Application Note:
Lead (Pb) Analysis using Capitainer®B10

Overview

This document is a guideline for laboratories implementing the analysis of lead (Pb) using ICP-MS from capillary blood collected on Capitainer®B10 microsampling card. This application is based on clinical validation of a method developed and subject to CLIA guidelines. miQro Lab Solutions, part of Capitainer, actively participates in two US-based proficiency testing programs (Wisconsin and Pennsylvania) for Pb analysis in venous blood.

Lead is a naturally occurring metal that can cause negative health effects. According to the CDC lead prevention program [1] there are “No safe levels of lead in children's blood.” Even low levels of lead that were once considered safe have been linked to harmful changes in intelligence, behavior, and health. The CDC uses a blood lead reference value (BLRV) of 3.5 micrograms per deciliter (µg/dL). This reference level helps identify children with blood lead levels (BLLs) higher than most children's levels. CDC estimates that approximately 500,000 children in the United States have BLLs at or above the BLRV.

The method described herein enables the collection of blood using a decentralized collection in addition to more conventional collection models. Capitainer®B10 dried microsamples are protected from contamination after capillary blood collection during both the drying and shipping process with the added advantage of no cold chain requirement. Results show excellent agreement between traditional venous blood analysis and Capitainer®B10 samples dried microsamples.

Features

- At-home self-sampling (adult & assisted pediatric)
- Room temperature stable – no cold chain required
- Compact device with minimal storage needs
- Protected sample disk reduces contamination risk
- Low and consistent Pb background in cellulose paper
- Detection limit: 0.5 µg/dL
- Background-corrected Pb measurements
- Strong correlation with venous samples (R^2 = 0.99)
- Pediatric-friendly – only two drops needed

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Method Highlights

Described below are the essentials of the ICP-MS method developed at miQro lab solutions. The methods described herein should be considered a guideline for your own validation and adaptation to your lab facilities including analytical equipment, reagents and supplies.

Capitainer® devices are designed for use with fresh blood. However, during early validation or method development, it is often practical to begin with previously collected venous blood and apply it to the cards by pipetting under laboratory conditions. For Capitainer®B10, apply 25 µL as a single drop to each inlet, simulating blood application from a finger prick, and let the cards dry at least overnight. Detailed card handling guidelines are provided in the appendix and at www.capitainer.com

Sample Preparation

Sample Extraction

To begin the reconstitution process, place the Capitainer® sample disc—containing the volumetric dried blood sample—into your tube. For lead testing, it is important to perform this step in a way that eliminates any risk of contamination, and we recommend using ceramic tweezers.

With the blood or blank disk in the conical tubes;

- Add 3 mL Ultra Pure Water with 0.01% Triton X-100
- Vortex 1-2 seconds and incubate on orbital shaker 360rpm for 30 minutes.
- Sonicate samples 30 minutes

Ice is added to the sonic bath to keep the temperature at approximately 20°C.

- Add additional 3 mL of 1% nitric acid producing 6 mL total volume.
- Vortexed for 1-2 seconds and wait for 30 minutes to allow any particulates and the extracted disk to settle to the bottom of the tube.

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Preparation of Calibration and Internal Standards

Preparation of ICP-MS Calibration Standards

An eight-point calibration set of Pb standards are prepared using a concentrated stock solution that contains Pb at 100 µg/mL. The final concentration of Pb in eight standard solutions ranged from 0.025 ng/mL to 10 ng/mL and a 0 ng/mL UHP blank.

Preparation of Bismuth (Bi) ICP-MS Internal Standard

A 1 ng/mL Bi internal standard is prepared from a 1 ug/mL stock solution.

Inductively Coupled Mass Spectrometric (ICP MS) Analysis

Organizing Samples for Automated Analysis

The following samples should be analyzed in the order described for optimal results.

1. 1.4 wash steps of 0.5% Nitric Acid, followed by 2.0% Nitric and 0.5 % HCl Acid mixture, a 1% Nitric acid, and then a repeat of the 0.5% Nitric acid. The standard curve is analyzed in reverse order with the zero-level followed by increasing levels of Pb (7 calibration standards). Two wash steps of 0.5% Nitric acid follow the calibration curve.
2. The extracted paper control specimens are analyzed next generally in the order of blanks, duplicate, followed by QC microsample specimens at each level, followed by 2 wash steps. We use WQC from BioRad here.
3. Prior to running the unknown specimens and additional blank paper analysis is recommended to give you three background blanks in total. The specimens are then analyzed in the order set by the laboratory.
4. We recommend 2 wash steps of 0.5% acid for every 10 samples to keep the ICP MS system clean and robust.
5. Each analysis is approximately one minute. 25% of all specimens are either blanks controls or standard curves.
6. A repeat of step 1, wash samples is used at the end of the analysis.

