LYMPHOCYTE SUBSET ENUMERATION

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1. INTRODUCTION

The aim of lymphocyte immunophenotyping is to enumerate and identify specific sets or subsets of lymphocytes. This phenotypic analysis is usually performed on blood specimens; however other body fluids may have to be examined.

2. SPECIMEN COLLECTION, TRANSPORT AND INTEGRITY

2.1. Specimen Collection.

2.1.1. Universal precautions should be strictly observed when collecting samples (see Safety Guidelines).

2.1.2. Any specimen which is unlabelled, or incorrectly labeled or of insufficient volume should be recollected.

2.1.3. A request form should accompany all specimens with patient identifier, age, sex, date and time of collection, name of requesting physician and source of specimen[9] and patient history.

2.1.4 EDTA or Sodium Heparin may be used.[9] **EDTA anticoagulated blood specimens are suitable if the specimen is to be processed within 24 hours of collection.** After which the sample becomes depleted of granulocytes**.** Sodium **Heparin anticoagulated blood specimens** are stable for 48 to 72 **hours.** If the WCC and differential is obtained from the same sample used for flow cytometry then EDTA is recommended.[9]

2.1.5. ***Dual platform***: **A total white cell count and differential and/or stained blood film should be performed at the laboratory initiating the request within the time frame specified by the manufacturer of the haematology instrument used. For distant laboratories and dispatch centres, a total white cell count and differential, and an unstained blood film should accompany each specimen.**

2.1.6**. *Single platform*: This is the preferred method.** It is based on adding a number of fluorescent microspheres to a known volume of sample enabling the determination of both absolute and percentage lymphocyte subsets in a single tube.[9]

2.2. Specimen Transport

2.2.1. Packaging, labeling and transport of specimens should comply with all current local, state, national and international regulations for the regions through which the specimens will pass.

2.2.2. Specimens should be maintained at 18°-22°C in a leak proof container. Temperatures below 4°C, and above 30°C must be avoided.

2.2.3. For deliveries longer than same day it is recommended to include a

minimum-maximum temperature sensor in the package to document whether or not extreme temperatures have been reached during shipping.[9]

2.3. Specimen Integrity

2.3.1. Visually inspect the specimen for clots, haemolysis or container defects. Where appropriate, recollect the sample if the specimen shows any visible signs of deterioration.

2.3.2. Specimens that are collected or transported outside of these guidelines should be treated with caution. It is recommended that specimen viability is greater than **75%[9]** for dual platform method. The deficiencies in the sample should be noted and the report should reflect the effect that these deficiencies may have on the results.

2.3.2 Non viable cells are significant source of false positive staining. Viability testing is recommended for samples tested more than 24 hours after collection or if there is obvious deterioration of the sample.[9]

3. SPECIMEN PROCESSING

3.1. The goal of sample preparation is to process a blood specimen into a representative sample suitable for analysis. All representative cellular and antigenic parameters of the lymphoid cells should be maintained. The more a cell is manipulated the greater is the opportunity of cell loss.[9]

3.2. CD45vs side scatter gating for lymphocytes shall be used. The single platform, whole blood lysis, stain, lyse no wash is recommended as it involves a minimum of manipulation.[9] Note that accurate pipetting is essential for the single platform method and a reverse pipetting technique is recommended.

3.3 Most manufacturers of monoclonal antibodies recommend 10-30 minutes staining time.[9]

3.4. Several commercial lysing techniques are available. Manufacturer's recommended protocol should always be followed unless data are available confirming that any modifications do not adversely affect results.

3.5. For a dual platform method a full blood count and differential should be performed before processing, and the cell concentration adjusted accordingly. One should aim for a cell concentration of up to 1×10^6 cells/ tube.

3.6. All tubes must include CD45.

3.7. Isotype controls are not required.

3.8. A suitable panel of antibodies should include CD45 CD3, CD4, CD8, CD16 and or CD56, CD19. If two tubes or more are used, CD3 must be included in each tube. The selection of antibodies used in the panel should be referenced.

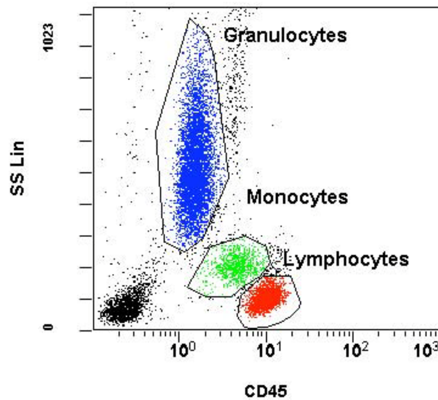
3.9. Any deviation from the manufacturer's recommended protocol should be verified and documented. Such deviations should show that the results are comparable with the manufacturer's recommended procedure.

