Efficacy of Dried Blood Spots for Assessing Biomarkers in Field Epidemiological Studies

By

Abhinendra Kumar

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Evaluation Report[1] of Ph.D. Viva-Voce

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8. **Name and Affiliation of the Examiner 1:**

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Recommendations of the Viva-Voce Board

1. **Date of Viva Voce Examination: 15.02.2021**

2. **Recommendations for the award of the Ph.D. degree: Recommended / Not Recommended**

(If Recommended, give summary of main findings and overall quality of thesis)
(If Not Recommended, give reasons for not recommending and guidelines to be communicated by Convener of the Doctoral committee to the student for further work)

1. Dried blood spots (DBS) can be utilized in field epidemiological studies for specific biomarker (generic DNA, IgG antibodies against infectious pathogens)
2. Thesis finally concluded, DBS should be transported before 24 hrs to laboratory for apolipoprotein analysis but for DNA & infection study it can be transport at RT before 72 hrs. of blood collection at field.
3. Overall thesis quality in good & accepted.

In case, Not Recommended, another date will be fixed by the Dean-Academic, CI, which shall not be earlier than a month after and not later than six months from the date of first viva.

3. **Name and Signature of the Viva Voce Board (Doctoral Committee & External Examiner):**

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15/6/21

Dean-Academic, CI

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Recommendations of the Viva Voce Committee

As members of the Viva Voce Committee, we certify that we have read the dissertation prepared by **Abhinendra Kumar** entitled "**Efficacy of Dried Blood Spot for Assessing Biomarkers in Field Epidemiological Studies**" and recommend that it may be accepted as fulfilling the thesis requirement for the award of Degree of Doctor of Philosophy.

	Signature with date
Chairman – Dr. R. A. Badwe	
Guide / Convener – Dr. Rajesh Dikshit	19.3.2021
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Date: 19.3.2021 **Signature**

Place: CCE (TMC), Navi Mumbai **Guide: Dr. Rajesh Dikshit**

[1] This page is to be included only for final submission after successful completion of viva voce.

CERTIFICATE

I certify that the thesis titled '**Efficacy of Dried Blood Spot for Assessing Biomarkers in Field Epidemiological Studies**' submitted for the degree of Doctor of Philosophy by **Mr. Abhinendra Kumar** is a record of the research carried out by him during the period September 2013 to December 2020 under my supervision. This work has not formed the basis for the award of any degree, diploma, associateship or fellowship at this or any other institute or university.

Date: 19.3.2021

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Dr. Rajesh Dikshit

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Place: Mumbai

Abhinendra Kumar

DECLARATION

I, hereby declare that the investigation presented in the thesis has been carried out by me. The work is original and has not been submitted earlier as a whole or in part for a degree / diploma at this or any other Institution / University.

Date: 19.03.2021
Place: Mumbai

Abhinendra Kumar

List of Publications arising from the thesis

Journal

- Optimization of extraction of genomic DNA from archived Dried Blood Spots (DBS): Potential application in epidemiological research & bio-banking. Kumar A, Mhatre S, Godbole S, Jha P, Dikshit R. **Gates Open Research** 14 Nov 2018, 2:57 (https://doi.org/10.12688/gatesopenres.12855.1)

- Kumar A, Mhatre S, Dikshit R. Utility of dried blood spots in detecting *helicobacter pylori* infection. **Indian J Med Microbiol** 2019; 37: 514-20

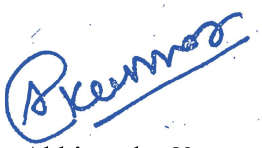
Conferences

- Presented "Efficacy of Dried Blood Spots in Field Epidemiological Studies" in 13th National Research Scholar Meet in Life Science held on 14th – 15th December 2017 at Advanced Centre for Treatment Research and Education in Cancer, Navi Mumbai

- Presented "Extraction of gDNA from Dried Blood Spots & its application in large scale epidemiological studies" in 61st Annual National Conference of Indian Public Health Association (IPHA) & First State Conference of IPHA Rajasthan held from 24th – 26th Feb 2017 organized by Department of Community Medicine and Family Medicine, AIIMS Jodhpur, Rajasthan.

Date: 19.03.2021

Place: Mumbai



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Date: 19.3.2021

Signature

Place: CCE (TMC), Navi Mumbai

Dr. Rajesh Dikshit

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Teachers affect eternity: no one can tell where their influence stop

This journey of education can't completed without immense support of my family, teachers, and friends. I am great thankful to all who directly & indirectly influence my carrier & life.

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Finally, I thank almighty God who has helped and blessed me through the entire voyage and have made this day possible.

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5.3 Importance to the Biomarker Research

This study provides the validation for the use of DBS methodology for gDNA, Infectious pathogen and apolipoproteins (apoA &apoB). No doubt, DBS does exhibit greater variation and assay error for the tested analytes, suggesting that DBS may have greater utility in group or population studies but may be less useful in individual clinical decisions than standard venipuncture samples. A particular strength of the present study was the demonstration through the use of time controls that DBS samples remain fairly constant over time.

The DBS method allows for biomarker assays to be implemented in studies using larger, community-based samples and those that target more vulnerable populations, broadening the applicability and generalizability of biomarker use in CVD research. Over the past decades, the DBS technique has established as a significant tool in epidemiological research, new born screening and drug discovery & monitoring.

5.4 Future perspective

In coming years, future of using DBS will be increases in large scale field epidemiological studies at population level. We have to plan to increase our work further to elaborate the testing of biochemical parameters such as HbA1c, total cholesterol, heavy metals, other infectious pathogens, hormones etc with the same manner as we did in this study to find out how much difference we observed while multiple groups comparison at different transport duration to establish correct transport duration with their stability.

5.5 Conclusion of the study

DBS could be a remarkable tool to measure the analytes/biomarkers (genomic DNA, antibodies raised against infection, apolipoproteins) even if it is stored at different thermal condition (4^0C or -20^0C) with varying transport duration up to 72 hours. The positive result showed that, the stability of biomarkers & feasibility of DBS can be utilize to conduct large

scale field epidemiological studies to know the disease burden & health challenges in community.

5.6 Implications of the study with recommendation

This study was conducted to analyze the utility & feasibility of Dried Blood Spot (DBS) in field epidemiological studies when there is limited availability of infrastructure to collect & process the venous blood. DBS has been used in research for 2-3 decades, specially for testing of phenylketonuria in neonates. Validity of DBS samples, preparation methodology, temperature, humidity, transportation time from one place to other, storage thermal condition all these parameters have some impact on analyte stability in DBS samples. With address to above parameters, our major aim & objective is to whether DBS can be useful tool to detect important biomarkers if is stored at different thermal condition within 0-24, 24-48 & 48-72 hours of transport duration. Because there is no such study is conducted where they show how the stability of biomarkers affected when DBS stored between 0-72 hours after preparation. Single analyte (genomic DNA, IgG antibody and apolipoproteins) compared 6 times with the reference standard to evaluate how the variation observed in the concentration of analyte, probably focus on which storage thermal condition & transport duration will be best for the DBS to answer many research questions. In this study, we observed good result for extraction of gDNA from all DBS at all stored temperature & transport duration but statistically mean concentration of gDNA at 4^0C storage slightly greater than at -20^0C, similarly, excellent result observed for detection of IgG antibody concentration against H. *pylori* at all stored temperature with sensitivity >87% & specificity >90%. Whereas, there is more variation seen in stability of apoliproteins with transport duration, the concentration of apolipoproteins decreases gradually with increase in transport duration from DBS preparation site to thermal storage site. Final recommendation for those researchers who wish to work

Ph.D. Thesis Abstract

Name: Abhinendra Kumar

Enrollment Number: HLTH09201304001

Title of PhD thesis: Efficacy of Dried Blood Spots for Assessing Biomarkers in Field Epidemiological Studies

Part 1: This is an observational study, the aim of this study is to evaluate the usefulness of DBS in field epidemiological study when DBS collected at field & transported to laboratory in 24, 48 & 72 hours, where DBS stored at 4^0C & -20^0C for longer duration. Standardization & validation of DBS samples have been done with plasma/venous blood through measuring some specific biomarkers (genomic DNA, IgG antibody against Helicobacter *pylori* infection, apolipoprotein (apoA & apo B). Blood spots prepared from venous blood on Whatman 903 cards, air dried it for 2-3 hours at room temperature, packaging in zip lock bag with 1-2gm desiccant sachets and stored at respective thermal conditions. All the statistical analysis done through STATA, Graph-pad PRISM software. Good quality DNA concentration obtained by DBS which can be utilized for downstream application such as gene amplification, sequencing but for genotyping it need modification in protocol. IgG antibody against Helicobacter pylori infection & apolipoproteins measured through serologically (ELISA) methodology. Excellent sensitivity (>90%) & specificity (>90%) observed for IgG antibody on comparison with venous blood. where the stability of apolipoprotein reduce on DBS when it measured beyond 48 hours of transport duration. Finally we concluded, DBS can be a potential & robust tool for extraction of genomic DNA & measurement of infectious pathogens in field epidemiological studies but further research warrant for apolipoprotein stability in future to explore the potential robustness of DBS.

Part 2. Guidelines develop to conduct the research on DBS samples for specific analytes. DBS should be transported to laboratory within 24 hours of transport duration (apolipoprotein analysis) whereas there is no specific impact on genomic DNA & IgG antibodies when it is transported within 72 hours of transport duration.

19.3.21

Abhinendra Kumar

Dr. Rajesh Dikshit (Guide)

Forwarded through:

Dr. S.D. Banavali
Dean (Academic)
T.M.C

Chapter1
Introduction

"As our world continues to generate unimaginable amounts of data, more data lead to more correlations, and more correlations can lead to more discoveries."

Hans Rosling

This thesis aimed to the analysis of biomarkers and the development of reference guidelines for the utility of dried blood spots in field epidemiological studies with primarily focuses on its application to regulate high sample throughput for biomarker analysis. This chapter introduces the fundamental concepts of the dried blood spot (DBS), its utility and includes both my own experience of performing the assays on DBS and results of a review of literature on published articles and updated throughout its duration. The review of the literature includes all the review and published an article on utility & compatibility of DBS for accessing biomarkers in field epidemiological studies.

This chapter includes the preparation of DBS, storage, and transportation. How to collect the blood samples on filter paper, what precautions to be taken during blood collection, quantity & quality of blood spots, validation of blood spots, factors affecting the blood volume. In the interest of conciseness, how DBS samples can be utilized to conduct field epidemiological & public health research when there is no refrigeration condition is available in field settings, DBS transport to laboratory even after long duration (>48 hours) & stored at the different thermal condition.

1.1 Background

The idea of sampling biological fluids on filter paper was proposed by Ivar Bang for nearly a century. The concept of capillary whole blood, obtained by heel or finger prick and blotted on to a blood collection card (Guthrie card), used to screen metabolic disease in populations of neonates was introduced in Scotland by Guthrie and Susie in 1963 (Guthrie R, 1963). This report is often regarded to be the first use of DBS. Dried Blood Spots (DBS) has been used for newborn screening for decades. DBS is a sampling method for whole blood where few drops of blood deposited over filter paper or protein saver card from heel prick, finger prick or venous blood. Other matrices can also be dried over filter paper such as (saliva, serum, urine, cerebrospinal fluid, DNA, buffy, plasma) referred to as Dried Matrix Spot (DMS) (Zimmer, Christianson, Johnson, & Needham, 2013).

The first introduction of the Guthrie test, the range of metabolic disorders, pharmacological studies, infectious diseases and basic biological investigations to which DBS has been applied has steadily increased(Parkin et al., 2012; Polley et al., 2015; Uzicanin A1, Lubega I, Nanuynja M, Mercader S, Rota P, Bellini W, 2011)(Guthrie & Susi, 1963)(Mcdade, Williams, & Snodgrass, 2007).

Historically this technique was introduced as it significantly simplified the collection, handling, and storage of blood samples compared to alternative technologies with minimal biohazard risk of particular importance here. DBS samples have been used in a wide range of applications to measure a large number of biomarkers and analytes such as infectious pathogens, vitamins, trace elements, glucose, specific antibodies and many others (Mei JV1, Alexander JR, Adam BW, 2001a). DBS provides a successful application where they offer an advantage over conventional plasma, serum, liquid whole blood. The paper substrate is cheap, readily available and easily stored and handled. DBS samples are generally considered non-hazardous, can be easily transported from remote areas to laboratory sites (Hamers, Smit,

Stevens, Schuurman, & Rinke de Wit, 2009). DBS has been demonstrated to be a particularly cheap, and reliable method of large scale testing in remote populations (L., 1966), it is a less invasive sampling method (heel prick, finger prick). Sample storage and transportation is easier and cheaper than venous blood as the dry specimen is robust, and generally does not need to be frozen or shipped in dry ice. The eluate is normally eluted from the sampling card into the solution before analysis using a mix of buffers and organic solvents and the lack of volume control during sampling is mainly solved by punching out a fixed part (typically diameter of 3-6 mm) of the spot. DBS collection process reduces the risk of infection and crucially reduced the blood volume required compared to liquid blood collection methods (Cassol et al., 1997). Some disadvantage to DBS sampling has been established, generally percentage of hematocrit, sample homogeneity, effect of temperature and humidity during transportation & storage, analyte recovery, filter paper characteristics, and anticoagulants (Ben LM van Baar, Tom Verhaeghe, Olivier Heudi, Morten Rohde, Simon Wood, Jaap Wieling, Ronald de Vries, Steve White, 2013; Ronald de Vries, Matthew Barfield, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij, Ben van Baar, Zoe Cobb, 2013; Timmerman et al., 2013; Zoe Cobb, Ronald de Vries, Neil Spooner, Stephen Williams, Ludovicus Staelens, Mira Doig, Rebecca Broadhurst, Matthew Barfield, Nico van de Merbel, Bernhard Schmid, Christoph Siethoff, Jordi Ortiz, Elwin Verheij, Ben van Baar, 2013).

Over the last 5 – 10 years there has been much interest in applying the advantage on the offer from DBS, when used as a microsampling technique, to a new application. In the present study, we aimed at using DBS as an alternative to traditionally used blood sampling to analyze biomarkers in epidemiological studies. The blood sampling conventionally the most common technique to be used in quantitative and qualitative analysis of biomarkers, but the conventional approach typically involves collecting whole blood from volunteers via a

venous cannula, then centrifuged under refrigeration to separate it into plasma and red blood cells fraction. Both plasma and red blood cells transferred into separate tubes, placed on ice and maintain frozen during transportation to, and during storage at, the site of analysis. The samples are easy to store and ship due to increased stability in dried samples and the minimal of space occupied by the sample cards. The storage and shipping of DBS samples are cost-effective as compared to liquid samples. On dehydration, bacteria and viruses also inactivated, decreasing the biohazard related to handling DBS samples. The advantages related to DBS could be beneficial in the collection of biological samples, however, there are still challenges that need to be addressed before a wide application of DBS is possible.

To obtain the possible sensitivity by using DBS samples is limited by the low volume of sample available, often difficult to pre-concentrate or reanalyze (Peter T Kissinger, 2011). Analysis of small sample volumes has partly been improved by the introduction of more sensitive analytical instruments(Wagner M, Tonoli D, Varesio E, 2016), but DBS analysis is still challenging in the detection and quantification of trace amounts of analytes. The DBS samples are normally transferred from the sampling material by punching out a part of the spot and eluting the sample with buffers or organic solvents, this step introduces an additional preparation step, increasing both preparation time and the probability for additional variability (Jager NG, Rosing H, Schellens JH, 2014; Meesters RJ1, 2013). Carry-over during the punch out of samples can lead to contamination hampering the quality of the analytical results, and cleaning of the puncher between every sample is often required. Another challenge is the incomplete elution of the sample together with the limited sample volumes available, which is critical for the sensitivity. The efficiency of transferring analyte from the material (termed as the elution efficiency) can be influenced by factors such as the analyte affinity to the paper, the sample material and the preparation protocol used (Liu G, Patrone L, Snapp HM, Batog A, Valentine J, Cosma G, Tymiak A, Ji QC, 2010). Additional time spent

on evaluating and optimizing the elution of sample from material, and in solution it is therefore expected A few papers have described automatic and on-line elution of DBS to speed up the elution process (Abu-Rabie P1, 2, Spooner N1, Chowdhry BZ2, 2015; Déglon J, Thomas A, Daali Y, Lauer E, Samer C, Desmeules J, Dayer P, Mangin P, 2011; Ooms JA1, Knegt L, 2011), but most laboratories are still using the traditional "punch and elute" procedure. The concentration of an analyte depends on the affinity of the analyte to plasma versus red blood cells, and the analyte concentration will, therefore, depend on the matrix analyzed. Plasma and serum samples have predominately used as matrices in bioanalysis and new reference values will be required for whole blood samples. The composition of capillary blood and venous blood is also different (e.g. due to intracellular and interstitial fluids) and caution must thus be taken when comparing venous blood samples and capillary blood samples (Mohammed et al., 2010).

1.2 Dried Blood Spots (DBS)

1.2.1 Utility of Dried Blood Spots

DBS is a well-established technique for collecting and storing blood samples. Since it is used for the detection of phenylketonuria in newborns, advances in analytical capability, particularly with mass spectrometry (MS) based techniques have enabled the utilization of DBS in prospective newborn screening programmes for a range of compounds including acylcarnitines, amino acids, organic acids, thyroxine and steroids (Carpenter & Wiley, 2002; Keevil, 2011). DBS has been widely used for the detection of a variety of biomarkers in epidemiological studies including infection, cytokines, amino-acids, vitamins (Butter, Hattersley, & Clark, 2001; Chaudhuri SN1, Butala SJ, Ball RW, 2009; Nelson KB1, Dambrosia JM, Grether JK, 1998; Skogstrand et al., 2005, Mwaba et al., 2003). With greater than 95% of newborns in the US included within the newborn screening program, DBS has become an important blood sampling method in the detection of treatable genetic and

metabolic disorders (Mei JV1, Alexander JR, Adam BW, 2001b). It can be used in large scale infectious epidemiological studies (Parker & Cubitt, 1999). These applications reveal the utility of DBS and their potential to study the range of analytes from low to high molecular weight. In large scale, epidemiological research DBS plays an important part to carry blood specimen in dry form which reduces the chance of contamination, infection, and cost.

1.2.2 Sampling material and devices

In the last few years, numerous innovations on DBS substrate material have done and the blood collection card format has also changed from various manufacturers such as Aligent, Whatman, Ahlstrom. DBS card has been approved as in vitro diagnostics medical device by FDA, comprising a specified collection medium, usually an untreated cellulose-based paper. The blood collection cards designed or redesigned for a variety of reasons which include: commencing newborn screening for the first time, additional screening test, requiring demographics and/or specimen detail for result interpretation, develop policies for current screening related to demographic, introducing laboratory automation which may require specific card features, introducing digital blood collection devices (cards) image storage, introducing long term blood collection devices (cards) /collection matrix storage, introducing tracking of each baby's blood collection devices (cards) journey (specimen tracking), commencing formalized collection of consent or decline for newborn screening.

Different cards are commercially available for microsampling of blood specimen collection, with other body fluids like saliva, urine, sputum. Most commonly used sampling materials Whatman® grade 903TM and the Ahlstrom grade 226 recommended by FDA for use in clinical research (Mei JV1, Zobel SD, Hall EM, De Jesús VR, Adam BW, 2010; Mei, Alexander, Adam, &

Hannon, 2018). These microsampling materials are made up of pure cellulose which is compatible with DBS sampling. Examples are the pure cellulose material FTA® DMPK-C

and the impregnated cellulose sampling cards FTA® DMPK-A and FTA® DMPK-B is available for pharmacokinetic drug discovering studies. The cellulose impregnated with some chemical that lyses the cells and denatures the proteins but is beneficial for quenching the enzyme activity in the blood sample. But sometimes the chemical treated cards also enhance the background signals during analysis (Clark GT1, 2011). Other cellulose-based indicating sampling cards are commercially available for colored or non-colored biological samples (e.g serum, plasma, saliva).

1.2.3 Properties of blood collection card

- Sampling card should be robust, compact and can be easily shipped.
- It should be free from any type of biological or chemical contaminants.
- Sampling card must be labelled with relevant information such as Name, ID, Date, Gender, and Address.
- Sampling card should have proper blood holding capacity.
- Cover design should be equipped with flap cover to eliminate any type of sample damage during assay performance.
- Blood sampling card should be labelled with instruction of blood collection procedure.
- It should be compatible with all type of blood samples & collection methods.
- Sampling card should retain all the measuring analyte in their intact form without any damage at any thermal condition.
- The substrate material of card should be embedded in such a format, that it should release all the analyte in solution while sample processing.

- It should be portable to carry from field to laboratory without any refrigeration
- The cost should be minimize to collect maximum blood samples.
- The diameter of circle on card should be separated at a distance, so that the spotted blood should not overlapped.

- The card should be labelled with barcode to identify the each patient details

Advancements to the substrate material and card format could assist many of the functions and considerations. The use of a more robust and rigid card which would sandwich the substrate in tough plastic material rather than cardboard, which has several advantages. (1) Due to rigidity and robustness of card, it offers more physical resistance to tearing and deforming while shipping and storage. Some cards provided with the flip cover over the spotting circle which also gives some physical protection from contamination during air dry. (2) A rigid card would greatly assist ease of automation because the used card can be bend and twist easily but in spite of that very little variability there in the shape and size of sample format, the easier it is reliable to automate. Today, there are several prototype plastic cards that have been produced by various cards and substrate manufactured and it is possible that the use of paper card developed by Robert Guthrie 5 decade ago for early detection of metabolic disorders. Guthrie also developed the first newborn screening (NBS) test for phenylalanine, the biochemical marker for phenylketonuria (Guthrie R, 1963). This invention introduced the New Born Screening among population using blood collected on filter paper (G. R, 1992).

In 1981, CDC established the filter paper evaluation services to observe the reliability and reproducibility of filter paper. CDC's New Born Screening Quality Assurance Program helped to establish the filter paper evaluation program in collaboration with Association of Public Health Laboratories (APHL)(Víctor R. De Jesús, PhD' Correspondence information about the author PhD Víctor R. De Jesús Email the author PhD Víctor R. De Jesús, Joanne V. Mei, PhD, Carol J. Bell, BS, MT (ASCP), W. Harry Hannon, 2010). Consequently, the Clinical and Laboratory Standards Institutes (CLSI) created and published a national benchmark for collecting blood on filter paper. This standard, CLSI LA4-A5, focus on issues associated with biological specimen collection, filter paper (Hannon WH, Whitley RJ, Davin

B et al. LA5–A4, Wayne, 2007). The approved standards describes the parameters required for manufacturing filter paper that provide homogeneous dried blood spots of uniform size (Hannon WH, Whitley RJ, Davin B et al. LA5–A4, Wayne, 2007).

Similar to other techniques of collecting blood, the filter paper blood collection device has a level of imprecision and variability that can be described by defined protocols. The standard outlines methods for reducing variables like the amount of blood spotted in preprinted circles on the paper and chromatographic effects that affect the volume of blood discovered in a typical 3.2mm punch from the spot (3.42 µl of blood) (Mei JV1, Alexander JR, Adam BW, 2001c). The effects of the newborn's hematocrit (percentage of red blood cells) on the sample aliquot cannot be controlled, and larger hematocrits will result in lower serum volumes in a standard punch (Mei JV1, Alexander JR, Adam BW, 2001c). Because several paper lots are in use at any given time, minimizing filter paper transitions from lot to lot is critical to assure uniformity of specimen collection, calibrators, quality control materials, and other reference materials on this matrix.

For almost 30 years, under a standardized methodology, NSQAP has been measuring filter paper performance using a DBS-based quantitative radioisotopic approach to quantify performance characteristics. (Barbara W. Adam, J. Richard Alexander, S. Jay Smith, Donald H. Chace, J. Gerard Loeber, L. H. Elvers, 2000; Mei JV1, Alexander JR, Adam BW, 2001c). The FDA has classified two commercial sources of blood collection filter paper as Class II medical devices: Whatman® Grade 903 and Ahlstrom Grade 226 filter papers. The two FDA approved filter papers have shown a comparable performance in analyte quantification (Mei et al., 2010).

The absorption time and spot size produced are also used to assess the absorption characteristics of the filter paper. While filter papers such as S&S 903 and Ahlstrom 226 are untreated, chemically treated cellulose papers such as FTA & FTA Elute, Whatman, and GE

Healthcare are also available. When these sheets come into contact with cells, they lyse them, deactivate enzymes, and give antimicrobial protection. This paper was created for the examination of nucleic acids and proteins, but it has also been used for drug quantification. (Barfield, Spooner, Lad, Parry, & Fowles, 2008; L. R., 2010; Spooner N, Lad R, 2009). The Clinical and Laboratory Standards Institute has certified that collection papers made from high purity cotton linters meet performance standards for sample absorption and lot-to-lot uniformity. The Centers for Disease Control and Prevention (CDC), which maintains an independent quality-control program, noted that, "The filter paper blood collection device has achieved the same level of precision and reproducibility that analytical scientists and clinicians have come to expect from standard methods of collecting blood, such as vacuum tubes and capillary pipettes"(Mei JV1, Alexander JR, Adam BW, 2001a).

1.3 Blood Collection Methodology

DBS can be prepared with 2 methods

(1) Venipuncture method (2) Capillary method.

1.3.1 Venipuncture blood collection method

In this method, blood is collected by trained staff through the penetration of the needle into veins using a syringe or with an evacuated tube system.

Selection of veins

Palpate the blood collection site and trace the veins with your finger. Select the cephalic vein, median cubital vein or basilic vein because there is a lack of resilience in thrombosed veins than arteries pulsate.

1.3.1a Procedure for venous blood collection

1) Subject position: The subject should be sit on chair or he/she can sit up on bed and arm should be hyperextend in relaxed position.

2) Selection site: Select the perfect vein by direct examination or through palpation on collection site. Generally basilic or median cubital vein is preferred for blood collection.

3) Tourniquet: Now apply tourniquet above the collection site, it should not leave more than 2 minutes and not too much tight. After apply tourniquet, the subject should make a fist without pumping the hand.

4) Collection of blood: Select the venipuncture site by palpating the collection area, alcohol wipe that area and leave it for dry. Once it is dry, use disposable syringes or vacuum system to draw the blood.

1.3.1b Preparation of DBS from venous blood (Grüner, Stambouli, & Ross, 2015)

1. Spot the collected anti-coagulated (EDTA) whole venous blood on the filter cards as soon as possible. Dry blood spots should not be prepared more than 24 hours following venipuncture.
2. Fill out the filter card with all of the information needed to identify the patient. Only one card should be spotted with a single person's blood.
3. Put on a pair of latex rubber gloves that are disposable.
4. Gently invert the blood collection tube 2–4 times before carefully opening the stopper.
5. Using a pipette with a disposable tip, aspirate 50 µl of whole venous blood. Transfer the blood to the center of one circle without touching the filter paper directly with the tip of the pipette. Try to fully saturate the circle.
6. Repeat this procedure to fill all required circles of the card.

1.3.2 Finger prick/capillary blood collection method

Normally, retractable lancet is preferred for capillary blood collections for safety reasons. Capillary blood collection technique specially design for children's and infants, because venipuncture methods might be discomfort to the children's due to pain or other major

complications. Some recommended depths for puncture, 1.5 mm for infants less than 6 months, 2.00 mm for children's and 2.5 mm for adults.

1.3.2a Procedure for capillary blood collection

1) The subject should be sit on chair or he/she can sit up on bed and arm should be hyperextend in relaxed position.

2) Select the finger and massage it properly 5-6 times with downward position of finger for proper blood flow.

3) Clean up the fingertip with 70% alcohol, wipe it with dry cotton or gauze.

4) Place the retractable lancet over the top of fingertip, make skin puncture at the center of finger pad. The puncture should be made perpendicular to the fingertip, so that the blood should not run down the ridges.

5) Wipe out first drop of blood which contain excessive amount of tissue fluid, collect blood drops in collection device by massaging the fingers, but avoid excess pressure otherwise squeeze tissue fluid in blood drop.

6) After collection of blood drops, put small gauze over puncture site for few minutes to stop bleeding from fingertip.

1.3.2b Dried Blood Spot preparation from capillary blood (Grüner et al., 2015)

Dried blood spot collection specially designed for children's or infants' disease diagnosis because a little amount of blood is directly transferred from fingertip or toes to filter paper. Fingertip is punctured with the help of retractable lancet, the first drop of blood wipeout which contains tissue fluid, continue to massage the fingertip with low pressure to form a big drop of blood and blood directly applies on collection card, blood drop should not overlap or overlay and it should be spread homogeneously on the circle. If the circle is not filled or improper blood collected on filter paper then DBS samples can't be used as per guideline of

valid and invalid DBS samples (source: CDC). In this study, we have used venous blood samples for the preparation of dried blood spots.

1. Because the first drop of blood may contain extra tissue fluids, wipe it away with a gauze pad. Increase blood flow at the puncture site by massaging the finger once more. Apply the next drop on one of the filter card's circles without touching the surface directly with your fingertip. Allow only capillary forces to soak the blood into the material of the filter.

2. Let the next large drop of capillary blood form on the finger-tip and collect it in the next circle. Continue this procedure until all necessary circles are filled or blood flow stops.