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We utilized common wash steps at the beginning of a run and at the end of the run using 3 different water blanks with varying acid content (from 0.5%, 1% Nitric acid solutions and 2% Nitric with 0.5% Hydrochloric. We also run a 0.5% nitric after every 10 samples. The large number of wash steps is to ensure there is no large baseline or buildup of contamination. We have examined carryover using the fast system and it is less than 1%.Twi wash steps after known high Pb standard helps ensure a better analysis and that the trace analysis of Pb is accurate and precise. When running patient samples, if very high patient result occurs, we will not only run that sample but run the next sample in the sequence if it is also slightly elevated due to possibilities of known carryover (which is small). We have also set up our ICP MS to be a robust system without unnecessary time spent cleaning and troubleshooting. We recently installed an inline filter from Glass Expansion to prevent clogging in the injector which is a time-consuming repair.

The volume of blood analyzed from a Capitainer B10 card is 10 µL which is the lowest volume of blood analyzed in the Pb screening space whether liquid or dry. Typically, those other assays range from 30-500 µL. The analytical method described below is also quite sensitive as the sample is diluted to 6000 µL (6 mL) for a 1 to 600 dilution factor. The net result is that our analysis is trace level measurement.

Importance of associating Patient Sample with Card Lot number
Background subtraction is critical to the correct determination of the final concentration of Pb in the specimens. The cards of different lot numbers may have slightly different concentrations of Pb due to the supply of paper from the manufacturer. QA/QC specimens, for example, may be prepared on a lot that is different than patient samples. Blanks that represent every specimen type must be analyzed in duplicate at a minimum and records of running averages should be stored.

Capitainer cards do not have excess blank paper with each sample, and each sample disk is independent of each other. Capitainer carefully controls the prepared disks in the card. Production batches of cards with certified background levels, as established by miQro Lab Solutions, are available to ensure optimal service setup.

As observed in the validation below, it is highly reproducible. This also is one of the reasons why a matrix matched with standard curve may not improve quantification.Since our blood is diluted 600-fold during extraction of 10 µL, the matrix most closely matches water based standard curve. However, we do use 3 standards from Bio Rad that has 3 levels between 3-37 µg/dL that are used to check the linearity and accuracy of our standard curve. That matrix is matched to the samples.

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ICP-MS Analysis
A Thermo Fisher iCAP RQ plus ICP MS with an Elemental Scientific 4DX autosampler / FAST system was used to analyze 2 mL of aqueous blood extract in approximately 1 min. Pb was analyzed using KED mode. The three most abundant isotopes of Pb were acquired at m/z 206, 207, and 208. Another isotope at m/z 210 was acquired, but it is at very low abundance. The instrument was configured to sum the intensities of all 3 major isotopes plus the trace amounts of the 210 isotopes. This total Pb value was used for all concentration calculations as the sum of the isotopes has a higher ion count and is more sensitive than any isotope measured alone.

Bi (bismuth) was used as the internal standard (1 ng/mL). In the fast system it is mixed equally in the injection port for quantitation. The ICP MS acquires 3 separate periods of analysis during each injection and the standard deviation for each period (RSD) calculated. The Bi signal is also monitored to determine any loss of sensitivity, system failures. Ion suppression does occur such that the signal for Bi may decline by 30% during the analysis. Periodic wash steps help to restore sensitivity and therefore it is recommended at every 8-10 injection.

Internal Calibration: The analyst identifies which samples are calibration standards and enters the concentration for each standard. These samples are run first (after wash steps) and on completion, provide calibration so that the measured ions of each element or isotope is converted to a concentration reported in ng/mL in the 6 mL extract. Additional calculations such as concentration adjustment for the specimen size (10 µL), background subtraction or conversion to µg/dL is done using excel after exporting the data as described in more detail below.

Data Processing
The raw data is exported as an xls file and saved in a workbook for each days run. On page 1, the calibration curve is recalculated and all blanks, linearity of the measurements are performed. The raw data post calibration data is sorted by blanks, QC specimens and unknown samples for result processing. For all groups of data on a separate page or the spreadsheet the concentration of Pb in the 6 mL is converted to the concentration of Pb in the 10 µL of blood expressed as µg/dL. The final concentration is obtained after blank subtraction.

