Serum Free Light Chain Analysis

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Introduction

In healthy individuals, plasma cells originating from the bone marrow produce immunoglobulin molecules composed of two heavy chains and two light chains. There are two types of light chains in humans. The κ light chains are encoded by the immunoglobulin κ locus on chromosome 2 and λ light chains are encoded by the immunoglobulin λ locus on chromosome 22 [1].

During B lymphocyte development, the light chains that are produced bind to heavy chains via interchain disulfide bonds and noncovalent interactions to form intact immunoglobulin molecules. However, light chains are usually produced in excess (up to 40 %) and those that do not bind to heavy chains enter the bloodstream as serum free light chains (sFLCs)[2]. The low molecular weight of the sFLCs (κ monomers: 22.5 kDa, λ dimers: 45 kDa) allows them to be filtered freely through the glomeruli. They are then almost completely reabsorbed in the proximal tubules via the megalin/cubulin scavenger receptors [3]. Trace amounts of immunoglobulins are secreted into the distal nephron and urethra along with secretory IgA as a mucosal defense mechanism, which may explain the 1–10 mg/24 h of FLCs found in the urine of normal individuals [4].

In plasma cell dyscrasias, such as in multiple myeloma (MM), the abnormally expanded clone of malignant plasma cells produces an excess of monoclonal immunoglobulins that are referred to as M-proteins or paraproteins. These M-proteins may be polymers, monomers, individual immunoglobulin chains of FLCs or heavy chains, or fragments of immunoglobulins. Very rarely occurring is the nonsecretory

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Table 1 M-proteins in multiple myeloma. [5, 6]

M-proteins	Incidence (%)	Incidence of uFLCs (%)
IgG	50	60
IgA	25	70
IgD	2	100
IgE	0.1	Most
IgM	1	100
Biclonal	1	Not applicable
Free light chain only	20	100
None detected[a]	< 1	Not applicable

[a] Defined by the absence of monoclonal immunoglobulins in serum and urine electrophoresis tests

myeloma with undetectable M-proteins [5, 6]. When excess sFLC production overwhelms the reabsorptive capacity of the kidney, elevated levels of FLCs are detected in the urine. The excessive FLCs bind to Tamm–Horsfall protein (also known as uromodulin) that is secreted in the distal tubule [7], and together, they precipitate as waxy casts [8]. The casts obstruct tubular fluid flow and disrupt basement membrane and interstitium [9], leading to a vicious cycle of deteriorating kidney function and loss of FLCs in the urine. The prevalence of each M-protein subtype and the incidence of finding urine FLCs (uFLCs) are summarized in Table 1.

History of Free Light Chain Detection

The presence of excessive uFLCs was first described in 1847 [10], when Dr. Bence Jones, a pathologist at St. George's hospital in the United Kingdom, published his findings on a patient with severe bone pain and fractures. The proteins in the patient's urine precipitated upon warming the urine from 40 to 60°C, and redissolved after heating to 75°C. Although the temperature-sensitive feature of these proteins was initially discovered by the patient's primary care providers, Dr. William MacIntyre and Dr. Thomas Watson, these proteins were named Bence Jones proteins (BJP) given the timing of publication [11]. The BJP were the first cancer marker in body fluids that were detectable by an in vitro test, a century before the discovery of any other. We now know that these temperature-sensitive BJP are only a subset of uFLCs, but the two terms have been used interchangeably. Over 150 years later, uFLCs remain an important diagnostic marker for MM but are now detected by electrophoresis and immunofixation analysis of the urine. For example, in light chain MM, the FLCs are the only M-proteins produced. Since sFLCs are constantly excreted through the kidney, they do not accumulate in the blood at a level sufficient to be detected by either serum protein electrophoresis (SPEP, analytical sensitivity of 500–2,000 mg/L) or serum immunofixation electrophoresis (sIFE, analytical sensitivity of 100–150 mg/L). By contrast, 24 h urine protein electrophoresis (UPEP)

and urine IFE (uIFE) are more sensitive than serum electrophoresis techniques and can detect FLCs in the urine at less than 20 mg/L [12]. A 24 h urine collection is always preferred over a random urine sample due to the higher protein yield and less subjectivity to the hydration status in 24 h urine samples.