3. Do not squeeze or "milk" the finger excessively if the blood flow is not sufficient to fill all the required circles of the filter card. If blood flow stops place a bandage on the finger-tip. Perform a second skin puncture on another finger if more blood is needed for the examination.

1.4 Dried Blood Spots preparation, drying and storage in our laboratory

1.4.1 Dried Blood Spot preparation

3ml - 4 ml venous blood collected in EDTA tube for the preparation of DBS. From single EDTA tube we have prepared paired DBS cards, each card contain 5 spots, all the 5 spots completely filled with venous blood. The blood specimen collection cards labeled before spotting of blood over the circles. Approx. 40-50µl blood apply at the center of specimen collection card with the help of pipette leave it for complete homogenously saturation of blood through capillary action. (Figure 1)

Figure 1. Prepared dried blood spots from venous blood.

1.4.2 Steps for Dried Blood Spot preparation

1) We have wiped the equipment's with 70% alcohol to prevent contamination in blood samples.

2) Labelled all the blood collection cards with subject ID, date of blood collection and time of blood collection

3) Apply 40 - 50µl blood on the center part of circle on Whatman 903 protein saver card with the help of pipette and the tip of pipette should not touch filter paper card.

4) Applied blood continuously on filter paper card until it is uniformly distributed over the entire circle.

5) Blood applied only on one side of the circle continuously and not overlaid the circle.

6) All the 5 circles of filter paper card filled completely with blood to avoid formation of invalid spots.

7) Recommended, continuously watching the blood until it is completely absorb by surface.

1.4.3 Drying of blood spots

Blood sampling cards placed in a Whatman card rack & air-dried at room temperature for 2-3 hours. The use of blower or heat for drying the cards is not recommended. Sometimes, DBS has been air-dried for several hours to immobilize on the matrix and preserve it, but as per our study protocol, we have dried the spotted cards for up to 0 – 72 hours according to our research question. We dried it into 3 phases, First, blood spots dried between 0 – 24 hours, Second, it is dried between 24 – 48 hours, Third, it is dried between 48 – 72 hours. Here we used word drying for 0-72 hours, which means we have dried all the spots for 2 hours only and then packed into ziplock bag and it was kept on RT for up to 0 – 24 hours, 24 – 48 hours and 48 – 72 hours, between these duration we have shifted our DBS samples to 4^0C and -20^0C (Figure 2)

Figure 2. Drying of blood spots in Whatman 903 rack at ambient temperature

1.4.4 Storage of Dried Blood Spots

After drying of blood spots at a different time interval, DBS cards were packed in an opaque sealed ziplock airtight plastic bag with 1 or 2gm desiccant pouches and stored at three different temperature -80^0C, -20^0C and 4^0C. These plastic ziplock bags were labeled with the biohazard marks. The bag was tightly packed to avoid moisture due to the accumulation of air. Even today, DBS is considered as non-infectious material by International Air Transport

Association (IATA) regulations (Polley et al., 2015). Desiccant pouches act as the adsorbent, which absorbs all the moisture content from the DBS surface to prevent it from spoilage. Bag was tightly packed to avoid moisture due to accumulation of air The International Air Transport Association still considers DBS to be noninfectious material (Polley et al., 2015). Desiccant pouches act as the adsorbent, which absorb all the moisture content from DBS surface to prevent it from spoilage.

1.5 Analysis of substrate material of blood collection card

The fundamental part for any dried blood spot preparation is the selection of substrate material or blood collection cards which can absorb blood continuously without any obstruction. For the last few years, several implementations have been done to improve the quality of substrate materials from various manufacturers such as Whatman, Aligent. The substrate material embedded with cellulosic material or the polymer-based paper is more robust, rigid, cheap and easily available. It is strongly recommended that the blood collection substrate should be specified by manufacturers in the collection card by checking batch to batch variability. Currently, only the International standard (CLSI NBS01-A6) is available for specification and evaluation of blood collection paper and their uniform absorption. Perkin Elmer 226, Whatman 903 from GE Healthcare Life Sciences, and Munktell TFN Neonatal Screening from Ahlstrom are manufacturing companies that satisfy this specification as per International standard.

1.5.1 Major challenges in Dried Blood Spot processing and analysis

1.5.1a How volume of hematocrit effect on DBS analysis

The blood hematocrit is one of the main issue related to analysis of DBS and is defined as the volume fraction of red blood cells in a blood sample. Its reference range is 0.35 – 0.50 but due to age and disease which can influence the amount of Red Blood Cells (RBC) which results is hematocrit values lie out of range(De Kesel, Sadones, Capiau, Lambert, & Stove,

2013). The viscosity of blood depends on amount of hematocrit volume, because when the volume of hematocrit increased it will not spread homogenously over the blood collection card and might be possible the blood components overlap to each other, this condition of DBS may be altered our result. The viscosity of the blood will thus be important in DBS sampling as it influences the spread of the sample on the sampling material.

A punch out from a DBS sample with low hematocrit blood will consequently contain a lower volume of sample than a punch out from a high hematocrit DBS sample, introducing bias in the quantitative analysis of DBS (Holub et al., 2006; Salis, Reith, Broadbent, & Medlicott, 2016; Vu, Koster, Alffenaar, Brouwers, & Uges, 2011). The blood hematocrit has in addition been reported to influence the recovery during the elution process (Youhnovski, Bergeron, Furtado, & Garofolo, 2011) and has been proposed to contribute to different degree of matrix effects (Meesters & Hooff, 2013). The analyte concentration in a blood sample is also influenced by the hematocrit, especially if the analyte distribution is significantly skewed to either blood cells or plasma..(Emmons & Rowland, 2010).

DBS area and hematocrit values have inverse relationship (De Kesel et al., 2013), because presence of hematocrit increases the viscosity of blood which causes blood to spread less over substrate, higher hematocrit values gives small blood spot area. The value of hematocrit varies between gender and age of subject. The obstruction caused by hematocrit can be overcome by applying fixed volume of blood over blood collection card and excise complete spot for analysis, but this is not possible with calibrated pipettes because due to viscosity of blood, complete blood will not spotted on collection card some amount of blood still present as residual on pipette tips.

This methods makes difference in calculation of hematocrit values.

Age/Sex	Hematocrit values (%)
0-2 years	28-67
2-12 years	34-45
12-18 years female	36-46
12-18 years male	37-49
Adult female	36-44
Adult male	41-50

Table 1. Hematocrit values (%) based on age and gender (Denniff & Spooner, 2010b)

1.5.1b Elution Challenges

Due to increased hematocrit values, the recovery of complete analyte reduces because of small concentrated blood spots (R. De Vries et al., 2013), De Vries have also studied if you store DBS for longer period it may limit the viability of some analytes.

1.5.1c Homogeneity Challenges

The blood sample should be completely spread over the marked spot on blood collection card. Chromatography on the substrate can impact the homogenous nature of the sample, with some analytes having greater affinity for the substrate than the blood and vice versa (O'Mara et al., 2011). This blood collection process is linked with affinity chromatography (S, Fanali, Paul R Haddad, 2013). Homogeneity affected by analyte, paper substrate and volume of hematocrit. Today there are many blood collection papers manufactured with different types of substrate for good analyte results. Some paper have very less impact even with high hematocrit values, where blood spread homogenously over filter paper. Such as Alhstrom paper, Whatman No. 3 paper, DMPK cards, Whatman 903protein saver card, Whatman FTA cards.

1.5.1d Stability of analyte

It have been already proved that analyte on DBS are more stable than in liquid form of blood specimen (Bowen, Hemberger, Kehler, & Evans, 2010; D'Arienzo et al., 2010). Generally, analyte on DBS stable for the months or even years if proper condition like temperature, humidity maintained because in dry form enzymes are in their inactive stage and unable to destroy (Denniff & Spooner, 2010a; Denniff, Woodford, & Spooner, 2013)

1.5.1e Storage and shipping of DBS cards

There is always controversial results appears on storage and shipping conditions of dried blood spots, because every analyte do not stable at all thermal condition. Therefore its challenging how we can transport and store DBS samples so that we can exploit the utility of DBS to recover maximum analyte with greater than >95% efficiency. As per published articles, probably DBS is transported at ambient temperature and stored at -20^{0}C or -80^{0}C. In the US, DBS cards are transported using the conventional postal service as DBS cards are considered as nonhazardous (SERVICE, 2013).

1.6 Biomarkers: definition, types and contribution

1.6.1 Biomarkers definition

It is a characteristics that is objectively measured and evaluated as an indicator of normal biological process, pathological process or therapeutic response to an intervention (Lewandowska, 2014). These are the indicators of exposure, effect, susceptibility, or clinical disease. The biomarkers is defined as the molecular alteration which is measurable in the cellular components of blood, tissue, fluids (Hulka BS, 1990).

They are biological substances found in blood, tissues, and other body fluids that indicate whether a process is normal or aberrant, or whether a disease condition exists. They can be used to see how body respond to any treatment of disease condition, they are also called as molecular marker or signature molecules.

Biomarkers have the potential to be used in risk stratification, early detection, selection of the best treatment and monitoring treatment response, surveillance, and drug monitoring and development in the case of disease. Biomarkers also used in other clinical scenarios, such as microbial identification and diagnostics. A biomarker could be a molecule, protein, gene or any other biological characteristics which can be measured accurately and can be reproducible and should be valid indicator of any disease with their outcome (Strimbu, Kyle; Tavel, 2010).

The "omics" era has resulted in unbiased discovery-based methodologies to identify biomarkers, including as proteomics, genomics, metabolomics, and epigenomics, which are utilised to find new biomarkers with clinical significance.

Proteomics is currently the most widely used omics approach for biomarker discovery. Splice variants and posttranslational modifications of proteins determine protein structure, function, localization, maturation, and turnover, all of which vary rapidly in response to environmental inputs.

1.6.2 Characterization of Biomarkers

The various biomarkers would be classified as Demonstration, Exploration, Characterization, and Surrogacy in a classification scheme proposed to FDA.

1.6.2a Exploration biomarkers

These are research and development tools applied to the preclinical setting without evidence necessarily linking the biomarker to clinical outcomes in humans.

1.6.2b Demonstration biomarkers

These biomarkers have been qualified in terms of preclinical sensitivity and specificity, and have been linked with clinical outcomes, but they have not been reproducibly demonstrated in clinical studies.

1.6.2c Characterization biomarkers

It demonstrated adequate preclinical sensitivity and specificity, and they are reproducibly linked with clinical outcomes.

1.6.2d Surrogate biomarkers

These biomarkers are used for drug development purposes, surrogate biomarkers include Cholesterol level, HIV load, blood pressure (Lewandowska, 2014).

Some researcher divide the biomarkers into 3 category, type 0, type1 & type2. Type 0 category measures the natural history of disease and correlate with clinical outcomes. Type 1 biomarkers usually determined the biological effect of a therapeutic interventions. Type 2 biomarkers are surrogate biomarkers (Lewandowska, 2014).

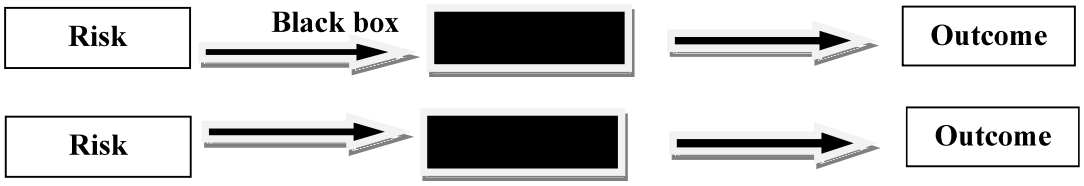
1.7 Potential contribution of biomarkers in epidemiological studies

A biomarker is a biological trait that can be measured precisely and consistently to offer an objective assessment of health or disease. Benefits of biomarkers include identification of therapeutic targets, monitoring of clinical intervention, and development of personalized or precision medicine. Biomarkers aid in the early detection, treatment, and management of a variety of noncommunicable diseases by providing a better knowledge of disease processes (NCDs) (Chrysanthi Skevaki MD, PhD. JoliceVanden Berg PhD. Nicholas Jones BSc. Johan Garssen MD, PhD. Peter Vuillermin MD, PhD. Michael Levin MD, 2016).

Typically, biomarkers are used currently to provide mechanistic insight of disease severity, with their future role in risk stratification/disease prediction. Today, many research studies is going on to identify predisposed biomarkers in children and adults.

Many pediatrics biomarkers are still in the discovery stage, with a long way to go before they can be evaluated and used in clinical trials. Biomarker discovery and validation in children with disease lags behind that in adults; given the early onset and hence potential lifetime influence of many NCDs, more research using children's cohorts should be conducted.

Biomarkers have an important applications in epidemiological research as well as in preventive medicine & environmental health. The major aim of epidemiological research is to identify the possible causes and risk factors associated with the particular disease condition. Because of insufficient considerations, the risk factor epidemiology has been some time referred as black- box epidemiology(P1., 1994). Cause effect relationship in modern epidemiology.



Epidemiological biomarkers characterized by cause effect relationship to know the disease etiology. Identification of biomarker is not a diagnostic test, it represents an indicator of transition phase or early change which could later converted to disease condition. In a study, the practical use of biomarker should be acceptable ethically(Ashford, 1994). In epidemiological studies basically 3 types of biomarkers(Brown & Reavey, 1994; Markers, 1987) are essentially useful to characterize the cause effect relationship for a particular disease conditions or likely to predict the risk of occurrence of disease in future. The characterize biomarkers such as biomarker of exposure, biomarker of effect and biomarker of susceptibility.

1.7.1 Exposure biomarkers

It could be a metabolite within body which can form compound with related event of exposure. The biomarkers of exposure to stable compound assessed by measuring their concentration in blood, serum, urine etc. Exposure biomarkers forms the adduct within body, development of DNA & protein adducts well studied in cancer epidemiology(Phillips, 2005; Poirier, 2004). Exposure biomarker include DNA, Protein, Hb, genes etc. Adducts formed in

DNA can be detect in White Blood Cells (WBC), biopsies of tissue or sometimes in urine also. Hb adduct indicate the complete transition events during the 4 month life of RBC.

1.7.2 Effect biomarkers

It is characterized by measurement of functional capacity or state or balance of body affected by impact of exposure. These effect biomarkers are potential indicator of transition phase or pre-clinical phase of any abnormality. These biomarkers are of 2 types, indicator biomarkers and non-indicator biomarkers (Grandjean, 1995). Specific biomarkers provide the evidence for disease prevention because they indicate a biological effect of a particular exposure, whereas non-specific biomarkers indicate biological effect due to multiple exposure phenomenon. There are variety of biomarkers applied to human research, the most commonly measures in peripheral blood cells for genetic alterations such as chromosomal aberrations, micronuclei and sister chromatid exchanges. In a recent publication by International Program on Chemical Safety (IPCS) reviews applications of biomarkers which includes adverse health effects: liver cell necrosis, low birth weight, decreased pulmonary function, and mental retardation("International Programme on Chemical Safety," 1993).

1.8 Why Validation of candidate biomarkers is important

"The more innovative a biomarker is, the more challenging it is to validate it"

The validation of single biomarkers aims to determine if there is existence of sufficient evidence for a potential clinical utility of a given candidate biomarker to ensure further investment in that candidate biomarker for clinical trial. Validation is very essential before moving forward with any candidate biomarker because a clinical study is expensive, time-consuming, and has a high demand for clinical samples. Potential characteristics of biomarkers to detect the disease & prognostic stages establish it as more robust and potent biological signature in medical science. The validation of any biomarker for a particular disease condition or prognostic stages require thousands of clinical trial.

In 2008, a thorough biomarker study validated the diagnostic assay's quality and clinical validity (Paulovich, Whiteaker, Hoofnagle, & Wang, 2010). Because the majority of biomarker candidates failed the clinical phase, Paulovich et al. termed biomarker validation the tar pit. As a result, the likelihood of a new biomarker candidate being adopted into clinical practise is quite low. However, there have been a slew of new biomarkers and diagnostic test platforms published in recent years. However, as Fontela et al. discovered, established diagnostic tests for infectious illnesses frequently fail to meet methodological quality standards and provide reliable reporting (Fontela et al., 2009). They employed the Quality Assessment of Diagnostic Accuracy Studies (QUADAS) and Standards for the Reporting of Diagnostic Accuracy Studies (STARD) tools to improve the reporting of diagnostic accuracy assays in tuberculosis, malaria, and HIV test quality.

It is the consequence of poorly designed biomarker discovery and validation phases without clear understanding the nuances of interpreting high dimensional data sets which often leads to biases and high false discovery rates(Ransohoff, 2005). Many candidate biomarkers has been discovered, which can easily discriminate statistically between cases and controls groups are often preferred without being tested. Strategies which allow a high number of candidate biomarkers to be analyzed with the highest throughput and the lowest possible costs are required for validation.

Based on the circulatory nature of blood in every part of our body, the measurements of blood components is valuable for to know the health state of population(Chen & Snyder, n.d.). In this study, we have selected the blood based identification of biomarkers from intravenous blood corresponding to their dried blood spots.

We have selected 3 broad categories and captured single biomarker from each category. First, **Molecular field** which includes all the molecular aspects which involves particularly genetic materials such as DNA or RNA and sometimes their translational product proteins, therefore

we have selected more robust biomarker genomic DNA (**gDNA**) and will observe the utility & feasibility of DNA in downstream applications. Second, **Infectious field** where we have tested the immunoglubulins (IgG) from DBS, we can test all the infectious pathogens which express IgG antibody because IgG antibody is more robust and stable immunoglobulins, and therefore we have tested IgG immunoglobulins against ***Helicobacter pylori*** infection. Third, **Biochemical field**, in biochemical category, we will test very important disease (Cardiovascular disease) biomarker such as **apolipoprotein A-I** and **apolipoprotein B-100**. Validation of various biomarkers extracted from dried blood spot is done with their corresponding blood plasma. But the discovery & validation of protein based biomarkers directly in serum is challenging due to complexity and availability of dynamic range of analytes in plasma. Some matters are complicated by the fact that potential candidate Low amounts of biomarkers are common, and they are frequently bound to carrier molecules. Consequently, high-abundance serum proteins may interfere and have a significant impact on test performance as well as the quality of the result.

1.8.1 Serum matrix effect

It plays a crucial role at the endpoint because it interfere with analytical technique by one or more components of the sample, which can lead to loss of sensitivity, specificity, assay robustness and high level of false positive and false negative results. In our study, we used ELISA methodology to detect antibody against infectious agent and Apo-lipoproteins. Today, the current gold standard for the biomarkers validation is classical ELISA. ELISA is frequently used for confirmatory studies since it has a relatively high throughput and is a versatile and sturdy technology. Basically ELISA performance strongly depends on antibody quality and skill of performer with experience. Because of the solid structure of ELISA, the immobilization of antigen to the solid matrix is altered, causing accuracy and repeatability

issues. However, ELISA development is costly, with long development lead times and high false positive rates.(Whiteaker et al., 2007).

1.8.2 Characterization of biomarkers through Dried Blood Spot

In epidemiological studies, the blood sampling method through DBS always preferred over venous blood for those analyte which is likely to give results compatible with results of plasma/serum. Drying a blood sample on filter paper is also an excellent way of collecting and preserving blood proteins. Traditional blood sample collection procedures tend to limit biomarker research to one or a few samples per person, focusing on comparing study groups. (Healthy and sick) of different people. According to a study of the literature, there are very few studies that follow biomarker levels in the same person through time using a large number of samples (so-called "longitudinal "studies). In this study, we have focused on the same pattern so called longitudinal study because we have collected the DBS from particular subject over different period of time interval and stored it at different temperature for 4-5 months. Assay were performed to see whether the results obtained from DBS is compatible with the results obtained from plasma or not. We have tested 3 biomarkers from DBS stored at 4^0C and -20^0C at different time point interval and compared it result with results obtained on plasma for same individual. This approach resolves the transportation and storage problems of DBS.

By allowing us to collect and maintain a blood sample anywhere and whenever we choose, DBS sample collection opens up a new paradigm for protein biomarker research and clinical usage. They provide us access to the domain of time. The concentration of most biomarker proteins in blood varies less from day to day in a normal person than it does between persons; a minor variation in the amount of a biomarker protein might be extremely significant for an individual while remaining within the population's normal range.

1.8.3 Advantage of using biomarkers

1. Biomarker act as the potent prognostic factor in disease manifestation.
2. Biomarkers provide necessary information regarding the disease condition.
3. Biomarker is used to treat the disease condition once the level of biomarker detected.
4. The reproducibility nature of biomarker make convenient to researcher and clinicians to conduct the large scale population based studies.
5. Biomarkers used as population screening, disease diagnosis, risk prediction, disease etiology, homogeneity of risk.
6. In clinical trials biomarkers plays very important role, classification of disease, establishment of dose-response relationship, Identification of disease mechanism with the effect of exposure(Schulte PA, 1993)
7. Reliability and Validity(Schulte PA, 1993) of biomarkers are very complex.
8. Based on reproducible nature of biomarkers, it can be precise the measurement. Biomarkers are less biased than the questionnaire based information.

1.8.4 Disadvantage of using biomarkers

1. Storage of biological specimen for long duration
2. Laboratory error may create false positive results during assay performance
3. It's very difficult to establish the normal range of biomarker due to large variability
4. Cannot perform maximum test due to low blood volume.

1.8.5 Limitation of using biomarkers

Generally the biological markers cross checks through so many hurdles before come to establish as potential and robust biomarker. The validity and reliability of any biomarkers depends on the sampling methods, handling of samples, storage of samples, and performance by laboratory person. Laboratory to laboratory the values of biomarker changes due to above all mentioned hurdles.

Chapter 2
Review of Literature

2.1 Evidences on Dried Blood Spots research

When performing diagnostic or epidemiological surveys, especially in remote areas in resource-poor settings, the facilities for processing blood samples and maintaining frozen samples frequently do not exist. This is particularly true for neglected tropical illnesses, which are often found in populations far from advanced diagnostic facilities. Dried blood spots (DBS) are a potentially effective and low-cost way to overcome these challenges. Finger-prick blood, for example, is simply and swiftly collected onto filter paper and delivered at room temperature. However, because blood sample quantities on filter paper are always small, careful assay validation is required to attain the best sensitivity and specificity.(Smit, Elliott, Peeling, Mabey, & Newton, 2014). Filter paper was first used as a in 1815 by the Swedish chemist Jo¨ ns Berzelius and later on in the 1940s, Heatley described the use of filter paper for incorporating antimicrobial solutions in Oxford, giving rise to antibiotic susceptibility disc testing(PF, 2001).

Almost all types of human body fluids (from blood to saliva, faeces to breast milk) have been stored on filter paper for a variety of biochemical assays, including newborn screening, genetic mutation screening, mass spectrometry-based metabolite determination, therapeutic drug monitoring, and detection of nucleic acids, antigens, and serological markers for infectious disease diagnosis. The World Health Organization's (WHO/Joint United Nations Programme on HIV/AIDS (UNAIDS) Treatment 2.0 initiative to achieve and sustain universal access to treatment, as well as the recent call for the use of DBS in diagnostics platforms for integrated mapping, monitoring, and surveillance of seven neglected tropical diseases, highlight the need for a review of DBS preparation, storage, and elution methodology to ensure best practice. (Solomon et al., 2012).

2.2 Use of Dried Blood Spot in Epidemiological studies

When conducting diagnostic or epidemiological surveys, especially in distant places with limited resources, facilities for processing blood and storing frozen samples are usually unavailable. This is particularly true for neglected tropical illnesses, which are frequently found in populations from advanced diagnostic facilities. Dried blood spots (DBS) offer a potentially effective and low-cost solution to these problems. Finger-prick blood samples are readily collected and transported on filter paper at room temperature (even by post). However, because blood sample quantities on filter paper are often tiny, assay validation is required to attain optimal sensitivity and specificity (Smit et al., 2014). DBS have been widely used in epidemiological studies particularly for infectious disease (AJ, 2008; Barbi, Binda, & Caroppo, 2019; BG, 2011; Gg, Stevens, Sa, Horsfield, & Ws, 2019; JE, 2011; Rl, Pw, Stevens, Schuurman, & Tf, 2009), HIV detection and monitoring, drug assays(JE, 2011), virology.

Dried blood spots used in research and diagnostic purpose for last 5 decades. Initially the idea of blood collection on filter paper was introduced by Ivor Bang. In 1913, Bang determined glucose from eluates of dried blood spot and later on measured nitrogen from filter paper by Kjeldahi method. Is was first introduced in 1960 for screening of phenylketonuria in neonates (Guthrie & Susi, 1963). Since then use of DBS tremendously increases in every field of science & technology. During these 5 decades researchers exploit DBS for its robustness & potential use in the field of research & development. But due to small volume of blood in DBS sample, there is limitation on sensitivity and specificity while screening and its use is restricted for other application for many years because due to dry condition of whole blood on filter paper many analyte can't give perfect result as expected.

Advances in analytical techniques, particularly in Polymerase Chain Reaction (PCR), Mass Spectrometry have overcome many of these problems and allowing the potential of stored

DBS to be realized by researchers (Parker & Cubitt, 1999). In 1969, phenylketonuria screening became mandatory for all newborns. Guthrie card samples from two to nine-day-old newborns have been routinely obtained in over 20 nations since then to screen for phenylketonuria, congenital hypothyroidism, and sickle cell diseases.(Basley & Smith, 1991; Henderson SJ, Fishlock, Horn ME, Oni L, 2019). DBS also used as detection of biomarkers pertaining to particular disease. Such as medium chain acyl CoA dehydrogenase (MCAD deficiency), human chorionic gonadotrophin (hCG) in Down syndrome, and HbA1c in insulin dependent diabetes, and the estimation of drug levels have been also been investigated so far (Ec, Kj, Hp, & Rf, 2019; Henderson et al., 2019; Henderson SJ, Fishlock, Horn ME, Oni L, 2019; Spencer, Macri, Carpenter, Anderson, & Krantz, 1993). DBS obtained from children's and adults have been used for sentinel surveillance of disease.

In this study, we have targeted evaluation of 3 biomarkers from DBS and compared it with their paired blood sample whole blood/plasma to reveal the utility of DBS in field epidemiological studies. In literature, there is no evidence of multiple comparison of DBS with their corresponding paired blood samples. We have prepared DBS and stored at different thermal condition (4^0C, -20^0C) after transport duration of 0-24 hours, 24-48 hours and 48-72 hours.

Compared single DBS sample 6 times to evaluate the change in concentration of measured biomarker while DBS stored after within 24 hours, 48 hours and 72 hours. We have included 3 biomarkers such as molecular biomarker (genomic DNA), infectious biomarker (*Helicobacter pylori*) and Biochemical biomarker (apolipoprotein: apoA & apoB). Limited literature exist for Molecular, Infectious and biochemical analysis with multiple comparison and its stability in dry form. Her we have review some article regarding evidence of such biomarkers. According to the Centers for Disease Control and Prevention (CDC), the filter paper blood collection device has attained the same degree of precision and consistency as

established methods for collecting blood, such as vacuum tubes and capillary pipettes. (Mei, Alexander, Adam, & Hannon, 2001).

2.3 Evidences on genomic DNA extraction from DBS

Genomic DNA (gDNA) are robust and potential source on DBS for biobanking in epidemiological studies. Studies on storage of dried blood spots over longer periods of time have shown varying results. In one study, sensitivity was reduced after 5 years of storage (Hwang et al., 2012), however in another study, sensitivity was raised after 4 years of storage because inhibitors eluted more easily in newer samples than in older samples (NACHER et al., 2003). In an study it shows, how humidity and temperature effect the DNA concentration on DBS filter paper (Firnert, Arez, Correia, Bjorkman, & Snounou, 1999). In research, a simple and inexpensive method of DNA extraction is desirable in order for the method to be feasible and sustainable in a resource-limited setting. Several methods for gDNA extraction from DBS are available, including a simple boiling method using TE buffer that was introduced by Bereczky et al. and demonstrated higher sensitivity compared to two established methods using Chelex and methanol.(Hwang et al., 2012). Other methods like chelex(Baidjoe et al., 2013, Hwang et al., 2012) and and InstaGene Matrix(Cox-Singh, Mahayet, Abdullah, & Singh, 1997, (Hwang et al., 2012) are based on substances which removes the PCR inhibitors. Other kits such as QIAamp DNA mini kit frequently used(C., Y.V., J.E., D., & S.V., 2013, Hwang et al., 2012) 6,15,16and are therefore relevant when comparing methods fsor DNA extraction from filter paper. Previously, many studies on DNA extraction from DBS were published, but few studies illustrated this process of testing and justifying the choice of one DNA extraction method over another.