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1. Sort the data by blanks, QC Positive Samples, and test / patient samples. If there is more than one lot used in the analysis, each should be grouped separately. We recommend using Excel workbook functionality to keep all original data on its unique page (Page 1 is raw data, Page 2 is sorted data, Page 3 is all blanks, Page 4 is all QCs, Page 5 is all test sample / patient, etc.

2. For each sample category (blanks, patient, QC), step 2 is to correct for the dilution of 10 µL in 6mL (6000 µL). This is a 600-fold dilution, therefore the result are multiplied by a factor or 600 to get ng/mL (µg/L). However, most Pb results are expressed at µg/dL which is a factor of 1/10th therefore, the factor is simply 60 instead of 600 to produce all concentrations in µg/dL. This should be done for all samples including blanks and QC.

3. The next step is important. This step determines the amount of Pb in the paper background based on the lot number used for the analysis. (If multiple lots are used, each should be calculated separately). The “median” not the mean of 3 or more analysis of blanks is calculated. The CVs should be within 25%. A running average of all blanks run during previous analysis should be saved in a separate spreadsheet. It is important for both quality control and to remove any outliers. The median of the running averages and the most current analysis is compared. A blank outlier may occur 1% of the time in any given lot. If the current blanks median is within 25% of the running averages, then either value can be used in the blank subtraction. If there is a large outlier in the current analysis, the running average should be used.

4. Background subtraction is done on QC specimens and patient/test samples. It is presuming all concentrations have been previously adjusted to µg/dL unit concentrations. The value for Pb in the background is subtracted from each sample or QC result giving the true sample concentration. We recommend that the 2-3 blanks analyzed be checked against a running average of all blank analysis. If within 10% the blanks from the run should be used. If not the median of the running average should be used. However, our experience shows excellent reproducibility but on occasion a spurious elevation of a blank is observed (less than 2% of the time).

Extraction efficiency correction. For older microsamples, we have found the extraction efficiency to be approximately 90%.However, when compared to liquid blood we have found that this step can cause a small bias of 5-10%. We therefore do not correct for extraction efficiency for fresh samples (not older than 2 weeks).

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Validation

The section below describes how we at miQro conducted our validation, and we recommend that any laboratory implementing the assay described above validate it in a similar manner.

The validation of the ICP-MS method is divided into two parts.

1. Analytical (basic analysis and mass spec measurement of Pb), QA/QC controls
2. Clinical validation which involves the comparison of venous blood (liquid) and venous blood added to B10 cards (dry microsample).

Analytical Validation

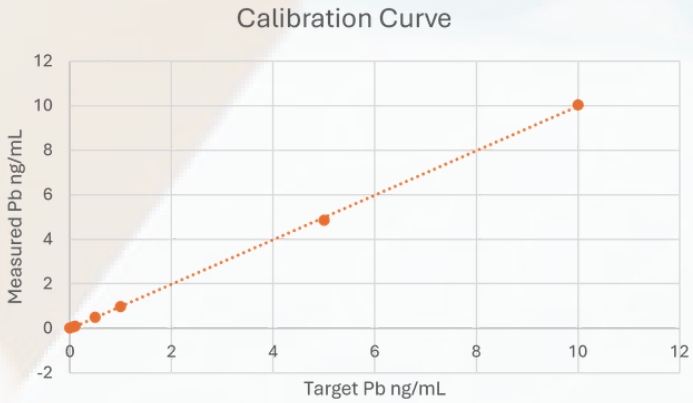
The analytical validation involves measurement of Pb in water, liquid blood and dried blood.

Calibration Curve

The analytical limits of detection for Pb are based on the calibration curve. Figure 1 shows the calibration curve, added concentrations and measured Pb in 6 mL of water expressed as ng/mL The correlation coefficient is 1, slope is 1 and intercept is -0.025 ng/mL The limit of detection is 0.001 ng/mL which is the amount of Pb in the UHP water and is at least 3 times the noise level of the instrument.

