Department of Clinical Pathology and Oncology
University of Veterinary Medicine Budapest

Quality Control for Fluorescence Activated Cell Sorting in Canine Tumour Immunophenotyping

Written by:
Connor Herst

Supervisor:
Edina Karai
PhD Student

Budapest, Hungary
2017

Table of Contents

1. Introduction

Developments in flow cytometry have allowed multi-parameter analysis of heterogenous cell populations which are applicable as a clinical service. (Owens and Loken, 1995). Three or four colour immunophenotyping assays are not uncommon in the laboratories running these experiments, allowing the simultaneous measurement of 5 or 6 parameters. (Nicholson et al., 1996). As the possible applications of flow cytometry have greatened with the increasing complexity of the process, so have the number of variables that need to be controlled. Standardisation and validation of the instrumentation, methodology, reagents and many other aspects are of utmost importance to ensure the technical quality of the results. (McCoy JP Jr, Carey JL and Krause JR, 1990). In a clinical setting, the need for quality assurance is of even greater importance. The centre for disease control and prevention (CDC) places flow cytometry in the category of high complexity laboratory testing meaning documentation of staff, qualifications and training as well as analytical accuracy, sensitivity, precision an QC are required. (Owens et al., 2000).

Flow cytometry is a method which for a long time, has been used for the diagnoses of non-Hodgkin lymphoma in human medicine. In the last decade, it has become a routine diagnostic tool in veterinary medicine, more so in the canine species than any other. (Guzera et al., 2014). One such veterinary oncology clinic that is utilising flow cytometry for the diagnosis of lymphoma in canine and feline species is the A.H.O.K., Veterinary Oncology and Haematology Centre, located in Budapest Hungary. Here Edina Karai, under the guidance of Prof. Dr. Péter Vajdovich D.V.M. Ph.D., has been developing a protocol for the diagnosis of haematopoietic tumours, as well as measuring the multidrug resistance factor in those patients found to be positive.

My aim is to outline a quality control protocol that suitably ensures the results of the above-mentioned assay are valid and reproducible. When planning a QC protocol, it is essential that you first spend some time understanding the principles and theories behind the tools and technology that will be used and be satisfied that the approach is scientifically sound. (Westgard, 2009). For this reason, the first portion of my article will be detailing the workings of the flow cytometer. Next, I will be discussing the considerations that must be taken specific to quality control of flow cytometry and finally I will show the results of the work that Miss Karai has produced and explain how they validate her study.

2. The flow cytometer in general

2.1. The Flow Chamber

The flow chamber is designed to carry cells from the sample, to the point of measurement, where the laser meets the cells (the interrogation point). A stream of fluid (water or buffer) known as sheath fluid is the medium in which the samples are carried. We refer to the 'fluidics' when we discuss sheath fluid and the system that drives it. Most commonly, air pressure drives the sheath fluid through the flow chamber and into the waste at a constant flow rate. Figure 1. Is a schematic of the fluidics system. It shows how air pressure forces sheath fluid into the flow chamber and injects a small amount of the sample into the stream. When the flow is completely laminar, the sample and the sheath fluid do not mix. The flow chamber narrows and the sample is forced into single file. (Novo and Ormerod, 2008)

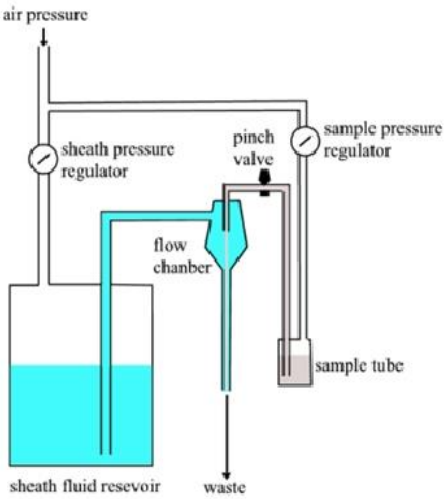

Figure 1.: The layout of fluidics (Novo and Ormerod, 2008)

2.2. The Optics system

The optics system refers to any area of the flow cytometer involved in measuring light such as the source, the lens, and of course, the filters. Each part is discussed in more detail below

2.2.1. The Light Source

The light source of a flow cytometer is a laser (light amplification by stimulated emission of radiation). They are nearly monochromatic, (meaning they exhibit a single wavelength of light), their radiation is highly directional (produced in a beam) and lasers are highly coherent, meaning the waves emitted have a constant relative phase. (Paschotta, 2008). Two principles on which flow cytometry depend are the measuring of scattered light and measuring of fluorescence (McCoy et al., 1990). Small changes in the directional origin of light would cause erroneous measurements of scatter, therefore a beam of light is essential to producing accurate measurements. To achieve peak fluorescence, a particular wavelength of light is required. Again, a laser is the suitable source of light to achieve this owed to its monochromatic nature.

2.2.2. Fluorescence

Fluorescence is a phenomenon that occurs when a compound (known as a fluorophore) absorbs energy from light. The electrons are raised from their ground state to a state of excitation. This excited state is unstable, the compound gives off energy to allow the electrons to reach their ground state again. A portion of this energy is heat loss, the remainder is lost as light emission. The light emitted by the compound is of a lower energy than the light absorbed (due to heat loss), this lower energy light has a longer wavelength than the higher energy absorbed light, thus a different colour is observed. (Novo and Ormerod, 2008).

The absorption spectrum of a fluorophore is the range of wavelengths of light that the compound is capable of absorbing. The wavelength of maximum absorption is usually the same as the excitation maximum. In other words, the wavelength that is absorbed at a maximum causes the molecule to fluoresce at a maximum intensity. (Davidson, 2015). The emission spectrum is the range of wavelengths of light that the fluorophore emits after excitation. (Figure 2.) At the upper end of the Absorption spectrum and the lower end of the emission spectrum, there is usually an overlap of wavelengths. “this overlap of excitation and emission intensities and wavelengths must be eliminated, in fluorescence microscopy” (Davidson, 2015). The same is true for fluorescence activated flow cytometry and this is achieved by the optic filters found in the channels that carry the light to the appropriate photodetectors.

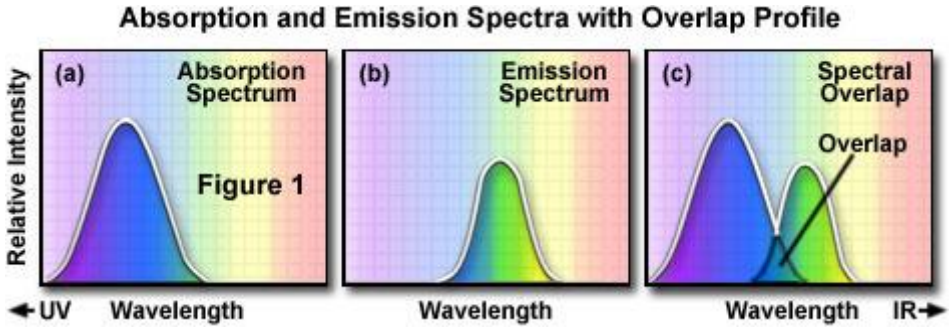

Figure 2. Davidson (2015)

We utilise fluorescence in flow cytometry by attaching these fluorophores to monoclonal antibodies (mAb’s). The mAb’s are specific to a cell type of interest (e.g. T-lymphocytes) and using a laser to excite these fluorophores, and a clever system of filters to sort the emissions from them, we can describe a population of cells.

2.2.3. A typical arrangement of filters and detectors

Figure 3 outlines the layout of a simple flow cytometer. It is suitable for measuring immunofluorescence from cells which have been incubated with labelled antibodies, such as fluorescein (FITC), phycoerythrin (PE) and PE-cyanine5 (PEcy5). Peak excitation of these fluorophores occurs at a range of approximately 450-600nm (ThermoFisher spectral viewer). A blue argon laser produces a wavelength of light that adequately excites these fluorophores. (Novo and Ormerod, 2008). As the laser meets the cells, light is scattered in many directions. The flow cytometer measures the forward scattered light (FS), side scattered light (SS) and light of fluorescent origin. The FS light is relative to the size of the cell, with larger cells producing more forwards scatter. A simple detector is sufficient to measure the FS as the intensity produced is ample. This detector sits behind a blocking bar to prevent the laser from registering on it (Bradford, 2012). The SS light is relative to the internal complexity of the cell. FITC, PE and PEcy5 have peak emissions of 516nm (green), 575nm (orange) and 668nm (red) respectively (ThermoFisher spectral viewer). Dichroic filters are very accurate colour filters that allow certain ranges of light to pass whilst reflecting others (Paschotta, 2008). In the setup seen in figure 3. there are three dichroic filters. The first will reflect all light with a wavelength less than 500nm onto a barrier. This barrier will allow only blue light to pass. Blue light seen here is proportional to SS rather than fluorescence. A photomultiplier tube (PMT) amplifies the intensity of this light before detection. All light above 500nm will land on dichroic filter 2. This will reflect light with a wavelength less than 560nm onto a barrier which allows only green light to pass onto the PMT. The green light is produced only by FITC, so the intensity of light measured by the detector in this location is proportional to the number of cells attached with a FITC marked antibody. All light wavelength above 560nm will reach dichroic filter 3. This filter reflects light below 620nm on a barrier for orange light. The light passing this barrier is representative of cells bound to PE marked antibodies. All light above 620nm reaches a barrier allowing only red light to pass. This light represents the cells bound to PEcy5. (Novo and Ormerod, 2008). Using this typical arrangement of filters and detectors, unknown cell populations can be easily described.

