

# 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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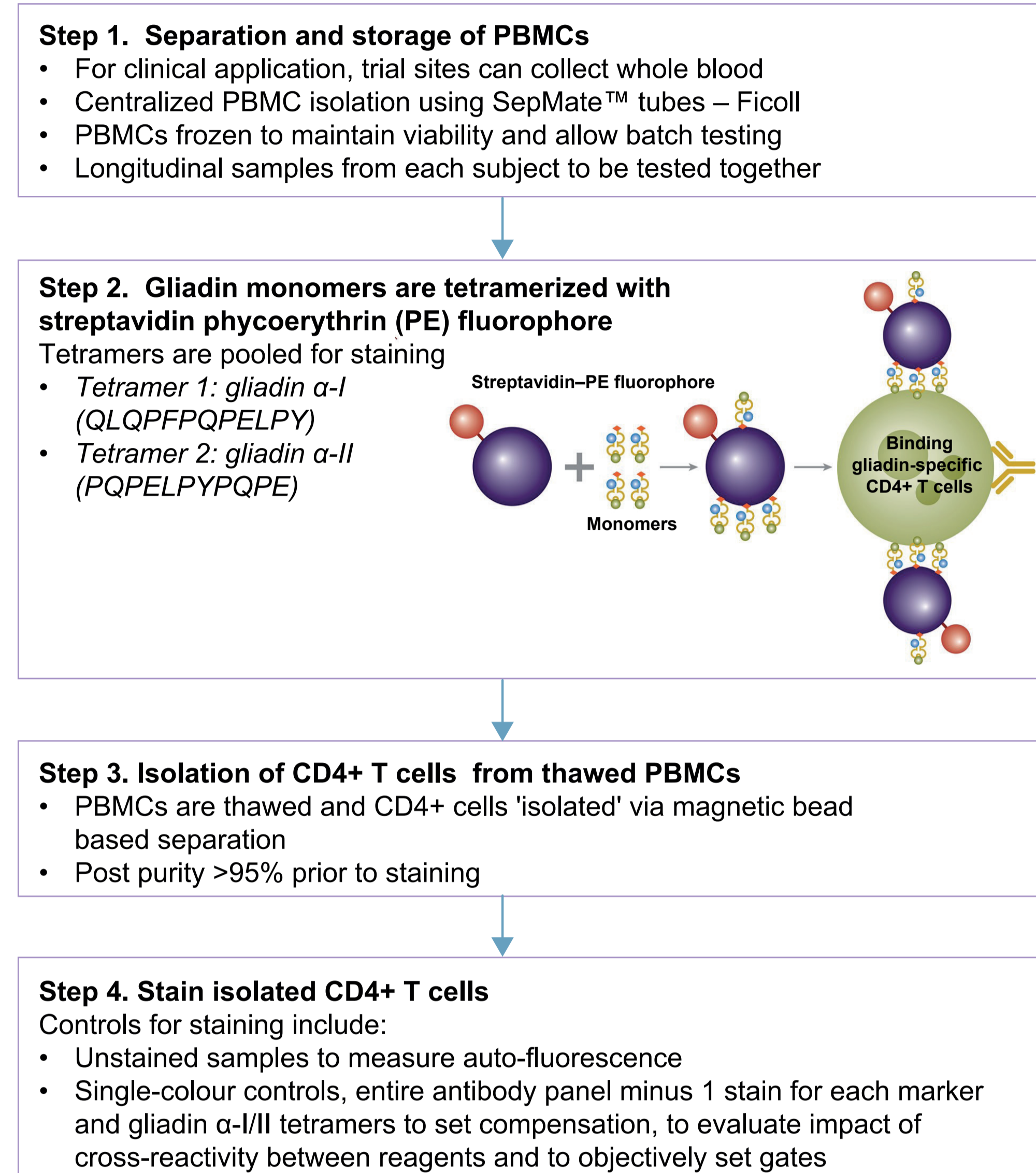
## Introduction

- 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  $\alpha$ -I/ $\alpha$ -II CD4+ T cells in the blood (Figure 1).

