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## Multiplex ImmunoFluorescence (mIF) Analytical Validation

Version 1.0

### 1. mIF Panels (s):

Panel Vectra 1 (Pancytokeratin AE1/AE3, CD3, CD8, CD68, PD1, PD-L1)

Panel Vectra 2 (Pancytokeratin AE1/AE3, CD3, CD8, GZB, CD45RO, FOXP3)

### 2. Technical platform(s):

This procedure describes an automated system for staining multiplex immunofluorescence paraffin sections using the Bond RX by Leica Biosystems and its Research Detection System 2. The Bond RX 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 Research Detection System 2 (DS9777) (enhance staining quality)
- Detection Buffer (1X TBS)
- OPAL PKI Blocking Buffer
- Bond Dewax Solution (AR9222)
- 100% alcohol
- Wash solution (AR9590)
- Epitope retrieval solution ER1 Low pH (AR9961) or ER2 High pH (AR9640)

### Panel 1 Antibodies

Antibody	Pancytokeratin	CD3	CD8	CD68	PD-1	PD-L1
Clone	AE1/AE3	-	C8/144B	PG-M1	EPR4877(2)	E1L3N
Vendor	Dako	Dako	Thermo Scientific	Dako	Abcam	Cell Signaling Technology
Catalog #	M351501-2	A045201-2	MS-457S (1 mg/mL)	M087601-2	AB137132	13684S (874 µg/ml)
Control Tissue	Tonsil/sample case					
Retrieval Method	Low target retrieval					
Dilution	1:600	1:500	1:50	1:100	1:3000	1:3000
Detection Kit	Perkin Elmer Opal Polymer HRP Ms + Rb					

## Panel 2 Antibodies

Antibody	Pancytokeratin	CD3	CD8	GZB	CD45RO	FoxP3
Clone	AE1/AE3	-	C8/144B	11F1	UCHL1	D2W8E
Vendor	Dako	Dako	Thermo Scientific	Leica Biosystems	Leica Biosystems	Cell Signaling Technology
Catalog #	M351501-2	A045201-2	MS-457s	PA0291	PA0146	98377S
Control Tissue	Tonsil/sample case					
Retrieval Method	Low target retrieval					
Dilution	1:600	1:500	1:50	1:1	1:1	1:100
Detection Kit	Perkin Elmer Opal Polymer HRP Ms + Rb					

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

Quality Control (QC) in histopathology involves both external and internal components for improving the standards of technical work as well as of reporting. These controls have been designed to enable monitoring of the technical assays performance, as well as the quality of individual samples, providing information at each step of the mIF protocol. All tissue specimens collected are reviewed by reference pathologists. At least, three types of QC activities for specimens collected are performed: a) histology/cytology examination of tissues and cells; b) tissue quality assessment of fresh specimens for immunohistochemistry and immunofluorescence; and, c) inclusion of all pathological data with Hematoxylin and eosin (H&E) stained and scanned images for assessment accuracy of the diagnosis.

H&E-stained sections from surgical resections and core needle biopsies are used to confirm the presence of tumor cells, as well as their abundance (tumor cellularity), fibrosis, necrosis and inflammatory cell components. H&E-stained sections from all formalin-fixed paraffin-embedded (FFPE) tissue diagnostic slides are scanned in the Aperio™ digital pathology scanner system and analyze for a pathological evaluation to choose the more representative tissue block specimen to perform the different assays (depending on tumor availability).

#### 5. **Critical pre-analytic variables**

Although the American Society of Clinical Oncology and College of American Pathologists (ASCO/CAP) has developed guidelines for handling tissues for IHC staining with some specific markers such as ER, PR and HER2 detection in breast cancer patients, these guidelines are not available for other surgical specimens or biomarkers and still less for mIF assays. In our experience, a good pre-analytic standardization is an important step to handling samples, and it is a modifiable factor to a proper assessment of the different biomarkers studies with mIF. Some recommendations, as tissue fixation quickly as possible after resection (less than 20 minutes as a prudent guideline to follow), recommended overall sample dimensions (1.5x1.5x0.4 cm as maximum for a good fixation), in an adequate volume of fixative (10-20 times the volume of the tissues for immersion fixation) and adequate time (6-18 hours for biopsy specimens and 24-72 hours for standard samples), can be the difference between higher or poorer quality of mIF staining. Another pre-analytical variable to be considered is the storage condition of FFPE blocks or prepared slides. However, limited studies addressing storage conditions of FFPE blocks and prepared slides tissue have been done. In our experience this is a critical point to consider in this assay. It is known that time and temperature are common factors to cause protein tissues degradation and loss of antigenicity in FFPE storage blocks and slides. The oxidation process of the tissue is a key factor involved in the protein tissues degradation and loss of antigenicity of different markers. Due to these facts, paraffin coating blocks before and after their use is a good practice to try to avoid any type of tissue antigenicity degradation. If it is not possible to obtain freshly cut sections for mIF, storage slides using vacuum sealed desiccator, paraffin coating, colder conditions (-4/-18°), as well as complete removal of water (presence of water both endogenously and exogenously plays a central role in loss of antigenicity) from those is highly recommended for their preservation. However, the optimal storage of unstained sections remains poorly defined, making freshly cut sections or sections stored for less than two months as the ideal to use to for mIF.

All the antibodies included in the panels were previously validated using conventional chromogenic immunohistochemistry (IHC) and in some cases using western blot technique. Positive and negative controls were used for PD-L1 IHC validation: HDLM-2 cell line, human mature placenta, human tonsil sample cases as positive controls, and PC3 cell line as negative control. For the tumour associated immune cell expression by IHC, human tonsil FFPE tissues with and without primary antibody were used as positive and negative controls for staining as a first step of the validation.

Similarly, each marker was tested and validated for IF in same tissues as IHC. As performed with the IHC staining, the correct titration in the single IF slides was chosen carefully to obtain a uniform, specific, and correct signal across all channel to produce a well balanced staining pattern during the multiplex staining. The correct signal from all the fluorophores, defined between 10 to 30 counts of intensity, needs to be maintained through the image to obtained good balance with all the stained markers. Once each target was optimized in single IF slides, multiplexed assay is used to generate multiple staining slides and each signal from the different marker was checked again to capture not only the correct signal expression but a similar thresholds of intensity. To detect possible variations in staining and optimal separation of the signal, positive and negative (auto- fluorescence) controls are included during each run of staining to make sure, especial with the internal positive control, that all the antibodies are working together. Auto -fluorescence controls with an expected spectral of 488 nm will be able to accurate remove the auto -fluorescence from all the label signals during the analysis process.