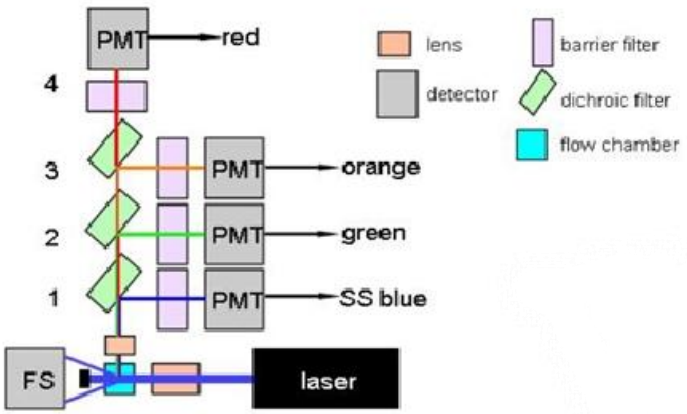

Figure 3:. Typical layout of a simple flow cytometer (Novo and Ormerod, 2008)

3. Monoclonal antibodies

As I discussed in the previous section, monoclonal antibodies (mAb's) are an essential aspect of FACS. They are the key molecules which recognise and bind the cells of interest allowing characterisation of an unknown cell population. In this section I will explain how monoclonal antibodies are produced as I feel it is a key component of FACS.

Definition of an antibody

> "Any of a large number of proteins of high molecular weight that are produced normally by specialized B cells after stimulation by an antigen and act specifically against the antigen in an immune response, that are produced abnormally by some cancer cells, and that typically consist of four subunits including two heavy chains and two light chains — called also *immunoglobulin*". (In Merriam-Webster's Dictionary, 2017)

Definition of Antigen

> "Any substance (such as an immunogen or a hapten) foreign to the body that evokes an immune response either alone or after forming a complex with a larger molecule (such as a protein) and that is capable of binding with a product (such as an antibody or T cell) of the immune response". (In Merriam-Webster's Dictionary, 2017)

Each antigen has specific antigen determinants known as epitopes, this is the area of the antigen that is recognized by the immune system and bound to by complementary determining regions found on the antigens. This is responsible for the antibody specificity. If an antigen has multiple epitopes, B-lymphocytes will produce many different antibodies from different cell lines. These are known as polyclonal antibodies (Nandkishor, 2015)

Monoclonal antibodies (mAb) are made by identical immune cells cloned from a unique parent cell. They bind the same epitope on the same antigens. Because of their high specificity, mAb's have a broad range of applications from diagnostic testing, analytical testing and even therapeutic treatment. Since their discovery scientists have been trying to isolate B-lymphocytes from specifically immunized animals to produce mAb's (Ward et al., 1999) This approach was unsuccessful as culturing B-lymphocytes is difficult and the synthesis of mAb's was short-lived and difficult. In 1975, George Kohler and Cesar Milstein achieved large scale production of mAb's by hybridizing B-lymphocytes with myeloma cells, (cells harvested from patients suffering multiple myeloma disease), into a special call known as a hybridoma. (Ward et al. 1999). The steps of producing mAb's are outlined below

1. Immunization
    - A mouse is immunized to the antigen of interest by injecting it intraperitoneally or subcutaneously multiple times, the immune system of the mouse will produce B-lymphocytes specific to the antigen. When the serum titre of antibodies is adequate, the mouse is killed and its spleen removed aseptically and lymphocytes harvested (Ward et al, 1999).
2. Cell fusion
    - The clean lymphocytes are mixed with the $HGPRT^-$ myeloma cells and are exposed to polyethylene glycol for a short period. The cells are washed and a mixture of free myeloma cells, free lymphocytes and the desired hybridoma cells remain. (Nandkishor, 2015)
3. Selection of Hybridomas
    - The mixture of cells described above is cultured in HAT Medium, a selective medium that will allow the growth of the hybridoma cells whilst the remaining types disappear over 7-10 days. (Ward et al, 1999)

4. Screening the products
    - The hybridomas are tested to see they produce the antibody of desired specificity. This is done using either ELISA or RIA most commonly. (Ward et al., 1999)
5. Cloning and propagation
    - The screened single hybridoma's are isolated and cloned. Two methods are commonly used to achieve this. The 'Limiting Dilution Method' achieves this by serially diluting the suspensions of hybridomas and dividing them into small wells containing only one single hybrid cell. This ensures that any propagation in each well is from a single cell line. (Ward et al., 1999)
    - The second method used is the "soft agar method" (Nandkishor, 2015) In this technique, many cells are grown in a semisolid medium forming colonies, these colonies are monoclonal in nature (Nandkishor, 2015)
6. Characterisation and storage
    - We must ensure the mAb's produced exhibit the desired specificity, this is achieved by subjecting them to biochemical and biophysical characterisation. We must also ensure the cell lines produced are stable enough to withstand freezing and thawing. (Nandkishor, 2015)

Once the mAb's are produced, we covalently bind fluorochromes to them (Novo and Ormerod, 2008, chapter 3.3.1.) and we are ready to utilise them.

4. Quality control in Fluorescence Activated Cell Sorting

4.1. Flow cytometry as a clinical service

When the purpose of flow cytometry is the diagnosis of tumours of haematopoietic and lymphoid tissue origin, quality control is of the utmost importance. The results that the laboratory collect will be used to decide what treatment is given, mistakes are very costly. Training and proficiency in the laboratory must be documented as must analytical accuracy, sensitivity, precision and many other parameters. In the following points, I have outlined the considerations the lab must make when offering flow cytometry as a clinical service

4.1.1. Sample handling

Laboratories must decide on what requirements there are for the collection, transport and handling of the sample. Clinical samples must always be appropriately labelled with, at the very least, a unique patient identifier, the test ordered and the date of sampling. Other data submitted may be age, gender, presumptive diagnosis, source of sample, name of physician, and recent treatment. (Owens et al., 2000).

4.1.2. Sample preparation

The sampling location must be considered when deciding the method of preparation. (Stewart and Stewart, 1994). It is important that this process yields single cells as oppose to small tissue clumps. The prepared product must contain optimally concentrated levels of the cells of interest for monoclonal antibody staining (Owens et al., 2000)

4.1.3. Accuracy

To put it simply, a test measurement is said to be accurate when the measured result is representative of the truth. In more technical terms, a test is accurate when the test value is approaching that of the absolute “true” value of the analyte being measured. (American association for clinical chemistry, 2008). Analytical accuracy is the process of comparing the test result with a “gold standard” reference value to ensure they are comparable. In haematology, we can compare healthy cell populations with automated equipment such as a haemocytometer for accuracy (Wooten and King, 1953). In abnormal populations, the comparison it trickier as haemocytometers may be unable to analyse the abnormal cell populations, therefore, in the case of haematopathology, the gold standard is morphology. The flow cytometric assessment of accuracy must be comparable with the morphology. Accuracy of the process can be validated by measuring previously characterised cells. A source of previously characterised cells could be cryopreserved samples from another

validated laboratory. An alternative source of samples for assessing accuracy are cases diagnosed by cytogenetics and molecular biology. (Owens et al., 2008)

4.1.4. Specificity

The American association for clinical chemistry defines specificity as the ability for a test to correctly exclude individuals who do not have a given disease or condition. Pertaining to monoclonal reagents, specificity is their ability to correctly recognise and attach their antigenic target. The manufacturers are themselves responsible for ensuring a reagent has the specificity claimed. When it comes to diagnosis of lymphoma with flow cytometry, result produced by the lab should be compared with morphological analysis of the sample and clinical presentation to appropriately assess specificity (Zagursky et al., 1995). It is recommended that each lab establish its own rate of discrepancy between flow panel and morphology, of ideally <5% (Owens et al., 2008). Other methods of assessing specificity of flow cytometric reagents include consensus workshops, notably Human Cell Differentiation Molecules, an organisation that create workshops to analyse reagents by sending them to a multitude of testing laboratories and analysing the returning data (Boumsell, 2016)

4.1.5. Sensitivity

The lower the minimum staining intensity above non-specific or negative staining that gives a positive result for a given reagent, the more sensitive that reagent is (Owens et al., 2008). The sensitivity depends on the titration of monoclonal reagents, the proper instrument set-up and calibration, the number of cells counted and the flow rate of the instrument. (Wittrup et al. 1994)

4.1.6. Precision

A test method is said to be precise when repeated analysis of the same sample gives similar results. Essentially, it is the measure of a tests repeatability. (American association for clinical chemistry, 2008). The guidelines set by the Clinical Laboratory Standards institute (CLSI) suggest minimum of 20 replicates are used to test precision. The materials used for this test can be normal peripheral blood, cell lines or CD chex. (CD chex are white cell controls available for purchase at https://www.streck.com/controls/cd-chex-plus/). According to Omerod, for each monoclonal antibody that is used the lab should collect a quantitative mean and standard deviation. These should be used to draw up a Levey-Jennings chart that is +- 2SD about the mean. If the same stained sample is run three times and the results all fall within +-2SD of the mean the test is said to be precise. We will quantify

precision using a coefficient of variation, (CV). CV is calculated by dividing the standard deviation by the mean, and multiplying by 100 to express it as a percentage (Westgard, 2008). The coefficient of variation (CV) varies with analyte concentration and is often higher at lower analyte concentrations (Stockham, 2008). The ability of a test or method to get the same result if a sample is analyzed multiple times, (Stockham, 2008)

We performed the "Repeatability" (within-run precision, intra-assay precision) Closeness of agreement between results of successive measurements carried out under the same conditions (short-term replication study). (Linnet and Boyd, 2006) (Westgard, 2008). Another, way of determining Precision is "Reproducibility", (between-run precision, interassay precision). Closeness of agreement between results of successive measurements carried out under different conditions (different times, operators, calibrators, reagent lots, etc). Also known as a long-term replication study. (Linnet and Boyd, 2006) (Westgard, 2008)

4.1.7. Analyte-Specific Reagents (ASR)

In the United States, all labs that perform physician ordered flow cytometric testing for human leukaemia and lymphoma immunophenotyping do so using only Analyte-specific reagents. (Regulated under CLIA 88). The FDA has the following definition of ASRs

> "antibodies, both polyclonal and monoclonal, specific receptor proteins, ligands, nucleic acid sequences, and similar reagents which, through specific binding or chemical reactions with substances in a specimen, are intended for use in a diagnostic application for identification and quantification of an individual chemical substance or ligand in biological specimens." (FDA Guidance for Industry and FDA Staff Commercially Distributed Analyte Specific Reagents (ASRs): Frequently Asked Questions, 2007)

Under the ASR rule, published in 1997 by the FDA, ASRs are subject to restrictions on sale, distribution, and use. It is recommended that all clinical laboratory use ASRs for their assays to ensure the products they are using are held to the highest standards.

