INSIGHTS IN CLINICAL PATHOLOGY

# Flow Cytometry: Introduction to Basics

***Kristina Meichner***, *DVM, DECVIM-CA (Oncology), DACVP (Clinical Pathology)*
*University of Georgia College of Veterinary Medicine, Athens, Georgia*

Flow cytometry is a diagnostic test that is increasingly being used in veterinary medicine to further characterize hematopoietic cells, including lymphocytes.[1] It is a laser-based analytic technique that measures multiple characteristics of cells based on their light-scatter and light-emitting properties, similar to laser-based in-house hematology analyzers.[2] However, in addition to measuring physical characteristics of cells such as size and internal complexity, as hematology analyzers do, flow cytometry measures fluorescence emitted from fluorochrome-labeled antibodies used in immunophenotyping leukocytes (**TABLE 1**).[1-3]

## CLINICAL INDICATIONS FOR FLOW CYTOMETRY

Flow cytometry is most often used to:

1. Determine if lymphoma or lymphoid leukemia is of B-cell or T-cell origin, also known as immunophenotyping (**TABLE 1**)
2. Identify prognostic markers in canine lymphoma to aid in prognosis (**TABLE 2**)
3. Distinguish between reactive and neoplastic lymphocytosis
4. Aid in differentiating lymphoid from myeloid leukemia
5. Further characterize mediastinal masses

Flow cytometry is not a stand-alone test and should always be interpreted in combination with the clinical history, physical examination findings, morphologic findings (i.e., cytology), and other pertinent diagnostic information.[1]

### Abstract

Flow cytometry is increasingly used in small animal veterinary medicine to further characterize leukocytes in dogs and cats, also known as immunophenotyping. Flow cytometry is commonly used to determine cell origin (i.e., B versus T cells) in canine lymphoma, identify markers associated with lymphoma prognosis, distinguish reactive from neoplastic lymphocytosis, differentiate thymoma from mediastinal lymphoma, and support or rule out lymphoma when cytology is inconclusive. Sample cellularity and quality are critical for this test. This article covers sample requirements and tips for sample collection.

Nazile Keskin/shutterstock.com

## Take-Home Points

- Flow cytometry is used to assign a lineage to leukocytes (i.e., T or B lymphocytes), also known as immunophenotyping.
- Identifying immunophenotype is critical for prognosis in canine lymphoma.
- In addition to the general immunophenotype, flow cytometry can identify other markers associated with prognosis in dogs with lymphoma.
- A sample with a sufficient number of viable cells is critical for this test, and recommendations on how to collect a sample for flow cytometry should be followed.
- Flow cytometry is not a stand-alone test and should always be interpreted in combination with clinical and other pertinent diagnostic information.

## Immunophenotyping of Lymphoma and Identification of Prognostic Markers

Knowing the immunophenotype is critical for prognosis in dogs with lymphoma and may also affect treatment decisions.[2,3] Technologies that can assign a certain lineage (i.e., B or T cell) to lymphocytes include immunohistochemistry (IHC), immunocytochemistry, flow cytometry, and polymerase chain reaction for antigen receptor rearrangement (PARR). In the past, IHC was considered the gold standard for immunophenotyping; however, it requires tissue biopsy. Flow cytometry has very high agreement (94%) with IHC for immunophenotyping of canine lymphoma.[8] It is also less invasive and less costly to the client, and in addition to immunophenotyping (i.e., B-cell versus T-cell lymphoma), it can identify markers (i.e., major histocompatibility complex [MHC] class II) or subtypes of lymphomas that affect prognosis (**TABLE 2**).[5-7] As a result, flow cytometry has largely replaced IHC.