### PANEL 1

<b>Singleplex staining CD8</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
07/17/2015	Tonsil-1 (positive control)	+ (Around germinal center)	
07/17/2015	Tonsil-1 (Auto-fluorescence)	-	
07/18/2015	Tonsil-2 (positive control)	+ (Around germinal center)	
07/18/2015	Tonsil-2 (Auto-fluorescence)	-	
07/19/2015	Tonsil-3 (positive control)	+ (Around germinal center)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	-	
<b>Singleplex staining AE1/AE3</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
07/17/2015	Tonsil-1 (positive control)	+ (Crypt epithelium)	
07/17/2015	Tonsil-1 (Auto-fluorescence)	-	
07/18/2015	Tonsil-2 (positive control)	+ (Crypt epithelium)	
07/18/2015	Tonsil-2 (Auto-fluorescence)	-	
07/19/2015	Tonsil-3 (positive control)	+ (Crypt epithelium)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	-	
<b>Singleplex staining CD68</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
07/17/2015	Tonsil-1 (positive control)	+ (Inside germinal center)	
07/17/2015	Tonsil-1 (Auto-fluorescence)	-	
07/18/2015	Tonsil-2 (positive control)	+ (Inside germinal center)	
07/18/2015	Tonsil-2 (Auto-fluorescence)	-	
07/19/2015	Tonsil-3 (positive control)	+ (Inside germinal center)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	-	
<b>Singleplex staining CD3</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
07/17/2015	Tonsil-1 (positive control)	+ (Around germinal center)	
07/17/2015	Tonsil-1 (Auto-fluorescence)		
07/18/2015	Tonsil-2 (positive control)	+ (Around germinal center)	
07/18/2015	Tonsil-2 (Auto-fluorescence)		
07/19/2015	Tonsil-3 (positive control)	+ (Around germinal center)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	+ (Around germinal center)	

<b>Singleplex staining PD-L1</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
05/19/2015	Cell Pellet (positive control)	+	HEK 293 cell line transfected PD-L1
05/19/2015	Cell Pellet (negative control)	-	HEK 293 cell line transfected PD-L1
05/19/2015	Cell Pellet (positive control)	+	
05/19/2015	Cell Pellet (negative control)	-	
05/19/2015	Tonsil (positive control)	+ (Crypt epithelium)	
05/19/2015	Placenta	+ (Trophoblast)	
07/17/2015	Lung-1 (positive case)	+ Tumor cells	Adenocarcinoma
07/17/2015	Lung-2 (negative case)	- Tumor cells	Adenocarcinoma
07/17/2015	Tonsil-1(positive control)	+ (Crypt epithelium)	
07/17/2015	Tonsil-1 (Auto-fluorescence)	-	
07/18/2015	Tonsil-2 (positive control)	+ (Crypt epithelium)	
07/18/2015	Tonsil-2 (Auto-fluorescence)	-	
07/19/2015	Tonsil-3 (positive control)	+ (Crypt epithelium)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	-	

In each batch and individual test, negative control slide (auto-fluorescence) is run in parallel, which contains the antibody or the antibodies used in test or in the panel without the fluorochromes to normalize the auto-fluorescence component during the image analysis.

## PANEL 2

<b>Singleplex staining AE1/AE3</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
07/17/2015	Tonsil-1 (positive control)	+ (Crypt epithelium)	
07/17/2015	Tonsil-1 (Auto-fluorescence)	-	
07/18/2015	Tonsil-2 (positive control)	+ (Crypt epithelium)	
07/18/2015	Tonsil-2 (Auto-fluorescence)	-	
07/19/2015	Tonsil-3 (positive control)	+ (Crypt epithelium)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	-	

<b>Singleplex staining CD3</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
06/08/2016	Tonsil-1 (positive control)	+ (Around germinal center)	
06/08/2016	Tonsil-1 (Auto-fluorescence)		
07/20/2016	Tonsil-2 (positive control)	+ (Around germinal center)	
07/20/2016	Tonsil-2 (Auto-fluorescence)		
08/10/2016	Tonsil-3 (positive control)	+ (Around germinal center)	
08/10/2016	Tonsil-3 (Auto-fluorescence)		

<b>Singleplex staining CD45RO</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
07/17/2015	Tonsil-1 (positive control)	+ (Around and inside germinal center)	
07/17/2015	Tonsil-1 (Auto-fluorescence)	-	
07/18/2015	Tonsil-2 (positive control)	+ (Around and inside germinal center)	
07/18/2015	Tonsil-2 (Auto-fluorescence)	-	
07/19/2015	Tonsil-3 (positive control)	+ (Around and inside germinal center)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	-	

<b>Singleplex staining CD8</b>			
<b>Date</b>	<b>Testing Tissue Cases</b>	<b>Result</b>	<b>Comments</b>
06/17/2016	Tonsil-1 (positive control)	+ (Around germinal center)	
06/17/2016	Tonsil-1 (Auto-fluorescence)	-	

08/18/2016	Tonsil-2 (positive control)	+ (Around germinal center)	
08/18/2016	Tonsil-2 (Auto-fluorescence)	-	
09/19/2016	Tonsil-3 (positive control)	+ (Around germinal center)	
09/19/2016	Tonsil-3 (Auto-fluorescence)	-	

**Singleplex staining Foxp3**

Date	Testing Tissue Cases	Result	Comments
07/17/2015	Tonsil-1 (positive control)	+ (Around and inside germinal center)	
07/17/2015	Tonsil-1 (Auto-fluorescence)	-	
07/18/2015	Tonsil-2 (positive control)	+ (Around and inside germinal center)	
07/18/2015	Tonsil-2 (Auto-fluorescence)	-	
07/19/2015	Tonsil-3 (positive control)	+ (Around and inside germinal center)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	-	

**Singleplex staining Granzyme B**

Date	Testing Tissue Cases	Result	Comments
07/17/2015	Tonsil-1 (positive control)	+ (Around germinal center)	
07/17/2015	Tonsil-1 (Auto-fluorescence)	-	
07/18/2015	Tonsil-2 (positive control)	+ (Around germinal center)	
07/18/2015	Tonsil-2 (Auto-fluorescence)	-	
07/19/2015	Tonsil-3 (positive control)	+ (Around germinal center)	
07/19/2015	Tonsil-3 (Auto-fluorescence)	-	

In each batch and individual test, negative control slide (auto-fluorescence) is run in parallel, which contains the antibody or the antibodies used in test or in the panel without the fluorochromes to normalize the auto-fluorescence component during the image analysis.

**6. Pre-Analytical performance characteristics:**

To address some pre-analytical issues, as storage conditions we retrieved 27 breast cases surgically resected from 2001 to 2016 and freshly cuttings were obtained.

<b>Multiple ImmunoFluorescence (mIF) Panel 1 pre-analytic test (archived blocks)</b>		
(i)	Parameters	AE1-AE3 <sup>+</sup> ; PD-L1 <sup>+</sup> ; CD3 <sup>+</sup> ; CD8 <sup>+</sup> , PD-1 <sup>+</sup> ; and, CD68 <sup>+</sup> : Positive expression on 5 areas (0.669 × 500 μm, 0.3345 mm <sup>2</sup> each) using Vectra Inform v.3.0 software
(ii)	Accuracy (≥ 8 years)	10/27 (37%) good stained (All markers).
(iii)	Pre-analytical variation	Loss of immunoreactivity or problems with cross talking reactions with AE1-AE3 <sup>+</sup> ; CD3 <sup>+</sup> ; and CD8 <sup>+</sup> .
(iv)	Pre-analytical sensitivity	Human Formalin-Fixed and Paraffin-Embedded (FFPE) Breast cancer tissues: Positive staining in 16/27 attempts (significant difference, <i>p</i> =0.011)

To address archive prepared unstained slides conditions, we retrieved unstained slides from those 16 samples stored in the room temperature around 2 years to compare with a freshly cut slides (6 days before the staining) from the same cases.

<b>Multiple ImmunoFluorescence (mIF) Panel 2 pre-analytic test (archived slides vs freshly cut slides)</b>		
(i)	Parameters	AE1-AE3 <sup>+</sup> ; AE1-AE3 <sup>+</sup> /PD-L1 <sup>+</sup> ; CD68 <sup>+</sup> /PD-L1 <sup>+</sup> ; CD3 <sup>+</sup> ; CD3 <sup>+</sup> /CD8 <sup>+</sup> , CD3/CD8 <sup>+</sup> /PD-1 <sup>+</sup> ; and, CD68 <sup>+</sup> : number of cells per 5 areas (0.669 × 500 μm, 0.3345 mm <sup>2</sup> each) using Vectra Inform v.3.0 software
(ii)	Accuracy	9/16 (56%) good stained (All markers).
(iii)	Pre-analytical variation	Loss of immunoreactivity or problems with cross talking reactions with AE1-AE3 <sup>+</sup> ; CD3 <sup>+</sup> ; and CD8 <sup>+</sup> .