4.2. Instrument Validation

For the results of a flow cytometry assay to be accurate it is essential the instrument is optimised ensuring laser alignment, proper function of photomultiplier tubes and optimal fluidics. "This is of particular importance in lymphoma and leukaemia immunophenotyping where subtle changes in scatter of light or fluorescence will allow for the identification of

aberrant populations". (Oldaker, see literature). The quality control of the instrument itself consist of two major parts, the initial set up of the machine and the daily monitoring of performance. (Purvis and Stelzer, 1998).

4.2.1. Initial instrument setup

Initial setup of the instrument is required after installation of the machine and after any major repairs have been performed. It should be carried out by a qualified service engineer. The initial setup establishes optimal laser alignment, fluidics, filter, lenses and log and linear amplification. Laser alignment occurs by focusing the laser beam so it properly meets the cell stream. It also ensures the emitted light signals are optimised with the filters and photodetectors ensuring the "brightest and tightest" signal whilst at the same time minimise the variation. Sub-optimal alignment will decrease the sensitivity of the instrument. (Purvis and Stelzer, 1998). Fluorescently stained polystyrene microspheres that exhibit extremely high uniformity with regards to size and fluorescence are available commercially. They replicate the size, fluorescence intensity and emission wavelength of biological samples. Figure 4. demonstrates how small the variation of fluorescence of these beads is. They can be used to appropriate cell-wide laminar flow, alignment between the laser beam and the cell at the interrogation point and proper adjustment of the laser power and PMT voltage. (Owens et al., 2000). Whilst they are designed to replicate the behaviour of cells as closely as possible, beads do not behave precisely as real cell samples. For this reason, it's prudent to run tests using samples of verified cell populations. (Henry and Segalove, 1952).

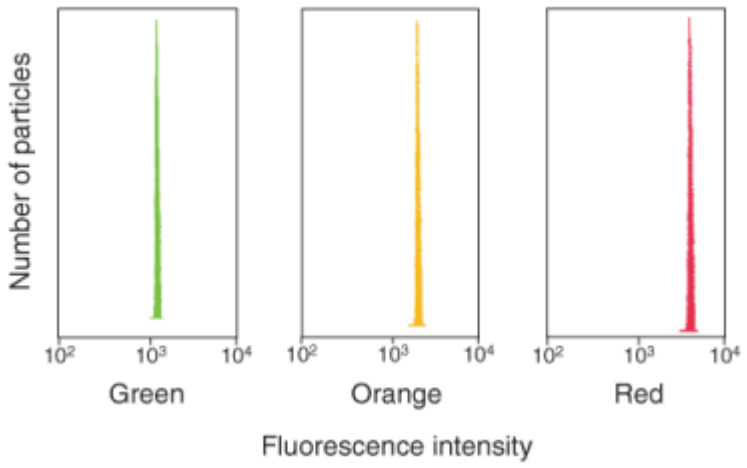

Figure 4.: flow cytometry alignment beads excited at 488 nm by an argon-ion laser and monitored in three emission channels. (Contributed by Carleton Stewart, Roswell Park Cancer Institute).

4.2.2. Establishing Acceptance values and daily monitoring

After every cold start, performance of the instrument is checked to ensure it falls between appropriate limits considering, **a.** the light scatter and resolution, **b.** the fluorescence sensitivity and resolution and **c.** the fluorescence compensation. Control beads will be run through the cytometer and the results plotted on Levey-Jennings graphs to visualise whether any fall outside of the acceptable ranges. In case any do, corrective action can be taken. (Oldaker, 2007)

a. Light scatter sensitivity and resolution

The mean forward scatter and side scatter channel numbers and the standard deviation should be recorded. These values are established by running the beads 20 times over a 5-day period using the same PMT setting. The calculated values will be used to create Levy-Jennings graphs which will be used to monitor the daily performance of the instrument. (Allen et al, 1969; Levy and Jennings, 1950).

b. Fluorescence sensitivity and resolution

To establish acceptance values for the daily monitoring of fluorescence sensitivity and resolution, the channel number and standard deviation of the calibration beads with a pre-determined laser power, filters, PMT voltage and gains (Owens et al., 2000). These values should be established by running the beads 20 times over 5 days.

c. Compensation

In a perfect world, all fluorophores would have distinct emission wavelength, this is far from the truth. (Figure 5.) Two commonly used fluorophores (Fluorescein (FITC) and Phycoerythrin (PE)) have a range of wavelengths that overlap one another. This area is known as Spectral overlap. As a result, each of the fluorophores will cause a positive result in, not only their channel, but the channel dedicated to the other. (Bushnell, 2015). Depending on the number, type of fluorochromes used in a particular staining protocol, we must correctly set the compensation to prevent any spill over from one signal to another (Bagwell and Adams, 1993). These set levels should be evaluated at the time of initial set up and regularly monitored.

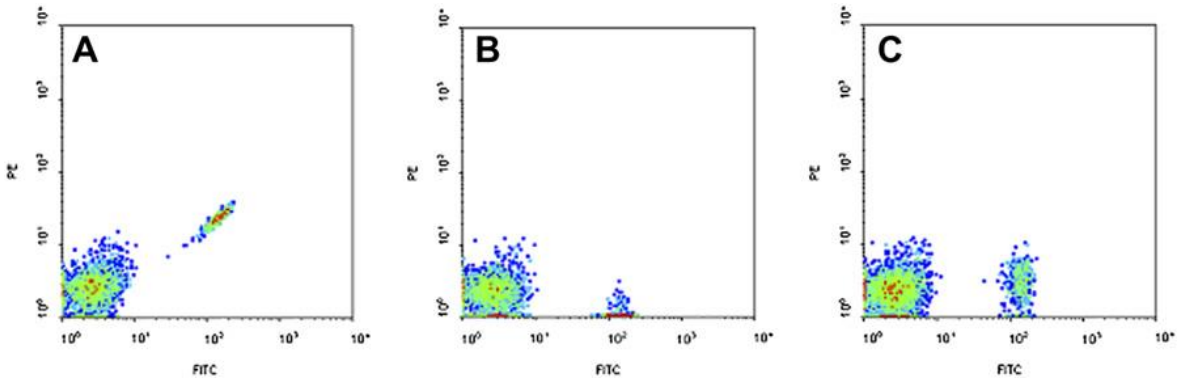

Figure 5.: Example of (A) uncompensated, (B) overcompensated and (C) properly compensated data (Owens et al. 2000)

4.2.3. Fluorescence Linearity and instrument correlation

Checking the linearity of fluorescence demonstrates that the signal measurements at the low and high end of the fluorescent scale are related to signal intensity. Ensuring that your instrument is giving a linear response is critical in measuring antigen density on the surface of cells. (Oldaker, 2007). It should be checked monthly or as frequently as the manufacturer of each specific flow cytometer recommends. For each bead used in checking linearity, acceptable mean fluorescence intensities should be established by running them 20 times over the course of 5 days. (Owens et al., 2000).

If more than one flow cytometer is being run in the same laboratory, it is required that the technician establishes equivalency of instrument output and monitor is at least 2 times a year (College of American Pathologist, Commission of Laboratory Accreditation, 2005). This is achieved by running the same stained samples on both machine and comparing results for equivalency (Owens et al. 2000). This is known as instrument correlation, table 1. shows an instrument correlation study carried out on 17 flow cytometers with the same samples

Table 1. (Oldaker 2007)

Example of an inter-instrument correlation quality control document using a stained sample run on seven instruments correlating the mean fluorescent intensity (MFI) of all markers. Categoric concordance of each marker mean required and fluorescent intensity concordance is required for acceptance

Inter-instrument correlation—leukemia/lymphoma

Date:_____

Instrument #	FC1	FC2	FC3	FC4	FC5	FC6	FC7	Concordance
Antibody	MFI	MFI	MFI	MFI	MFI	MFI	MFI	P = pass, F = fail
Kappa	N	N	N	N	N	N	N	P
Lambda	N	N	N	N	N	N	N	P
HLA-DR	N	N	N	N	N	N	N	P
CD2	N	N	N	N	N	N	N	P
CD3	N	N	N	N	N	N	N	P
CD4	N	N	N	N	N	N	N	P
CD5	N	N	N	N	N	N	N	P
CD7	N	N	N	N	N	N	N	P
CD8	N	N	N	N	N	N	N	P
CD10	N	N	N	N	N	N	N	P
CD11B	N	N	N	N	N	N	N	P
CD11C	MOD	MOD	MOD	MOD	MOD	MOD	MOD	P
CD13	DIM	DIM	DIM	DIM	DIM	DIM	DIM	P
CD14	N	N	N	N	N	N	N	P
CD16	N	N	N	N	N	N	N	P
CD19	N	N	N	N	N	N	N	P
CD20	N	N	N	N	N	N	N	P
CD22	N	N	N	N	N	N	N	P
CD23	N	N	N	N	N	N	N	P
CD33	MOD	MOD	MOD	MOD	MOD	MOD	MOD	P
CD34	N	N	N	N	N	N	N	P
CD38	DIM	DIM	DIM	DIM	DIM	DIM	DIM	P
CD45	DIM	DIM	DIM	DIM	DIM	DIM	DIM	P
CD56	N	N	N	N	N	N	N	P
CD64	N	N	N	N	N	N	N	P
CD117	DIM	DIM	DIM	DIM	DIM	DIM	DIM	P
	Acceptability criteria Categoric Concordance				Legend			
					BRI = BRI DIM = DIM MOD = MOD NEG = NEG	BRI, bright DIM, dim MOD, moderate NEG, negative Normal sample is non malignant case		

4.3. Reagent/antibody quality control

It is up to each clinical laboratory to establish the optimum antigen-antibody ratio for validation of each assay. This is especially important in case of lymphoma/leukaemia immunophenotyping, if too little antibody is present for a fixed amount of antigen it will result in a “dim” fluorescence intensity leading to erroneous interpretation of the assay. The optimisation is performed by titration using a 5-point, 2-fold serial dilution of the manufacturers recommendation. The titre exhibiting the highest signal-to-noise ratio is the optimum titre to use. (Purvis and Stelzer, 1998). When using a new antibody, a known positive sample should be identified for validation. Mostly this can be achieved by using normal blood samples as they contain cells that will express the target antigen, for example, normal T-cells express CD3, normal myeloid cells express CD33. Validation of antibodies targeting these antigens is straight forward. In cases where it is not easy to find positive samples, cryopreserved cells or commercial controls can be used. (College of American Pathologist, Commission of Laboratory Accreditation, 2005). In Addition to antibody verification, all other reagents that will be used in the assay must be validated prior to use. This includes buffers prepared by manufacturers or the lab, lysing reagents and any other used. (Oldaker, 2007).

