PRECISION FOR MEDICINE

Validation of a Gliadin α-I/α-II DQ2 Tetramer Flow Cytometric Assay a Biomarker to Assess Gluten Specific T cells in Celiac Disease

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INTRODUCTION

Gluten-challenge in subjects with celiac disease, results in a transient upregulation of gliadin-specific CD4+ T cells in the blood. Despite the increase, these cells are quite rare requiring a selective and sensitive assay for detection. We have developed a 12-color tetramer flow assay to enable detection and immunophenotyping of the gliadin α-I/α-II CD4+ T cells in the blood.

The assay was validated using healthy cryopreserved AccuCell™ PBMCs and CD4+ T cells spiked with T cells specific for gliadin α-I (QLQPFPQPELPY) or gliadin α-II (PQPELPYPQPE). The assay conditions were optimized for sensitivity, optimal signal:noise ratio, and detection of the gliadin α-I/α-II tetramers. The assay identifies gluten-specific (tetramer+) T cells and immunophenotypes the cells as either naïve or memory (CD4/CD3/CD45RA/CD62L/CCR7), activated (CD38), regulatory (CD39) and gut-homing (β7/α4). For validation, pre-set criteria were used to assess inter-assay, intra-assay, inter-operator precision and post-staining stability. Technical validation was successfully met; and the assay performs within acceptable precision parameters.

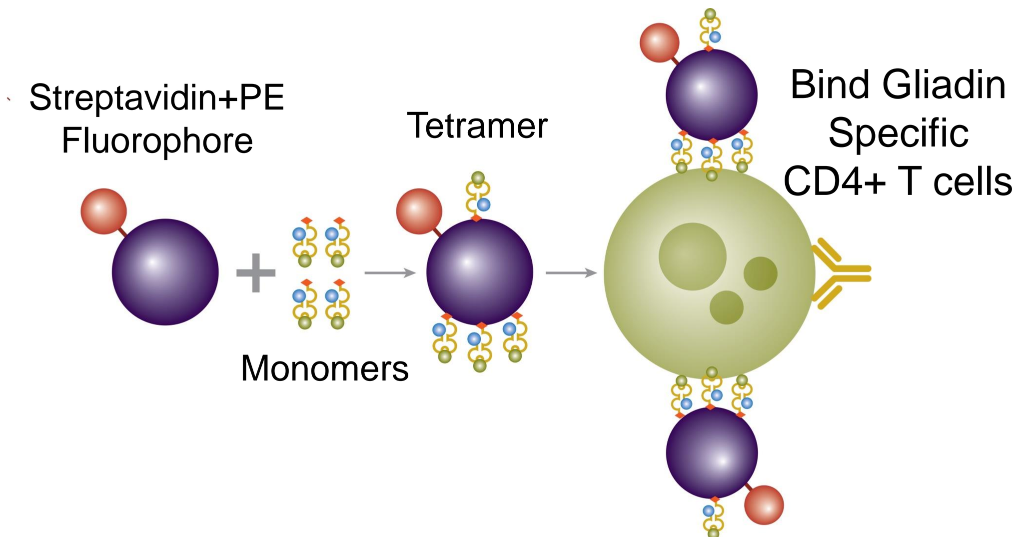
Tetramer positive T cells in the blood are rare events; therefore, this assay was developed and validated to be sensitive and selective. This assay will enable characterization of the gliadin α-I/α-II CD4+ T cells in the blood for celiac patients, providing a more comprehensive evaluation of response to new therapies and may reduce invasive biopsy-based measurements.

EXPERIMENTAL PROCEDURE

Step 1. Separation of PBMCs
SepMate™ tubes- Ficoll to enrich for PBMCs.

