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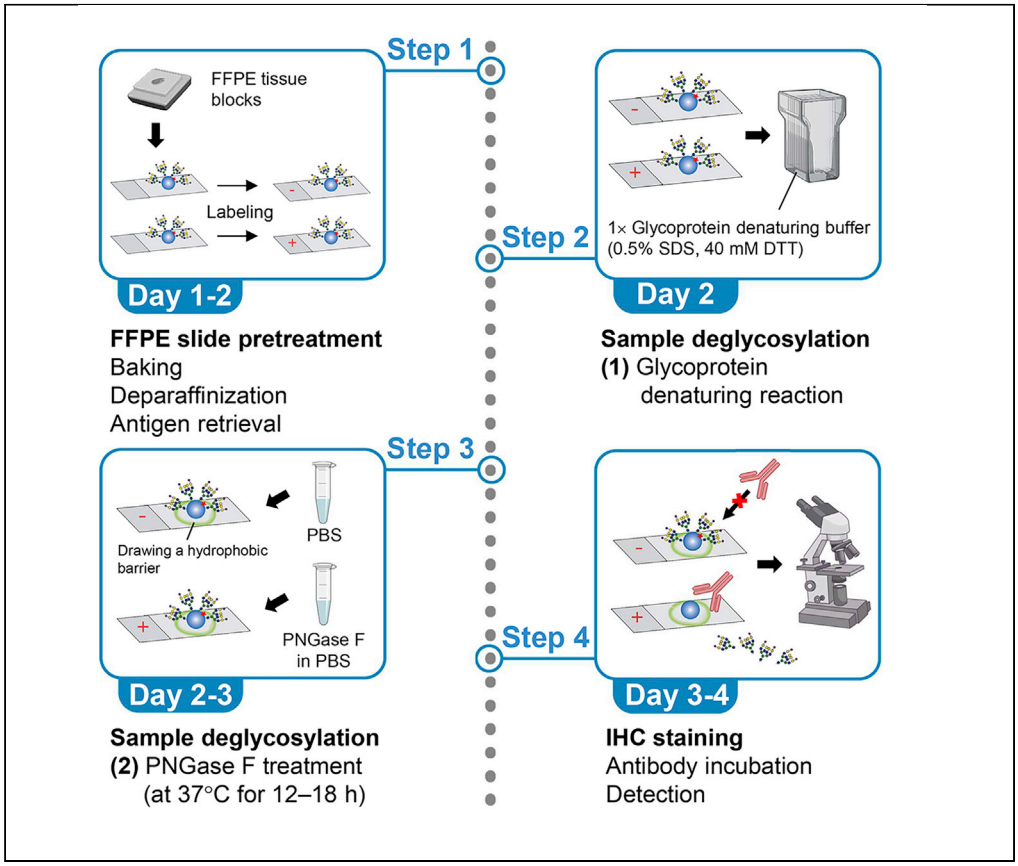
An optimized protocol for PD-L1 pathological assessment with patient sample deglycosylation to improve correlation with therapeutic response



Immunotherapy via PD-1/PD-L1 blockade is a promising strategy to eradicate cancer cells. However, the PD-L1 pathological level is inconsistent with the therapeutic response and is not a reliable biomarker to stratify patients for anti-PD-1/PD-L1 therapy. Here, we describe patient sample deglycosylation in an immunohistochemistry (IHC) assay to resolve this challenge. This protocol facilitates antigen retrieval by removing *N*-glycans from surface antigens on formalin-fixed paraffin-embedded (FFPE) tissue slides and can be applied in medical pathology for multiple cancer types.

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Highlights

Establishing a patient sample deglycosylation protocol incorporated into conventional IHC

Enzymatic removal of *N*-glycans from tissue sections enhances PD-L1 IHC detection

A more accurate assessment of PD-L1 improves correlation with therapeutic response

Sample deglycosylation in IHC assay can be applied in multiple cancer types

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An optimized protocol for PD-L1 pathological assessment with patient sample deglycosylation to improve correlation with therapeutic response

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SUMMARY

Immunotherapy via PD-1/PD-L1 blockade is a promising strategy to eradicate cancer cells. However, the PD-L1 pathological level is inconsistent with the therapeutic response and is not a reliable biomarker to stratify patients for anti-PD-1/PD-L1 therapy. Here, we describe patient sample deglycosylation in an immunohistochemistry (IHC) assay to resolve this challenge. This protocol facilitates antigen retrieval by removing *N*-glycans from surface antigens on formalin-fixed paraffin-embedded (FFPE) tissue slides and can be applied in medical pathology for multiple cancer types.

For complete details on the use and execution of this profile, please refer to Lee et al. (2019).

BEFORE YOU BEGIN

The protocol described below, a process termed sample deglycosylation in immunohistochemistry (IHC) assay, was developed to improve the accuracy of PD-L1 IHC detection and the correlation with therapeutic response, which in turn regains the ability of PD-L1 as a predictive biomarker to stratify patients for anti-PD-1/PD-L1 therapy. It takes a few additional steps that are directly incorporated into conventional IHC. This additional process is easy to manipulate and highly practical with a reasonable reaction time. Here, we describe the specific steps for performing an IHC assay using archived formalin-fixed paraffin-embedded (FFPE) blocks containing tumor tissues from patients with lung cancer. This protocol can also be adapted to other human tissue samples from tissue microarrays and FFPE cell blocks and applied to a large range of cancer types, such as breast, colon, rectal, prostate, and pancreatic cancers.

Institutional permissions

Note that institutional permission and oversight information for the human study to recruit human tissue samples should be acquired in advance from the relevant institutions. In this protocol, all

human tissue samples were collected following the guidelines approved by the Institutional Review Board at The University of Texas MD Anderson Cancer Center (LAB05-0127), China Medical University Hospital (CMUH106-REC1-145), Chang Gung Memorial Hospital (201800036B0), and The Affiliated Tumor Hospital of Harbin Medical University. Written informed consent was obtained from patients in all cases at the time of tissue sample collection.

Selection of archived FFPE tissue blocks with patient consent

Timing: 1 week

1. The following criteria for tissue collection must be met.
   a. Patients with lung cancer received or are undergoing either anti-PD-1 or anti-PD-L1 immunotherapy.
   b. All tissue samples are collected from patients before immunotherapy as archived FFPE tissue blocks.
   c. Patient clinical outcome, either progression-free survival (PFS) or overall survival (OS), is available for the cases selected. For those patients who are currently undergoing anti-PD-1/PD-L1 therapy, we defined the PFS as the length of time from the start of treatment until the date of correlation analysis with the PD-L1 pathological levels.

   Note: Most cases are typically acquired from needle biopsy rather than from surgical resection. Sometimes, an insufficient sample size of biopsy specimens leads to inaccuracy during histological evaluation (Focke et al., 2016). Moreover, thermal treatment by conventional IHC, together with additional sample deglycosylation steps, may increase the risk of section damage or detachment from slides, which may compromise the IHC readouts. If possible, prepare biopsy specimens of increasing sample sizes. See troubleshooting 1.

   Note: Two continuously cut tissue slides per patient are recommended. We normally use a pencil to label one slide with "+" and the other with "−", representing the process with and without sample deglycosylation, respectively.

   Note: Considering that the *N*-linked glycan structural hindrance to PD-L1 antibody detection against the antigen is a common issue in different fixed samples, we assume that the sample deglycosylation protocol should also work well to improve PD-L1 IHC detection when fixation methods other than formalin are used, such as alcohol-based fixatives (e.g., ethanol and methanol). Under the same digestion condition, the glycopeptidase PNGase F should also release the *N*-linked glycans by cleaving the covalent bond between the innermost amino sugar *N*-acetylglucosamine and the asparagine residue of targeted antigen on alcohol-fixed tissue sections.

   Note: The archived tissue blocks of pre-treatment will be utilized in a retrospective analysis to investigate the correlation between the expression levels of predictive biomarker candidates in tissue samples collected before treatment and the existing patient clinical outcome of post-treatment. Taking this protocol as an example, we evaluated the pathological assessment of PD-L1 in the pretreatment samples, processed with or without sample deglycosylation, in correlation to the existing data of patients' therapeutic response (PFS or OS) to anti-PD-1/PD-L1 therapy.

2. The patient has signed and dated a written informed consent at the time of tissue sample collection to partake in the study and publish identifiable images.

