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BEST PRACTICE

CLINICAL CYTOMETRY WILEY

TRBC1 in flow cytometry: Assay development, validation, and reporting considerations

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Abstract

The assessment of T-cell clonality by flow cytometry has long been suboptimal, relying on aberrant marker expression and/or intensity. The introduction of TRBC1 shows much promise for improving the diagnosis of T-cell neoplasms in the clinical flow laboratory. Most laboratories considering this marker already have existing panels designed for T-cell workups and will be determining how best to incorporate TRBC1. We present this comprehensive summary of TRBC1 and supplemental case examples to familiarize the flow cytometry community with its potential for routine application, provide examples of how to incorporate it into T-cell panels, and signal caution in interpreting the results in certain diagnostic scenarios where appropriate.

KEYWORDS
flow cytometry, leukemia, lymphoma, T-cell, validation

1 | INTRODUCTION

T-cell neoplasms are a diagnostically challenging group of diseases that require review of clinical presentation along with results from laboratory studies including histology and flow cytometry (Alaggio et al., 2022; Arber et al., 2022; Horna, Shi, Olteanu, & Johansson, 2021). Like most malignancies, demonstration of clonality greatly facilitates the diagnosis of T-cell neoplasms. To this end, molecular analysis of T-cell receptor (TCR) gene rearrangement is widely used as the gold standard of T-cell clonality assessment in clinical practice

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(Mahe et al., 2018; Syrykh et al., 2021; Tan et al., 2006). Although technologic advances such as next generation sequencing (NGS) have greatly improved sensitivity, sequencing-based methods continue to suffer from specificity issues, as they do not inherently discriminate between reactive and malignant T-cell clones.

Flow cytometry-based assays are a rapid, cost-effective diagnostic method for a wide array of hematolymphoid neoplasms. Despite extensive application in B-cell neoplasms, immunophenotypic analysis of T-cells traditionally has been more limited due to lack of reliable markers to detect neoplasms of the T-cell lineage (Tan et al., 2006). One of the key challenges lies in the exhaustive heterogeneity of the variable (V) domains of the TCR α-chain and β-chain that form the cornerstone of adaptive immunity. While evaluating the TCR Vβ repertoire can demonstrate clonality and is an established method for assessing αβ T-cell receptor diversity, this assay is not practical for routine clinical use due to its use of a large array of TCR Vβ-specific antibodies and incomplete coverage of the overall TCR Vβ repertoire (Feng et al., 2010; Morice et al., 2004).

Assessment of the T-cell receptor β chain constant region (TRBC) is an attractive alternative to TCR Vβ analysis for clonality assessment since only two mutually exclusive TRBC isoforms (TRBC1, TRBC2) exist. Indeed, numerous studies demonstrate that addition of a single anti-TRBC1 antibody to an 8–10 color T-cell-targeted panel improves the ability to assess clonality in αβ T-cells (Berg et al., 2020; Horna, Shi, Jevremovic, et al., 2021; Horna, Shi, Olteanu, & Johansson, 2021; Shi et al., 2019). These studies recommend the utilization of anti-TRBC1 in a manner very similar to how anti-kappa/lambda antibodies are used for B-cell and plasma cell analysis, with anti-TRBC1 performed in conjunction with multiple other T-cell antigens to evaluate different T-cell subsets and separate neoplastic and benign T-cell populations (Horna, Shi, Olteanu, & Johansson, 2021).

Given the marked potential of TRBC1 to improve T-cell analysis, there is great interest in its inclusion in clinical flow cytometry panels. Unfortunately, validation of new antigens remains challenging for many clinical laboratories despite recent improvements in flow cytometry assay development guidelines (CLSI, 2021). This manuscript describes a practical approach to incorporation of anti-TRBC1 into a T-cell immunophenotyping panel and includes common pitfalls that may be encountered during assay development and analysis. Additionally, a library of 13 case studies is included in the supplemental material and referenced throughout the text (Table 1). These cases are organized by theme and were chosen to illustrate various teaching points and considerations when including TRBC1 in flow panels.

2 | ASSAY DEVELOPMENT

The addition of TRBC1 can occur by using an "empty slot" in a current validated T-cell panel or reconfiguring a current T-cell panel to accommodate the inclusion of TRBC1. Either scenario constitutes an assay modification and should follow the recent CLSI H62 guidelines (CLSI, 2021). For some practical guidance, one can follow the ICCS Module 21: *Selection and Validation Strategy for Adding Antibodies to Flow Cytometry Panels* (Shah et al., 2021), as well as other related publications. However, since TRBC1 is a new marker mainly used to assess T-cell clonality and is different from many other immunophenotypic markers, additional work will be required to validate its clinical use. This may include steps such as establishing reference ranges of TRBC1+ percentages in T-cell populations observed in normal donors, confirming the ability to detect clonality, and establishing thresholds for abnormality. The decision to use TRBC1 in a screening tube or in an add-on tube rests with the laboratory and depends largely upon patient population and case composition.

