Plasma separation card (HemaSep-L) in quantitative LC-MS/MS amino acids analysis

Application note, MSD-HS-05-2022 - Medical Diagnostics

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Abstract

This application note presents the use of plasma separation cards (HemaSep-L) for free amino acid (AA) analysis. In this study, we compared AAs concentrations in the fraction containing red blood cells (RBC) and in plasma obtained using a HemaSep-L separation card with results obtained for plasma fraction obtained by centrifugation. Obtained results were also compared with concentrations established in the dried blood spot (DBS) samples obtained by the classical approach. The comparison was conducted using venous blood.

It was found that HemaSep-L allows the observation of changes in the AA composition in blood plasma, which is impossible with a standard TFN screening card used for DBS analysis.

The method is fast and requires smaller amounts of samples in comparison to classical blood fractionation based on centrifugation.

Introduction

Amino acids (AAs) are essential building blocks of every known life, including humans. They play an important role in organism homeostasis, so their circulated-free concentration should be carefully monitored. In human blood, amino acids are distributed between plasma and erythrocytes (RBC) based on their role and intrinsic properties. Free plasma AAs are typically more bioavailable but are also more prone to diet-related intraday fluctuations. AAs associated with erythrocytes are less susceptible to dietary composition, so they may serve as biomarkers of their long-term deficiency in the body. Monitoring the concentration of AAs in both plasma and RBCs fractions has its advantages and disadvantages, so it is optimal to monitor both parameters to fully assess the body's supply of individual amino acids.

The most commonly used method for obtaining plasma is centrifugation. HemaSep-L cards are also used to obtain RBC and plasma fractions for venous blood. The attractiveness of this method can be significantly enhanced by the possibility of using this card for the fractionation of blood spotted onto the card after finger pricking. It can then provide a medium for sample collection, fractionation, and storage.

This study aims to investigate whether the separation card can be used as a collection and fractionation medium to determine free (plasma) and blood cell-bound (RBC fraction) amino acids by LC-MS/MS.

Strengths: Fast and simple method for the determination of amino acids in plasma separated from red blood cells.

Experimental

Sample collection

Three sets of blood samples were collected to test the HemaSep-L card's ability to separate plasma from the RBC fraction.

1. Venous blood with K2EDTA (anticoagulant) was collected from one source to obtain plasma by means of centrifugation and the HemaSep-L separation card.
2. The capillary blood from the same source was collected directly on HemaSep-L separation card and standard filter paper (TFN screening card used to obtain classical DBS) after finger pricking (n = 3 drops).
3. Additionally, two sets of HemaSep-L separated samples were prepared for venous blood samples dripped onto the card using a micropipette (different volumes: 20 µL and 40 µL).

LC-MS calibration

LC-MS/MS calibration was carried out using standard solutions prepared in HPLC mobile phase (interpolation-based method). Internal standards were added to control the influence of matrix effects on the extraction and the esterification (derivatization) yield.

Preparation of samples collected with HemaSep-L/TFN cards

The sample preparation procedure for the plasma separation card and dried blood spot were identical.

1. 3 mm discs were cut out from TFN (Ahlstrom) and HemaSep-L cards. In the case of the separation card, discs were collected from two zones: the internal one (containing erythrocytes, RBC fraction) and the external one (plasma fraction) (Figure 1). Next, each disc was transferred to a well in a 96-well microplate.
2. Samples were extracted with 100 µL methanol solution containing isotopically enriched standard for each amino acid and centrifuged.
3. 30 µL of supernatant was transferred to a new 96-well plate and the solvent was evaporated under the nitrogen stream.
4. Next, butanol accompanied by acid was added to carry AAs derivatization to butyl esters.
5. Samples were dried under the nitrogen stream and reconstituted in a 150 µL solution of 0.1% (v/v) formic acid in a 95% MeOH mixture with water.

Preparation of venous blood plasma samples

1. 10 µL of plasma sample was pipetted to the well of the 96-well deep well plate.
2. Samples were precipitated first with a methanol solution containing isotopically enriched standards of AAs. In the next step, acetonitrile was added.
3. The 20 µL of supernatant was transferred to a new well in a 96-well plate. Next, the solvent was evaporated under the nitrogen stream.
4. After the derivatization of AAs with butanol, samples were dried and reconstituted in a 150 µL solution of 0.1% (v/v) formic acid in a 95% MeOH mixture with water.

