MAYO CLINIC

International Guidelines for the Diagnosis and Follow up of Sezary Syndrome/Mycosis Fungoides in Peripheral Blood

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Sezary Syndrome / mycosis fungoides group

- Cytometrists
  - Fiona Craig
  - Julia Almeida
  - Pedro Horna
  - Andrea Illingworth
  - Ulrika Johansson
  - Katherina Psarra
  - Michelle Pulitzer
  - Rick Torres
  - Susan Richardson
  - Sa Wang
  - Kristy Wolniak
  - Others
- Clinicians
  - Michael Girardi
  - Joan Guitart
  - Aaron Mangold
  - Julia Skarisbrick
  - Others

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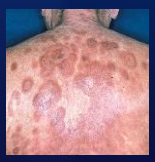
Sezary syndrome and Mycosis fungoides

Mycosis fungoides

- Patches and plaques
- Can eventually progress to involve lymph nodes and peripheral blood.



Sezary syndrome

- Erythroderma and blood involvement at presentation.
- Demonstration of peripheral blood involvement is essential for diagnosis.

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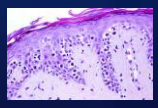
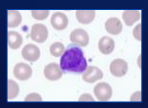
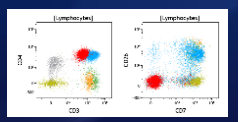
Sezary syndrome and Mycosis fungoides

- Morphologically indistinguishable skin, blood and lymph node involvement.
- SS and most MFs are phenotypically identical.
- MF and SS share the same staging system.
- Both entities are commonly accepted in the same clinical trials.







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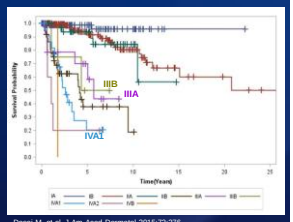
Role of blood tumor burden in the prognosis of MF/SS
2018 EORTC staging

STAGE I	**IA**: <10% BSA
	IB: >10% BSA
STAGE II	**IIA**: Lymphadenopathy (non-effaced)
	IIB: Skin tumors
STAGE III Erythroderma	**IIIA**: Sezary cell count < 250/µL
	IIIB: Sezary cell count ≥ 250/µL
STAGE IV	**IVA1**: Sezary cell count ≥ 1000/µL
	IVA2: lymphadenopathy (effaced)
	IVB: Visceral involvement

Olsen E, et al. Blood 2007;110(6):1713.
Scarisbrick JJ, et al. Eur J Cancer 2018; 93:47



Desai M, et al. J Am Acad Dermatol 2015;72:276

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Role of absolute Sezary cell counts in the follow up of patients with MF/SS
2018 EORTC recommendations

- Complete response (CR)
  - B2 (Stage IV) → B0
- Partial response (PR)
  - B2 (Stage IV) → ↓ ≥50%
- Progressive disease (PD)
  - B2 (Stage IV) → ↑ ≥50%
  - B0/B1 → B2 (Stage IV) AND ↑ ≥50%
- Relapse
  - CR → ↑ ≥1000 cells/µL
- Progressive disease (PD)
  - PR → ↑ ≥1000 cells/µL AND ↑ ≥50%

Adapted from: Scarisbrick JJ et al. Eur J Cancer 2018;93:47

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Flow cytometric monitoring of Sezary cells to assess response to therapy

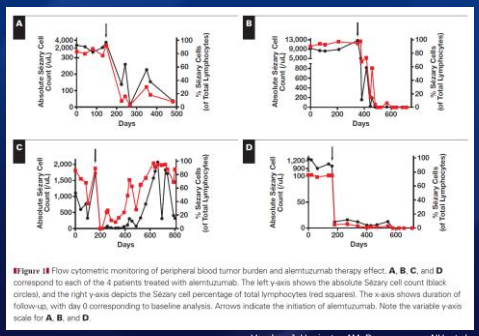

Figure 1 Flow cytometric monitoring of peripheral blood tumor burden and alemtuzumab therapy effect. **A**, **B**, **C**, and **D** correspond to each of the 4 patients treated with alemtuzumab. The left y-axis shows the absolute Sézary cell count (black circles), and the right y-axis depicts the Sézary cell percentage of total lymphocytes (red squares). The x-axis shows duration of follow-up, with day 0 corresponding to baseline analysis. Arrows indicate the initiation of alemtuzumab. Note the variable y-axis scale for **A**, **B**, and **D**.

