pSTAT3 Protocol Version 2

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Abstract

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Guidelines

Training Requirements

Able to accurately pipet load gel and dilute tissue samples and use Licor Odyssey Imaging Software

Materials

Mini Protean tetra cell by Bio-rad Laboratories
Power Pac 3000 by Bio-rad Laboratories
Mighty Small Transphor by Amersham
Dry bath Incubator by Daigger
Acrylamide BP1410-1 by Fisher Scientific
1.5M Tris pH 8.8 1610798 by Bio-rad Laboratories
0.5M Tris pH 6.8 161-0799 by Bio-rad Laboratories
10% SDS 161-0146 by Bio-rad Laboratories
APS (0.1g/1 ml H2O) 063219 by Fisher Scientific
dH2O BP2470-1 by Fisher Scientific
TEMED 17919 by Thermo Fisher Scientific
Tank buffer 161-0772 by Bio-rad Laboratories
APS BP179-25 by Fisher Scientific
PBS 28374 by Thermo Fisher Scientific
Laemelli Buffer 161-0737 by Bio-rad Laboratories
Molecular weight markers SM1811 by Thermo Fisher Scientific
ß -mercaptoethanol M3148 by Sigma Aldrich
70% alcohol 793213 by Sigma Aldrich
Methanol M3641 by Sigma Aldrich

- Microtubes 1.5ml by Contributed by users
- Microtubes 1.5ml with screw caps by Contributed by users
- Pippette by Contributed by users
- Pipette Tips by Contributed by users
- Pipette gel loading tips by Contributed by users
- 2L beakers by Contributed by users

  Mini Trans-blot filter paper 1703932 by Bio-rad Laboratories

  Nitrcellulose membrane 1.0um BA 79 by Sigma Aldrich
- Glass plates by Contributed by users
- Casting stands by Contributed by users
- Casting frame by Contributed by users
- Rubber sealers by Contributed by users
- 15 well combs 1.5um by Contributed by users

  SNAP ID 2.0 Protein Detection System by Emd Millipore

  Odyssey Imaging System by Licor

  TWEEN 20 P7949 by Sigma Aldrich

  Odyssey Blocking Solution P/N 927-40000 by Licor
- Primary Antibody by Contributed by users
- Secondary Antibody by Contributed by users
- 1 L bottles by Contributed by users
- 500 ml bottles by Contributed by users
- 50 ml conical tubes by Contributed by users
- 15 ml conical tubes by Contributed by users
- Pipettes by Contributed by users
- Pipette Tips by Contributed by users
- 2L beakers by Contributed by users
- Vacuum system by Contributed by users

Protocol

REAGENT PREPARATION

Step 1.

10 % APS

0.1g ammonium persulfate, dH_2O to 1 ml.

Tank Buffer

-100 ml tank buffer, dH_2O to 1 L.

Chill 4˚C

Transfer Buffer

- 100 ml buffer

- 200 ml Methanol

- 700 ml H_2O

Chill 4˚C

GEL PREPARATION

Step 2.

1. **GEL CASTING**

Wash plates well, wipe with 70% alcohol, dry. Put front glass plate with back glass plate & place in casting frame and close frame. Put rubber spacer in base of casting stand. Place casting frame with plates in casting stand & click into place.

2. **10% SEPARATING GEL**

7.5 ml Acrylamide

7.5 ml 1.5M Tris pH 8.8

1.2 ml 10% SDS

100 ul APS (0.1g/1 ml H_2O)

13.66 ml H_2O

40 ul TEMED (add last)

Mix – don't vortex

Pour gels using 6.75 mls of gel.

Layer with 1% SDS.

Allow to polymerize 1 hr

Rinse off SDS with distilled water 2x

3. **STACKING GEL**

2.25 ml Acrylamide

3.75 ml 0.5M Tris pH 6.8

0.6 ml 10% SDS

50 ul APS

8.33 ml H_2O

20 ul TEMED

Mix – don’t vortex

Pour gel until runs over frame

Place in the comb (no air bubbles).

Allow to polymerize 1 hr

SAMPLE PREPARATION

Step 3.

