ab205921 –

PD-L1 RabMAb®

antibody (clone 28-8)

Protocol Booklet

For Immunohistochemistry, Western Blot and Flow Cytometry Applications.

This product is for research use only and is not intended for diagnostic use.

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Immunohistochemistry Protocols

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BioGenex i6000 with off-line HIER:

IHC Reagents:

- Xylene (EM Science, Cat # UN1307)
- Ethanol (AAPER alcohol and Chemical Co)
- Wash Buffer (DAKO, Cat# S3006)
- Target Retrieval Solution, AR6, 10X (Dako, Cat#S1699) or Universal HIER antigen retrieval reagent (Abcam, Ab208572)
- Novolink Max Polymer Detection System (Leica, Cat# RE7260-CE)
- Peroxidase block (Dako Cat# S2003)
- Protein block (Dako Cat# X0909)
- Antibody Diluent (Abcam, Ab64211)
- DAB (DAKO Cat# K3468) or DAB Substrate Kit (Abcam, ab64238)
- 28-8 rabbit monoclonal antibody (Abcam # ab205921, final concentration 2 µg/mL)
- Isotype control rabbit monoclonal antibody (Abcam, ab172730, final concentration 2 µg/mL)

Equipment:

- BioGenex i6000 Autostainer
- BioCare Medical Decloaking Chamber™ Plus
- Leica autostainer ST5020 (for deparaffinization & rehydration)
- Leica Auto Coverslipper CV5030

HIER Method:

1. Deparaffinization & rehydration, on Leica autostainer ST5020: 2X Xylene at 5 min. each; 2X of 100% Ethanol at 2 min. each, 2X of 95% Ethanol at 2 min each, 2X of 70% Ethanol at 2 min each; dH_2O, 2 min.
2. Antigen retrieval by using Biocare Medical Decloaking Chamber Plus Dako AR6, heated to 110 ºC (P1) for 10 min, then move to the next step (P2 FAN ON at 95ºC; FAN OFF at 90 ºC).
3. Cool slide at room temp for 15 min, Rinse with water ~ 1min.

IHC Method:

1. Apply the “Peroxidase Block” for 10 min.
2. Wash 3X with IHC wash buffer.
3. Apply the “Protein Block” to the slides, and incubate 20 min. at RT.
4. Wash 3X with IHC wash buffer.
5. Apply the pre-diluted antibodies to the slides (see Reagents). Incubate 1 hr at R.T.
6. Wash 3X with IHC wash buffer
7. Add the “Post Primary Block” (NovoLink Kit) to the slides and incubate for 30 min.
8. Wash 3X with IHC wash buffer
9. Add the “NovoLink Polymer” (NovoLink Kit) to the slides, incubate for 45 min.
10. Rinse 3X with IHC wash buffer.
11. Add the DAB chromogen substrate, develop 5 mins.
12. Wash slides by rinsing with dH_2O for 5X at R.T.
13. Counterstain with Hematoxylin for 1 min at R.T.
14. Wash slides by rinsing with dH_2O for 5X at R.T.

De-hydration and Coverslipping:

1. Remove the slides from BioGenex i6000 autostainer.
2. Set up slides on Leica autostainer ST5020.
3. Dehydrate 2 min each: 1X with 70% Ethanol, 1X with 95% Ethanol, then 3X with 100% Ethanol.
4. Wash in Xylene for 3X at 2 min each.
5. Coverslip with Cytoseal Mounting Medium in Leica CV5030 coverslipper.