Vaughan J, Harrington AM, Parameswaran NH, et al. American Journal of Clinical Pathology 2012; 137:403.

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Clinical Perspective on Sezary cell testing by flow cytometry: Frustration amongst dermatologists and oncologists

- Hard to tell if an abnormal T-cell population was actually detected.
- Absolute counts are often not reported.
- The phenotype of the abnormal population is often not completely documented.
- The report includes an array of numbers and percentages which are hard to interpret.
- Lack of uniformity and consistency.
- Some clinicians are interested in accessing the flow cytometry histograms to render their own interpretation.

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Flow cytometric quantitation of Sezary cells

- **EORTC 2018 recommendation:**
  - "We propose that this [blood involvement by MF/SS] is determined using absolute flow counts of CD4+CD7- or CD4+CD26-."

  Scarisbrick JJ, et al. Eur J Cancer 2018; 93:47

- **Current flow cytometry practice:**
  - Comprehensive analysis of T-cell antigen expression (not only CD7 and CD26).
  - Different than normal approach.
  - Gating based on clusters/populations with homogenous phenotypic properties.

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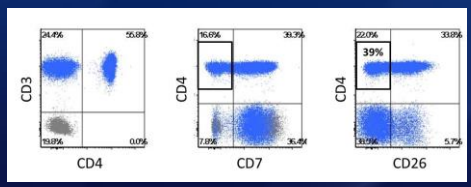
Case example: Reactive CD4 T-cells

- CD4 T-cells very commonly show reactive subsets with loss of CD7 and CD26.



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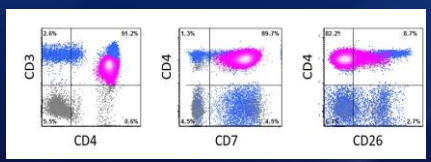
Case example: Sezary syndrome with "atypical" phenotype

- Cases of Sezary syndrome or mycosis fungoides with preserved expression of CD7 and/or CD26 are not rare.



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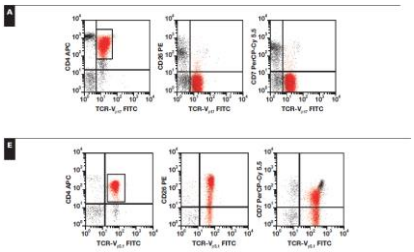
Classical and non-classical immunophenotypes of Sezary cells



Novelli M, et al. Am J Clin Pathol 2015;143:57

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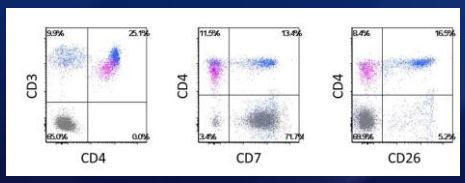
Case example: Low level involvement by Sezary cells.

- In the setting of low level involvement, CD7-/CD26- Sezary cells largely overlap benign/reactive CD4 T-cell subsets.



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Exclusive assessment of CD7(-) and/or CD26(-) on CD4+ T-cells is a suboptimal approach to detect and quantify Sezary cells

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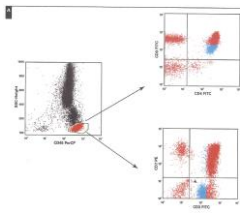
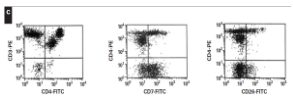
Early manuscripts documenting loss of CD7 and CD26 on Sezary cells.



