Flow cytometry immunophenotyping using commercial antibodies to maximize detection of PD-1 in immuno-oncology (I-O) clinical studies with anti-PD-1 check point inhibitors

Abstract# 2265

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INTRODUCTION

Precision for Medicine's translational biomarker expertise supports global clinical trials with flow cytometry. A biomarker strategy which includes flow cytometry to generate pharmacokinetic/ pharmacodynamic (PK/PD) models for rational design of dose escalation and expansion of clinical studies, may need to consider concomitant drugs for checkpoint inhibition like Pembrolizumab.

Precision has optimized the use of commercial anti-PD-1 and anti-IgG4 reagents to detect both free PD-1 and/or Pembro-bound PD-1 when patients are on study with a new molecular entity as either a monotherapy or in combination with concomitant checkpoint inhibitors. For I-O studies conventional efficacy endpoints combined with flow biomarker driven surrogate endpoints enhance therapeutic decisions for determining mechanism of action and biological response when incorporated in early-phase clinical studies.

Precision developed and qualified a flow immunophenotyping assay to quantify PD-1 on CD4+ and CD8+ T cells using a commercial PD-1 clone (PD1.3.1.3) and an anti-IgG4. This enabled detection of both free and drug bound PD-1 receptor. We compared detection of PD-1 in PHA stimulated healthy donors treated with saturating Pembrolizumab or no drug treatment. There was not a significant difference observed in PD-1 quantification in the presence or absence of Pembrolizumab, which demonstrated that our approach was able to accurately measure total PD-1 whether free or bound. As Pembrolizumab was titrated from the saturating concentration there was a dose-dependent decrease in detection of PD-1. We have used this assay to detect PD-1 with several new I-O therapies that combined standard anti-PD-1 checkpoint inhibitors, and in these clinical studies for advanced and rare solid tumor indications, we observed an average of 20-40% increase in PD-1 compared to no drug treatment.

Immuno-oncology clinical trials which incorporate checkpoint inhibitors like either PD-1 or PD-L1, the availability of commercial reagents are key considerations to detect these receptors when designing a sensitive, selective, and accurate biomarker assay.

BACKGROUND

Precision for Medicine (Precision) developed and qualified a multi-color flow immunophenotyping assays to quantify total PD-1 expression on CD4+ and CD8+ T cells in cryopreserved peripheral blood mononuclear cells (PBMCs).

Pembrolizumab (Pembro) is a programmed death receptor-1 (PD-1)-blocking antibody approved for the treatment of several oncology indications including (but not limited to) the following examples: Melanoma, Non-Small Cell Lung Cancer, Head and Neck Squamous Cell Cancer, Hodgkin Lymphoma, Gastric Cancer, Merkel Cell Carcinoma, and Renal Cell Carcinoma.

The detection of free PD-1 and Pembro-bound PD-1 was achieved by co-staining a partially competing αPD-1 antibody (clone PD1.3.1.3) with a biotinylated αHu-IgG4 antibody. The assay conditions were optimized for sensitivity, optimal signal:noise ratio, detection of free and drug bound receptor by titrating and testing various commercial αPD-1 antibody clones and tertiary reagents to detect biotinylated αHu-IgG4.

These flow assays will facilitate the evaluation of both free and drug bound PD-1 expression as a pharmacodynamic biomarker in T-cells when PD-1 blockade is being used.

METHODS

Step 1. Separation and Storage of PBMCs
- For clinical application, clinical sites collected patient whole blood
- Centralized PBMC isolation using SepMate™ tubes- Ficoll density gradient separation
- PBMCs cryopreserved and stored in vapor phase of LN2 to maintain viability
- Longitudinal samples from each subject intended to be batch tested together

Step 2. Thaw and Stain PBMCs
- PBMCs are thawed in complete medium, cells counted for viability
- Assay Controls included: single color controls for compensation controls
- Inter-assay healthy PBMC controls: full panel stain and fluorescence minus 1 or more markers, e.g. FMO stains, for objective setting of gates

Step 3. Detection of PD-1
- For patient PBMCs assumptions are they have Pembro-bound PD-1
- Healthy PBMC Inter-assay control –pretreated with and without 10ug/mL of Pembro for assay control
- Pembro bound receptor are detected using a biotinylated anti-Hu-IgG4 antibody, followed with staining of fluorochrome conjugated to anti-biotin antibody
- Free PD-1 receptors are quantified using a commercial anti-PD-1 antibody (Miltenyi clone PD1.3.1.3)
- Total PD-1 reported when evaluating PD-1 marker