Leica BOND RX with on-line HIER Protocol:

IHC Reagents:

- Bond Dewax Solution (Leica, Cat# AR9222)
- Bond Epitope Retrieval 1 (Leica, Cat# AR9961) or Universal HIER antigen retrieval reagent (Abcam, Ab208572)
- Bond Wash Solution (Leica, Cat# AR9590)
- Leica BOND Polymer Refine Detection kit (Leica, Cat#DS9800)
- Antibody Diluent (Abcam, Ab64211)
- 28-8 rabbit monoclonal antibody (Abcam # ab205921, final concentration 2 µg/mL)
- Isotype control rabbit monoclonal antibody (Abcam # Ab172730, final concentration 2 µg/mL)

Equipment:

- Leica BOND RX autostainer

IHC Method:

1. Heat and De-wax on Leica BOND RX.
2. Antigen retrieval on Leica BOND RX at 100ºC for 30 min using ER1 (Citra buffer, pH6 from Leica).
3. Apply the “Peroxidase block” (Refine Kit) for 10 min, followed by 3X rinse with IHC wash buffer.
4. Apply the diluted antibodies to the slides (see reagents). Incubate 1 hr. at R.T.
5. Wash 3X with IHC wash buffer
6. Add the “Post Primary Block” (Refine Kit) to the slides, incubate for 30 min.
7. Wash 3X with IHC wash buffer.
8. Add the “NovoLink Polymer” (Refine Kit) to the slides, incubate for 30 min.
9. Wash 3X with IHC wash buffer.
10. Add the DAB chromogen substrates (Refine Kit), Develop 10 mins.
11. Wash slides by rinsing with dH_2 for 5X at R.T.
12. Counterstain with Hematoxylin (Refine Kit) for 8 min. at R.T.
13. Wash slides by rinse with dH_2O for 5X at R.T.

De-hydration and coverslipping:

1. Remove the slides from Leica BOND RX.
2. Set up slides on Leica autostainer ST5020.
3. Dehydrate 2 min. each, 1 X with 70% Ethanol, 1X with 95% Ethanol, then 2X with 100% Ethanol.
4. Wash 2X in Xylene for 2 min each.
5. Coverslip with Cytoseal Mounting Medium in Leica CV5030 coverslipper.

Ventana Ultra with on-line HIER Protocol

IHC Reagents:

- Xylene (EM Science, Cat # UN1307)
- Ethanol (AAPER alcohol and Chemical Co)
- Universal HIER antigen retrieval reagent (Abcam, ab208572)
- Antibody Diluent (Abcam, Ab64211)
- Ventana reagents:
    - ChromoMap DAB kit: Cat # 760-159
    - Anti-Rabbit HQ: Cat # 760-4815
    - Anti-HQ HRP: Cat # 760-4820
    - Hematoxylin II: Cat # 790-2208
    - Bluing Reagent: Cat # 760-2037
- Anti PD-L1 antibody Clone 28-8 Abcam (ab205921)

Equipment:

- Ventana Ultra
- Leica Autostainer (Bake and deparaffinize, de-hydration)
- Biocare Decloaker

IHC Method:

1. Bake and deparaffinize on Leica Autostainer
    a. Bake at 65^{o}C for 10min
    b. Xylene: 3min, 3X
    c. 100% Ethanol: 2min
    d. 100% Ethanol: 1min
    e. 95% Ethanol: 1min
    f. 70% Ethanol: 1min
    g. PBS: 3min
2. Perform Antigen retrieval using Universal HIER antigen retrieval reagent (Abcam, Ab208572) in Biocare Decloaker at 110^{o}C for 10 min.
3. Load slides on Ventana Ultra (setup protocol as listed below).
4. Primary Antibody diluted in Antibody Diluent (Abcam, ab64211) and apply manually; ab205921 at 7.5 ug/ml, incubate for 1 hour at 37°C.

5. Secondary & Tertiary Abs; Anti-Rabbit HQ as linking antibody incubated at 37°C for 16 min. Anti-HQ-HRP antibody as enzyme conjugate incubated at 37°C for 16 min.
6. ChromoMap DAB used for detection.
7. Counterstained with Hematoxylin II in reaction buffer followed by Bluing reagent.

