**Protocol for Western Blot**

1. Spin down 1 min Max rpm (in eppendorf)
2. Withdraw and dry as much as possible.
3. Label tube one more set.
4. **“ALL in ICE”**
5. Prepare MPER 100uL and 10% Halt to each sample. (990uL MPER +10ul Halt Protease & Phosphatase inhibitor Single Cocktail 100x) for 10 samples. PLEASE prepare slightly more in order to make sure enough. Eg for 11 samples. RIPA solution not so good as will clump the cells.
6. Resuspend the samples using the pipette after adding the 100uL solution to protein.
7. Vortex and wait for 5-10 mins in 4C ice box (let cell lyse).

Prepare gel

1. Use glass 1.5mm and comb 15 well 1.5mm (green colour).
2. Clean with 100% EtOH and kimwipe the side of the glass +elevated edge.
3. Assemble the device the container and green clip.
4. Prepare Resolver and Stacker (follow the small paper on the bench; n= number of gel).

For 1.5mm- pipette resolver gel 7.5ml

Loading Dye pH ~6.8

1. Add Stacker solution over abit to make sure when insert comb no bubble trap.
2. Vortex the sample after 10 mins (lyse a while).
3. When Transfer SET at 80V 2hours (when transfer to PVDF. **PVDF must be activated**).

Prepare Buffer

1. Prepare 1xSDS Running Buffer and also 1x Transfer buffer.
   1. Running Buffer = 900mL H20 + 100ml of 10xTris/Gly/SDS
   2. 10% MetOH 1xTransfer Buffer = 800ml ddH20 +100ml of 10xTris/Glycine Buffer + 100ml MetOH (\* if use 20% MetOH will precipitate big size protein)
2. Pipette up and down the solution and on ice. Vortex the samples ~ 30 secs
3. Centrifuge the samples at 4C (min 5min At Max rpm) Use ~10-15min. All must in 4C until you boil it step.
4. After transfer prepare the 10 new Eppendorf for 10 cell lines.
5. Label accordingly 1-10 and arrange the sequence (Most resistant ->Most sensitive). Nicer sequence.
6. Withdraw all the supernatant (100uL) each spinned samples to the new Eppendorf (Use pipette 200uL).
7. Take out 96-well plate and Bradford solution to determine the standard and the samples concentration.
8. Pipette in 150uL Bradford for each well. Do triplicate for each sample or standard.
9. Standard 14-12-10-8-6-4-2-1-0 conc. (5 tips each time)
10. Pipette BSA into each standard.??

10uL-8uL-6uL-4uL-2uL

1. Pipette the samples - take out one by one from the ice box at a time. Pipette in 1uL of the samples into 96-well plates. When pipette out press one->the pipette in press all.
2. REMEMBER vortex 1-2sec then only pipette out 1uL. Put sample in ice box all the time.

# Also can pipette 2uL sample and add in 8uL MPER for dilution, instead of put in 1uL sample when dealing with too concentration when measuring std curve.

1. Read plate (Press “Quick Start” icon on the top bar). Then take out 6xSDS, 2xSDS and Protein ladder Eppendorf from fridge.

\***if self-prepare SDS, put in beta mercap when only that day want to use. 90uL beta mercap +910uL buffer SDS. Then boil 50C for few minutes as very viscous)**

1. Set up Excel and calculate the concentration. \*When calculate total ug in Excel (use 97uL because already used up 3uL for Bradford standard determination).
2. Load sample with SDS and MPER (calc Excel). Spin down
3. Boil the mixture for 10mins at 95-99C ( or 5 mins) (SET EARLY the black machine). **Put a heavy metal on top of the Eppendorf otherwise will pop out.**

* **Spin down and Can keep in -20C if want to run the next day. Take out and put at bench RT before load sample.**

1. Assemble the device and put inside the container.
2. Pour in the running buffer into the device till full overload.
3. Take out the comb carefully.
4. Pipette up down each well to equilibrate the buffer.
5. Add in Running buffer till the 2-gel “line” on the container.
6. Up down the Eppendorf. Quick spin (max 20secs)
7. Add 10uL of sample into each well. (min 50ug)
8. Loading dye to fill up all the empty well.
9. Protein ladder add in 2-3uL in each side to differentiate.
10. Set i)80V 30mins -to stack better

ii)120V 45 -60mins

1. Remove gel that transferred.
2. Check how protein was run on the orange machine.
3. Click L816->stain Free Gel->protein gel->stain free 45 sec or -> manual 1 min.
4. Prepare device and transfer buffer.
5. Put in the icepack (2icepack) into the container.
6. Pour in transfer buffer with gel inside + another into a new tray.
7. Dip the white sponge & black mesh into the transfer buffer.