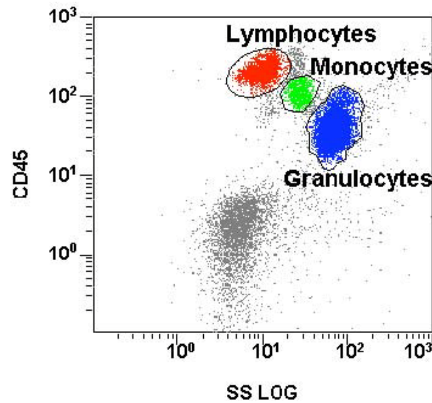
3.10. NOTE: Excess reagent may cause increased non-specific staining of negatives and may result in decrease of positive/negative resolution.

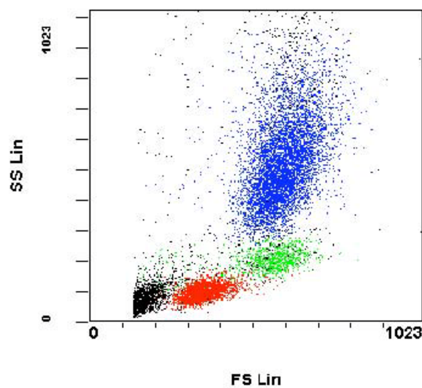
4. CONTROLS

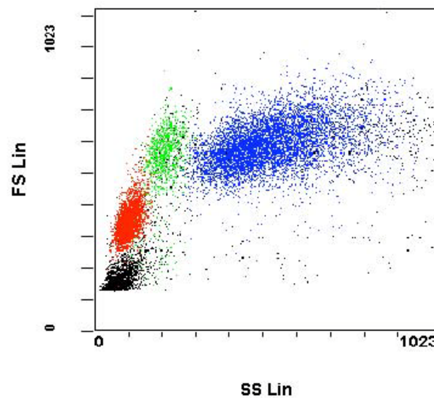
4.1. A method control should be prepared and run on a regular basis in parallel with patient samples. At a minimum, a positive reagent control should be prepared and run whenever a new batch of any reagent used in cell preparation and staining is initiated.

4.2 If a positive control is out of range the reason for the deviation must be determined.[9]









5. SAMPLE ANALYSIS

Primary gate shall be set on CD45 bright positive, low side scatter lymphoid populations.

5.1. Count at least 2500 gated lymphocytes in each sample. This number assures with 95% confidence that the result is within 2% of the “true” value (binomial sampling). NB: This sample mode assumes that the variability of determining replicates is < 2%.

5.2. The counting of 2500 gated lymphocytes to ensure reasonable statistical confidence may not be achievable in severely leukocytopenia specimens.

6. DATA REPORTING

6.1. Report all unique patient identifiers including name/code, medical record number and/or date of birth, laboratory ID/accession number and collection date/time as well as print date/time.

6.2. Report all data in terms of cluster of differentiation (CD) with a short description of the main antigen recognition characteristics3.

6.3. For blood specimens report all data as a percentage and absolute number of the population of interest within the gate as determined by the gating control.

6.4. Report data from all relevant antibody phenotyping combinations with corresponding reference

limits of expected normal values, e.g. CD3+8+ Suppressor/Cytotoxic T Cells ± absolute count and ± percentages.

6.5. Each laboratory should establish reference limits for the antigens being tested (see Appendix 2: Reference Range Determination).

7. QUALITY ASSURANCE

7.1. Analysis should include internal reliability checks of results, including:

- Optimally, the sum of CD3+% plus CD19+% plus CD3-CD16+ and/or CD56+ (the " lymphosum"4) should equal the purity of lymphocytes in the gate ± 5%, with a maximum variability of 10%. If the data are corrected for lymphocyte purity, then the lymphosum should be between 95 and 105% (minimally 90-110%). If the lymphosum is out of range, further investigation should be done.

- Optimally, the sum of the CD3+CD4+% plus CD3+CD8+% should be within the range of +3% to a maximum of [9]10%. Values outside these ranges warrant further investigation. For example, co-expression of CD4 and CD8 or increase in gamma/delta T cells.

