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## Immunohistochemistry (IHC) Analytical Validation

### Version 1.0

1. **Analyte(s):** CD3, CD4, CD45RO, CD57 (Leu-7), CD68, CD8, Foxp3, Granzyme B, PD-1, PD-L1

2. **Technical platform(s):**

This procedure describes an automated system for staining paraffin sections using the Bond Max by Leica Biosystems and its Polymer Refine Detection System. The Bond Max instrument enables small volumes of reagent (as little as 150 µl per slide) to be uniformly applied over the tissue sections on a slide and has continuous batch processing, allowing for independent start and finish times for each batch of 10 slides.

3. **Reagents, controls, and calibrators:**

Optimized reagents:

- Bond Polymer Refine Detection Kit (DAB) (DS9800) (enhance staining quality).
- Horseradish Peroxidase (HRP)
- Secondary antibody conjugated to a polymer
- Post Primary (anti-mouse IgG)
- Polymer (anti-rabbit IgG)
- Bond Dewax Solution (AR9222)
- 100% alcohol
- Wash solution (AR9590)
- Epitope retrieval solution (ER1 Low pH (AR9961) or ER2 High pH (AR9640))
- DAB (diaminobenzidine) and Hematoxylin reagents

MARKER	VENDOR (CAT#)	Clonality	CLONE	Epitope Retrieval	Dilution	Antibody incubation time
CD3	DAKO (cat#A0452)	Rabbit polyclonal	-	ER1 Low pH for 20'	1:100	15 min.
CD4	Novocastra (CD4-368-L-CE)	Mouse monoclonal	4B12	ER2 High pH for 20'	1:80	15 min.
CD45 RO	Leica Microsystems (PA0146)	Mouse monoclonal	UCHL1	ER1 Low pH for 5'	Ready to use	15 min.
CD57 (Leu-7)	BD Biosciences (347390)	Mouse monoclonal	HNK-1	ER1 Low pH for 20'	1:40	15 min.

<b>CD68</b>	DAKO (MO876)	Mouse monoclonal	PG-M1	ER1 Low pH for 20'	1:450	15 min.
<b>CD8</b>	Thermo Scientific (MS-457S)	Mouse monoclonal	C8/144B	ER1 Low pH for 20'	1:25	15 min.
<b>Foxp3</b>	BioLegend (320102)	Mouse monoclonal	206D	ER2 High pH for 20'	1:50	15 min.
<b>Granzyme B</b>	Leica Microsystems (PA0291)	Mouse monoclonal	11F1	ER2 High pH for 20'	RTU	
<b>PD-1</b>	Abcam (ab137132)	Rabbit monoclonal	EPR4877(2)	ER1 Low pH for 20'	1:250	15 min.
<b>PD-L1</b>	Abcam (ab205921)	Rabbit monoclonal	[28-8]	ER2 High pH for 20'	1:100	15 min.
<b>PD-L1</b>	Cell Signaling (13684)	Rabbit monoclonal	E1L3N	ER1 Low pH for 20'	1:100	15 min.
<b>PD-L1</b>	Spring (M4420)	Rabbit monoclonal	SP142	ER1 Low pH for 20'	1:100	15 min.
<b>PD-L1</b>	VENTANA (790-4905)	Rabbit monoclonal	SP263	ER1 Low pH for 20'	RTU	15 min.
<b>PD-L1 22C3</b>	DAKO (M365329-2)	Mouse monoclonal	22C3	ER2 High pH for 20'	1:50	15 min.

#### 4. **Quality control parameters for specimens:**

The quality control of the specimens for biomarker analysis is performed by trained pathologists and includes the histo-morphology analysis of the H&E and IHC slide, evaluation of appropriate staining of internal and external controls, and presence of artifacts.

Histopathological analysis of the H&E and IHC slide: The designee pathologist determine the quality of the tissue for biomarker analysis including presence of adequate number of viable malignant cells (minimum requirement, 100) (when applicable), fibrosis, necrosis, inflammatory cells, and tumor tissue area.