Arrange the sequence from bottom -> top

Black base>Black mesh>white sponge (2 layers better tighter)> membrane gel>PVDF

1. Attach the whole mixture to container. Align black colour to black.
2. Set 80V 2 hrs constant Volt.
3. Prepare blocking buffer

For Phosphorylated Ab. Use BSA. 5% BSA. Weigh 2g BSA crystal (bottle in 4C fridge) + 40ml TBST.

For normal Ab- Use Milk. 5% Blocking Buffer. Weigh 10g blocking buffer powder + 200ml TBST. Keep in 4C only 1 week. Better prepare fresh.

* TBST preparation. 50ml of 20xTBS +950ml ddH20 +1ml Tween 20.

1. Make sure the container for blocking clean and dry from EtOH.
2. Activate PVDF membrane using 100% MetOH.
3. Put in 5ml of Blocking buffer accordingly. BSA into container well for p-PLK4

|  |  |  |
| --- | --- | --- |
| **Types** | **Blocking Buffer** | **Amount** |
| pPLK4 ~110kb | BSA | 5ml |
| β-actin~42kb | Milk | 5ml |
| Total PLK4 ~110kb | Milk | 5ml |
| GAPDH ~30-40kb | Milk | 5ml |
| yH2AX ~15kDa | BSA | 5mL |
| PARP1 ~100kDa | Milk | 5mL |
| P53 ~53kDa | Milk | 5mL |
| P21 ~21kDa | Milk | 5mL |
| Histone H3 ~15kDa | Milk | 5mL |
| Cyclin D1 ~36kDA | Milk | 5mL |
| Cdc25c ~ 60-75kDa | Milk | 5mL |
| CDK1~34kDa | Milk | 5mL |
| Caspase 3~ 30-40kDa (35) | Milk | 5mL |
| Cleaved Caspase 3 ~17-19kDa | Milk | 5mL |
| FLT3 ~158-160kDa | Milk | 5mL |
| pFLT3 ~158-160kDa | BSA | 5mL |
| ERK1 ~42-44kDa | Milk | 5mL |
| pErK1 ~ 42-40 kDa | Milk | 5mL |
| Tubulin ~ 55kDa | Milk | 5mL |
| BRCA ~>250kDa | Milk | 5mL |
| MDM2 ~56kDa | Milk | 5mL |
| BAX~ 23kDa | Milk | 5mL |
| pAurora A/B/C ~35,40, 48kDa | BSA | 5ml |
| pLATS1 ~` 140kDa | BSA | 5mL |
| LATS1 ~ 140kDa | Milk | 5mL |
| YAP~ 65-78kDa | Milk | 5mL |
| pYAP ~65-78kDa | BSA | 5mL |
| Caspase 3 (D3R6Y) ~3,17,19 kDa both total and cleaved | Milk | 5mL |
| p-ATR (Ser428)~300kDa | BSA | 5mL |
| pATM (Ser1981)(D6H9)~350kDa | BSA | 5mL |
| P16 INK4A (D7C1M)~16kDa | BSA | 55mL |

1. Cut the membrane carefully. (Cut at the upper pink band for pPLK4 and PLK4; β-actin & GAPDH at lower another pink band)

