Published in final edited form as:

Methods Mol Biol. 2017 ; 1554: 253–262. doi:10.1007/978-1-4939-6759-9_18.

Validation of Anti-Mouse PDL-1 Goat Polyclonal Antibody Staining with Mouse PDL-1 In Situ Hybridization on Adjacent Sections of Cell Pellets and Mouse Tumors

Jacob Ricca, Mesruh Turkecul, Afsar Barlas, Dmitry Yarilin, Sho Fujisawa, Ning Fan, Matthew Brendel, Dmitriy Zamarin, Jedd D. Wolchok, and Katia Manova-Todorova

Abstract

Finding a valid antibody to detect mouse programmed death ligand 1 (PDL-1) by immunohistochemistry or immunofluorescence staining has been notoriously difficult. Successful validation of an antibody requires the use of multiple detection methods with the ability to compare appropriate positive and negative controls. Here, we describe in detail the protocols used to validate a mouse-specific PDL-1 antibody used in immunohistochemistry staining with an mRNA in situ hybridization on adjacent sections of mouse B16 tumor. This validation is supported by immunohistochemistry staining of PDL-1 on B16 cell pellets either treated or not treated with IFN-gamma.

Keywords

PDL-1; Antibody validation; PDL-1 mRNA; In situ hybridization; B16 melanoma cells; Interferon gamma; B16 mouse melanoma tumors; ViewRNA ISH; Lieca Bond RX

1 Introduction

PDL-1, which is constitutively expressed on most hematopoietic cells and some parenchymal cells, plays a major role in immune suppression through the interaction with its receptor, programmed cell death protein 1 (PD-1) [1, 2]. During an adaptive immune response, activated T-cells proliferate and release IFN- γ , which induces the upregulation of PDL-1 on activated macrophages. Subsequent binding of PDL-1 ligand to PD-1 on activated T-cells results in a signal cascade that ultimately suppresses T-cell proliferation and cytokine production, preventing an overactive immune response [2]. Though this function is important during normal biological activities, such as preventing auto-immunity during pregnancy, the PD-1/PDL-1 interaction is exploited by many cancers as an adaptive method of evading an anti-tumor immune response [2–4]. Many studies have shown a direct correlation between patient PD-L1 expression and response to anti-PD-1 checkpoint blockage immunotherapy [5–7]. Therefore, the ability to characterize PDL-1 expression and

⁶Most adult tissues will require overnight incubation in 4 % PFA. Avoid using conical bottom tubes since the tissue may get stuck on the bottom and the fixation will be uneven. Try to minimize the time between dissection and immersion in the fixative. Rinse the blood off of the tissue, as presence of blood can decrease fixation efficiency.

localization in tumors is necessary for understanding its interaction in cancer progression, and is undeniably valuable in developing more effective and/or appropriate therapies.

In order to confirm that the antibodies we use are indeed detecting PDL-1 protein, we employed RNA in situ detection of PDL-1 mRNA on adjacent sections of cell pellets and tissues. B16-F10 mouse melanoma cells were used for the validation of the antibodies were incubated with IFN- γ to induce PDL-1 expression. The same B16-F10 mouse melanoma cells were checked by FACS and confirmed positive (data not shown). Tissues used for validation were B16-F10 mouse melanoma tumors grown in mice and some of the mice were also treated with NDV (Newcastle Disease Virus) to induce PDL-1 expression. We confirmed presence of PDL-1 by several methods and consider antibodies validated for both immunohistochemical and immunofluorescent detection of mouse PDL-1.

Here, we present the automated mRNA in situ hybridization and for automated immunohistochemistry staining protocols we used to validate mouse-specific PDL-1 antibodies.

2 Materials

In situ hybridization experiments, it is crucial that target RNA molecules not be degraded before staining. All reagents should be molecular biology grade. The tissue needs to be removed immediately after the animal has been sacrificed and fixed in 4 % paraformaldehyde fixative.

2.1 Preparation of B16-F10 Mouse Melanoma Cell Pellets and B16-F10 Mouse Melanoma Tumors for mRNA In Situ Detection

1. 4 % Para formaldehyde (PFA), reagent grade, prepared in PBS at pH=7.4 (*see* Note 1).
2. Dissection tools.
3. Filter System, 0.22 μ m pore, CA membrane.
4. ParaPlast Plus Tissue Embedding Medium.