PARR is not recommended for lineage assignment as a first-line test. PARR assesses lymphocyte antigen receptor gene rearrangement diversity (often referred to as lymphocyte clonality), and its use should be restricted to differentiating reactive from neoplastic lymphoid proliferations if microscopic evaluation (i.e., cytology, histopathology) and immunophenotyping using flow cytometry, immunocytochemistry, or IHC are inconclusive.[8,9]

**TABLE 1 Antigens Commonly Used for Clinical Flow Cytometry**

| ANTIGEN | GENERALLY FOUND ON | COMMONLY USED IN | |
|---|---|---|---|
| | | DOGS | CATS |
| CD45 | All leukocytes | × | |
| CD18 | Neutrophils > monocytes > lymphocytes | × | × |
| CD3 | T cells | × | |
| CD5 | T cells | × | × |
| CD4 | Helper T cells, canine neutrophils | × | × |
| CD8 | Cytotoxic T cells | × | × |
| CD21 | B cells | × | × |
| CD22 | B cells | × | |
| CD25 | Activated T cells, B cells | × | |
| CD14 | Monocytes/macrophages | × | × |
| MHC class II | B cells, T cells, monocytes/macrophages | × | |
| CD34 | Hematopoietic progenitor cells | × | |

*MHC = major histocompatibility complex*

**TABLE 2 Examples of Published Prognostic Subtypes of Canine Lymphoma Based on Flow Cytometry Staining**[4-7]

| IMMUNOPHENOTYPE | FLOW CYTOMETRY | PROGNOSIS |
|---|---|---|
| B-cell lymphoma | ▪ CD21+, high MHC class II | Better prognosis (MST ~10 months) |
| B-cell lymphoma | ▪ CD21+, low MHC class II | Poorer prognosis (MST ~4 months) |
| T-cell lymphoma (T-zone lymphoma) | ▪ CD3+ and/or CD5+<br>▪ CD45-<br>▪ High MHC class II<br>▪ CD4, CD8: variable | More indolent behavior (MST ~21 months) |
| T-cell lymphoma | ▪ CD3+ and/or CD5+<br>▪ CD45+<br>▪ Low MHC class II, CD4+ | More aggressive behavior (MST 5–6 months) |

*MHC = major histocompatibility complex; MST = median survival time*

## Persistent Lymphocytosis: Differentiation Between Reactive and Neoplastic Causes

Differentiating a reactive lymphocytosis from one with a neoplastic cause can be difficult when small to intermediate lymphocytes in blood are only mildly to moderately increased (<15 000 to 20 000/µL).[10] In both, lymphocytes appear mature and well differentiated on cytology, and neoplastic cells are often indistinguishable morphologically from “normal” or reactive lymphocytes.

First, persistence (lasting more than 2 to 3 months) should be documented by ruling out transient physiologic or epinephrine-induced lymphocytosis, which usually occurs in young, very excited animals (more commonly cats) and usually resolves once the animal is allowed to calm down. Persistent reactive lymphocytosis is a common feature in dogs with chronic *Ehrlichia canis* infection. Lymphocytes are often granulated lymphocytes and CD8+ T cells. Non-neoplastic lymphocytosis can also be seen, albeit less pronounced, in hypoadrenocorticism (dogs), hyperthyroidism (usually cats), and immune-mediated diseases (especially immune-mediated hemolytic anemia in cats) as well as following vaccine administration. Lymphocytosis can also be paraneoplastic (e.g., thymoma).[10]

Chronic lymphocytic leukemia (CLL) should be considered if lymphocytes in circulation have atypical morphology, if flow cytometric analysis demonstrates a phenotypically homogeneous expanded lymphocyte population (with or without aberrant antigen expression), or if a phenotype typically present in low numbers has increased (such as B-cell CLL in dogs).[10,11] Dogs should be screened for *E canis* infection before performing supportive tests; *E canis* infection can cause homogeneous expansion of CD8+ T cells and can also rarely cause clonal expansion of lymphocytes.

Canine CLL is predominantly of T-cell origin; most are CD8+ T cells, and many cases have large granulated lymphocyte morphology on cytology (fine magenta cytoplasmic granules).[10-12] Most feline CLLs are also of T-cell origin, with CD4+ T-helper lymphocytes as the predominant subset.[13] B-cell CLL is more common in small-breed dogs, and approximately half of these affected dogs present with enlarged lymph nodes and/or splenomegaly.[14] Most peripheral blood B-cell expansion in cats is polyclonal; therefore, B-cell lymphocytosis in a cat is less likely neoplastic in origin.[15] A polyclonal non-neoplastic or preneoplastic expansion of B cells has also been reported in English bulldogs.[16] Clinical presentation and flow cytometry findings can overlap between B-cell CLL and the English bulldog B-cell lymphocytosis syndrome, and additional testing for clonality via PARR is necessary for further distinction.[14,16] CLL has a slow onset and a more protracted clinical course that is usually stable over several months to even years.[11-13,17,18]