(iv) Pre-analytical sensitivity	Human Formalin-Fixed and Paraffin-Embedded (FFPE) Breast cancer: Positive in 9/16 attempts (significant difference, $p=0.023$ )
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## 7. Analytical Performance characteristics:

<b>Multiple ImmunoFluorescence (mIF) Panel 1</b>	
(i) Parameters	AE1-AE3 <sup>+</sup> ; AE1-AE3 <sup>+</sup> /PD-L1 <sup>+</sup> ; CD68 <sup>+</sup> /PD-L1 <sup>+</sup> ; CD3 <sup>+</sup> ; CD3 <sup>+</sup> /CD8 <sup>+</sup> , CD3/CD8 <sup>+</sup> /PD-1 <sup>+</sup> ; and, CD68 <sup>+</sup> : number of cells per 5 areas (0.669 × 500 μm, 0.3345 mm <sup>2</sup> each) using Vectra Inform v.3.0 software
(ii) Accuracy	20/20 (100%) is the ratio of cases expressing positive types and subtypes of cells in tonsil tissues
(iii) Precision	Inter-pathologist scoring concordance using Vectra Inform v3 algorithm image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares
(iv) Analytical sensitivity	Human Formalin-Fixed and Paraffin-Embedded (FFPE) tonsil: Positive in 10/10 attempts
(v) Analytical specificity including interfering substances	Human Formalin-Fixed and Paraffin-Embedded (FFPE) placenta: Negative in 5/5 attempts
(vi) Reportable range of assay results for assay system	By Vectra Inform v.3.0 software range observed in tonsils tissue is 0-5,000 for each subpopulation of cells.
(vii) Establishment of appropriate quality control & improvement procedures	Lot variation analysis of antibodies and Vectra Opal kits; calibration of equipment; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); external validation is not available

<b>Multiple ImmunoFluorescence (mIF) Panel 2</b>	
(i) Parameters	CD3 <sup>+</sup> ; CD3 <sup>+</sup> CD8 <sup>+</sup> Granzyme B <sup>+</sup> , CD3 <sup>+</sup> CD8 <sup>+</sup> CD45RO <sup>+</sup> ; and CD3 <sup>+</sup> Foxp3 <sup>+</sup> number of cells per 5 areas (0.669 × 500 μm, 0.3345 mm <sup>2</sup> each) using Vectra Inform v.3.0 software
(ii) Accuracy	20/20 (100%) is the ratio of cases expressing positive types and subtypes of cells in tonsil tissues
(iii) Precision	Inter-pathologist scoring concordance using Vectra Inform v3 algorithm image analysis: 10/10 expression of >10, >100, >500 and >1,000 cells in averaged five 1 mm <sup>2</sup> squares
(iv) Analytical sensitivity	Human Formalin-Fixed and Paraffin-Embedded (FFPE) tonsil: Positive in 10/10 attempts
(v) Analytical specificity including interfering substances	Human Formalin-Fixed and Paraffin-Embedded (FFPE) placenta: Negative in 5/5 attempts
(vi) Reportable range of assay results for assay system	By Vectra Inform v.3.0 software range observed in tonsils tissue is 0-5,000 for each subpopulation of cells.
(vii) Establishment of appropriate quality control & improvement procedures	Lot variation analysis of antibodies and Vectra Opal kits; calibration of equipment; 10 run of tissue and cell lines controls in 10 days inter-technologist (100% concordance); external validation is not available

## 8. Analytical Data and Analyses

Analysis of the region of interests (ROIs) using the mIF assay is performed quantitatively with the different cells phenotypes. The individual cells (defined by nuclei [DAPI] staining) identified by the cell segmentation tool are subjected to the phenotyping pattern recognition learning algorithm tool to characterize co-localization of the various cell populations using different panels by the software (InForm, PerkinElmer). Positive and negative

(auto-florescence) controls are included during the analysis to obtain uniform, specific, and accurate positive signal across all channels with a threshold between 10 to 30 counts of intensity without any auto-fluorescence signal interference. The individual cell report created by InForm will be processed by Spottfire software (TIBCO, PerkinElmer) to create a final data report expressing the results as number of cells/mm<sup>2</sup> from each individual cell phenotyping population.

Spearman's test is used to detect differences in continuous variables between the different staining batches and between mIF and chromogenic IHC quantifications. The statistical software program IBM SPSS (version 22; Armonk, NY) was used to perform the computations for all analyses.

<b>Multiplex staining Panel 1 (Pancytokeratin AE1/AE3, CD8, CD68, CD3, PD1, PD-L1)</b>								
Date	Tissue	Results						Comments
		AE1/AE3+ (n/mm <sup>2</sup> )	CD3+ (n/mm <sup>2</sup> )	CD3+CD8+ (n/mm <sup>2</sup> )	PD1+ (n/mm <sup>2</sup> )	CD68+ (n/mm <sup>2</sup> )	AE1/AE3+PD-L1+ (%)	
TMP-IL-1	Lung	2628.6	1919.5	757.7	958.9	985.1	0.16	ADC
TMP-IL-2	Lung	4379.9	1045.7	427.2	427.7	1422.9	0.01	ADC
TMP-IL-3	Lung	1773.8	1527.3	473.1	954.8	830.6	0	SCC
TMP-IL-4	Lung	3712.9	808.5	241.5	306.8	779.3	0.04	SCC
TMP-IL-5	Lung	2880.0	503.0	97.6	186.0	519.2	0	ADC
TMP-IL-6	Lung	4463.4	465.0	108.6	63.2	277.0	10.66	ADC
TMP-IL-7	Lung	2773.9	2335.8	438.7	322.6	442.9	25.02	ADC
TMP-IL-8	Lung	3264.9	2279.3	1188.6	699.2	859.8	84.51	ADC
TMP-IL-9	Lung	9033.4	828.6	235.7	325.4	244.4	39.85	SCC
TMP-IL-10	Lung	3975.0	1835.3	748.9	402.3	638.8	52.91	SCC
TMP-IL-11	Lung	3708.2	712.1	316.1	149.5	165.4	67.54	ADC
TMP-IL-12	Lung	5234.7	2097.4	544.8	385.3	671.3	43.27	ADC
TMP-IL-13	Lung	4093.6	283.7	51.3	23.3	329.0	0.047	ADC
TMP-IL-14	Lung	4678.0	2388.3	1362.6	284.1	888.7	35.86	SCC
TMP-IL-15	Lung	2157.9	1641.5	987.7	37.3	389.8	0.97	ADC
TMP-IL-16	Lung	3768.1	397.2	114.1	1.3	340.1	9.07	ADC
<b>Average</b>	-	3907.89	1316.76	505.89	345.48	611.52	23.12	-
<b>Median</b>	-	3740.50	1286.50	432.95	314.70	579.00	9.87	-
<b>Standard deviation</b>	-	1659.35	763.36	402.62	300.13	336.86	27.71	-
<b>Max</b>	-	9033.40	2388.30	1362.60	958.90	1422.90	84.51	-
<b>Min</b>	-	1773.80	283.70	51.30	1.30	165.40	0.00	-

**RESULTS:**

<b>Week 1 Panel 1 Run 1 09/09/2015</b>	Results Multiplex IF
TONSIL	+
TMP-IL-1 lung cancer	+ (99%)
<b>Week 2 Panel 1 Run 2 09/16/2015</b>	Results
TONSIL	+
TMP-IL-1 lung cancer	+ (99%)
<b>Week 3 Panel 1 Run 3 09/21/2015</b>	Results
TONSIL	+
TMP-IL-1 lung cancer	+ (99%)