2.1 | Antibody panel design and selection of antibody/fluorochrome pairing

2.1.1 | Establishing panel components

TRBC1 is a part of the TCRαβ-CD3 complex expressed only on mature αβ T-cells; γδ T-cells are inherently negative for TRBC1 and TRBC2 hence their inclusion may result in erroneous interpretation as TRBC1-negative clonal T-cells. This is particularly important for CD8+ and CD4-CD8− T-cell analysis, as γδ T-cells predominantly lack CD4/CD8 but a subset can express CD8. (Figure 1; Case #4; Case #5). For this reason, a general T-cell panel should at the very minimum include CD3, CD4, CD8, and CD45 and a mechanism to limit TRBC1 analysis to αβ T-cells via exclusion of γδ T-cells through the use of anti-TCRγδ or anti-TCRαβ (Figures 2 and 3; See gating strategy section below). In contrast, panels designed to evaluate certain CD4-positive T-cell subsets (e.g., Sezary cell assessment) may not require this step, as CD4 expression in normal and malignant γδ T-cells is vanishingly rare. As a neoplastic T-cell population may not be readily identified by a monotypic TRBC1 expression pattern if mixed with normal/reactive T-cells, specific markers on the disease of interest should be included to increase the sensitivity of identifying the clonal neoplastic T-cell population that reflects the laboratory's desired testing strategy and patient population (Case #6). This may include pan-T cell antigens such as CD2, CD5, and CD7; CD26 for mycosis fungoides/Sezary syndrome (Horna, Shi, Olteanu, & Johansson, 2021); CD25 for adult T-cell leukemia/lymphoma (Craig & Foon, 2008); CD10 for T-follicular helper cell lymphoma (Craig & Foon, 2008); and CD57, CD16, and CD56 for T-large granular lymphocytic leukemia (T-LGLL) (Case #9). Use of cytoplasmic CD3 and TRBC1 may be useful in the diagnosis of surface CD3-negative mature T-cell neoplasms or neoplastic immature T-cells (Horna et al., 2022).

2.1.2 | Antibody clone selection

The selection of antibody clones should be based on published data and/or experiments you have performed using multiple clones in comparison. TRBC1 clone JOVI.1 is the only available clone at this time

TABLE 1 List of case studies included in the supplemental section. Diagnosis, sample type, TRBC1 interpretation, clonal status, and teaching points for each are included. Flow cytometric plots, gating strategies, and clinical information can be found with the cases.

Theme	Case	Diagnosis	Sample	TRBC1	Clonality	Teaching points
Normal	1	Normal	LN	Polytypic	Non-clonal	Example of reactive lymph node. Note that CD4−, CD8− TCRγδ− T-cells (presumably TCRαβ+ T-cells) also show a biphasic TRBC1 pattern.
	2	Normal	BM	Polytypic	Non-clonal	Example of a normal bone marrow. TRBC1 is useful to determine if populations with immunophenotypic changes are clonal.
	3	Normal	Thymus	Negative on sCD3− thymocytes; Polytypic on sCD3+ T-cells	Non-clonal	Surface TRBC1 expression correlates with sCD3 expression in thymic tissue. Notice the diagonal appearance of their relationship in plots and recognize this is inherent rather than an issue with compensation.
Pitfalls	4	Increased γδ T-cells	BM	Negative on γδ T-cells; Polytypic on CD8+, γδ− T-cells	Non-clonal	A subset of TCRγδ+ T-cells express CD8, which can lead to false elevation of TRBC1−, CD8+ T-cells. Notice TRBC1 expression in all CD8+ T-cells versus CD8+, TCRγδ− T-cells, which may lead to misinterpretation.
	5	Residual Sezary	PB	Negative	Clonal	Multiple abnormal subclones can be present. Rare cases of TCRαβ+ double-negative clonal T-cells can be seen with TRBC1 after excluding TCRγδ+ T-cells.
	6	EBV+ T-cell lymphoma	BM	Positive	Clonal	The 15/85 threshold alone is not always reliable to detect abnormal populations and assessment of other antigens or subpopulations may be needed. Care must be taken to not miss cell populations of large size.
	7	Polytypic T-cells	PB	Dim positive but polytypic	Non-clonal	Proximity of TRBC1 and CD3 results in a characteristic pattern with CD3 versus TRBC1. Interpretation of TRBC1dim populations with bright CD3 must be interpreted with caution in the absence of other abnormalities as they may be due to cross-reactivity of anti-TRBC1 with TRBC2.
Mature T-cell neoplasms	8	Sezary syndrome	PB	Dim positive	Clonal	T-cell lymphomas may show dim TRBC1 which may be due to low levels of TRBC1 (often seen with decreased CD3 expression) or a TRBC2+ population. TRBC1-dim populations may be missed with automatic thresholds.
	9	T-LGLL	PB	Positive	Clonal	CD8+ T-LGLL and γδ+ T-cells can show overlapping immunophenotype including dim CD8, loss of CD5, and expression of CD16.
	10	T-PLL	PB	Negative	Clonal	Straightforward case with restricted CD4+ T-cells. Additional gating strategies are needed to isolate the clonal subset. TRBC1 may serve to verify that the "normal" population excluded is polytypic, which is relevant if enumeration is desired (e.g., Sezary)
	11	AITL	LN	Negative	Clonal	