   ⚠ CRITICAL: Tissue in the archives is accessed only when the patient consents to the research study.

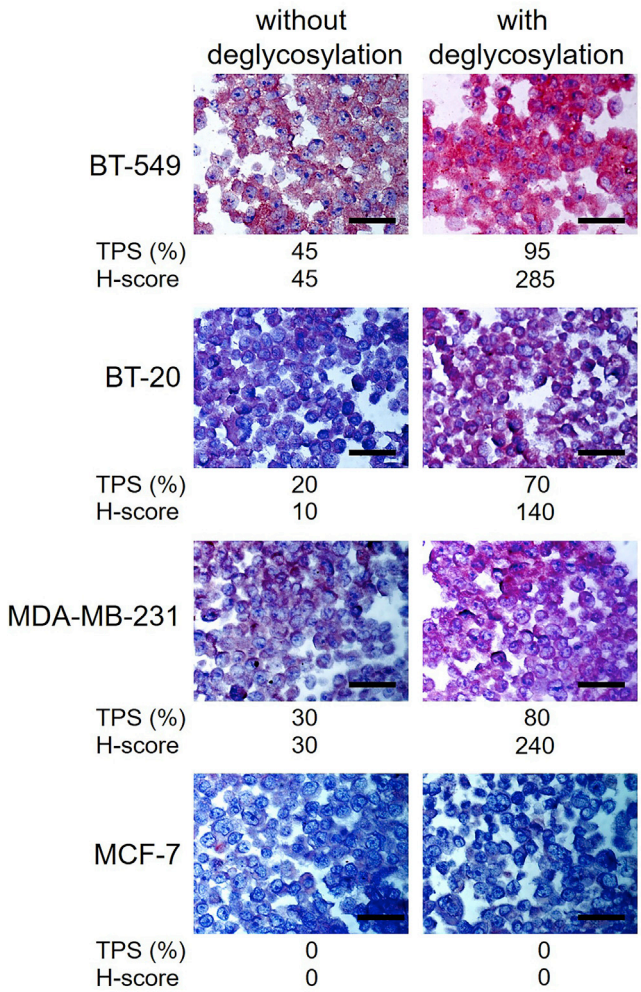

Figure 1. PD-L1 IHC detection, processed with or without deglycosylation, in breast cancer cells prepared from FFPE cell blocks

Representative images of TPS and H-score of IHC staining for BLBC (BT-549, BT-20, and MDA-MB-231) and non-BLBC (MCF-7) cancer cell samples, processed with or without deglycosylation by PNGase F treatment. Scale bars, 50 μm. Figure reprinted with permission from (Lee et al., 2019).

Cell preparation for FFPE cell blocks

Timing: 1 week

Note: Preparing FFPE cell blocks from cell lines is a helpful and less costly method of providing known positive and negative controls to evaluate enzyme efficiency and mimic the histologic processing of tissue samples used in IHC. Here, we set up positive and negative controls using FFPE cell blocks of breast cancer (Figure 1); PD-L1 expression is known in basal-like breast cancer cells that are used as a positive control (BLBC; BT-549, BT-20, and MDA-MB-231) but is limited in MCF-7 non-BLBC cells that are used as a negative control (Ali et al., 2015; Li et al., 2016). See troubleshooting 2.

Note: Step 3a is optimized for adherent cell lines. Suspension cell lines do not require attachment for growth; thus, this step can be skipped.

3. Collect the cells for preparing positive and negative controls.
   a. Harvest the cells by scraping them into media or by trypsinization of the cells with 0.05% trypsin-EDTA (ethylenediaminetetraacetic acid) solution in a 37°C humidified incubator with 5% CO_2 for 3–10 min, varying depending on the cells of interest, followed by inactivation of the trypsin with complete media.
   b. Count and spin down 10–30 × 10^6 cells in a sterile 15-mL centrifuge tube at room temperature (20°C–25°C) at 200 g for 5 min.
   c. Aspirate the media off the cell pellet.
   d. Resuspend the pellet with 10 mL of PBS, gently pipette up and down 2–3 times to break up cell clumps, spin down at room temperature (20°C–25°C) at 200 g for 5 min, and aspirate the PBS.
   e. Fix the pellet with 10 mL of 10% formalin at 4°C overnight (12–18 h).
   f. Spin down at room temperature (20°C–25°C) at 200 g for 5 min and aspirate the formalin.
   g. Resuspend the pellet with 10 mL of PBS, gently pipette up and down 2–3 times to break up cell clumps, spin down at room temperature (20°C–25°C) at 200 g for 5 min, and aspirate PBS.
   h. Add 10 mL of 70% ethanol as a safe holding solution.
   i. Seal the tube with parafilm to prevent evaporation.
   j. Store the tube at 4°C.
4. Drop off the cell solution from step 3j at the core facility for the following standard procedure.
   a. Trim the fixed cell samples from step 3j to fit into histology cassettes.
   b. Processing using an automatic tissue processor by immersing the samples in a series of solutions including ethanol, xylene, and paraffin wax. All steps are processed at room temperature (20°C–25°C) except the immersion of paraffin wax at 60°C.
      i. 70% ethanol, two times: 20 min, stirrer off; 20 min, stirrer on (low)
      ii. 95% ethanol, one time: 15 min, stirrer on (low)
      iii. 100% ethanol, two times: 15 min, stirrer on (low); 25 min, stirrer on (low)
      iv. Xylene, two times: 15 min, stirrer on (low); 25 min, stirrer on (low)
      v. Paraffin wax (60°C), two times: 30 min each, stirrer off
   c. Embed the samples in paraffin blocks to create FFPE cell blocks.
   d. Place blocks on ice for cooling.
   e. Cut the blocks into 4-μm slices using an automatic microtome at room temperature (20°C–25°C).
   f. Float the paraffin ribbon in a warm water bath (40°C–45°C).
   g. Place a microscope slide into the water bath and mount sections onto the slide.
   h. Air dry the slides overnight (12–18 h) at room temperature (20°C–25°C) ready for use.

Note: If the signal on FFPE cell samples is weak or the positive control does not work, see troubleshooting 3.

Preparation of solution and container for deparaffinization

Timing: 15–30 min

5. Prepare clean staining jars and fill them with each of the following solutions:
   a. Xylene (6×)
   b. 100% ethanol (3×)
   c. 95% ethanol (3×)
   d. 80% ethanol (2×)
   e. Distilled H_2O

Note: If dewaxing solutions (xylene and ethanol) have been reused many times, they tend to foul easily as a result of residual wax. We recommend preparing these solutions as fresh as possible, a maximum of 10 slides per 10 mL processed, to effectively completely remove paraffin wax and achieve good staining results.

⚠ CRITICAL: Xylene is a highly toxic, flammable substance and should be handled with care in a fume hood. For the use and disposal, be careful not to inhale the xylene vapor. Wear personal protective equipment such as chemical safety goggles, gloves, and masks. Turn off any ignition sources and turn on any ventilation devices to reduce the risk of xylene vapor combustion.

Preparation of the enzyme and device for sample deglycosylation

Timing: 15–30 min

6. Before deparaffinization, make sure that there is enough of the recombinant glycopeptidase, peptide-*N*-glycosidase F (also known as PNGase F), to obtain a final concentration of 5%.

   Alternatives: This protocol is optimized for the use of the commercially available PNGase F from New England BioLabs (Cat#P0704). However, it should be also applicable to that from different vendors, such as from Promega (Cat#V4831) or Sigma-Aldrich (Cat#G5166). We have not evaluated it using other PNGase F proteins, but we extrapolate that optimal concentrations may differ between sources.