<b>Multiplex staining Panel 2 (Pancytokeratin AE1/AE3, CD3, CD8, GZB, CD45RO, Foxp3)</b>						
Date	Tissue	Results				Comments
		AE1/AE3+ (n/mm <sup>2</sup> )	CD3+ (n/mm <sup>2</sup> )	CD3+CD8+GZB+ (n/mm <sup>2</sup> )	CD3+Foxp3+ (n/mm <sup>2</sup> )	
TMP-IL-1	Lung	1874.1	2132.2	29.5	279.5	ADC
TMP-IL-2	Lung	3533.7	1428.0	14.3	267.0	ADC
TMP-IL-3	Lung	872.9	1928.9	111.0	403.1	SCC
TMP-IL-4	Lung	1695.5	948.8	16.1	244.2	SCC
TMP-IL-5	Lung	2751.9	724.9	7.9	148.1	ADC
TMP-IL-6	Lung	3516.5	363.3	7.8	77.8	ADC
TMP-IL-7	Lung	2331.1	2356.4	33.0	452.4	ADC
TMP-IL-8	Lung	1657.4	2614.2	211.4	253.4	ADC
TMP-IL-9	Lung	7818.8	403.6	31.5	39.2	SCC
TMP-IL-10	Lung	3295.7	1410.5	43.5	233.6	SCC
TMP-IL-11	Lung	3079.0	743.8	3.7	123.6	ADC
TMP-IL-12	Lung	4403.8	1818.9	48.5	420.9	ADC
TMP-IL-13	Lung	4659.5	820.6	30.1	197.9	ADC
TMP-IL-14	Lung	5281.1	2437.4	119.6	618.7	SCC
TMP-IL-15	Lung	1999.3	1960.8	203.6	283.5	ADC
TMP-IL-16	Lung	4367.1	352.3	43.4	32.5	ADC
<b>Average</b>	-	3321.09	1402.79	59.68	254.71	-
<b>Median</b>	-	3187.35	1419.25	32.25	248.80	-
<b>Standard deviation</b>	-	1728.59	790.05	66.52	159.64	-
<b>Max</b>	-	7818.80	2614.20	211.40	618.70	-
<b>Min</b>	-	872.90	352.30	3.70	32.50	-

<b>Week 1 Panel 2 Run 1 09/05/2016</b>	
TONSIL	+
TMP-IL-1 lung cancer	+ (99%)

<b>Week 2 Panel 2 Run 2 09/12/2016</b>	
TONSIL	+
TMP-IL-1 lung cancer	+ (99%)

<b>Week 3 Panel 2 Run 3 09/29/2016</b>	
TONSIL	+
TMP-IL-1 lung cancer	+ (99%)

## 9. Analytical reproducibility

### 9.1. Data summary

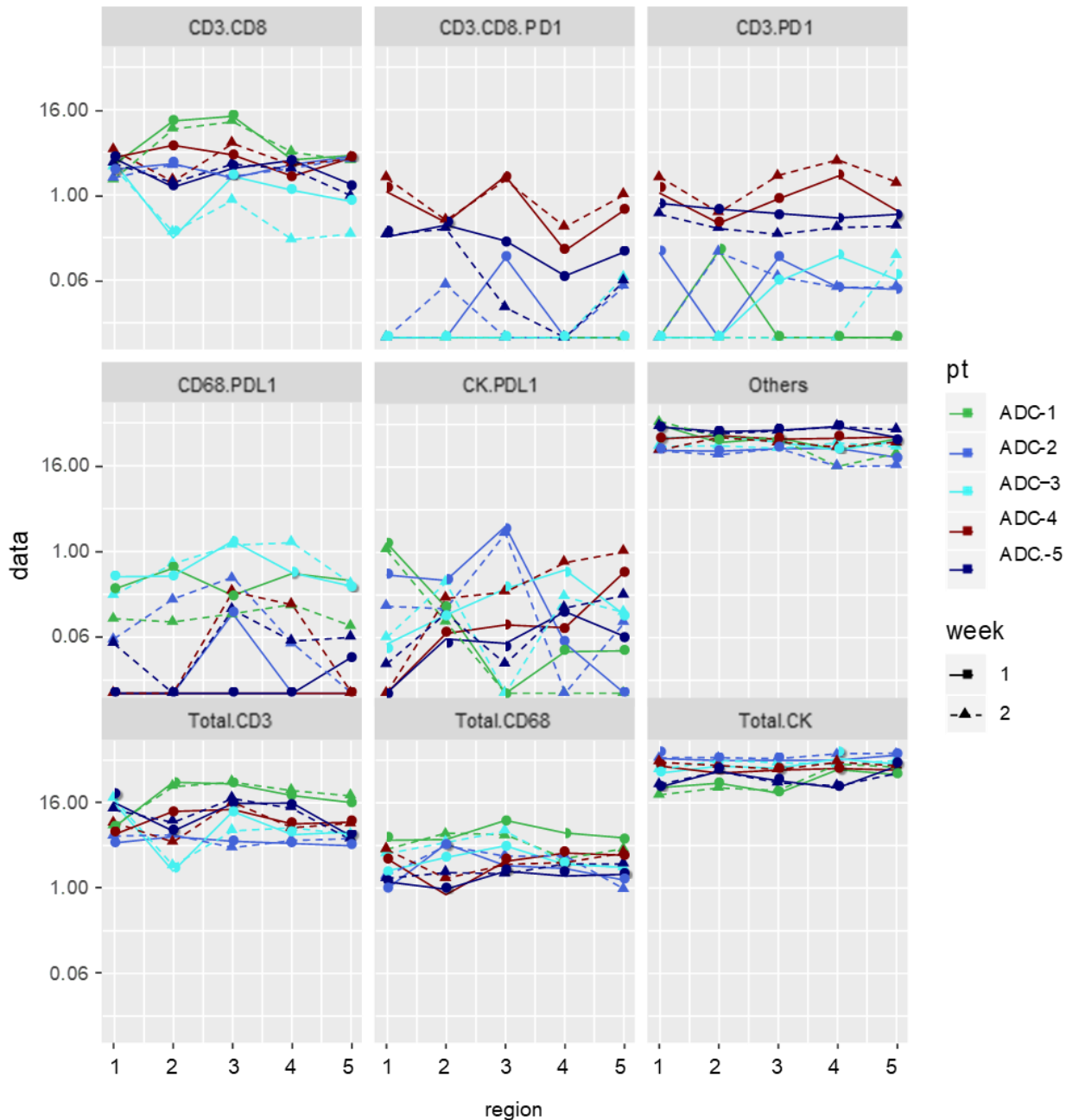
To provide a statistical evaluation of analytical reproducibility of mIF data, 10 lung FFPE tumor samples (5 Lung Adenocarcinomas (ADC-1, ADC-2, ADC-3, ADC-4 and ADC-5) and 5 Lung Squamous Cell Carcinomas (SCC-1, SCC-2, SCC-3, SSC-4 and SCC\_5) were analyzed. Each consecutive sample was subjected to automated staining (Leica Bond RX) at two time points with one week interval ( Week 1 and Week 2) using the same Opal 7 kit and panel's marker. . Five ROIs were obtained from each sample that overlap with the sequential mIF slides (from each staining batch), to quantify each cell phenotype at the same location of the specimens. Six markers plus DAPI were stained in the panel (AE1/AE3, CD3, CD8, PD-1, PD-L1 and CD68) and nine cell phenotypes were measured as following: malignant cells (Total Pancytokeratin, CK+), malignant cells expressing PD-L1+ (CK+PD-L1+); T lymphocytes (Total CD3; pan T-cell marker including, cytotoxic T cells CD3+CD8+, antigen experienced T cells CD3+PD-1+, and other CD3+ T cells); cytotoxic T cells (CD3+CD8+); antigen experienced T cells (CD3+PD-1+; Tumor Associated Macrophages , (TAMs, Total CD68+); TAMs expressing PD-L1+ (CD68+PD-L1+) and other cells (include in this category endothelial cells, fibroblast, neutrophils and