4.4. Viability

Regarding immunophenotyping with flow cytometry, the viability is the percentage of cells that are alive and therefore suitable for analysis. Assessment of viability is critical in assessing leukaemia and lymphoma samples because cell membrane integrity affects the antigen expression. (Oldaker, 2007). Analysis of dead cells can lead to misdiagnosis due to non-specific binding of antigens. We must identify the non-viable cells so a gating strategy can be established to exclude data collection from them. This is achieved using a fluorescent dye which will be taken up by the dead cells and identified by the instrument. Commonly used dyes are 7-amino-actinomycin D (7-AAD) (Schmid et al., 1992) or propodium iodide (PI) (Sasaki et al, 1987). Figure 6. demonstrates a gating strategy being employed using two common fluorescent dies for dead cell discrimination

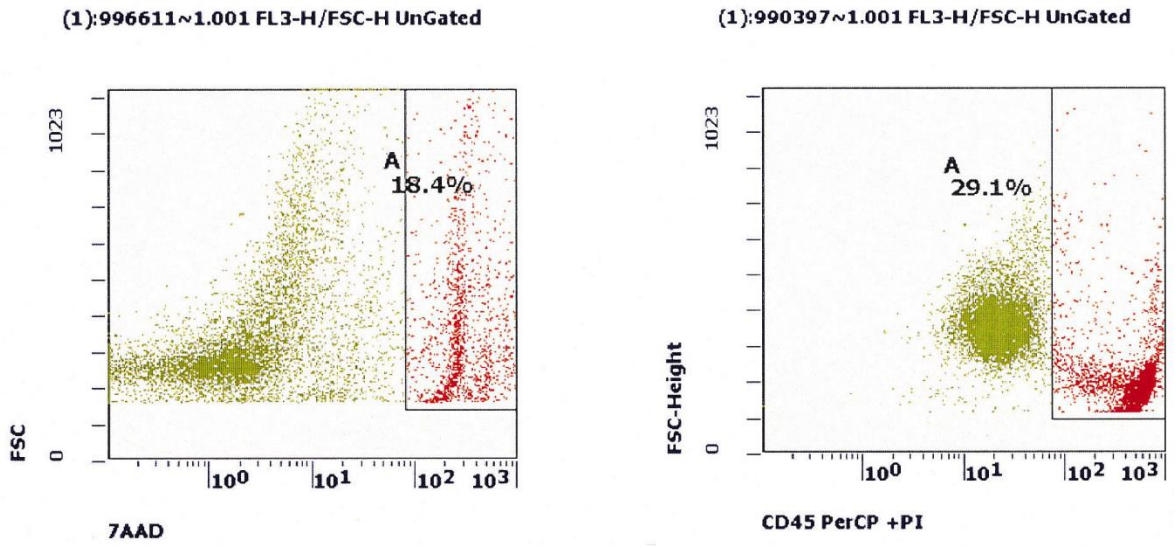

Figure 6.:. Dead cell discrimination on two lymphoma tissues. (A) 7-AAD vs. Forward Scatter with 18.4% dead cells. (B) CD451PI vs. FSC with 29.1% dead cells. (Owens et al., 2000)

4.5. Isotype controls

Antibodies can non-specifically bind to molecules other than their target antigen binding site, usually cellular proteins. This undesirable binding results in meaningless background fluorescence being picked up which can lead to false interpretation of the results. (Bushnell, 2016. http://expertcytometry.com/what-is-an-isotype-control/). Isotype controls are a type of negative control that feature the same FC-region as the antibody of interest but with a variable region that should not bind antigen. Any fluorescence monitored from this isotype control antigen can be attributed to non-specific binding and can be deducted as background noise (absoluteantibody.com focus on negative and isotype controls). Generally, commercially available antigens have been selected by screening for the IgG class as this

usually exhibits the least non-specific binding. We must keep in mind the fact that some IgG subclasses will be more problematic as they exhibit more non-specific binding. In general, the order of 'stickiness' goes IgG2b>IgG2a>IgG1. (Owens et al., 2000). Due to their carbohydrate structure, some antigenic targets (CD15 and CD57) will only generate IgM class antibodies. IgM commonly exhibits high levels of nonspecific binding and negative isotype control will be particularly important in this case. Another time that isotype control is highly recommending is for cytoplasmic staining. Non-specific staining is heavily influenced by cell size. (Jacobberger and Bauer, 2000).

4.6. Data analysis and interpretation

Gating is one of the most important processes in multi-parameter data analysis. Figure 7. compares results from the same assay but with different gating strategies. The left panel shows gating by forward scatter vs side scatter. The cell populations are indistinguishable from one another. In the right-hand panel, CD45 perception is plotted against side scatter. 3 distinct cell populations are visible, this is clearly the correct gating strategy to employ in this case. Careful choice in gating strategy is an essential aspect of the interpretation of flow cytometry.

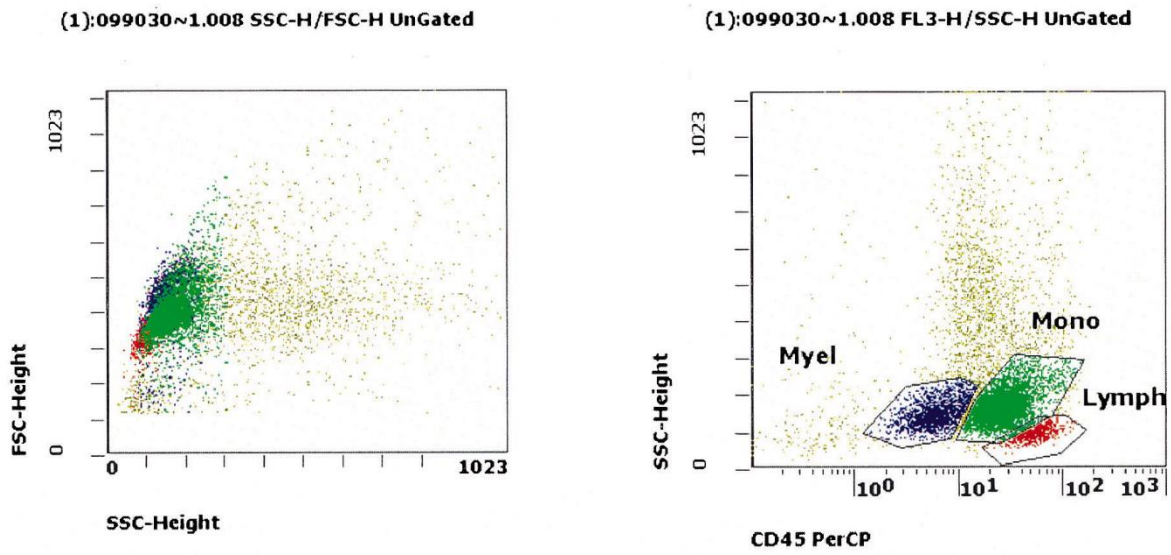

Figure 7.: Comparing Gating Strategies (Owens et al., 2000)

The final interpretation of flow cytometric data depends on an experience diagnostic interpreter, usually a pathologist, reviewing all data available. Drawing conclusions from flow cytometry is an experienced based skill. The final interpretation should include evaluation of every aspect of the assay results. (Davis et al. 1997)

5. Materials and methods

5.1. Patients and samples

Samples were collected in the Veterinary Haematology and Oncological Centre, Budapest, between 2015 and 2017. Fifteen canine patients were included in this study (7 males and 8 females) with multicentric lymphoma. The mean age of the dogs was 7.2 years (±2.4). The breed of the dogs was diverse (6 mongrels, Bull mastiff, Bichon havannese, Yorkshire terrier, Argentine dog, French bulldog, Weimaraner, Doberman, Hungarian pointing dog, Labrador retriever) (Table 2.)

The initial diagnosis was made by examination, blood tests (complete blood count, clinical chemistry profile, acid-base analysis), two-sided chest x-ray and abdominal ultrasonography. All dogs were staged and sub-staged according to the scheme established by the World Health Organization (WHO) (Moulton and Harvey, 1990) (Table 2.)

Table 2.: Clinical stage and sub-stage at the time of biopsy of the 15 canine patients.

case no	breed	age	Sex	subtype of lymphoma (IHC)	Stage	Sub-stage
2838-3088	mongrel	6.49	male	B-cell lymphocytic lymphoma	5	b
3956-4335	bull mastiff	8.26	female	diffuse large B-cell lymphoma	4	a
1563-1675	bichon havannese	9.35	female	diffuse large B-cell lymphoma	3	b
4049-4433	yorkshire terrier	8.91	male	diffuse large B-cell lymphoma	4	a
4424-4828	argentine dog	6.25	male	diffuse large B-cell lymphoma	4	b
4772-5172	mongrel	7.94	male	large B-cell lymphoma	4	a
4796-5196	french bulldog	4.67	male	diffuse large B-cell lymphoma	5	a

4819-5221	mongrel	9.27	female	diffuse large B-cell lymphoma	4	b
4923-5923	weimaraner	6.56	female	periferial T-cell lymphoma	4	a
5020-5433	doberman	4.42	male	large cell immunoblastic lymphoma	4	a
5090-5508	mongrel	11.02	female	diffuse large B-cell lymphoma	4	a
5132-5553	mongrel	11.09	female	diffuse large B-cell lymphoma	4	a
5343-5749	mongrel	3.94	female	diffuse large B-cell lymphoma	4	a
5351-5756	hungarian pointing dog	7.25	female	diffuse large B-cell lymphoma	4	a
5546-5945	labrador retriever	3.03	male	diffuse large B-cell lymphoma	5	a

Lymph node samples were collected by fine needle aspiration (FNA) from prescapular or popliteal lymph node. Tumours were classified according to WHO classification by immunhistopathology. We compared the immunhistopathology results with the flow cytometric results (Table 3.).