TABLE 1 (Continued)

Theme	Case	Diagnosis	Sample	TRBC1	Clonality	Teaching points
						T-cell clonality in surface CD3-negative populations cannot be assessed by surface TRBC1 expression; other supporting features should be used to determine neoplasia.
TCUS	12	TCUS × 2	PB	Negative	Clonal	Small TRBC1-restricted populations are common and frequently show a cytotoxic immunophenotype with upregulation of CD57 and downregulation of CD5 and/or CD7. These TCUS cases should be interpreted with caution.
	13	Sezary & TCUS	PB	Positive	Clonal	Abnormal T-cell clones often show more antigenic aberrancies than reactive TCUS. Incidental TCUS may be present in the background of a hematolymphoid neoplasm and must be interpreted cautiously.

Abbreviations: AITL, angioimmunoblastic T-cell lymphoma; BM, bone marrow; EBV, Epstein–Barr virus; LN, lymph node; PB; peripheral blood; s, surface; TCUS, T-cell clones of uncertain significance; T-LGLL, T-large granular lymphocytic leukemia; T-PLL, T-prolymphocytic leukemia.

and is used in published studies (Capone et al., 2022; Delfau-Larue et al., 2000; Horna, Shi, Olteanu, & Johansson, 2021).

2.1.3 | Fluorochrome selection

Fluorochrome selection is based on the antigen expression on the cells of interest, specific known fluorochrome characteristics, existing panels, and clinical purpose. In general, an antibody against a weakly expressed antigen should be conjugated with a bright fluorochrome, and vice versa. We suggest that the TRBC1 antibody be conjugated to a fluorochrome, which shows good separation between TRBC1-positive cells and TRBC1-negative cells (high signal/noise ratio) which is critical for the assessment of T-cell clonality. In our experience, the TRBC1-FITC showed the best S/N ratio but TRBC1-PE also performed well. Consideration should also be given to spillover/spreading that may reduce the resolution between TRBC1-positive and TRBC1-negative populations. Please see the variety of fluorochrome-antibody pairs in the supplemental case examples.

2.2 | Antibody optimization and performance verification

This process includes validating the intended use, finding the optimal concentration (or titer) of an antibody, and minimizing background fluorescence and steric hindrance of the antibody. The published literature and the manufacturer's package insert usually provide useful information to guide the design of the staining protocols.

2.2.1 | Antibody specificity

The specificity of the antibody can be tested using appropriate negative and positive controls. Since the expression of TRBC1 is limited to a subset of αβ T-cells, internal negative and positive controls are easily found in most specimens (discussed in detail below; also see Figures 2 and 3). The γδ T-cells can be detected in most specimens and can be used as a CD3+ TRBC1-negative control.

2.2.2 | Antibody titration

Titration is an option for achieving an optimal signal-to-noise ratio (S/N) or staining Index (SI). It also often reduces antibody reagent cost of antibodies. The best titer is determined through a serial dilution and examination of the S/N ratio or the Staining index (SI) to provide objective assessment of staining performance. An example of a TRBC1 titration study is provided in Figure 4. Please see more detailed ICCS Quality & Standards Module #7: *Quality of Reagents – Monoclonal Antibodies* (Hulspas et al., 2018).

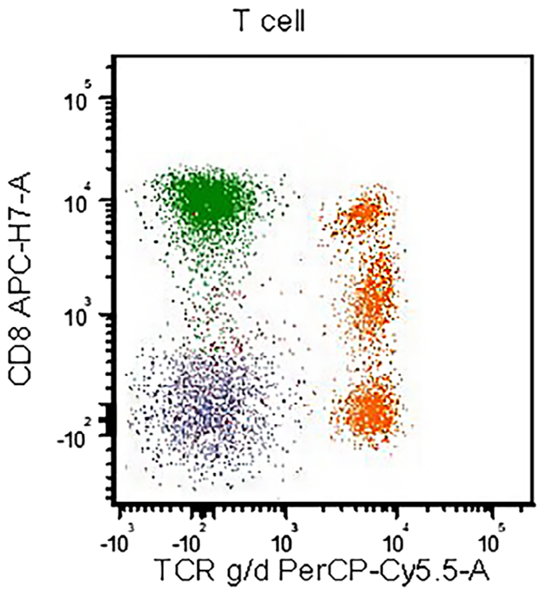

FIGURE 1 Usually, γδ T-cells have dim CD8 expression and do not overlap with αβ T-cells based on CD8 expression. In this example, the γδ T-cells show various levels of CD8 expression including a subset with strong CD8 that overlaps with the αβ T-cells. This case illustrates the need to remove the γδ T-cells from TRBC1 analysis, as gating on all CD8+ T-cells will include γδ T-cells and skew the TRBC1 distribution, potentially masking a clonal TRBC1+ population. [Color figure can be viewed at wileyonlinelibrary.com]

2.2.3 | Antibody quality control

The laboratory's quality control program should be followed to verify the antibody performance over time.