Protocol on Ventana Ultra:

1. Antibody [Selected].
2. 1st Antibody Manual Application [Selected].
3. Warm up Slide to 37 °C from Very Low Temperatures (Primary Antibody).
4. Hand Apply (Primary Antibody), and Incubate for 60 Minutes.
5. Linking Antibody [Selected].
6. 2nd Antibody [Selected].
7. Warm up Slide to 37 °C from Very Low Temperatures (2nd Antibody).
8. Apply One Drop of [Anti-Rabbit HQ] (Detection #1), and Incubate for 16 min.
9. Enzyme conjugate [Selected].
10. Apply One Drop of [Anti-HQ HRP] (Conjugate #1), and Incubate for 16 min.
11. DAB [Selected].
12. Counterstain [Selected].
13. Use RB for Counterstain [Selected].
14. Apply One Drop of [HEMATOXYLIN II] (Counterstain), and Incubate for 8 min.
15. Post Counterstain [Selected].
16. Use RB for Post Counterstain [Selected].
17. Apply One Drop of [BLUING REAGENT] (Post Counterstain), and Incubate for 4 min.

De-hydration and coverslipping:

1. Remove the slides from Ventana Ultra.
2. Wash slides with Dawn soap.
3. Rinse with DI water.
4. Set up slides on Leica autostainer:
    a. Dehydrate with 70% Ethanol, 95% Ethanol, 3X 100% Ethanol, 2 min each.
    b. Rinse in Xylene 3X 2min.
5. Cover-slipped with Cytoseal Mounting Medium manually.

Dako Omnis

Deparaffinization

Two-Phase-Deparaffinization-IHC	Solvent: Clearify Clearing Agent	Transport Liquid: DI Water	Temperature: 25° C	Incubation (top): 10 seconds	Incubation (bottom): 1 minute	# cycles: 1
Two-Phase-Deparaffinization-Wash-IHC	Reagent: DI Water	Incubation: 5 seconds	# cycles: 1			

Antigen-Retrieval (Demasking)

Demasking-IHC	Reagent: EnVision FLEX TRS, Low pH	Temperature: 97°C	Incubation: 30 min.	cooling liquid: DI water

Staining:

Step	Reagent:	Incubation:	# cycles:
Wash	Wash-Buffer	2:40 min.	2
Primary Antibody	**PD-L1 (1:400)**	**1 hour**	
Wash	Wash-Buffer	2 min.	10
Endogenic Enzyme Blocking	**EnV FLEX Peroxidase-Blocking Reagent**	**3 min.**	
Wash	Wash-Buffer	2 min.	10
Secondary Reagent	**EnV FLEX + Rabbit Linker**	**10 min.**	
Wash	Wash-Buffer	2 min.	10
Labeled Polymer	**EnV FLEX/HRP**	**20 min.**	
Wash	Wash-Buffer	2 min.	10
Wash	Wash-Buffer	2 min.	10
Wash	DI Water	31 seconds	1
Wash	Wash-Buffer	2 min.	10
Substrate-Chromogen	**EnV FLEX Substrate Working Solution**	**5 min.**	
Wash	Wash-Buffer	2 min.	10
Wash	DI Water	31 seconds	1
Wash	Wash-Buffer	2 min.	10

Counter-Staining

Staining	Hematoxylin	3 min.	
Wash	Wash-Buffer	2 min.	10
Wash	Wash-Buffer	2 min.	10

Western Blot Protocols

Western Blot protocol for Odyssey System

Materials:

- RIPA: 25 mM Tris pH 7.6/150 mM NaCl/1% NP-40/1% Na-deoxycholate/0.1% SDS
- HALT (Thermo#87786) protease inhibitor cocktail, 100X
- Phosphatase inhibitor, 100X
- Benzonase
- Control antigen: rHuB7-H1/Fc
- SDS-PAGE: 4-20% Tris/glycine
- Laemmli buffer
- Nitrocellulose
- Transfer buffer: Tris/glycine/20% methanol
- DPBS
- PBS-T: DPBS + 0.05% Tween20
- DTT, dithiothreitol
- Prestained protein standards
- 4-20% gels
- LiCor# 927-40000: blocking buffer for blots, used undiluted for blocking and dilute antibodies
- Primary Antibody: Anti-PDL1 (Clone 28-8)
- Isotype Control: Rabbit IgG (ab172730)
- IRDye800CW-Donkey anti-Mouse IgG (H+L)
- IRDye800CW-Donkey anti-rabbit IgG (H+L)
- LiCor Odyssey Infrared imaging system (CLx), direct near-infrared fluorescence detection system

Preparation of lysates:

1. Three T-175 flasks of tumor cells at >90% viability (or CHO-expressing PDL1) are harvested by scraping and collected by centrifugation at 500 x g for 5 min.