5.2. Sample preparation

Tumour samples were collected by fine needle aspiration (FNA) from lymph nodes of a dogs with suspected lymphoma. FNA samples were immersed into dissociation medium containing Dulbecco's Modified Eagle Medium (DMEM), 200 U/ml collagenase type II, and 0.6 U/ml dispase (Gibco, Life Technologies, USA). Cells were incubated approximately 30 minutes at 37^{o}C, 1 minute vortexing in every 15 minutes. Cells were separated by 40 µm cell strainer. Isolated cells were centrifuged at 300 g.

Lymph node and bone marrow samples were taken under general anesthesia. The dogs were anesthetized (propofol /: AstraZeneca Co., Cambridge, UK / 5 mg/bwkg iv., isoflurane

/Abbott Ltd., Budapest, Hungary / 1.5–2.5 V/V%, fentanyl / Gedeon Richter Plc., Budapest, Hungary / by constant rate infusion 0.01 to 0.04 mg/bwkg/h) and one of the enlarged lymph nodes was excised for routine histological and immunohistochemical examination. Bone marrow aspirates were taken from the iliac crest (*crista iliaca externa*) using a Jamshidi needle. The aspirates were smeared and stained with a conventional panoptic procedure (May-Grünwald and Giemsa /Sigma-Aldrich Co., Saint Louis, Missouri, USA/).

5.3. Immunophenotyping

We used CD (cluster of differentiation) markers to determine the immunophenotype of the samples. There were 3 CD markers for every sample (CD3 T cell lymphocyte marker, CD21 B cell lymphocyte marker and CD45 pan-leukocyte marker) and there were other CD markers in some cases (CD11/18 marker for monocytes and macrophages, CD34 recognizes endothelial cells and haematopoietic stem cells). We also used the isotype controls of the CD markers (mIgG1, rIgG2a, rIgG2b). To investigate whether the patient had diffuse large B cell lymphoma or T cell lymphoma we needed at least 2 markers (CD3, CD21) and their isotype controls (mouse IgG1). 2 test tubes (T1, T2) were prepared with 1.200.000 cells in 200 µl Phosphate-buffered saline (PBS). The isotype controls (mIgG1-FITC, mIgG1-PE) were added into the T1 tube and the CD3-FITC, CD21-PE markers were added into the T2 tube. These tubes were incubated for 30 minutes at 37°C. The reaction was stopped by 1 ml of cold (4^{o}C) PBS and 5 minutes spinning at 300 g, the cell pellets were re-suspended in 270 µl PBS containing 0.8 µl 7-AAD (7-aminoactinomycin D, Sigma-Aldrich, USA). Samples were stored on ice, and were measured within 4 hours on a FACScan flow cytometer (Becton Dickinson Biosciences, San Jose, CA, USA). To specify the immunophenotype of the dogs, we used the other monoclonal antibodies and their isotype controls with the same method.

5.4. FACS analysis

Lymphoid cells were selected by their size and granularity (gate 1=R1); viable cells were gated based on 7-AAD fluorescence (gate 2=R2). 10 000 7-ADD negative cells were collected. The immunophenotyping dot plots present CD3-CD21- cells, CD3+CD21- cells, CD3-CD21+ cells or CD3+CD21+ cells. We used the "BD cellquest" software which shows the percentage of cells which fall into the different gates.

In our first analysis, we compared the percentages measured by the flow cytometer with the result of the immunhistopathology (Table 3.). Table 4a. shows the results for the

immunophenotyping of four samples measured with three parallel markers. Each sample is repeated three times. Table 4b. shows our calculated values of these samples averages, standard deviations and coefficients of variation. Lastly, we performed a test of precision over time. Multiple samples were measured every 5 minutes, over a 25-minute period to measure how the results varied with time. (Table 5.)

5.5. Histopathology and immunohistochemistry

Tissues were routinely processed and sectioned at 3 microns. They were stained with hematoxylin and eosin. Serial sections of each case were placed on positively charged slides for further immunohistochemical labelling. All cases were labelled immunohistochemically for B and T cell antigens following a routine protocol. For immunophenotyping of T cells, CD3 CD79a was used. For B-cell labelling, rabbit anti-CD316 or mouse anti-CD79a17 was used (DAKO Ltd., High Wycombe, UK). After diagnosing lymphoma, the tumours were graded according to the WHO classification scheme applied for canine lymphoma (Valli et al., 2011). The cases were grouped into diagnostic categories: low-grade B-cell; high-grade B- and T-cell; low-grade T-cell; moreover, we subdivided the types (Valli et al., 2013). Grouping was determined by histological grade (based on mitotic rate/400 field, with low-grade 0–5, intermediate 6–10, and high-grade >10) (Valli et al., 2013). The grading system was based on the original article written in 1986 (Carter et al., 1986) with additional further suggestions (Valli et al., 2013). To assess the proliferation status of the tumour the percentage of Ki67 positive cells was calculated after immunostaining the sections with the Ki67 marker MIB-118 (DAKO Ltd., High Wycombe, UK). Approximately, minimum of 150 (up to 500) cells were counted on each slide in 5 different zones of the section.

5.6. Statistical analysis

To compare the results of immunophenotyping by flow cytometry or immunohistopathology, we used the Pearson's correlation by R Statistic program. The Pearson correlation coefficient is a measure of the strength of the linear relationship between two variables. (Jacob Benesty et al., 2009) We correlated the two types of measurements (IHC with FACS) (Table 3).

Calculation of coefficient of variation (CV): standard deviation divided by mean, multiplied by 100 (expressed as %) with repeated measurements by three parallel dilutions (Table 4.) and by repeated measurements over time (Table 5.).

6. Results

6.1. Flow assisted cell sorting Vs Immunohistochemistry

In our first study, we compared the immunohistochemistry (IHC) results with our FACS measurements. We found that the correlation between the T-cell marker ($CD3_{IHC}$ compared to $CD3_{FACS}$) and B-cell markers ($CD79a_{IHC}$ compared to $CD21_{FACS}$)- measurements were, R=0.910931863 ($p<0.00001$) and R=0. 604082148 ($p=0.00236$), respectively. (Table 3.)

Table 3: Percentages of the T-cell marker ($CD3_{IHC}$ compared to $CD3_{FACS}$) and B-cell markers ($CD79a_{IHC}$ compared to $CD21_{FACS}$).

case no	$CD3_{FACS}$ (%)	$CD21_{FACS}$ (%)	$CD3_{IHC}$ (%)	$CD79a_{IHC}$ (%)
2838-3088	3	94	30	70
3956-4335	0	22	15	85
1563-1675	2	73	10	90
4049-4433	1	60	11	89
4424-4828	1	17	35	65
4772-5172	1	87	12	88
4796-5196	0	61	19	81
4819-5221	0	98	18	82
4923-5923	99	0	93	7
5020-5433	2	60	13	87
5090-5508	2	21	38	62
5132-5553	4	41	12	88
5343-5749	1	60	20	80
5351-5756	2	80	17	83
5546-5945	3	61	18	82
mean	8.07	55.67	24.07	75.93
median	2.00	60.00	18.00	82.00
SD	24.33	28.74	20.22	20.22
R (B-cell marker)	0.910931863	R (T cell marker)	0.604082148	

Two-tailed probability:	p< 0.00001	Two-tailed probability:	P=0.002362	

6.2. Quantifying Imprecision

During the second analysis we calculated Imprecision (CV percentages) of FACS analysis by determining the immunophenotype of four different samples by labelling and measuring three parallels in different tubes (Table 4a.). Imprecision values are showing great CV differences with those markers which had markedly small value (Table 4b).

Table 4a: The results of three parallel marking with FACS CD markers in case of four tumour samples.

case no	sample	date	CD5-PE %	CD3-FITC %	CD21-PE %	CD45-FITC %
5782-6192	blood	9.26.2017		1	0	81
		9.26.2017		0	0	57
		9.26.2017		1	1	72
5798-6211	FNA	10.2.2017	0		68	44
		10.2.2017	2		46	44
		10.2.2017	3		60	43
5818-6227	FNA	10.6.2017		1	96	98
		10.6.2017		1	93	82
		10.6.2017		1	92	54
3907-6230	FNA	10.11.2017		1	35	37
		10.11.2017		0	15	19
		10.11.2017		1	45	47

Table 4b: The coefficient of variation (CV%) in four tumour samples with different FACS CD markers (three parallel marking)

Case No	CD marker (%)	Source - sample	Mean	SD	CV%
5782-6192	$CD21_{FACS\ (}$%)	Blood	0.33	0.47	141.42

5782-6192	$CD45_{FACS}$ (%)	Blood	70.00	9.9	14.14
5798-6211	$CD21_{FACS}$ (%)	FNA	58.00	9.09	15.68
5798-6211	$CD45_{FACS}$ (%)	FNA	43.67	0.47	1.08
5798-6211	$CD5_{FACS}$ (%)	FNA	1.67	1.25	74.83
5818-6227	$CD21_{FACS}$ (%)	FNA	93.67	1.70	1.81
5818-6227	$CD45_{FACS}$ (%)	FNA	78.00	18.18	23.31
3907-6230	$CD21_{FACS}$ (%)	FNA	31.67	12.47	39.39
3907-6230	$CD45_{FACS}$ (%)	FNA	34.33	11.59	33.74

The mean CD-values and the imprecision CV %-s significantly inversely correlated with each other (r= -0,79870906, p= 0.00994828) (Figure 8).