2.2.4 | Performance of the new antibody and panel

Once the antibody is optimized, the new panel should be tested by comparing the results with those of the existing panel, as well as unstained control, single stained control, and fluorescence minus one (FMO; mixture of all antibodies except one). These experiments should verify the instrument settings (proper voltage, compensation) and facilitate the identification and investigation of potential problems associated with a panel such as incorrect compensation or suboptimal antibody performance in the fully stained panel, as well as any other issues visible on the dot plot combinations. Of note, CD3 and TRBC1 are both part of the TCR complex, hence their physical proximity and biological interaction leads to a characteristic diagonal pattern (Figures 2 and 3). This is a normal finding and should not be interpreted as a compensation issue (Shi et al., 2019).

3 | ASSAY VALIDATION

The necessary components of the validation will vary depending on the manner in which TRBC1 is being introduced. Many labs will have an already-validated T-cell panel in which they wish to add to or exchange a marker for TRBC1 (assay modification). Some labs will want to validate an entirely new panel as part of a new assay validation. Much detail regarding the different components has been covered in other ICCS Q&S modules such as Module 21: *Selection and validation strategy for adding antibodies to flow cytometry panels* and Module 25: *A summary of validation considerations with real-life examples using both qualitative and semi quantitative flow cytometry assays* (Oldaker et al., 2022; Shah et al., 2021). A brief overview of validation components and TRBC1-specific details is provided below. Please note, all sample sizes are referencing a suggested minimum, and may need to be adjusted to the lab-specific setting. A practical example of one laboratory's approach to the incorporation of TRBC1 into their panels is provided for reference in the Supplemental Section.

3.1 | Accuracy

Although each lab has a different composition of frequently tested specimen types, it is recommended that at least 20 samples from various tissue types representing a spectrum of disease types as well as normal specimens should be tested (CLSI, 2021). The abnormal specimens should be CD3+ TCRαβ+ with a clear pathologic diagnosis of T-cell lymphoma/leukemia, and they should include both TRBC1+ and TRBC1- cases. Ideally, the abnormal cases should have confirmed clonality by other ancillary testing strategies such as TCR gene rearrangement results or TCR Vb flow cytometry assay and/or a confirmed clinical diagnosis of a T-cell neoplasm. If this is not possible, correlational studies with other labs with a validated TRBC1 panel should be considered. Desirable criteria for acceptance include at least 95% concordance.

3.2 | Precision

Depending on the extent and type of validation (new validation vs. method modification to include TRBC1 into an already validated panel), the requirement for precision experiments may vary. In general, the steps for assay modification will be similar to the validation of new assays: *intra-assay precision*: one stained sample with at least 3 replicates in a single run; *inter-assay precision*: three to five samples analyzed in triplicate; *inter-operator precision*: follow the inter-assay precision protocol per each performing employee; *inter-instrument precision*: follow the inter-assay protocol for each instrument the assay is performed on (Devitt et al., 2023). See table A6 in CLSI H62 for further guidance and additional suggestions regarding number of samples and replicates (CLSI, 2021). This situation lends itself well to consideration of factorial design for precision studies. Of course, the final decision for the extent of precision experiments lies with the medical director of the laboratory.