Figure 1. Experimental procedure



PBMC, peripheral blood mononuclear cell

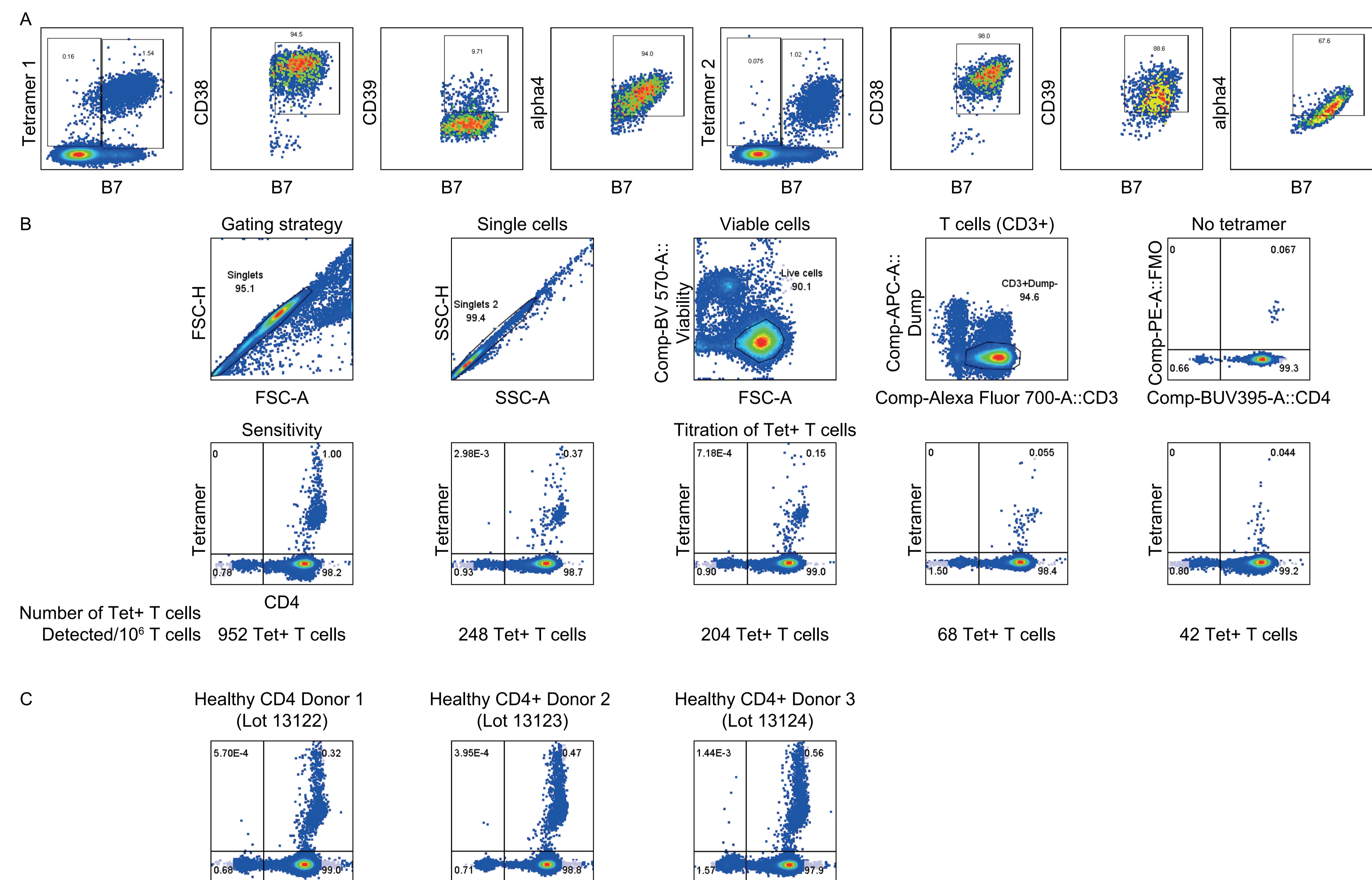
- The assay was validated using cryopreserved AccuCell™ peripheral blood mononuclear cells (PBMCs) and CD4+ T cells from healthy subjects spiked with cloned T cells isolated from subjects with celiac disease that were specific for gliadin  $\alpha$ -I (QLQFPQPPELQPE) or gliadin  $\alpha$ -II (PQPELPYPQPE).
- The assay conditions were optimized for sensitivity, optimal signal:noise ratio and detection of the gliadin  $\alpha$ -I/ $\alpha$ -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 ( $\beta$ 7/ $\alpha$ 4). 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%