7. Prepare humid chambers; the number depends on the slide numbers placed. Place a paper towel in the bottom of the slide-staining chamber and wet it with distilled H_2O. Cover the chamber with a clear or black lid since there are no light-sensitive stains in this protocol. Keep it at room temperature (20°C–25°C) until use.
8. Prepare a 37°C incubator with a small water bath for a PNGase F enzymatic reaction in a humid chamber overnight (12–18 h). We use a cell culture incubator in air supplemented with 5% CO_2; another appropriate tissue culture incubator could be used instead.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
PD-L1 antibody (clone 28-8 mAb) (1:100)	Abcam	Cat#ab205921; RRID:AB_2687878
Goat anti-rabbit IgG antibody (H+L), biotinylated (1:200)	Vector Laboratories	Cat#BA-1000; RRID:AB_2313606
Biological samples		
Human pre-immunotherapy archived tissue samples from 68 males and 27 females between 25–92 years old (mean ± SD, 59.29 ± 11.18; median 59.00)	Lee et al. (2019)	N/A
Chemicals, peptides, and recombinant proteins		
PNGase F	New England BioLabs	Cat#P0704
Sodium dodecyl sulfate (SDS)	Sigma-Aldrich	Cat#L5750
DL-dithiothreitol (DTT)	Sigma-Aldrich	Cat#D0632
Sodium chloride (NaCl)	Fisher Scientific	Cat#BP358212
Potassium chloride (KCl)	Fisher Scientific	Cat#BP366-500
Sodium phosphate dibasic (Na_2HPO_4)	Sigma-Aldrich	Cat#S0876
Potassium phosphate monobasic (KH_2PO_4)	Sigma-Aldrich	Cat#P5379
Formalin solution, 10%	Fisher Scientific	Cat#SF98-4
Xylenes	MDACC	N/A
Ethyl alcohol (ethanol)	MDACC	N/A
Paraffin wax	Leica Biosystems	Cat#39602004
Citrate buffer (pH 6.0), concentrate	Thermo Fisher Scientific	Cat#005000
Hydrogen peroxide (H_2O_2) solution	Sigma-Aldrich	Cat#H1009
Methanol	Fisher Scientific	Cat#A433S
Normal goat serum blocking solution	Vector Laboratories	Cat#S-1000-20

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
AEC chromogen substrate (3-amino-9-ethylcarbazole)	Sigma-Aldrich	Cat#A5754
N,N-dimethylformamide	Sigma-Aldrich	Cat#D4254
Sodium acetate anhydrous	Sigma-Aldrich	Cat#S2889
Glacial acetic acid	Sigma-Aldrich	Cat#695092
Mayer's hematoxylin	Agilent Technologies	Cat#S330930-2
Aqueous mounting medium	Agilent DAKO	Cat#S1964
Trypsin-EDTA solution (0.5%, 10×)	Thermo Fisher Scientific	Cat#15400054
Critical commercial assays		
VECTASTAIN Elite ABC-HRP kit	Vector Laboratories	Cat#PK-6100
Experimental models: Cell lines		
BT-549	ATCC	ATCC#HTB-122
BT-20	ATCC	ATCC#HTB-19
MDA-MB-231	ATCC	ATCC#HTB-26
MCF-7	ATCC	ATCC#HTB-22
A549	ATCC	ATCC#CCL-185
Calu3	ATCC	ATCC#HTB-55
Software and algorithms		
GraphPad Prism (version 7)	GraphPad Software	https://www.graphpad.com/
Other		
Parafilm M wrapping film	Fisher Scientific	Cat#13-374
PAP (peroxidase-antiperoxidase) pen	Electron Microscopy Sciences	Cat#71310
Glass slide microscope	Olympus, model: BX41	N/A
Automatic tissue processor	Leica Biosystems, model: Peloris II	N/A
Automatic microtome	Leica Biosystems, model: RM2255	N/A
Staining jar, rack, dish	N/A	N/A
1.7-mL microcentrifuge tube	N/A	N/A
15-mL centrifuge tube	N/A	N/A
Vortex	N/A	N/A
pH meter	N/A	N/A

MATERIALS AND EQUIPMENT

10× Phosphate-buffered saline (PBS)		
Reagent	Final concentration	Amount
NaCl	1.37 M	80 g
KCl	27 mM	2 g
Na_2HPO_4	100 mM	14.4 g
KH_2PO_4	18 mM	2.4 g
H_2O	N/A	Up to 1 L

Mix and adjust the pH with HCl to 7.4. Store at room temperature (20°C–25°C) for up to 1 year. Dilute stock solution 10:1 to make a 1× working solution.

1× Glycoprotein denaturing buffer			
Reagent	Final concentration	Stock concentration	Amount
SDS	0.5% (w/v)	5% (w/v)	100 µL
DTT	40 mM	1 M	40 µL
H_2O	N/A	N/A	900 µL
Total	-	-	1 mL

SDS can be made as a 0.5% working solution or as a 5% stock (see other solutions below). Store at room temperature (20°C–25°C) for 6 months. Right before use, add 40 µL of DTT (1 M) per 1 mL of 0.5% SDS buffer. Buffers should be scaled up linearly to accommodate larger reaction volumes.

Note: If glycoprotein denaturing buffer becomes turbid, see troubleshooting 4.

Other solutions

- 5% PNGase F: Add 5 μL of PNGase F stock solution to 95 μL of 1× PBS and mix well. The required volume of PNGase F working solution should be scaled up linearly. Prepare 5% PNGase F solution fresh prior to use. Store the stock solution at -20°C for up to 12 months.
- 5% SDS: Dissolve 5 g of SDS in 80 mL of H_2O. The solution may be gently heated (50–60°C) using a hot plate until the powder completely dissolves. Adjust the total volume to 100 mL with additional H_2O. Store the stock solution at room temperature (20°C–25°C) for 6 months.

⚠ CRITICAL: SDS is a fine powder that is easy to disperse and may cause respiratory tract irritation if inhaled. Be careful and wear an appropriate mask when weighing SDS.

- 1 M DTT: Dissolve 1.54 g of DTT in 8 mL of H_2O in a 15-mL centrifuge tube. Adjust the total volume to 10 mL with an additional H_2O. Vortex and prepare 1-mL aliquots. Stocks may be kept for 1 year at -20°C.
- 3-amino-9-ethylcarbazole (AEC) chromogen substrate stock solution (2.5%): Dissolve 0.25 g of AEC in 10 mL of *N,N*-dimethylformamide. Store at 4°C for up to 6 months.
- AEC chromogen substrate working solution (0.125%): Add 50 μL of 2.5% AEC stock solution and 1 μL of 30% H_2O_2 solution to 1 mL of 33 mM acetate buffer. Mix well. Prepare AEC working solution fresh prior to use.

Reagent	Final concentration	Amount
2.5% AEC stock solution	0.125% (w/v)	50 μL
30% H_2O_2	0.03% (w/v)	1 μL
33 mM acetate buffer	-	1 mL

- 33 mM acetate buffer: Dissolve 2.72 g of sodium acetate anhydrous in 1 L of H_2O. Mix and adjust the pH with glacial acetic acid to 5.0. Store at room temperature (20°C–25°C) for up to 6 months.

STEP-BY-STEP METHOD DETAILS

Note: Positive and negative controls of FFPE cell samples should be included from step 1 side by side with human archived FFPE tissue samples.

Pretreatment of FFPE tissue slides (days 1 and 2)

Timing: 5–7 h (day 2)

This section describes three major steps—slide baking, deparaffinization, and antigen retrieval—for the appropriate preparation of FFPE tissue slides prior to sample deglycosylation and IHC staining procedures.

1. Slide baking
   a. Place the slides on a staining rack in a 40°C oven overnight (12–18 h).
   b. Bake the slides in a dry oven at 58°C–65°C for 2–3 h the next day (day 2).
   c. Cool the slides to room temperature (20°C–25°C) before proceeding to the next step.

Note: To minimize tissue impairment or loss during the IHC staining process with additional sample deglycosylation steps, we highly recommend placing slides for a longer incubation

time at 40°C prior to baking at 58°C–65°C to help with tissue adhesion. For tissues that do not lie perfectly flat on slides, we have performed 40°C incubation for 3 days and reached experimental completion. See troubleshooting 1.

2. Deparaffinization and rehydration
   a. Deparaffinize or dewax slides in xylene solution for 10 min in each container, 6 times (5 min in each container if slide numbers are less than five).
   b. Rehydrate the slides by sequentially dipping them into a series of ethanol baths:
      i. 100% ethanol, 10 dips per container, 3 times
      ii. 95% ethanol, 10 dips per container, 3 times
      iiii. 80% ethanol, 10 dips per container, 2 times
   c. Wash the slides by rinsing them 3 times with distilled H_2O.

Alternatives: Xylene can be substituted with Histo-clear solution (National Diagnostics; Cat#HS200), which is less toxic, less flammable, and biodegradable and thus friendlier to the environment than xylene, although it is more expensive.