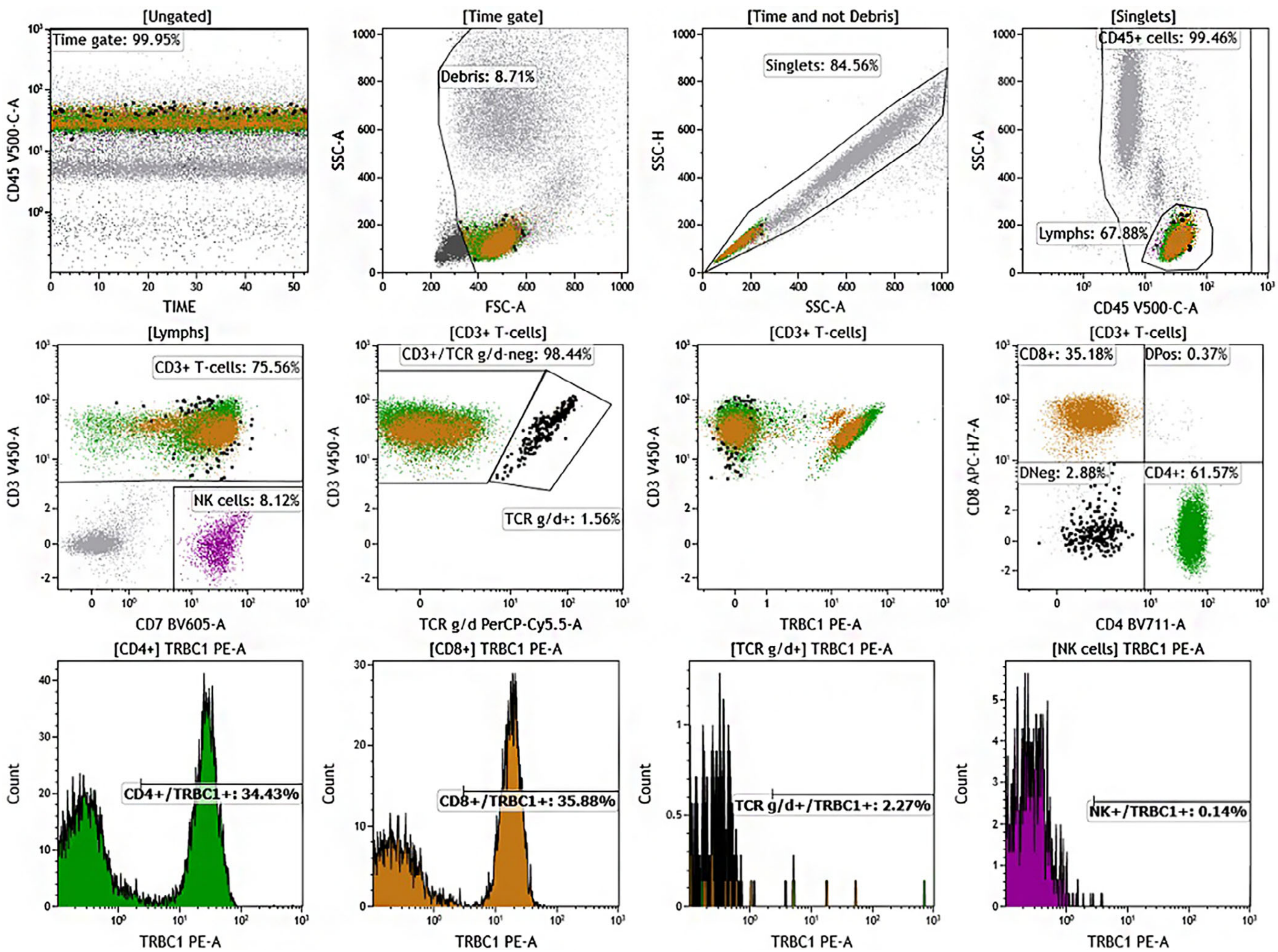

FIGURE 2 Gating **OUT** the γδ T-cells. Example of T-cell panel and gating strategy using TCR-γδ on a normal peripheral blood sample. The gating strategy includes time, debris exclusion, singlet inclusion, and focusing on CD45+ lymphocytes (top row). Various plots are shown with CD3, CD4, CD8, TRC γδ, and TRBC1 (middle row). TRBC1 expression is bimodal on CD3+ CD4+ T-cells (green) and CD3+ CD8+ T-cells (orange), and is inherently negative on γδ T-cells (black) and NK-cells (purple) (bottom row). These provide good internal positive and negative controls. Notice the direct correlation between TRBC1 and surface CD3 expression (middle row, 3rd from left). [Color figure can be viewed at wileyonlinelibrary.com]

3.3 | Diagnostic sensitivity and specificity

Using the accuracy dataset, create 2 × 2 concordance tables to determine diagnostic sensitivity and specificity (CLSI, 2021; Oldaker et al., 2022).

3.4 | Selectivity

Much of the work required for demonstrating that the antibodies and panels are identifying the targeted populations of interest occurs during panel design, antibody selection, clone choice, and antibody titration. Antibody specificity sheets are a useful tool, and an example of a TRBC1 antibody specificity sheet is shown in Figure 5. All decisions regarding the antibody/clone choice should be summarized within the validation summary.

3.5 | Detection capability

These panels are primarily used for detecting clonality in cases suspected of having a T-cell abnormality, and not intended to be used as a screening panel. The TRBC1 findings should not be interpreted in isolation but rather in the context of additional immunophenotypic features. It should be noted that the LOB, LOD, and LLOQ are challenging to define for these assays due to the fact that there are many variations in abnormal T-cell phenotypes and it is often difficult to separate "normal" from "abnormal", as reported for assays for other diseases designed to detect minimal residual involvement. If the panel is used for monitoring therapeutic response or screening purposes, the sensitivity should be established following rare event detecting SOPs, but this is outside the scope of this module.

