Development and technical validation of tetramer staining for use as a biomarker for assessing gluten-specific T cells in clinical studies

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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.

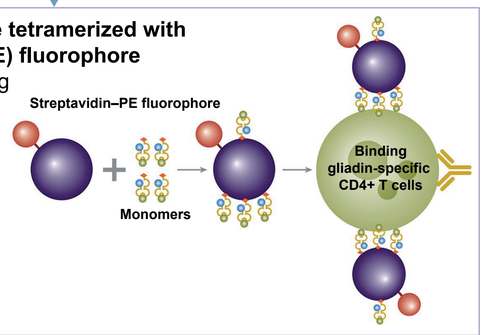
Method and results

- We have developed a 12-colour tetramer flow assay to enable detection and immunophenotyping of the gliadin α-I/α-II CD4+ T cells in the blood (**Figure 1**).

Figure 1. Experimental procedure

Step 1. Separation and storage of PBMCs
- For clinical application, trial sites can collect whole blood
- Centralized PBMC isolation using SepMate™ tubes – Ficoll
- PBMCs frozen to maintain viability and allow batch testing
- Longitudinal samples from each subject to be tested together

Step 2. Gliadin monomers are tetramerized with streptavidin phycoerythrin (PE) fluorophore
Tetramers are pooled for staining
- *Tetramer 1: gliadin α-I (QLQPFPQPELPY)*
- *Tetramer 2: gliadin α-II (PQPELPYPQPE)*



Step 3. Isolation of CD4+ T cells from thawed PBMCs
- PBMCs are thawed and CD4+ cells 'isolated' via magnetic bead based separation
- Post purity >95% prior to staining

Step 4. Stain isolated CD4+ T cells
Controls for staining include:
- Unstained samples to measure auto-fluorescence
- Single-colour controls, entire antibody panel minus 1 stain for each marker and gliadin α-I/II tetramers to set compensation, to evaluate impact of cross-reactivity between reagents and to objectively set gates

Step 5. T cell tetramer full antibody panel
This includes CD3, CD4, CD38, B7, tetramer, CD45RA, CD62L, Alpha4, CCR7, CD39, lineage depletion (CD11c, CD14, CD19, CD56) and live/dead viability dye