Apparatus

All LC-MS/MS measurements were carried out using a 4000 API (SCIEX) system equipped with HPLC and an autosampler.

LC instrument conditions

HPLC column: Zorbax Eclipse XDB-C18 4.6x50 mm

Column temperature: 40 °C

Autosampler temperature: 6 °C

Injection volume: 5 µL

Flow rate: 0.8 mL/min

Mobile phase: A: 0.1%FA in water

B: 0.1%FA in MeOH : ACN (1:1)

Needle wash: 1. H2O : IPA (1:1)

2. H2O : MeOH (9:1)

Gradient	Time(min)	%B
	0.5	3
	4.5	50
	5.1	95

Stop time: 5.8

MS Instrument conditions

Source parameters

GS 1: 50 psi

GS 2: 45 psi

Curtain gas: 45 psi

Ion Spray voltage: 5000 V (ESI+)

Source temperature: 300°C

Acquisition

The multi-reaction monitoring (MRM) method was used for positive ion mode. All method parameters, such as m/z values for precursor and product ion in MRM transitions, retention time (RT), collision energy (CE), and declustering potential (DP), are presented in table 1.

AA/ IS	SRM transitions	RT (min)	CE (V)	DP (V)
Ala / ($^{13}C_3$,^{15}N)-Ala	146.3→43.9 / 150.4→46.9	2.90	21	40
Arg / ($^{13}C_6$,$^{15}N_4$)-Arg	231.3→69.9 / 241.4→75.1	2.20	40	60
Asn/ ($^{13}C_4$,$^{15}N_2$)-Asn	189.4→87.1 / 195.5→91.9	2.53	18	40
Gln / ($^{13}C_5$,$^{15}N_2$)-Gln	203.4→186.2 / 210.3→192.1	2.60	15	36
Glu / ($^{13}C_5$,^{15}N)-Glu	260.3→83.9 / 266.3→89.0	6.30	50	30
Gly / ($^{13}C_2$, ^{15}N)-Gly	132.3→75.9 / 135.2→79.0	2.58	15	35
His / ($^{13}C_6$,$^{15}N_3$)-His	212.3→110.0 / 221.5→118.1	1.80	25	60
Ile / ($^{13}C_6$,^{15}N)-Ile	188.4→86.2 / 195.5→91.9	5.60	18	50
Leu / ($^{13}C_6$,^{15}N)-Leu	188.4→86.2 / 195.5→91.9	5.70	30	50
Lys / ($^{13}C_6$,$^{15}N_2$)-Lys	203.4→186.3 / 211.4→193.3	1.90	15	50
Met / ($^{13}C_5$, ^{15}N)-Met	206.3→103.9 / 212.2→109.0	4.55	18	50
Phe / ($^{13}C_9$,^{15}N)-Phe	222.2→103.0 / 232.4→111.0	6.00	50	50
Pro / ($^{13}C_5$,^{15}N)-Pro	172.4→69.9 / 178.3→75.1	3.23	40	50
Ser / ($^{13}C_3$,^{15}N)-Ser	162.4→105.9 / 166.5→109.9	2.60	15	45
Thr / ($^{13}C_4$,^{15}N)-Thr	176.3→73.7 / 181.2→78.1	2.85	20	50
Trp / ($^{13}C_{11}$,$^{15}N_2$)-Trp	261.3→132.0 / 274.4→171.3	6.20	50	45
Tyr / ($^{13}C_9$,^{15}N)-Tyr	238.3→136.3 / 248.2→145.2	4.40	22	40
Val / ($^{13}C_5$,^{15}N)-Val	174.3→71.9 / 180.4→77.1	4.30	20	50
Cit / ($^{13}C_5$)-Cit	232.3→215.3 / 237.4→220.3	2.73	23	50
Orn / ($^{13}C_5$)-Orn	189.3→172.1 / 194.3→177.2	1.20	16	50

Table 1. MS/MS acquisition parameters for detection of selected amino acids (AA) and their isotopically enriched counterparts used as internal standards (IS)

Results

Effectiveness of the blood fractionation by HemaSept-L card

As a first step, it was checked whether two fractions (containing RBC) and plasma were obtained and what was the efficiency of the separation. It was assumed that the plasma fraction obtained would contain free amino acids whose concentrations would be proportional to the amino acid concentrations of the plasma obtained by centrifugation of blood from the same source.