## Differentiation Between Lymphoma and Leukemia

Differentiating between leukemia and stage V lymphoma can be difficult and is usually based on clinical course, lack or presence of significant lymphadenopathy, degree of blood and bone marrow involvement, and immunophenotypic characteristics determined by flow cytometry (i.e., CD34 expression, presence or absence of lymphoid and myeloid markers, or MHC class II expression). It is important to note that neoplastic cells in acute leukemia (“blasts”) can be easily misinterpreted as being lymphoid in origin based on morphology alone. Therefore, flow cytometry is

critical in lineage assignment as lymphoma and leukemia can present with either or both peripheral blood and nodal involvement; the prognostically important information is the type of malignancy present, rather than where it is found.

A detailed review of leukemia classification is beyond the scope of this article. In general, however, diagnosis and classification of canine acute leukemia require a combination of clinical and clinicopathologic data, cytomorphologic criteria, immunophenotyping by flow cytometry, and cytochemical staining.[10,19] It is noteworthy that 64% (16/25) of dogs with acute myeloid leukemia demonstrated clonal and biclonal B- or T-cell receptor rearrangements in 1 study.[20] Therefore, PARR is currently not recommended to distinguish between acute leukemia of myeloid or lymphoid origin in dogs.

## Distinction Between Mediastinal Lymphoma and Thymoma

Flow cytometry is a noninvasive test to distinguish lymphoma from thymoma in dogs and cats with a mediastinal mass, usually in combination with clinical findings, diagnostic imaging, and cytology. Given that these 2 diseases are treated completely differently (i.e., systemic chemotherapy for lymphoma versus surgical resection of thymoma), pretreatment distinction between these entities is critical. Typically, more than 10% of lymphocytes aspirated from a thymoma are small $CD4^+/CD8^+$ double-positive T cells, whereas that proportion is much lower in mediastinal lymphoma (<2%).[21] Thymoma can be associated with expansion of normal T cells in the peripheral blood. Circulating T cells will most commonly be a combination of mature $CD4^+$ and $CD8^+$ T cells, and in some cases T cells that do not express either antigen.[22]

## Further Characterization of "Tricky" Nodes

The characterization of cytologically "tricky" nodes can be clarified with flow cytometry. Such nodes include, for example, enlarged lymph nodes with a predominance of small lymphocytes or an expansion of intermediate lymphocytes for which distinction between a "normal" lymph node and a hyperplastic lymph node from a small- or medium-sized lymphoma is not possible based on cytology. Other examples include some variants of canine T-cell lymphoma (aggressive and indolent) and small-cell B-cell lymphoma. Occasionally, lymphoma cells can be very fragile and difficult to prepare for cytologic diagnosis (i.e., a smear with too many ruptured cells with rare intact large lymphocytes concerning for lymphoma). In these cases, a lymph node aspirate for flow cytometry can support a lymphoma diagnosis.

Flow cytometry is a noninvasive test to distinguish lymphoma from thymoma in dogs and cats with a mediastinal mass, usually in combination with clinical findings, diagnostic imaging, and cytology.

## SAMPLE REQUIREMENTS AND COLLECTION

Until sample preservation can be further optimized, clinical flow cytometry in veterinary medicine requires intact, viable cells. Therefore, a freshly collected sample should be submitted or shipped to the laboratory on the same day or overnight for Monday through Friday delivery. Laboratories performing flow cytometry may offer overnight shipping labels. Friday collection is discouraged unless it is unavoidable.

Cells deteriorate more slowly when kept cool; therefore, it is recommended to keep the sample refrigerated until it is shipped and to include a cooling source such as an ice pack in the shipment. Samples must not be frozen.

Samples are best when collected from the primary disease site. If the primary disease is affecting lymph nodes (e.g., multicentric lymphoma), submit a lymph node aspirate (not peripheral blood). If the primary disease is in the blood (e.g., leukemia), submit peripheral blood (not a lymph node sample).

A pertinent patient history and a cytology or histopathology report, if already performed, should always be included with the submission, as it will assist the laboratory in interpreting the flow cytometry results. For peripheral blood samples, include a recent

complete blood count (CBC) if available or request a CBC from the lab performing the flow cytometry.