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

PBMC, peripheral blood mononuclear cell

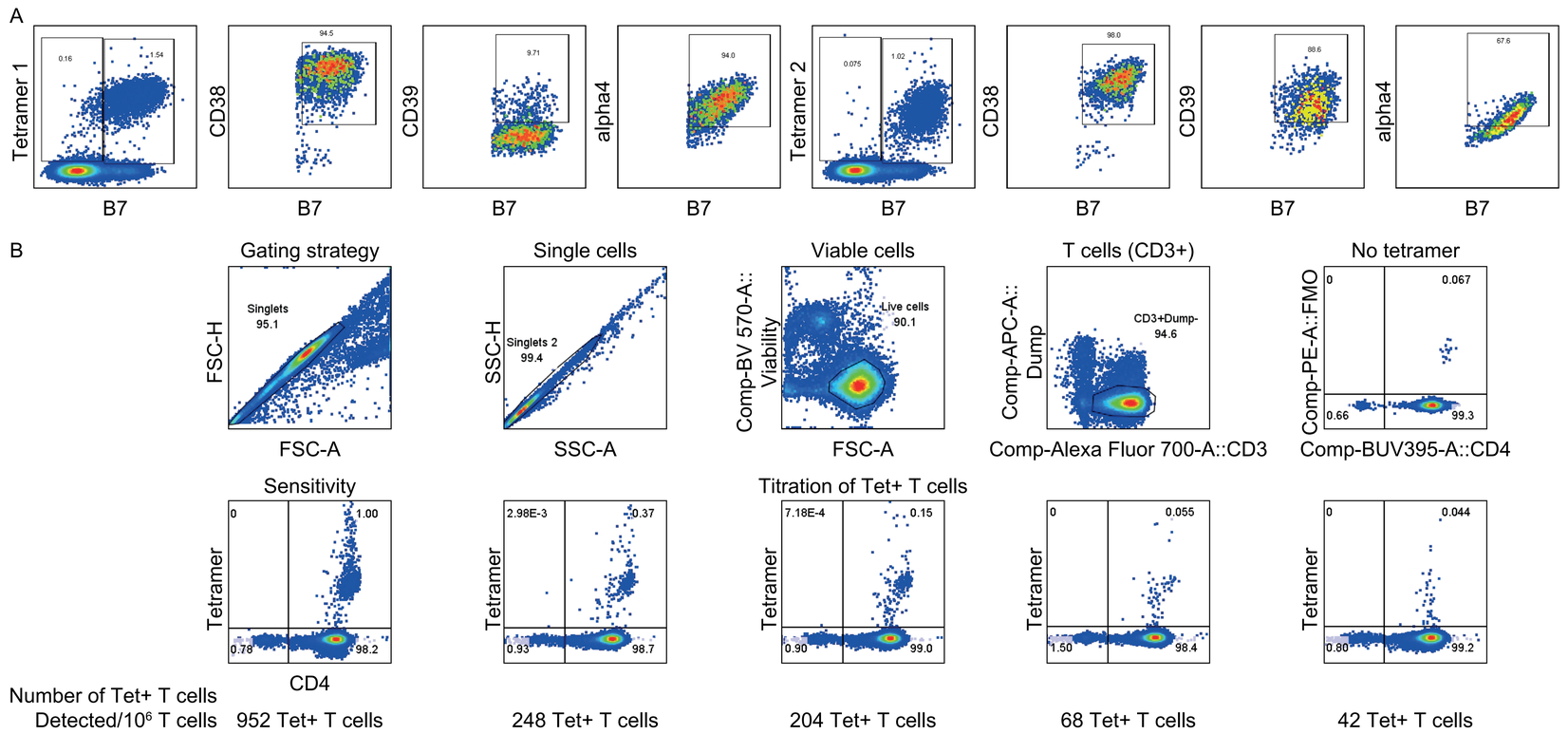
- The assay was validated using cryopreserved AccuCell™ peripheral blood mononuclear cells (PBMCs) and CD4+ T cells from healthy subjects spiked with cloned T cells isolated from subjects with celiac disease that were specific for gliadin α-I (QLQPFPQPELPY) or gliadin α-II (PQPELPYPQPE).
- The assay conditions were optimized for sensitivity, optimal signal:noise ratio and detection of the gliadin α-I/α-II tetramers. The assay identifies gluten-specific (Tet+) 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, and inter-operator precision and post-staining stability (**Table 1**).

Table 1. Assay validation parameters with pre-set acceptance criteria

Validation parameters	Definition	Acceptance criteria
Intra-assay precision	Comparison of replicates using the same test on the same sample in the same experiment	≤30%
Inter-assay precision	Comparison of reproducibility of the same test on the same sample across separate experiments	≤40%
Intra-operator precision	Comparison of same test on the same sample by different operators	≤40%
Post-staining stability	Comparison of staining stability on the day of staining to 24 hours post-staining	≤40%

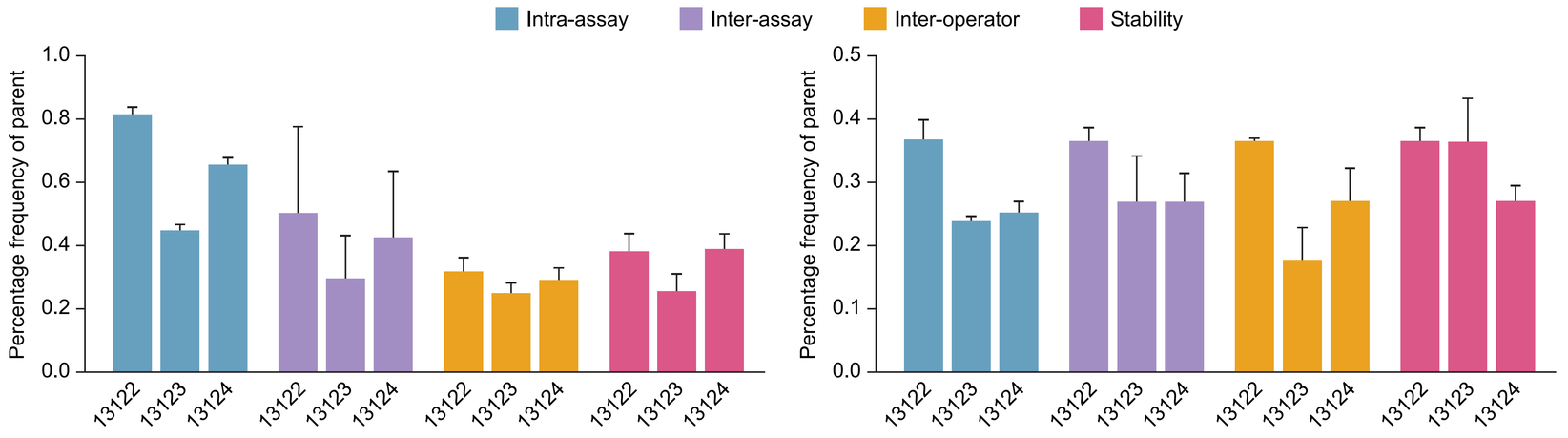
- Primary T cell clones isolated from the peripheral blood of subjects with celiac disease were used as positive controls for validation and were specific for tetramer 1 (gliadin α-I; QLQPFPQPELPY) or tetramer 2 (gliadin α-II; PQPELPYPQPE) (**Figure 2A**). Cells that bound tetramer 1 did not bind tetramer 2 and those that bound tetramer 2 did not bind tetramer 1 (data not shown). Cells were stained with the antibody panel described in **Figure 1** step 5, gated on CD4+ cells, and show staining characteristics similar to what has been described.[1,2]