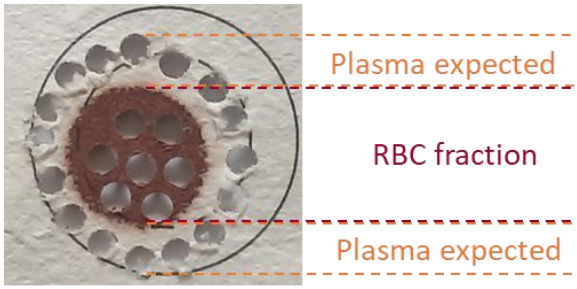

Figure 1. Picture of blood spot after taking sections from zones containing RBC and expected plasma for LC-MS/MS analysis.

From one spot of capillary blood collected from a finger prick, 3 mm disks were cut out. It was possible to obtain 7 discs from RBC fraction and 13 discs from the plasma fraction (Figure 1).

When cutting discs, we observed that the standard semi-automatic DBS puncher used in our laboratory (MS Ekspert auto DXS I) does not accurately cut discs from the plasma zone, leaving uncut pieces of material on the card. One disc from the plasma zone fell apart and was not used for the measurements.

The problem was not observed while cutting discs from cards containing RBC, e.g., internal fraction obtained with HemaSep-L card and DBS obtained with standard TFN screening cards.

The efficiency of amino acid fractionation (plasma-RBC fraction) was monitored by tracking the relative changes in peak areas for each amino acid corrected by the areas obtained for the isotopically enriched counterparts.

As observed in figure 2, for most AAs, except for arginine (Arg), the concentration is about twice as high in the RBC fraction as in the plasma fraction. Serine (Ser) and glutamic acid (Glu), concentrations were significantly higher (up to 8-fold) than in the plasma fraction. Based on a comparison of the relative areas of the peaks, it can be concluded that the amino acid content of the fractions obtained with the HemaSep-L card will vary and be higher in the RBC fraction.

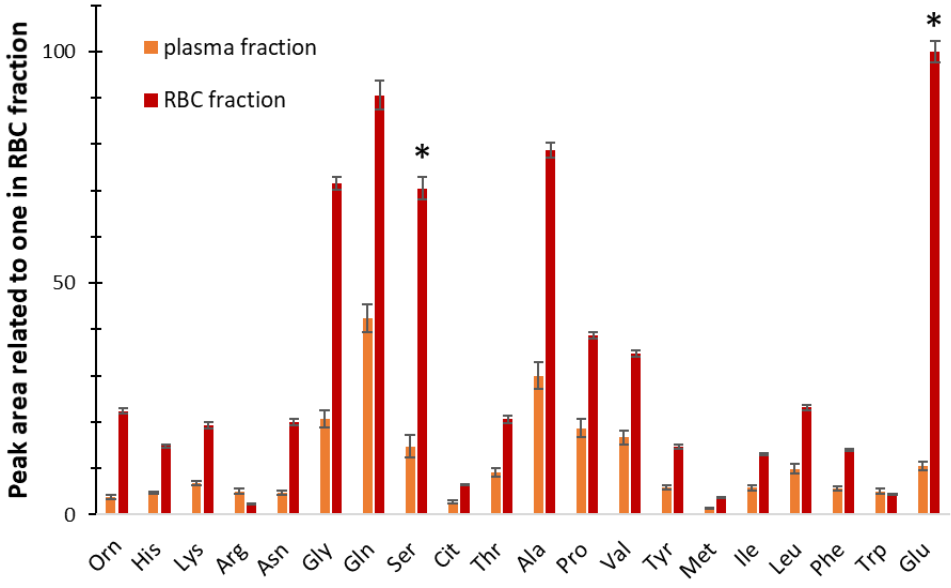

Figure 2. Relative peak areas (vs. the highest peak area for glutamic acid in RBC fraction) were obtained in plasma and RBC fraction by LC-MS/MS.