An adequate cell concentration is critical for flow cytometry analysis. For lymph node/organ/mass samples, take an aspirate. Then, *gently* mix the aspirated cells with approximately 1 mL of isotonic saline supplemented with approximately 10% to 20% of the patient's serum in a plain tube without any additives (**BOX 1**). Cellularity for flow cytometry is usually adequate when the cell–fluid mixture turns from clear to opaque (**FIGURE 1**).

For peripheral blood, bone marrow, and body cavity fluids, submit at least 1 mL of specimen in an EDTA (purple top) tube. Certain specimens, such as cerebrospinal fluid (CSF), are often not suitable for flow cytometry given their relatively low cellularity even in the presence of a pleocytosis (increased nucleated cell count in CSF) and the relatively low volume that can be safely collected from a patient. If flow cytometry on CSF is being considered, it is recommended to contact the laboratory first to discuss whether the specimen is suitable.

## SUMMARY

Flow cytometry is a noninvasive diagnostic tool to further characterize hemolymphatic cells in dogs and cats. Following a cytologic diagnosis of lymphoma, flow cytometry can determine B-cell or T-cell origin and can identify prognostic markers and subtypes in canine lymphoma to aid in prognosis. It also aids in lymphoma diagnosis when cytology is inconclusive and

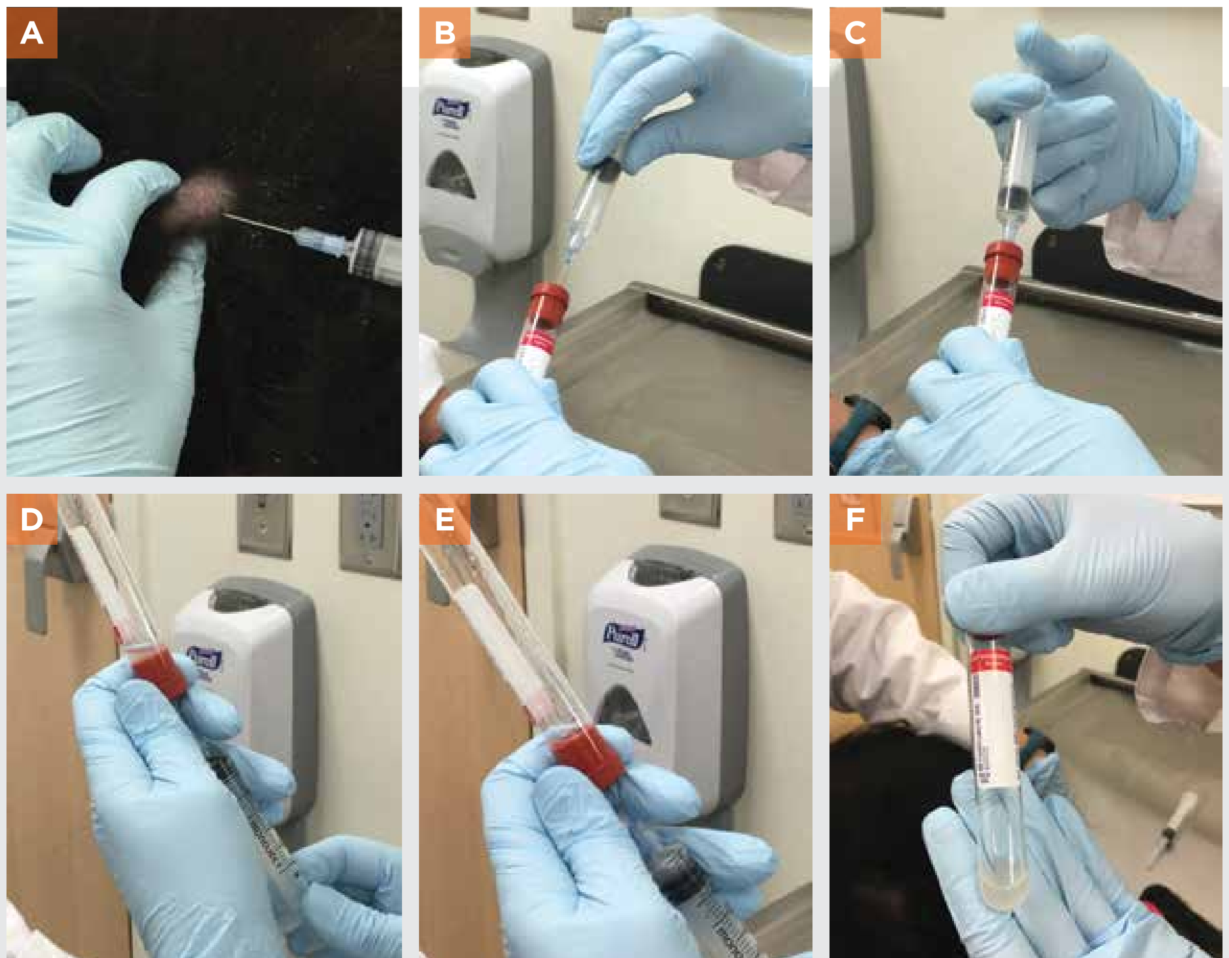