¹4 % Para formaldehyde (PFA) fixative must be used fresh, maximum 48 h after being made. It should be kept at 4 °C. It is not recommended to freeze aliquots of PFA. Most commercially available preparations are not suitable for mRNA in situ hybridization, especially if they contain methanol to prolong shelf-life. Buffered formalin is not an optimal fixative for in situ hybridization. Never use warm PFA; always chill it on ice before use. To prepare PFA, use a chemical hood and follow these steps:

- a. Weigh Para formaldehyde powder (4 g Para formaldehyde for 100 ml fixative).
- b. Preheat deionized water to 68 °C in Erlenmeyer flask (~60 ml).
- c. Take flask off the hot plate, add PFA powder and mix quickly by agitation.
- d. Clear milky PFA solution with 0.1 N NaOH (4–5 drops will be sufficient, keep continued agitation of the flask).
- e. Add 25 ml water to pre-cool and then add 10 ml of 10 $\times$ PBS.
- f. Use pH paper to make sure the pH = 7.4. (Fixative can damage pH meter electrodes.)
- g. Bring the volume to 100 ml with deionized water.
- h. Filter through 0.22 μ m filter system, cool on ice before use.

5. Ethanol: 70 %, 95 % and absolute.
6. HistoClear, a xylene substitute (xylene can also be used).
7. Tissue embedding cassettes.
8. FisherBrand Superfrost/Plus slides 2"×3".
9. 10× Difco FA Buffer (PBS).
10. Microtome for cutting paraffin-embedded tissue sections (Leica).

2.2 Automated mRNA In Situ Hybridization with ViewRNA eZ Probe to Detect Mouse PDL-1 mRNA

1. All of the following reagents are provided by Affymetrix:
 - a. ViewRNA eZ Probe: Mm PDL-1 (*see* Note 2).
 - b. ViewRNA eZ-L Detection 1-plex (Red) (*see* Note 3).
2. All of the following reagents are provided by Leica Biosystems:
 - a. Leica Bond dewax solution.
 - b. Leica Bond wash buffer (10×) (*see* Note 4).
 - c. Leica Bond epitope retrieval 2.
 - d. Leica Bond Enzyme Pretreatment kit (*see* Note 5).
 - e. Leica Bond opened containers 30 ml.
 - f. Leica Bond titration containers and inserts.
 - g. Deionized (Milli-Q) water.
 - h. Absolute alcohol.

2.3 Automated Immuno-Fluorescence Detection of PDL-1 Protein

1. All of the following reagents were provided by Leica Biosystems:
 - a. Bond polymer refine detection kit.
 - b. Leica Bond dewax solution.
 - c. Leica Bond wash buffer (10×) (*see* Note 4).
 - d. Leica Bond epitope retrieval 2.
 - e. Leica Bond antibody diluent.

²Store the probe at −20 °C and use it at 1:20 dilution. If signal is strong and background is high, reduce the concentration to 1:30 or 1:40.

³Store the kit at −20 °C and equilibrate at room temperature for 2 h before each use.

⁴Dilute 10× wash buffer; nine parts deionized water and one part 10× wash buffer before filling the bulk container.

⁵Dilute the enzyme from enzyme pretreatment kit in the enzyme buffer from the same kit. 1:1000 dilution was used in this experiment. Experiment with different dilutions (1:500, 1:1000, 1:2000, etc.) and observe the signal of the positive control probe. Use the dilution that gives the best signal-to-noise ratio for positive control probe. Test the enzyme dilution for each tissue type.

- f. Leica Bond titration containers and inserts.
 - g. Deionized (Milli-Q) water.
 - h. Absolute alcohol.
2. Primary antibody: Goat anti-PDL-1 goat polyclonal antibody (R&D).
 3. Linker antibody: AffyPur Fab fragment of Rabbit anti-goat IgG (H + L) (Jackson ImmunoResearch).
 4. Series of alcohols for dehydration: 70 % Ethanol, 95 % Ethanol, Absolute Ethanol.
 5. HistoClear.
 6. Permount.

2.4 Data Acquisition and Review

1. Zeiss Imager upright epifluorescent microscope, equipped with Zeiss AxioCam HRc color camera and Zeiss AxioCam 506 monochrome camera.
2. Panoramic Flash Scanner (3DHistech, Budapest, Hungary) with and its viewing software, Panoramic Viewer.

3 Methods

Preparation of B16-F10 mouse cell pellets and B16 mouse melanoma tumors for in situ detection.

All steps should be carried on ice or at 4 °C, where specified, using reagents prechilled on ice.