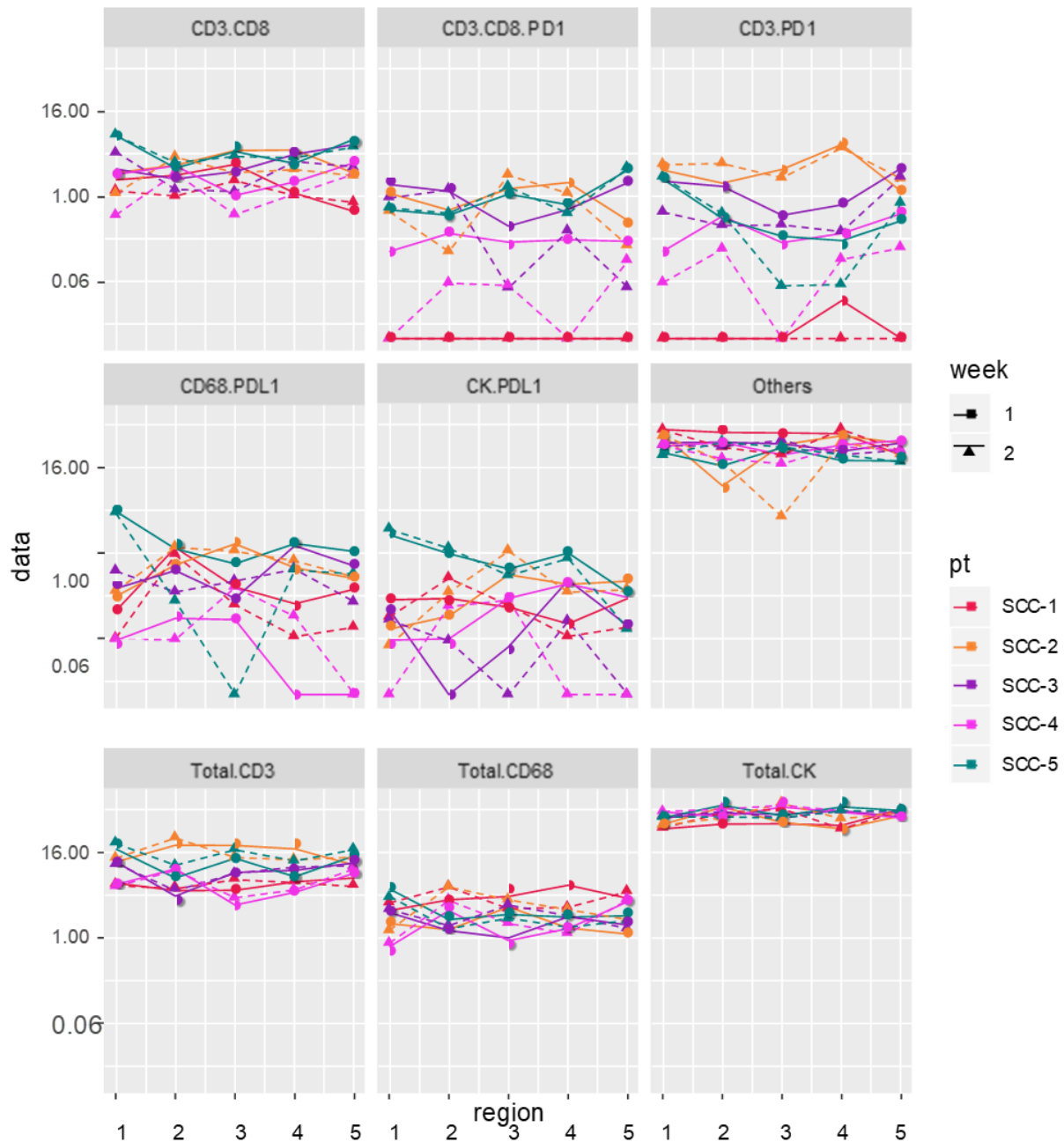
cells that are not expressing any of the markers in the panel). The percentage of each phenotype was calculated by dividing the number of total nucleated cells on each consecutive panel and used for the analysis of this data. Statistical analyses were carried out with the R software program (version 3.3.0, released May 2016; Vienna, Austria; URL <https://www.R-project.org/>).

### 9.2. Analytical reproducibility between times

Trellis plots, of each patient sample showing the nine cell phenotypes analyzed by ROS and weeks, were generated to evaluate the reproducibility and consistency of the data. As shown in **Figure 1** high consistency and reproducibility was observed between Week 1 and Week 2 in each patient sample for both ADC and SCC samples.

**Figure 1. Trellis plots by ROIs and Week 1 and 2.** The solid line with the square symbol represents data from Week 1 and the dash line with the triangle symbol from Week2. Patients are color coded and data shown is in log scale.