2. Cells are washed twice with cold DPBS to remove media. Cells are resuspended with 0.9 mL of RIPA buffer that contains HALT and phosphatase inhibitors at 1X each. Cells are placed on ice for 10 min for lysis, and then centrifuged at 4K rpm for 20 min at 4°C.

3. The supernate contains the lysate and is aliquoted (100 µL) and frozen at -80°C until use. In some cases the lysate may contain sufficient DNA that it will cause viscosity problems when running gels, this can be overcome by adding 1 µL of Benzonase for each 200 µl lysate and incubating on ice for 15 min.

4. An aliquot of the lysates (3, 6 and 12 µL) are heated in Laemmli buffer and are run on 4-20% Tris/glycine gels and stained with Coomassie blue. The staining intensity of the lysates can be used to adjust the aliquot size to ensure equal loading of the lysates for use on the blots.

WB protocol:

1. Lysate and standard samples are heated at 95°C for 5 min in Lamelli buffer (typically 10 uL of lysate and 15 µl Laemmli buffer). If reduced samples are needed then fresh DTT is added to the sample at 50 mM.

2. The 4-20% Tris/glycine gels are run at 180 volts and then blotted onto nitrocellulose at 300 mM Amps constant current in Tris/glycine/methanol transfer buffer for 1 hr.

3. Blots are blocked with Odyssey blocking buffer for 1 hr at room temp, 100 rpm mixing. Alternatively, they can be blocked with 1% BSA-PBS-tween.

4. Blots can be stored overnight at 4°C in blocking buffer.

5. PDL1 RabMAb product (ab205921, clone 28-8) is diluted to 1/2000 to 1/4000 in blocking buffer and probed for 2 hrs at room temp.

6. When preabsorbing with excess recombinant antigen the samples are added to the primary antibody and incubated for 1 hr prior to placing it on the blot. The blots are washed three times with PBS-T for 10 min.

7. Odyssey secondary antibodies are diluted in Odyssey blocking buffer at 1/10,000 and probed for 1 hr, these have to kept in the dark and are covered with foil.

8. The blots are washed with Odyssey blocking buffer for 10 min and kept covered in foil. The blots are placed face down on the Odyssey CRx instrument and scanned according to instrument protocols. The software allows for exporting images that are of the users defined intensity, therefore both overexposed proteins and underexposed proteins can be captured.

Note: Odyssey secondary antibodies are donkey polyclonal antibodies that have been pre-absorbed to remove cross-reactivity and chemically linked to a near infrared dye. This system has the advantages of a wider dynamic range and less background than chemiluminescence. If the Odyssey system is not available a system using HRP-conjugated secondary antibodies and chemiluminensce detection should be able to reach the same sensitivity, but may have more background autofluoresence. A colorimetric substrate was not sensitive enough to detect proteins in the lysates when using HRP-conjugated secondary antibodies.

Western Blot protocol for endogenous PD-L1 expression

Materials:

- 1%SDS Hot Lysate buffer: 10 mM Tris-HCl pH 8.0/1% SDS/1 mM Na-Orthovanadate
- SDS-PAGE: 4-20% Bis-Tris
- 2X Sample buffer: 124.9 mM Tris-HCl (pH 6.8)/2.5% SDS/0.04% Bromophenol Blue/25% Glycerol
- Dithiothreitol (DTT): 0.1 M
- PVDF: pore size 45 µm
- Transfer buffer: Tris/glycine/10% methanol
- Prestained protein standards
- PBS
- TBST: TBS + 0.1% Tween 20
- Blocking buffer: 5% non-fat milk in TBST, used for blocking and to dilute antibodies
- Primary antibody: Anti-PD-L1 [28-8]
- Secondary antibody: Goat anti-rabbit IgG H&L (HRP) (ab97051)
- Optiblot ECL detect kit (ab133406)
- Optimax X-Ray film processor (PROTEC)

Preparation of lysates:

1. One T-150 flask of tumour cells at > 80% confluence are harvested by scraping and collected by centrifugation at 500 x *g* for 5 min.