Pause Point: The slides can be kept in distilled H_2O at room temperature (20°C–25°C) for up to 2 h and then antigen retrieval can be continued on the same day.

3. Heat-induced antigen retrieval
   a. Immerse the slides in citrate buffer (10 mM, pH 6.0).
   b. Heat the slides in a microwave oven.
      i. 1,000 W for 2 min
      ii. 200 W for 8 min
   c. Cool down slides for 1–2 h to room temperature (20°C–25°C) before proceeding to the next step.
   d. Wash the slides with PBS for 5 min, 2 times.

Note: Antigen retrieval used prior to IHC staining on FFPE tissue sections is an effective method of unmasking the epitopes for antibody binding. It can be performed by heat (e.g., microwave, autoclave, pressure cooker, and water bath) or enzyme treatment (e.g., pronase, trypsin, and pepsin). Here, we recommend using a heat method to denature tissue sections at 95°C–100°C, for instance, microwave heating in the present protocol.

Pause Point: The slides can be kept in PBS at room temperature (20°C–25°C) for up to 2 h and then continued with sample deglycosylation with PNGase F enzymatic digestion on the same day.

Sample deglycosylation (day 2 continued and day 3)

Timing: 4 h (day 2)

The principle of heat-induced antigen retrieval to expose epitopes is in line with the glycoprotein denaturing reaction step, as described in the manufacturer's deglycosylation protocol: https://www.neb.com/protocols/2014/07/31/pngase-f-protocol. Taking advantage of the consistency between these two steps, which can both cause protein denaturing to facilitate enzymatic digestion, we developed a new protocol by incorporating sample deglycosylation into a conventional IHC assay after antigen retrieval. This section describes how to perform the sample deglycosylation protocol in an IHC assay in two major steps, glycoprotein denaturing reaction and PNGase F treatment.

4. Glycoprotein denaturing reaction
   a. Discard the PBS.

b. Fill the staining jar with enough glycoprotein denaturing buffer to cover the slides.
c. Incubate the slides at room temperature (20°C–25°C) for 3 h.

Note: Since we have performed pretreatment of heat-induced antigen retrieval on FFPE tissue sections, an additional heating reaction is not recommended in the glycoprotein denaturing reaction in this protocol to minimize possible tissue damage. We immerse slides in glycoprotein denaturing buffer at room temperature (20°C–25°C) for up to 3 h to complement the effectiveness of pretreating heat-induced antigen retrieval. Although we have not directly compared the heating reaction of FFPE tissue slides in the denaturing reaction condition to that used here, our approach should also be applicable to the heating reaction at 100°C for 10 min in the denaturing reaction condition, according to the manufacturer's protocol and to the results of recent reports that successfully applied the thermal denaturation strategy (Mei et al., 2021; Xu et al., 2021).

Note: To minimize evaporation during the process, we use parafilm to seal the container; this could be substituted with a staining jar with a lid.

d. Discard the glycoprotein denaturing buffer.
e. Wash the slides with PBS for 5 min, 4 times.
f. Immerse the slides with PBS and proceed with the PNGase F treatment procedure immediately.

⚠ CRITICAL: SDS as an anionic denaturing detergent in the glycoprotein denaturing buffer that is known to inhibit PNGase F enzymatic activity. To prevent PNGase F inactivation in the next major step, it is important to wash away residual SDS on tissue sections thoroughly after the denaturing reaction. As the SDS concentration will be much diluted after four repeats of slide washing with PBS, we have not tested it by adding a non-ionic detergent, such as a final concentration of 1% of Nonidet P-40 (NP-40), in washing buffer; this may also help to counteract the inhibitory effect of SDS on PNGase F activity. See troubleshooting 2.

5. PNGase F treatment
   a. Remove a maximum of 4–6 slides per 2–3 patients from the container at one time.
   b. Drain and shake off the excess PBS.
   c. Carefully wipe around the specimen on each slide.
   d. Create a hydrophobic barrier around the slide specimen with a PAP pen.

Note: The PAP pen is a special marking pen that draws a thin film-like water repellent circle around a specimen on a slide. This hydrophobic barrier keeps staining liquid pooled on the tissue sections for using less reagents per section. The marking circle can be optionally removed by xylene or xylene substitutes after the staining procedure.

Alternatives: In addition to the PAP pen used in this protocol (Electron Microscopy Sciences; Cat#71310), similar pens are available from other manufacturers, such as Abcam (Cat#ab2601) or Thermo Fisher Scientific (Cat#008899).

   e. Add enough of the appropriate solution to cover specimens processed with or without deglycosylation.
      i. With deglycosylation: add 5% PNGase F solution to the specimen on the slide labeled with "+".
      ii. Without deglycosylation: add PBS solvent alone to the specimen on the slide labeled with "−".

⚠ CRITICAL: Considering that the storage buffer of PNGase F contains 50% glycerol and that an over 5% glycerol concentration may interfere with PNGase F activity, PNGase F

is limited to 1/10 (or less) of the total reaction volume, as recommended by the manufacturer, to keep the final glycerol concentration equal to (or less than) 5%. In this protocol, we use PNGase F at 1/20 of the total reaction volume to make a 5% PNGase F working solution in a 2.5% glycerol final concentration, that is, 5 μL of PNGase F per 100 μL of PBS. See troubleshooting 2.

Note: We recommend making fresh PNGase F solution each time. Depending on the number of tissue sections carried out, the PNGase F solution should be scaled up linearly to accommodate larger reaction volumes.

⚠ CRITICAL: Full coverage of solutions on tissue sections is important. We recommend applying the average volume of 200 μL per section on a slide. However, for large sections (e.g., surgical specimens), more than 200 μL should be used; for small sections (e.g., needle biopsy specimens), less than 200 μL may be enough.

f. Lay slides flat with the specimen facing upward in a humid chamber.
g. Place the humid chamber in a 37°C incubator in air supplemented with 5% CO_2 for 12–18 h.

Note: Treatment of PNGase F overnight for 12–18 h ensures the complete removal of glycan moieties from the surface antigens, although we can also test the incubation time for less than 12 h to facilitate the process. This deglycosylation protocol is optimized for PD-L1 pathological assessment; however, PNGase F treatment conditions, including enzyme concentration and incubation time, may vary depending on the protein of interest. Further optimization is highly recommended.

h. Remove the humid chamber with slides from the incubator and place it on a bench the next day (day 3).
i. Drain and shake off excess solution from the slides.
j. Place the slides in a clean staining jar filled with PBS.
k. Wash the slides with PBS for 5 min, 2 times.
l. Immerse the slides with PBS.

⏸ Pause Point: The slides can be kept in PBS at room temperature (20°C–25°C) for up to 2 h and then continued with an IHC staining procedure the same day.

IHC staining: Blocking and primary antibody incubation (days 3 and 4)

Timing: 1 h (day 3)

After sample deglycosylation has completed, the tissue sections can be processed for the subsequent steps using a routinely used, conventional IHC method, unless otherwise noted. Tissue sections should not be allowed to dry out during the incubation process. Given that 200 μL results in an average coverage of the entire tissue section on a slide, the required working volume of each solution should be calculated and prepared before the step.

6. Peroxidase and non-specific antigen blocking
   a. Block endogenous peroxidase
      i. Incubate the slides with 3% hydrogen peroxide solution (H_2O_2) for 10 min in a humid chamber at room temperature (20°C–25°C).
      ii. Wash the slides with PBS for 5 min, 3 times.
   b. Block non-specific antigens
      i. Incubate the slides with 10% blocking buffer (normal goat serum in PBS) for 30 min in a humid chamber at room temperature (20°C–25°C).

ii. Wipe off excess blocking buffer from the slides.

7. Incubation with primary antibody
   a. Prepare a 1:100 dilution of anti-PD-L1 rabbit monoclonal antibody in PBS.
   b. Apply the diluted primary antibody to the tissue sections on the slides.
   c. Incubate the slides in a humid chamber at 4°C overnight (12–18 h).
   d. Wash the slides with PBS for 5 min, 3 times (day 4).

Note: The optimal dilution ratio and incubation time may vary between antibodies. The first time a new primary antibody is tested, we recommend 4°C overnight (12–18 h) incubation to allow more time for the antibody to bind to the antigen at a lower titer. Here, we use a 1:100 antibody-to-PBS ratio, that is, 10 μg/mL of anti-PD-L1 antibody (clone 28-8; Abcam, Cat#ab205921); the concentration could be further adjusted to 2 μg/mL in a 1:500 dilution ratio (Mei et al., 2021).