Edelman J, et al. Diminished CD3 Expression Is Useful for Detecting and Enumerating Sézary Cells. American Journal of Clinical Pathology. 2000;114:467-477.



Jones D, et al. Absence of CD26 expression is a useful marker for diagnosis of T-cell lymphoma in peripheral blood. American Journal of Clinical Pathology. 2001;115:885-892.

- Other immunophenotypic abnormalities besides CD7 and CD26 are fairly common.

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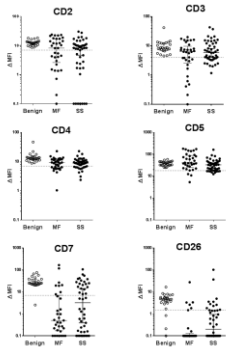

Phenotypic abnormalities of Sezary cells

- 79 blood specimens from 52 patients with MF/SS.
- 27 patients with no hematologic malignancy.
- Doted lines: Approximate threshold where abnormality is visually evident.

Data from: Horna P, et al. Cytometry B Clin Cytom. 2019 May;96(3):234.

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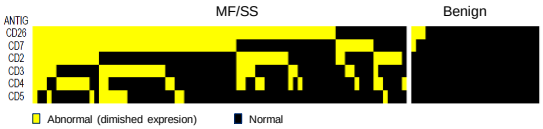
Phenotypic abnormalities of Sezary cells



Antigen	% of cases with antigen loss
CD26	81%
CD7	65%
CD2	41%
CD3	35%
CD4	30%
CD5	15%

Data from: Horna P, et al. Cytometry B Clin Cytom. 2019 May;96(3):234.

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Antibody panel design

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Antibody panel design
Basic principles

- Single tube panel.
  - No single antigen can by itself accurately identify Sezary cells.
  - Diagnostic utility of each antibody is highly dependent its combination with other useful antibodies in a single analysis tube.
  - 6-8 color flow cytometry is now broadly available and accesible.
- Selection based on the reported utility of different antigens to discriminate Sezary cells from reactive/benign CD4+ T-cells.
  - Review of the literature and experience from contributors.
- Favor basic T-cell antigens.
  - Assay that can be adopted widely (not just in specialty labs).

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Recommended minimal 6-color Sezary panel

Marker	Normal expression	Sézary cells
CD3	**Bright positivity** on T-cells. Negative on other cells.	Positive. **Slight dim expression in 40-80% of cases.** Rare partial or complete negativity.
CD4	**Bright positivity** on subset of T-cells. Dim positive on monocytes.	Positive. **Slight dim expression in 30-50% of cases.** Rare partial or complete negativity.
CD7	Positive on CD4 T-cells, with **variable loss in reactive subsets.** Bright positive on essentially all NK cells.	**Partially or completely negative in 50-80% of cases.**
CD8	Bright positivity on subset of T-cells. Some CD4/CD8 double positive T-cells and CD4/CD8 double negative T-cells might be dim for CD8.	Almost always Negative.
CD26	Variably positive on CD4 T-cells, with **variable loss in reactive subsets.**	**Partially or completely negative in 80-100% of cases.**
CD45	Bright positive on all lymphocytes.	Bright positive, **rare dim expression.**

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Additional markers of potential diagnostic utility

Marker	Normal expression	Sezary cells
CD2	**Bright positivity** on T-cells. Positivity on a subset of NK cells. Negative on other lymphoid cells.	**Slight dim expression in 40-70% of cases.** Rare partial or complete negativity.
CD5	**Bright positivity on CD4 T-cells**; variable expression on CD8 T-cells. Negative on other lymphoid cells,	**Slightly dim expression inconsistently reported in 10-30% of cases.** Rare partial or complete negativity.
CD38	Variably and/or partial positivity on CD4-positive T-cells	**Negative in most cases.**
CD158k (KIR3DL2)	Largely negative on CD4-positive T-cells.	**Positive on 20% to 80% of cases**, might depend on the antibody utilized.
CD164	Largely negative to dim positive on CD4-positive T-cells.	**Variable overexpression** in most cases.
CCR4	Positive on a minor subset of CD4 T-cells.	Variable degree of **overexpression** in most cases.
CD279 (PD-1)	Negative or variably positive on a subset of CD4-positive T-cells.	Variable degree of **overexpression** in most cases.