1. Sacrifice the mouse in accordance with the Animal Protocol at your institution.
2. For tumors: Dissect out the B16-F10 mouse melanoma tumors in PBS and fix by immersion in freshly prepared 4 % Para formaldehyde (PFA).
3. For cells: Fix the B16-F10 cell pellets by immersion in freshly prepared 4 % Para formaldehyde (PFA) made in PBS.
4. Gently rock tumor and cell pellet the samples in a flat-bottom tube filled with 4 % PFA overnight at 4 °C. The volume of PFA solution should be at least 20 times the volume of the sample.
5. Wash twice in ambient temperature tap water, 15 min each.
6. Place all samples in 70 % Ethanol and keep at 4° C until ready for processing.
7. Load the tissues onto Leica ASP6025 tissue processor with the following schedule (*see Note 7*):

⁷If possible include a delay step (90 % Ethanol) before your tissue processor schedule begins, the tissues can safely wait in 90 % Ethanol until the processor is ready to start so that the procedure finishes at convenient time for embedding. Otherwise, the tissues may stay longer in hot paraffin and deteriorate (overcook and dry out).

- a. 90 % Ethanol 30 min, ambient temperature.
 - b. 95 % Ethanol 30 min ambient temperature.
 - c. 95 % Ethanol 30 min, ambient temperature.
 - d. Absolute Ethanol 30 min, ambient temperature.
 - e. Absolute Ethanol 30 min, ambient temperature.
 - f. Absolute Ethanol 30 min, ambient temperature.
 - g. HistoClear 40 min, ambient temperature.
 - h. HistoClear 40 min, ambient, temperature.
 - i. HistoClear 40 min, ambient temperature.
 - j. ParaplastPlus 40 min at 59 °C.
 - k. ParaplastPlus 40 min at 59 °C.
 - l. ParaplastPlus 40 min at 59 °C.
8. Embed tissue in paraffin molds and cool on a cold plate for minimum 30 min.
 9. Paraffin-embedded samples can be stored in 4 °C refrigerator.
 10. Prepare 5-µm-thick sections using a paraffin microtome and mount them on FisherBrand Superfrost/Plus slides. Let the slides air dry until the tissue sections do not look wet for several hours at ambient temperature (*see* Note 8).

3.1 Automated In Situ Hybridization with ViewRNA eZ Probes for Mouse PDL-1 on Leica Bond RX

Automated in situ detection was performed with ViewRNA eZ-L Red kit using Leica Bond RX processor (*see* Note 9).

- 1 Leica Bond RX routine factory based “ViewRNA Dewax 1” protocol.
- 2 ViewRNA eZ heat pretreatment protocol using ER2 buffer at 95 °C for 15 min.
- 3 ViewRNA eZ-L enzyme pretreatment protocol using 17 µg/ ml (1:1000) Leica Bond Enzyme diluted in Leica enzyme buffer at 40 °C for 15 min. Prepare fresh before each experiment (*see* Note 10).

All of the following steps are based on Affymetrix ViewRNA eZ-L Red protocol V2.

⁸To start sectioning, the paraffin blocks need to be at room temperature. Trim the block by removing as many as 15-µm slices until desired depth for section collection has been reached. Incubate the block submerged in ice-water mixture for 30 min to cool down and rehydrate. Immediately collect 5 µm sections using brand new clean microtome blade and float them on a clean water bath. Fish the sections on clean charged slides. Use gloves during sectioning to prevent RNase contamination. Dry the slides at room temperature for few hours and bake them for 1 h at 60 °C oven. Store the sections at –20 °C freezer for long-term storage (more than 1 month).

⁹It is highly recommended that probe cleaning kit is run before and after each ISH experiment. Avoid running other IHC or IF experiments with Leica Bond RX at the same time as the ISH experiment.

¹⁰Proteinase K is a hemolytic serine protease that partially reverses the cross-linking effect of aldehyde fixation, removing the masking proteins that hinder riboprobe binding and permeabilizes the tissue. Too low concentration of Proteinase K may not expose the nucleic acids enough and too high a concentration may over-digest the tissue and the target is lost. Proteinase K concentration, incubation duration, and temperature should be optimized for each riboprobe and tissue sample. Other proteolytic enzymes like pepsin, trypsin, and pronase E could also be tested.