Evaluations of IHC controls: An external positive and negative controls are added to each run. The negative control is the same tissue used as positive control without primary antibody (diluent). The designee pathologist will evaluate if the staining of the controls is appropriate in intensity and presence of positive cells in the correct pattern and subcellular location (e.g. membrane, nuclear, cytoplasmic).

Evaluation of artifacts: These would include presence of folds, background staining, endogenous pigments, edge artifact, crush artifacts, necrosis that may interfere in the IHC evaluation of the biomarker.

We determine that a sample is not evaluable (NE) if: the specimen is inadequate; sample has less than 100 viable malignant cells (when applicable); presence of extensive staining artifact; or inadequate staining of external and/or internal IHC controls. When the specimen is considered NE, the reasons of this determination are annotated in the report table.

#### 5. **Any critical pre-analytical variables:**

To avoid pre-analytical variables related to handling of the tissue (poor tissue preservation or non-standard fixation) the IHC lab recommends to use our SOP protocols for formalin fixation and cell pellet processing.

Run-to-run variation: For every run the staining in the positive control is assessed by standard microscopy evaluation to have the appropriate staining pattern and intensity by comparing the original validation slide (positive control) with the positive control slide used in each run. In case that the staining of control tissue are not appropriate, the IHC slides stained in that run are considered not qualified.

If the internal control fails (for IHC staining performed in tissues containing internal positive control for the biomarker tested), we request a different FFPE tissue from the same case and repeat the staining. If the external control failed, the next step is to run the assay with sections from the same or another blocks with positive control tissue to exclude the use of control tissue with poor processing. In case of repeated failure we thoroughly investigate the parameters of the staining protocols and substitution of fresh reagents; if necessary recalibration and maintenance of the equipment is performed. In case that all the steps fail to correct the problem, re-optimization and revalidation of the assays is performed before we repeat the staining.

## 6. Analytical Performance characteristics:

Antibody (Clone)	Parameter	Accuracy	Precision	Analytical sensitivity	Analytical specificity (including interfering substances)	Reportable range of assay results for the assay system	Establishment of appropriate quality control & improvement procedures
PD-L1 (28-8)	Programmed death ligand 1 protein expression by IHC in malignant cells membrane in lung cancer tissues	10/10 (100%) is the ratio of cases positive for >50% membrane staining	Inter-pathologist scoring concordance : 10/10 on >50% expression	Human embryonic kidney 263 cell line transfected with PD-L1 gene and HDLM2 cell line known to be PD-L1 positive by Western Blot: Positive in 4/4 attempts	Human embryonic kidney 263 cell line non-transfected with PD-L1 gene and PC3 cell line known to be PD-L1 negative by Western Blot: Positive in 4/4 attempts	By microscope observation, range 0-100% in malignant cells; positive >50%	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
PD-L1 (22C3)	Programmed death ligand 1 (PD-L1) protein expression by IHC in malignant cells membrane in lung cancer tissues	9/10 (90%) is the ratio of cases positive for >1% membrane staining	Inter-pathologist scoring concordance : 9/10 (90%) on >1% expression	Human embryonic kidney 263 cell line transfected with PD-L1 gene and HDLM2 cell line known to be PD-L1 positive by Western Blot: Positive in 4/4 attempts	Human embryonic kidney 263 cell line non-transfected with PD-L1 gene and PC3 cell line known to be PD-L1 negative by Western Blot: Positive in 4/4 attempts	By microscope observation, range 0-100% in malignant cells; positive >1%	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
PD-L1, (SP142)	Programmed death ligand 1 (PD-L1) protein expression by	10/10 (95%) is the ratio of cases positive for	Inter-pathologist scoring concordance : 10/10	Human embryonic kidney 263 cell line transfected	Human embryonic kidney 263 cell line non-transfected	By microscope observation, range 0-100% in	Lot variation analysis of antibodies and kits; 10 run of tissue and cell