In some studies it is shown that DNA is quite stable and is useful decades after collection (Hardin et al., 2009; Nagy et al., 2010). Even ribonucleic acid (RNA), which is far less stable than DNA in solution, appears to be stable on the dried blood spot cards(Gauffin, Nordgren,

Barbany, Gustafsson, & Karlsson, 2009; Haak et al., 2009). In a study published by J.A Lane, concluded that after modification of some steps in gDNA extraction we can increase gDNA yield up to 6 – 10 folds(Lane & Noble, 2010). In another study researchers show that DNA is stable up to 11 years on DBS stored on ambient temperature and they perform PCR based applications where all the samples amplified PAX8 and beta-globin gene(Chaisomchit, Wichajarn, Janejai, & Chareonsiriwatana, 2005). It is always questionable whether we can perform epigenetic research by using DBS or not, gDNA methylation process require substantial amount of DNA, in a study by using mass spectrometry gDNA methylation have been perform by researcher by using 3mm diameter punches of DBS (N. C. Wong, Morley, Saffery, & Craig, 2008). Magnetic microspore is an another technique used for gDNA extraction from whole blood, frozen blood, dried blood(Gong & Shengying, 2014). Application of gDNA from DBS is always challenging for researchers because the reproducibility of gDNA from DBS is partial, but no doubt DBS samples could be a rich source of productive research. However the amount of gDNA from DBS is quite small, in-spite of that scientist explore its utility by performing sequencing, genotyping, GWAS (Genome Wide Association Studies) (Hollegaard, Grove, Grauholm, Kreiner-Møller, et al., 2011). The amount of gDNA extracted from DBS varies depending on the number of spots and methodology used; approximately 60 ng of genomic DNA (gDNA) can be extracted from a 3.2-mm punch of a DBS sample. (Hannelius et al., 2005).

Internationally, storage policies for residual neonatal dried blood spot (DBS) samples vary, but several countries keep residual samples in repositories for research purposes. (B.L. et al., 1996; de Carvalho, dos Santos, dos Santos, Vargas, & Pedrosa, 2007; K., 2003; Olney, Moore, Ojodu, Lindegren, & Hannon, 2006). Previous studies used whole-genome amplified DNA (wgaDNA) for genotyping with some success, but only a limited number of polymorphisms could be tested in most cases. (B.L. et al., 1996; Hollegaard, Grove, Thorsen,

Nørgaard-Pedersen, & Hougaard, 2009; Lovmar, Fredriksson, Liljedahl, Sigurdsson, & Syvänen, 2003; Olney et al., 2006; Sjöholm, Dillner, & Carlson, 2007; Zygalaki et al., 2005). There is limited study which compare extracted gDNA concentration from DBS when stored at different temperature and different transport duration. In our study we have extracted gDNA 7 times from single blood source (DBS) stored at standard condition, 4^0C and -20^0C with different transport duration (0-24 hours, 24-48 hours and 48 – 72 hours) and compared it with goldstandard. In an another study, the quality of DNA extracted from liquid or dried blood is not adversely affected by storage at 48 C for up to 24 h(Halsall et al., 2008).

In a study, gDNA from DBS is used for PCR based molecular assay (Hughes D, Hurd C, 1996) to determine its applicability in epidemiological studies. DBS represents itself as a genetic repository because they are used for extraction of gDNA for PCR (Gupta, Jayasuryan, & Jameel, 1992). Blood samples on filter paper broadly used for last 50 years (Petrini, Olivieri, Corbetta, & Cerone, 2012). Since last 50 years DBS have been adopted in disease screening (Guthrie R, 1963), genetic analysis (Prior, Hlghemlth, Friedman, & Perry, 1990), drug monitoring (Lindström B, Ericsson O, Alván G, Rombo L, Ekman L, Rais M, 1985). Technically very small amount of blood approx. 50µl needed to fill single circle on Whatman cards and only portion of DBS used for multiple tests. Sometimes similar portion of DBS used for multiple disease screening test.

Initially DNA extracted from DBS in 1987 (McCabe ER, Huang SZ, Seltzer WK, 1987). After reporting of gDNA extracted from DBS (Mccabe, 1991) scientist explore to analyze very small quantity of gDNA. Some study demonstrated that gDNA can be amplified with thousands of base pairs from very small portion of DBS (Caggana, Conroy, & Pass, 1998; Chaisomchit S1, Wichajarn R, Chowpreecha S, 2003; Chaisomchit S1, Wichajarn R, Janejai N, 2005). Limited study also shows target Single Nucleotide Polymorphism (SNP) analysis done on whole genome amplification of DNA (wgaDNA) from DBS (Chang, Ryu, & Sung,

2007a; Ida & Sjo, 2007; Malmberg, Dobeln, & Kere, 2005; Norgaard-pedersen, 2009; Sørensen KM1, Jespersgaard C, Vuust J, Hougaard D, Nørgaard-Pedersen B, 2007), wgaDNA from DBS also used in array based SNP genotyping(Hollegaard et al., n.d.; Hollegaard, Grove, Grauholm, Kreiner-møller, et al., 2011; Mv, Grove, Thorsen, & Dm, 2018; Paynter et al., 2006). Due to improvement in gDNA extraction technique from DBS, it permits to extract even $1/8^{th}$ inch part of DBS which is equivalent to 3µl of whole blood(Jinks DC1, Minter M, Tarver DA, Vanderford M, Hejtmancik JF, 1989).

To study the genetic basis of inheritance there is a need of large amount of gDNA but unfortunately amount of gDNA is limited from DBS. In this situation amplification of small amount of gDNA by whole genome amplification of DNA (wgaDNA) will be suitable, this method is based on multiple displacement amplification technology (Syvanen, 2006). There is lack of evidence that suggested wgaDNA from gDNA which is stored on filter paper in form of DBS can be reliable use in genetic inheritance and sequence analysis. But literature shows the utility of wgaDNA from gDNA can be used for single nucleotide polymorphism(SNPs) genotyping including genome wide scanning (Chang, Ryu, & Sung, 2007b; Hollegaard et al., 2008; Norgaard-pedersen, 2009). DNA have all necessary information in coded form which is required for translation process in protein synthesis for cell structure and its functionality.

Genetic biomarker exist as DNA biomarker, DNA tumor biomarker and general biomarkers. Some researcher state that DNA is stable over an individual's life time and biomarker explicit this stability termed as "DNA Biomarkers". Single nucleotide polymorphisms (SNPs), short tandem repeats (STRs), deletions, insertions and other variation on the DNA sequence level are among this group. SNPs are the most commonly used type of DNA variation due to the availability of high-throughput molecular biological facilities. "DNA Tumor markers" are formed specifically in cancerous or tumor cells. Molecular basis of cancer is change or

mutation in DNA sequence at cellular level and these changes measured in tumor or cancerous cells. “General Biomarkers” include protein, RNA, and other metabolites which can be measured in tissue, fluids. DBS have some limitation that it contains very less amount of blood and genetic material but it can be overcome by whole genome amplification of gDNA (wgaDNA) to increase the copy of gDNA to perform all downstream applications from PCR to Single Nucleotide Polymorphism (SNP) (Hollegaard, Grauholm, et al., 2009).

DNA Biomarkers are stable and reproducible, which can be measured several times from same sample and may be used in prospective & retrospective studies. A part from downstream applications of gDNA from DBS, genetic biomarkers can also be used for diagnosis, prognosis and prediction if personalized medicines (Buyse et al., 2011; Karl Egerer, Eugen Feist, 2009; Keedy et al., 2018; Krijgsman O1, Roepman P, Zwart W, Carroll JS, Tian S, de Snoo FA, Bender RA, Bernards R, 2012; Study, 2008). Diagnostic biomarkers used to determine the severity of disease, the most important diagnostic biomarker are screening biomarkers (Karl Egerer, Eugen Feist, 2009).

Prognostic biomarkers help to predict the transition phase of disease under proper treatment condition, such as Mamma Print, DNA tumor marker of breast cancer prognosis used in surgery which provide information of chance of low or high risk of metastatic condition, which guide to physicians how to treat the patients. These types of DNA biomarkers require validation for their predictive capability. In USA Food and Drug Administration (FDA) declared MammaPrint as an invitro diagnostic multivariate index assay (Slodkowska EA1, 2009).

In our study, we will focus on concentration, stability, integrity and purity of gDNA extracted from DBS stored at 4^0C and -20^0C with different transport duration and validate it with gold standard (whole blood) to evaluate its feasibility in field epidemiological studies and its downstream application.

2.4 Evidences on testing of infectious pathogen from DBS

DBS is not only restricted to DNA extraction but it has many application as done with whole blood**.** Evidences are available on testing of infectious agents from DBS but limited evidence is available which supports multiple testing of paired DBS which is stored at standard condition, 4^0C and -20^0C with varying transport duration for detection of infectious biomarkers. Here we will described some literature which supports DBS can be exploit for testing of infectious pathogens. In a study, Vemu Lakshmi described the detection of HIV-1 from DBS through PCR application (Lakshmi, Sudha, Rakhi, Anilkumar, & Dandona, 2011). De Vries showing the identification of cytomegalovirus (CMV) from DBS by amplifying its DNA using real-time PCR and evaluating sensitivities in triplicate for DBS with CMV DNA loads of 5-4, 4-3, and 3-2 log (10)copies/ml, respectively. This means that in the therapeutically relevant concentration range, sensitivity limitations exist.(J. J. C. de Vries, Claas, Kroes, & Vossen, 2009).

Any analyte that can be analysed in whole blood, serum, or plasma may theoretically be measured in dried blood on filter paper. Only the analytes to be analysed from dried blood must be stable during drying and selectively liberated from the paper during elution. Furthermore, stability studies of analytes during storage are required before large-scale epidemiological use of it. Many analytes, including DNA, are stabilised by the dried blood matrix, allowing measurement of both phenotype (biochemical marker) and genotype (mutation or polymorphism) from a small volume of blood. (Lakshmy, 2008). Recent performance evaluation studies using commercially available and in-house enzyme immunoassays demonstrated that collecting DBS for measles diagnostics is a feasible and reproducible alternative to phlebotomy.(Chakravarti, Rawat, & Yadav, 2003; De Swart et al., 2001; El Mubarak et al., 2004; Helfand et al., 2001). Blood products, such as serum from DBS for infectious disease serology, such as measles and rubella, have been extracted using a

variety of techniques. Some of the protocols are suitable for use in the field, while others necessitate the use of laboratories with adequate equipment.

Discs are excised from DBS and soaked in elution buffer in all techniques, which aids in the diffusion of serum components from the rehydrated filter paper matrix. (Chanbancherd et al., 1999; Condorelli et al., 1994; Farzadegan, Noori, & Ala, 1978; Riddell, Leydon, Catton, & Kelly, 2002). Protocols may differ in the number and diameter of discs for elution, depending on the subsequent serological immunoassay (El Mubarak et al., 2004; Fortes et al., 1989; Helfand et al., 2001; Inborn, Screening, & Hospital, 1998). In a study paired DBS blood samples have been used and compared it with plasma samples for the detection of hepatitis B virus in resource limited settings, correlation coefficient ($r = 0.92$) were observed between DBS and plasma blood samples and there is no significant changes in DBS viral load after storage at room temperature after 12 weeks (Stene-Johansen et al., 2016).

In a review, Paul N. Newton describe, focusing on the evaluation of nucleic acid and serological assays for diagnosis of infectious diseases using dried blood spots (DBS) compared with recognized gold standards. DBS samples have sensitivities and specificities that are comparable to or slightly inferior to gold standard sample types. Uncritical use of DBS, incorrect statistical analysis, and a lack of uniform methodology, on the other hand, revealed significant flaws. By making laboratory-based diagnostic tests more accessible, DBS have the potential to empower healthcare workers. Three investigations compared DBS hepatitis C (HCV) serology to serum or plasma and found that it has a high sensitivity and specificity (> 98%). (Croom et al., 2006; Judd et al., 2003). In comparison to serum, DBS detection of dengue nucleic acid was likewise extremely sensitive (> 90.7 percent). Because HIV is an RNA virus, proviral HIV-1 DNA testing is often used to diagnose infants. The Roche Amplicor and Roche Cobas Taqman (Basel, Switzerland) tests for DBS were examined in six investigations, with sensitivities and specificities ranging from 97 to 100

percent and 99.6 percent and 100 percent, respectively (A., S., & E., 2010; G.G., G., S.A., P., & W.S., 2005; Patton et al., 2007; Stevens, Erasmus, Moloi, Taleng, & Sarang, 2008).

Approx. 985 paired DBS samples were examined for human papillomavirus (HPV), hepatitis C virus, Helicobacter pylori, and JC polyomavirus in a multiplex serology (JCV), The median correlation values for high-titer (i.e., H. pylori, HCV, JCV) and low-titer (i.e., H. pylori, HCV, JCV) antibodies in serum and DBS were 0.88 (range, 0.80–0.90) and 0.79 (range, 0.72–0.85), respectively, for high-titer (i.e., H. pylori, HCV, J). DBS provide a reliable alternative to serum or plasma for detection of antibodies against diverse infections by multiplex serology. Epidemiologic connections with established risk factors for HPV antibodies were as significant for DBS as for serum. DBS do not require blood centrifugation and can be stored and shipped at room temperature, making field work for sero-epidemiologic studies easier, especially in areas with limited technical infrastructure. (Waterboer et al., 2012).

Many studies have been published so far for the detection of infectious pathogens, but in our study we will perform the test 7 times to detect IgG antibody concentration against *Helicobacter pylori* infection from DBS and compared it with gold-standard. We want to study sensitivity, specificity, correlation coefficients and pattern of variation in concentration between DBS and plasma samples when it is stored at standard condition, 4^0C and -20^0C at 3 transport duration between 0-24 hours, 24-48 hours and 48-72 hours. The storage and transport duration of DBS samples is not yet standardized because still researchers get different results with different storage conditions. We need to specify the correct storage condition and transport duration of DBS for every analyte because all the biomarker do not stable on a single storage temperature but in general most researchers preferred -20^0C is an ideal temperature for longer period DBS storage.

2.5 Evidences on estimation of Apolipoproteins (apoB, apoA) from DBS

Apolipoprotein A1 (ApoA1) is the primary protein associated with high-density lipoprotein (HDL) particles, and plays a central role in reverse cholesterol transport (Sorci-Thomas & Thomas, 2013). The levels of HDL cholesterol (HDL-C) and ApoA1 are inversely related to the risk of coronary artery disease. (CAD) (Walton, 2009).

The principal protein component of low-density lipoprotein (LDL) is apolipoprotein B (ApoB) (LDL). Although the amount of cholesterol in LDL varies, each LDL carries exactly one ApoB protein. As a result, compared to LDL cholesterol, ApoB is a better predictor of circulating LDL. ApoB has been shown to be equally effective in the presence of LDL particles as evaluated by nuclear magnetic resonance spectroscopy (Cole et al., 2013). ApoB is strongly associated with increased risk of developing cardiovascular disease (CVD) and often outperforms LDL-C at predicting risk of coronary heart disease (Sierra-Johnson et al., 2009; Thompson & Danesh, 2006). Research have been published on detection of Apo-lipoproteins and their ratio for the prediction of cardio-vascular disease from venous blood but there is limited study have been done so far on detection of Apo-lipoproteins from dried blood spots (DBS).

In a neonatal screening study, the aim was to detect young families with the dominantly inherited familial hypercholesterolemia through identification of Apo-AI and Apo-B lipoproteins. The DBS was stored up to 20 days at 4^0C. The apoA/apoB ratio was higher in female than in male neonates ($p>0.001$), as is also true for adults (Wang, Dudman, & Wllcken, 1989) Earlier we developed a radial immunodiffusion (RID) method for measuring Apo-B directly in dried blood spot samples(Dudman, Blades, Wilcken, & Aitken, 1985). In a study where, the immunorectivity of Apo-B decline with time of storage at 4^0C (Blades, Dudman, & Wilcken, 1987; Kronenberg, Lobentanz, König, Utermann, & Dieplinger, 1994).

In a study, researchers looked at how long-term storage, as well as multiple freezing and thawing, affected the observed values of lipoprotein[a] Lp[a], apolipoproteins B, and AIV, using plasma samples held at -80^{0}C, -20^{0}C, and 4^{0}C, total and high density lipoprotein (HDL) cholesterol and triglycerides were determined. After 24 months, samples maintained at -80°C and -20°C revealed substantial changes in Lp[a], with a mean drop of 7% and 1376, respectively ($P < 0.01$). In contrast, apolipoproteins B and A-IV decreased continuously over time ($P < 0.05$). Only the measured values of Lp[a] and apolipoprotein B were affected by multiple freezing and thawing. With the exception of Lp[a] after 18 and 24 months ($P <0.05$), there was no variation in any of the parameters between samples maintained at -80°C and -20^{0}C at any time. Immunoblotting with Apo[a] detection was possible from samples under each storage condition after a 24-month storage period. Only samples held at -20^{0}C or -80^{0}C were found to have apoB and apoA-IV (Kronenberg et al., 1994).

Recent studies suggested that plasma concentrations of apolipoprotein B (apoB) and apolipoprotein A-I (apoA-I) may be superior(Walldius & Jungner, 2006; Walldus et al., 2004).

Limited study published on measurement of apoA, apoB and its ratio from DBS and their correlation with corresponding plasma samples. In field epidemiological studies collection of DBS, drying and transportation to laboratory is very precautious because for measurement of apolipoproteins we have to maintain cold chain throughout the transportation. The concentration of apoliproteins decreases with time periods.

In our study, we have performed estimation of apoB, apoA and their ratio (apoB/apoAI) from DBS stored at standard condition, -20^{0}C and 4^{0}C with varying transport duration (0-24 hours, 24-48 hours and 48-72 hours). After estimation, we will compare results from DBS 7 times with gold standard (plasma) sample. We have paired DBS samples, from single EDTA tube we prepare 7 DBS cards and stored at different temperature as describe above. These 7 DBS

cards derived from same blood, which means we have compared single blood samples 7 times with plasma at various transport duration to evaluate the fluctuation in concentration of apolipoproteins (apoB, apoA). In a published study, apoB were measured from 208 paired DBS & plasma samples, they find out very strong significant correlation between plasma and DBS. Our major aim is that, whether we can replace plasma or whole blood samples with alternative dried blood spot (DBS) in field epidemiological studies or not.

2.6 Hypothesis

Dried blood spots are the alternative effective tool for biomarker measurements in field epidemiological studies

2.7 Aim

To study the utility of dried blood spot in field epidemiological studies

2.8 Objectives of the study

2.8a Primary objective

Biomarkers assessment from dried blood spots.

1. Standardization and validation of following Biomarkers (genomic DNA, *Helicobacter pylori*, apolipoproteins (apoB, apoA, apoB/apoA ratio) measured from Dried Blood Spots stored at standard condition.
2. Establish optimal transport and storage condition for Dried Blood Spots to evaluate its utility in epidemiological studies

2.8b Secondary objective

Development of mandatory guidelines for preparation, transport & storage of dried blood spots.

2.9 Statistical Analysis

All statistical analysis is done by using software STATA 15.0, Excel, Graph-pad software.

Chapter 3
Assessment of Biomarkers from Dried Blood Spot (DBS) Samples

3.1 Evaluation of Genomic DNA (gDNA) from Dried Blood Spots

3.1A Standardization and validation of extraction of genomic DNA (gDNA) from DBS* stored at standard condition.

3.1A.1 Introduction

As per my experience in the laboratory, the extraction of gDNA from DBS is a challenging task. In this study, we have extracted gDNA from paired Dried Blood Spots (DBS) samples and compared them with the gold-standard (whole blood). We aimed to check the compatibility of extracted gDNA from DBS with venous blood. First, for standardization purposes, we have validated the extracted gDNA from DBS* stored at the standard condition with the venous blood. Second, for comparison purposes, we have compared the gDNA extracted from paired DBS samples stored at a compromised thermal condition (4^0C & -20^0C) at a particular time-period with gold-standard blood samples. Several research papers published so far, on the extraction of gDNA from DBS with their downstream applications, but we have compared extracted gDNA from DBS which is air-dried between time-periods 0-24 hours, 24-48 hours and 48-72 hours & stored at 4^0C and -20^0C. gDNA is the most robust and potential sample source for biobanking and epidemiological studies due to its feasibility.

3.1A.2 Materials & methods

Required materials for extraction of gDNA from DBS & Venous Blood

- Prepared valid DBS cards (Figure 4)
- 6mm single hole punch plier (Figure 5)
- Phosphate Buffer Saline pH 7.4
- genomic DNA extraction kit from blood (Qiagen, Spin column based)
- genomic DNA extraction kit from blood (Invitrogen, Magnetic bead based)
- Pipettes (10µl, 100 µl, 200 µl, 1000 µl) (Eppendorf)
- Waterbath (Trishul Equipment, Sr. No. 5460311)
- Centrifuge (Eppendorf 5810R)

- Spectrophotometer (Nanodrop, Thermofisher)
- Fluorometer (Qubit 3.0, Thermofisher)

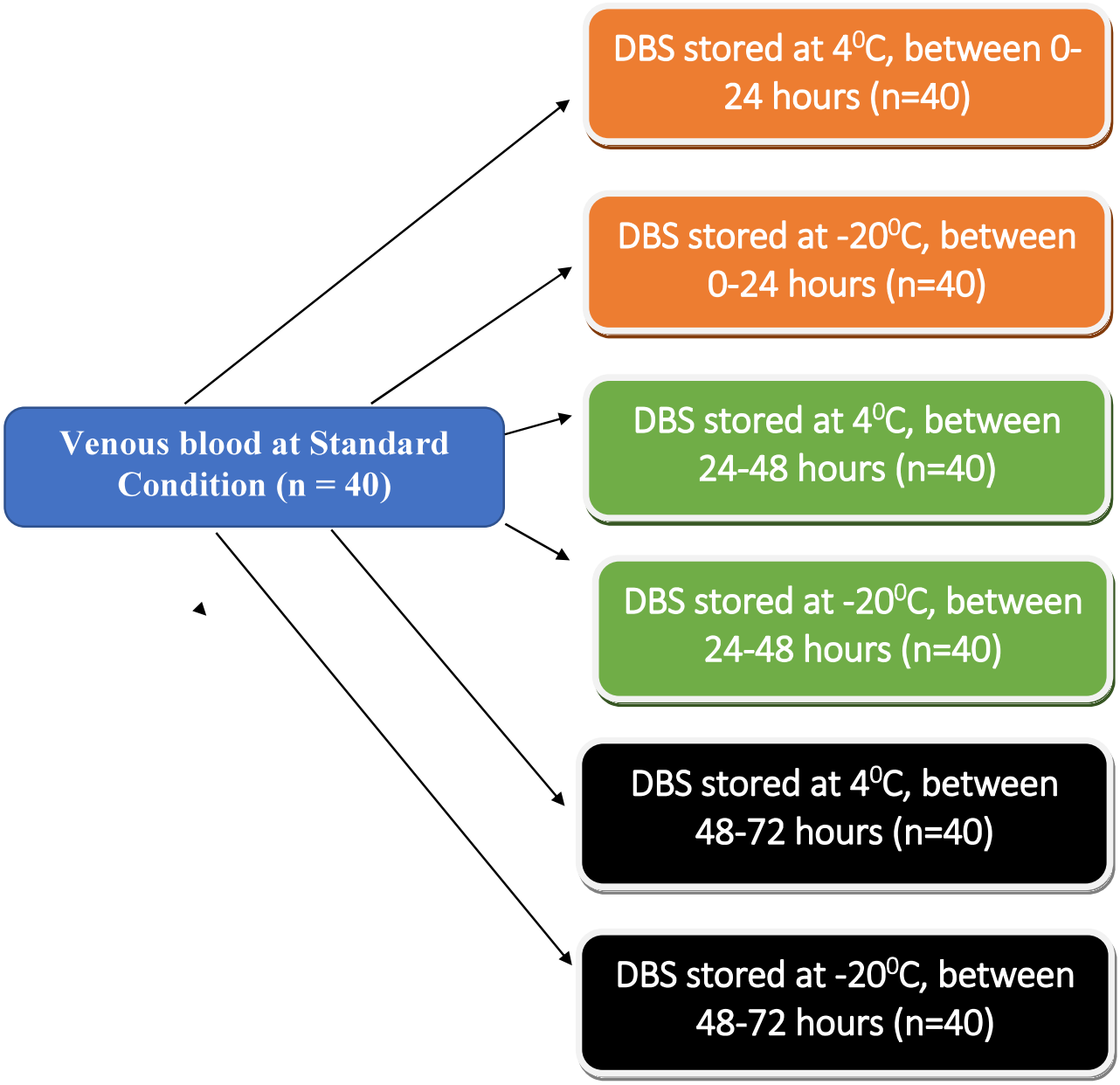


Figure 3. Outline for DBS preparation from venous blood and its storage condition

3.1A.3 Dried blood spot terminologies

Standard condition*: Blood samples stored at -80^{0}C

DBS*: Dried blood spot stored at -80^{0}C immediately

DBS 1: Dried blood spot samples stored at 4^{0}C within 0-24 hours of transport duration of DBS

DBS 2: Dried blood spot samples stored at -20^{0}C within 0-24 hours of transport duration of DBS

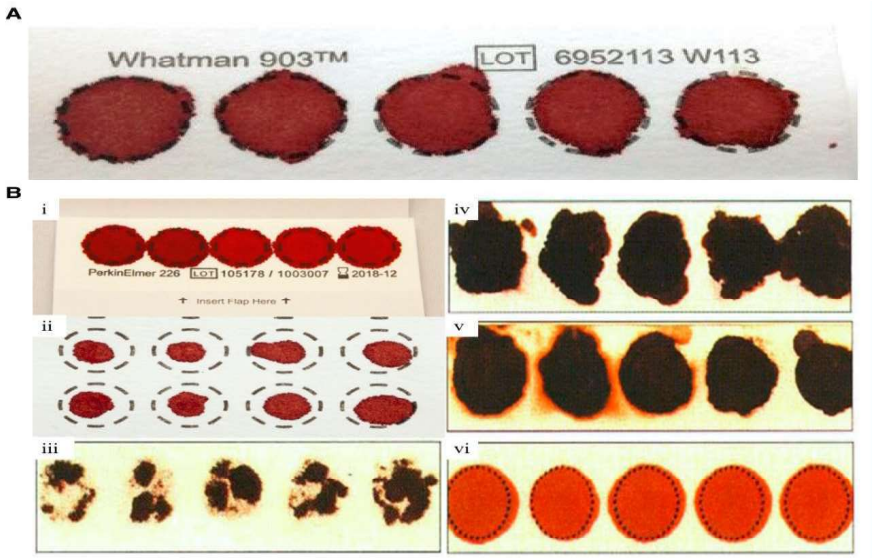
DBS 3: Dried blood spot samples stored at 4^{0}C within 24-48 hours of transport duration of DBS

DBS 4: Dried blood spot samples stored at -20^{0}C within 24-48 hours of transport duration of DBS

DBS 5: Dried blood spot samples stored at 4^{0}C within 48-72 hours of transport duration of DBS

DBS 6: Dried blood spot samples stored at -20^{0}C within 48-72 hours of transport duration of DBS

Figure 4. Valid / Invalid Dried Blood Spot cards **(WHO, 2005)**



(A) Valid specimen. DBS with complete filled circle with proper air dry with no hemolyzed blood or serum ring. **(B) Invalid DBS Specimen. (i)** DBS with overlapping of spotted blood. **(ii)** DBS with insufficient filled blood. **(iii)** DBS with incomplete absorption which reduces blood volume. **(iv)** DBS that is potentially rubbed and develop scratches. **(v)** DBS with hemolyzed or contaminated blood. **(vi)** DBS with improper air drying before packaging in ziplock bags.

Study Design

A prospective observational study conducted to evaluate the feasibility of DBS samples in large scale epidemiological study. Venous blood samples collected from the on-going Head & Neck case-control study between Jan 2018 – April 2018 which is approved by the Institutional Ethical Committee (IEC), Tata Memorial Hospital Mumbai. Venous blood samples utilize to prepare DBS samples to observe the robustness of DBS alternative to venous blood.

Sample Size Estimation

Sample size calculated in software STATA 12 (two power correlation) with 95% CI, 0.05 (α) level of significance, 0.8 power (1-β), with expected (r=0.9) at standard condition & estimated (r=0.7) at stored condition, approximately 80 paired sample size obtained for the study.

Study Setting

2.00 ml - 3.00 ml venous blood collected in EDTA tube by trained staff at TMH under feasible condition & transported to CCE laboratory immediately under maintained cold chain within 1-2 hours. DBS prepared on same day with proper air dried at RT, plasma separated from remaining blood and stored at standard condition (-80^{0}C). DBS samples stored at different temperature at varying time points (0-24 hours, 24-48 hours & 48-72 hours). Laboratory experiments were conducted after 4-5 months of storage of DBS samples. All the experiment performed in our CCE laboratory at optimum temperature 22^{0}C – 25^{0}C with complete proper laboratory infrastructure & precaution.

3.1A.4 Processing of Dried Blood Spots for gDNA extraction

For standardization purposes, 2 blood spots excised from the blood collection card of size 6mm in diameter with the help of a single hole punch plier. The excised blood spots put into a 1.7ml centrifuge tube and added with 200µl Phosphate Buffer Saline (PBS, Gibco with Ref No. 10010-023) with pH 7.4 & kept it overnight at 370C in Waterbath (Trishul Equipment, Sr. No. 5460311). This process helps to adsorption of PBS by cotton linter on blood spots which might be useful to complete the extraction of eluate from filter paper to carry out an easy gDNA extraction process.

Figure 5. 6mm steel punch plier used for excision of DBS

3.1A.5 Genomic DNA (gDNA) extraction methodology

We aimed to extract the maximum amount of gDNA from DBS to evaluate its feasibility in molecular epidemiology, therefore we have adopted 2 methods.

1) Column based gDNA extraction (QIAamp DNA kit, Qiagen Catalog no. 56304)

2) Magnetic bead based gDNA extraction (ChargeSwitch Forensic DNA Purification Kit, Invitrogen Catalog No. CS11200).