#7% - PLK4 12% PARP1

GAPDH p53

yH2AX p21

Cyclin D1

1. Shake for 30mins-1 hr in shaker.

**#BOOK the Chemiblot Machine upfront!!!!!**

1. Put in antibody following ratio:-

|  |  |  |
| --- | --- | --- |
| **Antibody** | **Ratio** | **Amount** |
| pPLK4 ~110kDa (Kinexus) | 1:250 | 5uL:5000uL BSA |
| β-actin~42kDa | 1:1000 | 5uL:5000uL Milk |
| Total PLK4 ~110kDa | 1:5000 | 1ul:5000uL Milk |
| CDK1 ~34kDa | 1:500-1000 | 5uL:5000uL Milk |
| Cdc25c ~60-75kDa (CST) | 1:1000 | 5uL:5000uL Milk |
| GAPDH ~30-40kb (see whether conjugated or not. If yes, no need 2ndary) | 1:5000 | 1ul:5000uL Milk |
| GAPDH ~30-40kDa Conjugated | 1:20,000 | - |
| HPK1 ~97kDa (ab33910) | 1:5000 | 5uL:5000uL Milk |
| Tubulin | 1;10,000 | ? |
| p-ATR ~300kDa | 1:1000 | 5uL:5000uL BSA |
| p-ATM~350kDa | 1:1000 | 5uL:5000uL BSA |
| p16INK4A ~16kDa | 1:1000 | 5uL:5000uL |

\*reference gene- very concentrate, thus ratio larger.

1. Put in shaker overnight 4C. Not too fast.
2. Next day, withdraw all the blocking buffer in the container and keep at -20C. Can be used 2-3x & must not keep > 2 weeks. If put in 4C must add ini Sodium azide-only last for 1-2 weeks.
3. Membrane must be moist. Thus, pour TBST (enough to dip and shake) into each container.
4. Shake 5 mins and discard all solution. Replenish new TBST. Repeat this process for 4-5x.
5. Pour out the last solution.
6. Prepare secondary antibody 1:2500 Anti-Rabbit IgG HRP Conjugate (take from common western Ab bx 4-Orange and Red cap eppendorf) (1uL in 2500uL milk solution. So, if 4 containers, need 8uL in 20ml milk solution.

* If background too much-> use lesser secondary antibody.

|  |  |
| --- | --- |
|  | Secondary Antibody |
| PLK4 | **Anti-Rabbit** |
| GAPDH | **Already Conjugated** |
| Gamma H2AX | **Anti-mouse** |
| PARP | **Anti-Rabbit** |
| P53 (Santa Cruz) | **Anti-mouse** |
| P21 F-5 sc 6246 (Santa Cruz) | **Anti-mouse** |
| Cyclin D1 | **Anti-Rabbit** |
| p-PLK4 | **Anti-Rabbit** |
| CDK1 (ab131450) | **Anti-Rabbit** |
| HPK1 (ab33910) | **Anti-Rabbit** |
| Cdc25c (5H9) | **Anti-Rabbit** |
| MDM2 SMP14 sc 965 (Santa Cruz) | **Anti-Mouse** |
| BAX (Santa Cruz) | **Anti-Mouse** |
| p-ATR | **Anti-Rabbit** |
| p-ATM | **Anti-Rabbit** |
| P16INK4A | **Anti-Rabbit** |

\*

1. **Shake for 1-2hours. MUST not more than 2 hours.** If needed, after 2 hours, pour out the solution then replenish with TBST and put awhile in 4C room or fridge.
2. Withdraw the secondary antibody solution. Wash with TBST 5 mins and repeat the wash 4-5x.
3. Final wash-pour out the solution.
4. Dry the PVDF on the tissue but do not touch the side.
5. Drop the Western HRF Substrate solution. Dap at the side on the transparent paper to even the solution. Then 2nd time drop the solution to dampen the PVDF.
6. Slightly wet the black cover class machine and put on those membrane PVDF with the transparent paper on it.
7. Click L816 >cheminiluminescent Blot> press exposure> for one minute

* Must capture the first black and white image & second the band image. (to compare)
* > Click send/save> transfer to pendrive.

## Choose Colorimetric Blot

* Auto-> Rapid Exposure

Normalize the band and rerun the gel. Based on the calculated excel normalized volume. Use back the diluted sample in -20C fridge to normalized.

Setting for the Running Gel.

**\*80V Time: 30mins; Amp 3A; Watt300w**

**01 Step 01**

**\*120 Time 1hr30mins; Amp 3A; Watt 300w**

**02 Step 02**

ChemiBlot Software

* Open the scn file-> Crop the band-> custom
* Export for publication ->600dpi-> export-> save as tiff file

PLK4i-

|  |  |
| --- | --- |
| **Increase** | **Reduce** |
| P21 | pPLK4 |
| Cleaved PARP | PLK4 |
| YH2AX | Cyclin D1 |
| P53 (depends) | cMyc |
|  | Cdc25c |

MDM2 p53

p53 cdc25c mitosis -> cell die

p53 cdc25c mitosis -> cell proliferate