	IHC in malignant cells membrane and inflammatory cells in lung cancer tissues	>1% membrane staining; 10/10 (100%) is the ratio of cases positive for >50% membrane staining and 8/10 (80%) 10% inflammatory cells	(100%) >50% expression in malignant cells and 8/10 (80%) for 10% expression in inflammatory cells	with PD-L1 gene and HDLM2 cell line known to be PD-L1 positive by Western Blot: Positive in 4/4 attempts	with PD-L1 gene and PC3 cell line known to be PD-L1 negative by Western Blot: Positive in 4/4 attempts	malignant cells; positive >50%; range in inflammatory cells 0-100%; positive >10%	lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
<b>PD-L1, (SP263)</b>	Programmed death ligand 1 (PD-L1) protein expression by IHC in malignant cells membrane in lung cancer tissues	10/10 (100%) is the ratio of cases positive for >25% membrane staining	Inter-pathologist scoring concordance : 10/10 (100%) on >25% expression	Human embryonic kidney 263 cell line transfected with PD-L1 gene and HDLM2 cell line known to be PD-L1 positive by Western Blot: Positive in 4/4 attempts	Human embryonic kidney 263 cell line non-transfected with PD-L1 gene and PC3 cell line known to be PD-L1 negative by Western Blot: Positive in 4/4 attempts	By microscope observation, range 0-100% in malignant cells; positive >25%	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
<b>PD-L1 (E1L3N)</b>	Programmed death ligand 1 (PD-L1) protein expression by IHC in malignant cells membrane in lung cancer tissues	10/10 (100%) is the ratio of cases positive for >50% membrane staining and 19/20 (95%) for 1% staining	Inter-pathologist scoring concordance : 10/10 (100%) on >50% expression and 19/20 (95%) on 1% expression	Human embryonic kidney 263 cell line transfected with PD-L1 gene and HDLM2 and H4006 cell lines known to be PD-L1 positive by Western Blot: Positive in 5/5 attempts	Human embryonic kidney 263 cell line non-transfected with PD-L1 gene and PC3 cell line known to be PD-L1 negative by Western Blot: Positive in 4/4 attempts	By microscope observation, range 0-100% in malignant cells; positive >50% and >1%	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
<b>CD3 (A045229-2)</b>	CD3 positive T lymphocytes; number of cells per 5 random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	10/10 (100%) is the ratio of cases expressing positive lymphocytes type of cells in tonsil tissues	Inter-pathologist scoring concordance using Aperio image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged	Human formalin-fixed and paraffin-embedded tonsil: Positive in 10/10 attempts	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external

			five 1 mm <sup>2</sup> squares				validation is not available
<b>CD8 (144B)</b>	CD8 positive T lymphocytes cytotoxic; number of cells per 5 random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	10/10 (100%) is the ratio of cases expressing positive lymphocyte s type of cells in tonsil tissues	Inter-pathologist scoring concordance using Aperio image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares	Human formalin-fixed an paraffin-embedded tonsil: Positive in 10/10 attempts	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
<b>CD4 Clone (4B12)</b>	CD4 positive T lymphocytes helper; number of cells per 5 random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	10/10 (100%) is the ratio of cases expressing positive lymphocyte s type of cells in tonsil tissues	Inter-pathologist scoring concordance using Aperio image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares	Human formalin-fixed an paraffin-embedded tonsil: Positive in 10/10 attempts	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
<b>CD45RO (UCHL1)</b>	CD45RO positive T lymphocytes memory; number of cells per 5 random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	10/10 (100%) is the ratio of cases expressing positive lymphocyte s type of cells in tonsil tissues	Inter-pathologist scoring concordance using Aperio image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares	Human formalin-fixed an paraffin-embedded tonsil: Positive in 10/10 attempts	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
<b>PD-1 (EPR4877-2)</b>	Programmed death-1 (PD-1) positive inflammatory cells (mostly lymphocytes); number of cells per 5	10/10 (100%) is the ratio of cases expressing positive lymphocyte s type of	Inter-pathologist scoring concordance using Aperio image analysis: 10/10	Human formalin-fixed an paraffin-embedded tonsil: Positive in	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist

	random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	cells in tonsil tissues	expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares	10/10 attempts			(100% concordance); calibration of instruments; external validation is not available
<b>GZB (11F1)</b>	Granzyme (GZB) positive T lymphocytes cytotoxic; number of cells per 5 random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	10/10 (100%) is the ratio of cases expressing positive lymphocytes type of cells on tonsil tissues	Inter-pathologist scoring concordance using Aperio image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares	Human formalin-fixed an paraffin-embedded tonsil: Positive in 10/10 attempts	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
<b>Foxp3 (206D)</b>	FOX-P3 positive T lymphocytes regulatory; number of cells per 5 random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	10/10 (100%) is the ratio of cases expressing positive lymphocytes type of cells in tonsil tissues	Inter-pathologist scoring concordance using Aperio image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares	Human formalin-fixed an paraffin-embedded tonsil: Positive in 10/10 attempts	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
<b>CD57 (HNK-1)</b>	CD57 positive T lymphocytes natural killer; number of cells per 5 random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	10/10 (100%) is the ratio of cases expressing positive lymphocytes type of cells in tonsil tissues	Inter-pathologist scoring concordance using Aperio image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares	Human formalin-fixed an paraffin-embedded tonsil: Positive in 10/10 attempts	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available

<b>CD68 Clone PG-M1)</b>	CD68 positive macrophages ; number of cells per 5 random square (1-mm <sup>2</sup> ) using Aperio (Leica) cytoplasmic algorithm	10/10 (100%) is the ratio of cases expressing positive macrophages type of cells in lung cancer and adjacent non-malignant lung tissues	Inter-pathologist scoring concordance using Aperio image analysis: 10/10 expression of >10, >100, >500, and >1,000 cells in averaged five 1 mm <sup>2</sup> squares	Human formalin-fixed an paraffin-embedded tonsil: Positive in 10/10 attempts	Human formalin-fixed and paraffin-embedded placenta Negative in 10/10 attempts	By Aperio image analysis, range observed in tonsils tissues is 0-5,000 cells	Lot variation analysis of antibodies and kits; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); calibration of instruments; external validation is not available
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## Analytical Data

<b>ANALYTE</b>	<b>PD-L1 (Clone 28-8)</b>		
Date	Testing Tissue Cases	Results	Comment
09/16/2015	Cell Pellet 293 transfected (positive control)	+	
09/16/2015	Cell Pellet 293 wild type (negative control)	-	
09/16/2015	Cell Pellet CST HDLM-2 (positive control)	+	
09/16/2015	Cell Pellet CST PC3 (negative control)	-	
09/16/2015	Normal-term Placenta	+ (trophoblastic cells)	
09/30/2015	Tonsil #1	+ (Crypt epithelium)	
09/30/2015	Tonsil #2	+ (Crypt epithelium)	
09/30/2015	Tonsil #3	+ (Crypt epithelium)	
<b>ANALYTE</b>	<b>PD-L1 (Clone 22C3)</b>		
10/18/2016	Cell Pellet 293 transfected (positive control)	+	
10/18/2016	Cell Pellet 293 wild type (negative control)	-	
10/18/2016	Cell Pellet CST HDLM-2 (positive control)	+	
10/18/2016	Cell Pellet CST PC3 (negative control)	-	
10/18/2016	Normal-term Placenta	+ (trophoblastic cells)	
10/18/2016	Tonsil #1	+ (Crypt epithelium)	
10/18/2016	Tonsil #2	+ (Crypt epithelium)	
10/18/2016	Tonsil #3	+ (Crypt epithelium)	
10/18/2016	Lung Ca whole section #1	+ (80%)	
10/18/2016	Lung Ca whole section #2	+ (90%)	
10/18/2016	Lung Ca whole section #3	-	
12/01/2016	Lung Ca TMA3	+ 68/185 (37%)	
<b>ANALYTE</b>	<b>PD-L1 (Clone SP142)</b>		
10/31/2014	Cell Pellet 293 transfected (positive control)	+	
10/31/2014	Cell Pellet 293 wild type (negative control)	-	
10/31/2014	Cell Pellet CST HDLM-2 (positive control)	+	
10/31/2014	Cell Pellet CST PC3 (negative control)	-	
11/03/2014	Normal-term Placenta	+ (trophoblastic cells)	
11/03/2014	Tonsil #1	+ (Crypt epithelium)	
11/03/2014	Tonsil #2	+ (Crypt epithelium)	
11/03/2014	Tonsil #3	+ (Crypt epithelium)	
11/05/2015	Lung Ca whole section #1	-	ADC
11/05/2015	Lung Ca whole section #2	+ (40%)	ADC
11/05/2015	Lung Ca whole section #3	-	ADC
11/05/2015	Lung Ca whole section #4	+ (30%)	ADC

