Western blot_v001

Introduction

Get started by giving your protocol a name and editing this introduction.

Materials

- 1,5M Tris pH 8.8
- 1M Tris pH 6.8
- PAA 30%
- ddH2O
- 10% SDS
- 10% APS
- TEMED
- Loading Dye (Lämmli Buffer)
  - 250mM Tris pH6.8
  - 10% SDS
  - 5% ß-Mercaptoethanol
  - 50% Glycerol
  - Bromphenolblue
- Running Buffer (Tris- Glycine - SDS)
  - 25mM Tris
  - 192mM Glycine
  - SDS 0.1%
- Transfer Buffer (Towbin)
  - 25mM Tris
  - 192mM Glycine
  - Methanol 20%
- TBS-T (Tris buffered saline with Tween)
  - 10mM Tris
  - 150mM NaCl
  - 0,05% Tween 20
  - in H_2O

Procedure

Separation gel

Table1

	A	B	C	D	E	F	G
1		10%			12.5%		
2	**1M Tris pH 8.8**	3.75	11.25	22.5	3.75	11.25	22.5
3	**PAA 30%**	3.33	10	20	4.17	12.5	25
4	**Water**	2.72	8.15	16.3	1.88	5.65	11.3
5	**10% SDS**	0.1	0.3	0.6	0.1	0.3	0.6
6	**10% APS**	0.1	0.3	0.6	0.1	0.3	0.6
7	**TEMED**	10µl	30µl	60µl	10µl	30µl	60µl
8	**Volume in ml**	**10**	**30**	**60**	**10**	**30**	**60**

Stacking gel

Table2

	A	B	C	D
1	**1M Tris pH 6.8**	1.25	2.5	5
2	**PAA 30%**	1.3	2.6	5.2
3	**Water**	7.6	15.2	30.4
4	**10% SDS**	0.1	0.2	0.4
5	**10% APS**	0.1	0.2	0.4
6	**TEMED**	10µl	20µl	40µl
7	**Volume in ml**	**10**	**20**	**40**

Protocol

Day 1

1. Cast the Gels using the Tabels above.

   first, cast the Separation Gel mixing all the solutions mentioned*

   (*to decide what percentage of gel you need figure out how big (in kDa) your protein of interest is. for Proteins ~100kDa 10-12,5% is recommended, for proteins with smaller size, use higher percentage, for bigger, less)
   Pour the liquid solution to the gap between the two glassplates.

   Add a small amount of Isopropanol on top of the liquid gel to get a straigth line.

   Let the gel polymerize

   Cast the stacking gel by mixing all solutions well

remove isopropanol and add the liquid stacking gel solution into the gap between the glassplates on top of the separating gel.
Put a comb on top of the gels to get wells in your Gel.
(note: Gels can be kept about 4 weeks at 4°C in a bag with a little bit of water to keep them wet)

2. Set up Gel running Chamber (https://www.youtube.com/watch?v=XnEdmk1Sqvg)

3. Prepare Samples

   by mixing your samples (30µg) with loading dye (Lämmli Buffer)
   equalize protein concentrations* in each samples by diluting the samples with H_2O
   (*to determine protein concentrations, use either Bradford test listed in the protocolls list or BCA Kit - manual in the Kit)
   heat up Samples including loading dye for 5 minutes to 95°C
   (note: after preparing the samples, they are stable and can be frozen for later use)

4. Add Ladder and Samples to the Gel

5. Run the gel:

   following Setting work with every gel and sample:
   Step 1: 20 min 80V
   Step 2: 70min 125V

6. Remove the Gel and transfer it to a Membrane (Nitrocellulose, wet transfer)

   Equibrilate Filterpapers, gel and membrane in blotting buffer
   set up a Sandwich with:
   2Filter papers > gel > membrane> 2 filter papers

7. Run at 90V for 70min at 4°C

8. (optional) perform a ponceau red staining to check transfer

9. Block membrane with either 5% BSA in TBS-T or 5% Milkpowder in TBS-T for 1 hour

10. Incubate the membrane with primariy ABs at 4°C

Primary Antibodies

	A	B	C	D	E	F	G	H
1	Antibody	Reactivity	Host	RRID	Company	#Cat	Blocking in	Dilution in
2	K-Ras Monoclonal Antibody (9.13)	Human, Mouse, Rat	Mouse	AB_2532192	fisher	415700	5% milk in TBST	1:500 in 1% BSA in TBST
3	p44/42-phospho MAPK (pErk1/2) Antibody	H M R Hm Mk Mi Dm Z Pg Sc	Rabbit	AB_331772	cell signaling	4376S	5% BSA in TBST	1:1000 in 1% BSA in TBST
4	ERK1/2	H M R Hm Mk Mi Dm Z B Dg Pg Ce	Rabbit	AB_390779	Cell signaling	4695S	5% milk in TBST	1:1000 in 1% BSA in TBST
5	Phospho-Akt (Ser473) (D9E) XP® Rabbit mAb #4060	H M R Hm Mk Dm Z B	Rabbit	AB_2315049	Cell Signaling	4060S	5% BSA in TBST	1:2000 in 1% BSA in TBST
6	Akt Antibody #9272	H M R Hm Mk C Dm B Dg Pg GP	Rabbit	AB_329827	Cell Signaling	9272S	5% milk in TBST	1:1000 in 1% BSA in TBST
7	GAPDH (14C10) Rabbit mAb #2118	H M R Mk B Pg	Rabbit	AB_561053	cell signaling	2118	5% milk in TBST	1:2000 in 1% BSA in TBST

Day 2

11. wash membrane 3x with TBS-T for 15 Minutes (~15ml)

12. Incubate with secondary antibody (note: the secondary AB depends on the source of the primary antibody (e.g. mouse) it needs to be HRP conjugated/designed for westernblot)

Table3

	A	B	C	D	E
1	Antibody	RRID	Company	#Cat	Dilution in
2	Anti-mouse IgG, HRP-linked Antibody #7076	AB_330924	Cell Signaling	7076S	1:1000 in 5% milk in TBST
3	Anti-rabbit IgG, HRP-linked Antibody #7074	AB_2099233	Cell Signaling	7074S	1:2500 in 5% milk in TBST

13. wash 3x with TBS-T for 15 minutes (~15ml)

14. develope signal using pierce ECL western (Thermo scientific, 32209)

15. aquire signal with Fusion Fx Vilber Lourmat developing machine.