Note: There are four PD-L1 antibodies in IHC assays that have been approved by the US Food and Drug Administration (FDA) for in vitro diagnostic use, including clones 28-8, SP142, 22C3, and SP263 (Buttner et al., 2017; Adam et al., 2018). Besides clone 28-8 used here, this protocol has been successfully applied to clone SP142 (Mei et al., 2021). We expect these assays to be an effective general approach for other membrane proteins that are frequently glycosylated at different levels.

IHC staining: Secondary antibody incubation and detection (day 4 continued)

Timing: 3–4 h

8. Incubation with biotin-conjugated secondary antibody
   a. Prepare a 1:200 dilution of anti-rabbit secondary antibody in PBS.
   b. Apply the diluted secondary antibody to the tissue sections on the slides.
   c. Incubate the slides in a humid chamber for 1 h at room temperature (20°C–25°C).
   d. Wash the slides with PBS for 5 min, 3 times.
9. Incubation with peroxidase-conjugated avidin-biotin complex (ABC-HRP conjugate)
   a. Prepare a 1:100 dilution of ABC-HRP conjugate in PBS.
   b. Apply the diluted ABC-HRP conjugate to the tissue sections on the slides.
   c. Incubate the slides in a humid chamber for 1 h at room temperature (20°C–25°C).
   d. Wash the slides with PBS for 5 min, 3 times.
10. Incubation with AEC chromogen substrate
   a. Prepare fresh AEC chromogen substrate working solution (0.125%).
   b. Incubate the slides in a staining dish filled with the working solution for 5–10 min at room temperature (20°C–25°C).
   c. Rinse the slides with distilled H_2O, 3 times.

Note: The enzyme HRP catalyzes appropriate chromogenic substrates, such as AEC and 3,3′-diaminobenzidine (DAB), into visible colored products to facilitate IHC detection. Here, we use AEC, an aqueous chromogen that is relatively less toxic than is DAB, but DAB can be used as well (Mei et al., 2021; Xu et al., 2021).

11. Counterstaining
   a. Incubate the slides in Mayer's hematoxylin for 20–30 s.
   b. Rinse the slides with distilled H_2O, 3 times.
12. Mounting
   a. Apply aqueous mounting medium to the tissue sections on the slides.
   b. Place the coverslip on top.
   c. Lay the slides on a flat surface to dry prior to viewing them under a microscope.

Note: The immunostained images can be viewed under a glass slide microscope (Lee et al., 2019) or digitally by digital slide scanners, such as Aperio Digital Pathology Slide Scanners (Leica Biosystems) (Mei et al., 2021; Xu et al., 2021).

⚠ CRITICAL: Unlike DAB, AEC is soluble in ethanol and xylene. Specimens that have been stained in AEC cannot be dehydrated with ethanol and cleared with xylene before mounting; a suitable aqueous, but not organic, mounting medium should be used for permanent record-keeping.

Pause Point: After drying, IHC slides can be stored at room temperature (20°C–25°C).

EXPECTED OUTCOMES

Several anti-PD-L1 antibodies and their corresponding IHC platforms have been approved by the FDA for diagnostic tests to guide anti-PD-1/PD-L1 treatment; however, inter-assay heterogeneity in PD-L1 IHC detection has also been reported (Pinato et al., 2019). Such diversity may partly result from the presence of the *N*-glycan structure of PD-L1 that hinders its detection by anti-PD-L1 antibodies to varying degrees. In this protocol, we use one of the approved PD-L1 antibodies, clone 28-8, as an example to describe a detailed method of enzymatic removal of *N*-glycan moieties from FFPE tissue sections on the slides and subsequently evaluate PD-L1 levels by conventional IHC staining. Once completed, using the deglycosylation procedure to facilitate antigen retrieval, one can expect to observe an alteration in the anti-PD-L1 signal in certain populations of samples, leading to a more accurate assessment of the PD-L1 pathological level to correlate with patients' therapeutic response.

Indeed, PD-L1 IHC detection, as determined either by tumor proportion score (TPS), defined by the percentage of PD-L1-positive membrane staining of tumor cells (Figure 2) (Lantuejoul et al., 2020; De Marchi et al., 2021), or a histochemical score (H-score; see also quantification and statistical analysis) (Detre et al., 1995), is enhanced after deglycosylation when processed with PNGase F treatment in lung cancer patient cases 6, 7, and 11 but was not changed in case 8 (Figure 3). Here, we illustrate these expected outcomes in a statistical analysis using the TPS and H-score of PD-L1 from a cohort of lung cancer patients (n = 44), as shown in Figures 4 and 5. The TPS of samples processed with deglycosylation increases significantly compared with that of those without deglycosylation ($p < 0.0001$; Figure 4A). The fold changes in TPS after deglycosylation can be categorized into two comparable groups: (1) cases whose PD-L1 TPS did not change (50.0%) and (2) those that increased (50.0%), among which 18.2% increased by more than 2-fold (Figure 4B). Similar patterns can also be observed in the evaluation of PD-L1 H-score (Figures 4C and 4D). For the correlation analysis between PD-L1 pathological levels and clinical response rates, such as progression-free survival (PFS), deglycosylation of tissue samples can significantly improve the p values between patients' PFS and the PD-L1 levels, as determined by the TPS (Figure 5A; $p = 0.017$ vs. $p = 0.460$) or H-score (Figure 5B; $p = 0.016$ vs. $p = 0.362$). In addition to tissue samples from patients with lung cancer, we expect that the sample deglycosylation protocol can be used as a general approach to be applied to multiple cancer types. Indeed, in a cohort of patients (n = 95) with different types of cancer, including lung, head and neck, esophageal, and bladder, we observe the similarly improved PD-L1 IHC detection (either TPS or H-score) (Figure 6) and correlation with therapeutic response (either PFS or OS) (Figure 7) after sample deglycosylation. See troubleshooting 5.

QUANTIFICATION AND STATISTICAL ANALYSIS

Pathologists will evaluate the IHC results independently using an established semiquantitative approach to assign an H-score to tumor samples (Detre et al., 1995).The H-score of PD-L1 is randomly chosen from 10 fields at 400× magnification and the average is calculated by adding the results of multiplication of the membrane staining intensity to the percentage of PD-L1-positive

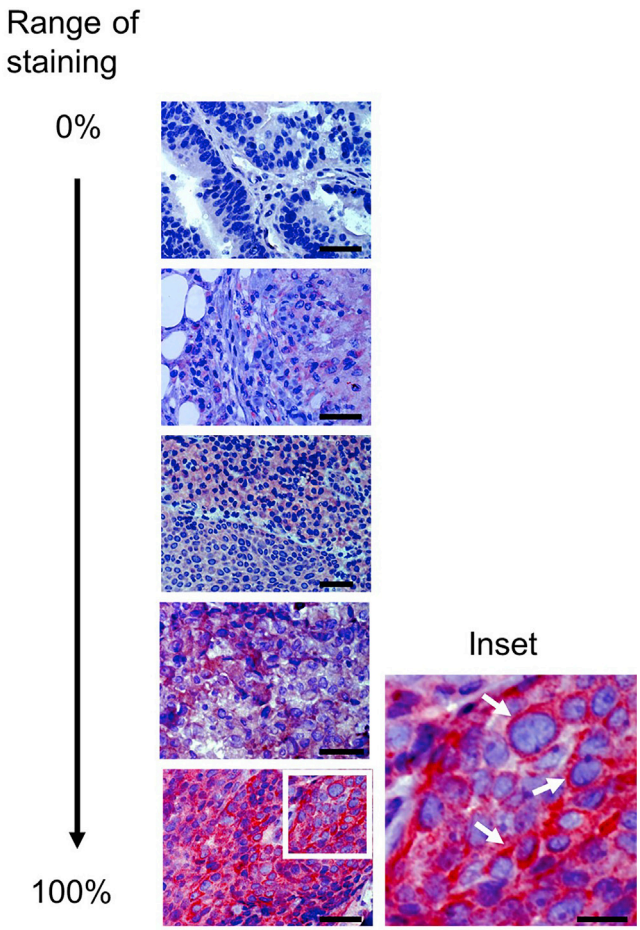

Figure 2. Standard for the percentage of PD-L1 TPS in IHC detection

Representative images of PD-L1 TPS, defined by the percentage of PD-L1 membrane staining of positive tumor cells, in lung cancer patients. Samples displayed varying percentages of the stained cells, ranging from negative (0%) to strongly positive (100%) staining of the tumor cells. Scale bars, 50 μm. Inset: PD-L1 membrane staining (arrows); scale bar, 20 μm. Figure reprinted with permission from (Lee et al., 2019).

cells (% TPS) at each staining intensity level. The intensity level is scored as 0, 1, 2, or 3 for the presence of a negative, weak, intermediate, or strong signal, respectively. The following formula is used:

$$\text{H-score} = [1 \times (\%\ \text{TPS at intensity level 1}) + 2 \times (\%\ \text{TPS at intensity level 2}) + 3 \times (\%\ \text{TPS at intensity level 3})]$$

The statistical analyses used in this protocol are performed using the GraphPad Prism program (version 7; Prism Software, Inc., San Diego, CA, USA). These include but are not limited to the Wilcoxon signed-rank test for comparing two groups of matched samples and the Pearson correlation test for determining the linear correlation between two variables. A p value of < 0.05 was considered statistically significant.