**FIGURE 1.** Preparing a tissue aspirate sample for flow cytometry. **(A)** Prepare a 5-mL syringe with a 22- or 20-gauge needle and gently aspirate the tissue with suction. **(B and C)** Eject the aspirated contents into a plain, additive-free tube already containing a mixture of saline and patient serum (see **BOX 1** for more details). **(D and E)** Flush the aspirate into the saline–serum solution several times to recover all the cells, inverting the tube to mix well. Repeat aspiration and mixing steps as necessary until the solution becomes cloudy. **(F)** When the solution–cell mixture turns cloudy, enough cells are collected.

### BOX 1 Preparing Patient Serum for Flow Cytometry Samples

- Collect serum: Take whole blood from the patient, let it clot, and spin to collect ~0.2 mL (200 µL) of serum. Serum from another patient of the same species can be used instead.
- Fill an additive-free (plain) tube (i.e., plain serum tube or screw-top tube, not a serum separator tube) with ~1 mL of isotonic (0.9%) saline.
- Add the 0.2 mL of serum to the 1 mL of saline and mix to create a solution with ~20% serum.

assists in differentiating a reactive from a neoplastic lymphocytosis. Cell concentration and cell viability are critical for this test, and a freshly collected sample should be submitted to the laboratory within 24 hours. Flow cytometry is not a stand-alone test and should always be interpreted in combination with clinical and other diagnostic findings. TVP