Homogeneity of amino acid concentration in both fractions

The fractionation efficiency was verified by examining the distribution of amino acid concentration in both obtained fractions for punctured finger samples whose volume is usually in the range of 20 - 40 µL (VDBS) and for spotted venous blood samples with a controlled volume (20 and 40 µL).

The comparison was performed for both fractions (Figure 3A-B). The CV for AAs' concentrations in RBC fraction ranged between 4-8%. The RBC fraction is very homogenous. The CV for AAs in the plasma fraction was much higher, in the range of 20% - 34%(Ser). Lower concentrations of AAs and longer migration distances (frequently observed in capillary electrophoresis) can cause a high CV in plasma fraction (Figure 3C). Still, established AA concentrations in both fractions agree with normal distribution.

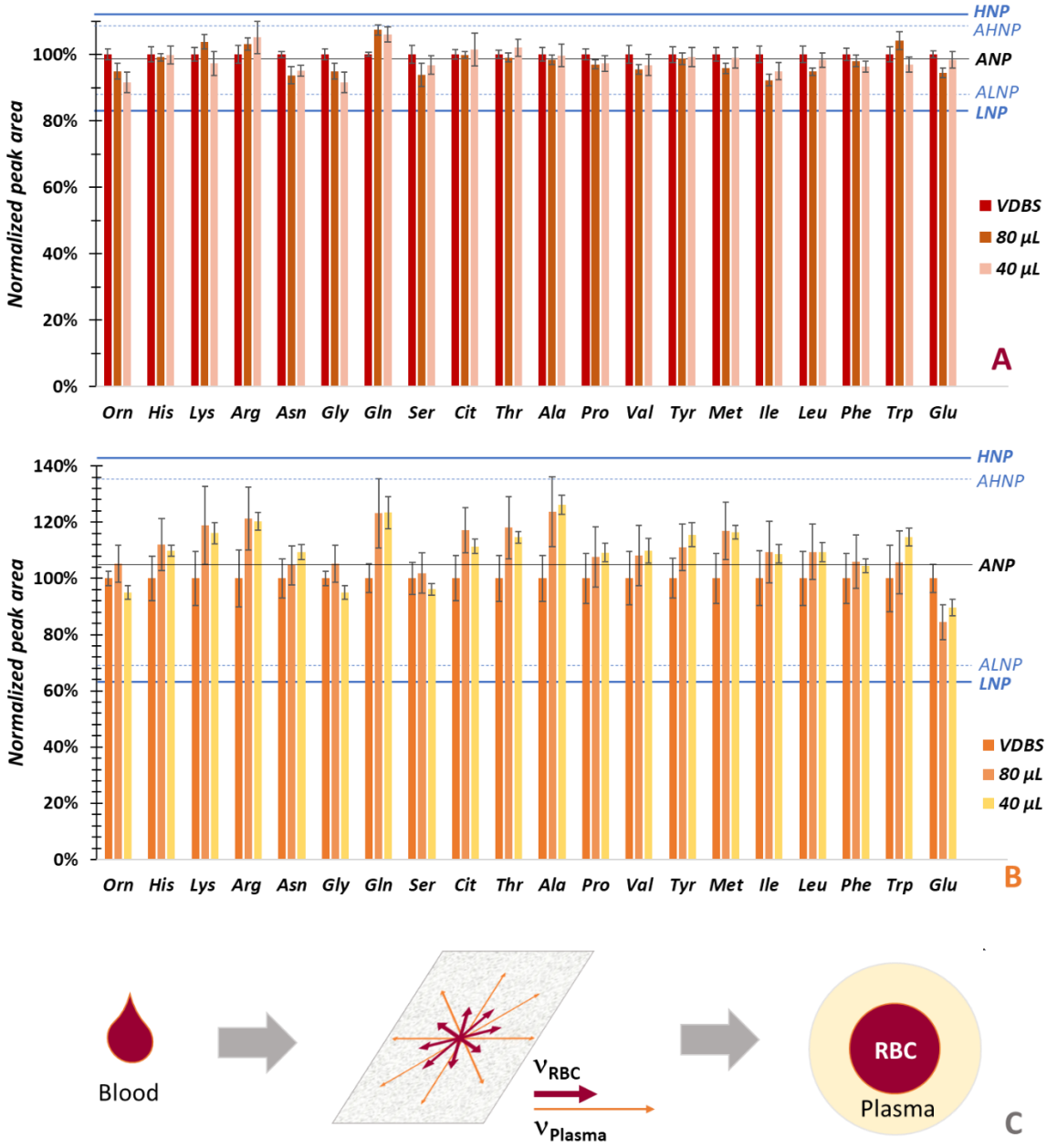

Figure 3. Distribution of amino acids in collected sections of RBC (A) and plasma (B) fractions obtained by HemaSep-L separation card with indicated highest (HNP) and the lowest normalized peak (LNP) areas and averaged values for all determined AA permitted by Student test for □=0.05. Visualization of the mobility of compounds attached to RBC and plasma components (C) through separation card.

Accuracy of AAs' concentrations in HemaSep-L plasma fraction

In the last step, the method for determination of AAs in plasma fraction was validated by comparison of AAs' concentrations in plasma fraction obtained by HemaSep-L card with concentrations of AAs' determined in plasma samples obtained with centrifugation (Figure 4A-B). For most AAs, the difference between measured values was in the range of +/- 20%, except for serine. The