However, even with additional sensitivity, the techniques of UPEP and uIFE still have limitations. First, the presence of uFLCs is the combined consequence of overproduction of sFLCs and insufficient proximal tubular reabsorption. Normal kidneys can process up to 10–30 g/24 h of FLCs [13] and in order for any uFLCs to be detected, the κ sFLCs need to reach a median level of 113 mg/L, and λ sFLCs need to reach 278 mg/L [14]. Second, the identification of monoclonal uFLCs in UPEP and uIFE is based on subjective interpretation of the electrophoresis results. Concentrated urine samples may show ladder banding (multiple, unidentified protein bands that mimic the rungs of a ladder), which may be mistakenly interpreted as monoclonal FLCs. On the other hand, polyclonal FLCs found in heavy proteinuria may mask a band of monoclonal FLC.

Another practical concern is the challenge of obtaining a 24 h urine collection. Poor compliance with sample collection significantly hampers the practicality of UPEP and uIFE [15]. Sample batching in the laboratory and the need for a subjective interpretation of results may also delay diagnosis.

Therefore, the search for alternatives to UPEP and uIFE is warranted, and sFLC quantification has emerged as a more direct and objective measurement of M-protein overproduction without the inconveniences of a 24 h urine collection [16]. Consequently, the sFLC assay is recommended by the International Myeloma Working Group (IMWG) consensus panel as an adequate substitute for uIFE [16].

Kinetics of Free Light Chains

Normal individuals have a median level of κ sFLCs of 7.3 mg/L with a 95% range spanning 3.3–19.4 mg/L, and a median λ FLC level of 12.7 mg/L (95% range 5.7–26.3 mg/L) based on data from Katzmann et al. [17, 18]. Both κ and λ FLCs are mainly cleared through the kidney and thus their kinetics are dependent on the balance between production and renal clearance. The normal production rate of FLCs is approximately 500 mg/24 h [2, 19]. The κ light chains are produced at approximately twice the rate as the larger λ light chain dimers [19]. The κ light chains are cleared with a half-life of 2–4 h at 40% of the glomerular filtration rate (GFR), while the dimeric λ light chains are cleared with a half-life of 3–6 h at 20% of the GFR. Some polymerized light chains are cleared at an even slower rate [20, 21]. Despite the complex contributing factors to variability in half-life and clearance, there is a relatively consistent balance between κ and λ levels in normal subjects (i.e., the κ:λ ratio) [18].

Normal individuals may show a rise in the half-life of both κ and λ FLCs due to minor renal impairment seen with aging. In advanced renal failure, pinocytosis by reticuloendothelial cells starts to play a major role in the removal of FLCs, and the half-life

of sFLCs can be prolonged to 2–3 days. The pinocytosis process equally absorbs κ and λ, so the half-life of κ FLCs is no longer different from that of λ FLCs [22, 23]. Increased polyclonal immunoglobulin production can result in a 30–40 fold increase of both κ and λ FLC concentrations, but the κ:λ ratio usually remains unchanged [24, 25]. In contrast, patients with monoclonal gammopathies produce excessive amounts of monoclonal FLCs, frequently accompanied by bone marrow suppression of the alternate FLC type, and thus show greater changes in the κ:λ ratio [26].

Serum Free Light Chain Quantification

Analytical Methodologies

The attempts to quantify sFLCs were initially hindered by the difficulties of producing antibodies specific to FLCs that do not cross-react with bound light chains found in intact immunoglobulins. The breakthrough in 2001 that developed such antibodies [17] finally enabled the application of a quantitative immunoassay technique in the diagnosis and monitoring of monoclonal gammopathies.

Somewhat surprisingly, the monoclonal FLCs found in patients with monoclonal gammopathies are actually heterogeneous. Genetic recombination, allotypic and isotypic variation, and somatic hypermutation of the variable regions after antigen exposure all contribute to the heterogeneity of the FLC molecules. The FreeLite® assay from The Binding Site® was the first sFLC assay developed and it uses human BJP to immunize sheep for polyclonal antibody isolation. These polyclonal antibodies can detect a wide variety of epitopes found in the monoclonal FLCs produced by patients with monoclonal gammopathies. The polyclonal antihuman-FLC antisera are adsorbed with IgG to remove antibodies that react with bound immunoglobulin light chains. The remaining antibodies therefore recognize regions that are only exposed when the light chain is in the free form. Both the constant and variable domains of the light chain contain such regions [17].