For Standardization and comparison purpose, DBS of size 6mm X 2 spots was used to extract gDNA, but from molecular application point of view we used 6mm x 1 spot to 6mm x 4 spots to extract maximum amount of gDNA. In our trial phase, we have done some small modifications in recommended protocol for complete recovery of gDNA from DBS.

3.1A.6 Column based gDNA extraction from DBS

We used QIAamp DNA kit (Qiagen, Catalog no. 56304), processed DBS was incubated in a waterbath (Trishul Equipment, Sr. No. 5460311) at 85°C for 10min with 180 µL cell lysis

buffer ATL (Lysis buffer supplied with Qiagen kit), then 20 µL Proteinase K was added and incubated it at 56°C for 1 hour to denature the proteins. 3-4 µL RNAse was added immediately after to degrade RNA, then 200 µL buffer AL (Lysis buffer supplied with Qiagen kit) added & mix thoroughly by vortexing and incubated at 70°C in a waterbath (Trishul Equipment with Sr. No. 5460311) for 10 min. Buffer AL helps in complete cell lysis and binding of gDNA with the silica gel of column provided in the Qiagen kit. gDNA was then immediately precipitated by adding 200 µL of 70% v/v ethanol. The solution was then transferred into a spin column (supplied with Qiagen kit) & centrifuged (Eppendorf 5810R) at 8000 rpm for 1 min. Spin column have capacity to load approximately 600 microliter sample at a time, but generally we have approx. 1.2 or 1.4ml solution with gDNA we therefore performed the process 2-3 times. In this process gDNA becomes bound with the column which is then washed with 700 µL buffer AW1 (Wash buffer with low concentration of quinidine) followed by 700 µL AW2 (Wash buffer with Tris based ethanol solution used for removal of salts) buffer. Buffers AW1 & AW2 remove unwanted impurities from the gDNA. The empty tube was then centrifuged at 14000 rpm for 3 min for complete removal of ethanol from the gDNA. Finally the gDNA was eluted with 30 µl-60 µl of pre-incubated elution buffer (AE).

3.1A.7 Magnetic bead based gDNA extraction from DBS

We used ChargeSwitch Forensic DNA Purification Kit (Thermofisher, Catalog No. CS11200). With processed DBS, 1ml lysis buffer was added with 10 µL of Proteinase K in a tube containing blood spots, vortexed and incubated at 55ºC in a waterbath (Trishul Equipment Sr. No. 5460311) for 1 hour. Blood spots were removed after complete cell lysis and 200 µL of purification buffer added, followed by 20 µL magnetic beads. The solution was then gently mixed, left for 5 minutes, and then incubated on a Magna Rack (Thermofisher, catalog no. AM10027) for 1 minute. The supernatant was removed after

complete binding of the pellet to the magnet. The pellet containing gDNA was then washed with 500 µL wash buffer (W12) 3 times and finally gDNA eluted with 30-60 µL Elution Buffer (E5).

3.1A.8 Validation

We have validated gDNA extraction from DBS with whole blood which is stored at standard condition (-80^{0}C).

3.1A.9 Comparison of gDNA extraction

(**1**) Genomic DNA extracted from whole blood stored at standard condition compare with DBS stored at 4^{0}C (DBS1, DBS3, DBS5) & -20^{0}C (DBS2, DBS4, DBS6)

(**2**) Genomic DNA extracted from DBS* stored at standard condition compare with DBS stored at 4^{0}C (DBS1, DBS3, DBS5) & -20^{0}C (DBS2, DBS4, DBS6)

<table>
<tr><th colspan="2">whole blood stored at standard condition*</th><th colspan="2">DBS stored at Standard condition*</th><th colspan="3">DBS stored at different thermal condition with varying transport duration</th></tr>
<tr><td rowspan="8">n = 40</td><td rowspan="8">-80^{0}C</td><td rowspan="8">n = 40</td><td rowspan="8">-80^{0}C</td><td>Transport Duration</td><td>Thermal condition</td><td>Number of Samples</td></tr>
<tr><td rowspan="2">Between 0-24 hours</td><td>4^{0}C</td><td>n = 40</td></tr>
<tr><td>-20^{0}C</td><td>n = 40</td></tr>
<tr><td rowspan="2">Between 24 – 48 hours</td><td>4^{0}C</td><td>n = 40</td></tr>
<tr><td>-20^{0}C</td><td>n = 40</td></tr>
<tr><td rowspan="2">Between 48 – 72 hours</td><td>4^{0}C</td><td>n = 40</td></tr>
<tr><td>-20^{0}C</td><td>n= 40</td></tr>
</table>

Table 2. Paired blood samples stored at different thermal condition with varying transport duration

3.1A.10 Standardization of gDNA extraction from DBS

We have performed the gDNA extraction procedure as per protocol is given in the gDNA extraction kit (Qiagen, Invitrogen), but due to the small yield of gDNA from existing protocol we have done some modification in DBS processing methods to increase the yield of gDNA with the same kit. Genomic DNA extracted from paired DBS samples is validated with whole

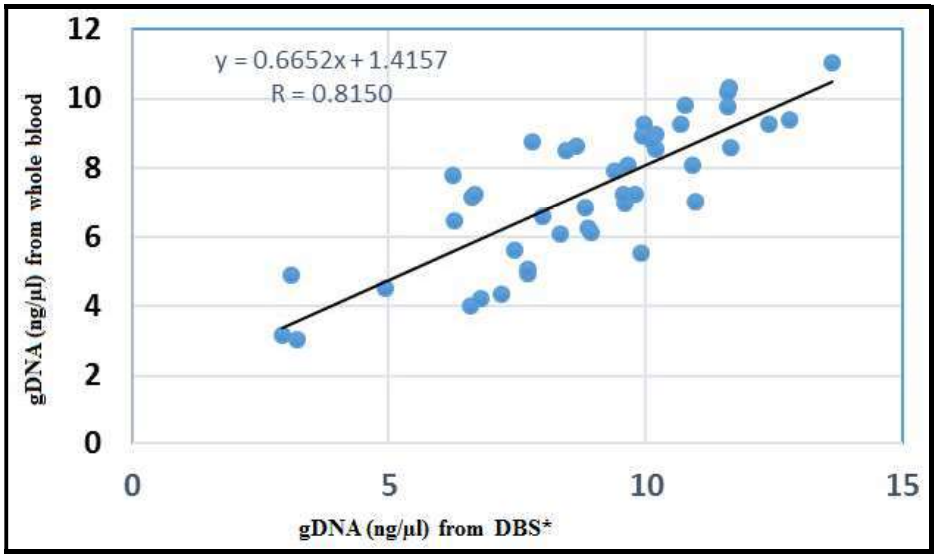
blood. The average gDNA amount is obtained from spot size of 6mm X 2 spots of DBS which is equivalent to 17 - 18µl of whole blood. We have validated our results with equal volume of blood samples from DBS & whole blood.

3.1A.11 Results: T-test[2], Scattered plot[3], Bland-Altman plot[4]

Blood samples stored at standard condition*	Number of Observations	Mean	Standard Deviation	95% CI	p - value
Whole blood	40	8.8245	2.4907	8.0672 – 9.5818	p>0.001
DBS*	40	7.2857	2.0329	6.6676 – 7.9037	

Table 3. T-test, mean comparison of genomic DNA concentration between DBS* and whole blood stored at standard condition

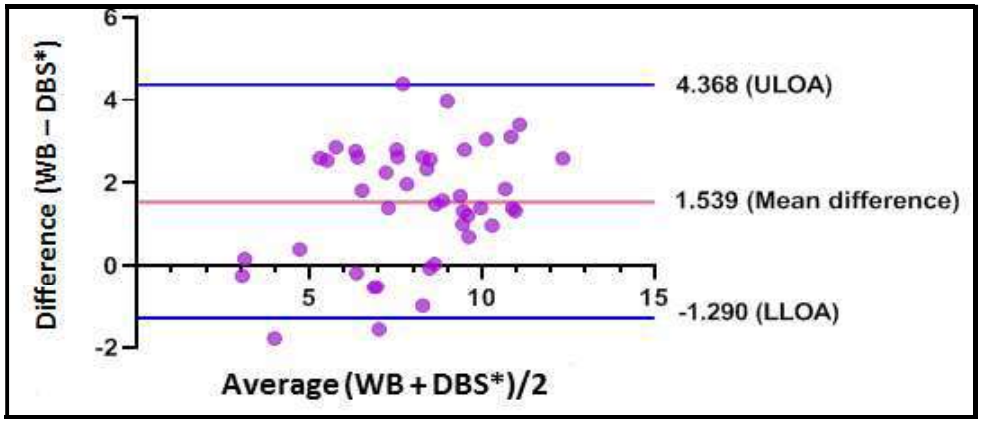
Figure 6. Prepared scattered plot to compare correlation coefficient of gDNA concentration between whole blood & DBS* stored at standard condition.



Scattered plot of gDNA between whole blood & DBS*, with fitted regression equation shows correlation coefficient (r = 0.8150, p>0.001)

[2] Paired t-test used to compare difference of mean value of gDNA between whole blood and DBS stored at standard condition.
[3] Scattered plot were used to compare correlation coefficient of gDNA concentration between whole blood & DBS stored at standard condition
[4] Bland-Altman plot were used to evaluate bias between the mean difference and average of two measurementmethods (whole blood and DBS stored at standard condition) of gDNA concentration.

Figure 7. Prepared Bland-Altman graph, a plot of difference of gDNA concentration between whole blood & DBS* samples stored at standard condition.



BA plot: A plot of difference between whole blood & DBS* stored at standard condition.

BA Plot Interpretation: BA plot analysis is a simple way to evaluate the bias between the mean difference and agreement between the two measurement methods of same variable in paired samples. Here, we have plotted the graph of gDNA between Whole blood & DBS* stored at standard condition. Above figure showed, result measured by whole blood may be 1.290 units below or 4.368 units above the DBS*. Majority of gDNA concentration scattered close to 0 on measurement scale, although the difference is significant but plotted graph shows slight difference observed between both the measurements. If the difference between the two measurement values decreases then the band-width of the agreement limit also closer to zero, which shows there is no difference observed in measurement methods.

3.1A.12 Quantification of genomic DNA

The concentration of gDNA (ng/µl) quantify by using Qubit 3.0 and the quality of extracted genomic DNA checked by technique 0.8% agarose gel electrophoresis (AGE) (Figure 12, 13)

3.1A.13 Protocol modifications for standardization purpose

Modification 1) Excise 6mm X 2 spots of DBS with the help of a 6mm punch plier (Fig). Transfer 2 blood spots into a 1.7ml eppendorf centrifuge tube with the help of pointed forceps, add 150µl - 200µl PBS (Phosphate Buffer Saline) with pH 7.4. Incubate the eppendorf tube

for 3 – 4 hours at 37^0C OR else incubate it overnight at room temperature for complete extraction of whole blood from filter paper.

Modification 2) The cell lysis step initially it recommended (manufacturer) for 1-hour incubation in the water bath at 56^0C but we have increased the time more than 30 minutes for complete and proper cell lysis for release of all gDNA from cells into the supernatant.

Modification 3) Increases the incubation time for proper binding of gDNA with magnetic beads present in supernatant.

3.1B Evaluation & comparison of gDNA concentration between whole blood and DBS stored at 4^0C (DBS1, DBS3, DBS5), & -20^0C (DBS2, DBS4, DBS6).

As per our objective, we have compared genomic DNA (gDNA) concentration between whole blood samples stored at the standard condition and DBS samples stored at 4^0C, -20^0C at different transport duration between 0-24 hours, 24-48 hours and 48-72 hours.

3.1B.1 Result: T-test[5], ANOVA, Scattered plot, Bland-Altman plot

3.1B.1a T-test

T-test was conducted to observe the mean difference between two groups in terms of their average gDNA concentration, a total of 6 combinations compared. Statistically significant p-value calculated at 0.05 significance level with a 95% confidence interval.

Table 4. T-test, mean comparison of genomic DNA concentration between whole blood and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5)

Blood samples storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p-value
Whole blood (-80^0C)	Immediate	40	8.824	2.490	8.067 – 9.581	
DBS1 (4 Degree Celsius)	Between 0-24 hours	40	5.292	2.479	4.539 – 6.046	p>0.001
DBS3 (4 Degree Celsius)	Between 24-48 hours	40	4.948	2.341	4.236 – 5.660	p>0.001
DBS5 (4 Degree Celsius)	Between 48-72 hours	40	4.707	2.275	4.015 – 5.399	p>0.001

[5] Paired t-test used to compare difference of mean value of gDNA between whole blood and DBS stored at 4^0C.

Table 5[6]. T-test, the mean comparison of gDNA concentration between whole blood and paired DBS samples stored at -20^{0}C (DBS2, DBS4, DBS6)

Blood samples storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
Whole blood (standard condition*)	Immediate	40	8.8245	2.4907	8.0672 – 9.5818	p>0.001
DBS2 (-20 Degree Celsius)	Between 0-24 hours	40	4.5092	2.4981	3.7497 – 5.2687	
Whole blood (standard condition*)	Immediate	40	8.8245	2.4907	8.0672 – 9.5818	p>0.001
DBS4 (-20 Degree Celsius)	Between 24-48 hours	40	4.2490	2.3270	3.5416 – 4.9565	
Whole blood (standard condition*)	Immediate	40	8.8245	2.4907	8.0672 – 9.5818	p>0.001
DBS6 (-20 Degree Celsius)	Between 48-72 hours	40	4.2638	2.3613	3.5459 – 4.9817	

3.1B.1b ANOVA (Analysis of Variance) Test: gDNA analysis

Analysis of variance is a statistical model to test if there is a significant difference exists between means of multiple group comparison. We have compared the mean of extracted genomic DNA between whole blood and DBS samples stored at temperature 4^{0}C (DBS1, DBS3, DBS5) & -20^{0}C (DBS2, DBS4, DBS6) with transport duration (between 0-24 hours, between 24-48 hours, between 48-72 hours).

Table 6[7]. ANOVA-test, multiple comparison of group mean of gDNA, between whole blood and paired DBS samples stored at 4^{0}C & -20^{0}C irrespective of all 3 transport duration.

Whole blood storage condition	DBS samples stored condition irrespective of transport duration	F-value	p value
Standard condition (n = 40)	4 degree Celsius (n = 120)	28.66	p>0.001
Standard condition (n = 40)	-20 degree Celsius (n = 120)	37.85	p>0.001

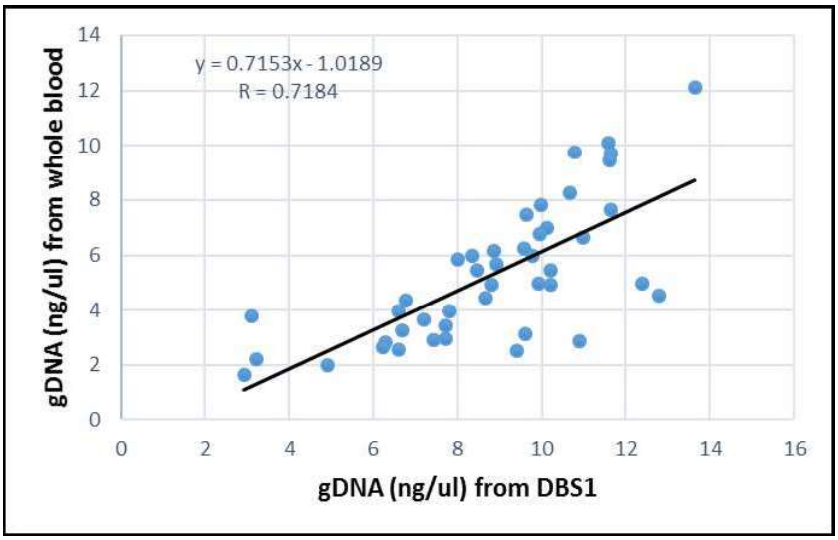
[6] Paired t-test used to compare difference of mean value of gDNA between whole blood and DBS stored at -20^{0}C.

[7] Oneway ANOVA test was conducted to compare mean concentration of gDNA of all groups of DBS with whole blood

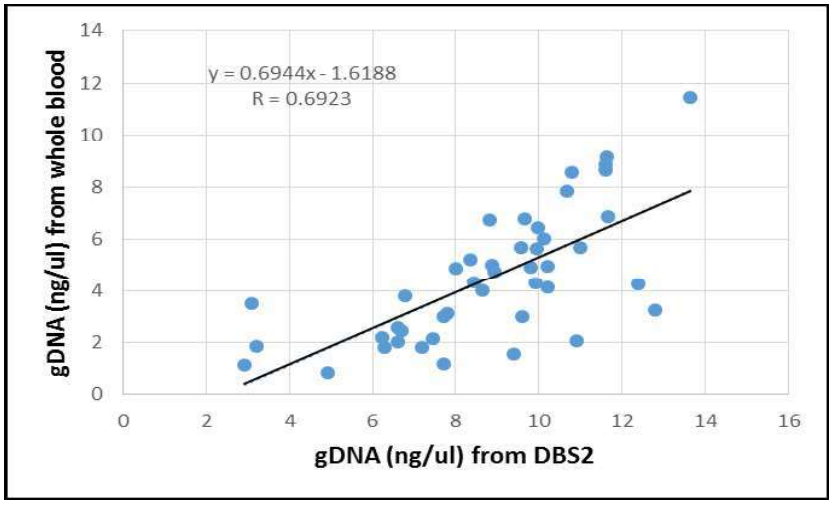
3.1B.1c Scattered plot (Correlation coefficient): genomic DNA

We have measured the correlation coefficients by creating a scattered plot of gDNA concentration between DBS stored at different thermal conditions with varying transport duration and whole blood.

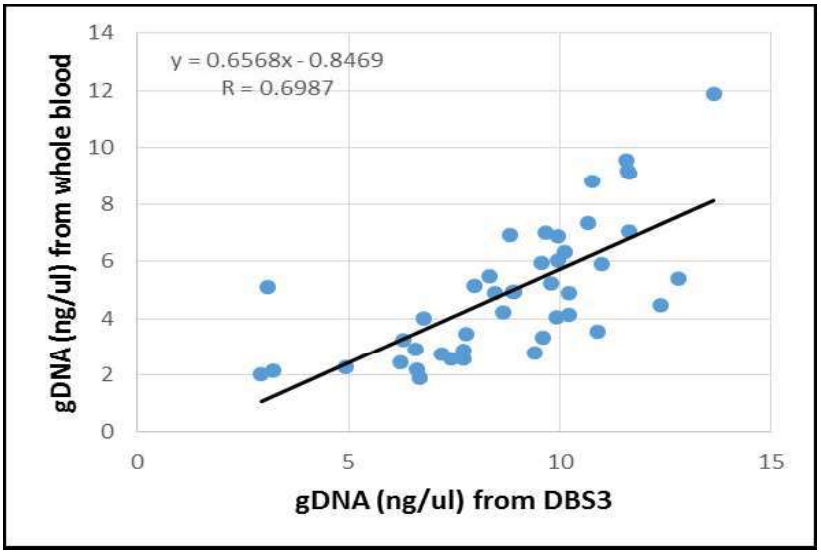
Figure 8. Prepared scattered plot to compare gDNA concentration between whole blood and DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).



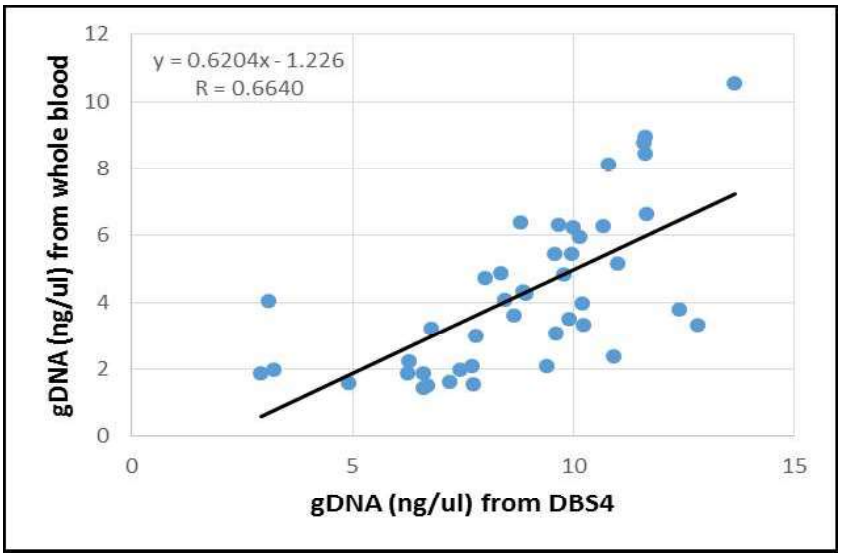
(Figure 8A) Scattered plot of gDNA between whole blood & DBS1, with fitted regression equation shows correlation coefficient (r = 0.7184, p>0.001)



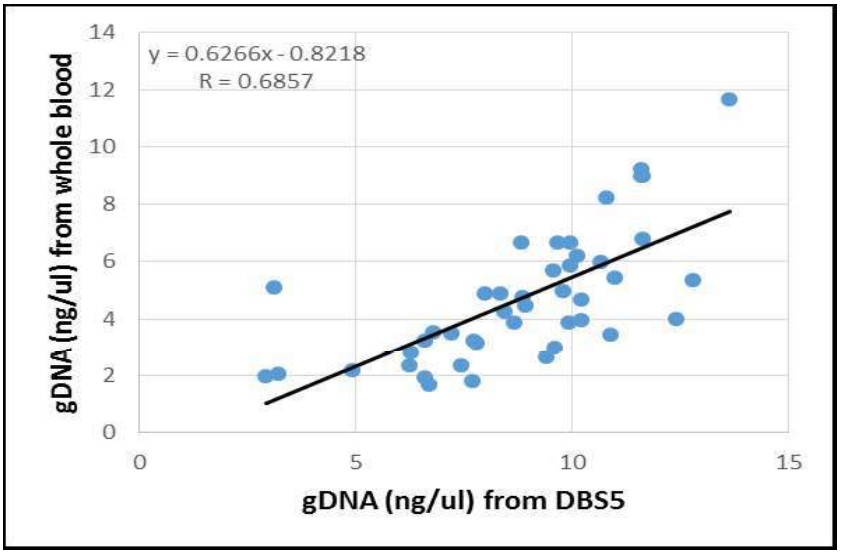
(Figure 8B) Scattered plot of gDNA between whole blood & DBS2, with fitted regression equation shows correlation coefficient (r = 0.6923, p>0.001)



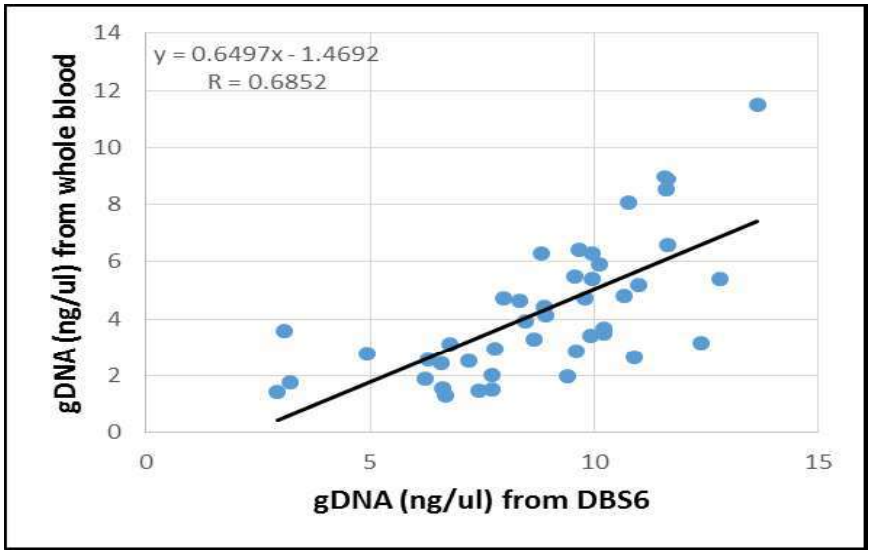
(Figure 8C) Scattered plot of gDNA between whole blood & DBS3, with fitted regression equation shows correlation coefficient (r = 0.6987, p>0.001)



(Figure 8D) Scattered plot of gDNA between whole blood & DBS4, with fitted regression equation shows correlation coefficient (r = 0.6640, p>0.001)



(Figure 8E) Scattered plot of gDNA between whole blood & DBS5, with fitted regression equation shows correlation coefficient (r = 0.6857, p>0.001)



(Figure 8F)[8] Scattered plot of gDNA between whole blood & DBS5, with fitted regression equation shows correlation coefficient (r = 0.6852, p>0.001)

[8] Scattered plot created of gDNA concentration between whole blood & all DBS groups (DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6)

3.1B.1d Bland Altman Plot: A plot of difference between two measurement tools

The measurement of the variables always implies some degree of error. While comparing the two measurement methods, it could be interesting to assess the degree of agreement between the two methods. There is no correct statistical approach to assess the degree of agreement. Many studies focus on the correlation coefficient (r) between variables to assess the degree of agreement, but the correlation only gives the relationship between variables but not differences, and this is not recommended the correct method to measure the degree of agreement between two measurements. To overcome this problem, in 1981 Eksborg introduced the new concept to measure the degree of an agreement but later it was re-proposed in 1983 by two scientist Martin J. Bland and Douglas G. Altman based on quantification of the agreement by measuring mean difference and limit of agreement between two measurement methods.

This plot was first introduced by Bland & Altman (BA) in 1983. This plot is used to describe the agreement between two quantitative measurements by plotting the limit of agreement. The statistical limits are calculated by the mean and standard deviation of the differences between the two measurements. A graphical approach is used for assumptions of normality of difference.

We have created a graphical plot of the difference of gDNA against their mean, between gold standard (venous blood/plasma) and DBS stored at standard condition, 4 degrees & -20 degree celsius to evaluate the limit of agreement. BA plot recommended that 95% of data points should lie within ±2SD of mean difference, and this is the most common way to present the BA plot but it can also be plotted as a percentage. Ideally, the measurements between the two methods should be the same for a perfect correlation. The BA plot simply represents every difference between two paired methods against the average of the measurement. Plotting difference against mean allows us to investigate any possible

relationship between a true value and measurement error. The mean of the two measurements is the best estimation (J. M. Bland & Altman, 2010). In a study it was observed that the first method marked as a 'standard' or 'reference' point for the plot of difference (Krouwer, 2008)), but although it was controversial because a plot of the difference against a "reference measurement" will always show a relation between difference and magnitude when there is none (Bland JM1, 1995). Drawing a regression line of differences helps in detecting a proportional difference ((M. Bland, 2015; Ch, 2003; Peter Armitage, Geoffrey Berry, 2001).

3.1B.1e Bias and agreement limit

The visual examination of graphical plots allows to evaluating the global agreement between two methods of measurement. If differences are normally distributed, then more precisely 95% of the differences must lie between M-2SD (Lower Limit of Agreement) & M+2SD (Upper Limit of Agreement). The systematic bias only can be significant when the line of equality is not within the confidence interval of the mean difference but the agreement interval is too wide or narrow is based on biological, clinical and analytical goals. The calculation of 95% CI evaluates how precise our estimates are. If the line of equality is not in the defined interval, then there is a systematic significant difference exist, i.e the second method constantly has under or overestimated compared to the first one.

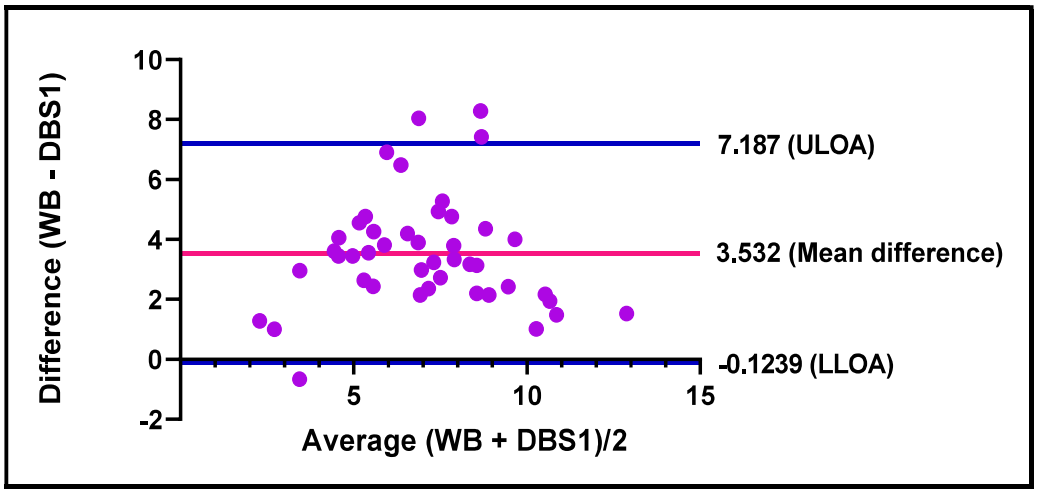
The 95% CI of a limit of agreement allows us to estimate the size of the possible sampling error (Blandab DouglasG. Altmanab, 1986). The 95% CI corresponds to the observed value minus t standard error to the observed value plus t standard error, where t is the value of t distribution ("Medcalc manual," 2018) with an (n-1) degree of freedom. Overall the 95% CI of the mean difference and limit of the agreement simply described the possible error in the estimate due to sampling error. The measurement of the difference in percentage will be helpful when there is an increase in variability of the differences as the magnitude of the measurement increases. BA plot method proposed in 1983 is widespread now, there is one

article which is published in Lancet ("Medcalc manual," 2018) has been cited more than 30000 times by peer-reviewed scientific articles (Lei & Vorechovsky, 2002).

