INTRODUCTION

The immaturity-associated antigen terminal deoxynucleotidyl transferase (TdT) is a nuclear DNA polymerase that is present primarily in immature lymphoid cells of the B and T cell lineages. In healthy precursor B cells, TdT expression levels are highest during IGH gene rearrangements in early progenitor B cells (hematogones) and gradually decrease with increasing B cell maturation (1). Similarly, immature thymic T cells express TdT, which is lost during T cell maturation (2). In clinical flow cytometry, presence of TdT expression is commonly utilized in the diagnosis of acute lymphoblastic leukemias (ALL) as strong TdT nuclear staining allows differentiating ALL from other mimicking entities such as Burkitt lymphoma (see WHO Classification) when used in combination with lineage- and maturation-specific antibodies (3, 4). In addition, the absence or presence of TdT may not always be helpful to distinguish lymphoid from myeloid neoplasms, particularly in acute myeloid leukemia (AML) cases with t(8;21)(q22;q22) [RUNX1-RUNX1T1] common recurrent genetic abnormality (5). In general, AML cases tend to have weaker TdT expression levels than ALL cases (6). TdT positivity has also been reported in high-grade B-cell lymphoma, with MYC and BCL2 and/or BCL6 rearrangements, leading to a dilemma in diagnosis and classification in some cases (7). Finally, steroid-induced loss of immaturity-associated antigens (CD10, CD34, and TdT) during induction therapy has been reported for B-ALL (8) and T-ALL (2), potentially impacting the utility of TdT staining in minimal residual disease (MRD) detection (1-4, 8). It is important to note that in the WHO 2016 classification of hematopoietic and lymphoid tumors, TdT is not used to assign lineage to a neoplastic population, due to the lack of specificity for lymphoid cells (9).

In contrast to antigens anchored on the cell membrane, TdT is a molecule that is only present in the cell nucleus; therefore, staining of TdT must be performed intracellularly after permeabilizing both the cell membrane and nuclear membrane. Due to known inconsistencies in nuclear/cytoplasmic staining techniques, achieving reliable nuclear TdT staining patterns can be challenging and impacting the correct interpretation of flow results. Common technical issues include achieving only poor distinction of a TdT-positive population from high non-specific background staining, insufficient permeabilization of the cell membrane and/or nuclear membrane, and the lack of adequate positive or negative controls based on
internal and external control material. Therefore, it is vital for clinical flow cytometry laboratories to develop a standardized and tightly controlled procedure that ensures optimal nuclear TdT staining.

GOAL FOR THIS MODULE
The goal for this ICCS-initiated TdT survey was to gain a better understanding of the technical and interpretational challenges of flow cytometric testing for TdT. The survey was based on 18 questions developed by the Quality & Standards Committee, which were answered by 81 clinical laboratories in the US, Canada, as well as Europe. Interpretation of the technical data was somewhat challenging due to the inherent variability between laboratories regarding different instrument platforms, different staining protocols and selection of controls as well as interpretation of results. The following survey analysis represents more of a current state of TdT staining across the various clinical flow cytometry laboratories with a summary for each question and some suggestions and guidance for optimization if indicated. As mentioned before, although TdT is not used as a definite marker for lineage assignment, its clear association with immaturity and thus maturation-arrested leukemias may still add useful diagnostic information in certain cases. Flow cytometry laboratories should optimize and standardize TdT staining based on best practices by using optimized instrument settings with a validated antibody, meeting defined acceptability criteria based on both internal and external controls, and excluding technical artifacts in order to produce accurate and reproducible results. The questions asked in this survey aimed to highlight the wide range of practices related to flow cytometric TdT testing. The answers given in response to the TdT survey are practical examples that illustrate common pitfalls and potential strategies to achieve optimal results.
Results and Comments:

The majority of the participating laboratories appear to perform more than 100 acute leukemia cases per year. This may indicate higher level of testing complexity among the majority of participating laboratories.
Results and Comments:

These results indicate that the flow expertise of the participating clinical laboratories extends to both pediatric and adult acute leukemia and is not biased towards only one patient cohort.
Results and Comments:

Most laboratories (70%) are routinely setting up TdT staining for every patient with more than 15-20% blasts, suggesting that TdT is a routine component of their diagnostic acute leukemia panels. Staining immaturity markers (e.g. CD10, CD34, TdT) helps identifying aberrant expression patterns. However, it remains unclear whether TdT results are necessary to diagnose acute leukemia upfront, or if this approach is workflow-related (e.g. utilizing the initially optimal viability in case TdT staining results are subsequently needed). Based on the answers provided, TdT staining is not routinely included in MRD diagnostics (14%). Overall, these results highlight how TdT testing is currently utilized in multiple disease settings.
Results and Comments:

Out of 81 participating laboratories, only 8 indicated that they do not perform intracellular TdT staining. Only one laboratory mentioned technical problems with TdT staining, while the other 7 laboratories cited other non-technical reasons for not including TdT staining in their acute leukemia immunophenotyping panels, which may include laboratories not performing routine TdT testing as it is not required for classification of acute leukemias.
Results and Comments:

The vast majority of laboratories (84%) uses the identical antibody clone (HT-6) for their intracellular TdT staining, purchased from major vendors with fairly even distribution (36% Beckman Coulter, 26% Dako, 22% SuperTech). Another 15% of laboratories use the clone E17-1519 from BD Biosciences. Both clones are monoclonal antibodies. These results suggest a high level of consistency in the used TdT antibodies, decreasing the likelihood of technical problems stemming from a heterogeneity of used clones. However, the TdT-6 clone seems to be exclusively used conjugated with a dim fluorochrome, FITC, while the E17-1519 clone from BD is also used with the brighter APC or PE conjugates. This difference may potentially impact brightness as well as the separation between TdT+ and TdT negative cells (signal/noise ratio).

Considering that TdT staining is often difficult to interpret (negative versus dim+), it is surprising that only a few laboratories chose a brighter fluorochrome. Maybe their existing panel design requires TdT to be in that particular channel (FITC), or maybe their vendors do not offer brighter fluorochrome options with a better signal/noise ratio.
Results and Comments:

Most labs (85%) did not report any issues with the antibody; however, some labs (15%) experienced dimmer staining intensities than expected, which impacted separation of dimly stained blasts from background staining. It was also reported that some TdT antibodies worked best when used with the vendor-specific intracellular staining protocols/product/reagents. Very dim staining intensities were more commonly observed with FITC-conjugated antibodies, but at times also observed with brighter fluorochromes. As TdT expression can indeed be dim in some patients, it is important to compare the TdT expression intensity of the leukemic population to truly positive and negative controls. It should be noted that another possible technical reason for achieving only dim or suboptimal staining may include suboptimal voltage settings with the antigen-negative populations “crushed against the axis”, making it challenging to discriminate the cells as a distinct population. This suboptimal voltage was seen on some of the plots submitted by the participants and confirms that a suboptimal voltage setting may not allow for dimly staining cell populations to be identified as a TdT-positive populations (see Q&S Module for Optimizing Voltage Settings: [https://www.cytometry.org/web/modules/module2.pdf](https://www.cytometry.org/web/modules/module2.pdf))

Selected comments from the survey responses

- problem with BD APC E17-1519 clone was dimmer than expected
- Supertech TdT-6 sometimes dim staining, difficult to determine neg vs dim+
- Supertech TdT-6 tends to be dimmer and sometimes it is hard to separate from the negatives
- BC clone with FITC - problems but no details
- Biorad clones (mixture): false positives with monocytic populations
- BD E17-151 with FITC: The antibody works only when used with BD intrasure kit
- Supertech TdT-6 FITC it is always variable, partially positive in 60% of cases
- BC clone with FITC: very dim results on positive population, high background from mature cells, overall poor resolution
- BC clone with FITC: dim expression
- BC clone poor sensitivity
- BD E17-1519 APC dim reaction
"Other" include:
1. Nordic Fix and perm
2. "BD FACS Lyse and then permeabilize with reagent B; reagent A didn't seem as good as BD FACS Lyse".