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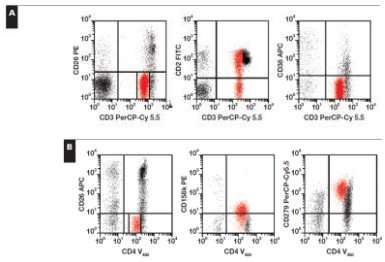
Additional markers of potential diagnostic utility



Novelli M, et al. Am J Clin Pathol 2015;143:57

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Additional markers of potential diagnostic utility

- Naïve-memory phenotypic markers:
  - CD62L, CD27, CD28, CD197 (CCR7), CD45RA, CD45RO.
  - Sezary cells have a highly heterogenous naïve-memory phenotype.
  - Homogeous expression of these antigens on any particular case might be the most useful feature for gating.
- Therapeutic targets:
  - CD30, CD52, CD279 (PD-1), CD194 (CCR4).
  - No standard approach to assess the expression of these antigens and their clinical significance.
- Antigens relevant for other T-cell lymphoproliferative disorders:
  - CD5, CD10, CD25, CD16, CD56, CD57, TCRαβ, TCRγδ.
- Antigens relevant to other lymphoid subsets:
  - CD19, kappa, lambda

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Antibody clone selection

- **No specific reagent or vendor recommended**.
  - Many commercially available reagents have been extensively tested and are likely to work well.
  - Supplementary information will be provided with list of reagents utilized by contributors to this consensus.
- **The CD26 reagent should be carefully selected and titrated**
  - Some reported issues with signal-to-noise ratio.

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Fluorochrome selection

- CD3, CD7 and CD26 should be matched with bright fluorochromes.
- CD4 and CD8 are typically brightly expressed and should fare well with moderately bright fluorochromes. However, prioritize CD4 over CD8.
- CD45 best coupled with dimmer and less discriminatory fluorochrome.

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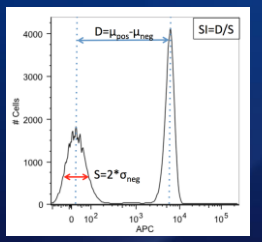
Assessment of Staining Adequacy

Goal

- CD3 and CD4 staining should discriminate slightly dim subsets from bright positive.
- CD7 and CD26 staining should discriminate dim or negative subsets from bright positive.



However…

- Current measures of staining adequacy do not adequately address the capacity to identify antigen loss.
- The dispersion of the log-normal fluoresence curve cannot be adequately described based on calculations provided by most analysis softwares.

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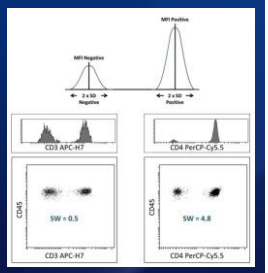
Assessment of Staining Adequacy
Modified stain window

Modified stain window

- Based on the mean and standard deviation of log-normal Gaussian curves.
- Estimates the width of the space between the curves, relative to the width of each curve, as visually appreciated on a log scale plot.
- Complicated formula due to log transformed data.



Preliminary plan for manuscript

- Provided and Excel sheet to calculate the SW based on conventional linear MFI and SD.
- Recommend mSW >1 for CD3, CD4 and CD7 in at least 8 of 10 normal peripheral blood specimens.
- CD26 needs to be visually evaluated (no good normal positive control).

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Specimen processing and event acquisition

Processing

- Broad variability in staining and lysing practices. No specific recommendations.
- Refer to ICCS quality standards: Module #1: Lysing Methods and Reagents for Flow cytometric immunophenotyping.