2. Cells are washed twice with cold PBS to remove media. Cells are re-suspended with 0.5 ml of boiled 1%SDS Hot Lysate buffer and placed in a water bath (at 95 °C) for 15 min for lysis.

3. Cell clusters are broken by ultrasonic cell disruptor till lysates become clear (Ultrasound time 3 sec, 10 sec interval, ultrasonic 5~15 times, ultrasonic power: 40 kW), followed by centrifugation at 15,000 x *g* for 10 min.

4. The supernatant is collected and mixed with 2X Sample buffer. Generally, we test the protein concentration and then mix lysates in 2X sample buffer to reach 2 mg/ml of total protein, followed by storage at -20 °C until use.

WB protocols:

1. Tested samples (20 ug) are heated at 100 °C for 5 min.

2. The 4-20% Bis-Tris gels are run at 150 volts and then blotted onto PVDF at 72 volts constant voltage in Tris/glycine/methanol transfer buffer for 1 hour.

3. Blots are blocked with 5% Milk at 4°C overnight, shaking gently. Alternatively, blots can be blocked at room temp for 1 hour.

4. PD-L1 RabMab antibodies (ab205921) is diluted to 1/100 in blocking buffer and then the diluted antibodies are added to the blots for one-hour-incubation at room temp.

5. The blots are washed three times with TBST for 10 min each.

6. Secondary antibodies (ab97051) are diluted in blocking buffer at 1/20,000 and added to the blots incubated for 1 hour.

7. After 3 cycles of washing with TBST, the blots are covered with Optiblot ECL detect kit (ab133406). The films are subsequently developed by Optimax X-Ray film processor (PROTEC).

Flow Cytometry Protocols

Materials:

- Primary Antibody: Anti-PDL1 (Clone 28-8)
- Isotype Control: Rabbit IgG (ab172730)
- Secondary Antibody: Donkey anti-Rabbit (Fab)'2-APC or Donkey F(ab')2 Anti-Rabbit IgG - H&L (Alexa Fluor® 647) (ab150071)
- FACS Buffer: 1% BSA / 0.1% NaAzide in PBS (buffer)
- 96 well u-bottom plates, plate sealers, 50mL conical Tubes
- Human Gamma-Globulin
- L2987 (PD-L1 High) and A549 (PD-L1 Low) human tumor cells

Cell Preparation:

1. L2987 and HT29 cells are harvested using 0.25% Trypsin.
2. Cells are counted, then centrifuge at 300G for 5 min.
3. Wash cells in 10mL buffer.
4. Bring cells to 10×10^6 cells/mL in buffer.

Staining:

1. Block Fc-receptors using 20µL / mL human gamma-globulin
2. Incubate 20 min, on ice
3. Transfer 100µL (1×10^6 cells) per well
4. Add 1µg / well anti-PDL1 or isotype
5. Incubate plate 30 min, on ice, in dark
6. Add 100µL buffer, centrifuge 300G for 5 min, decant
7. Add 100µL buffer, centrifuge 300G for 5 min, decant
8. Dilute Secondary mAb 1:66 (45µL stock + 2.95mL buffer)
9. Add 100µL diluted secondary mAb to each well
10. Incubate plate 20 min, on ice, in dark
11. Add 100µL buffer, centrifuge 300G for 5 min, decant
12. Add 100µL buffer, centrifuge 300G for 5 min, decant
13. Add 100µL 1% formaldehyde, incubate 20 min on ice in dark
14. Add 100µL buffer, centrifuge 300G for 5 min, decant
15. Add 200µL buffer per well, seal plate and store in fridge
16. Read samples on BD FACS CANTO

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