- 4 ViewRNA eZ-L Mm-PDL-1 probe (1:20 dilution in Affymetrix hybridization buffer) hybridization at 40 °C for 3 h (*see Note 11*).
- 5 ViewRNA eZ-L Amp R1 for 1 min followed by ViewRNA eZ-L Amp R1 for 60 min.
- 6 Bond wash buffer: multiple post hybridization washes.
- 7 ViewRNA eZ-L Rinse six times for 5 min each.
- 8 Bond Wash buffer ten times 0:00 min each.
- 9 ViewRNA eZ-L Amp R2 for 1 min followed by ViewRNA eZ-L Amp R2 for 15 min.
- 10 Bond wash buffer: multiple post hybridization washes.
- 11 ViewRNA eZ-L Amp R3 for 1 min followed by ViewRNA eZ-L Amp R3 for 15 min.
- 12 Bond wash buffer: multiple post hybridization washes.
- 13 ViewRNA eZ-L Amp R4 for 1 min followed by ViewRNA eZ-L Amp R4 15 min. Amp R4 was diluted 1:500 in amplification diluent provided in the Affymetrix kit. Prepare fresh Amp R4 before each experiment.
- 14 Bond Wash buffer ten times 0:00 min each.
- 15 ViewRNA eZ-L Mixed Red for 5 min followed by ViewRNA eZ-L Mixed Red for 40 min.
- 16 Bond Wash buffer ten times 0:00 min each.
- 17 Deionized water wash 0:00 s.
- 18 ViewRNA eZ-L Hematoxylin two times for 7 min each.
- 19 Deionized water wash 0:00 s.
- 20 Bond Wash buffer 0:30 min.
- 21 Deionized water wash four times 0:00 s each.
- 22 Incubate the slides in 5 µg/ml DAPI solution for 5 min.
- 23 Bond wash buffer rinse.
- 24 Mount cover slips using Fluorescence mounting media and keep the slides in the dark at –20 °C (*see Note 11*).
- 25 Check the slides under bright field or fluorescence microscope to determine the quality of the staining.

¹¹It is very important to mount the slides with aqueous mounting media that contains glycerol and antifading reagent. Glycerol is an anti-freeze and prevents the media from freezing allowing the slides to be stored at –20 °C or –80 °C freezer for longer storage. Mowiol[®] is a proprietary blend of polyvinyl alcohols and serves as antifading reagent and polymerizes keeping the coverslip in place, making the use of nail polish unnecessary. Antifade reagents prolong the life of fluorescence molecules and prevent bleaching during imaging. Mowiol[®] and glycerol are water soluble and coverslips can be easily removed in PBS and slides can be processed with other staining protocols after imaging and/or storage.

3.2 Automated Immunohistochemistry Detection of PDL-1 Protein

Automated detection was performed using Leica Bond RX processor with the following steps based on Protocol F using Bond Polymer Refine Detection kit:

1. Leica Bond RX routine factory based “Bake and Dewax” protocol.
2. Heat Induced Antigen Retrieval using ER2 buffer at 100 °C for 20 min.
3. Peroxide Block 5 min (*see* Note 12).
4. Bond Wash buffer three times 0:00 min each.
5. Primary antibody incubation for 60 min at 2 µg/ml concentration (*see* Note 13).
6. Bond Wash buffer three times 0:00 min each.
7. Incubate with linker antibody at 2.6 µg/ml concentration for 8 min.
8. Bond Wash buffer three times for 2 min each.
9. Incubate with polymer for 8 min.
10. Bond Wash buffer five times 0:00 s each.
11. Deionized water wash 0:00 s.
12. Incubate with Mixed DAB Refine for 0:00 s followed by Mixed DAB Refine for 10 min.
13. Deionized water wash for three times 0:00 s each.
14. Incubate with Hematoxylin for 10 min.
15. Bond Wash buffer three times 0:00 s each.
16. Deionized water wash 0:00 s.
17. Slides are dehydrated in series of alcohols, cleared with histoclear and mounted with Permount.

3.3 Data Acquisition and Review

1. Digitize the slides using Panoramic Flash scanner from 3DHitech in both brightfield and fluorescent modes.
2. View the scanned slide with Panoramic Viewer software and take sample snapshot images (Fig. 1).

¹²Endogenous peroxidases/pseudoperoxidases in the tissue will cause TSA reagents to deposit in regions where there is no probe binding giving rise to false-positive signal. High concentration of H₂O₂ will saturate the endogenous enzymes and eliminate this source of background.