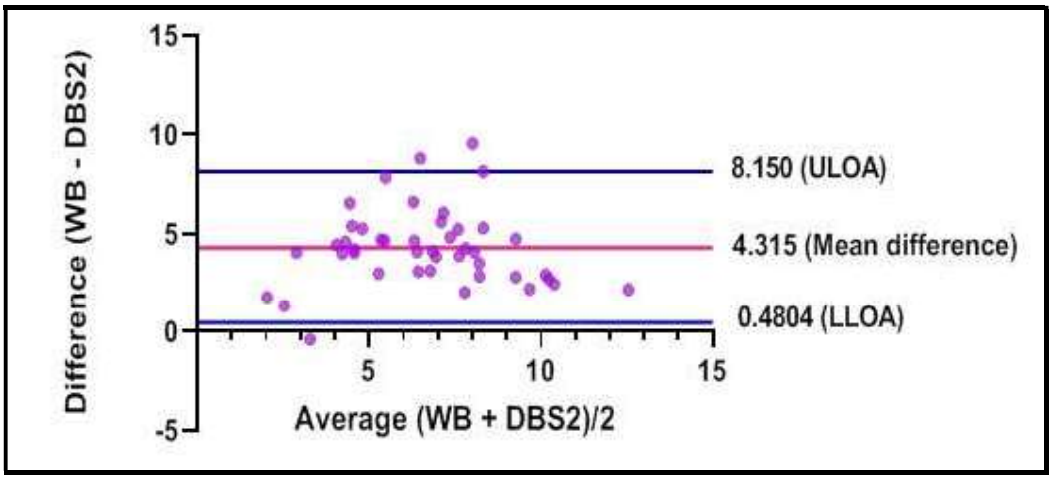
Table 7. Bland-Altman, a plot of difference of gDNA concentration between whole blood (WB) & DBS stored at 4^0C (DBS1, DBS3, DBS5) and -20^0C (DBS2, DBS4, DBS6).

Sample stored thermal condition	Group comparison	N	SD	Mean difference (d) of group	95% CI	
					UCL	LCL
Standard condition*	Whole blood* & DBS*	80	1.4268	1.5388	4.3353	-1.2577
4 degree Celsius	Whole blood* & DBS1	80	1.8437	3.5315	7.187	-0.123
	Whole blood* & DBS3	80	1.8590	3.8759	7.562	0.190
	Whole blood* & DBS5	80	1.8781	4.1172	7.841	0.393
-20 degree Celsius	Whole blood* & DBS2	80	1.9341	4.3152	8.150	0.480
	Whole blood* & DBS4	80	1.9574	4.5754	8.456	0.694
	Whole blood* & DBS6	80	1.9063	4.5606	8.340	0.781

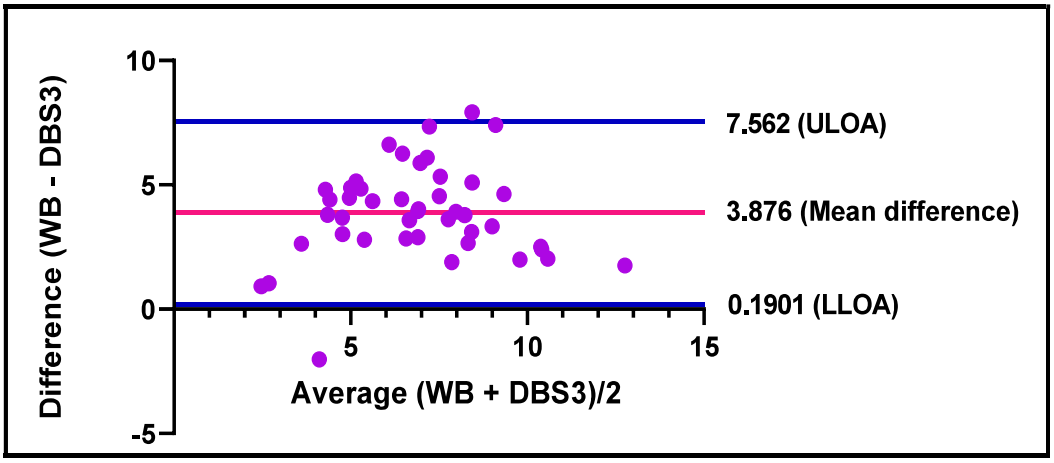
Figure 9. Prepared Bland-Altman graph, a plot of difference of gDNA concentration between WB & DBS stored at 4^0C & -20^0C with the representation of the limits of agreement with range -1.96 to +1.96



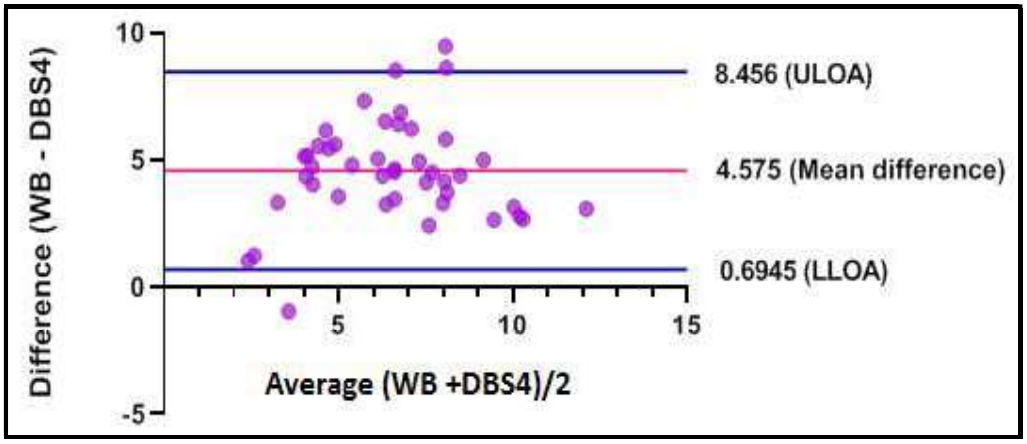
(Figure 9A) Plot of difference between whole blood & DBS1 against their average



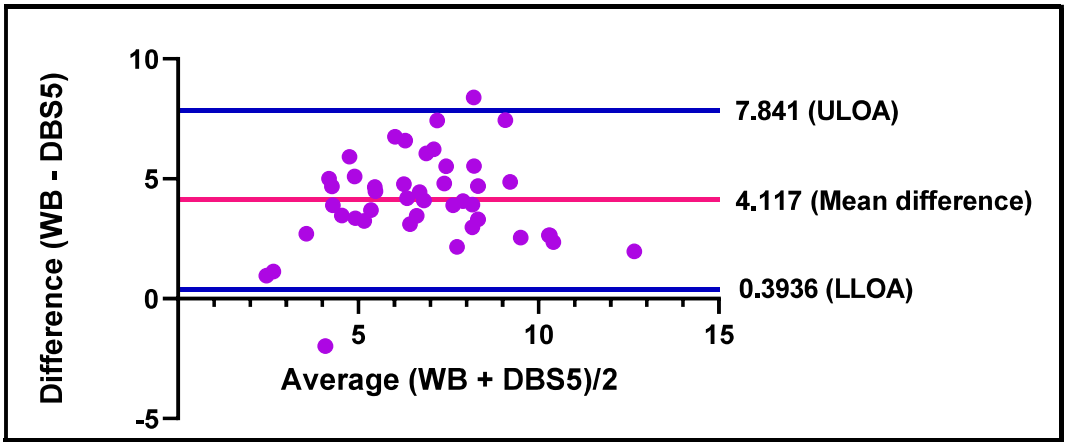
(Figure 9B) Plot of difference between whole blood & DBS2 against their average



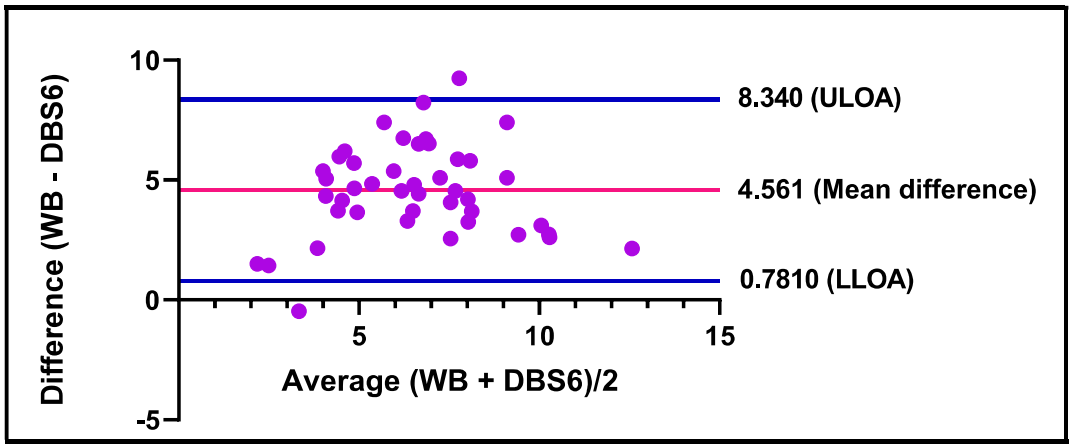
(Figure 9C) Plot of difference between whole blood & DBS3 against their average



(Figure 9D) Plot of difference between whole blood & DBS4 against their average



(Figure 9E) Plot of difference between whole blood & DBS5 against their average



(Figure 9F)[9] Plot of difference between whole blood & DBS6 against their average

3.1C Evaluation and comparison of genomic DNA (gDNA) concentration between DBS* stored at standard condition and DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

3.1C.1 Results: T-test, ANOVA, Scattered plot, Bland-Altman plot

Compare gDNA extracted from all DBS group stored at 4^0C & -20^0C with DBS* stored at standard condition

Table 8[10]**.** T-test result, the mean comparison of gDNA concentration between DBS* sample stored at standard condition and all paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5).

Blood samples storage condition	Transport duration	N	Mean	SD	95% CI	p - value
DBS (Standard condition*)	Immediately	40	7.2857	2.0329	6.6676 – 7.9037	p>0.001
DBS1 (4 degree celsius)	Between 0-24 hours	40	5.2929	2.4798	4.5390 – 6.0468	
DBS (Standard condition*)	Immediately	40	7.2857	2.0329	6.6676 – 7.9037	p>0.001
DBS3 (4 degree celsius)	Between 24 – 48 hours	40	4.9486	2.3412	4.2368 – 5.6604	
DBS (Standard condition*)	Immediately	40	7.2857	2.0329	6.6676 – 7.9037	p>0.001
DBS5 (4 degree celsius)	Between 48 - 72 hours	40	4.7072	2.2758	4.0153 – 5.3991	

[9] Bland-Altman plot (GraphPad) of gDNA created to observe the bias between whole blood and DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately.
[10] Paired t-test (STATA) used to compare difference of mean value of gDNA between DBS* stored at standard condition and DBS stored at 4^0C (DBS1, DBS3 & DBS5)

Table 9[11]. T-test result, the mean comparison of gDNA concentration between DBS* sample stored at standard condition and all paired DBS samples stored at -20^{0}C (DBS2, DBS4, DBS6)

Blood samples storage condition	Transport duration	N	Mean	SD	95% CI	p - value
DBS (Standard condition*)	Immediately	40	7.2857	2.0329	6.6676 – 7.9037	p>0.001
DBS (-20 degree celsius)	Between 0-24 hours	40	4.5092	2.4981	3.7497 – 5.2687	
DBS (Standard condition*)	Immediately	40	7.2857	2.0329	6.6676 – 7.9037	p>0.001
DBS (-20 degree celsius)	Between 24 – 48 hours	40	4.2490	2.3270	3.5416 – 4.9565	
DBS (Standard condition*)	Immediately	40	7.2857	2.0329	6.6676 – 7.9037	p>0.001
DBS (-20 degree celsius)	Between 48 – 72 hours	40	4.2638	2.3613	3.5459 – 4.9817	

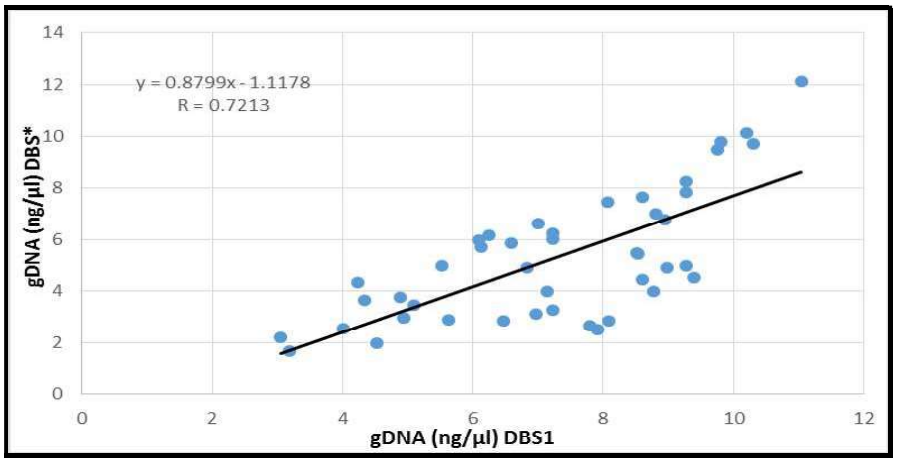
Table 10[12]. ANOVA test result, compared gDNA concentration between DBS* samples stored at standard condition with all paired DBS samples stored at 4^{0}C & -20^{0}C irrespective of all 3 transport duration

DBS sample storage condition	DBS samples stored condition irrespective of transport duration	F-value	p value
Standard condition (n = 40)	4 degree Celsius (n = 120)	11.63	p > 0.001
Standard condition (n = 40)	-20 degree Celsius (n = 120)	17.08	P > 0.001

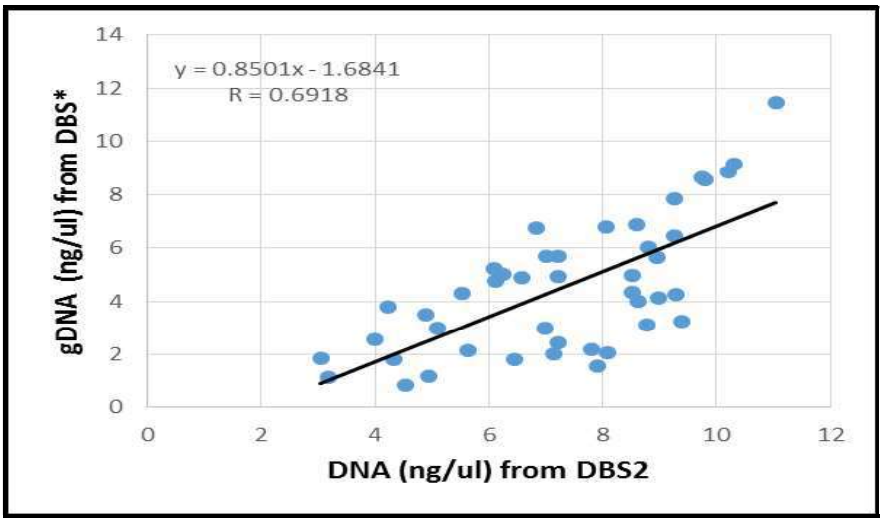
[11] Paired t-test (STATA) used to compare difference of mean value of gDNA between DBS* stored at standard condition and DBS stored at -20^{0}C (DBS2, DBS4 & DBS6)

[12] Oneway ANOVA test (STATA) was conducted to compare mean concentration of gDNA between DBS* stored at standard condition and all DBS groups stored separately at 4^{0}C & -20^{0}C.

Figure 10[13]. Prepared scattered plot to compare gDNA concentration between DBS* stored at standard condition and DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6)

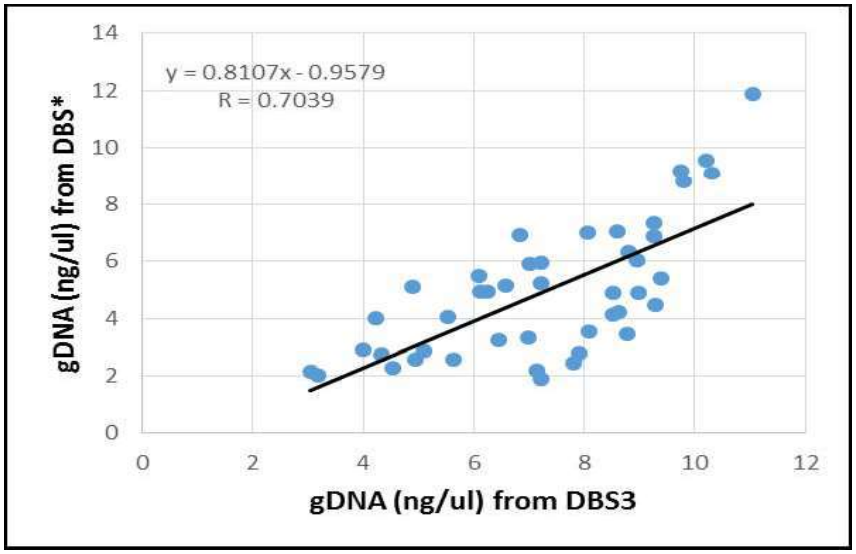


(**Figure 10A**) Scattered plot of gDNA between DBS* & DBS1, with fitted regression equation shows correlation coefficient (r = 0.7213, p>0.001)

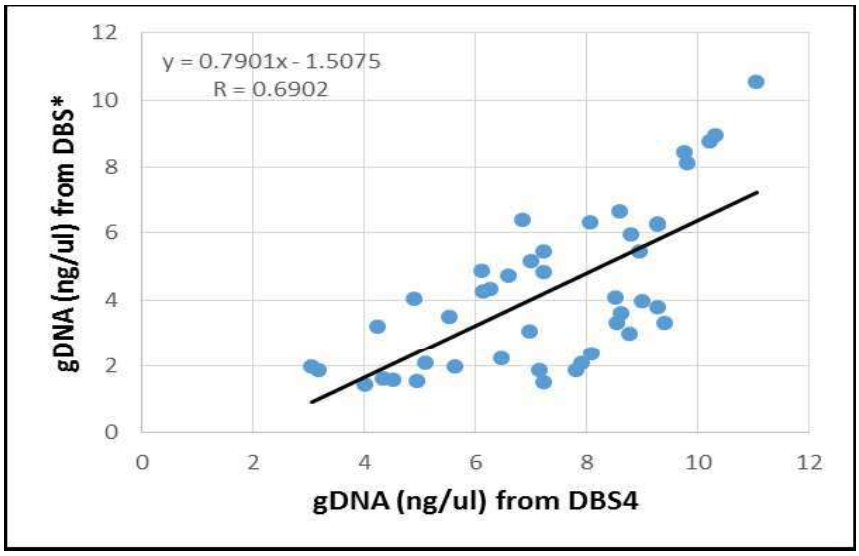


(Figure 10B) Scattered plot of gDNA between DBS* & DBS2, with fitted regression equation shows correlation coefficient (r = 0.6918, p>0.001)

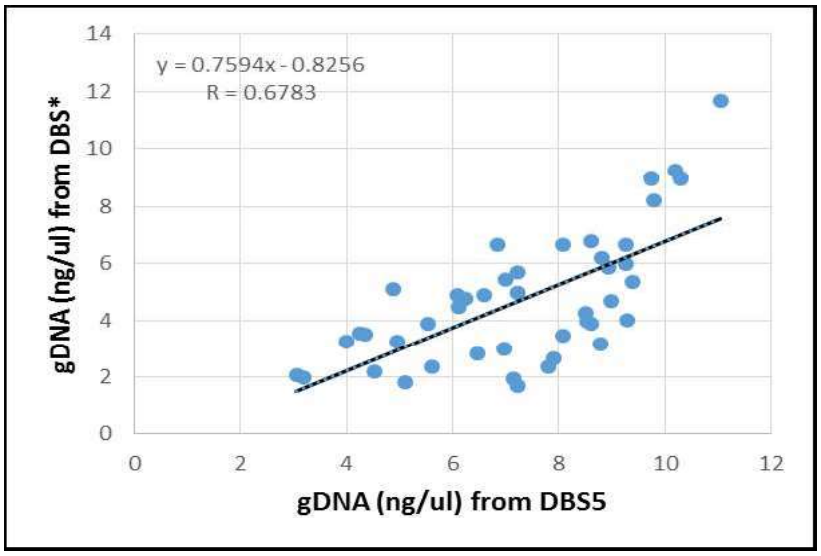
[13] Scattered plot (Excel) created of gDNA concentration between DBS* & all DBS groups (DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6)



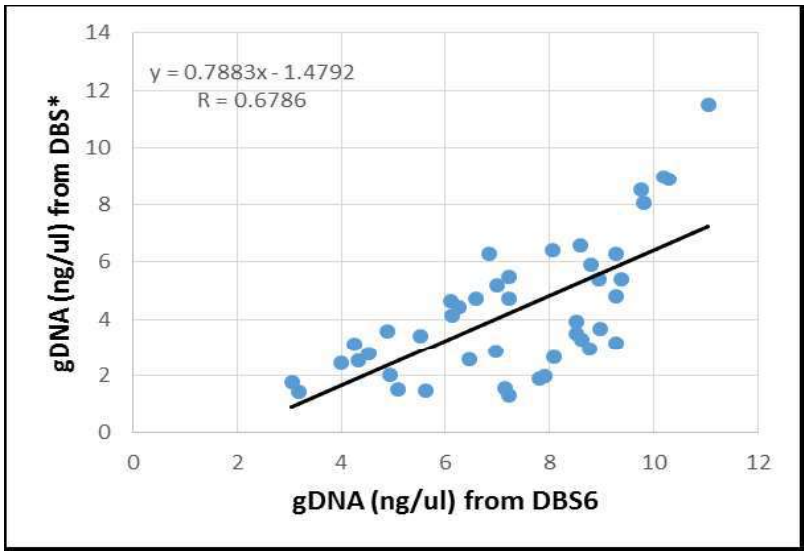
(**Figure 10C**) Scattered plot of gDNA between DBS* & DBS3, with fitted regression equation shows correlation coefficient (r = 0.7039, p>0.001)



(**Figure 10D**) Scattered plot of gDNA between DBS* & DBS4, with fitted regression equation shows correlation coefficient (r = 0.6902, p>0.001)



(**Figure 10E**) Scattered plot of gDNA between DBS* & DBS5, with fitted regression equation shows correlation coefficient (r = 0.6783, p>0.001)

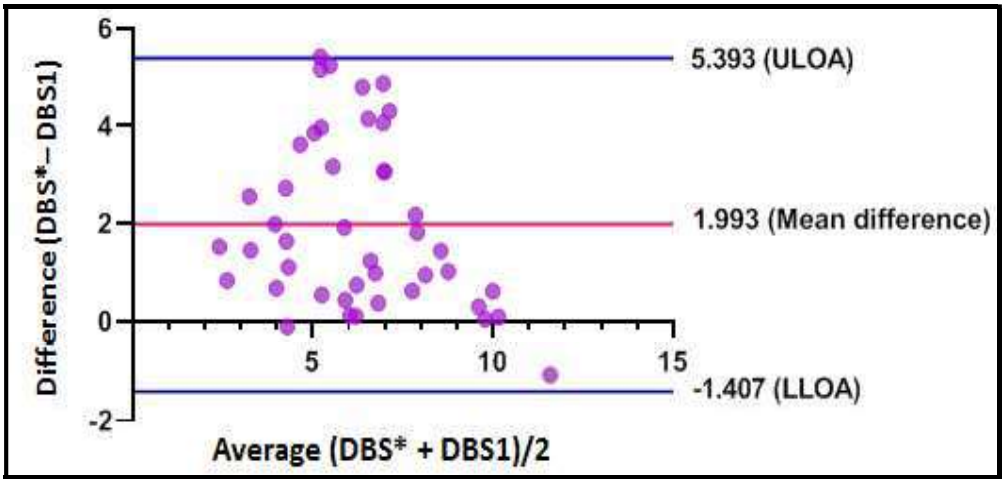


(**Figure 10F**) Scattered plot between DBS*& DBS6, with fitted regression equation shows correlation coefficient (r = 0.6786, p>0.001)

Table 11. Bland-Altman plot of difference of gDNA concentration between DBS* stored at standard condition & DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

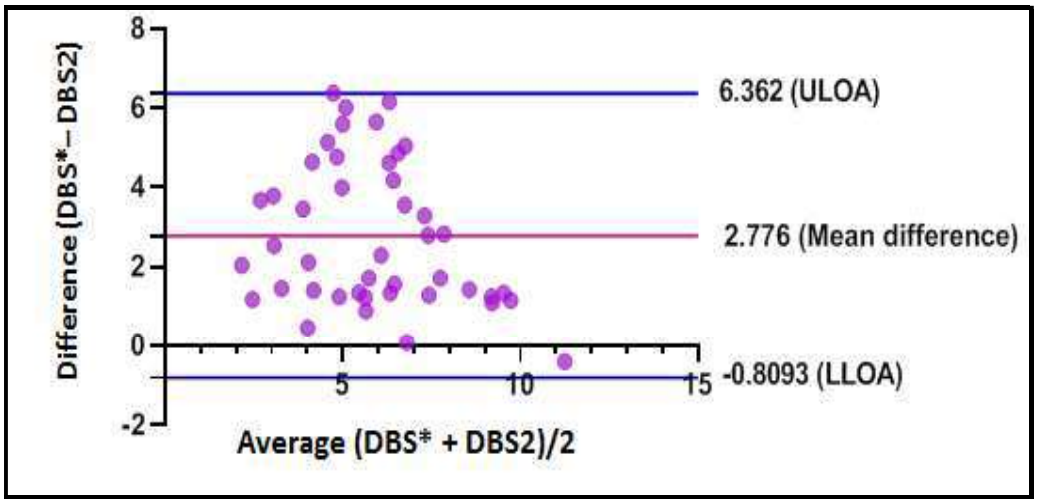
Sample stored thermal condition	Group comparison	N	Standard deviation (s)	Mean difference (d) of group	95% CI UCL	95% CI LCL
Standard condition* & 4 degree Celsius	DBS* & DBS1	80	1.7148	1.9927	5.3539	-1.3684
	DBS* & DBS3	80	1.6873	2.3370	5.6441	-0.9700
	DBS* & DBS5	80	1.7223	2.5784	5.9541	-0.7972
Standard condition* & -20 degree Celsius	DBS* & DBS2	80	1.8085	2.7764	6.3212	-0.7683
	DBS* & DBS4	80	1.7171	3.0366	6.4021	-0.3289
	DBS* & DBS6	80	1.7665	4.6970	8.1594	1.2346

Figure 11[14]**.** Prepared Bland-Altman graph, a plot of difference of DNA concentration between DBS* stored at standard condition & all DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6) against their average with the representation of the limits of agreement with range -1.96 to +1.96.