LIMITATIONS

Our protocol provides a starting point for researchers who want to carry out sample deglycosylation in IHC studies, although it is not ubiquitously applied to a broad spectrum of all glycosylation types. There are two main types of glycans bound to proteins that have been extensively analyzed in recent decades, *N*-linked and mucin-type *O*-linked. Considering that PD-L1 is a primarily *N*-linked

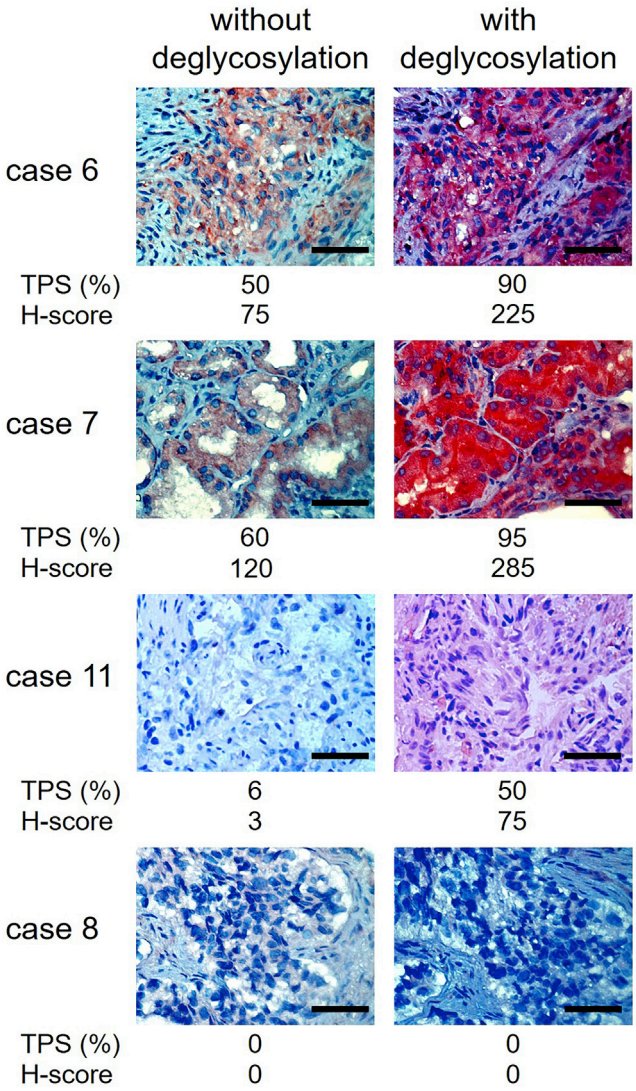

Figure 3. PD-L1 IHC detection, processed with or without deglycosylation in lung cancer patients

Representative cases of TPS percentages and H-score values representing PD-L1 protein expression from IHC staining. Results are from the archived FFPE tumor tissue blocks before treatment from patients with lung cancer who received (cases 7, 8, and 11) or are undergoing (case 6) anti-PD-1/PD-L1 immunotherapy, processed with or without deglycosylation by PNGase F. Scale bars, 50 μm. Figure reprinted with permission from (Lee et al., 2019).

glycoprotein (Li et al., 2016), this protocol, which uses the universal *N*-glycosidase PNGase F, is of specific value for investigating the enzymatic removal of *N*-glycan, but not *O*-glycan, from the cell surface PD-L1 antigen.

Since the steric hindrance of *O*-glycans may interfere with antigen-antibody recognition for clinical diagnosis, similar to the issue of the *N*-glycan structure in PD-L1 (Wang et al., 2020), this protocol should not be efficient enough to provide precise pathological readouts for proteins that harbor both *N*-linked and *O*-linked glycosylation, such as immune checkpoint protein T-cell immunoglobulin mucin-3. *N*-glycans is removed completely by PNGase F treatment, but it currently remains difficult to deglycosylate all *O*-glycans from a glycoprotein; the substantial heterogeneity with a lack of a common-core *O*-glycan structure makes studies challenging (Wilkinson and Saldova, 2020).

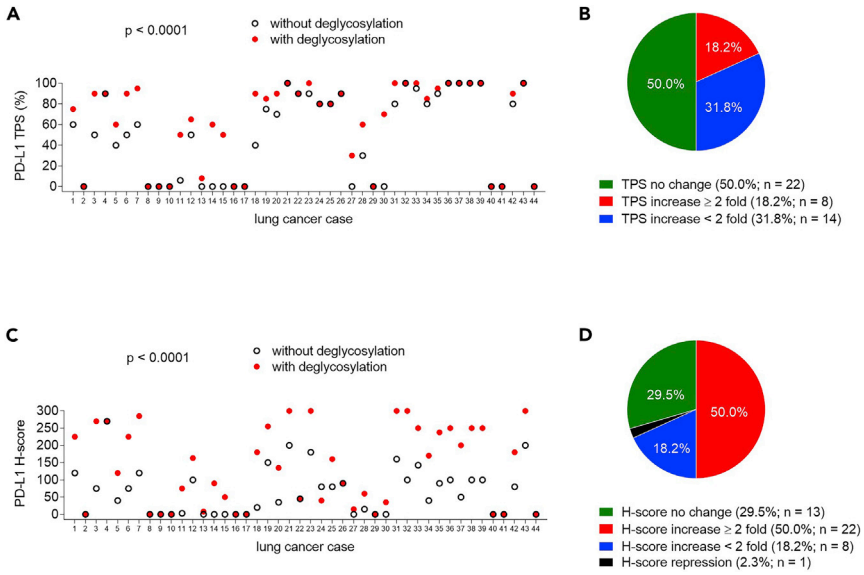

Figure 4. Increased PD-L1 signal after deglycosylation in a significant number of lung cancer patient tissue samples

(A) The percentage of PD-L1 TPS from IHC staining of archived FFPE tumor tissue blocks before treatment from patients with lung cancer who received or are undergoing anti-PD-1/PD-L1 immunotherapy (n = 44), processed with or without deglycosylation by PNGase F treatment.

(B) A pie chart highlighting the fold change in PD-L1 TPS after *N*-linked glycosylation removal through PNGase F treatment from (A).

(C) The value of PD-L1 H-score from IHC staining of patient tissue slides, processed with or without deglycosylation from (A).

(D) A pie chart highlighting the fold change in PD-L1 H-score after *N*-linked glycosylation removal through PNGase F treatment from (C). (A and C) Results were analyzed by the Wilcoxon signed-rank test.

TROUBLESHOOTING

Problem 1

The sample deglycosylation steps increase the risk of tissue lifting from slides (before you begin step 1; major step 1).

Potential solution

To keep more tissue on the slides after they have been subjected to the harsh treatments of the protocol, we recommend selecting biopsy samples of archived FFPE tissue blocks of increasing size as often as possible. To yield more accurate sample detection and reliable results, we recommend using well-processed tissue samples that are cut evenly and freshly at a universal thickness; however, not all conditions can be perfectly met, especially as this protocol requires archived patient tissue samples that may come from collaborations across multiple clinic centers following various operating procedures. Therefore, when tissue samples are obtained from different locations, proper slide drying before deparaffinization is necessary to improve tissue attachment to slides, followed by incubating samples in a 40°C oven for a longer duration, at least overnight for 12–18 h, and then baking at 58°C–65°C for at least 2 h.