In the FreeLite® assay, polyclonal antibodies are purified and then digested with pepsin to produce the antigen-binding $F(ab')_2$ fragments. These fragments are then adsorbed onto polystyrene latex particles that generate signal when cross-linked to FLCs in serum samples. The light scatter or absorbance from these immune complexes can be measured using either nephelometry or turbidimetry on multiple platforms. Figure 1 shows the instrument design for measuring turbidimetry and nephelometry. When using rate nephelometry, the lower limit of detection for the assay has been reported to reach 1.5 mg/L for κ and 3 mg/L for λ FLCs [18], which is a substantial improvement in sensitivity compared to serum and UPEP or immunofixation [27].

Another sFLC immunoassay, the N latex FLC assay (Siemens®), was introduced in 2011 [28]. It uses mouse-derived monoclonal antibodies covalently coupled to polystyrene particles as opposed to polyclonal antibodies in the FreeLite® assay. The monoclonal antibodies used in the N latex FLC assay are specific to an epitope within a constant domain present in κ and λ FLCs and are expected to have

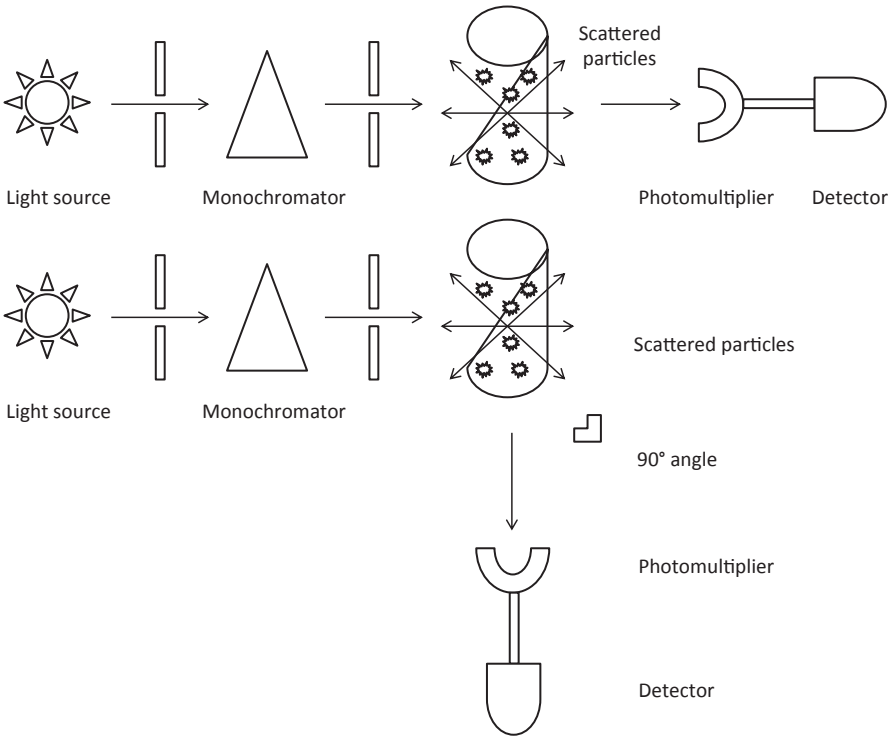


Fig. 1 **a** In turbidimetry measurement, the absorbance of light intensity through the suspended particles is quantified. **b** In nephelometry measurement, the detector is positioned at an angle different from the plane of the light source, and the intensity of the scattered light is quantified

equivalent immunoreactivity for all the light chain variable regions [29, 30]. Similar to the polyclonal assay, the antibody-targeted constant domain is only exposed when the light chains are not in a bound state. This assay uses nephelometry and has been claimed to provide improved precision across its analytical range in comparison to the FreeLite® assay [31].

A number of enzyme-linked immunosorbent assay (ELISA) based methods for sFLC measurements are also available, although not widely used in clinical labs. The design of a sandwich ELISA is shown in Fig. 2. The ELISA-based assays are able to provide high assay sensitivity and can also be automated to allow multiple sample handling [27, 30]. However, no commercial sFLC assay in the automated ELISA format is currently available.