Figure 1

	Pb ng/mL targeted	Pb ng/mL measured
zero std	0	0.001
SCA1-50p	0.05	0.044
SCA1-100p	0.1	0.089
SCA1-500p	0.5	0.485
SCA1-1n	1	0.969
SCA1-5n	5	4.849
SCA1-10n	10	10.038
	correlation	1.000
	slope	1.000
	y-intercept	-0.025



In terms of sample analysis, with a 600-fold dilution the range required, a 0.001 ng/mL is 0.6 ng/mL or 0.06 µg/dL for the lower range while a 10 ng/mL is detected at 6000 ng/mL or 600 µg/mL for the upper detection limit. This represents a dynamic range of 1000.

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Background Pb Reproducibility

For any elemental analysis, especially Pb, the lab must consider all potential sources of contamination which include the cellulose filter paper used to absorb liquid blood. At Capitainer we evaluated several different lots and two grades of paper provided by the supplier, Ahlstrom. We identified the lowest Pb levels in one specific lot of Ahlstrom paper sheets from which we manufacture the cards. The lab has analyzed hundreds of blank, unspotted disks from several different Capitainer production lots and have found that for the sample disk made from Ahlstrom 222 paper and used in B10 cards, the background contribution to the signal is in the range of 0.3-0.5 µg/dL. This value is the calculated equivalent amount of Pb extracted from blank disk as if it were a patient sample. The CVs for the paper disk lots are consistently ~20% for trace levels of background. More importantly, we have found that in only 1% of the analysis of blank cards, there will be an elevation more than 2 times the baseline level (200%), a clear outlier for which we do not know the source (tube, manufacturing, dust). We therefore recommend that at least 3 blanks be run per essay. A running average for a specific lot should be kept. The value of each analysis should fall within the range of the running average. If one outlier is found, either the point discarded or the running average used. If 2 or 3 points are found outside the median value, then the running average should be used for background subtraction. Then the QC values should be checked.

Clinical Validation

The clinical validation of the assay was performed using venous blood from proficiency testing programs in Wisconsin and Pennsylvania. Both programs are used for CLIA certification and are reported to CMS. The most recent data set from Wisconsin, which represents the most optimized method to date (reported here) is shown as two groups of data. Five blood specimens were shipped to miQro Lab Solutions. On receipt within 1 day, aliquots of blood were applied to Capitainer®B10 cards and dried overnight. For analysis, both the original liquid blood was analyzed, 50 µL as well as one of the Capitainer B10 discs.

In addition, as part of our routine method we analyze one, two or all 3 sets of Bio Rad's lyophilized blood Pb standard as our control. Each set of results for Liquid and Dry compared to Wisconsin's reported results (which is the mean of all program participants) and one final set of data that examines liquid versus dry for the same blood samples.

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Venous 50 µL Liquid Blood Microsample
QC blood from Bio Rad labeled as A, B and C specified the following concentrations in each of these standards, at 2.95, 12.95 and 37.3 µg/dL. We labeled these as target concentrations. The average CV for the analyses was 2.9%. Comparison of the target value with our experimental results at 3 levels had correlation of 0.999 and a slope of 1.06.

For proficiency testing blood of the 5 samples of venous blood, the average CV's were 3.7%. Comparison of the target value with our experimental results for 5 different blood samples had correlation of 0.999 and a slope of 0.99. All data is shown in Figure 2.