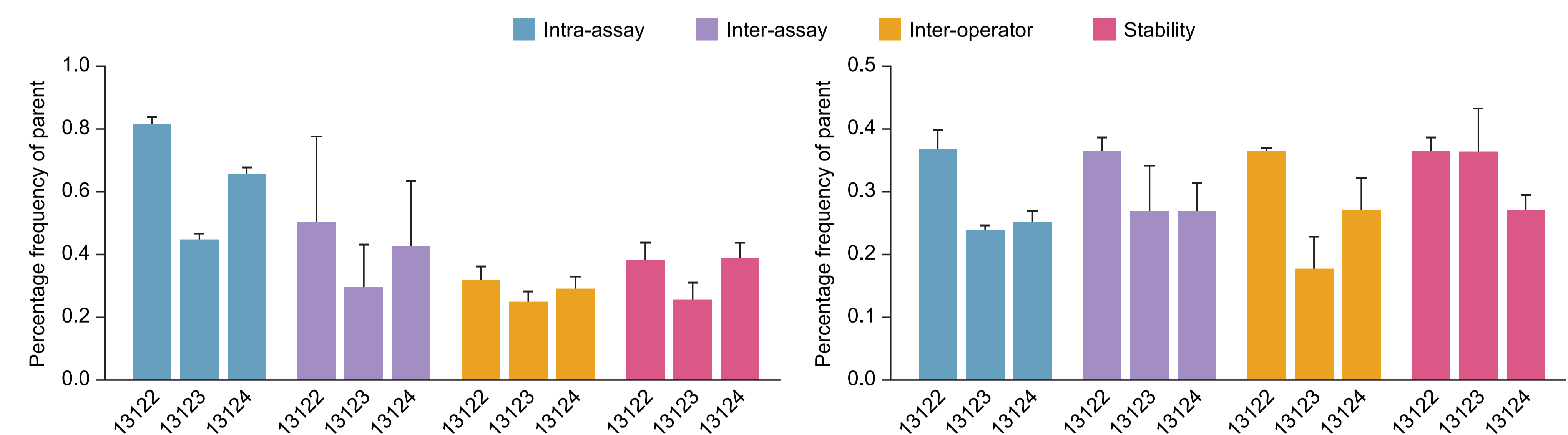
- Primary T cell clones isolated from the peripheral blood of subjects with celiac disease were used as positive controls for validation and were specific for tetramer 1 (gliadin  $\alpha$ -I; QLQFPQPPELQPE) or tetramer 2 (gliadin  $\alpha$ -II; PQPELPYPQPE) (Figure 2A). Cells that bound tetramer 1 did 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.<sup>1,2</sup>

Figure 2. Evaluating sensitivity of gliadin  $\alpha$ -I/ $\alpha$ -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  $\alpha$ -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  $\alpha$ -I/ $\alpha$ -II tetramer positive

Figure 3. Tetramer staining is reproducible and stable. (A) The frequency of  $\alpha$ -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  $\alpha$ -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 $\alpha$ -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 $\alpha$ -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  $\alpha$ -I/ $\alpha$ -II tetramer positive

- Titrating primary,  $\alpha$ -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  $\alpha$ -II tetramer. One million CD4+ T cells from healthy 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  $\alpha$ -II tetramer (Figure 2C). The cells were also stained with the gliadin  $\alpha$ -I tetramer to demonstrate specificity of the Gliadin 2 Clone 14 primary T cells to the gliadin  $\alpha$ -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  $\alpha$ -I and gliadin  $\alpha$ -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  $\alpha$ -I/ $\alpha$ -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  $\alpha$ -I/ $\alpha$ -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

- Han A et al. *Proc Natl Acad Sci USA* 2013;110:13073-8.
- 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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