correlation of obtained concentrations of AAs in both types of plasma was investigated (Figure 4B). It was found that concentrations established for plasma fractions are in good agreement (r^2>0.95, Figure 4B with concentrations obtained for plasma samples (serine was excluded from comparison)

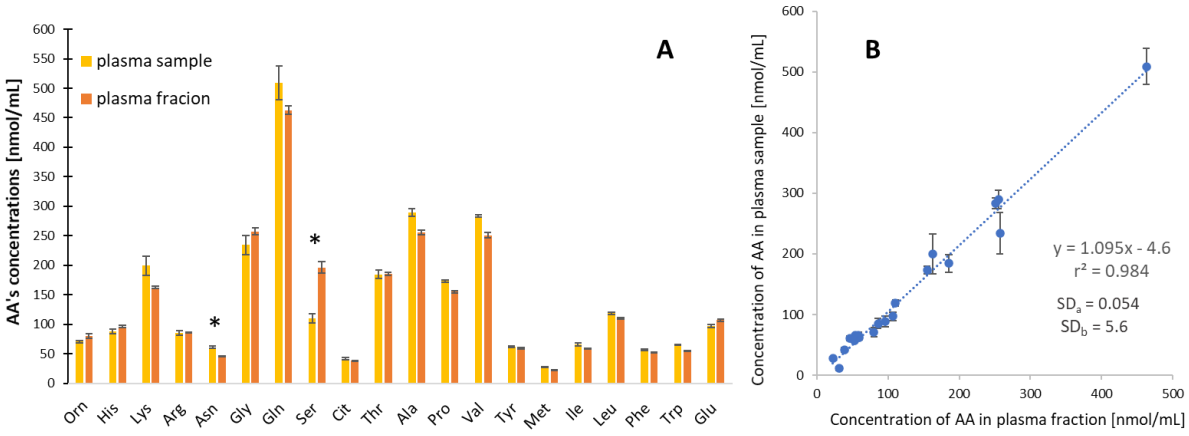

Figure 4. Comparison of established AA's concentrations in a plasma sample and plasma fraction obtained by HemSep-L (A) with established correlation (B) for obtained results by two methods (for n = 3 blood samples).

Monitoring of changes for AAs' concentrations in HemaSep-L plasma fraction

Capillary blood from the punctured finger of the patient/volunteer was spotted on the separation HemaSep-L card and the standard TFN screening card for 5 consecutive days at three-time points (8.00 am fasting, 12.00 pm, and 4.00 pm).

This allowed for illustrating the variability of the concentration of free amino acids in the daily cycle and during the working week. The experiment gives a lot of exciting data. The results highlighted the high amino acid concentration variability throughout the study period and the relatively low variability between fasting points.

A higher relative variability characterized the concentration of amino acids in the plasma over the entire period under study concerning the erythrocyte fraction and DBS.

The changes in amino acid concentration depend on AA's properties and the patient's metabolism. This could be very easily observed in the figure. 5. In the case of asparagine (Asn), the concentration is stable in RBC fraction and varies during a day in plasma fraction. These differences cannot be observed for samples obtained with standard TFN screening cards.