Step 4. Full Panel Stain
- For PBMCs full flow panel staining in flow panel containing CD45, CD3, CD4, CD8, and PD-1 to assess PD-1 expression in CD4+ and CD8+ T cells.
- PBMCs are stained, washed and prepared for acquisition on flow cytometer

Step 5. Cell Acquisition and Detection with BD LSRFortessa™ 5-laser 20-parameter system

FLOW ASSAY DEVELOPMENT

Figure 1. Schematic illustration of the strategy utilized in the development of a flow method for the detection of Total PD-1

Free PD-1 and Pembro-bound PD-1 was achieved by co-staining a partially competing PD-1 antibody (clone PD1.3.1.3) with a biotinylated Human-IgG4 antibody. Together these measurements enable Total PD-1 to be quantified.

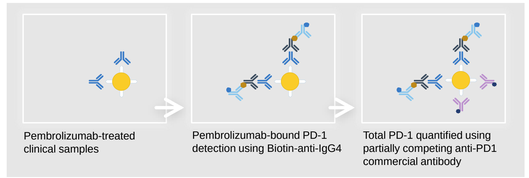


Figure 2. Identification of a Commercial Noncompeting Antibody (NCA) to Pembrolizumab

- Healthy donor PBMCs were either treated with 10µg/mL of Pembrolizumab or not treated with Pembro. Each sample was stained with three different commercial PD1 antibodies conjugated to Phycoerythrin (PE).
- These results represent the frequency (%) PD-1+ cells on CD4+ T cells.

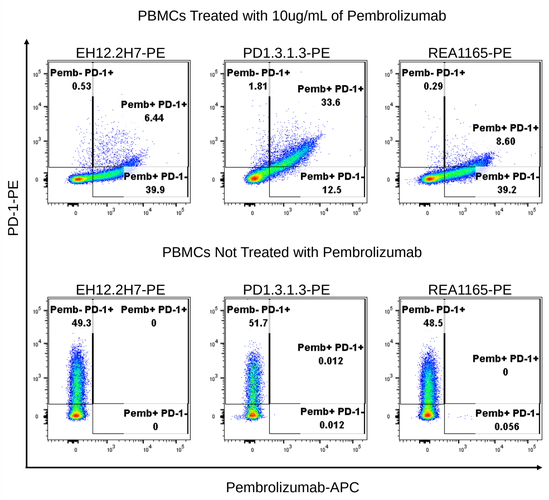


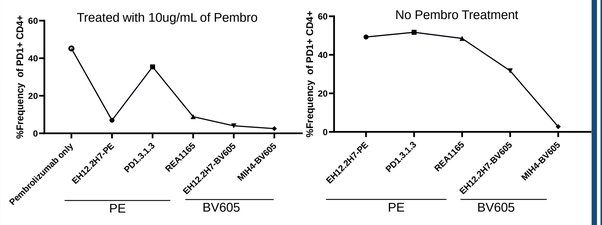
Table 1. % Frequency of PD1+ CD4+ T cells on healthy PBMCs

PBMCs Treated with 10ug/mL of Pembrolizumab			
Antibody Clone	Pembro – PD-1+	Pembro+ PD-1+	Pembro+ PD-1–
EH12.2H7	0.53	6.44	39.9
PD1.3.1.3	1.81	33.60	12.5
REA1165	0.29	8.60	39.20

PBMCs Not Treated with Pembrolizumab			
Antibody Clone	Pembro – PD-1+	Pembro+ PD-1+	Pembro+ PD-1–
EH12.2H7	49.30	0.00	0.00
PD1.3.1.3	51.70	0.012	0.012
REA1165	48.50	0.00	0.056

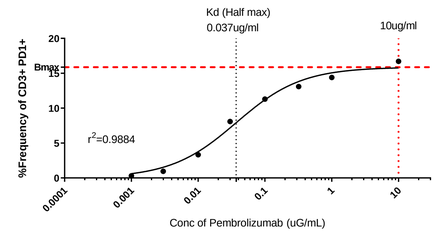
Figure 3. Summary of Commercial Noncompeting Antibody Screening for Pembrolizumab

- Miltenyi's clone PD1.3.1.3 can be used as a **Partial** Noncompeting antibody for detection of Pembrolizumab
- PD-1 detection was weaker with BV605 for clones EH12.2H7 and MIH4



