Western Blotting Protocol

Date _____

* Non-specific binding sites on the nitrocellulose are blocked by immersing the membrane in blocking buffer for a period of 20-30 minutes at room temperature.

* The membrane is then incubated in primary antibody for 20-30 minutes at room temperature with gentle mixing. (There is no need to wash the blot before adding primary antibody). Be sure you have enough volume to cover the blot (usually ~ 10 mL for a minigel and ~ 20 mL for a large gel).

    * The Primary Antibody is diluted in Blocking buffer.
        * polyclonal antibody is usually in the range of 1:500-1:3000,
        * monoclonal antibody is usually 1 μg/mL final conc.

* The blot is then washed five times (2 min. each) at room temperature in TBST to remove unbound antibody.

* The blot is incubated for 20-30 minutes in secondary antibody at room temperature with gentle mixing.

    * protein-A peroxidase conjugate (BMB) is used at 1:3000 and is diluted in Blocking Buffer,
    * protein-A/G+peroxidase conjugate (Pierce) is used at 1:10,000 & is diluted in Blocking Buffer,

* Finally, the blot is washed 5 times (2 minutes each) in a large volume of TBST.

* Chemiluminescence is carried out by incubating the blot in chemiluminescence substrate for 1 minute. After 1 minute, it is quickly blotted on whatman 3MM paper to remove the excess liquid and is placed inside a page protector sheet and exposed to X-ray film for a minute or two depending on the intensity desired.

    * The ECL reagent (from Amersham) is prepared by mixing equal amounts of ECL reagent solution #1 and solution #2. (~ 2 mL each for a minigel or ~ 4 mL each for a large gel).

Blocking Buffer	**TBST**	**1 liter 10X**
TBST + 5 % non-fat dry milk	50 mM Tris pH 7.2	500 ml of 1M
	150 mM NaCl	300 ml of 5 M
(prepare this fresh each time;	0.2 % Tween-20	20 ml of 100%
do not add azide for storage since		
azide inhibits the peroxidase)		

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