11/05/2015	Lung Ca whole section #5	+ (30%)	Sq
11/05/2015	Lung Ca whole section #6	-	ADC
11/05/2015	Lung Ca whole section #7	+ (90%)	ADC
11/05/2015	Lung Ca whole section #8	+ (30%)	Sq
11/05/2015	Lung Ca whole section #9	+ (90%)	ADC
11/05/2015	Lung Ca whole section #10	-	ADC
11/05/2015	Lung Ca whole section #11	+ (80%)	Sq
11/05/2015	Lung Ca whole section #12	+ (80%)	Sq
11/05/2015	Lung Ca whole section #13	+ (90%)	ADC
11/05/2015	Lung Ca whole section #14		
02/01/2015	Lung Ca TMA3	78/185 (42%)	
<b>ANALYTE</b>	<b>PD-L1 (Clone SP263)</b>		
10/10/2016	Cell Pellet 293 transfected (positive control)	+	
10/10/2016	Cell Pellet 293 wild type (negative control)	-	
10/10/2016	Cell Pellet CST HDLM-2 (positive control)	+	
10/10/2016	Cell Pellet CST PC3 (negative control)	-	
10/14/2016	Normal-term Placenta	+ (trophoblastic cells)	
10/14/2016	Tonsil #1	+ (Crypt epithelium)	
10/14/2016	Tonsil #2	+ (Crypt epithelium)	
10/14/2016	Tonsil #3	+ (Crypt epithelium)	
11/20/2015	Lung TMA3	+ 89/185 (48%)	
<b>ANALYTE</b>	<b>PD-L1 (Clone E1L3N)</b>		
03/23/2014	Cell Pellet 293 transfected (positive control)	+	
03/23/2014	Cell Pellet 293 wild type (negative control)	-	
03/23/2014	Cell Pellet H4006 (positive control)	+	
03/23/2014	Cell Pellet CST HDLM-2 (positive control)	+	
03/23/2014	Cell Pellet CST PC3 (negative control)	-	
03/10/2016	Normal-term Placenta #1	+ (trophoblastic cells)	
03/10/2016	Normal-term Placenta #2	+ (trophoblastic cells)	
03/10/2016	Tonsil #1	+ (Crypt epithelium)	
03/10/2016	Tonsil #2	+ (Crypt epithelium)	
03/10/2016	Tonsil #3	+ (Crypt epithelium)	
03/10/2016	Lung whole section #1		
03/10/2016	Lung whole section #2	-	
03/10/2016	Lung whole section #3	-	
03/10/2016	Lung whole section #4	+ (10%)	
03/10/2016	Lung whole section #5	-	
04/11/2014	Lung TMA3	+ 67/185 (36%)	
<b>ANALYTE</b>	<b>CD3</b>		
04/10/2014	Tonsil #1	+ (T-cell pattern)	
04/10/2014	Tonsil #2	+ (T-cell pattern)	
04/10/2014	Tonsil #3	+ (T-cell pattern)	
04/10/2014	Tonsil #4	+ (T-cell pattern)	
04/10/2014	Tonsil #5	+ (T-cell pattern)	
04/10/2014	Tonsil #6	+ (T-cell pattern)	
04/10/2014	Tonsil #7	+ (T-cell pattern)	
04/10/2014	Tonsil #8	+ (T-cell pattern)	
04/10/2014	Tonsil #9	+ (T-cell pattern)	
04/10/2014	Tonsil #10	+ (T-cell pattern)	
<b>ANALYTE</b>	<b>CD8</b>		
03/03/2014	Tonsil #1	+ (T-cell pattern)	
03/03/2014	Tonsil #2	+ (T-cell pattern)	
03/03/2014	Tonsil #3	+ (T-cell pattern)	