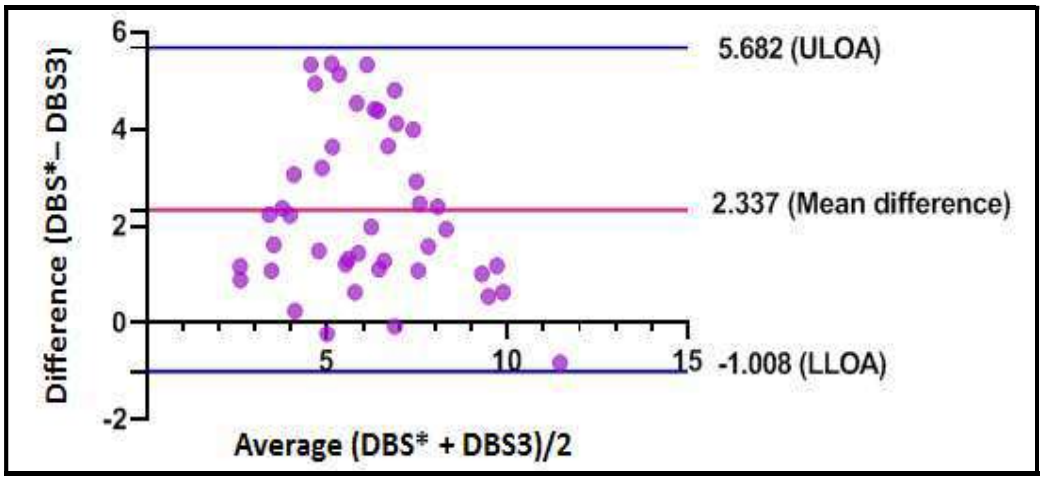


(**Figure 11A)** A plot of difference between DBS* & DBS1 against their average

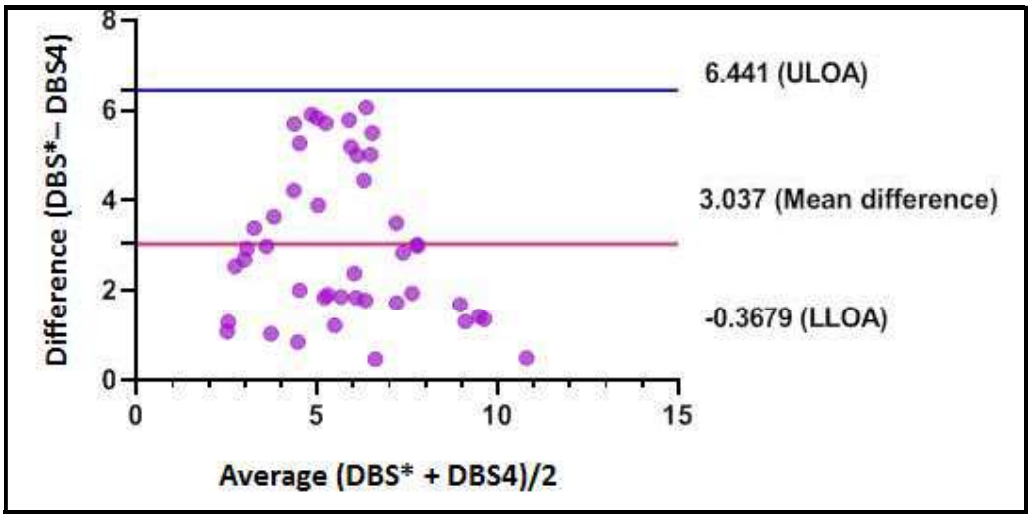
[14] Bland-Altman plot (GraphPad) of gDNA created to observe the bias between DBS* stored at standard condition and DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately



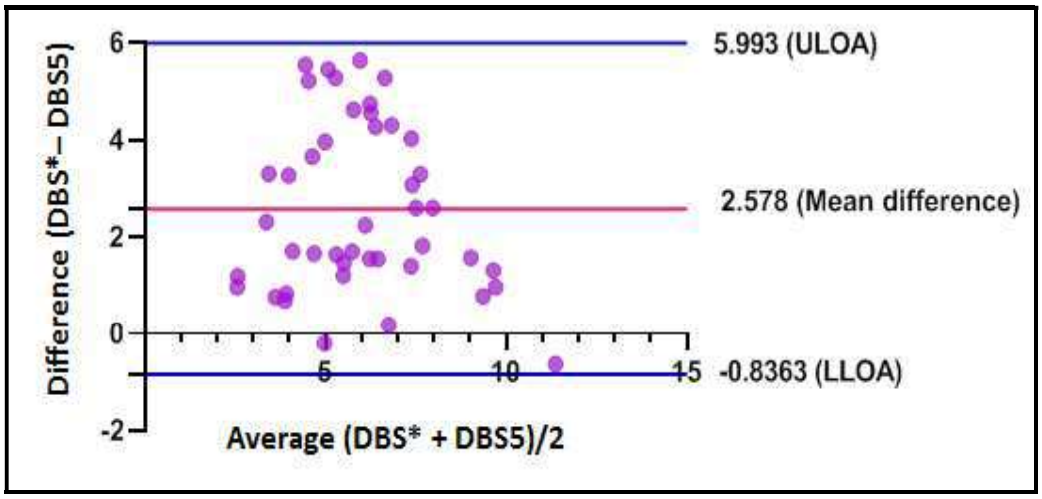
(**Figure 11B)** A plot of difference between DBS* & DBS2 against their average



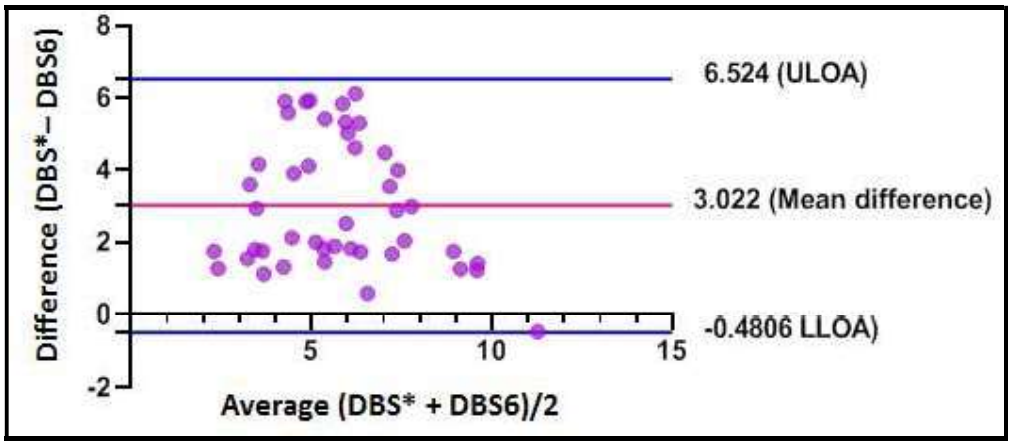
(**Figure 11C)** A plot of difference between DBS* & DBS3 against their average



(Figure 11D) A plot of difference between DBS* & DBS4 against their average



(Figure 11E) A plot of difference between DBS* & DBS5 against their average



(Figure 11F) A plot of difference between DBS* & DBS6 against their average

We have done the standardization on extraction of gDNA from DBS* stored at standard condition, and observed (r = 0.8150) good correlation coefficient with the gold standard (whole blood). Similarly, we have compared the gDNA extracted from all DBS samples stored at 4^0C & -20^0C with blood samples (whole blood & DBS*) stored at standard conditions.

3.1C.1a Evaluation of Genomic DNA concentration

3.1C.1b Evaluation and comparison of gDNA concentration between whole blood stored at standard condition & paired DBS samples stored at 4^0C (DBS1, DBS3, DBS4) & -20^0C (DBS2, DBS4, DBS6).

Comparison have been done with gold standard (whole blood) and we have obtained correlation coefficient between whole blood & DBS1 (r = 0.7184), whole blood & DBS2 (r = 0.6923), whole blood & DBS3 (r = 0.6987), whole blood & DBS4 (r = 0.6640), whole blood & DBS5 (r = 0.6857) and whole blood & DBS6 (r = 0.6852). As per categorization of values of minimum to maximum correlation coefficients -1 to +1, we have obtained strong correlation coefficient between whole blood and DBS at both the stored temperature irrespective of transport duration. At 4^0C correlation coefficient lie between (0.6857 – 0.7184) whereas at -20^0C is lies between (0.6852 – 0.6923), correlation coefficients explore clear

picture that we can store DBS samples at both the temperatures, but DBS storage at 4^0C better than -20^0C.

As per epidemiological point of view, we can collect DBS samples in field settings and transport to laboratory within 3 days of DBS preparation, but it will be better if these blood samples transported within 24 hours of transport duration because we got the maximum correlation coefficient (r = 0.7184) within 24 hours of transport duration and sample stored at 4^0C.

3.1C.1c Evaluation and comparison of gDNA concentration between DBS* stored at standard condition & DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6)

We have obtained excellent correlation coefficient (r = 0.8150) between whole blood & DBS* stored at standard condition. Whereas, good correlation coefficient obtained between DBS* & DBS1 (r = 0.7213), DBS* & DBS2 (r = 0.6918), DBS* & DBS3 (r = 0.7039), DBS* & DBS4 (r = 0.6902), DBS* & DBS5 (r = 0.6783), DBS* & DBS6 (r = 0.6786) respectively. In the above results obtained by comparing gDNA concentration in 2 phases, first compared with whole blood stored at standard condition and second compared with DBS* stored at standard condition. Overall results reveals, not too much difference observed in correlation coefficients (r = 0.6783 – 0.7213) at 4^0C by comparing with both blood samples (whole blood & DBS*) at standard condition similarly almost same results (r = 0.6640 – 0.6923) obtain with DBS while they were stored at -20^0C irrespective of their transport duration.

3.1C.1d T-test mean comparison: gDNA

We have conducted a t-test to compare the means of gDNA extracted from DBS stored at 4^0C & -20^0C with the gold standard. At standard condition, we have compared means between DBS* (Mean±SD 7.2857±2.0329) and whole blood (8.8245±2.4907), and there is a statistically significant difference observed (p > 0.001). Similarly, we have compared the

group means of gDNA concentration from all DBS samples stored at 4^0C & -20^0C with gold-standard, all group means are statistically significant with p-value (p>0.001).

3.1C.1e Analysis of Variance (ANOVA) test: gDNA

We have done multiple comparison of gDNA concentration from DBS stored separately at 4^0C & -20^0C irrespective of their transport duration.

ONEWAY-ANOVA test has been conducted to compare gDNA concentration from all DBS samples stored separately at 4^0C & -20^0C irrespective of their transport duration. Total 120 paired DBS samples stored at 4^0C & 120 paired DBS samples stored at -20^0C. All DBS samples stored at 4^0C & -20^0C compared with gold standard (whole blood & DBS*). At -20^0C stored condition, we have obtained slightly large F value (F = 37.85) as compared to at 4^0C (F = 28.66), but p-value (p>0.001) at both the temperature are statistically significant.

Table 12. Compare percentage change in gDNA concentration between gold-standard and all DBS samples stored at 4^0C & -20^0C**.**

Samples at standard condition	Percentage change in DBS* as compared to plasma		Percentage change in DBS as compared with samples stored at standard condition					
	-80^0C		**4^0C**			**-20^0C**		
	Plasma/WB	DBS*	DBS1	DBS3	DBS5	DBS2	DBS4	DBS6
Plasma/WB	0%	17%	40%	44%	46%	48%	51%	51%
DBS*	17%	0%	27%	32%	35%	38%	41%	41%

Figure 12. Agarose gel electrophoresis of extracted gDNA from dried blood spots. Figure shows highly intense band with little smear.

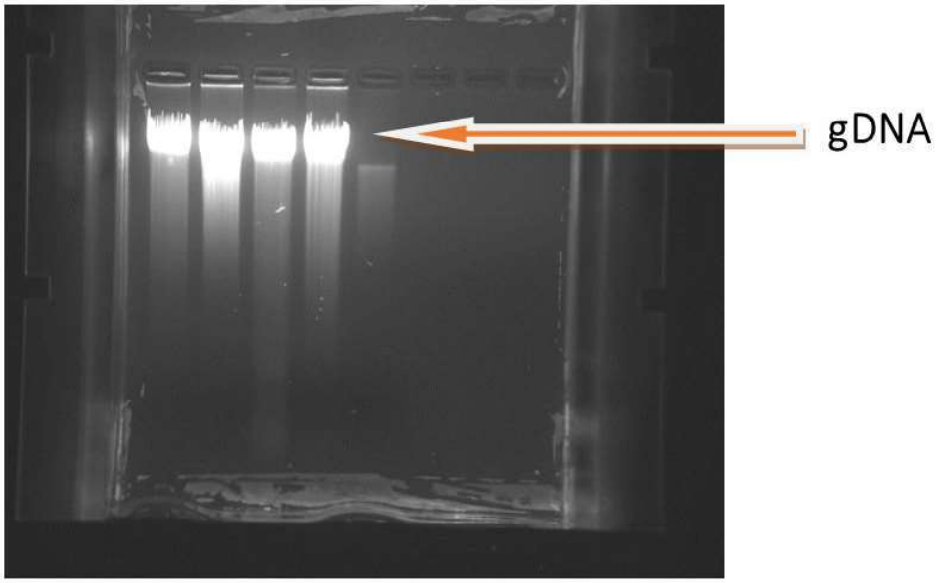
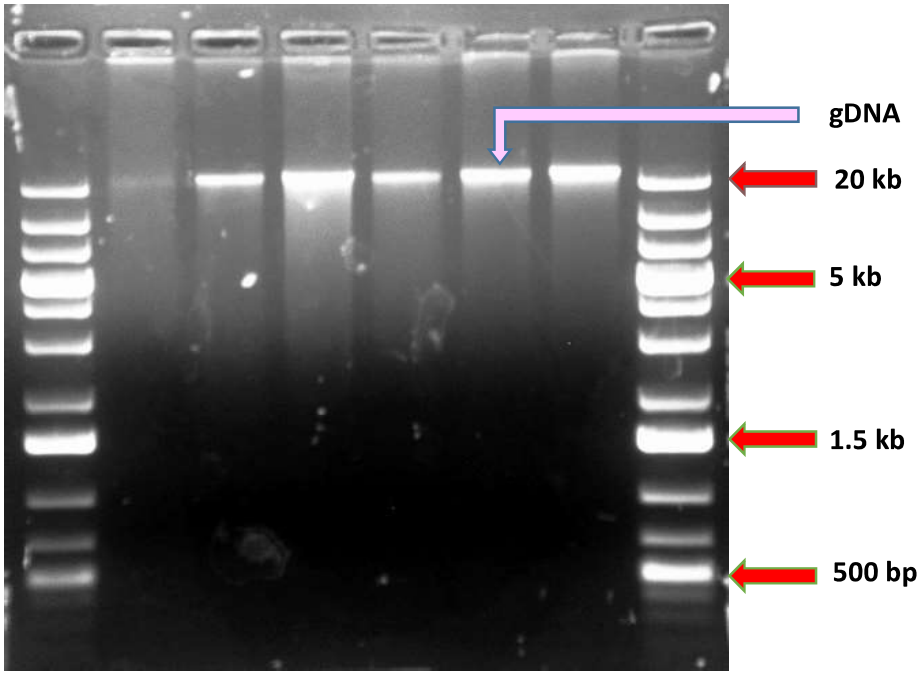


Figure 13. Agarose gel electrophoresis of gDNA extracted from dried blood spots with 1kb plus DNA marker at extremes.



3.1C.1f Discussion: gDNA evaluation from DBS

In literature, shreds of evidence exist on gDNA extraction from DBS to date, but there are limited articles available on the feasibility of paired DBS samples stored at different temperatures (4^0C & -20^0C) after 0-24, 24-48 & 48-72 hours of transport duration. We have compared a total of 6 times gDNA concentration extracted from DBS with the gold standard (whole blood & DBS*).

Our results show, the clear statistical significant ($p>0.001$) difference in gDNA concentration in all DBS samples stored at 4^0C & -20^0C as compared to the gold standard, but quantitatively less variation observed in mean concentration gDNA from DBS as compared to the gold standard (whole blood & DBS*). On average, (4.9829±2.3656) at 4^0C & (4.3406±2.3954) at -20^0C gDNA concentration obtained from size 6mm x 2 blood spots. In few studies, researchers demonstrated the extraction of DNA from DBS and evaluate DNA stability at various thermal conditions, but in our study, we have compared gDNA concentration 6 times from paired DBS samples to observe the variation and stability in gDNA concentration while they are stored at different thermal conditions at various transport duration.

In a study, the researcher has tried to increase gDNA yield from DBS and find out there is a 6 – 10 fold increase in gDNA concentration, but not mentioned storage condition (Lane, Noble, & Ph, 2010). Another study reveals that there is no difference observed in DNA concentration between DBS and fresh blood samples while stored for up to 24 hours at 4^0C (Halsall et al., 2008b).

During the standardization phase, we aimed to extract the maximum amount of gDNA from DBS, therefore, we have extracted gDNA from 1- 4 dried blood spots and quantify its concentration range 2.16ng/µl – 24ng/µl. Besides this, we have compared gDNA concentration between all stored DBS samples and gold-standard. For this comparison, 2

blood spots of size 6mm diameter compared with 17 - 18µl whole blood (gold-standard), because approximately 8.7±1.9µl blood contains by a single spot of 6mm DBS sample (Hewawasam, Liu, Jeffery, Gibson, & Muhlhausler, 2018).

The purity of genetic material (gDNA) measured by 260/280 ratio, ideally this ratio lies between 1.8 - 2.0 that indicates a pure form of gDNA, whereas we have observed gDNA purity 1.8 – 2.9 (260/280 ratio) from DBS. This variation in the purity of gDNA might be possible due to the hindrance of cotton linter present on blood collection cards, cell debris of dry blood components, or as per the protocol of gDNA extraction 5-10%, gDNA might be lost during the experiment. The final elution of gDNA completed in 30 microliter buffer, therefore total yield obtained 63.6ng – 720ng with a mean ratio (194.73 +- 106.14). The large variation observed in gDNA extraction from blood spots (Kumar, Mhatre, Godbole, Jha, & Dikshit, 2018). We aimed to reveal the utility of DBS in field epidemiological research. The gDNA extracted from DBS can be utilized for downstream applications such as Polymerase Chain Reaction (PCR), PCR based sequencing, PCR based genotyping, etc. DNA is stable biomarkers on DBS samples even if samples stored at 4^0C or -20^0C after particular transport duration.

Researchers have been shown that we can perform amplification through PCR of gDNA extracted from DBS even with 3mm x 3 blood spots (Chaisomchit, Wichajarn, Chowpreecha, & Chareonsiriwatana, 1997) and it can be a probe for single nucleotide polymorphism (SNP)(Catsburg et al., 2007). In another study, it supports the application of gDNA from DBS in GWAS (Genome-Wide Association Studies) where it shows that SNP genotyping is more robust of unamplified gDNA than genotyping of amplified gDNA from DBS(St. Julien et al., 2013).

T-test has been conducted to compare the means of gDNA concentration between all DBS samples & gold standard (whole blood & DBS*). Statistically significant p-value ($p>0.001$) observed between all DBS samples stored at the compromised condition and gold-standard (whole blood & DBS*) samples stored at standard condition. On average, the concentration of gDNA from DBS stored at 4^0C is better than DBS stored at -20^0C. The excellent correlation coefficient ($r = 0.81$) observed between whole blood and DBS at standard conditions, whereas, on average it is slightly less when DBS stored at 4^0C ($r=0.70$) & at -20^0C ($r=0.67$) as compared to the standard condition. While, almost similar correlation observed between DBS stored at 4^0C ($r=0.70$) & at -20^0C ($r=0.68$) as compared to DBS* stored at standard condition. From the overall analysis, we have concluded that DBS can be stored at 4^0C or -20^0C even within 3 days of transport duration, on average there is no change observed in gDNA concentration. Good correlation coefficient observed between these paired blood samples but correlation at 4^0C is quite better than at -20^0C.

We have calculated the percentage difference in gDNA concentration, only 17% mean difference observed between DBS* & whole blood at standard condition, whereas this difference fairly increases over time when DBS stored at 4^0C such as DBS1 = 40%, DBS3 = 44% and DBS5 = 46%, and at -20^0C DBS2 = 48%, DBS4 = 51% and DBS6 = 51%. With the increase in transport duration, the percentage difference increases between all DBS stored at compromised condition & whole blood. From observed results on gDNA, DBS can be transported before 24 hours & can be stored at 4^0C or -20^0C for a shorter time but for the longest time, it should be stored at -80^0C.

ANOVA (Analysis of Variance) tests conducted to evaluate multiple comparisons of gDNA concentrations to observe variability between and within groups. gDNA concentration compared between all DBS stored at the compromised condition and whole blood irrespective of transport duration. ANOVA result shows more variability in gDNA

concentration within a group at -20^{0}C than 4^{0}C. Less variability observed within the group when all DBS samples compared with DBS* stored at standard condition, statistically, significant p-value observed (p>0.001) at 4^{0}C (F=11.63) and -20^{0}C (F = 17.08).

3.1C.1g Bland-Altman graph (BA plot)

BA graph is plotted between mean differences of two groups against their average. This graph is prepared to observe the gDNA concentration difference between DBS stored at the compromised condition and gold standard (whole blood & DBS*). BA plot describes the limit of agreement between two quantitative measurements, which is measured by a lower limit of agreement (LLOA) d-1.96*SD, and upper limit of agreement d+1.96*SD (ULOA). BA plot never reveals, whether the agreement is suitable or sufficient, it simply quantifies the bias and limit of agreement in which 95% of the differences lie between both the measurements. In an ideal BA plot, the graphical representation of the mean difference should be narrow, which implicates that the measurement by both the methods is equal and close to zero.

3.2 Evaluation of IgG immunoglobulin against *Helicobacter pylori* infection from Dried Blood Spot (DBS)

3.2A Standardization and validation of estimation of IgG status against Helicobacter pylori infection from DBS stored at standard condition.

3.2A.1 Introduction

Helicobacter pylori is a 2.3-3µm long twisted or helical gram-negative bacteria responsible for 80-90% of B-gastritis cases and suspected to a major cofactor for the development of gastric and duodenal ulcers. The classical detection methods of Helicobacter pylori like culturing from mucous membrane biopsies or a urease test are only successful in case of a relative high germ count. The colonization of gastric and duodenal mucosa membranes with Helicobacter pylori can also be detected serologically using an enzyme immunoassay (ELISA) or by performing western blot.

In this section, we have targeted infection-causing blood-based biomarkers. We aimed to evaluate the IgG (immunoglobulins) antibody concentration status against Helicobacter pylori infection in human blood measured from DBS. Epidemiologically the prevalence of *H.pylori* in general population is >65%.

Previously some seroepidemiologic studies have been published but they have not compared the single source of blood samples multiple times at different time points and storage temperature.

Initially, we have standardized protocol from DBS to evaluate the IgG immunoglobulin concentration and validate it with the gold standard (plasma) sample. Both paired blood samples (DBS* and plasma) stored at standard conditions (-80^{0}C).

3.2A.2 Material & methods

Commercial available ELISA kits (IBL, Germany) used for the estimation of IgG antibody from DBS & plasma.

3.2A.3 Principle of the Assay

It is a solid phase enzyme linked immunoassay (ELISA) based on sandwich principle. The wells are coated with antigen. Specific antibodies of the sample binding to the antigen coated wells are detected by a secondary enzyme conjugated antibody specific for human IgG. After the substrate reaction the intensity of the color developed is proportional to the amount of IgG-specific antibodies detected. Results of samples can be directly using the standard curve.

3.2A.4 Reagent preparation

All the reagents are readymade to use, only wash buffer diluted in a ratio 1:10. Wash buffer contain some crystals which is dissolved by putting it at 37^0C for few minutes.

3.2A.5 Dilution of samples

As recommended, serum/plasma samples diluted with diluent buffer in a ratio 1:101

3.2A.6 Elution of plasma from DBS

ELISA assay components prepared as per given instruction by the manufacturer and diluted plasma samples in ratio 1:101. Excise a single spot of DBS from blood collection card with size 6mm in diameter, put it into 24 well non-treated cell culture plate, add 500µl assay diluent buffer, cover the plate and put it on the shaker at 200rpm for 120 minutes (Figure 14). All the components on DBS eluted in the diluent buffer, this supernatant directly used as a sample for the assay.

Figure 14. Processing of 6mm single DBS sample for serology

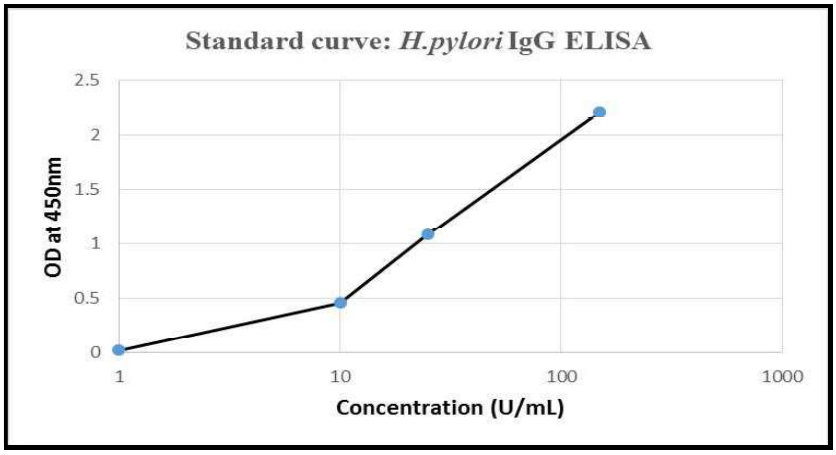
3.2A.7 Assay protocol: Testing IgG antibody against *H. pylori* infection from DBS (ELISA)

1. Pipetted 100µl of each standard/diluted samples into respective wells of Mircotiter plate.
2. Cover the plate with adhesive foil and incubate it for 60min at 18-25 ^{0}C.
3. Remove the adhesive foil, discarded the incubation solution. Wash the plate 3x times with 300µl wash buffer (1:10). Washing is done on TECAN multichannel micro-plate washer.
4. Pipetted 100µl of enzyme conjugate into each well, cover the plate with adhesive foil & incubate it for 30 min at 18-25 ^{0}C.
5. Remove the adhesive foil & discarded incubation solution. Wash the plate 3x times with 300µl of wash buffer. Remove excess solution by tapping the inverted plate on paper towel.
6. Pipetted 100µl of TMB substrate solution into each well, & incubate it for 20 min at 18-25 ^{0}C in dark place.
7. Stop the substrate reaction by adding 100µl of TMB stop solution into each well. Mix it well by gentle shaking the plate till color changes from blue to yellow.
8. Measured optical density with spectrophotometer at wavelength 450 nm within 60 min after adding stop solution.

Cut of Index (COI) calculation

$$\text{COI} = \frac{\text{Optical density of sample}}{\text{Optical density of standard B}}$$

Figure 15. Standard curve: *Helicobacter pylori* IgG ELISA



Calibration of standards for standard curve preparation: *H pylori* IgG ELISA

3.2A.8 Result: T-test, Scattered plot, Bland-Altman plot

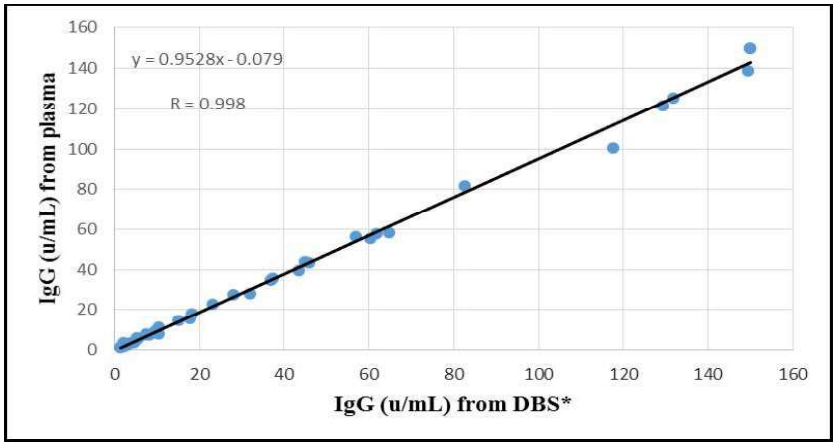
Table 13[15]. T-test result, mean comparison of IgG concentration between plasma and DBS* stored at standard condition.

Samples stored at standard condition	Number of Observations	Mean	Standard Deviation	95% CI	p - value
Plasma	40	36.681	45.892	22.728 - 50.633	p <0.001
DBS*	40	34.869	43.805	21.551 - 48.187	

T-test has been conducted to compare group means of IgG immunoglobulin between DBS* & plasma stored at standard conditions. Mean of plasma (36.681±45.892) with 95 % CI (22.728 - 50.633), is comparable with the mean of DBS* (34.869±43.805) with 95% CI (21.551 - 48.187). Means are statistically significant with p-value ($p < 0.05$), but they are highly correlated with correlation coefficient ($r = 0.9981$) at standard condition.

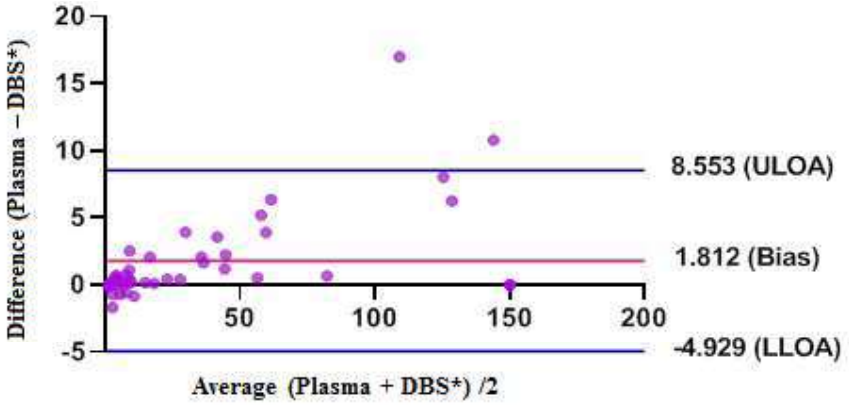
[15] Paired t-test (STATA) used to compare mean difference of IgG antibody against H. *pylori* between plasma and DBS* stored at standard condition.

Figure 16[16]**.** Prepared scattered plot to evaluate correlation coefficient of IgG antibody against *H. pylori* infection between plasma and DBS* stored at standard condition.



At standard condition, excellent correlation coefficient (r = 0.9980) obtained between plasma & DBS*

Figure 17[17]**.** Prepared Bland-Altman graph, a plot of difference of IgG concentration against *H. pylori* between plasma & DBS* stored at standard condition, plotted against their average.



[16] Scattered plot (Excel) were used to compare correlation coefficient of IgG concentration between plasma & DBS* stored at standard condition

[17] Bland-Altman plot (GraphPad) of IgG created to evaluate bias between plasma & DBS* stored at standard condition

3.2B Evaluation & comparison of IgG (U/mL) antibody concentration between plasma & paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6)

3.2B.1 Results: T-test, ANOVA, Scattered plot, Bland-Altman plot

Table 14[18]. T-test result, the mean comparison of IgG concentration between plasma and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5).