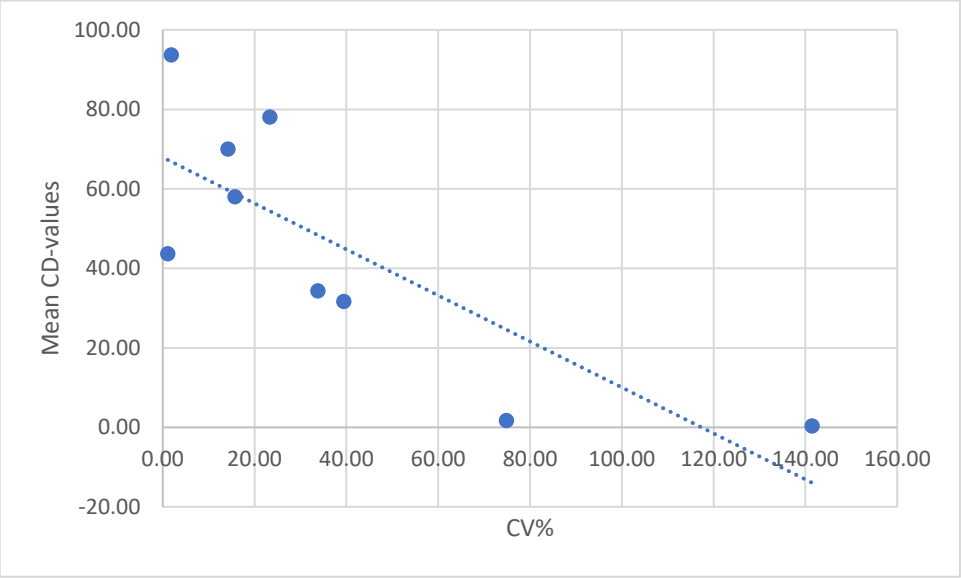

Figure 8.: Comparison between imprecision CV%-s and mean CD-values

6.3. Quantifying Imprecision with repetition

The third part of our study was designed to evaluate imprecision of FACS analyses in time dependent manner. CV % was also determined. We measured the percentage of the CD markers at the zero minute and every five minutes thereafter. We repeated the measurements 5 times, every 5 minutes after the initial combination with the CD markers. We also see large CV values in cases where the marker measurements had very low averages. We performed one sample Student's t-test (which suspects data of non-equal variance) to show the differences between the measurements of the "0" time, and the different time points (5, 10,

15, 20, 25 min.). The 10, 15 and the 25 min. time point measurements showed significant difference compared to the "0" time point (p=0.0105). The immunophenotype CD marker positivity percentages showed marked and gradual decrease in different time points (Table 5.). The percentage of the labelled cells decreased (Figure 9., Figure 10., Figure 11.) but the dead cell ratio increased (Figure 12., Figure 13..).

Table 5: The coefficient of variation (CV%) in case of different tumour samples (FNA, blood, bone marrow) and with different FACS CD markers. (repeated measurement ten times)

		0. min	5. min	10. min	15. min	20. min	25. min	Mean	SD	CV%
FNA test 1	$CD21_{FACS}$ (%)	60	0	0	0			15.00	25.98	**173.21**
	$CD45_{FACS}$ (%)	98	91	50	22			65.25	30.98	**47.48**
FNA test 2	$CD21_{FACS}$ (%)	85	78	50	48	7	18	47.67	28.44	**59.67**
	$CD45_{FACS}$ (%)	95	87	75	53	49	35	65.67	21.53	**32.79**
blood test 1.	$CD3_{FACS}$ (%)	89	88	84	80	80	48	78.17	13.93	**17.83**
blood test 2.	$CD3_{FACS}$ (%)	88	88	86	82	80		84.80	3.25	**3.83**
bone marrow test 1.	$CD34_{FACS}$ (%)	12	7	5	0			6.00	4.30	**71.69**
	$CD45_{FACS}$ (%)	13	8	8	3			8.00	3.54	**44.19**
bone marrow test 2.	$CD34_{FACS}$ (%)	11	2	0				4.33	4.78	**110.41**
	$CD45_{FACS}$ (%)	13	3	0				5.33	5.56	**104.21**
	T test		0.0729	0.0105	0.0121	0.1263	0.0187			

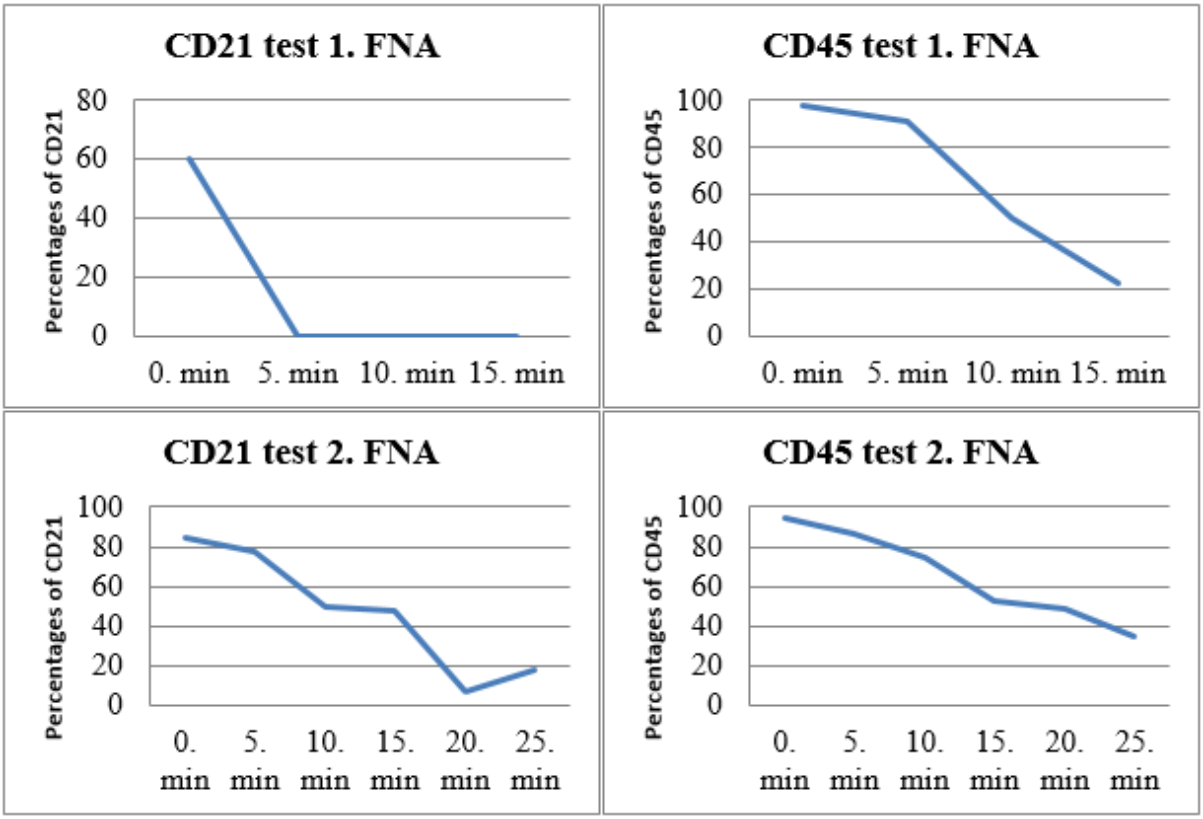

Figure 9..: The graphs present the decreasing percentages of CD markers (CD21 and CD45) in case of FNA sample.

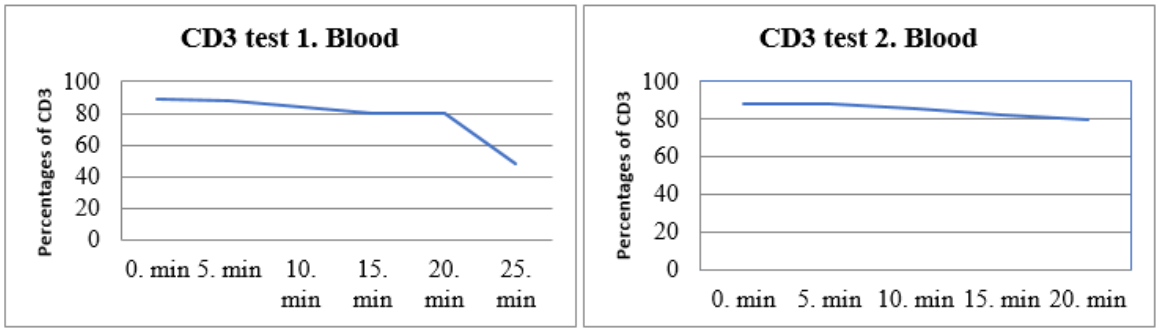

Figure 10.. The graphs present the decreasing percentages of CD3 marker in case of blood sample.