It should also be noted that HemaSep-L separation card allowed for easy separation of amino acids dominant in erythrocytes (Asn, Glu, Ser, Orn) or plasma (Arg), respectively.

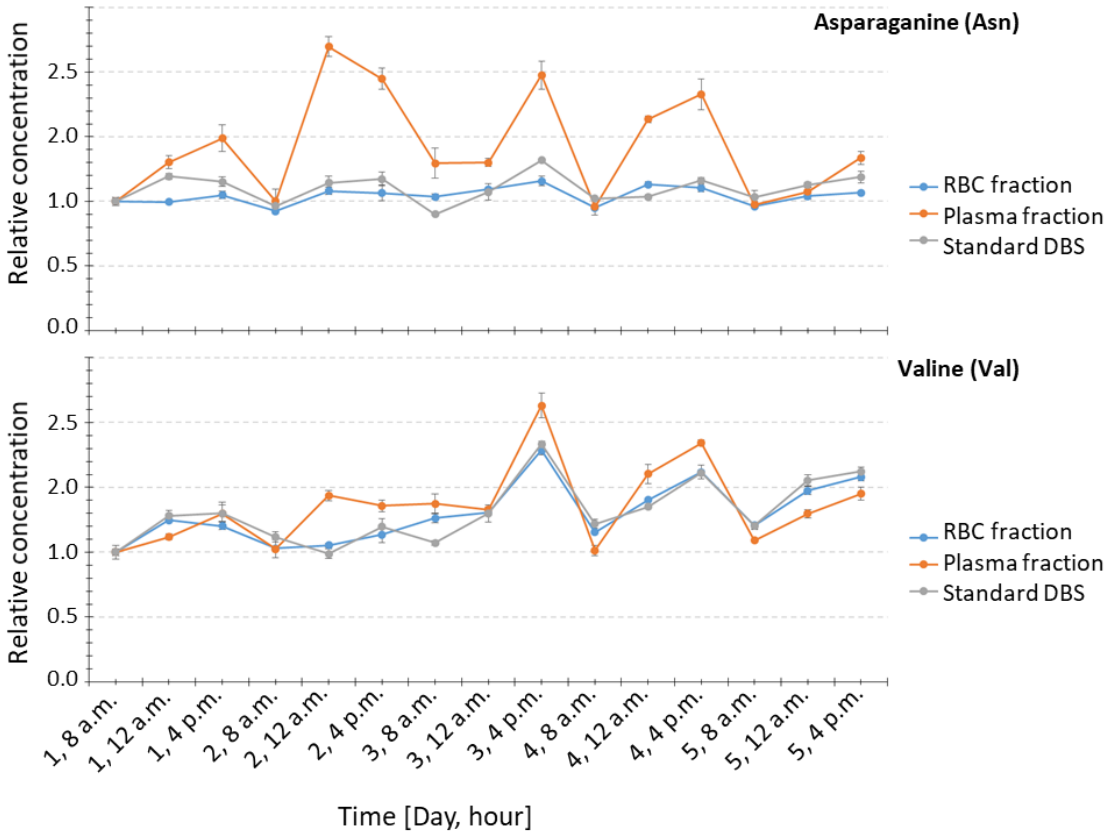

Figure 5. A comparison of the results obtained during monitoring Asn and Val in one patient's blood was collected using different methods.

Conclusions

The method based on applying the HemaSept-L card for blood fractionation combines the advantages of dried blood spot analysis with the ability to use a collection card to fractionate a blood sample by using steric/affinity interactions and capillary effects. This new tool enables simple, low-volume blood sampling (from the punctured finger) and simultaneous monitoring of any endo or exogenous compounds in plasma and erythrocyte fractions of a single sample.

The applied approach allows for information on short-term and long-term changes in the concentration of free amino acids. It should be noted that the application of the HemaSep-L separation card requires separated calibration for each fraction (RBC and plasma).

The condition of the plasma fraction disc-cutting knife is critical and influences precision. The tool must be sharp to ensure the correct shape of the disc. HemaSep-L allows the observation of changes in the AA composition in plasma, which is impossible with a standard DBS card.

GET IN TOUCH:

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