**Results and Comments:**

Based on the reported variation in fix/perm kits used in this TdT survey, it appears that most labs do not match the intracellular protocol with an antibody provided by the same vendor. This is not necessarily a requirement, but it should be noted that each lab needs to validate if their antibody is compatible with the intracellular fix/perm procedure. Appropriate external and internal controls need to be used in order to verify accuracy of the results.

Choosing the appropriate fixation and permeabilization reagents depends on the target and its subcellular location. While staining of cytoplasmic antigens can be achieved with mild permeabilization buffers, optimal staining of nuclear antigens (as is the case with TdT) requires stronger permeabilization buffers capable of dissolving nuclear membranes. Also, conventional tandem dyes are not recommended for intracellular staining due to their large size that impedes membrane transport.
Results and Comments for Q8 and Q9:
Two thirds of the participating laboratories did not perform an initial bulk-lyse step, regardless of whether they stained peripheral blood or bone marrow.
Results and Comments:

It is concerning that 45% of the labs do not use an external control in a disease setting that can be characterized by negative or dim-staining intensities, particularly in the context of relevant background staining due to other TdT-expressing cell populations.

These potential pitfalls highlight that it is critical to confirm that the antibody is performing as expected by verifying that the assay accurately identifies true TdT+ cells, which need to be correctly and reproducibly distinguished from true TdT-negative cell populations and non-specific background staining. External controls also can verify that all the other components of the assay (including instrument set-up, antibody performance and specimen preparation) are optimally performing before a patient sample is run.
Results and comments:
The majority of respondents (58%) are using lymphocytes as internal negative control, while 17% are using granulocytes, which may not be the appropriate cell population due to higher background staining. The remaining 26% are using other internal controls, such as isotype, other events, all other CD45+ population besides the population in question, hematogones/immature B cells, or unstained cells.

Internal controls should be used whenever possible and can often be utilized from assessing staining intensities in other healthy cell populations present in the same sample. The selection of internally negative control populations may depend on the type of antigen expressed. As an example, a good internal TdT-negative control that is already present in a bone marrow sample, are typically mature lymphocytes, while monocytes and granulocytes are less appropriate, since they exhibit more non-specific staining/higher background. Some participants showed good examples of how this approach can be very useful to verify optimal assay performance (e.g. hematogones as internal TdT+ control versus TdT-negative mature lymphocytes).
Results and Comments:

Determining acceptability criteria for accurate and reproducible TdT assay performance varied between the different labs. The variability in responses highlights the challenges with systematic assessment due to lack of guidance, standardized procedures or established assessment criteria. This was illustrated by very inconsistent answers, and in particular the variable use of acceptability criteria for the TdT assay in clinical cases. This suggests that there is no consensus on how to optimize the instrument, how to process the specimen, which controls to use and how to interpret the results for ensuring optimal intranuclear TdT staining.

These issues may be addressed by developing a solid quality control (QC) program. This typically includes an initial validation process that addresses/includes assay-specific instrument optimization, antibody titration, assay-specific specimen preparation, selection of appropriate external and internal controls and a targeted analysis template for optimal data interpretation. Based on these achieved results, the lab needs to determine the standards and acceptability criteria and monitor the assay performance to ensure accurate and reproducible results over time.

Some suggested acceptability criteria include:

- Lot-to-lot comparisons (in use versus new antibody) should produce similar staining patterns
- Evaluating TdT staining on internal controls, such as hematogones (TdT+ control) against other WBC subsets such as mature lymphocytes (TdT-negative control).
Selected survey responses for assessments of antibody acceptability included:

- Use Streck CD CHex TdT plus control for TdT lot to lot comparison
- Testing biannually on TdT+ patient as confirmed by IHC
- Verify positive staining on hematogones if present
- Run on EQA sample
- Use already validated panel an protocols (Euroflow)
- Lot to lot comparison (use MFI as acceptance criteria)
- Isotype control (IgGx)
- Normal blood
- Compare positive and normal healthy samples
- Negative on CD19+ lymphocytes
- Use internal control as part of every patient run
- TdT is part of a cocktail of antibodies (checked with normal patient and WBC are checked for expected expression)
- Protocols recommended by NOPHO
- Weekly QC on Streck QC material
- As part of internal QC
- Perform shipment lot QC before using on patients
- Intracellular testing is performed with isotypes
- Compare new lot against in use and verify staining intensity and % population of interest
- Verify positive staining based on pos sample for new lot
- Blasts cells with isotypic control
- Check all CD45+ populations besides the population in question
- Run new TdT comparing patient control and previous TdT lot
- Weekly TdT control
- Samples from UK NEQAS
- Test new lot with pos patient
- Stain 10 normal bone marrow samples with identifiable lymphoblast population and verified by its expression of CD45, CD34 and B-cell markers
Results and Comments:

Most laboratories (over 60%) do not change their FS/SS settings when running TdT-stained cells, although there is a definite decrease in the FS/SS signal after the fixation and permeabilization procedure due to significant shrinkage of the cell membrane. This might either indicate that the general instrument set-up is already optimized for panels that include fix/perm steps, because some laboratories stated that they routinely fix cells for all panels. Or it may indicate a potential pitfall, as some of the cells of interest may fall below certain gates or below the discriminator when they are not sufficiently represented on scale. The separation of cell populations may also not be as optimal when using suboptimal FS/SS settings (see examples below).

Example of optimal use of the FS/SS plot (left) versus suboptimal FS/SS settings (right)
Results and Comments for Q14 and Q16:

71 out of 81 laboratories reported that they experience some type of potential pitfall or issue with TdT staining; only 12% did not answer this question. The vast majority of these laboratories (>70%) self-reported challenges in interpreting dimly positive TdT results, 24% identified problems with either external (%) or internal (%) controls, followed by antibody validation, the antibody itself or specimen processing. One third of these labs gave more specific explanations, mostly focusing on various technical reasons for insufficient separation of TdT positive and negative cells. These included: shift in FS/SSC properties, problem of non-specific (or falsely positive) background staining in granulocytes, monocytes or AML subtypes, too small panels that do not allow for identification of other CD45+ and other lineage-specific cells, not bright enough fluorochrome options, lack of commercially available controls comprised of TdT-negative and TdT+ cells, too high costs for purchasing external controls, or insufficient permeabilization with certain permeabilization buffers leading to weak signal strength.
Selected responses from the survey participants:

- FS vs SS shifts so that all populations are close together, making the separation harder.
- It would be helpful to have commercially available controls with internal negative and positive control cells.
- Granulocytes picking up TdT stain.
- Non specific staining in Acute Monocytic Leukemia.
- Uses BD FACS LYSE AND THEN PERMEABILIZE WITH RGT B; RGT "A" DIDN'T SEEM AS GOOD AS BD FACSLYSE.
- Separation between positive and negative could be better using intrasure kit.
- For samples suspected having AML/MDS we do not fix and permeabilize for TdT. We just add TdT after the membrane staining and after washing. We keep in mind that neutrophils stain for TdT.
- We have been getting false positive results for TDT on monocytic populations, including disease populations. We have been running anti-TdT by Dako which appears to perform better.
- Our method is dated (dual colour acute panel) and we look to changing the protocol from dual to multi in the near future. In terms of last qu (correlation of flow with IHC), to confirm, I have selected no as the lab does not correlate but there are MDT meetings with medical staff who discuss results from all areas there.
- We follow the EuroFlow SOPs for cellular staining.
- The signals are not bright enough we prefer to add a little bit more ab (e.g. 7 microliter instead of 5 microliter) we always add CD19 and CD2 on the Tdt tubes to be able to use the other population as a negative control.
- We have tried with BD Tdt, BC Tdt (pool) and supertech Tdt. Supertech Tdt is better than other two but doesn't give 100% positivity.
- CD45 recently included, aids analysis as CD45 weak/neg blast population can be gated, with plots looked at through that gate.
- Supertechs is the only Ab that we had consistently good results with.
- Would like brighter positive fluorescence.
- Very dim results on Positive population. High background from mature cells. Overall poor resolution.
- Dim expression that sometimes is indistinguishable from internal control.
- As mentioned in the comments about processing we have validated in our laboratory the step when we add the perm (solution B) and the cocktail at the same time that there is no difference compared to surface staining, fix, perm, cytoplasmic stains. It saves us about 30 minutes in processing time.
- ?Budget to purchase external control.
- We tested a BD anti-tdt antibody with significantly poorer separation. We discarded that. It can be challenging to interpret a dim expression when the blast population is >90% of the leukocytes. This is particularly a problem when the tdt-expression is aberrant on myeloblasts.
- Positive control material not readily available.
- No real issues. I've tried other commercial controls that have not worked well, but the CRISP cells have always been fine.
Results and Comments:

As TdT staining intensities can be absent in some cases, this question sought to clarify whether responding laboratories correlated their flow-based TdT expression analysis with another method, such as immunohistochemistry (IHC). Majority of the respondents (66%) indicated that they do not correlate their flow-based TdT result with an IHC result, with only a third of the laboratories regularly confirming their results.

Q17 (Please upload typical TdT control plot(s) and Q18 (Please upload typical plot(s) from a TdT+ patient)

Out of the total 81 survey responses submitted, 46 responses (57%) were received without plots while 35 responses included de-identified original patient data analysis plots from their laboratories. Review of all plots submitted for this survey was somewhat a challenge as they were comprised of different platforms, different specimen preparations and based on a variety of different and often unknown patient specimens. This did not allow for a fair comparison regarding the quality of the preparation & analysis. However, several plots indicated the following issues, which may affect the interpretation of TdT testing:

1. Settings for FS and SS were often not optimized. It is recommended to use the entire plot space for optimal separation of populations.
2. PMT voltage and compensation issues, potentially producing false-positives or false negative results for TdT.
3. Using 1 parameter vs 2 parameter plots. Typically 2 parameter plots are more informative and should be used whenever possible.

4. Internal populations not identifiable (not enough cells acquired, not color-coded etc.)

5. Incorrect cursor setting and overall suboptimal determination between negatives (based on internal control, lymphs) and true positives.

6. Specimen preparation issues (too much aggregation, cells look poorly clustered, etc).

Below are some selected examples of informative plots:

**A - Q17 example for Internal TdT Controls:**
This example demonstrates a sequence of various plots which include several gating plots (TIME, viability, singlet gating, CD45 gating) and more focused plots focusing on internal control plots (CD34dim+, low SS/TdT+ hematogones and CD45++/TdT-negative/CD3+ T-cells).
B - Q18 example for TdT+ patient:
This example demonstrates a series of plots similar to the previous control sample (TIME, viability, singlet gating, CD45 gating) and shows a CD45dim+/TdT+/CD19 B-cell population while the internal controls cells (CD45++/CD19+ mature lymphs) show no TdT staining.
C - Q17/18 examples of TdT interpretation using internal controls:

ALL with CD34+/CD10+ lymphoblasts: TDT is considered positive on the majority of the CD10+ lymphoblasts (green), using the lymphocytes (red) as internal negative control, MPO clearly negative.

AML with CD34+/CD117+ myeloblasts showing dim TdT staining using lymphocytes (red), monocytes (blue) and granulocytes (purple) as internal negative controls. MPO was also dim positive.

AML with CD34+/CD117+ myeloblasts showing no TdT staining. Note the slightly increased TdT staining on granulocytes (purple) versus lymphocytes (red), which suggests using the granulocytes as internal negative control rather than lymphocytes when assessing TdT staining on myeloblasts.
FINAL DISCUSSION AND KEY POINTS

This survey brought to light the many different approaches of flow cytometric TdT testing, achieving results that often appeared difficult to interpret based on the plots submitted. Although it is somewhat difficult to evaluate all aspects of technical and interpretational problems of TdT testing based on this limited survey, we have summarized the key points that address the critical aspects of an optimized TdT protocol:

1. The instrument needs to be optimized for cytoplasmic staining (FS and SS needs to be adjusted to bring the populations on scale after fixation).
2. The TdT antibody appears to be working sufficiently well for most labs but needs to be validated in conjunction with the Fix & Perm procedure used with the TdT antibody.
3. The validation protocol should include the definition of acceptability criteria based on selected control material (internal and external controls).
4. Regular QC and lot-to-lot comparisons should be monitored based on previously defined acceptability criteria.
5. Standardized analysis template may help with consistent interpretation of the data.

REFERENCES:


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