Figure 2

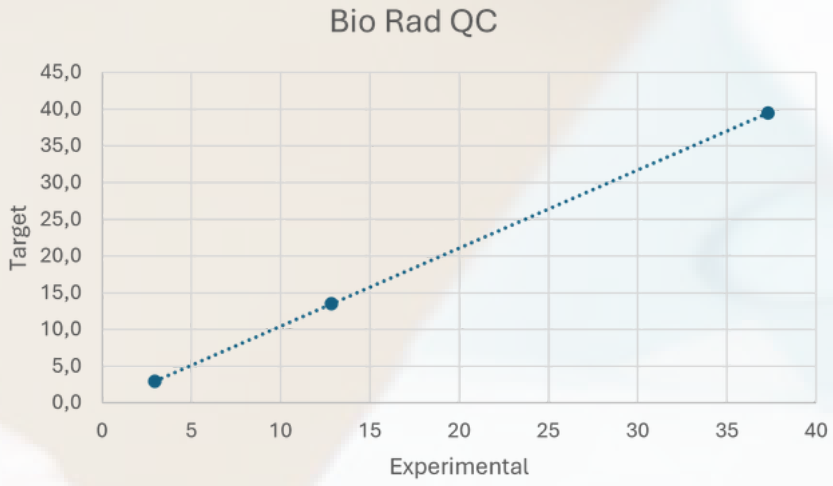
Venous Blood

50 µL Liquid Lyophylized Standard
median

Target	Experimental
2.95	2.9
12.85	13.5
37.3	39.5

correlation	0.9999994
slope	1.06
y-intercept	-0.18

average %CV at all levels 2.9
3 separate analyses on 3 different days

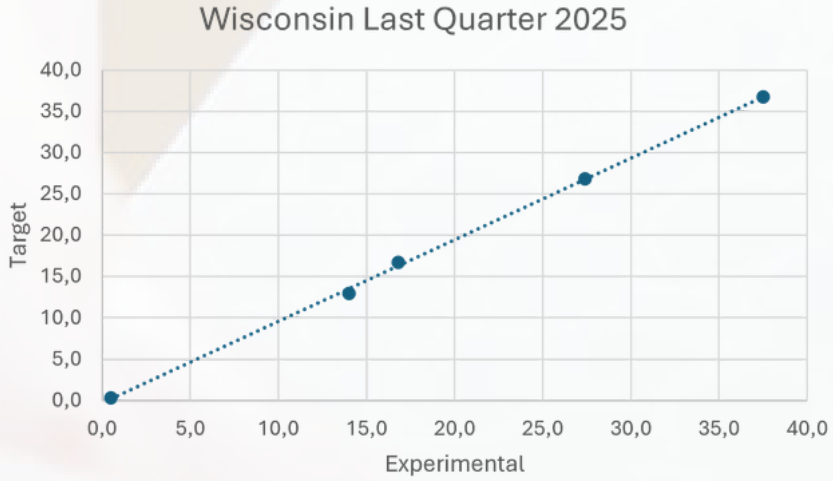


50 µL Liquid Blood Proficiency
median

Target	Experimental
16.8	16.7
27.4	26.8
0.5	0.3
14.0	13.0
37.5	36.8

correlation	0.99966004
slope	0.99
y-intercept	-0.32

average % CV at all levels 3.7
3 separate analyses on 3 different days



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Capitainer®B10 Dried Microsample
QC blood from Bio Rad labeled as A, B and C specified the following concentrations in each of these standards, at 2.95, 12.95 and 37.3 µg/dL. We labeled these as target concentrations. The average CV for the analyses was 4.7%. Comparison of the target value with our experimental results at 3 levels had correlation of 0.999 and a slope of 1.08.

For proficiency testing blood of the 5 samples of venous blood, the average CV's were 4.6%. Comparison of the target value with our experimental results for 5 different blood samples had correlation of 0.999 and a slope of 1.02. All data is shown in Figure 3.