Step 2. Gliadin Monomers are Tetramerized with Phycoerythrin (PE) Fluorophore

1. Tetramer 1: gliadin α-I (QLQPFPQPELPY)
2. Tetramer 2: gliadin α-II (PQPELPYPQPE)



Step 3. Isolation of CD4+ T cells from thawed PBMCs
The clinical application of this assay will include whole blood collections and cryopreservation of PBMCs for batch flow testing. The PBMCs are thawed and using a negative depletion magnetic bead based separation CD4+ T cells are isolated. Post purity >95% prior to full panel staining.

Step 4. Stain isolated CD4+ T cells
Unstained, single color controls, FMOs for surface markers and Gliadin α-I/II Tetramers

T-cell Tetramer Full Panel Includes:
CD3, CD4, CD38, B7, Tetramer, CD45RA, CD62L, Alpha4, CCR7, CD39, Lineage depletion (CD11c, CD14, CD19, CD56) & Live/Dead viability dye

Step 6. Detection with BD LSRFortessa™ 5-laser 18-color system

OPTIMIZED SENSITIVITY OF GLIADIN α-I/α-II TETRAMERS

Figure 1. Sensitivity of Detection of Gliadin α-II Tetramers

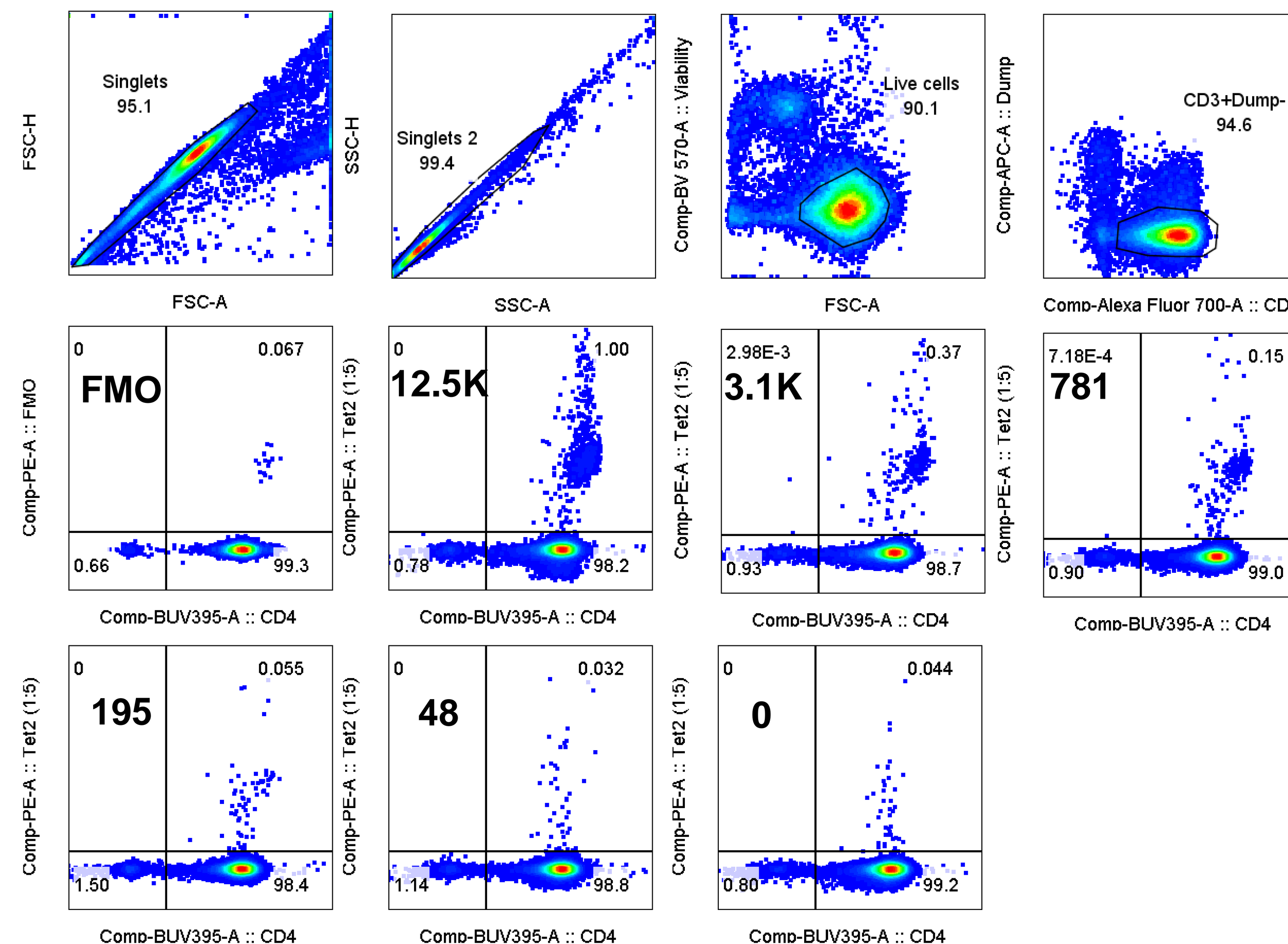


Table 1.

Number of Primary T cells per 1 Million CD4+ T cells	Frequency % of Gliadin α-II Tetramer + Cells	Cell Count of Recovered Tetramer Positive Cells
FMO+12,500 (No Tetramer)	0.067%	18
12,500	1.000%	952
3,125	0.370%	248
781	0.150%	204
195	0.055%	68
48	0.032%	44
0	0.044%	42

Figure 1: Data is representative of titrating primary T cells from Celiac patients (Gliadin 2 Clone 14) in CD4+ T cells. This primary Gliadin T-cell clone is Gliadin α-II tetramer positive when stained alone or spiked in previously cryopreserved CD4+ T cells. One million CD4+ T cells were spiked with 5 cellular concentrations of the Gliadin 2 Clone 14 primary T cells. The five cell concentrations included 12.5K, 3.1K, 781, 195, 48, and no cells as a negative control. Included was an FMO control for objective gating. The cells were stained with the full panel including the Gliadin α-II tetramer. The frequency (%) of Gliadin a-II Tetramer+ CD4 T cells and the cell count are summarized, see **Table 1** above. This data is representative of several independent experiments for the Gliadin α-I/II tetramers. These optimized assay conditions established the number of primary T cells, 3.1K, that were spiked in 3 lots of isolated CD4 T cells used in the assay validation.