Figure 2. Evaluating sensitivity of gliadin α-I/α-II tetramers. (A) Primary gluten-specific CD4+ T cell clones used for validation have a phenotype consistent with gluten-specific T cells. (B) Detection of gliadin α-II tetramers+ T cells spiked in CD4+ T cells from a subject without celiac disease. (C) CD4+ T cells from different healthy donors does not impact detection of Tet+ T cells.



Tet+, gliadin α-I/α-II tetramer positive

Figure 3. Tetramer staining is reproducible and stable. (A) The frequency of α-I tetramer+ CD4+ T cells (Gliadin 1 Clone 4) spiked into three different CD4+ T cell samples isolated from individual healthy controls (13122, 13123 and 13124). (B) The frequency of α-II tetramer+ CD4+ T cells (Gliadin 2 Clone 2) spiked into three different CD4+ T cell samples isolated from individual healthy controls (13122, 13123 and 13124).



The error bars are the %CV for the intra-assay (n=3), inter-assay (n=7) and inter-operator (n=4) precision, and 24-hour post-staining stability (n=4).
%CV, percentage coefficient of variation

Table 2. Assay criteria were met for the validation parameters

Tetramer (Tet+ T cell clone)	Healthy CD4 Donor#	Intra-assay Mean	Intra-assay %CV	Inter-assay Mean	Inter-assay %CV	Inter-operator Mean	Inter-operator %CV	Stability Mean	Stability %CV
Gliadin α-I tetramer (Gliadin 1 Clone 4)	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
Gliadin α-II tetramer (Gliadin 2 Clone 2)	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

%CV, percentage coefficient of variation; Tet+, gliadin α-I/α-II tetramer positive

- Titrating primary, α-II tetramer positive T cell clone (Gliadin 2 Clone 14) into CD4+ T cells from a healthy subject. Included was a No Tetramer (no tetramer + full antibody panel) control for objective gating. All other samples were stained with the full antibody panel including the gliadin α-II tetramer. One million CD4+ T cells from heathy subject were spiked with five cellular concentrations of the Tet+ T cells (bottom panel, L to R: 12.5K, 3.1K, 781, 195, 48 [not shown]) and no cells as a negative control) (**Figure 2B**).
- CD4 T cells from three different healthy controls were spiked with 3.1K of Gliadin 2 Clone 14 primary T cell clone and stained with gliadin α-II tetramer (**Figure 2C**). The cells were also stained with the gliadin α-I tetramer to demonstrate specificity of the Gliadin 2 Clone 14 primary T cells to the gliadin α-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.
- The data shown in **Figure 3** for the gliadin α-I and gliadin α-II tetramers are summarized in **Table 2**. The Z-score test was used to identify outliers for the inter-assay precision for G1C4. Based on the Z-score test and the inter-assay precision, the results for the CD4+ tetramer+ G1C4 T cell clone are within the validation acceptance criteria.

Conclusions

- This gliadin α-I/α-II DQ2 tetramer flow cytometric 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.

References

1. Han A *et al. Proc Natl Acad Sci USA* 2013;110:13073–8.
2. Sarna VK *et al. Gastroenterology* 2018;154:886–96.

Disclosures

GMS, WM and JE are employees of Takeda. LK, AB and DP are employees of Precision for Medicine. WK has no disclosures to report.

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