- Replicate results within a panel (e.g. CD3+%) for the same sample should be within 3% for CD45 v SS gating.
- 7.2. Light scatter patterns should be examined for each tube within the panel for variation from tube to tube. Similarly, the number of gated events and/or time to collect data should not vary greatly from tube to tube.
- 7.3. Potential sources of error which are not necessarily covered by the above reliability checks may include inappropriate gating leading to exclusion of relevant cells, tubes in a panel run in the wrong order, inappropriate cut-offs between negative and positive cells and calculation or transcription errors. Individual laboratories may require procedures to cover such possibilities.
- 7.4. Regulatory bodies currently require that a laboratory keeps all equipment maintenance and calibration records, staff training records, up-to-date method protocols, daily operator/reagent records, verification of transcription of results from machine printouts, procedures for amendment of results and checks by supervisors/pathologists.
- 7.5. The laboratory must belong to and participate in a recognized external quality assurance program with regular review of the results.

8. DATA STORAGE[9]

8.1. Both the Data stored and the methods used to obtain the data must be thoroughly documented.

8.2. All primary files, worksheets and report forms must be retained for 2 years or as required by state or local regulations which ever is longer.

8.3. After the retention period disposal is at the discretion of the Laboratory Director.

8.4. QA and QC data files should include all parameters, analysis regions and analytical results used to verify performance (instrument and method).

8.5. Data may be stored as paper hard copy and/or archival files.[9]

9. DATA REPORTING[9]

9.1 For each of the lymphocyte and T cell population populations the percentage and absolute numbers should be reported.

- **8. REFERENCES**

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9. APPENDIX 2: DETERMINATION OF REFERENCE RANGES

9.1. Definitions

9.1.1.Reference values: Set of values for any measured quantity.

9.1.2.Reference interval: Classically, the range of values found in 95% of a reference population of healthy individuals without overt clinical disease.

NOTE: Age, sex and race are factors known to influence reference intervals.

9.2. Procedure for Determining Reference Ranges

9.2.1.Statistical methods, both parametric and nonparametric, and graphical methods are discussed in detail in references 6-8. Only a brief summary of the steps involved is presented here.

9.2.2.Parametric methods The steps of parametric methods are to:

- Collect data on randomly chosen set of representative individuals (e.g. 50 healthy individuals representative of patient population.
- Inspect frequency distribution of values obtained.
- If frequency distribution is Gaussian, use appropriate statistical techniques to estimate 95% confidence interval and use endpoints of interval as the reference range.
- If frequency distribution is non-Gaussian, back transform endpoints of 95% confidence interval to obtain reference range, (e.g. log X, of (X + C), square root X, arcsin X) and proceed as in step 3.
- • If no satisfactory transformation can be identified, use nonparametric methods which do not depend on the exact distribution of the data.

- 9.2.3.Nonparametric methods

- Collection of data on randomly chosen set of representative individuals.

- Arrangement of data in ascending or descending order.

- Use of appropriate nonparametric techniques to identify desired limiting percentiles (e.g. 2.5 and 97.5) to desired confidence level.

9.2.3.1. Nonparametric methods are most appropriate when data does not show a Gaussian distribution and cannot be so transformed. However, they are very sensitive to outliers, and final ranges chosen may be highly dependent on methods used for removing outliers6-8.

9.3. Pitfalls in Determining Flow Cytometric Reference Ranges

9.3.1.Each laboratory should determine its own reference range using its particular preparation method and instrumentation because significant laboratory to laboratory differences related to these variables have been reported.

9.3.2. Note that Red Cross Donors are suitable for reference ranges where the donor clinic collects the sample before the bag of blood is collected. If this is not the case the samples are not suitable to establish reference ranges for T, B and NK cells. Lymphocyte subsets are known to be different before and after donation.

9.3.3.However, quite large data sets are technically required to carry the above described methods for reference range determination, typically >300 for parametric methods and >120 for establishing a nonparametric interval with 90% confidence. Until more standardised methodology allows pooling of data among laboratories (hence this document), this is clearly an unrealistic expectation.

9.3.4.Other variables, besides sample size, can be considered. These include collection containers, anticoagulants and red cell lysis reagents which may show lot to lot variation or deteriorate over time. Other factors are age, diurnal variations, stress, diet, smoking, exercise and drugs(9,10).

9.3.5.One practical solution to the dilemma is to accumulate and analyse reference data in smaller sets (e.g. 10-20 individuals), which can then also be pooled and analysed. If the last two sets of pooled data are found to give the same reference range within experimental error, this gives increased confidence that the reference range selected is not unduly affected by the small sample size.