Specimen Considerations

The measurement of FLCs in both blood and urine has been well studied. The preferred specimen for measuring FLCs is serum, although it has been shown statistically that no significant difference exists in measurements of FLCs in

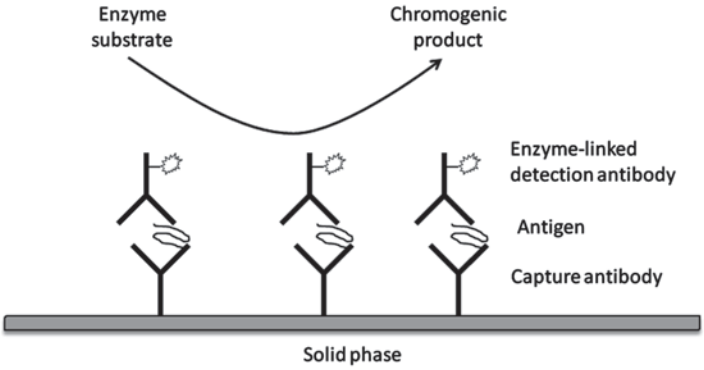


Fig. 2 In a sandwich ELISA, the solid phase is prepared with a known quantity of capture antibody. After blocking of the nonspecific binding sites, the antigen-containing sample is applied. Enzyme-linked detection antibodies are then added to the reaction after washing off the unbound antigens. The unbound antibody–enzyme conjugates are removed through washing. Enzyme substrates are then added to generate signals that are measured to determine the quantity of antigens present in the sample

lithium–heparin plasma and serum among six subjects with and without M-protein [32]. Similar findings were reported among nine MM patients with abnormal sFLC κ:λ ratios, whose serum samples with and without additives resulted in comparable measurements [33]. However, both of the above studies were limited in sample size and more data is necessary to confirm equivalence between serum and plasma measurements. In assessing sample stability, manufacturers of the FreeLite® assay have reported that samples collected for sFLC measurements are stable for up to 3 days at room temperature. The stability of the serum sample for up to 24 h at room temperature as well as at 37°C has been confirmed by one study, which also reported comparable sFLC results upon storing samples at −20°C for 12 weeks [33].

A study comparing the utility of urine FLC measurement as an alternative to urine electrophoresis of FLCs found increased sensitivity using the FreeLite® assay, although with overestimation in FLC concentrations in 75 % of the cases, evidenced by the kappa and lambda concentrations exceeding the total urine protein measurement [34]. It is unclear whether the overestimation was in part due to falsely low total urine protein measurements (secondary to the known imprecision and variability of urine protein assays), miscalibration of the serum free light chain assay, or other factors. Additionally, in light chain multiple myeloma (LCMM) cases, where there are excess FLCs in urine, uFLC measurement by FreeLite® was found to be largely uncorrelated with sFLCs following treatment [35]. Furthermore, residual renal function and reabsorption capacity by renal tubules make quantification of uFLCs an unreliable measure for diagnosis, thus leading to potential misdiagnosis in early and advanced myeloma [36]. Thus, the IMWG has not recommended the use of uFLCs for monitoring [16]. In contrast to FreeLite® assays, a new monoclonal-antibody-based assay has been shown to detect specific κ and λ FLCs in urine samples, much like the comparative uIFE method [37]. However, the utility of this monoclonal-antibody assay in urine awaits further testing and stringent quantitative analysis.

Reference Ranges

The most widely used reference ranges for interpreting sFLC levels were published in 2002 by Katzmann et al. and made use of fresh and frozen sera from 127 healthy donors between the ages of 21 and 62 (68 women and 59 men), as well as sera from 155 subjects (78 women and 77 men) between 51 and 90 years of age, for a total of 282 reference individuals. The reference interval for the sFLC κ:λ ratio, based on the central 95% of the study population, was determined to be 0.3–1.2 (Table 1). However, since 5% of the reference population is outside of the central 95% interval, the authors rationalized that using all 282 donors (i.e., 100% of reference individuals) would be of greater diagnostic value, and reported a sFLC κ:λ ratio range of 0.26–1.65. The effect of widening the reference interval increased the estimated specificity from 95 to 100% with a subtle decrease in sensitivity from 98 to 97%. The study did not find any correlation between sex or age and sFLCs. However, the authors noted that sFLC κ and sFLC λ values were generally higher above 80 years of age, but this qualitative observation was not preserved when the data were normalized to cystatin C [18]. Hence, a clear age-stratified reference interval could not be established as the age-related changes could be due to impaired renal function.