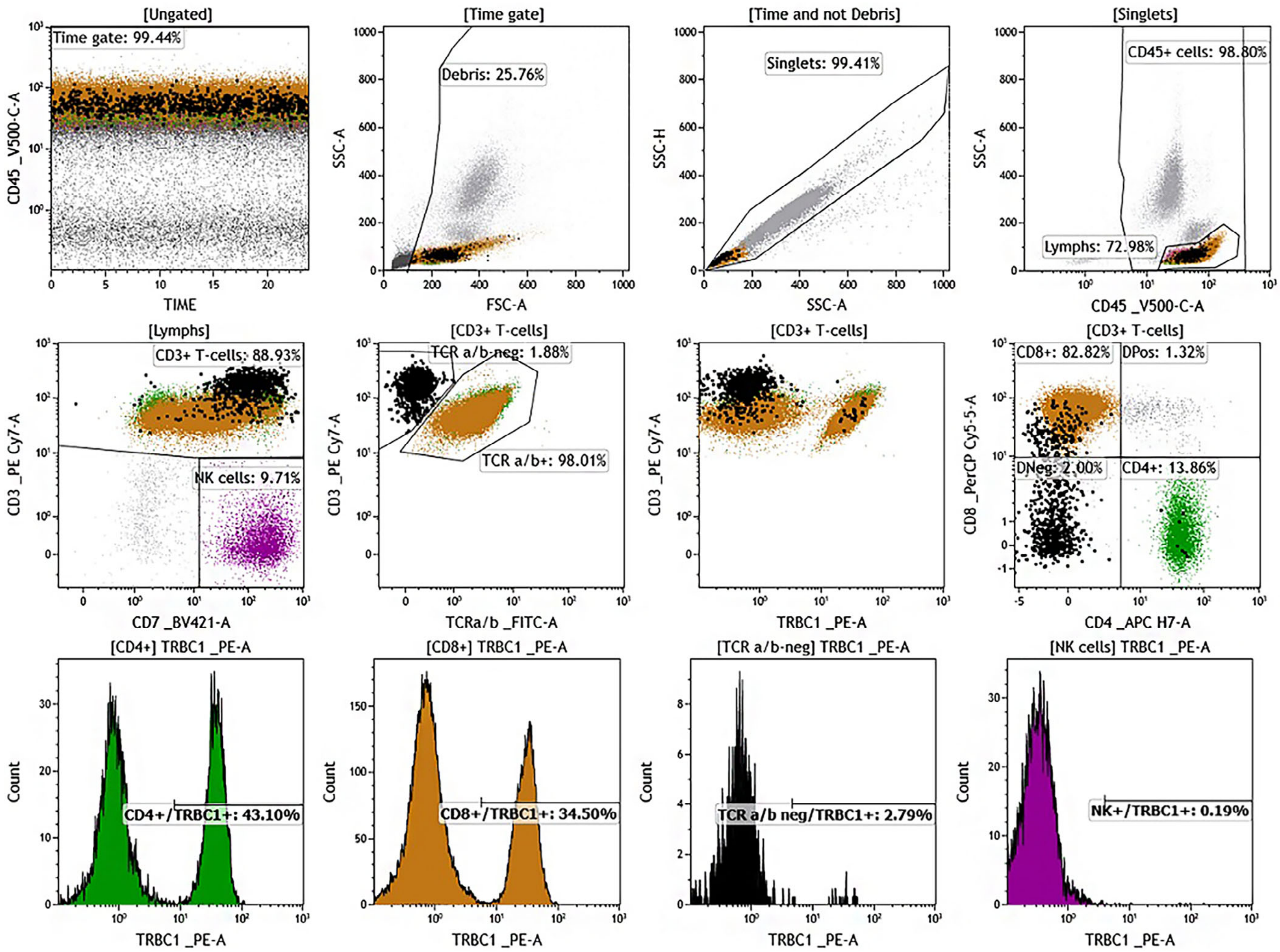

FIGURE 3 Gating **IN** the αβ T-cells. Example of T-cell panel and gating strategy using TCR-αβ on a normal peripheral blood sample. The gating strategy includes time, debris exclusion, singlet inclusion, and focusing on CD45+ lymphocytes (top row). Various plots are shown with CD3, CD4, CD8, TRC αβ, and TRBC1 (middle row). TRBC1 expression is bimodal on CD3+ CD4+ T-cells (green) and CD3+ CD8+ T-cells (orange), and is inherently negative on αβ-negative (i.e., γδ+) T-cells (black) and NK-cells (purple). These provide good internal positive and negative controls. Notice the direct correlation between TRBC1 and surface CD3 expression (middle row, 3rd from left). [Color figure can be viewed at wileyonlinelibrary.com]

3.6 | Stability

Cocktail stability studies should be performed if the new antibody is used as part of a cocktail. Specimen stability studies need not be repeated so long as sample types remain the same (O'Donahue et al., 2019).

3.7 | Reference ranges

Published reference ranges are available for TRBC1-positivity in various T-cell subsets (summarized in Table 2). The lab should consider running the assay on normal/non-neoplastic samples to verify the published reference ranges and establish the threshold that will be considered abnormal. We recommend a validation cohort with at least 20 "normal" (no reason to suspect a T-cell neoplasm) specimens, recognizing that different regulatory bodies may have different requirements. The 20 specimens should include different tissue types (bone marrow, peripheral blood, lymph nodes, fluids) even though there are no statistically significant differences in %TRBC1+ populations in these samples (Shi et al., 2019). Moreover, since T-cells are heterogenous with multiple normal/reactive subsets, and the subsets have different %TRBC1+ reference ranges (Muñoz-García et al., 2021), a separate cut-off for each subset could be an option.

4 | STRATEGIES FOR IDENTIFICATION OF CLONAL POPULATIONS

4.1 | Gating strategies

All gates should follow the appropriate initial hierarchy (e.g., time, debris exclusion, singlets, CD45) to keep a consistent strategy and to verify the location of known populations (mature vs. immature, etc.).