Acquisition

- Wide variability of number of events acquired and type of events counted (survey to be included in manuscript).
- Recommend minimum acquired events to approximate average of most reference institutions:
  - 20,000 lymphocytes
  - 100,000 leukocytes
  - 200,000 total events
- 500,000 might be required to detect low-level residual disease.
- Goal: Analytical sensitivity of 1% of white blood cells.

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Gating strategies

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Gating strategies
Basic principles

- Contemporary approach to gating on lymphoid subsets.
- Identification of Sezary cells based on comprehensive immunophenotypic analysis (not just CD7 and CD26).
- Gating on Sezary cells based on the identification of an immunophenotypically abnormal cluster (different than normal).
- No specific template or gating order. Too much variability between analysis softwares and practice preferences.

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Gating Strategy
The basics

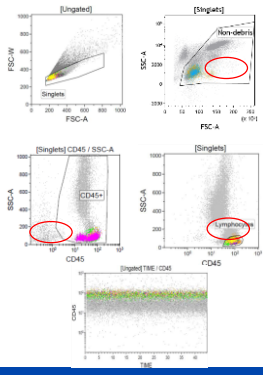
- Exclude doublets and debris
- Gate on leukocytes and lymphocytes
- Time-based plot

Pitfalls:

- Tumor cells with high light scatter.
- Tumor cells dim/negative for CD45
- Monocytes might be counted as abnormal lymphocytes or viceversa.

Safeguards:

- Plot and/or back gate all CD3-positive events.
- Confirm phenotype of monocytes.



Gating Strategy
Gate on T and NK cells

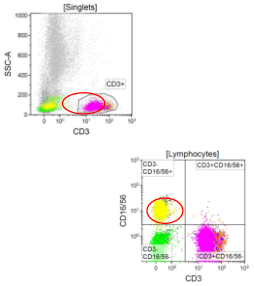
- T-cells based on inclusive CD3-positivity
- NK cells based on CD3(-)/CD7(+) or CD3(-)/CD15+CD16(+)

Pitfalls:

- CD3 dim/negative T-cells might be missed or gated as NK cells.

Safeguards:

- Plot and/or back gate all CD4-positive lymphocytes.
- Confirm phenotype of NK cells.



Gating Strategy
Gate on CD4+ T-cells (and CD8+ T-cells)

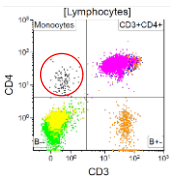
- CD4vsCD3 and CD8vsCD3, or
- CD3-positive T-cells on CD4vsCD8

Pitfalls:

- Monocytes may appear as CD4-dim.
- Rare CD4-/CD8- or CD8+ Sezary cells.

Safeguards:

- Confirm/back gate CD4 dim events as monocytes.
- Phenotype CD8 T-cells.
- Abnormal CD4-/CD8- or CD8+ subset may require additional work up.



Gating Strategy
Gate on Sezary cells

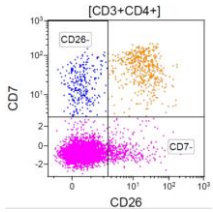
- Plot CD4 T-cells on CD7vsCD26.
- Examine clustered populations on other plots.
- Examine aberrant subsets detected on other plots, on CD7vsCD26

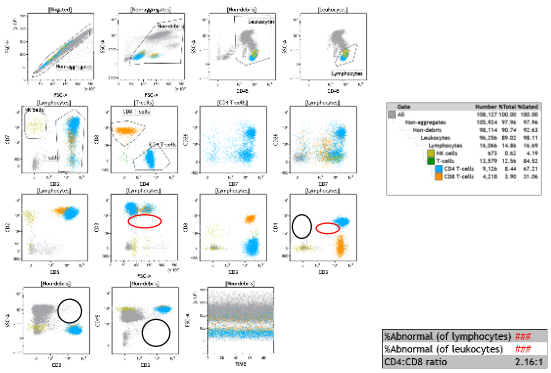
Pitfalls:

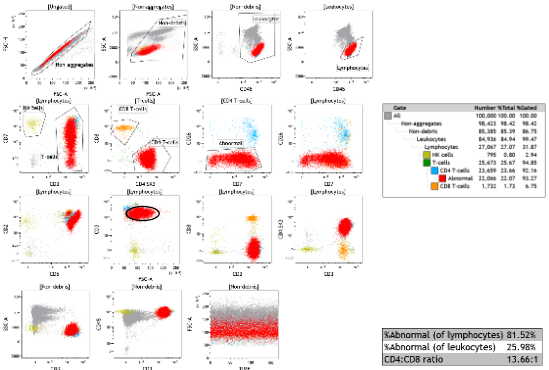
- CD7 and CD26 loss overlaps with reactive subsets.
- Phenotypically complex Sezary cells might be underestimated.

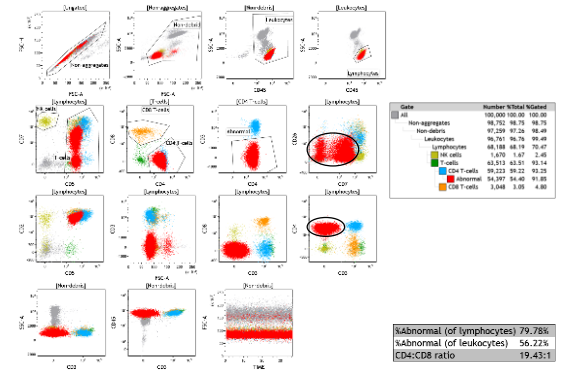
Safeguards:

- Always look for additional abnormalities besides loss of CD7 and/or CD26.
- Consider prioritizing other abnormalities (dim CD3, dim CD4, high light scatter, dim CD45) over CD7/CD26 loss.









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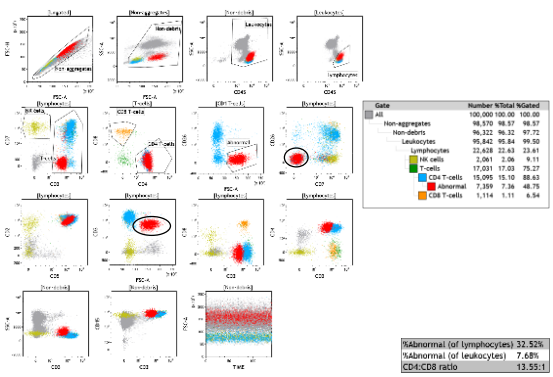

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Estimating absolute Sezary cell counts

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Estimating absolute numbers of Sezary cells
Dual platform

- Correlation with white blood cell counts of obtained separately from an automated blood analyzer.
- **Correlation with absolute lymphocyte count (ALC)**
  - % abnormal cells of lymphocytes x ALC
  - ALC might be falsely low due to large neoplastic cells counted as monocytes.
  - Risk of underestimating Sezary cells
- **Correlation with white blood cell count (WBC)**
  - % abnormal cells of CD45+ leukocytes x WBC
  - Neutrophils and monocytes might be lost during some processing protocols.
  - Risk of overestimating Sezary cells.

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Estimating absolute numbers of Sezary cells
Alternative methods

- **Dual platform based on mononuclear cells**
  - % abnormal cells of lymphocytes and monocytes x (ALC + AMC)
  - Overcomes loss of neutrophils and limitations of analyzer.
  - Strategy not yet tested.
- **Single platform (beads of volumetric)**
  - Direct quantitation of abnormal cells.
  - Lyse/no-wash might affect staining.
  - Not widely available / limited experience
- **Dual flow assay**
  - Second quantitative flow assay for lymphoid subsets (IVD or other).
  - Different processing and gating might result in different proportion of lymphoid subsets.

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Estimating absolute numbers of Sezary cells

- No agreement on a single method.
- Dual platform is currently the most commonly utilized method.
- Some propose that correlation with WBC should be considered first.
- Pitfalls of each method should be addressed during test validation.