Blood samples storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
Plasma (standard condition)	Immediate	40	36.681	45.892	22.728 - 50.633	p>0.0637
DBS1 (4 Degree Celsius)	Between 0-24 hours	40	30.297	39.106	18.408 - 42.187	
Plasma (standard condition)	Immediate	40	36.681	45.892	22.728 - 50.633	p>0.0345
DBS3 (4 Degree Celsius)	Between 24-48 hours	40	29.488	37.154	18.192 - 40.784	
Plasma (standard condition)	Immediate	40	36.681	45.892	22.728 - 50.633	p>0.0095
DBS5 (4 Degree Celsius)	Between 48-72 hours	40	26.742	31.357	17.208 - 36.275	

Table 15[19]. T-test result, mean comparison of IgG concentration plasma and paired DBS samples stored at -20^0C (DBS2, DBS4, DBS6).

Blood samples storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
Plasma (standard condition)	Immediate	40	36.681	45.892	22.728 - 50.633	p > 0.620
DBS2 (-20 Degree Celsius)	Between 0-24 hours	40	35.145	44.325	21.669 - 48.621	
Plasma (standard condition)	Immediate	40	36.681	45.892	22.728 - 50.633	p > 0.136
DBS4 (-20 Degree Celsius)	Between 24-48 hours	40	31.888	40.765	19.494 - 44.282	
Plasma (standard condition)	Immediate	40	36.681	45.892	22.728 - 50.633	p > 0.245
DBS6 (-20 Degree Celsius)	Between 48-72 hours	40	32.193	41.966	19.434 - 44.952	

[18] Paired t-test (Excel) used to compare mean difference of IgG antibody between plasma and DBS stored at 4^0C

[19] Paired t-test (Excel) used to compare mean difference of IgG antibody between plasma and DBS stored at -20^0C

Table 16[20]**.** ANOVA-test result, multiple group mean comparison of IgG antibody concentration between plasma and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

Plasma sample as Gold standard storage condition	DBS samples stored condition irrespective of transport duration	F-value	p value
Standard condition (n = 40)	4 degree Celsius (n = 120)	0.83	p>0.477
Standard condition (n = 40)	-20 degree Celsius (n = 120)	0.26	p>0.851

T-test has been conducted to compare mean between all DBS groups with plasma. At 0.05 significance level, statistically non-significant p-value (p>0.0637) observed between plasma & DBS1, and significant p-value observed for DBS3 (p>0.0345) and DBS5 (p>0.0095) at 4^0C celsius of storage. Whereas, at -20^0C storage, non-significant p-values observed between plasma & DBS2 (p > 0.620), DBS4 (p > 0.136) and DBS6 (p > 0.245). At -20^0C DBS storage, there is no group mean difference observed between plasma & DBS, but at 4^0C storage, only DBS1 shows non-significant p-value but not by other DBS samples. ANOVA test has been conducted between plasma & all 6 DBS groups. Statistically non-significant p-value observed at 4^0C (p > 0.477) and -20^0C (p > 0.8519), with their corresponding F-value (F = 0.83) and (F = 0.26). Transportation of DBS samples even on 3rd day, have not disrupt the integrity and stability of IgG immunoglobulins on DBS samples.

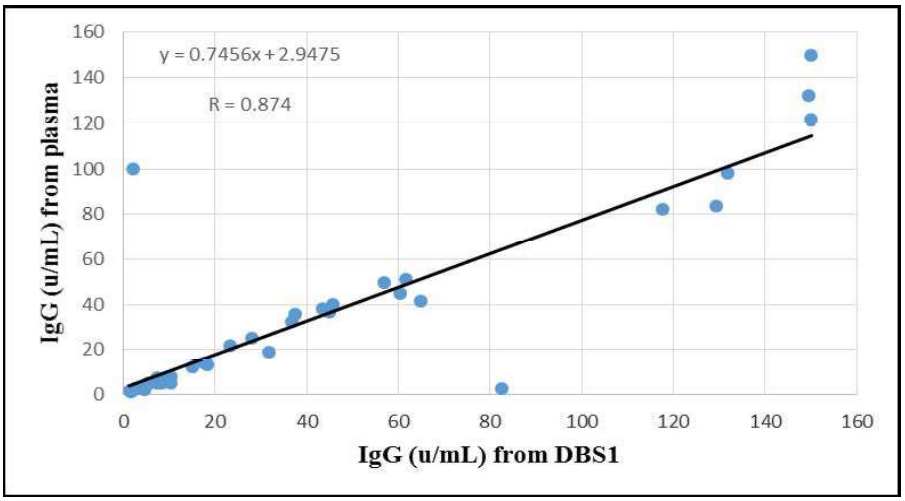
Table 17[21]**.** Compared Sensitivity, Specificity of test between plasma & all DBS groups.

Blood sample comparison		Sensitivity	Specificity	Kappa	PPV	NPV
Plasma	DBS1	87.5%	95%	80	95.45	86.36
Plasma	DBS2	87.5%	90%	77.17	91.3	85.7
Plasma	DBS3	87.5%	95%	81	95.45	86.36
Plasma	DBS4	87.5%	86.36%	81	95.45	86.36
Plasma	DBS5	100%	75%	77	82.75	100
Plasma	DBS6	87.5%	95%	81	95.45	86.36

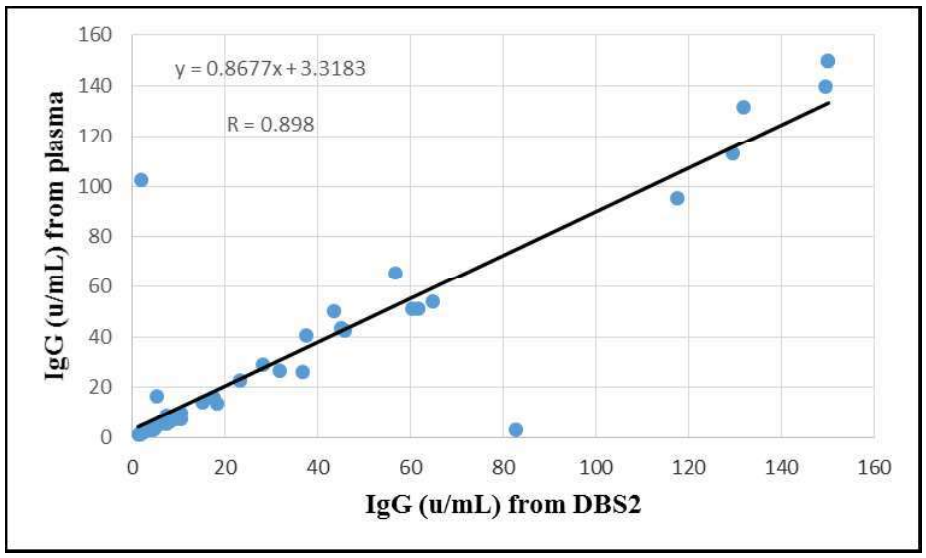
[20] Oneway ANOVA test was conducted to compare mean concentration of IgG antibody conc. between plasma & DBS stored at 4^0C & -20^0C.

[21] 2x2 table created to evaluate the sensitivity/specificity/PPV/NPV between DBS & plasma samples

Figure 18[22]. Prepared scattered plot to compare correlation coefficient between plasma and DBS stored at 4^0C (DBS1, DBS3, DBS5) and -20^0C (DBS2, DBS4, DBS6).

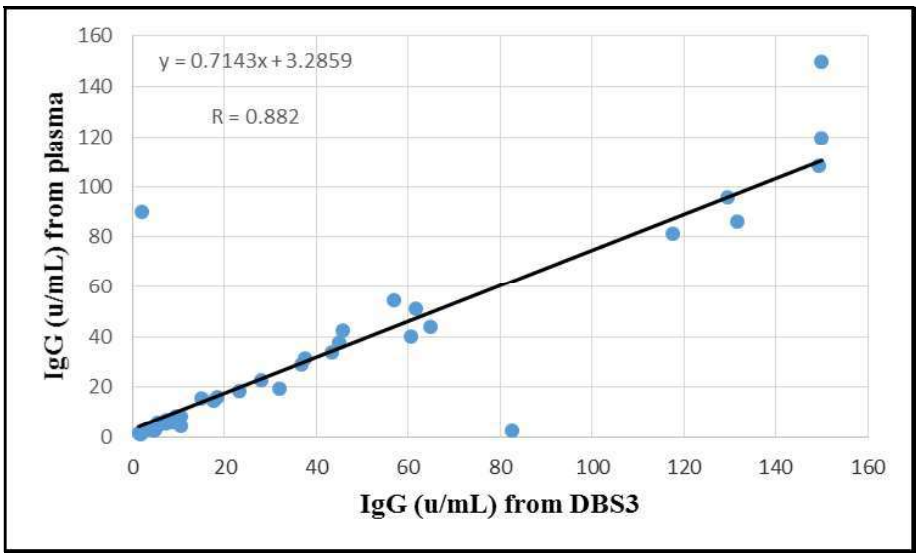


(Figure 18A) Scattered plot for estimation of IgG antibody between plasma & DBS1 with correlation coefficient (r = 0.8740).

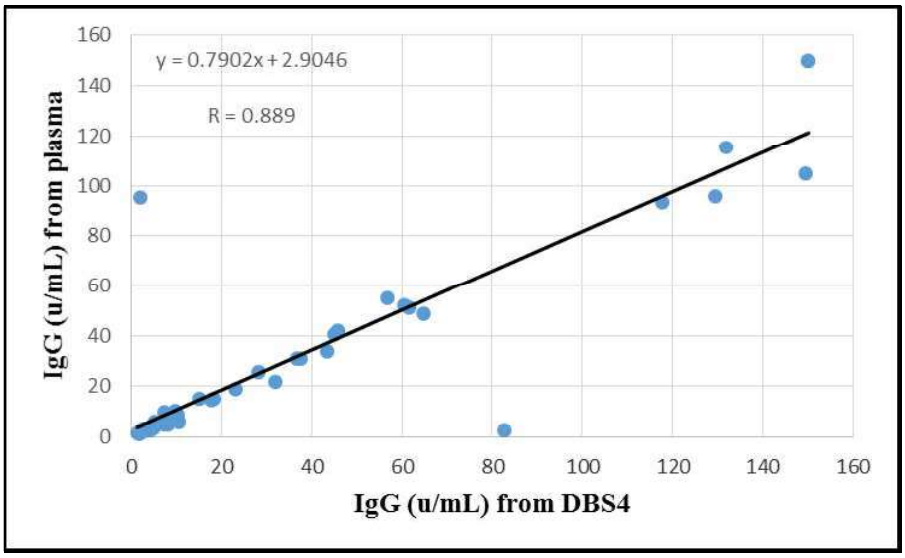


(Figure 18B) Scattered plot for estimation of IgG antibody between plasma & DBS2 with correlation coefficient (r = 0.8980)

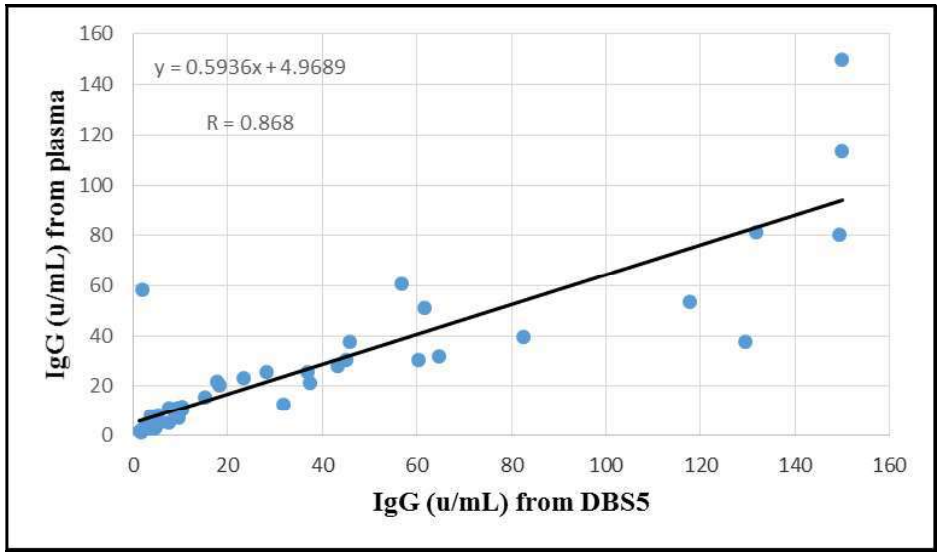
[22] Scattered plot (Excel) created to evaluate correlation coefficient of IgG conc. between plasma & all DBS groups stored at 4^0C & -20^0C



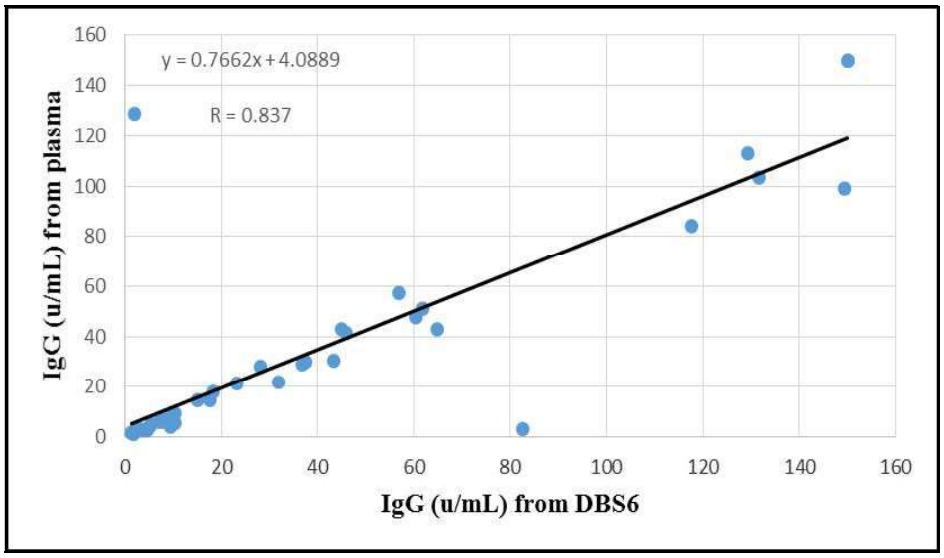
(Figure 18C) Scattered plot for estimation of IgG antibody between plasma & DBS3 with correlation coefficient (r = 0.8820)



(Figure 18D) Scattered plot for estimation of IgG antibody between plasma & DBS4 with correlation coefficient (r = 0.8890)

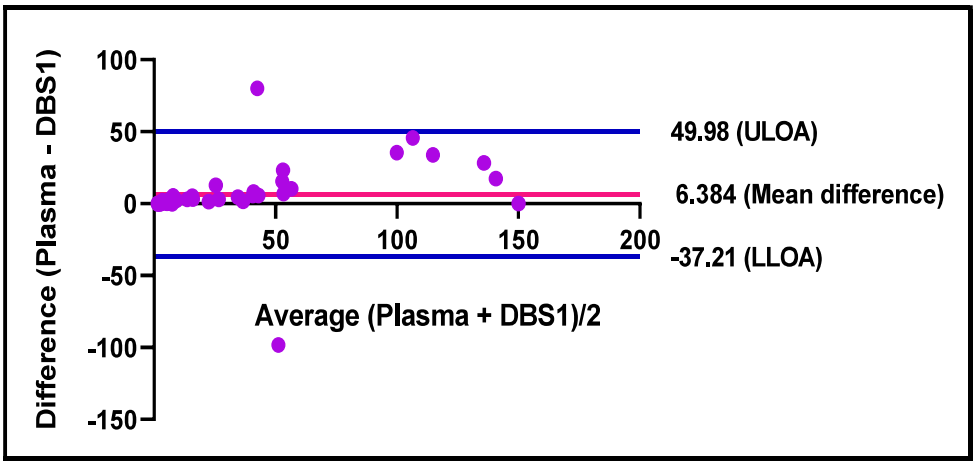


(Figure 18E) Scattered plot for estimation of IgG antibody between plasma & DBS5 with correlation coefficient (r = 0.8680)

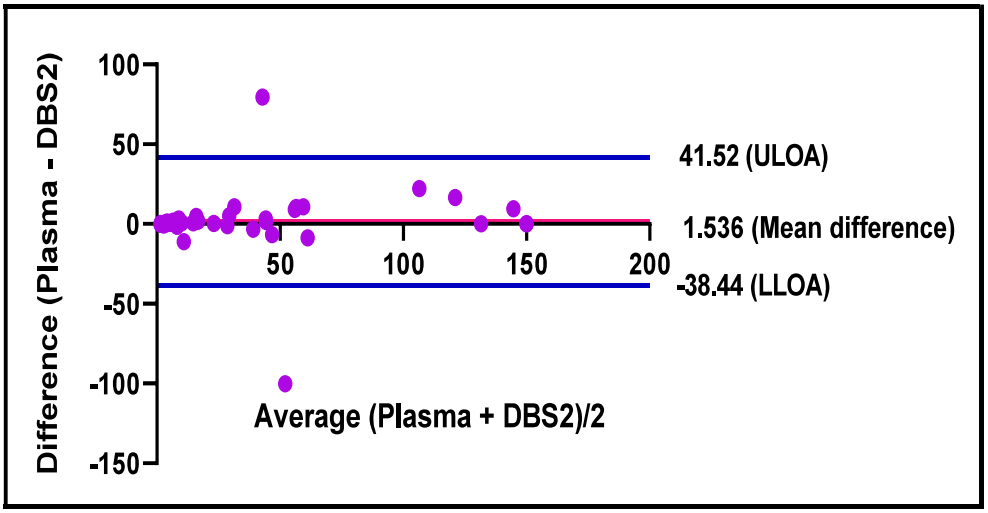


(Figure 18F) Scattered plot for estimation of IgG antibody between plasma & DBS6 with correlation coefficient (r = 0.8370)

Figure 19[23]. Prepared Bland-Altman graph, a plot of difference of IgG concentration between plasma & DBS, plotted against average of plasma & DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

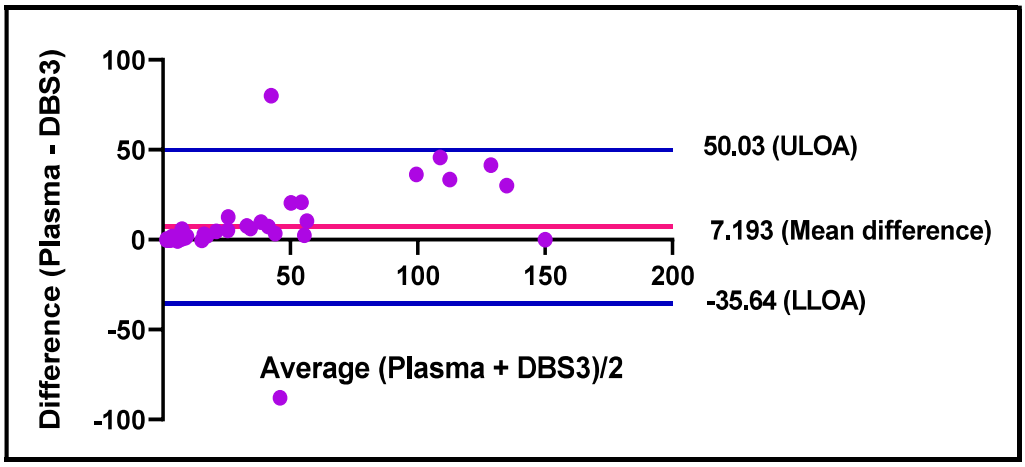


(**Figure 19A)** BA plot (IgG) of difference between plasma & DBS1

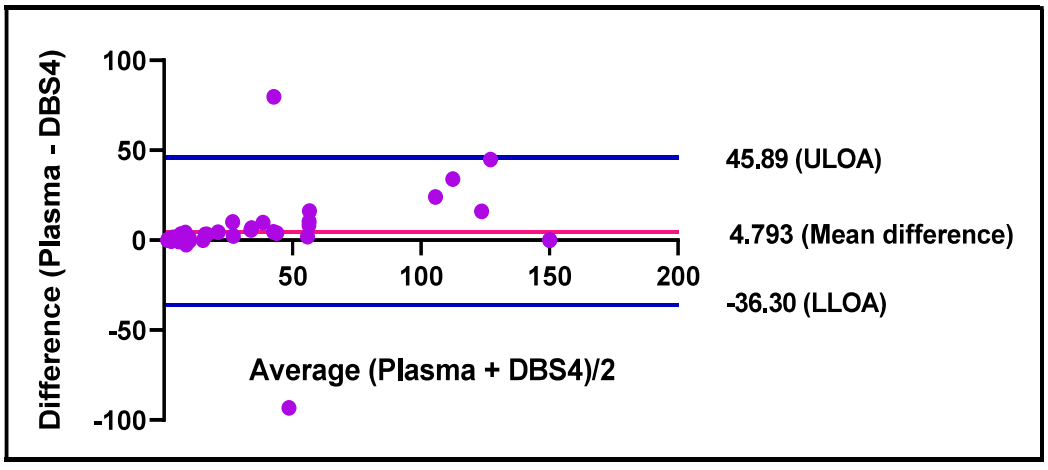


(Figure 19B) BA plot (IgG) of difference between plasma & DBS2

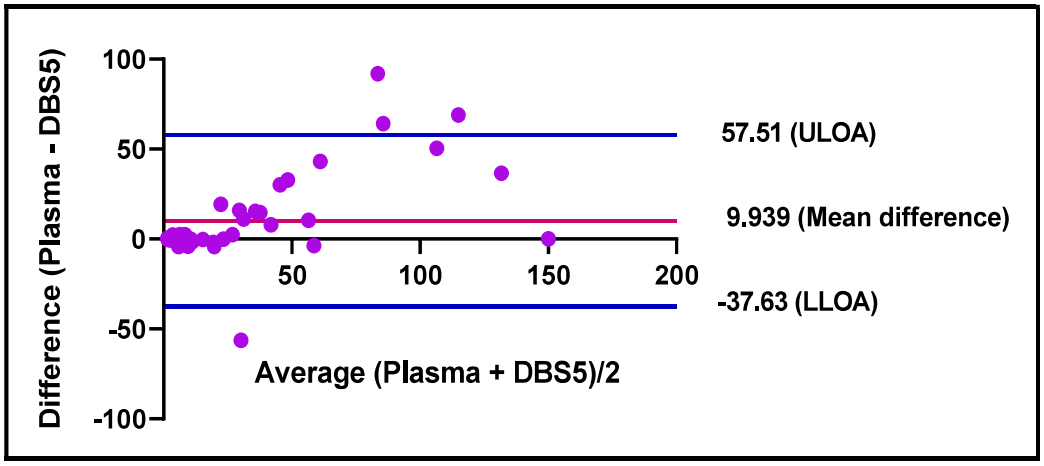
[23] Bland-Altman plot (GraphPad) of IgG antibody created to evaluate the bias between plasma and all DBS groups (DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 group separately).



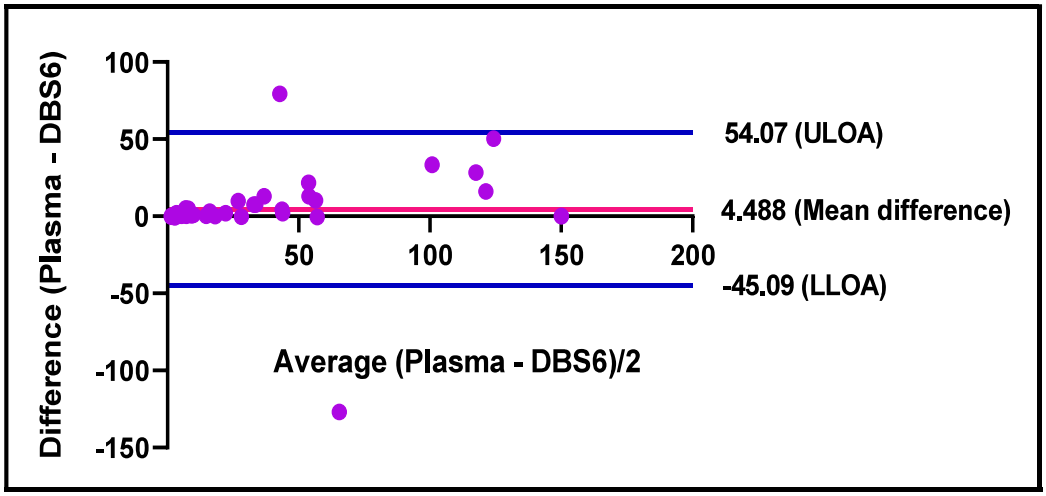
(Figure 19C) BA plot (IgG) of difference between plasma & DBS3



(Figure 19D) BA plot (IgG) of difference between plasma & DBS4.



(Figure 19E) BA plot (IgG) of difference between plasma & DBS5



(Figure 19F) BA plot (IgG) of difference between plasma & DBS6

3.2C Evaluation & comparison of IgG (U/mL) antibody concentration between DBS* & paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6)

3.2C.1 Results: T-test, ANOVA, Scattered graph and Bland-Altman plot

Table 18[24]. T-test result, the group mean comparison of IgG concentration between DBS* sample stored at standard condition and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5).

Sample storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
DBS (standard condition)	Immediate	40	34.86956	43.8056	21.551 - 48.187	p > 0.154
DBS (4 Degree Celsius)	Between 0-24 hours	40	30.29785	39.10659	21.669 - 48.621	
DBS (standard condition)	Immediate	40	34.86956	43.8056	21.551 - 48.187	p > 0.085
DBS (4 Degree Celsius)	Between 24-48 hours	40	29.48851	37.15444	19.494 - 44.282	
DBS (standard condition)	Immediate	40	34.86956	43.8056	21.551 - 48.187	p > 0.016
DBS (4 Degree Celsius)	Between 48-72 hours	40	26.74207	31.35703	19.434 - 44.952	

Table 19[25]. T-test, the group mean comparison of IgG concentration between DBS* sample stored at standard condition and paired DBS samples stored at -20^0C (DBS2, DBS4, DBS6)

Sample storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
DBS (standard condition)	Immediate	40	34.86956	43.8056	21.551 - 48.187	p > 0.926
DBS (-20 Degree Celsius)	Between 0-24 hours	40	35.14566	44.32572	21.669 - 48.621	
DBS (standard condition)	Immediate	40	34.86956	43.8056	21.551 - 48.187	p > 0.319
DBS (-20 Degree Celsius)	Between 24-48 hours	40	31.8889	40.76599	19.494 - 44.282	

[24] Paired t-test (STATA) used to compare mean difference of IgG conc. between DBS* stored at standard condition and DBS stored at 4^0C (DBS1, DBS3 & DBS5)

[25] Paired t-test (STATA) used to compare mean difference of IgG conc. between DBS* stored at standard condition and DBS stored at -20^0C (DBS2, DBS4 & DBS6)

DBS (standard condition)	Immediate	40	34.86956	43.8056	21.551 - 48.187	p > 0.462
DBS (-20 Degree Celsius)	Between 48-72 hours	40	32.1939	41.96661	19.434 - 44.952	

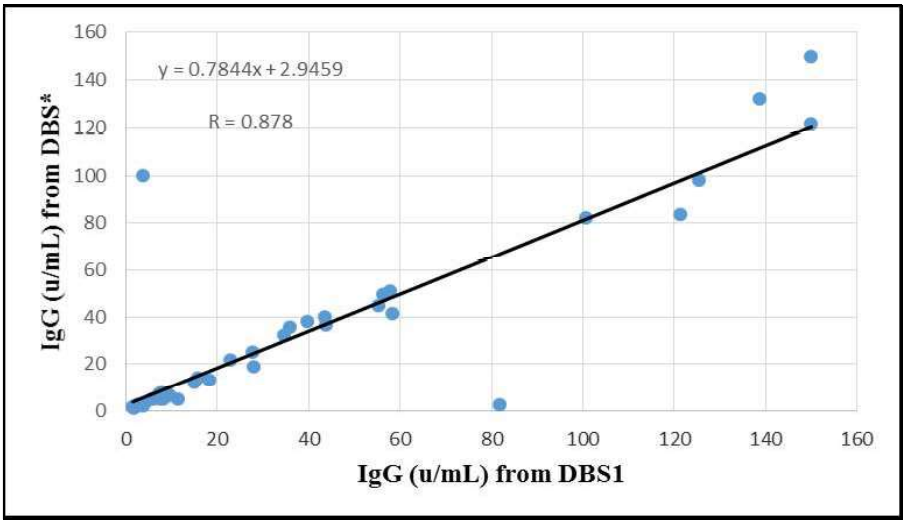
Table 20[26]. ANOVA-test result, the multiple group mean comparison of IgG antibody concentration between DBS* stored at standard condition and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

DBS* sample stored condition	**DBS samples stored condition irrespective of transport duration**	**F-value**	**p value**
Standard condition (n = 40)	4 degree Celsius (n = 120)	0.60	p>0.618
Standard condition (n = 40)	-20 degree Celsius (n = 120)	0.16	p>0.926

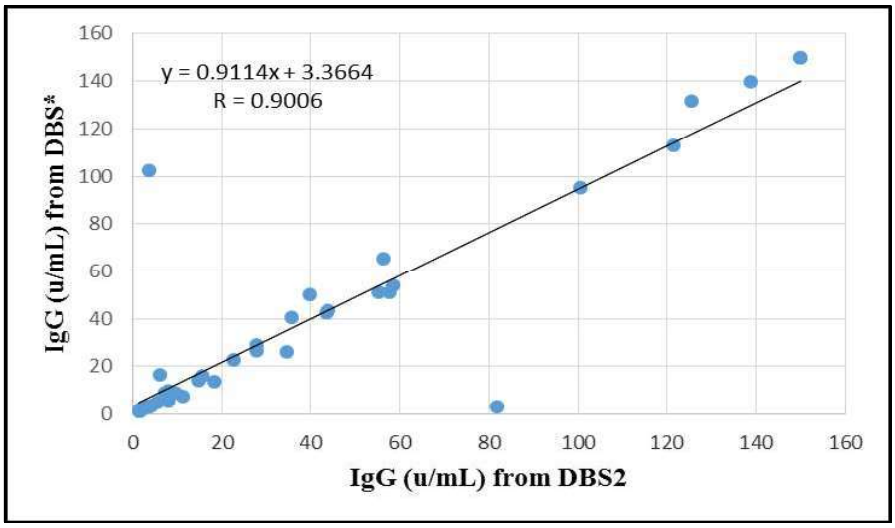
T-test has been conducted to compare group mean for IgG immunoglobulins concentrations between all DBS with DBS* stored at standard condition. At 4^0C with 0.05 significance level, statistically non-significant p-value observed between DBS* & DBS1 (p>0.154), and DBS3 (p>0.085), while significant p-value observed for DBS5 (p>0.016). While at -20^0C, strongly non-significant p-value observed for DBS2 (p >0.926), DBS4 (p >0.319), and DBS6 (p > 0.462). ANOVA test conducted for multiple group comparison for IgG immunoglobulins concentrations between DBS* stored at standard condition with all 6 groups of DBS. Statistically non-significant p-value observed at 4^0C (p >0.6185) & -20^0C (p >0.9263) with their corresponding F- value (F = 0.60) & (F = 0.16).