Problem 2

How to control PNGase F performance consistently across experiments (before you begin steps 3 and 4; major steps 4 and 5).

Potential solution

In general, the experimental conditions during the PNGase F incubation period should be well maintained, such as a proper temperature at 37°C, full coverage of solutions on tissue sections, and humidity with a wet paper towel in the chamber overnight (12–18 h).

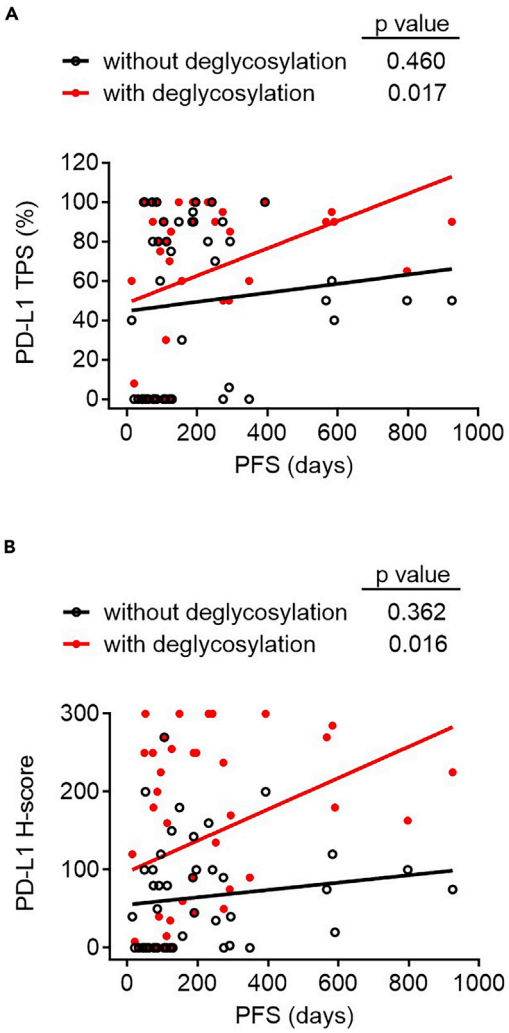

Figure 5. Positive association of PD-L1 detection after deglycosylation with response to anti-PD-1/PD-L1 therapy in lung cancer patients

(A and B) Pearson correlation test between PD-L1 TPS (A) or PD-L1 H-score (B) in lung cancer patient tissue samples, processed with or without deglycosylation (Figure 4; n = 44), and the corresponding PFS from anti-PD-1/PD-L1 therapy. Figure reprinted with permission from (Lee et al., 2019).

Ensure that PNGase F enzymatic activity is not inhibited using the following approach: first, completely remove the wax from the tissue sections because incomplete removal of paraffin can interfere with enzymatic access to *N*-glycans; fresh dewaxing solutions are highly recommended. Second, keep the final concentration of glycerol additive in the storage buffer of PNGase F equal to (or less than) 5% to avoid excess glycerol, which will result in a loss of PNGase F enzymatic activity. Third, make certain to thoroughly wash away any residual SDS after the denaturing reaction as SDS detergent applied in the glycoprotein denaturing buffer is an excellent inhibitor of PNGase F. You can also add 1% NP-40 to washing buffer to counteract detergent inhibition. We recommend using PNGase F from the same catalog and lot numbers to ensure consistent enzymatic cleavage. However, if not applicable, check the enzymatic activity using proper controls (before you begin steps 3 and 4) when you use a new batch of PNGase F.

Problem 3

The signal on FFPE cell samples is weak, or the FFPE-positive control does not work. (before you begin steps 3 and 4)

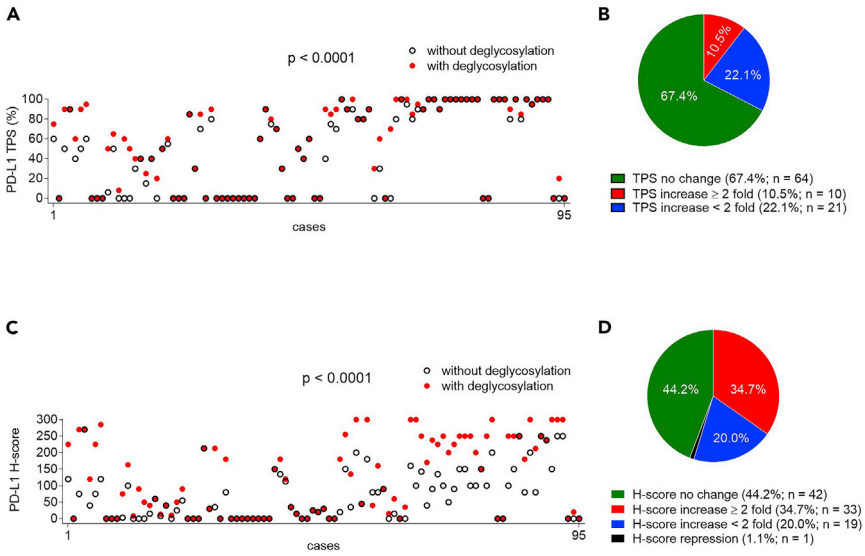

Figure 6. Increased PD-L1 signal after deglycosylation in a significant number of tissue samples from patients with different types of cancer

(A) The percentage of PD-L1 TPS from IHC staining of archived FFPE tumor tissue blocks before treatment from patients with different types of cancer who received or are undergoing anti-PD-1/PD-L1 immunotherapy (n = 95), processed with or without deglycosylation by PNGase F treatment.

(B) A pie chart highlighting the fold change in PD-L1 TPS after *N*-linked glycosylation removal through PNGase F treatment from (A).

(C) The value of PD-L1 H-score from IHC staining of patient tissue slides, processed with or without deglycosylation from (A).

(D) A pie chart highlighting the fold change in PD-L1 H-score after *N*-linked glycosylation removal through PNGase F treatment from (C). (A and C) Results were analyzed by the Wilcoxon signed-rank test. Figure reprinted with permission from (Lee et al., 2019).

Potential solution

To create FFPE cell samples with less cell integrity damage and better preservation of cell membrane proteins such as PD-L1, cells should be harvested by gentle scraping or trypsinization by optimizing both the duration of trypsinization and the concentration of trypsin. If a FFPE-positive control is not effective, namely that the anti-PD-L1 signal is not enhanced in BLBC-positive control cells (Figure 1) after deglycosylation with PNGase F, check all of the conventional IHC staining and sample deglycosylation steps thoroughly, including troubleshooting for consistency and accuracy. Storage of FFPE sections that were cut more than 1 month previously is usually not recommended, as older sections tend to produce poor signals.

Problem 4

Glycoprotein denaturing buffer becomes turbid (Materials and equipment).

Potential solution

The storage temperature of glycoprotein denaturing buffer that is composed of SDS is critical. SDS solution should always be stored at room temperature (20°C–25°C) and not refrigerated to avoid precipitation. However, the cloudy precipitate can be redissolved if the solution is warmed up at 37°C.

Problem 5

Does sample deglycosylation impact on the selection of PD-L1 scoring methods (expected outcomes).