## References


1. Meichner K, Stokol T, Tarigo J, et al. Multicenter flow cytometry proficiency testing of canine blood and lymph node samples. *Vet Clin Pathol*. 2020;49(2):249-257. doi:10.1111/vcp.12843
2. Burkhard MJ, Bienzle D. Making sense of lymphoma diagnostics in small animal patients. *Vet Clin North Am Small Anim Pract*. 2013;43(6):1331-1347. doi:10.1016/j.cll.2015.05.008
3. Moore AS. Treatment of T cell lymphoma in dogs. *Vet Rec*. 2016;179(11):277. doi:10.1136/vr.103456
4. Deravi N, Berke O, Woods JP, Bienzle D. Specific immunotypes of canine T cell lymphoma are associated with different outcomes. *Vet Immunol Immunopathol*. 2017;191:5-13. doi:10.1016/j.vetimm.2017.07.008
5. Rao S, Lana S, Eickhoff J, et al. Class II major histocompatibility complex expression and cell size independently predict survival in canine B-cell lymphoma. *J Vet Intern Med*. 2011;25(5):1097-1105. doi:10.1111/j.1939-1676.2011.0767.x
6. Seelig DM, Avery P, Webb T, et al. Canine T-zone lymphoma: unique immunophenotypic features, outcome, and population characteristics. *J Vet Intern Med*. 2014;28(3):878-886. doi:10.1111/jvim.12343
7. Avery PR, Burton J, Bromberek JL, et al. Flow cytometric characterization and clinical outcome of CD4+ T-cell lymphoma in dogs: 67 cases. *J Vet Intern Med*. 2014;28(2):538-546. doi:10.1111/jvim.12304
8. Thalheim L, Williams LE, Borst LB, Fogle JE, Suter SE. Lymphoma immunophenotype of dogs determined by immunohistochemistry, flow cytometry, and polymerase chain reaction for antigen receptor rearrangements. *J Vet Intern Med*. 2013;27(6):1509-1516. doi:10.1111/jvim.12185
9. Keller SM, Vernau W, Moore PF. Clonality testing in veterinary medicine: a review with diagnostic guidelines. *Vet Pathol*. 2016;53(4):711-725. doi:10.1177/0300985815626576
10. Avery AC, Avery PR. Determining the significance of persistent lymphocytosis. *Vet Clin North Am Small Anim Pract*. 2007;37(2):267-282. doi:10.1016/j.cvsm.2006.11.001
11. Workman HC, Vernau W. Chronic lymphocytic leukemia in dogs and cats: the veterinary perspective. *Vet Clin North Am Small Anim Pract*. 2003;33(6):1379-1399. doi:10.1016/s0195-5616(03)00120-7
12. McDonough SP, Moore PF. Clinical, hematologic, and immunophenotypic characterization of canine large granular lymphocytosis. *Vet Pathol*. 2000;37(6):637-646. doi:10.1354/vp.37-6-637
13. Campbell MW, Hess PR, Williams LE. Chronic lymphocytic leukaemia in the cat: 18 cases (2000-2010). *Vet Comp Oncol*. 2013;11(4):256-264. doi:10.1111/j.1476-5829.2011.00315.x
14. Bromberek JL, Rout ED, Agnew MR, Yoshimoto J, Morley PS, Avery AC. Breed distribution and clinical characteristics of B cell chronic lymphocytic leukemia in dogs. *J Vet Intern Med*. 2016;30(1):215-222. doi:10.1111/jvim.13814
15. Rout ED, Labadie JD, Curran KM, Yoshimoto JA, Avery AC, Avery PR. Immunophenotypic characterization and clinical outcome in cats with lymphocytosis. *J Vet Intern Med*. 2020;34(1):105-116. doi:10.1111/jvim.15650
16. Rout ED, Moore AR, Burnett RC, et al. Polyclonal B-cell lymphocytosis in English bulldogs. *J Vet Intern Med*. 2020;34(6):2622-2635. doi:10.1111/jvim.15913
17. Comazzi S, Gelain ME, Martini V, et al. Immunophenotype predicts survival time in dogs with chronic lymphocytic leukemia. *J Vet Intern Med*. 2011;25(1):100-106. doi:10.1111/j.1939-1676.2010.0640.x
18. Rout ED, Labadie JD, Yoshimoto JA, Avery PR, Curran KM, Avery AC. Clinical outcome and prognostic factors in dogs with B-cell chronic lymphocytic leukemia: a retrospective study. *J Vet Intern Med*. 2021;35(4):1918-1928. doi:10.1111/jvim.16160
19. Stokol T, Schaefer DM, Shuman M, Belcher N, Dong L. Alkaline phosphatase is a useful cytochemical marker for the diagnosis of acute myelomonocytic and monocytic leukemia in the dog. *Vet Clin Pathol*. 2015;44(1):79-93. doi:10.1111/vcp.12227
20. Stokol T, Nickerson GA, Shuman M, Belcher N. Dogs with acute myeloid leukemia have clonal rearrangements in T and B cell receptors. *Front Vet Sci*. 2017;4:76. doi:10.3389/fvets.2017.00076
21. Lana S, Plaza S, Hampe K, Burnett R, Avery AC. Diagnosis of mediastinal masses in dogs by flow cytometry. *J Vet Intern Med*. 2006;20(5):1161-1165. doi:10.1892/0891-6640(2006)20[1161:dommid]2.0.co;2
22. Wikander YM, Knights K, Coffee C, et al. CD4 and CD8 double-negative immunophenotype of thymoma-associated lymphocytes in a dog. *J Vet Diagn Invest*. 2020;32(6):918-922. doi:10.1177/1040638720948628

**Kristina Meichner**

Dr. Meichner received her DVM degree from the Ludwig-Maximilian University of Munich in Germany. Following a small animal rotating internship, she completed a residency in medical oncology, followed by a residency in clinical pathology at North Carolina State University. Dr. Meichner is currently an associate professor in the department of pathology at the University of Georgia College of Veterinary Medicine, as well as director of the clinical pathology and clinical flow cytometry laboratories. Her research interests focus on immunology and canine cancer, especially the characterization of canine hematopoietic tumors and the investigation of alternative treatment strategies.