Figure 2. Staining Verification of Gliadin α-II Tetramers

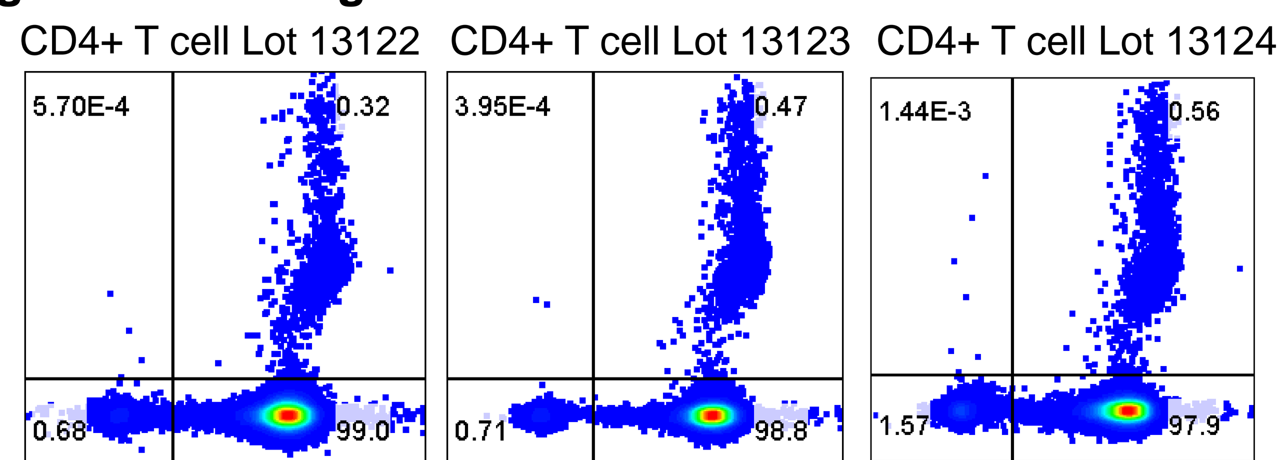


Figure 2: Data representative of 3 CD4 T cells lots spiked with 3.1K of Gliadin 2 Clone 14 primary T cells and stained with Gliadin α-II tetramer. The cells were also stained with the Gliadin a-I Tetramer to demonstrate specificity of the Gliadin 2 Clone 14 primary T cells to the Gliadin a-II Tetramer (not shown).

This study established the sensitivity of the tetramer staining on the three CD4 lots spiked with Gliadin 2 Clone 14 primary T cells used in the validation study.

Table 2. Validation Parameters with pre-set Acceptance Criteria

Validation Parameters	Acceptance Criteria
Intra-Assay Precision	≤30%
Inter-Assay Precision	≤40%
Intra-Operator Precision	≤40%
Post Staining Stability	≤40%