Figure 3

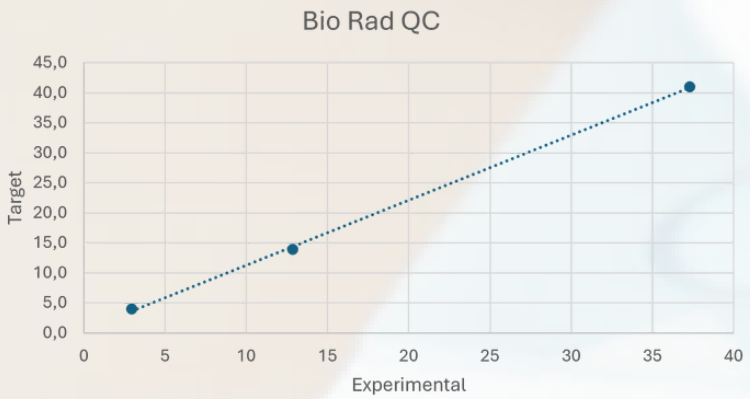
Capitainer B10

10 µL B10 Dry Lyophylized Standard
median

Target	Experimental
2.95	4.0
12.85	13.9
37.3	41.0

correlation	0.99978165
slope	1.08
y-intercept	0.48

average %CV at all levels 4.7
2 separate analyses on 2 different days

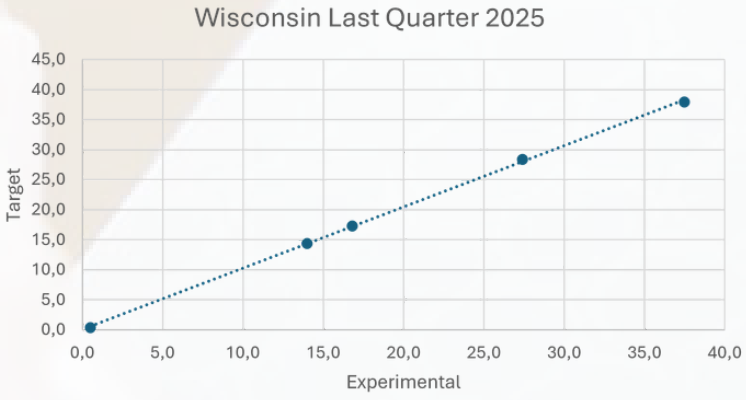


10 µL B10 Dry Blood Proficiency
median

Target	Experimental
16.8	17.3
27.4	28.4
0.5	0.4
14.0	14.4
37.5	38.0

correlation	0.99980326
slope	1.02
y-intercept	0.05

average % CV at all levels 11.3less outlier 4.6
3 separate analyses on 3 different days



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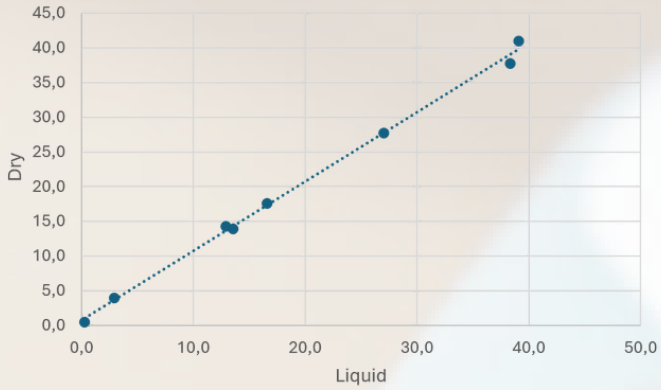
Liquid versus Dry
Comparing all samples analyzed, QC and proficiency blood, the correlation between venous liquid and Capitainer B10 dry venous blood was 0.999 with a slope of 0.999. The data is shown in Figure 4.

Figure 4

Venous (Wet versus Dry)

	Target	Liquid Experimental	Dry Experimental
Qc LV 1	2.95	2.9	4.0
Qc LV 2	12.85	13.5	13.9
Qc LV 3	37.3	39.1	41.0
Wisconsin 11	16.8	16.6	17.6
Wisconsin 12	27.4	27.0	27.7
Wisconsin 13	0.5	0.3	0.5
Wisconsin 14	14	12.9	14.3
Wisconsin 15	37.5	38.3	37.8
		correlation	0.999
		slope	0.999
		y-intercept	0.78



Special Considerations
Sample Stability is one area that is often asked regarding the analysis. The biomarker, Pb, is a very stable element and does not degrade. In terms of blood extracted, ICP-MS works by combusting all molecules to their elements in the ionized plasma flame. There is a possibility of generation of neutral molecules such as oxides that might interfere and therefore, we use the Kinetic energy displacement (KED) mode that separates energetic ions and reduces neutrals or unenergetic ions improving the detection of Pb and other elements.

As biomarkers, Pb, is stable, the main issue that could affect quantification is extraction efficiency from the disk. We have found this to be consistently above 95% for B10. This correlates well with other filter paper-based analysis on Capitainer cards as well as other types of cards that use cellulose matrices. We have analyzed dried blood proficiency samples that were stored at room temperature for more than 3 months and did not see significant differences in results.

The CV% of the established method is approximately 5% at the decision criterion of 3.5 µg/dL.
The highest CV values occur in blank paper samples due to trace levels of Pb, where CVs are around 20%. However, because these background levels are far below the decision point, their variability contributes only minimally to the total method CV at 3.5 µg/dL.

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Summary

With the described method, lead can by using background controlled Capitainer card lots confidently quantify blood lead levels in the range of 0.6-600 µg/dl, well below the CDC cutoff at 3.5 with a CV% less than 5% at the decision criteria.

Analysis of dried microsamples collected with Capitainer®B10 delivers results on par with traditional liquid blood testing.

Baseline correction is required, but background levels are low and consistent within by miQro controlled lots of Capitainer® cards. Capitainer® is a protected card that minimize the risk of sample contamination.

The volume of blood collected is low meaning it is pediatric friendly. For children, assisted sampling is preferably done by the parent. Self-sampling can be done by any adult.

Pb analysis of a dried blood microsample collected using a Capitainer®B10 card is fit for purpose for environmental toxicology and screening.