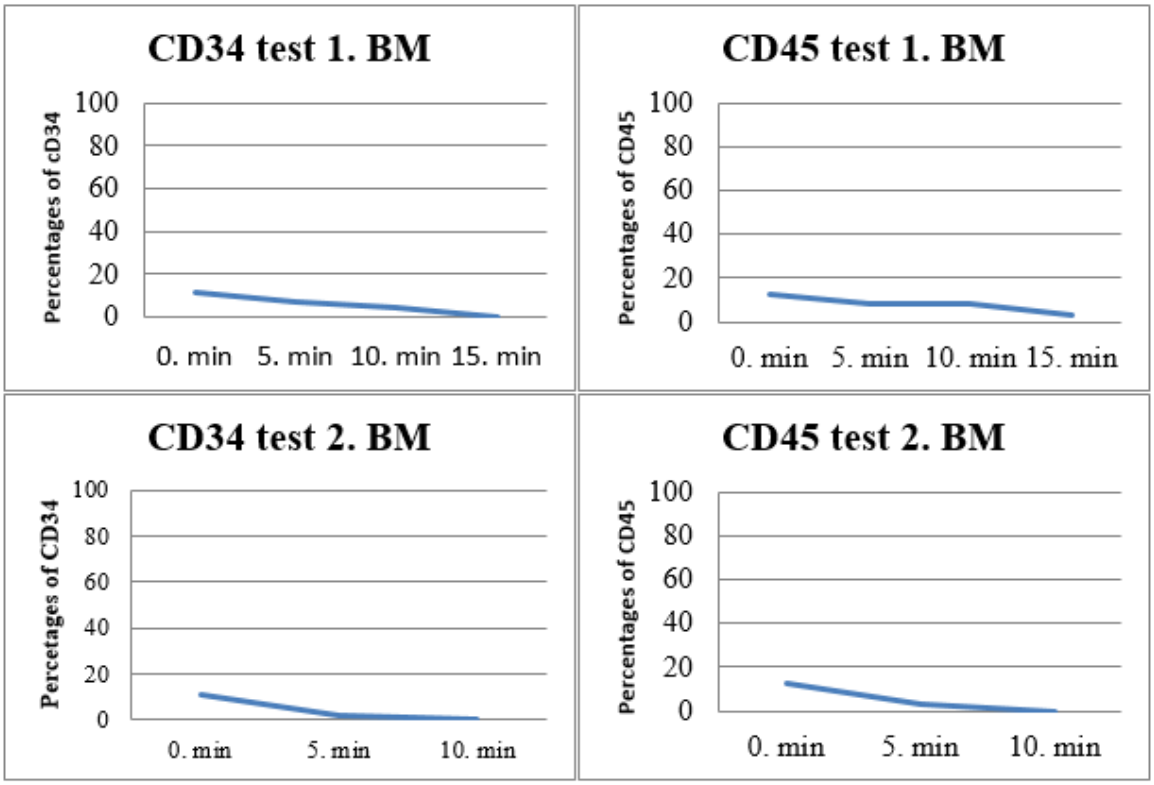

Figure 113.: The graphs present the decreasing percentages of CD markers (CD34 and CD45) in case of bone marrow sample.

Figure 12..: The dot plots of the immunophenotyping by the flow cytometer at "0" time point in case of blood sample. This figure shows the FSC-SSC panel in the first column with R1 gate (R1=lymphoid cells). The second panel shows the living and dead cell ratio with R2 gate (R2=living cells gate). The third panel presents the percentages of isotype controls (mIgG1-FITC, mIgG1-PE) and CD3-CD21 makers.

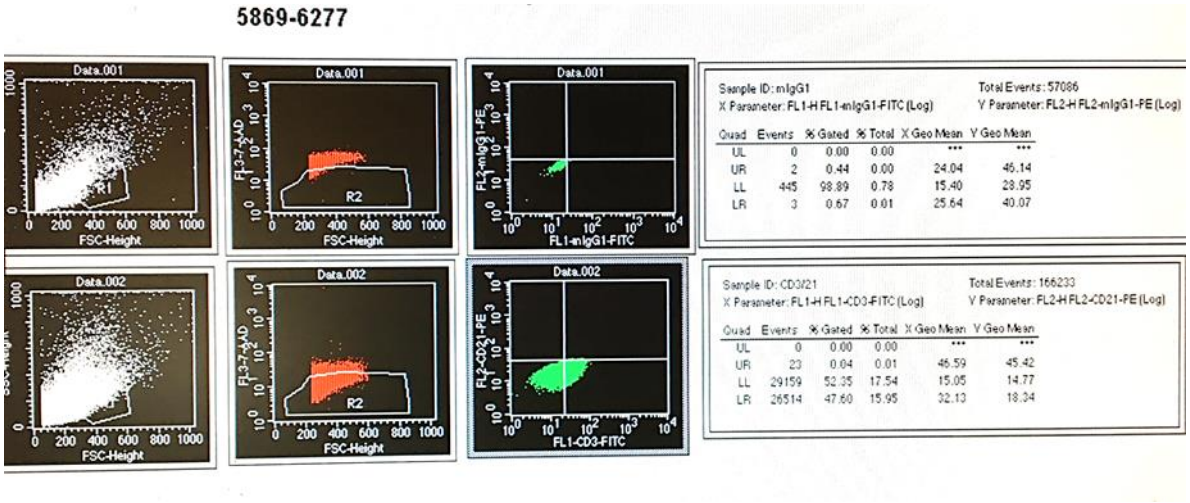

Figure 13.: The dot plots of the immunophenotyping by the flow cytometer at 25 min. time point in case of blood sample. This figure shows the FSC-SSC panel in the first column with R1 gate (R1=lymphoid cells). The cell population is aggregated compared to the dot plot of 0 min. time point. The second panel shows the living and dead cell ratio with R2 gate (R2=living cells gate). The dead cell ratio is markedly increased. The third panel presents the percentages of isotype controls (mIgG1-FITC, mIgG1-PE) and CD3-CD21 makers. The percentage of CD3 marker significantly decreased compared to the percentages 0 min. time point.

7. Discussion

Looking at the table of results seen in heading 6.1. we can see a good correlation between the results from our FACS study and the "gold standard" immunohistopathology results. The comparison between the two methods was significant, as is shown by the calculated Pearson correlation coefficients. This correlation is particularly pronounced in the T-cell lymphoma diagnosis comparison where the R value is 0.88. We have proved that FACS analysis is a reliable method to distinguish B-cell and T-cell lymphoma/leukaemia.

Diagnosis of lymphoma and leukaemia using immunohistopathology is a slow process, requiring days to yield results. FACS can be completed in just some hours. With the confirmation of its reliability, it will be extremely useful for fast diagnosis. Treatment protocols will be decided more quickly and therapy will begin sooner, hopefully leading to more success in treating these deadly diseases.

Under heading 6.2. we see the results from our test designed to calculate how precise our assay is. As explained in the method, we measured the same samples 3 times, having marked

them with four different mAb's. The results from these repeated assays are seen in table 4a. The calculations describing the tests precision are seen in 4b. In our results, we have quite a large range of CV's. In two thirds of cases, the CV is below 34%. This shows our process in quite precise. In two of the cases, the CV is up above 74%. This clearly is an unreasonably high level of imprecision and would question the validity of our results. In both cases, the mean calculation is below 2%. Figure 8. shows how the CV is negatively correlated with the mean (r= -0,79870906, p= 0.00994828). This finding means that the sensitivity of our method is limited and it decreases markedly when the CD measurement is lower than 30%.

Our results for the measurement of precision over time were surprising. We did not expect to see such significant differences between the 10-, 15- and 25-minute time point measurements compared with the 0-minute time point measurement. Figures 12. and 13. show how the number of dead cells counted markedly increased with time. We hypothesise that the increasing number of dead cells with time is caused by the FACS cleaning fluid. Unfortunately, the use of this fluid is necessary between the measurements to prevent sample contamination.

During repeated measurements, the number of dead cells is undoubtedly and markedly increasing. As mentioned in the Literature review analysis that the dead cells can lead to misdiagnosis due to non-specific binding of antigens and possibly cause false results. We must always take care to consider this fact if carrying out repeat assays on the same sample.

8. Summary

Flow cytometry is a method which for a long time, has been used for the diagnoses of non-Hodgkin lymphoma in human medicine. In the last decade, it has become a routine diagnostic tool for diagnosis of tumours of haematopoietic and lymphoid tissue origin in veterinary medicine.

The aim of this paper was to demonstrate the validity of FACS for the diagnosis of canine lymphoma.

Fifteen canine patients were included in this study with multicentric lymphoma. All dogs were staged and sub-staged. The immunophenotype of the patients was determined by FACS analysis and by immunhistochemistry (IHC). We compared IHC results with our FACS measurements. We also calculated averages, standard deviations and coefficients of

variation of four different samples by labelling and measuring three parallels in different tubes. We performed a test of precision over time. Multiple samples were measured every 5 minutes, over a 25-minute period to measure how the results varied with time.

The comparison between the two methods (IHC and FACS) was significant ($p<0.00001$, $p=0.00236$). Imprecision values, in case of four tumour samples, are showing great CV differences with those markers which had markedly small mean value. The mean CD-values and the imprecision CV %-s significantly inversely correlated with each other ($r= -0,7987$). The imprecision quantification at 10, 15 and the 25 min. time point measurements showed significant difference compared to the "0" time point ($p=0.0105$). During the FACS analysis the percentage of the labelled cells decreased but the dead cell ratio increased.

In our study we have proven that the FACS analysis is accurate by demonstrating a strong correlation with previously validated methods, in this case immunohistopathology. We can confidently use this method in a clinical environment, distinguishing B-cell and T-cell lymphoma/leukemia and helping to make decisions on treatment protocols. The sensitivity of the method is limited when the percentage of CD marker is less than 30% and the FACS cleaning fluid can influence the repeated measurements using the same FACS tubes. Baring this in mind, I still strongly feel that FACS has an important place in routine diagnostic methods of canine lymphoma and leukaemia.