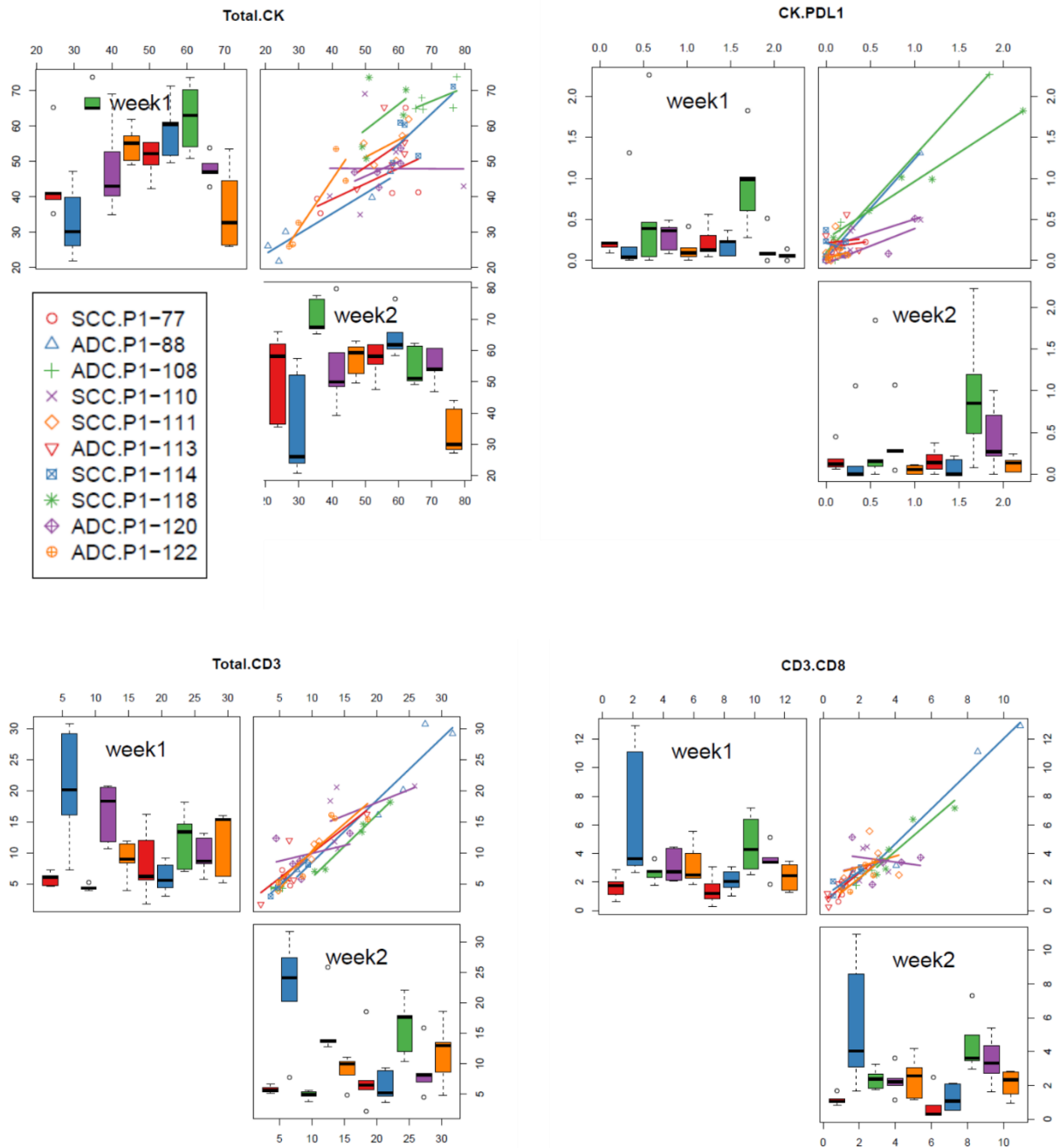


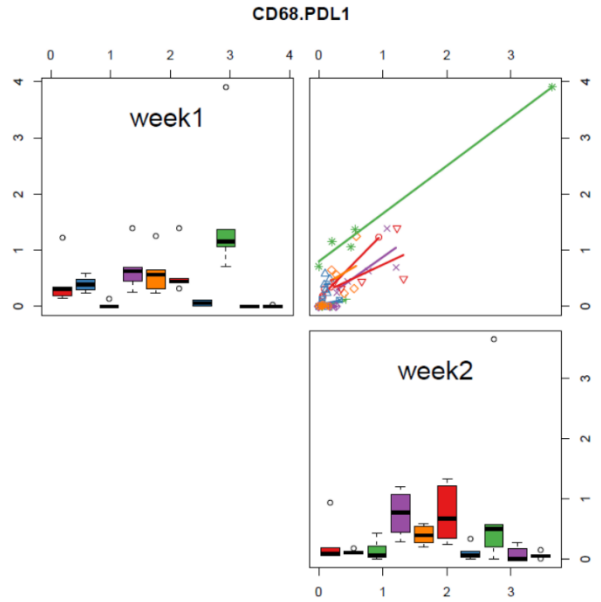
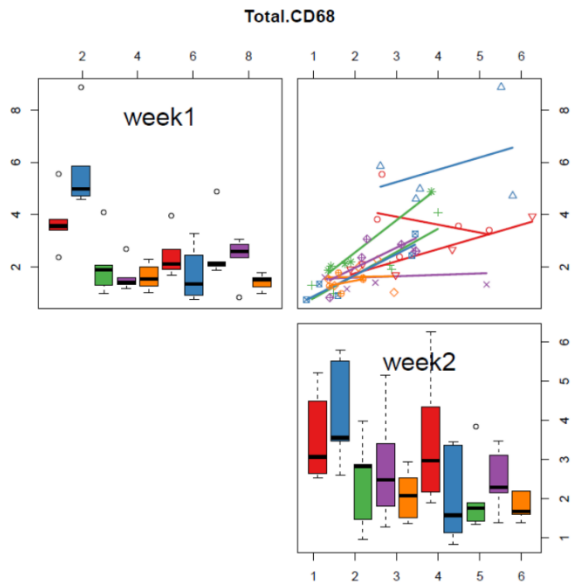
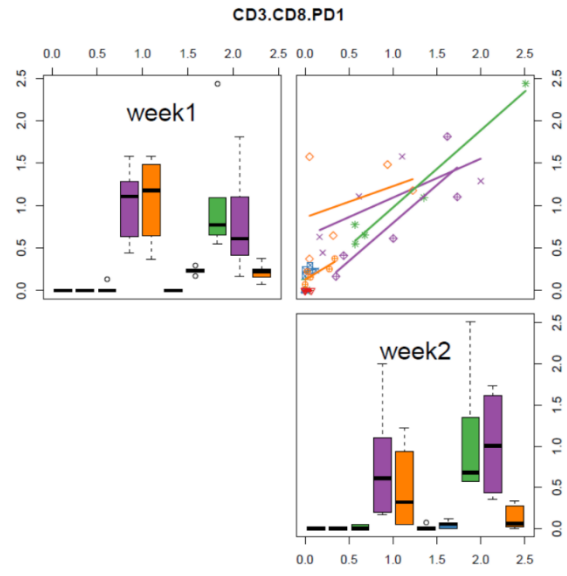
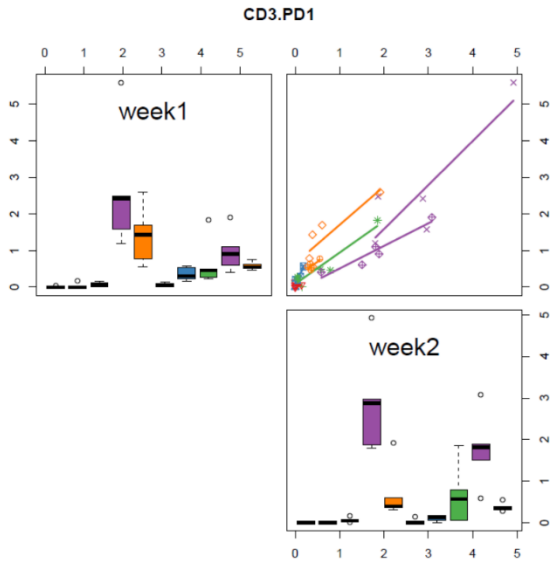


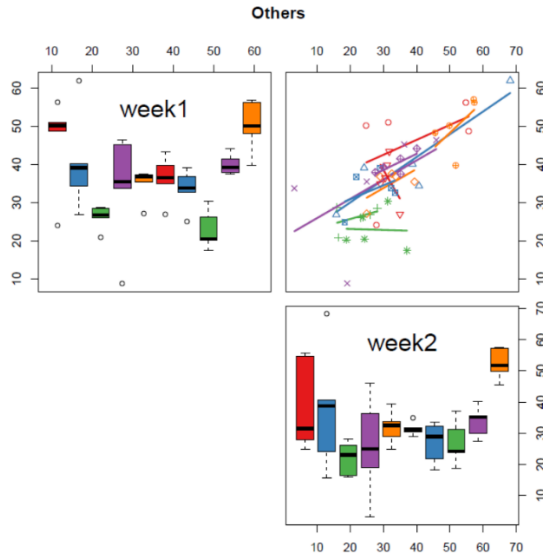
### 9.3. Analytical reproducibility within each sample: Correlation analyses.

To assess the correlation for each phenotype within each patient sample, Spearman's rank correlation coefficient was calculated for the five measurements (5 ROIs) between Week 1 and 2 for each sample. Overall a high consistency for the majority of the phenotypes was observed between weeks (Week 1 and 2) as shown in **Figure 2**.

**Figure 2.** The boxplots for each cell phenotype for Week 1 and Week 2 are shown on diagonal and scatter plots for each pair of weeks are shown with linear regression, line labeled in a different color and symbol for each sample. Data in percentage in original scale.