Potential solution

There are several PD-L1 scoring assessments currently applied in clinical trials, including TPS, combined positive score, and tumor-infiltrating immune cell score, depending on the varying antibody

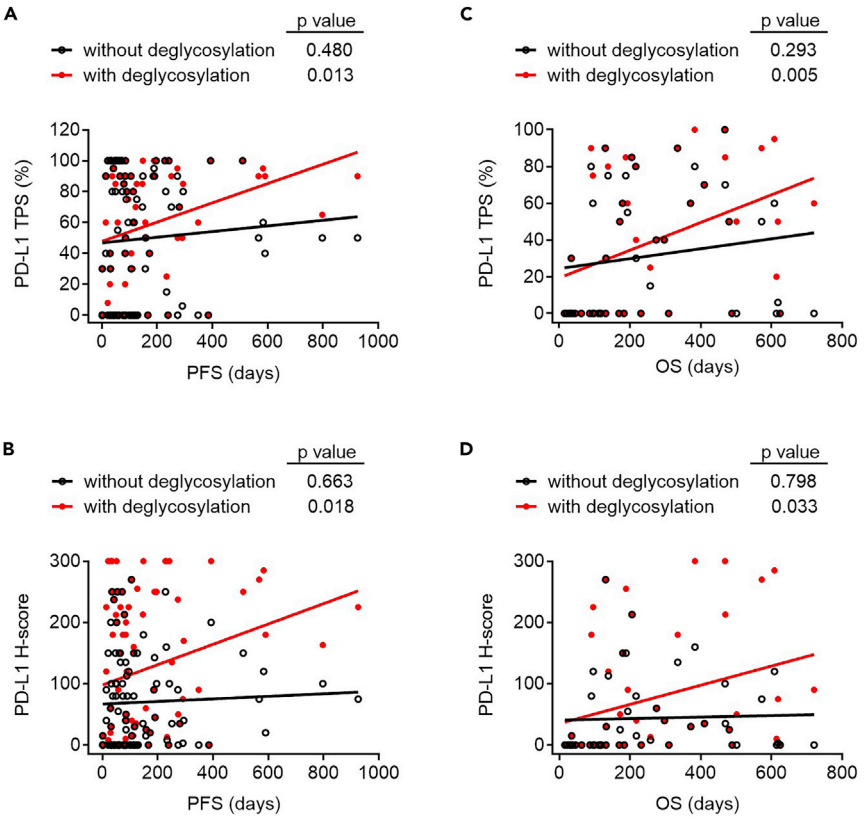

Figure 7. Positive association of PD-L1 detection after deglycosylation with response to anti-PD-1/PD-L1 therapy in patients with different types of cancer

(A and B) Pearson correlation test between PD-L1 TPS (A) or PD-L1 H-score (B) in patient tissue samples, processed with or without deglycosylation (Figure 6; n = 95), and the corresponding PFS from anti-PD-1/PD-L1 therapy.

(C and D) Pearson correlation test between PD-L1 TPS (C) or PD-L1 H-score (D) in patient tissue slides processed with or without deglycosylation and the corresponding OS from anti-PD-1/PD-L1 therapy (Figure 6; n = 49 with the OS available). Figure reprinted with permission from (Lee et al., 2019).

clones and cancer types. For example, the assays with clones 28-8, 22C3, and SP263 in lung cancer are regularly reported using TPS (Lantuejoul et al., 2020). In addition, pathologists also utilize a semi-quantitative approach to assign an H-score for laboratory-developed preclinical studies. Based on our results, both TPS and H-score evaluated in this sample deglycosylation protocol present the similarly improved PD-L1 IHC detection and correlation with therapeutic response, which may suggest that this protocol could be applicable with the use of different pathology scoring methods of PD-L1. However, for the purpose of clinical settings, we recommend following the current guidelines for PD-L1 expression testing in the clinic to evaluate TPS rather than H-score if there is a discrepancy between these two scoring methods.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Mien-Chie Hung (mhung@cmu.edu.tw).

Materials availability

There are no newly generated materials associated with this protocol.

Data and code availability

This study did not generate any datasets or code.

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AUTHOR CONTRIBUTIONS

Conceptualization, Y.-N.W., H.-H.L., and M.-C.H.; Investigation, Y.-N.W., H.-H.L., and W.X.; Writing - Original Draft, Y.-N.W., H.-H.L., and Y.-Y.C.; Writing - Review & Editing, Y.-N.W., H.-H.L., Y.W., and M.-C.H.; Funding acquisition, M.-C.H.; Supervision, M.W., D.Y., and M.-C.H.

DECLARATION OF INTERESTS

H.-H.L., Y.-N.W., and M.-C.H. are listed as inventors on a patent application (International Patent Application No. PCT/US2019/036073 based on U.S. Provisional Patent Application No. 62/681,929, entitled "Detection of Immune Checkpoint Molecules by Deglycosylation") submitted by The University of Texas MD Anderson Cancer Center. All other authors declare no non-financial or financial competing interests.

REFERENCES

Adam, J., Le Stang, N., Rouquette, I., Cazes, A., Badoual, C., Pinot-Roussel, H., Tixier, L., Danel, C., Damiola, F., Damotte, D., et al. (2018). Multicenter harmonization study for PD-L1 IHC testing in non-small-cell lung cancer. Ann. Oncol. *29*, 953–958.

Ali, H.R., Glont, S.E., Blows, F.M., Provenzano, E., Dawson, S.J., Liu, B., Hiller, L., Dunn, J., Poole, C.J., Bowden, S., et al. (2015). PD-L1 protein expression in breast cancer is rare, enriched in basal-like tumours and associated with infiltrating lymphocytes. Ann. Oncol. *26*, 1488–1493.

Buttner, R., Gosney, J.R., Skov, B.G., Adam, J., Motoi, N., Bloom, K.J., Dietel, M., Longshore, J.W., Lopez-Rios, F., Penault-Llorca, F., et al. (2017). Programmed death-ligand 1 immunohistochemistry testing: a review of analytical assays and clinical implementation in non-small-cell lung cancer. J. Clin. Oncol. *35*, 3867–3876.

De Marchi, P., Leal, L.F., Duval Da Silva, V., Da Silva, E.C.A., Cordeiro De Lima, V.C., and Reis, R.M. (2021). PD-L1 expression by tumor proportion score (TPS) and combined positive score (CPS) are similar in non-small cell lung cancer (NSCLC). J. Clin. Pathol. *74*, 735–740.

Detre, S., Saclani Jotti, G., and Dowsett, M. (1995). A "quickscore" method for immunohistochemical semiquantitation: validation for oestrogen receptor in breast carcinomas. J. Clin. Pathol. *48*, 876–878.

Focke, C.M., Decker, T., and Van Diest, P.J. (2016). The reliability of histological grade in breast cancer core needle biopsies depends on biopsy size: a comparative study with subsequent surgical excisions. Histopathology *69*, 1047–1054.

Lantuejoul, S., Sound-Tsao, M., Cooper, W.A., Girard, N., Hirsch, F.R., Roden, A.C., Lopez-Rios, F., Jain, D., Chou, T.Y., Motoi, N., et al. (2020). PD-L1 testing for lung cancer in 2019: perspective from the IASLC pathology committee. J. Thorac. Oncol. *15*, 499–519.

Lee, H.H., Wang, Y.N., Xia, W., Chen, C.H., Rau, K.M., Ye, L., Wei, Y., Chou, C.K., Wang, S.C., Yan, M., et al. (2019). Removal of N-linked glycosylation enhances PD-L1 detection and predicts anti-PD-1/PD-L1 therapeutic efficacy. Cancer Cell *36*, 168–178 e4.

Li, C.W., Lim, S.O., Xia, W., Lee, H.H., Chan, L.C., Kuo, C.W., Khoo, K.H., Chang, S.S., Cha, J.H., Kim, T., et al. (2016). Glycosylation and stabilization of programmed death ligand-1 suppresses T-cell activity. Nat. Commun. *7*, 12632.

Mei, J., Xu, J., Yang, X., Gu, D., Zhou, W., Wang, H., and Liu, C. (2021). A comparability study of natural and deglycosylated PD-L1 levels in lung cancer: evidence from immunohistochemical analysis. Mol. Cancer *20*, 11.

Pinato, D.J., Mauri, F.A., Spina, P., Cain, O., Siddique, A., Goldin, R., Victor, S., Pizio, C., Akarca, A.U., Boldorini, R.L., et al. (2019). Clinical implications of heterogeneity in PD-L1 immunohistochemical detection in hepatocellular carcinoma: the blueprint-HCC study. Br. J. Cancer *120*, 1033–1036.

Wang, Y.N., Lee, H.H., Hsu, J.L., Yu, D., and Hung, M.C. (2020). The impact of PD-L1 N-linked glycosylation on cancer therapy and clinical diagnosis. J. Biomed. Sci. *27*, 77.

Wilkinson, H., and Saldova, R. (2020). Current methods for the characterization of O-glycans. J. Proteome Res. *19*, 3890–3905.

Xu, J., Yang, X., Mao, Y., Mei, J., Wang, H., Ding, J., and Hua, D. (2021). Removal of N-linked glycosylation enhances PD-L1 detection in colon cancer: validation research based on immunohistochemistry analysis. Technol. Cancer Res. Treat. *20*, 15330338211019442.