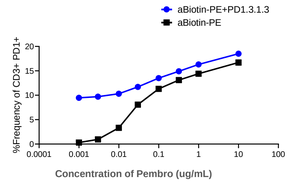
	Pembro Only	EH12.2H7-PE	PD1.3.1.3-PE	REA1165-PE	EH12.2H7-BV605	MIH4-BV605
Pembro Treated	45.2	6.97	35.41	8.89	4.00	2.50
No Pembro	N/A	49.30	51.7	48.50	31.70	2.71

Figure 4. Saturation curve of Pembrolizumab: Healthy PBMCs treated with Pembro (0.001-10ug/mL) detected with Biotin α-IgG4 and α-Biotin-PE



- The maximum specific binding (Bmax) and half-maximum binding (Kd) of Pembrolizumab were calculated using specific binding with hill slope.
- Bmax = 10ug/mL
- Kd (Half-max) = 0.037ug/mL

Figure 5. % Frequency of PD1+ CD3+ T cells on healthy PBMCs treated with a dose titration Pembro (.001-10ug/mL), Biotin α-IgG4, α-Biotin-PE, and Miltenyi clone PD1.3.1.3



%PD-1+ Frequency

Pembro ug/mL	αBiotin-PE +PD1.3.1.3	αBiotin-PE
0.000	9.15	0.23
0.001	9.47	0.31
0.003	9.71	0.96
0.010	10.30	3.33
0.030	11.70	8.08
0.100	13.50	11.30
0.320	14.90	13.10
1.000	16.30	14.40
10.000	18.50	16.70

RESULTS: CLINICAL FLOW CYTOMETRY RESULTS

Figure 6. PD-1 Detection in CD4+ and CD8+ T cells: Healthy PBMCs treated with 10ug/mL of Pembro before staining. For detection of Total PD-1, all samples were stained with Biotin α-IgG4, α-Biotin-PE, and staining with Miltenyi clone PD1.3.1.3

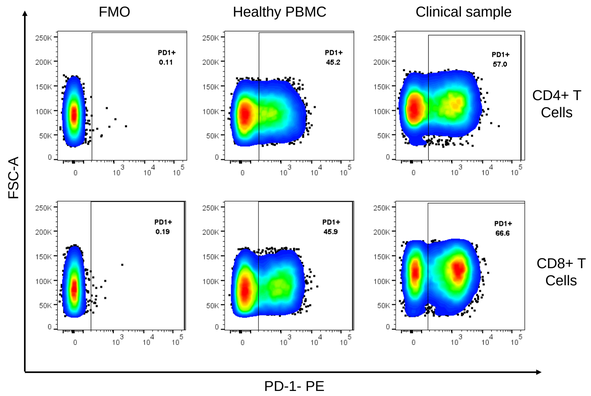
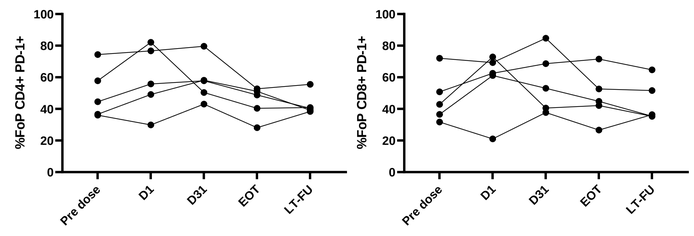
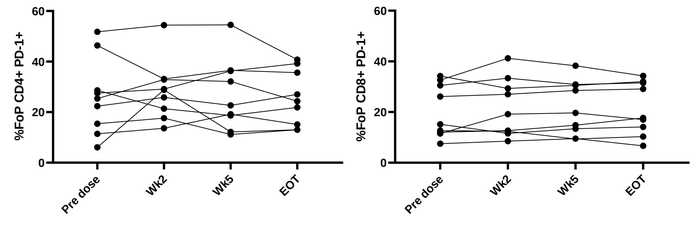


Figure 7. %Frequency of PD1+ in CD4+ and CD8+ T cells in Clinical PBMC Samples

- Patients with advanced solid tumor metastatic cancer treated with immunotherapy in combination with Pembrolizumab



- Non-Small Cell Lung cancer patients treated with an immunotherapy in combination with Pembrolizumab



CONCLUSION

- The advantage of PD-1 using this methodology is, PD-1 expression can be determined independently of PD-1 receptor status: both free and drug-bound PD-1 are accounted for in this detection method.
- The flow panel performed as expected and no interference in PD-1 detection due to Pembrolizumab PD-1 blockage was observed.
- This method of PD-1 detection can be used in any flow panel where the context of use is intended for clinical studies which include anti-PD1 check-point inhibitors.

ACKNOWLEDGEMENTS

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