9. Bibliography

- Allen, J.R., Earp, R., Farrell, C.E., Gruemer, H.D., 1969. *Analytical bias in a quality control scheme*. Clin. Chem. 15, 1039.
- American association for clinical chemistry, *how reliable is laboratory testing?* (2008) [Available at] http://labtestsonline.org.uk/understanding/features/reliability/start/1/
- Benesty J., Chen J., Huang Y., Cohen I. (2009) *Pearson Correlation Coefficient. In: Noise Reduction in Speech Processing. Springer Topics in Signal Processing*, vol 2. Springer, Berlin, Heidelberg
- Boumsell, (2016). *The HLDA Workshops* [Available at] http://www.hcdm.org/index.php/about-hcdm
- Bradford, 2012. *Flow Cytometry Basics Part 1* [Available at] https://www.youtube.com/watch?v=y9-mojIXU_I
- Bushnell, 2015. *An Introduction to spectral overlap and Compensation protocols in flow cytometry* [Available at] https://bitesizebio.com/13696/introduction-to-spectral-overlap-and-compensation-flow-cytometry-protocol/
- Carter RF, Valli VE, Lumsden JH. *The cytology, histology and prevalence of cell types in canine lymphoma classified according to the National Cancer Institute Working Formulation*. Can J Vet Res. 1986;50:154-64.
- College of American Pathologists, *Commission of Laboratory Accreditation. General inspection checklist.* Northfield (IL): College of American Pathologists; 2005.
- Davidson (2015). *Optical Microscopy Primer – Physics of Light and Colour.* Available at: https://micro.magnet.fsu.edu/primer/lightandcolor/fluoroexcitation.html (accessed 01/10/2017)
- Flatland B, Freeman KP, Friedrichs KR, Vap LM, Getzy KM, Evans EW, Harr KE. *ASVCP quality assurance guidelines: control of general analytical factors in veterinary laboratories.* Vet Clin Pathol. 2010 Sep;39(3):264-77.
- *Fundamentals of Veterinary Clinical Pathology*. 2nd ed. Ames, IA: Blackwell Publishing; 2008:3–52.
- Guzera M, Cian F, Leo C, Winnicka A, Archer J. *The use of flow cytometry for immunophenotyping lymphoproliferative disorders in cats: a retrospective study of 19 cases*. Vet Comp Oncol. 2016 Aug;14 Suppl 1:40-51. doi: 10.1111/vco.12098. Epub 2014 Jun 2.

- Henry, R.J., Segalove, M., 1952. *The running of standards clinical chemistry and the use of the control chart*. J. Clin. Pathol. 5, 305.
- Hooijberg E, Leidinger E, Freeman KP. *An error management system in a veterinary clinical laboratory*. J Vet Diagn Invest. 2012 May;24(3):458-68
- In Merriam-Webster's dictionary, 2017, https://www.merriam-webster.com, accessed 20/10/2017)
- Levy, S., Jennings, E.R., 1950. *The use of control charts in the clinical laboratory.* Am. J. Clin. Pathol. 20, 1059.
- Linnet K, Boyd JC. *Selection and analytical evaluation of methods—with statistical techniques*. In: Burtis CA, Ashwood ER, Bruns DE, eds: Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. 4th ed. Philadelphia, PA: Elsevier Inc; 2006:353–407.
- McCoy JP Jr, Carey JL, Krause JR. *Quality control in flow cytometry for diagnostic pathology, I: cell surface phenotyping and general laboratory procedures*. Am J Clin Pathol 1990; 93(Suppl 1):S27–37.
- Moulton JE, Harvey JW (1990) Tumours of lymphoid and hematopoetic tissue. In: Moulton JE (ed) *Tumours of Domestic Animals*. 3 rd ed., University of California Press, California, pp 231-301.
- Nandkishor, 2015, *Monoclonal Antibodies: Production, Advantages and Limitations*. [Available at] http://www.biologydiscussion.com/antibodies/monoclonal-antibodies-production-advantages-and-limitations/10068 (accessed 28/09/2017)
- Novo and Ormerod (2008). *Flow Cytometry: A Basic Introduction.* [Online] Available at: http://flowbook.denovosoftware.com/ (accessed 01/10/2017)
- Owens et al. (2000), *Validation and quality control of immunophenotyping in clinical flow cytometry* (paper saved on pc)
- Paschotta, 2008) article on *'dichroic mirrors'* in the Encyclopedia of Laser Physics and Technology, accessed on 2017-11-21
- Purvis N, Stelzer G. *Multi-platform, multi-site instrumentation and reagent standardization. Cytometry* 1998;33:156–65
- Sasaki, D., Bumas, S., Engleman, E., 1987. *Discrimination of viable and nonviable cells using propidium iodide in two color immunofluorescence*. Cytometry 8, 413–420.

- Schmid, I., Krall,W., Uittenbogaart, C., Braun, J., Giorgi, J., 1992. *Dead cell discrimination with 7-amino-actinomycin D in combination with dual color immunofluorescence in single laser flow cytometry*. Cytometry 13, 204–208.
- Stewart, C.C., Stewart, S.J., 1994. *Cell preparation for the identification of leukocytes.* In: Darzynkiewicz, A., Robinson, J., Crissman, H. (Eds.). Methods in Cell Biology, Vol. 41. Academic Press, New York, pp. 39–60.
- US Centers for Medicare & Medicaid Services (CMS). *Medicare, Medicaid and CLIA programs; laboratory requirements relating to quality systems and certain personnel qualifications. Final rule*. Fed Regist 2003;16.
- Valli VE, Kass PH, San Myint M, Scott F. *Canine lymphomas: association of classification type, disease stage, Tumour subtype, mitotic rate, and treatment with survival*. Vet Pathol. 2013;50:738-748.
- Valli VE, San Myint M, Barthel A et al. *Classification of canine malignant lymphomas according to the World Health Organization criteria*. Vet Pathol. 2011;48:198-211.
- Ward et al. 1999, *Large scale production of monoclonal antibodies* [Available at] https://www.ncbi.nlm.nih.gov/books/NBK100189/
- Westgard (2009). *QC – The Planning Process*. [Available at] https://www.westgard.com/lesson3.htm (Accessed 18/10/2017)
- Westgard and Barry (2009). *Basic QC Practices*. [Available at] https://www.westgard.com/qcapp10.htm (Accessed 20/10/2017)
- Westgard JO. *Method validation—the data analysis tool kit.* In:Westgard JO, ed: Basic Method Validation. 3rd ed. Madison, WI:Westgard QC, Inc; 2008:72–80. Stockham SL, Scott MA.
- Westgard JO. *Method validation—the replication experiment*. In:Westgard JO, ed: Basic Method Validation. 3rd ed. Madison, WI: Westgard QC, Inc; 2008:114–122.
- Wittrup, K.D., Westerman, R.J., Dessai, R., 1994. *Fluorescence array detector for large-field quantitative fluorescence cytometry* 16, 206–213.
- Wooten, I.D.P., King, E.J., 1953. *Normal values for blood constituents: inter-hospital differences*. Lancet 1, 470.
- Zagursky, R.J., Sharp, D., Solomon, K., Schwartz, A., 1995. *Quantitation of cellular receptors by a new immunocytochemical flow cytometry technique*. BioTechniques 18 (3).

10. Acknowledgements

I would first and foremost like to thank Edina Karai, she has spent more hours than I am even aware, preparing samples, running the cytometer and producing the results. I am enormously grateful to her for time she spent teaching me the about flow cytometry and working on this paper.

Dr. Péter Vajdovich is the man responsible for kindling my interest in oncology and clinical laboratory science. He has always made me welcome to his haematology and oncology clinic where the equipment for this study is kept, and from where he oversaw the project. I have learnt an awful lot at his clinic and in his classes. I am extremely thankful to him.

Finally, I would like to acknowledge my parents, Lauren and Michel Herst. They have supported me throughout my education, without them I would not be where I am today.

HuVetA

ELECTRONIC LICENSE AGREEMENT AND COPYRIGHT DECLARATION*

Name: Connor Herst

Contact information (e-mail): Connorherst@hotmail.co.uk

Title of document (to be uploaded): Quality Control for Fluorescence Activated Cell Sorting in Canine Tumour Immunophenotyping

Publication data of document: published 2017

Number of files submitted: 1

By accepting the present agreement the author or copyright owner grants non-exclusive license to HuVetA over the above mentioned document (including its abstract) to be converted to copy protected PDF format without changing its content, in order to archive, reproduce, and make accessible under the conditions specified below.

The author agrees that HuVetA may store more than one copy (accessible only to HuVetA administrators) of the licensed document exclusively for purposes of secure storage and backup, if necessary.

You state that the submission is your original work, and that you have the right to grant the rights contained in this license. You also state that your submission does not, to the best of your knowledge, infringe upon anyone's copyright. If the document has parts which you are not the copyright owner of, you have to indicate that you have obtained unrestricted permission from the copyright owner to grant the rights required by this Agreement, and that any such third-party owned material is clearly identified and acknowledged within the text of the licensed document.

The copyright owner defines the scope of access to the document stored in HuVetA as follows (**mark the appropriate box with an X**):

- [X] I grant unlimited online access,
- [ ] I grant access only through the intranet (IP range) of the University of Veterinary Medicine,
- [ ] I grant access only on one dedicated computer at the Ferenc Hutÿra Library,
- [ ] I grant unlimited online access only to the bibliographic data and abstract of the document.

Please, define the **in-house accessibility of the document** by marking the below box with an **X**:

[X] I grant in-house access (namely, reading the hard copy version of the document) at the Library.

If the preparation of the document to be uploaded was supported or sponsored by a firm or an organization, you also declare that you are entitled to sign the present Agreement concerning the document.

The operators of HuVetA do not assume any legal liability or responsibility towards the author/copyright holder/organizations in case somebody uses the material legally uploaded to HuVetA in a way that is unlawful.

Date: Budapest, 30 day 11 month 17 year

Author/copyright owner signature

HuVetA Magyar Állatorvos-tudományi Archívum – Hungarian Veterinary Archive *is an online veterinary repository operated by the Ferenc Hutÿra Library, Archives and Museum. It is an electronic knowledge base which aims to collect, organize, store documents regarding Hungarian veterinary science and history, and make them searchable and accessible in line with current legal requirements and regulations.*

HuVetA relies on the latest technology in order to provide easy searchability (by search engines, as well) and access to the full text document, whenever possible.
Based on the above, HuVetA aims to:

- *increase awareness of Hungarian veterinary science not only in Hungary, but also internationally;*
- *increase citation numbers of publications authored by Hungarian veterinarians, thus improve the impact factor of Hungarian veterinary journals;*
- *present the knowledge base of the University of Veterinary Medicine Budapest and its partners in a focussed way in order to improve the prestige of the Hungarian veterinary profession, and the competitiveness of the organizations in question;*
- *facilitate professional relations and collaboration;*
- *support open access.*