- Typical load is 20 µg total protein

- Typical amount loaded is 20 µl/well

1. **PREPARE 2X BUFFER**

950µl Laemelli Buffer with 50 µl B-mercaptoethanol

2. **SAMPLE CALCULATION and DILUTION**

- sample total protein 10 µg/µl

- prepare diluted samples in screw cap tubes so that the tops can't pop open during denaturing

- 3 ul sample

- 12 µl SDS (use to make up difference in volume)

- 15 µl Laemelli Buffer (this is always half of the total volume)

3. **DENATURING of SAMPLES**

Boil sample in screw cap vial for 1-2 minutes then place on ice until ready to load. (gently boiling H2O or Dry bath set at 100°C)

ELECTROPHORESIS

Step 4.

1. **ASSEMBLE ELECTROPHORESIS TANK**

Assemble tank as shown in BioRad MiniProtean 3 Manual

Pour Pre-Chilled Tank Buffer into the tank.

Fill upper chamber and pour excess into lower chamber to 2 in deep.

2. **LOAD SAMPLES**

Using gel loading tips, Pipet 5 µl molecular weight marker into first well then pipet samples into gel wells (20 µl/well).

Run at 50 mAmps/gel.

Stop when dye front reaches bottom of gel.

TRANSFER

Step 5.

1. Remove gel from electrophoresis unit. Soak gel for 5 minutes in transfer buffer.

2. Cut nitrocellulose membrane the size of the gel and soak for 5 minutes in transfer buffer.

3. Build sandwich in transfer cage as below (prepare in tray ½ full of transfer buffer) and roll out air bubbles.

Sponge

Blotter Paper 2 sheets

Gel

Membrane

Blotter Paper 2 sheets

Sponge

GREY CAGE ⇒

4. Place cages in tank. Fill tank with transfer buffer.

5. Place lid with electrodes on tank, Making sure the grey side of the cages are facing the **red** electrode.

6. Run overnight in cold room with at 80 mAmps.

SNAP ID IMMUNOBINDING REAGENT PREPARATION

Step 6.

1. **PBS**

This reagent is made according to the directions provided by Pierce; 1 packet BupH Phosphate Buffered Saline Packs, dH_2O to 500 ml

2. **PBS/ 0.1%Tween 20 Rinse Buffer**

2packet BupH Phosphate Buffered Saline Packs, dH_2O to 1L, 1 ml Tween 20

3. **Wash Buffer**

1L PBS + 1ml Tween 20

4. **Block Solution Odyssey 927-40000**

5. **1˚ Buffer; Block Solution/0.1%Tween 20**

100ml Block + 100µl Tween 20

6. **2nd Buffer: Block Solution/0.1%Tween/0.1%SDS**

30ml Block/Tween + 30µl Tween 20

PRE-WET SNAP ID SYSTEM

Step 7.

1. Wet the white suface of the blot holder with H2O
2. Place blot in center; protein side down
3. Remove air bubbles with roller
4. Place spacer on blot & remove air bubbles w/roller
5. Close & squeeze firmly
6. Open lid & place in chamber/align tabs w/notches
7. Close & latch lid

BLOCK

Step 8.

1. Add 30ml LiCor blocking soln, sit 5 min
2. Apply vacuum until empty, TURN OFF vacuum

PRIMARY ANTIBODY INCUBATION

Step 9.

1. Add 30µl 1° antibody/3ml Block+ 3ul Tween 20
2. sit for 10 min
3. Turn on vacuum
4. With vacuum running wash 3X30ml in wash buffer: :PBS-0.1%Tween 20
5. Turn vacuum off

SECONDARY ANTIBODY INCUBATION

Step 10.

1. Add 2µl 2nd antibody to 3ml Block+ 3µl Tween+3µl 10%SDS
2. *COVER from light*
3. Sit 10 min
4. Turn on vacuum
5. With vacuum on wash 3X30ml in wash buffer: :PBS-0.1%Tween 20
6. Turn OFF vacuum
7. Remove blot

DRY BLOT

Step 11.

1. Lay the membrane on filter paper
2. Put membrane/filter paper in black box, put in dark until dry (4 hours – over night)

SCAN ON ODYSSEY IMAGING SYSTEM

Step 12.

1. Make sure surface and glass is clean and dry

2. Place membrane on LiCOR

3. Place clean glass plate on top of membrane3. Select appropriate channel(s) 700, 800

4. Preset on Membrane

5. Select appropriate channel(s) 700, 800

6. Select area to scan and start scan

Warnings

CAUTIONARY NOTES/SPECIAL CONSIDERATIONS

Add TEMED last and mix gently, do not vortex.

Membrane must remain wet, do not let it dry.