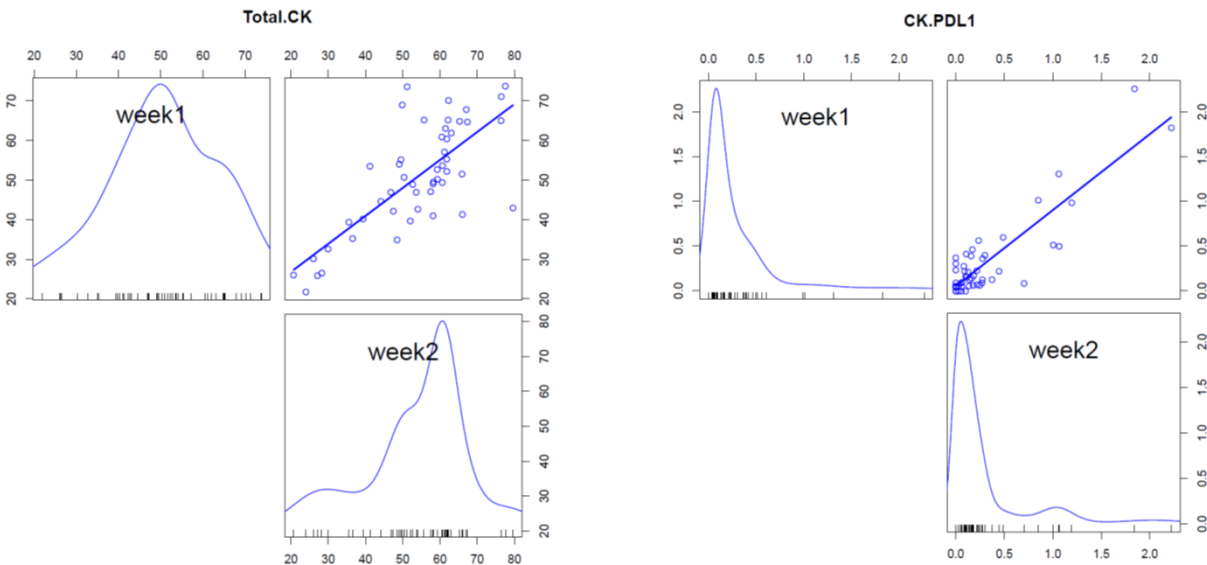


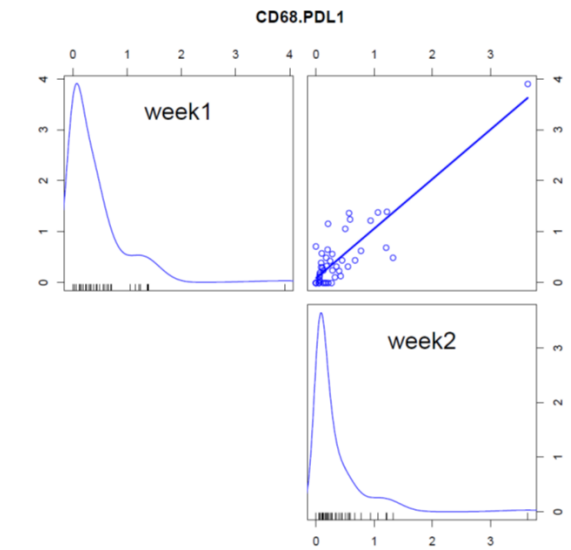
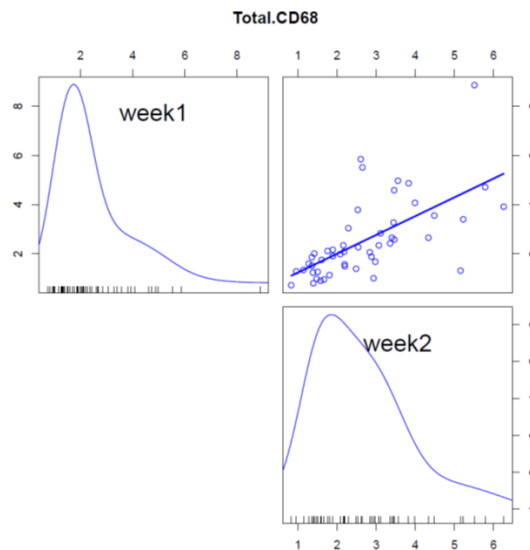
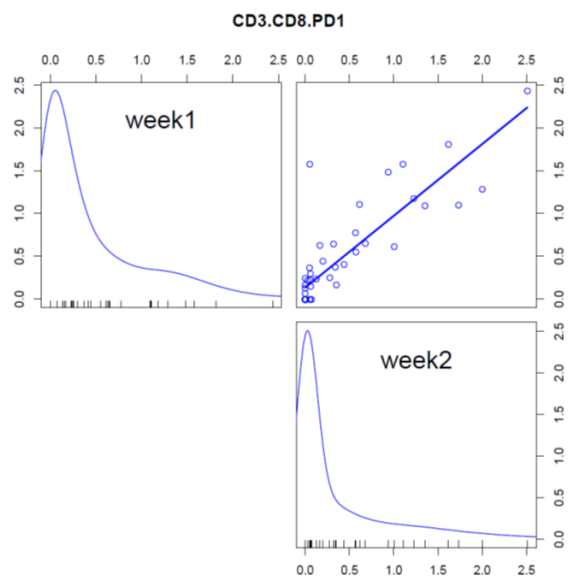
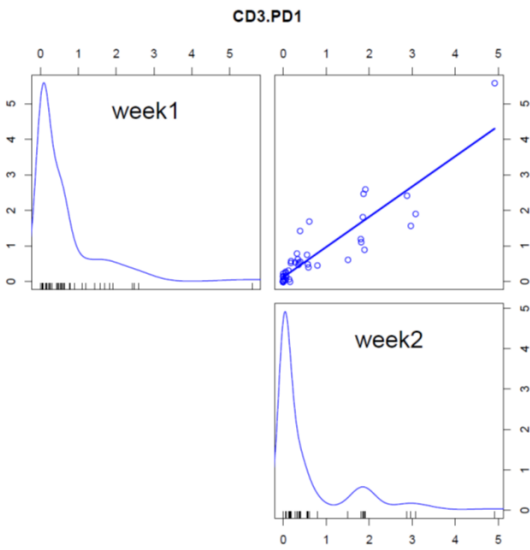
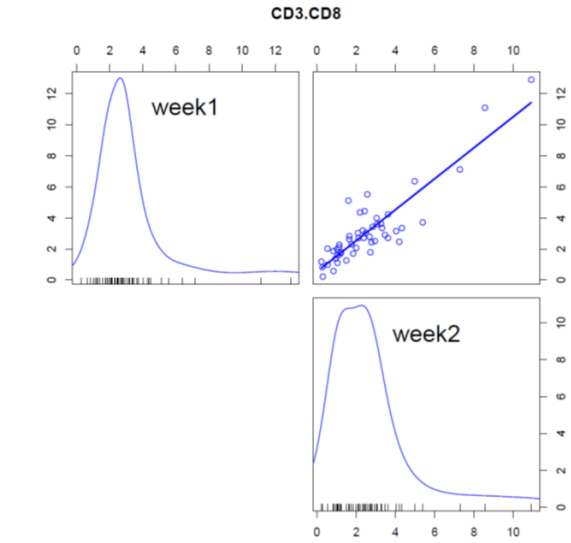
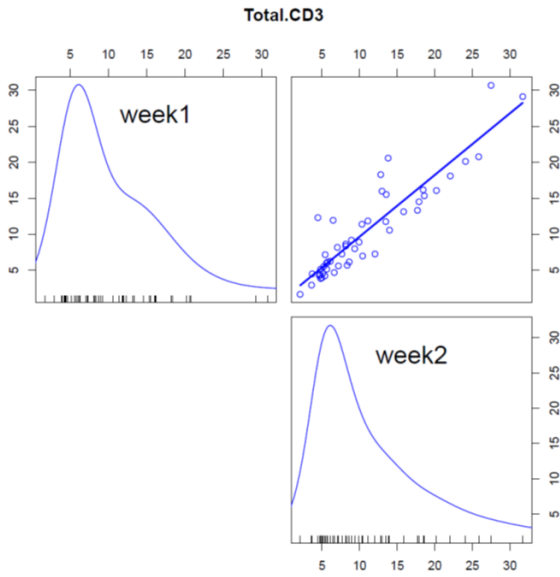