More recently, age-dependent reference intervals have been established from 169 healthy donors (73 women and 97 men) between 17 and 66 years of age [33] (Table 1). Although the reference intervals are not significantly different from those of Katzmann et al. the smaller sFLC κ was found to be generally more elevated in subjects 60 years old and above, resulting in an elevated sFLC κ:λ ratio in this subgroup. Interestingly, no apparent age-dependent trend was observed for sFLC λ in the study. The subtle age-dependent trend for sFLC κ:λ was preserved when normalized to creatinine, suggesting that the increase in sFLC κ and sFLC κ:λ ratio was not purely due to renal impairment with age [33]. However, other studies have not been able to identify such trends.

The overproduction of monoclonal FLCs in patients with proliferative plasma cell disorders can result in renal pathology [26, 38]. Such renal complications can alter the half-life and clearance of sFLCs (see section on kinetics of free light chains), thereby warranting a separate reference interval in individuals with kidney injury. Consequently, the proposed sFLC κ:λ ratio reference range for patients with renal failure is 0.37–3.1 instead of the 0.26–1.65 range reported by Katzmann et al. [18, 38]. This higher reference range increases the specificity of the assay for MM patients with renal failure from 93 to 99% without loss in sensitivity (100%) [38].

The reference values established by Katzmann et al. are also recommended by FreeLite® manufacturers and frequently cited in the literature [16, 18, 39–43]. However, the imprecision between reagent lots has led laboratories to establish their own reference ranges (Table 2). For example, a study based on a population of 162 healthy donors (87 males and 75 females) in Italy aged 18–66 years, deduced a unique set of reference ranges from those previously found by Katzmann et al. using an American population. Utilizing the FreeLite® assay and nephelometer from the same manufacturer as previous studies, a central 95% reference range for the sFLC κ:λ ratio (Table 1) was higher than previously obtained by Katzmann

Table 2 Reference ranges for serum free light chains from published studies

Study	sFLC κ (mg/L)	sFLC λ (mg/L)	sFLC κ:λ	Comments
Katzmann et al. [18] *n* = 127 healthy donors(68 women + 59 men; 21–62 years of age) and*n* = 155 donors (78 women + 77 men; 51–90 years of age)	3.3–19.4	5.7–26.3	0.3–1.2	Reference interval established from the central 95% interval. Diagnostic interval for sFLC κ:λ was 0.26–1.65 and included 100% of the reference population (see main text). sFLCs were quantified with FreeLite® reagents from The Binding Site Ltd on the DadeBehring BNII nephelometer. Intraassay and interassay coefficient of variability (CVs) were 7.9 and 8.7%, respectively, for κ sFLCs. The λ sFLC intraassay and interassay CVs were 10 and 7%, respectively. These references ranges are also recommended by FreeLite® manufacturers.
Pattenden et al.[45] *n* = 126 ostensibly healthy donors	6.2–35.3 (AU400) 4.1–41.0 (BNII)	2.7–20.6 (AU400) 11.6–45.7 (BNII)	0.7–3.4 (AU400) 0.3–1.3 (BNII)	Local reference ranges from central 95% interval. Two instruments BNII (DadeBehring BNII) and AU400 (Olympus AU 400) were used for measurements, both utilizing the FreeLite® reagents.
Hansen et al.[33] *n* = 170 ostensibly healthy individuals (73 women + 97 men;17–66 years of age)	All: 4.0–21.0 60 years: 3.4–20.9 ≥60 years: 7.5–20.8	8.1–25.0	All: 0.35–1.37 60 years: 0.36–1.26 ≥60 years: 0.53–1.60	Reference interval established from the central 95% interval. sFLCs were quantified with FreeLite® reagents from The Binding Site Ltd on the Dade-Behring BNII nephelometer Intraassay and interassay CVs were 5.3% (1.2–11 %) and 5.0% (0.45–15%) for κ sFLCs respectively. Intraassay and interassay CVs were 2.2% (0.3–5.5%) and 3.9% (0–15.5%) respectively, for λ sFLCs. Total analytical variation was 7.3 and 4.5 % for κ and λ respectively.