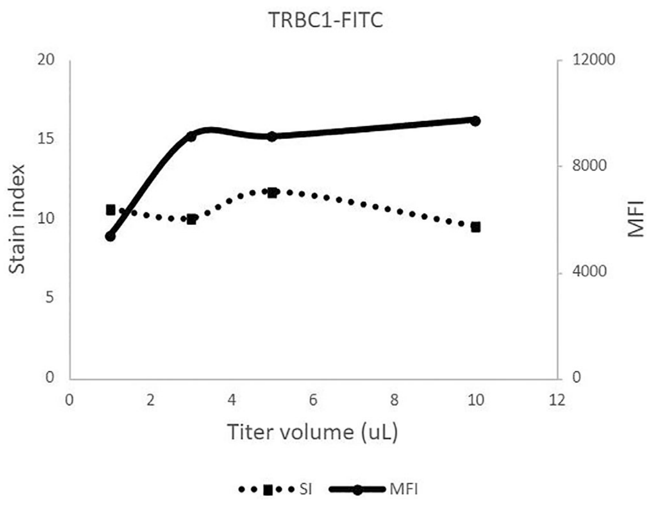

FIGURE 4 Antibody titration using TRBC1-FITC. Stain index (SI) is plotted against titer volume. As antibody concentration increases, the staining index increases as the positive signal increases. Too much antibody causes non-specific background staining, decreasing the stain index. The optimal antibody concentration results in the peak stain index, representing good separation between the positive and negative populations (5 uL in this example).

4.1.1 | Use of anti-TCRγδ antibody

A gate is placed around the TCRγδ-negative CD3+ T-cells, followed by various combinations of other relevant antibodies as discussed in the panel design section (Figure 2).

4.1.2 | Use of anti-TCRαβ antibody

The use of αβ can be used to similarly isolate the population of interest, in this case via inclusionary gating (Figure 3). A gate is placed around the αβ CD3+ T-cells, and from there, the downstream steps remain the same.

4.2 | Identifying clonal populations

4.2.1 | Internal controls and TRBC1 threshold establishment

Most samples will have internal control populations which can be used to demonstrate normal polytypic distribution or negative staining for TRBC1. Figures 2 and 3 show various T-cell subsets demonstrating expected polytypic staining as well as expected negative staining in non-T-cell lymphocytes and γδ+ T-cells (also, Cases #1–3). While determination of the TRBC1 negative/positive threshold using internal controls is recommended, one must note that there may be TRBC1(dim) populations may be present which may obscure the two populations. These TRBC1(dim) populations may be a malignant population (Case #8) or normal/reactive T-cells (possibly TRBC2-positive T-cells; Case #7).

4.2.2 | Identification of abnormal T-cells

Template design should include multiple combinations of the T-cell antigens and TRBC1 to better identify possible phenotypic abnormalities in potential subsets, which can increase the detection capability (Case #10). Generally, the candidate abnormal population is identified using a combination of various T-cell antigens ("difference from normal" method) and TRBC1 is used to verify the clonality using the threshold method described above, but plots showing TRBC1 versus an antigen of interest (e.g., CD26 in Sezary cell detection) may also be useful to quickly identify a clonal population (see Case Studies).

5 | INTERPRETATION OF RESULTS AND REPORTING CONSIDERATIONS

The interpretation of TRBC1 as clonal, or indicative of a clonal process, may rely on individualized thresholds established by the laboratory but use of the 15%/85% threshold can provide a quick screening method for detecting a prominent clonal TRBC1-restricted population (Horna, Shi, Olteanu, & Johansson, 2021). In addition to the common patterns of being either positive or negative for TRBC1, there are cases where T-cells show a dim expression of TRBC1. In these cases, if the dim expression is consistent across an abnormal group of cells, and if more than 50% of these cells fall between the normal ranges for TRBC1-negative and TRBC1-positive cells, this dim expression may also be considered clonal (Berg et al., 2020). TRBC1-dim populations that otherwise normal expression of CD3 and other T-cell antigens most likely show non-specific staining for TRBC1 on TRBC2-positive T cells and should be interpreted as TRBC1-negative. Evaluation with anti-TRBC2 in the future will likely resolve this conundrum (Horna, Shi, Olteanu, & Johansson, 2021) (Case #7).

Of note, because TRBC1 and CD3 are part of the TCR-αβ complex, TRBC1 assessment is not possible in abnormal non-γδ T-cells that lack surface CD3 (Case #3; Case #11). In such cases, cytoplasmic TRBC1 has been shown to be useful in detecting abnormal populations that express cytoplasmic CD3 only, or alternative gating strategies utilizing other pan T-cell antigens may need to be employed (Horna, Otteson, Shi, et al., 2021). Overall, reporting of TRBC1 expression should be similar to how kappa/lambda restriction is described for B-cell and plasma cell populations with a clear description of an abnormal population and characterization as TRBC1-negative, TRBC1-positive, TRBC1(dim), or TRBC1-indeterminate (see Case Studies). Reporting out percentages of T-cells that are positive or negative for TRBC1 is not recommended as it can be confusing to clinicians and patients.