03/03/2014	Tonsil #4	+ (T-cell pattern)	
03/03/2014	Tonsil #5	+ (T-cell pattern)	
03/03/2014	Tonsil #6	+ (T-cell pattern)	
03/03/2014	Tonsil #7	+ (T-cell pattern)	
03/03/2014	Tonsil #8	+ (T-cell pattern)	
03/03/2014	Tonsil #9	+ (T-cell pattern)	
03/03/2014	Tonsil #10	+ (T-cell pattern)	
<b>ANALYTE</b>	<b>CD4</b>		
04/10/2014	Tonsil #1	+ (T-cell pattern)	
04/10/2014	Tonsil #2	+ (T-cell pattern)	
04/10/2014	Tonsil #3	+ (T-cell pattern)	
04/10/2014	Tonsil #4	+ (T-cell pattern)	
04/10/2014	Tonsil #5	+ (T-cell pattern)	
04/10/2014	Tonsil #6	+ (T-cell pattern)	
04/10/2014	Tonsil #7	+ (T-cell pattern)	
04/10/2014	Tonsil #8	+ (T-cell pattern)	
04/10/2014	Tonsil #9	+ (T-cell pattern)	
04/10/2014	Tonsil #10	+ (T-cell pattern)	
<b>ANALYTE</b>	<b>CD45RO</b>		
04/15/2014	Tonsil #1	+ (T-cell pattern)	
04/15/2014	Tonsil #2	+ (T-cell pattern)	
04/15/2014	Tonsil #3	+ (T-cell pattern)	
04/15/2014	Tonsil #4	+ (T-cell pattern)	
04/15/2014	Tonsil #5	+ (T-cell pattern)	
04/15/2014	Tonsil #6	+ (T-cell pattern)	
04/15/2014	Tonsil #7	+ (T-cell pattern)	
04/15/2014	Tonsil #8	+ (T-cell pattern)	
04/15/2014	Tonsil #9	+ (T-cell pattern)	
04/15/2014	Tonsil #10	+ (T-cell pattern)	
<b>ANALYTE</b>	<b>PD-1</b>		
04/23/2014	Tonsil #1	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #2	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #3	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #4	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #5	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #6	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #7	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #8	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #9	+ (some lymphocytes in T-cell region)	
04/23/2014	Tonsil #10	+ (some lymphocytes in T-cell region)	
<b>ANALYTE</b>	<b>Granzyme B (GZB; Clone 11F1)</b>		
04/15/2014	Tonsil #1	+ (cytoplasmic in lymphocyte of T-cell region)	