Table 2 Continued

Study	sFLC κ (mg/L)	sFLC λ (mg/L)	sFLC κ:λ	Comments
Altinier et al.[44] $n=162$ healthy donors (87 males + 75 females; 18–66 years of age)	4.52–22.33	4.84–21.88	0.65–2.36	References ranges were determined for females and males separately but no statistical differences were found. References ranges validated with 43 subjects with polyclonal hypergammaglobulinemia FLCs were quantified with FreeLite® reagents from The Binding Site Ltd on the Siemens BNII nephelometer. Intraassay CV was 8.56–12.18% and interassay CV was 16.09% for κ sFLCs. Intraassay CV was 9.69–16.50% and interassay CV was 16.72% for λ sFLCs.
Hutchison et al. [38] $n=142$ patients with dialysis dependent renal failure (39% males) of which 41 hadconfirmed MM (56% males)	None proposed	None proposed	0.3–3.1	Reference range for sFLC κ:λ ratio was proposed based on observation that increasing the upper limit increased specificity for multiple myeloma in patients with renal failure

et al. [44]. However, both studies show that a statistically significant relationship between sFLCs and age or between sFLCs and sex does not exist or could not be derived. The study based in Italy validated the reference values in 43 subjects with polyclonal hypergammaglobulinemia, which were within the normal population range used in the study. Upon using the sFLC κ:λ ratio of 0.26–1.65 as defined by Katzmann et al. and as recommended by the manufacturer, 6 of the 43 subjects (14%) were incorrectly classified as having possible sFLC κ clonal proliferation. Thus, validating local reference values is important for appropriate clinical evaluation [44]. Additionally, reference ranges will vary depending on the instrument used for measuring sFLCs. For example, measurement of sFLCs from identical samples using the Olympus AU 400 versus Dade-Behring BNII required instrument-specific reference ranges for appropriate interpretation (Table 1) [45].

Reference ranges are dependent on a multitude of factors originating from biological and analytical variation. The former has been assessed using index of individuality, which in its simplified form is defined as the ratio between within-subject variation and between-subject variation, and has been reported to be 0.32 and 0.23 for κ and λ, respectively, when using the FreeLite assay [33]. It should be noted that

an index of individuality <0.6 indicates that an analyte shows marked between-subject variation in the population, and as such population-based reference ranges are not as useful and may not detect all cases of monoclonal gammopathy. When the index of individuality is low, the more appropriate reference values may be the patient's own previous measurements during times of health, which practically speaking may not be available. Even if prior measurements are available, analytical variation between assays (i.e., due to lot-to-lot variation in reagents or different assay instrumentation) may render serial measurements to be of little use. In summary, defining appropriate reference ranges for each patient is challenging and may require not only local laboratory validation, but also monitoring of consistency in instrument and reagent performance, as well as serial measurement for individualized results interpretation.

Interpretation of Serum Free Light Chain Analysis

The concentration of sFLCs offers an indication of disease course. In individuals with a normal rate of sFLC production, about twice as many κ as λ light chains are produced. However, the renal clearance of the larger sFLC λ (a dimer) is slower than the monomeric sFLC κ, which results in measured sFLC κ:λ ratio around a median of 0.6 if one uses the reference range 0.26–1.65 published by Katzmann et al. (Table 1)[16, 36, 46]. A sFLC κ:λ value outside the reference interval would be considered abnormal and supports further assessment of clonality (Fig. 3). For example, using these reference ranges, sFLC κ:λ ratio >1.65 is consistent with a clonal population producing excess κ sFLCs, whereas a sFLC κ:λ ratio <0.26 is indicative of excess λ sFLC production [16, 26, 41].

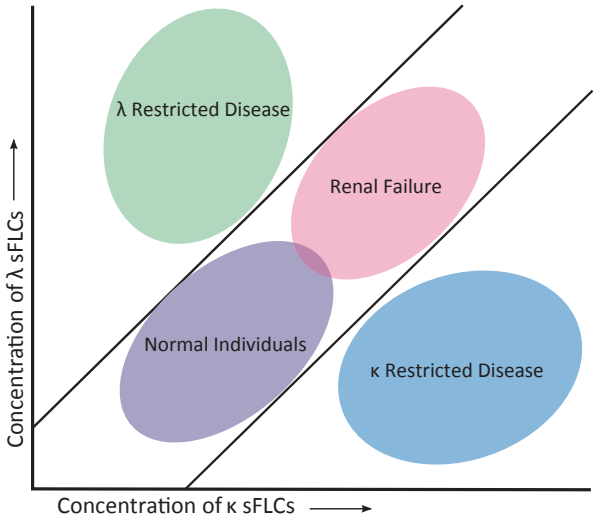


Fig. 3 Concentration-dependent relationship between κ and λ sFLCs. The *black diagonal lines* enclose regions that capture sFLC κ:λ ratios whose values fall within established reference ranges for normal individuals or individuals with renal failure. Outside this region are individuals with λ or κ restricted disease

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