¹³If the IgG concentration is not written on the tube or on antibody specification sheet, contact the vendor to inquire about the IgG concentration. Antibodies come in different formulations such as whole serum, crude extract, affinity purified IgG, ascites fluid, or culture supernatant. Each of the above formulations has completely different IgG concentrations. Each lot of the same antibody may have different IgG concentrations. Working with dilutions, instead of IgG concentration will be misleading. When using an antibody for the first time titrate the concentration of the antibody. In general, most of the antibodies will perform optimally at IgG concentration between 0.5 and 10 µg/ml. Testing 1, 5, and 10 µg/ml will give you a pretty good estimate of the optimum concentration. An optimum concentration will give strong signal with minimum nonspecific background staining. If the signal is weak and there is no background, consider increasing the antibody concentration and/or incubation time. If the signal is very strong with relatively high background consider, decreasing the antibody concentration and/or increase blocking.

Acknowledgments

We thank the members of the Molecular Cytology Core Facility for discussions and support. We would also like to thank Dr. Willie Mark from Mouse Genetics Core Facility at MSKCC for providing help and advice on mouse breeding. This work was supported by CCSG P30 CA 008748 grant from NCI.

References

1. Zitvogel L, Kroemer G. Targeting PD-1/PD-L1 interactions for cancer immunotherapy. *Oncoimmunology*. 2012; 1(8):1223–1225. DOI: 10.4161/onci.21335 [PubMed: 23243584]
2. Sharpe AH, Wherry JE, Ahmed R, et al. The function of programmed cell death 1 and its ligands in regulating autoimmunity and infection. *Nat Immunol*. 2007; 8:239–245. DOI: 10.1038/ni1443 [PubMed: 17304234]
3. Pardoll DM. The blockade of immune checkpoints in cancer immunotherapy. *Nat Rev Cancer*. 2012; 12(4):252–264. DOI: 10.1038/nrc3239 [PubMed: 22437870]
4. Chen DS, Mellman I. Oncology meets immunology: the cancer-immunity cycle. *Immunity*. 2013; 39(1):1–10. DOI: 10.1016/j.immuni.2013.07.012 [PubMed: 23890059]
5. Topalian SL, Hodi FS, Brahmer JR, et al. Safety, activity, and immune correlates of anti-PD-1 antibody in cancer. *N Engl J Med*. 2012; 366:2443–2454. DOI: 10.1056/nejmoa1200690 [PubMed: 22658127]
6. Grosso J, Horak CE, Inzunza D, et al. Association of tumor PD-L1 expression and immune biomarkers with clinical activity in patients (pts) with advanced solid tumors treated with nivolumab (anti-PD-1; BMS- 936558; ONO-4538). *J Clin Oncol (Meeting Abstracts)*. 2013; 31(15):Suppl 3016.
7. Gettinger SN, Shepherd FA, Antonia SJ, et al. First-line nivolumab (anti-PD-1; BMS-936558, ONO-4538) monotherapy in advanced NSCLC: safety, efficacy, and correlation of outcomes with PD-L1 status. *J Clin Oncol (Meeting Abstracts)*. 2014; 32(15):Suppl 8024.

Antibody and mRNA Probe Validation: Mouse-specific PDL-1

B16 Melanoma Cell Line Tumor in Mouse

Validation- Controls on B16 Cell Pellet

Fig. 1.

Validation of PDL-1 antibody with PDL-1 in situ hybridization. The top six panels are adjacent paraffin sections of B16 mouse melanoma cell line tumor in mouse. The first pair of panels is 10× and 60× images of Immunohistochemistry using PDL-1 goat polyclonal antibody. The second pair of panels is 10× and 60× images of mouse PDL-1 mRNA in situ hybridization staining imaged with bright field (using fast red as a chromogen). The last pair of panels is 10× and 60× images of mouse PDL-1 mRNA in situ hybridization staining imaged with epifluorescence microscopy (using fast red as red fluorophore). The bottom six panels are adjacent paraffin sections of B16 mouse melanoma cell line pellets. The top two panels are immunohistochemistry staining using PDL-1 goat polyclonal antibody imaged

with bright field and 60× magnification (left panel is cells treated with IFN-gamma and right panel is nontreated control cells). Bottom four panels are mouse PDL-1 mRNA in situ hybridization staining imaged with bright field (first panel) and fluorescence microscopy (second panel) (left two panels are cells treated with IFN-gamma and right two panels are nontreated control cells)

Author Manuscript

Author Manuscript

Author Manuscript

Author Manuscript