VALIDATION OF GLIADIN TETRAMER ASSAY

Figure 3. Gliadin α-I & α-II Tetramer Staining

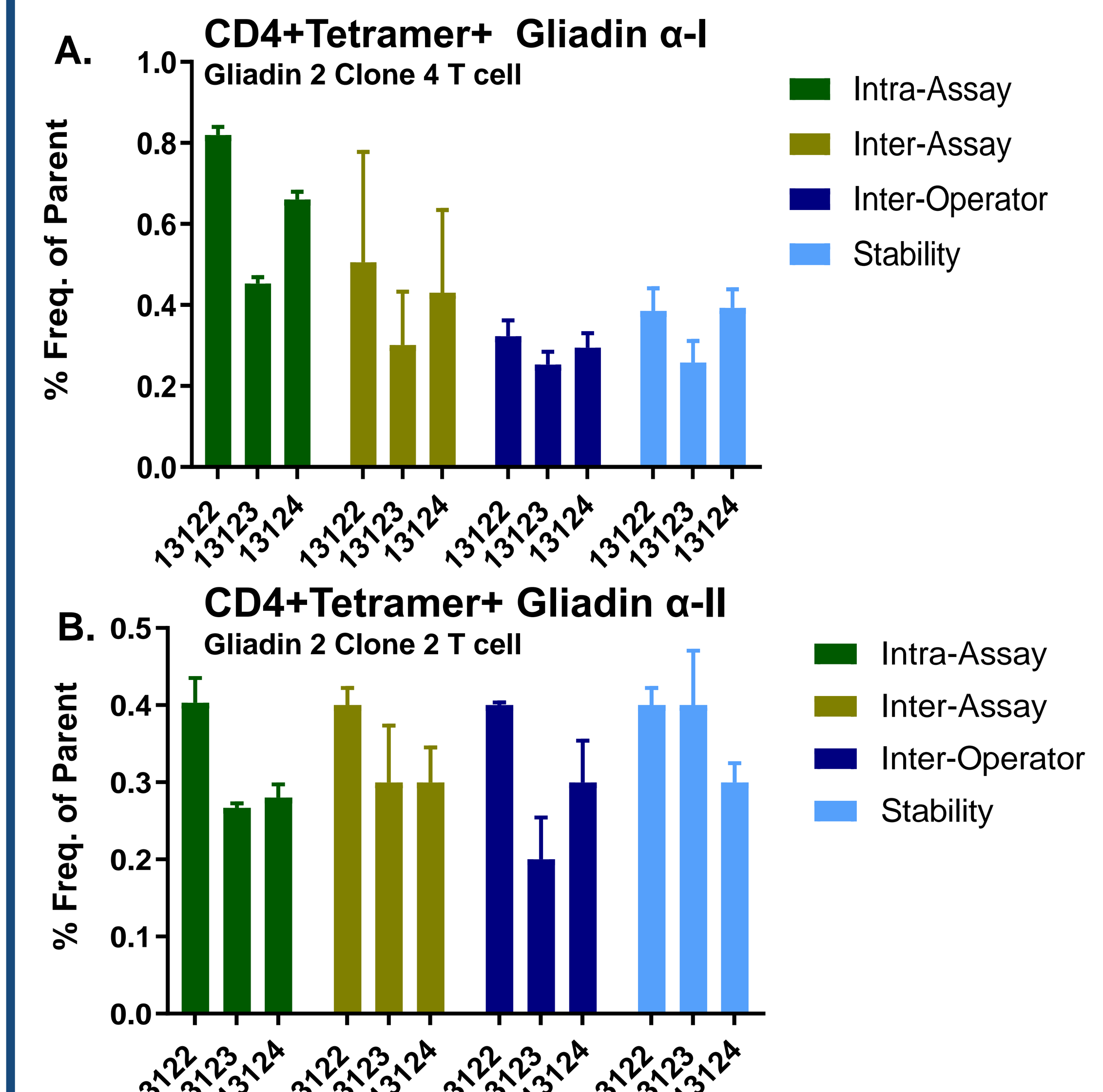


Figure 3: Figure **3A** represents the frequency of CD4+Tetramer+ cells acquired for each of the 3 CD4+ T cell lots (13122, 13123 and 13124) spiked with Gliadin 1 Clone 4 (G1C4) T cells and stained with Gliadin α-I tetramer.

Figure **3B** represents the frequency of CD4+Tetramer+ cells acquired for each of the 3 CD4+ T cell lots (13122, 13123 and 13124) spiked with Gliadin 2 Clone 2 (G2C2) T cells and stained with Gliadin α-II tetramer.

The error bars represent the %CV for the Intra-Assay (n=3), Inter-Assay (n=7) and Inter-Operator (n=4) Precision, and 24hr post-staining Stability (n=4).

Table 3.CD4+Tetramer+ Frequency Mean and %CV

		Intra-Assay		Inter-Assay		Inter-Operator		Stability	
Population	CD4 Lot	Mean	%CV	Mean	%CV	Mean	%CV	Mean	%CV
FoP CD4+Tet+ Gliadin α-I tetramer G1C4	13122	0.8	2.4	0.5	54.0	0.3	12.1	0.4	14.7
	13123	0.5	3.4	0.3	43.8	0.3	12.6	0.3	20.6
	13124	0.7	3.0	0.4	47.5	0.3	12.0	0.4	11.7
FoP CD4+Tet+ Gliadin α-II tetramer G2C2	13122	0.4	8.0	0.4	5.6	0.4	0.9	0.4	5.6
	13123	0.3	2.2	0.3	24.5	0.2	27.1	0.4	17.7
	13124	0.3	6.2	0.3	15.1	0.3	18.0	0.3	8.2

Table 3. These data are the cell frequencies in Figure 3A & 3B of CD4+Tetramer+ cell Mean and %CV for each of the Gliadin α-I and Gliadin α-II tetramers.
Z-score test was used to identify outliers for the inter-assay precision for G1C4. Since the frequency of the CD4+Tetramer+ population is <5% the inter-assay and based on the Z-score test the inter-assay precision results for the CD4+ Tetramer+ G1C4 T cell clone are within the validation acceptance criteria.
Summary: The assay criteria were met for the validation parameters tested.

CONCLUSION

This gliadin α-I/α-II DQ2 tetramer flow assay was developed and validated to be sensitive and selective. Technical validation of the assay was successfully met and the assay performs within the precision parameters.

This assay will enable characterization of the gliadin α-I/α-II CD4+ T cells in the blood for celiac patients, providing a more comprehensive evaluation of response to new therapies and may reduce invasive biopsy-based measurements.

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