water
Store at 4˚C

1X TTBS

10X TTBS	100 ml
Milli-Q Water	900 ml

Store at 4˚C

Procedure

1. Run Western gels in pre-chilled 1X Running Buffer at 200 V 60 min in ice bucket.

2. Transfer to PVDF membrane:
   a) Pre-wet PVDF in 50% methanol, starting at one end and slowly proceeding across membrane
   b) Equilibrate in 1X Transfer Buffer ~ 5 min
   c) Set-up transfer apparatus
   d) Transfer in pre-chilled 1X Transfer Buffer at 100 V 60 min with ice pack and apparatus in ice bucket during transfer

3. Block membrane in 20 ml 5% NFDM, 1X TTBS 60 min on shaker.

4. Detect membrane in 2 ml (1˚ Ab diluted in 5% NFDM, 1X TTBS) in sealed bag on rocker overnight.

5. Rinse membrane 3 x 5 min with 1X TTBS.

6. Combine 2 drops Vectastain Reagent A in 10 ml 1X TTBS; mix.

7. Add 2 drops Vectastain Reagent B, mix and incubate at RT 30 min.

8. Meanwhile, incubate membrane in 10 ml (2˚ Ab + 1X TTBS) 30 min, shaking.

9. Rinse membrane 3 x 5 min with 1X TTBS.

10. Incubate membrane in 10 ml Soln. ABC (from step 7) 30 min, shaking.

11. Rinse membrane 2 x 5 min with 1X TTBS.

12. Rinse membrane 1 x 5 min with Tris pH 9.5.

13. Combine 2 drops BCIP/NBT Reagent 1 with 5 ml Tris pH 9.5 and mix.

14. Add 2 drops BCIP/NBT Reagent 2 and mix.

15. Add 2 drops BCIP/NBT Reagent 3 and mix.

16. Incubate membrane with mixture until desired staining intensity is achieved.

17. Rinse membrane 3 x 5 min with ddH_2O and air dry.