5.1 | T-Cell clones of uncertain significance

While TRBC1 greatly aids in detection of abnormal T-cells, its use also has led to increased detection of small clonal T-cell clones of unknown significance (T-CUS) in patients without an underlying T-cell neoplasm. T-CUS incidence varies between 6% and 41%, depending on

Antibody Specificity Verification

Antibody:	TRBC1	Vendor:	Caprico Biotechnologies
Fluorochrome:	PE	Catalog #:	4133022
Panels:	T-CLPD	Clone:	JOVI.1
Use:	Identify T-cell clonality	Isotype:	IgG2a

Sample #	Expected Positive Expression	Actual Results	Pass/Fail	Expected Negative Expression	Actual Results	Pass/Fail
FC-23-107	Subset reactive T-cells	Positive	Pass	Subset reactive T-cells; B-cells	Negative	Pass
FC-23-115	Subset reactive T-cells	Positive	Pass	Subset reactive T-cells; B-cells	Negative	Pass
FC-23-151	TRBC1+ Neoplastic T-cells	Positive	Pass	B-cells, NK-cells	Negative	Pass
FC-23-187	X	X	Pass	CD3-negative TCLPD	Negative	Pass

Comments:	TRBC1-PE marks as expected

Evaluation Date:	3/23/2023	Tech:	XX
Medical Director:	XX		

Titration**

Manufacturer Volume/Test (uL):	5	Date:	2/6/2023
Number of Tests:	50	Tech:	XX
Amount provided (ug):	0.2	Titered Volume:	XX
Total Volume (mL):	250		
Concentration (ug/mL):	50		

**Refer to titration experiment

FIGURE 5 An example of an Antibody Specificity Sheet for TRBC1 antibody.

TABLE 2 Reference ranges for TRBC1 positivity in CD4+ and CD8+ T-cell subsets in published studies and verified at Stanford University.

	CD4+ T-cells	CD8+ T-cells
Shi et al., 2019	22%–82%	17%–57%
Muñoz-García et al., 2021	24%–62%	8.3%–61%
Novikov et al., 2019	36%–53%	18%–61%
Stanford ($n = 20$)	33%–52%	26%–52%

the population studied and methodology used to identify T-cell clonality (Delfau-Larue et al., 2000; Horna, Shi, Jevremovic, et al., 2021). The incidence of T-CUS rises with age and given the normal function of T-cells, T-CUS is favored to represent reactive T immunoclones as they commonly exhibit features of activation and terminal differentiation including downregulation of CD5, CD2, CD3, and CD7 or upregulation of CD57 and CD56 (Case #12; Case #13).

Given that several studies have demonstrated that clones constituting less than 20% of total lymphocytes or 400 cells/μL of blood are highly prevalent in patients without T-cell malignancy and show no particular disease association, it is unclear if reporting these populations is necessary or even harmful (Chin-Yee et al., 2022; Kroft & Harrington, 2022; Shi et al., 2020). The risk of overdiagnosis of a T-cell malignancy is particularly higher in older patients presenting with unexplained cytopenia or post-transplant patients who present with oligoclonal reactive cytotoxic T-cell proliferations (Chin-Yee et al., 2022; Kroft & Harrington, 2022). Most reactive T-CUS cases are CD8+ or CD4+/CD8+ double-positive, hence the decision to report these populations must be balanced with the risk of triggering unnecessary laboratory workup and misdiagnosis (Shi et al., 2020).

CD4+ T-CUS cases also exist but are rare; hence if found, they should raise strong suspicion for clinically significant T-cell neoplasm, and careful evaluation for the possibility of a T-cell lymphoproliferative disorder must be performed irrespective of the clone size (Kroft & Harrington, 2022; Shi et al., 2020). Overall, clinical/laboratory correlation is essential when evaluating T-CUS and guidance regarding these populations will likely evolve as more laboratories gain experience in encountering them in routine practice.

6 | SUMMARY

Assessing samples for T-cell neoplasms by flow cytometry has long been problematic. The enviable surface light chains on B-cells rocketed flow cytometry into the spotlight as an essential component in the workup of hematologic samples. Assessment of clonal T-cells has lagged behind, relying on varying levels of marker expression in a "different from normal" capacity, often providing non-specific or equivocal interpretations. The advent of TRBC1 finally brings clonal T-cell assessment by flow cytometry into the spotlight. While there remain pitfalls and shortcomings, the existence of such a marker for assessing T-cell clonality is extremely promising. With TRBC2 on the horizon, the prospect of soon having complementary surface markers on T-cells is realistic. We present this comprehensive summary of TRBC1 and supplemental case examples to familiarize the flow cytometry community with its potential for routine application, provide examples of how to incorporate it into T-cell panels, and to signal caution in interpreting the results in certain diagnostic scenarios where appropriate. Initial work was published as a Quality & Standards Module for the International Clinical Cytometry Society (Illingworth et al., 2023), and has been substantially modified in content and form for this manuscript.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflicts of interest.

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SUPPORTING INFORMATION

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