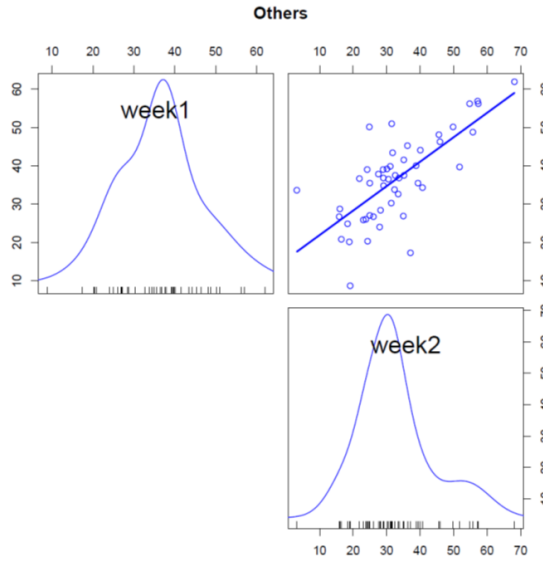
#### 9.4. Analytical reproducibility across samples: Correlation analyses

To show the correlation across all samples, scatter plots for each cell phenotype comparing Week 1 and Week 2 were generated. As shown in **Figure 3** high concordance along all cell phenotypes was observed. In addition, the Spearman's rank correlation coefficient were calculated for pairs of weeks. As shown in **Figure 4**, six out of the nine cell phenotypes had Spearman's rank correlation coefficients  $\geq 0.7$ . The remaining three cell phenotypes had Spearman's rank correlation coefficients between 0.64 and 0.70.

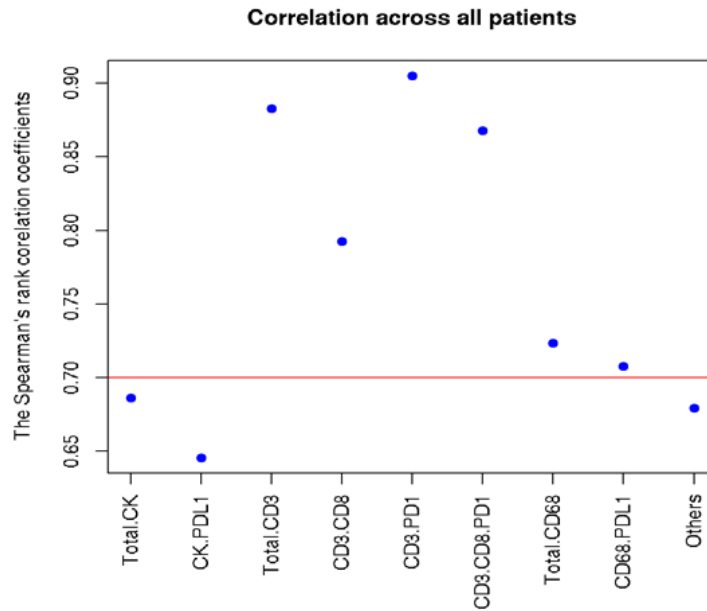
**Figure 3.** Scatter plots for each cell phenotype for Week 1 and Week 2. Density plot for each week are shown on diagonal; scatter plots for pairs of weeks are shown with linear regression labeled. Data is in original scale.







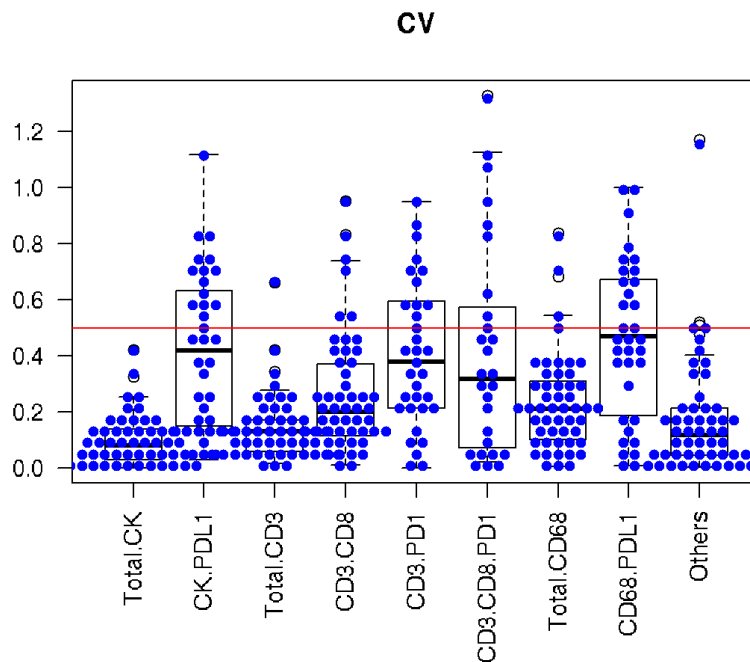
**Figure 4.** Dotplot of Spearman's rank Correlation coefficients for each cell phenotype for pairs of weeks



**9.5. Coefficient of Variation (CV) within sample and within region**

To determine the analytical reproducibility in the cell phenotypes, the Coefficient of Variation (CV) within each region of each sample was calculated (only 2 values from 2 weeks), thus, for each marker in total 50 CVs (number of ROIs\*number of samples). As shown in **Figure 5** all cell phenotypes had median CV <0.5, therefore showing a high reproducibility within sample and region.

**Figure 5.** CVs for each cell phenotype per ROIs.



### 9.6. Conclusion

Combining results from Trellis plots, Spearman's rank correlation coefficient, scatter plots and CVs it was observed that overall the different phenotypes studied have high analytical reproducibility between consecutive sample staining at different time points. Phenotypes cells as Total.CD3+, C3+CD8+, CD3+PD1+, C3+CD8+PD1+ and CD68+PD-L1+ had high correlation both within samples (median > 0.5) and across samples (median > 0.7). As expected malignant cells, malignant cells expressing PD-L1, macrophages CD68+ as well as others cells (fibroblast, endothelial cells, neutrophils cells not identify by some marker used) had lower correlation either within samples (median < 0.5) or across samples (median < 0.7) when are normalized by the total number of cells from the ROIs. We observed that the variation of this lower correlation either within samples or across samples is minimum. However, the low expression in markers such as PD-1 and PD-L1 in certain samples poses a challenge in accurately quantifying these markers. The low correlation observed could be caused by the different geographic distribution of these cell phenotypes between the intervals of the cutting sample. It has been well documented that the malignant cells in different levels and regions of the tumor exhibits 'random' expression of PD-L1, and this factor likely accounts for this low correlation. The total number of cells from each ROI is also variable depending on the characteristic of the tumor in different levels, an additional factor that can further impact the interpretation of the results when normalizing the different phenotypes with these numbers.

## References

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