04/15/2014	Tonsil #2	+ (cytoplasmic in lymphocyte of T-cell region)	
04/15/2014	Tonsil #3	+ (cytoplasmic in lymphocyte of T-cell region)	
04/15/2014	Tonsil #4	+ (cytoplasmic in lymphocyte of T-cell region)	
04/15/2014	Tonsil #5	+ (cytoplasmic in lymphocyte of T-cell region)	
04/15/2014	Tonsil #6	+ (cytoplasmic in lymphocyte of T-cell region)	
04/15/2014	Tonsil #7	+ (cytoplasmic in lymphocyte of T-cell region)	
04/15/2014	Tonsil #8	+ (cytoplasmic in lymphocyte of T-cell region)	
04/15/2014	Tonsil #9	+ (cytoplasmic in lymphocyte of T-cell region)	
04/15/2014	Tonsil #10	+ (cytoplasmic in lymphocyte of T-cell region)	
<b>ANALYTE</b>	<b>Foxp3 (Clone: 206D)</b>		
04/14/2014	Tonsil #1	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #2	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #3	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #4	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #5	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #6	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #7	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #8	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #9	+ (nuclei of lymphocytes in T-cell region)	
04/14/2014	Tonsil #10	+ (nuclei of lymphocytes in T-cell region)	
<b>ANALYTE</b>	<b>CD57 (Clone: HNK-1)</b>		
04/10/2014	Tonsil #1	+ (lymphoid cells/germinal center-interfollicular region)	
04/10/2014	Tonsil #2	+ (lymphoid cells/germinal center-interfollicular region)	
04/10/2014	Tonsil #3	+ (lymphoid cells/germinal center-interfollicular region)	
04/10/2014	Tonsil #4	+ (lymphoid cells/germinal center-interfollicular region)	
04/10/2014	Tonsil #5	+ (lymphoid cells/germinal center-interfollicular region)	
04/10/2014	Tonsil #6	+ (lymphoid cells/germinal center-interfollicular region)	
04/10/2014	Tonsil #7	+ (lymphoid cells/germinal center-interfollicular region)	
04/10/2014	Tonsil #8	+ (lymphoid cells/germinal center-interfollicular region)	

04/10/2014	Tonsil #9	+ (lymphoid cells/germinal center-interfollicular region)	
04/10/2014	Tonsil #10	+ (lymphoid cells/germinal center-interfollicular region)	
<b>ANALYTE</b>	<b>CD68 (Clone: PG-M1)</b>		
07/10/2014	Tonsil #1	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #2	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #3	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #4	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #5	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #6	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #7	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #8	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #9	+ macrophages-tingibles bodies/germinal center region)	
07/10/2014	Tonsil #10	+ macrophages-tingibles bodies/germinal center region)	

### Data Analysis

Data analysis was performed by performing analysis on the malignant cells and on the immune cells. Standard microscopic evaluation is performed along with digital image analysis.

### SCORING:

Analyte	Standard Microscopy Evaluation	Digital Image Analysis	Analysis in Malignant Cells	Analysis in Immune Cells
<b>PD-L1 (Clone 28-8)</b>	Performed	Not routinely performed	Percentage of positive cells	Not routinely performed/ Project specific
<b>PD-L1 (Clone 22C3)</b>	Performed	Not routinely performed	Percentage of positive cells	Not routinely performed/ Project specific
<b>PD-L1 (Clone SP142)</b>	Performed	Not routinely performed	Percentage of positive cells	Not routinely performed/ Project specific
<b>PD-L1 (Clone SP263)</b>	Performed	Not routinely performed	Percentage of positive cells	Not routinely performed/ Project specific
<b>PD-L1 (Clone E1L3N)</b>	Performed	Not routinely performed	Percentage of positive cells	Not routinely performed/ Project specific
<b>CD3</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )
<b>CD8 (Clone 144B)</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )
<b>CD4 (Clone 4B12)</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )
<b>CD45RO (Clone UCHL1)</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )
<b>PD-1 (Clone EPR4877-2)</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )
<b>GZB (Clone 11F1)</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )

<b>FOXP (Clone 206D)</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )
<b>CD57 (Clone HNK-1)</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )
<b>CD68 (Clone PG-M1)</b>	Not routinely performed	Performed (Aperio)	n/a	cell density (number of positive cells/mm <sup>2</sup> )

n/a: not applicable

## References

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*[Signatures] and [dates]*