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The flow cytometry report

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The flow cytometry report

Required elements

- Presence of absence of abnormal T-cells
- Phenotype of abnormal T-cells
- Estimated absolute number of abnormal T-cells per µL of blood.
- Interpretation.

Optional, depending on needs of clinical group

- CD4:CD8 ratio
- Percentage of CD4 T-cells negative for CD7, CD26 and/or both.

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Flow cytometry report
Example of a positive result

Interpretation:
An abnormal T-cell population detected.
The abnormal T-cell population has abnormal expression of CD3(dim), CD7(dim to negative), CD26(absent), CD45(dim) with normal expression of CD2, CD4, CD5, and CD45; without CD8 or CD56. The abnormal population represents 12.2% of the total white cells. The population is consistent with previously diagnosed involvement by Sezary/Mycosis Fungoides.

Absolute clone size for CTCL like population: 0.68 thousand cells/microliter

Specimen Source: Peripheral Blood
Cell Concentration: 5.6 million/mL
CBC (12/4/2017) WBC: 5.4 K/uL

Specimen Viability: 98%
Specimen Quality Comment: Specimen adequate.
Flow Cytometry Antibodies Used: Analysis performed using anti- CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD45, CD56 and CD279 antibodies. Intensities of monoclonal antibodies not specifically mentioned above are within normal ranges. Number of antibodies used = 10.

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Flow cytometry report
Example of a negative result

Interpretation:
No abnormal T-cell population detected.

T cells 5.2% of WBC

T cell content:
%CD4+CD3+ 62.9%
%CD8+CD3+29.5%
CD4/CD8 ratio: 2.1
CD4 positive CD7 negative 9.6 as % CD4
CD4 positive CD26 negative 39.7 as % CD4
CD4 positive CD7 and CD26 negative 6.4 as % CD4

Specimen Source: Peripheral Blood
Cell Concentration: 8.49 million/mL
CBC 01/25/2018 WBC: 8.4 K/uL
Specimen Viability: 97.5%
Specimen Quality Comment: Specimen adequate.
Flow Cytometry Antibodies Used: Analysis performed using anti- CD2, CD3, CD4, CD5, CD7, CD8, CD26, CD45, CD56 and CD279 antibodies. Intensities of monoclonal antibodies not specifically mentioned above are within normal ranges. Number of antibodies used = 10.

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Assay validation, optimization and ongoing quality monitors

Work in progress

- ICCS quality standards will be extensively referenced.
- Validation of the qualitative and quasi-quantitative components of the test.
- Clinical and analytical sensitivity and specificity.
- Reproducibility.
- Reportable range.

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Assessment of T-cell clonality

Not a requirement, but might be useful in selected cases.

- **TCR gene rearrangement study (molecular):**
  - Biomed-2 primers.
  - Common false positive results.
  - Subjective interpretation.
  - No direct correlation with phenotypic subset.
- **TCR V-β repertoire analysis (separate flow cytometry test):**
  - IOTest Beta Mark TCR Vβ repertoire kit (Beckman Coulter).
  - 24 antibodies on 8 separate tubes, plus custom gating antibodies for each case.
  - High cost, demanding logistics and required expertise is a limiting factor.
- **TCR C-β restricition (TRBC1 expression by flow cytometry):**
  - Needs further study.

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Main Conclusions

- Identification and quantification of Sezary cells should be based on the identification of immunophenotypically abnormal T-cell subsets, based on comprehensive immunophenotypic analysis, and in alignment with contemporary flow cytometry practices.
  - Relying exclusively on CD7(-) and/or CD26(-) subsets on CD4+ T-cells is not recommended.
- At a minimum, a 6-color single tube analysis should be utilized, including:
  - CD3, CD4, CD7, CD8, CD26 and CD45.
- At a minimum, the report should include:
  - The presence of absence of an abnormal T-cell population.
  - A detailed phenotype of the abnormal population.
  - The calculated absolute number of abnormal cells/µL.
  - Intepretation.
- Several knowledge gaps are identified, needing further study.

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Thank you!

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