[26] Oneway ANOVA test was conducted to compare mean concentration of IgG antibody between DBS* stored at standard condition & all groups of DBS stored at 4^0C & -20^0C.

Figure 20[27]. Prepared scattered plot to compare correlation coefficient between DBS* stored at standard condition and other DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

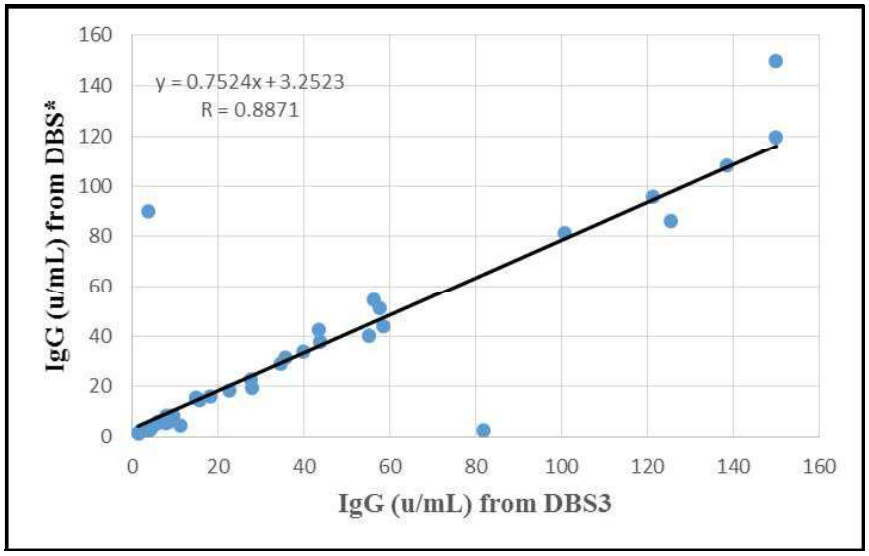


(**Figure 20A)** Scattered plot between DBS* & DBS1 with correlation coefficient (r =0.8780)

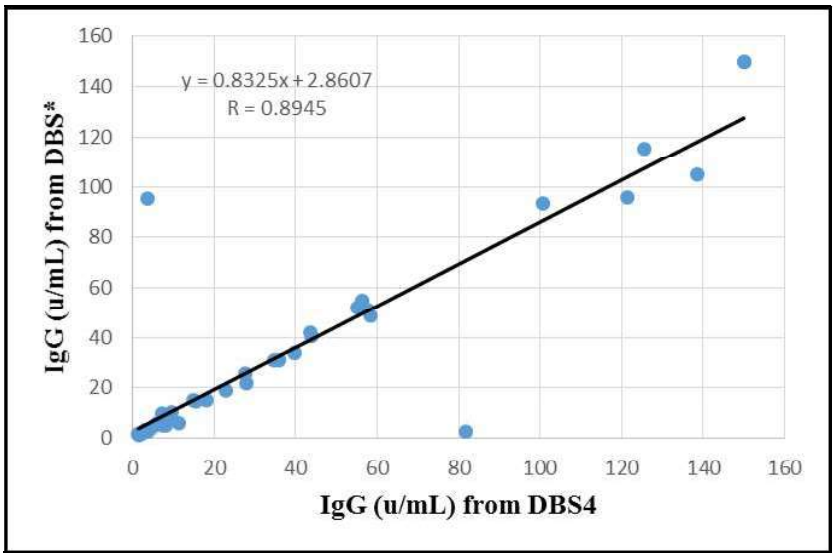


(Figure 20B) Scattered plot between DBS* and DBS2 with correlation coefficient (r =0.9006)

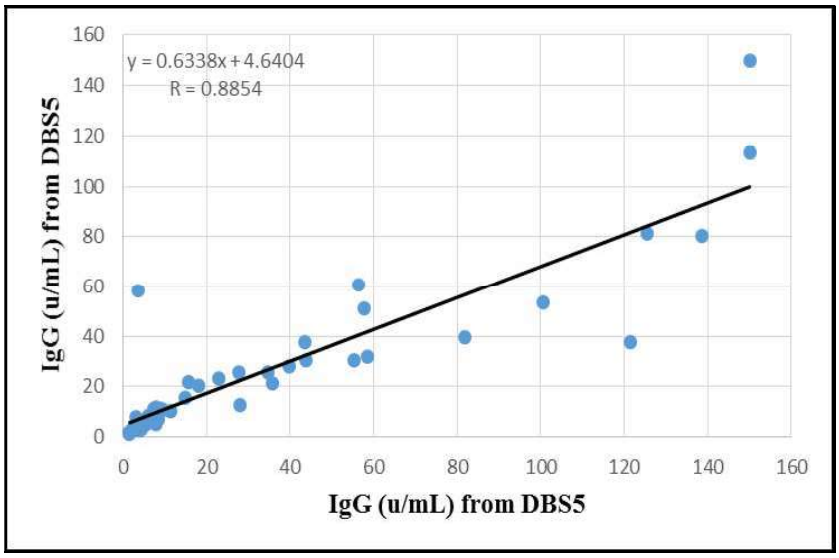
[27] Scattered plot (Excel) created of IgG antibody conc. between DBS* stored at standard condition & all DBS groups (DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately)



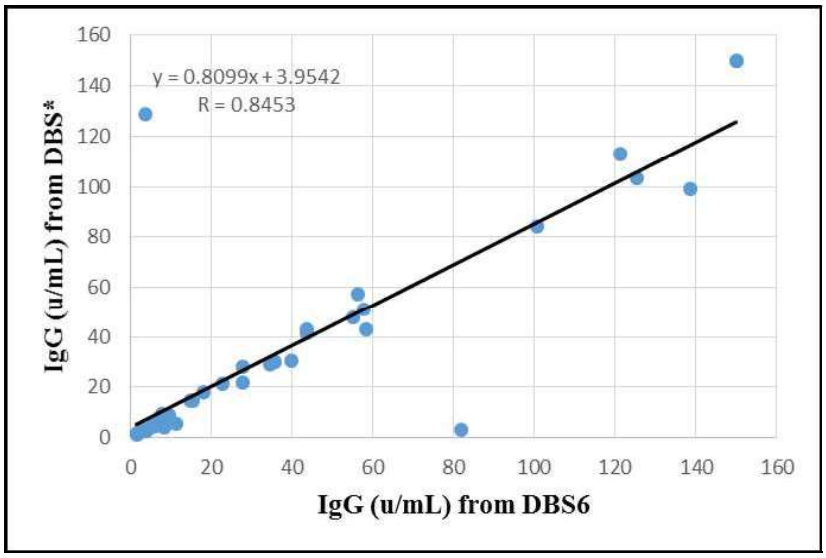
(Figure 20C) Scattered plot between DBS* and DBS3 with correlation coefficient (r =0.8871)



(Figure 20D) Scattered plot between DBS* and DBS4 with correlation coefficient (r =0.8945)

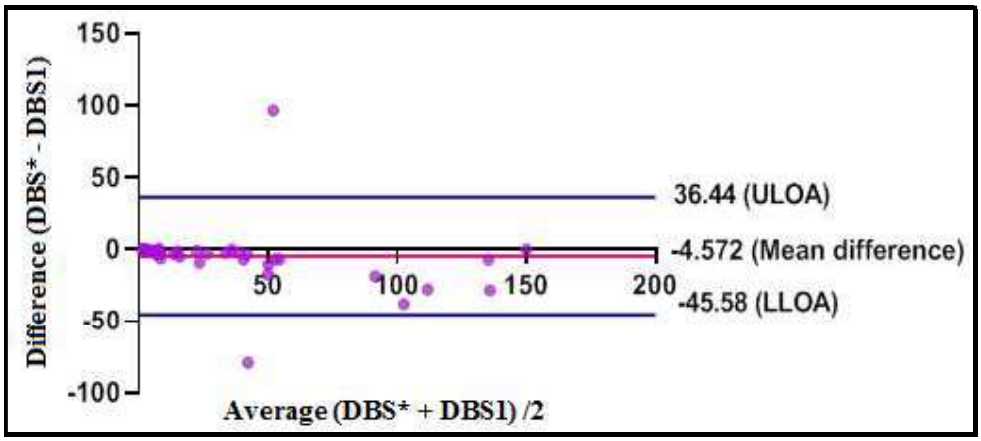


(Figure 20E) Scattered plot between DBS* and DBS5 with correlation coefficient (r =0.8854)

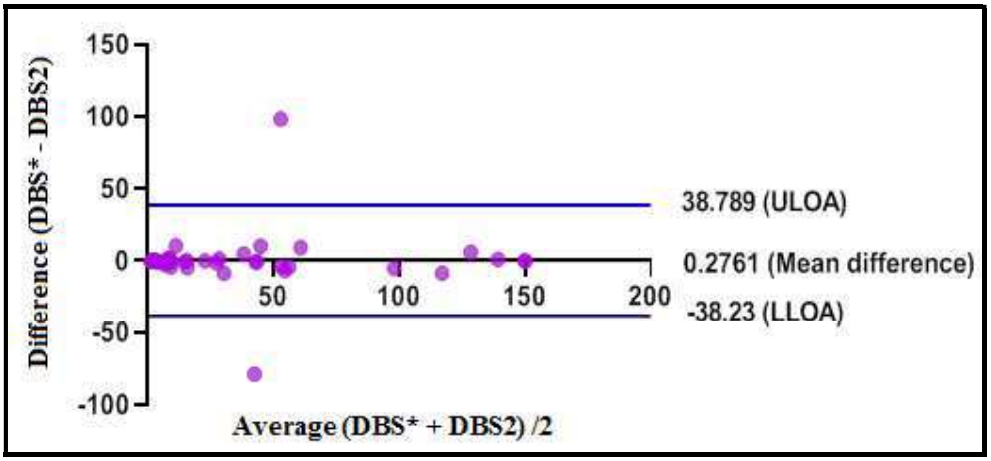


(Figure 20F) Scattered plot between DBS* and DBS6 with correlation coefficient (r =0.8453)

Figure 21[28]**.** Prepared Bland-Altman plot to evaluate the IgG antibody concentrations difference between DBS* stored at standard condition and DBS stored at 4^0C (DBS1, DBS3, DBS5) and -20^0C (DBS2, DBS4, DBS6).

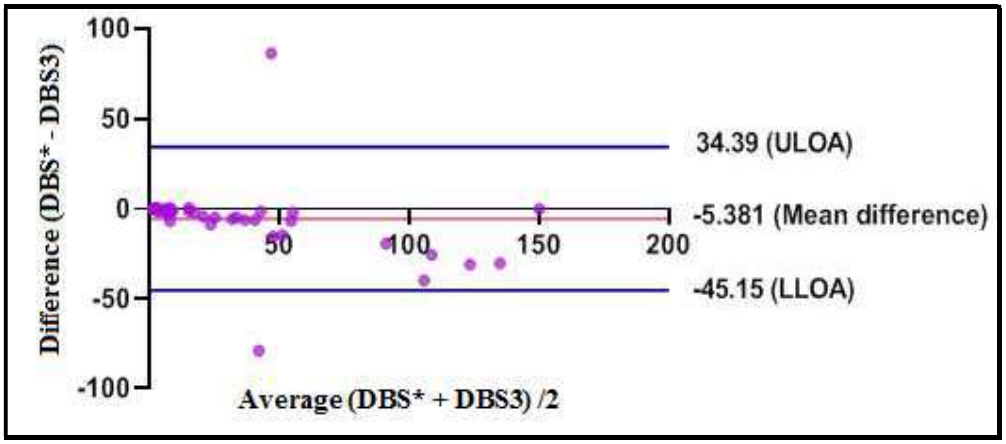


(Figure 21A) BA plot showing difference in IgG concentration between DBS* & DBS1

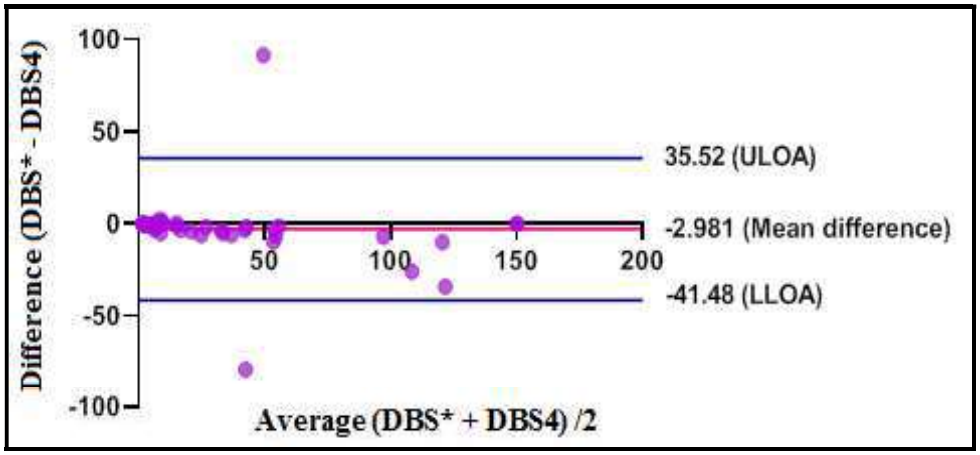


(Figure 21B) BA plot showing difference in IgG concentration between DBS* & DBS2

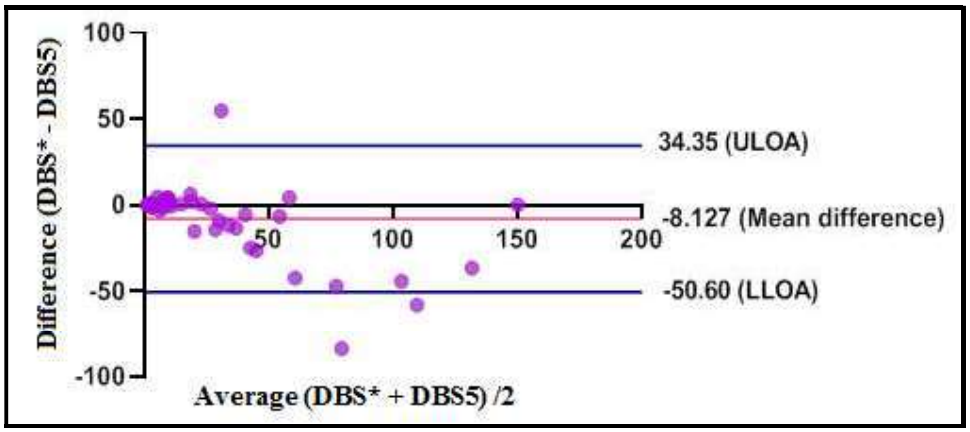
[28] Bland-Altman plot (GraphPad) of IgG antibody created to observe the bias DBS* stored at standard condition and DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately



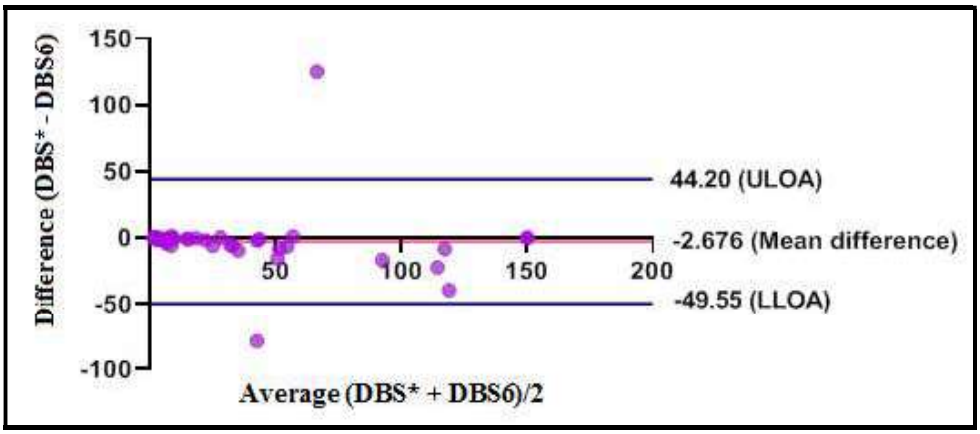
(Figure 21C) BA plot showing difference in IgG concentration between DBS* DBS3



(Figure 21D) BA plot showing difference in IgG concentration between DBS* & DBS4



(Figure 21E) BA plot showing difference in IgG concentration between DBS* & DBS5



(Figure 21F) BA plot showing difference in IgG concentration between DBS* & DBS6

Table 21. Compare percentage change in IgG concentration between Plasma and DBS samples stored at 4^0C & -20^0C.

Samples at standard condition	Percentage change in DBS* as compared to plasma		Percentage change in DBS as compared with samples stored at standard condition					
	-80^0C		**4^0C**			**-20^0C**		
	Plasma/WB	DBS*	DBS1	DBS3	DBS5	DBS2	DBS4	DBS6
Plasma/WB	0%	5%	17%	19%	27%	4%	13%	12%
DBS*	5%	0%	13%	15%	23%	0.79%	8%	7%

3.2C.2 Discussion: Evaluation of IgG immunoglobulin from DBS

The good correlation observed between plasma & DBS* at standard condition (Figure 16) & all paired DBS samples (Figure 18). More than 85% sensitivity & specificity observed with good kappa agreement (Table 17). The highest sensitivity and specificity obtained up to 48 hours of transport duration while DBS stored at 4^0C, but if the DBS sample stored at -20^0C before 48 hours, it does not improve any sensitivity and specificity. The robustness & stability of the IgG immunoglobulin on filter paper can be utilized to test the infectious pathogens from DBS in field epidemiological research.

We have evaluated the percentage difference between paired DBS samples & gold-standard (plasma & DBS*). At standard condition, only 5% difference observed in mean values between DBS* & plasma. While comparing this difference at compromised condition i.e at 4^0C storage, 17%,19%, and 27% difference observed in DBS1, DBS3 & DBS5 samples with plasma, whereas at -20^0C storage this difference was 4%,13% & 12% for DBS2, DBS4 & DBS6. With the comparison of all DBS samples with plasma, at 4^0C we can interpret that the percentage difference increases on increase in transport duration, but less difference observed at -20^0C storage as compared to at 4^0C. But when this difference calculated between all the DBS samples & DBS* stored at the standard condition, we have observed that at 4^0C & -20^0C the percentage difference is decreased as compared to plasma samples. (Table 21).

The laboratory experiments performed on testing of IgG immunoglobulins against the infectious pathogen (*H. pylori*) from DBS after 4-5 months of storage at 4^0C & -20^0C and we get an excellent result from all stored DBS samples. Prepared scattered plots for the assessment of correlation coefficients between all DBS & gold-standard (plasma & DBS*) samples. An excellent correlation coefficient (r=0.9980) observed between plasma & DBS* at standard conditions. Similarly, on average, a good correlation (>85%) also observed between all DBS (DBS1, DBS2, DBS3, DBS4, DBS5, DBS6) samples and plasma,

while >88% correlation observed between all DBS samples & DBS* stored at standard condition.

There is very little difference exist between plasma and DBS samples stored at a different temperature at varying time points, but the result shows good correlation coefficients, strong sensitivity & specificity with the decrease in percentage difference between DBS & gold-standard. Finally, it is concluded that we can store DBS samples at -20^0C or 4^0C (If -20^0C is not available) for testing of infectious pathogens even within 72 hours of transport duration of DBS, because in the dry condition the integrity of IgG antibody concentration remains stable on DBS.

ANOVA test result shows that there is very little variation observed between and within-group of DBS samples with a low F value at -20^0C than at 4^0C (Table 20). In conclusion, DBS can be utilized as an alternative biological sample source in field epidemiological studies to grant future research on infectious disease-causing agents.

3.3 Evaluation of apolipoprotein-AI (apoA-I) from Dried Blood Spot

3.3A Standardization and validation of estimation of apolipoprotein-A (apoA) from DBS stored at standard condition.

3.3A.1 Introduction

Human apolipoprotein AI (Apo-AI) comprise about 70% of the high-density lipoproteins (HDL) protein mass. Apo-AI a 243 amino acid molecule that contains a series of highly homologous amphipathic alpha-helices, is a 28-kDa single polypeptide that lacks glycosylation or disulfide linkage (Silva et al., 2007). In human plasma, about 5-10% of Apo-AI is in a lipoprotein unassociated condition. Apo-AI plasma concentration is one of the greatest predictors of cardiovascular disease vulnerability (Noma, 2019).

The major protein associated with high-density lipoprotein (HDL) particles, apolipoprotein A1 (ApoA1), is involved in reverse cholesterol transport (Sorci-Thomas & Thomas, 2013). The concentrations of HDL cholesterol (HDL-C) and ApoA1 are inversely associated to the risk of coronary heart disease (CAD) (Walton, 2009). There are a variable number of ApoA1 proteins per HDL particle. As a result, ApoA1 is not a perfect surrogate marker for HDL particles. Similarly, there are highly variable numbers of ApoA1 proteins and the cholesterol in particles of HDL. This heterogeneity resulted in unique clinical findings for ApoA1 in comparison to HDL-C. More highly than HDL - C, increased ApoA1 concentrations have been associated with lower risk of a first myocardial infarction (McQueen et al., 2008). Low ApoA1 levels, but not HDL-C levels, are associated with preclinical atherosclerosis as measured by computed tomography estimated coronary artery calcium (CAC) scoring. (Sung, Wild, & Byrne, 2013)

3.3A.2 Materials & methods

Commercial available Human Apo-A ELISA kit (Assay pro, Catalog Number EA5301-1, Lot Number: 02821825) is used.

3.3A.3 Principle of the Assay

The Assaymax human apolipoprotein AI ELISA (Enzyme Linked Immunosorbent Assay) kit is designed for detection of Apo-AI in human plasma, serum, saliva, urine, cell culture, cell lysate samples. This is a quantitative sandwich enzyme immunoassay technique that measures human Apo-AI in less than 4 hours.

3.3A.4 Reagent preparation

Prepared all the reagent, standard solution, and samples as per kit protocol. The assay performed at room temperature (20-25^{0}C)

3.3A.5 Dilution of samples

All the testing DBS and plasma samples diluted as per suggested in kit protocol. We have diluted samples in a ratio 1:100000

3.3A.6 Elution of plasma from DBS

We have excise 6mm single DBS with the help of punch plier and place it into 24 well not treated cell culture plate (Nunc). Add diluent (provided in the kit) into each well and put the plate on the shaker for 2 hours at room temperature**.**

3.3A.7 Assay Protocol: Assay is performed at 20 - 25^{0}C (ELISA apolipoprotein AI)

1. Initially prepared all the reagents, standards and samples as instructed in protocol.
2. Add 50µl sample or standard into each well, cover the plate with adhesive sealing tape and incubate plate for 2 hours.
3. After incubation, wash the plate with 300µl wash buffer 6 times with the help of multichannel ELISA plate washer (TECAN), invert the plate and decant the content, hit 4-5 times on absorbent material for complete removal of liquid.
4. Add 50µl biotinylated human apolipoprotein-A1 antibody to each well, cover the plate with sealing tape and incubate the plate for 1 hour.
5. Wash the plate with same procedure.

6. Add 50µl SP conjugate to each well and cover well with sealing tape, incubate plate for 30 minutes.
7. Wash the plate with same procedure.
8. Add 50µl Chromogen substrate material to each well for 12 minutes or until blue color appears.
9. Add 50µl stop solution into each well, color will change from blue to yellow.
10. Read absorbance on ELISA plate reader (TECAN) at wavelength 450nm immediately.

3.3A.8 Results: T-test, Scattered plot, Bland-Altman plot

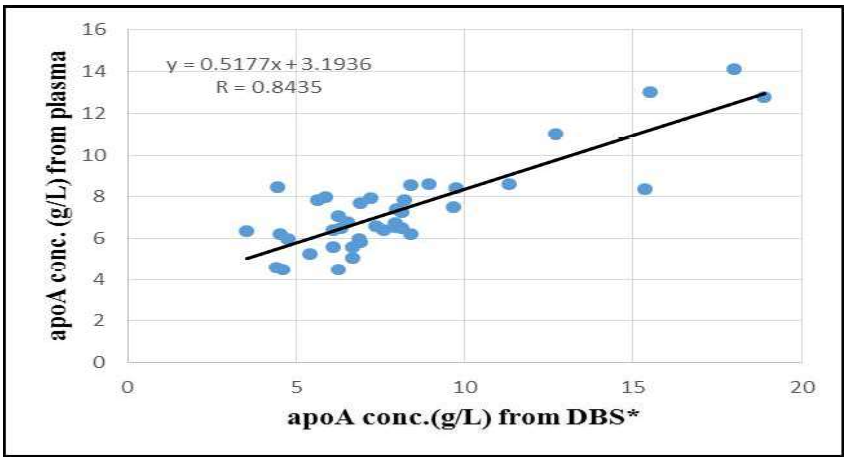
Comparison of apolipoprotein-AI concentration between plasma and DBS* stored at standard condition.

Table 22[29]. T-test result, the mean comparison of apolipoprotein-A (apoA) concentration between plasma and DBS* stored at standard condition.

Samples stored at standard condition	N	Mean	Standard Deviation	95% CI	p - value
Plasma	40	8.061	3.550	6.926 - 9.197	p> 0.0405
DBS	40	7.366	2.179	6.669 - 8.063	

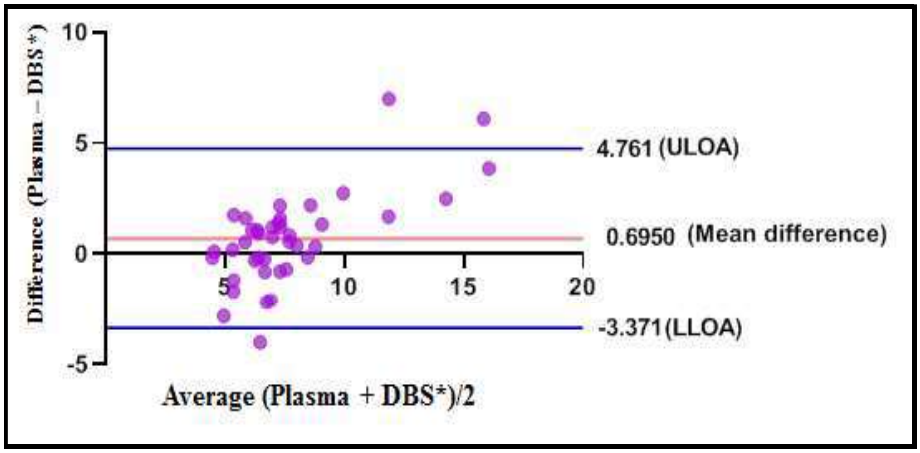
[29] Paired t-test (STATA) used to compare mean difference of apoA conc. between plasma and DBS* stored at standard condition

Figure 22[30]. Prepared scattered plot to compare correlation coefficient of apoA concentration between plasma and DBS* stored at standard condition.



Scattered plot between plasma & DBS* stored at standard condition, with fitted regression equation shows correlation coefficient (r = 0.8435, p>0.001)

Figure 23. Prepared Bland-Altman graph, a plot difference of apoA concentration between plasma and DBS* stored at standard condition.



T-test has been conducted to compare the mean of apolipoprotein (apoA) between plasma and DBS* stored at standard conditions. At standard condition, statistically significant (p>0.0405) difference observed between DBS* & plasma with good correlation coefficient (r = 0.8435).

[30] Scattered plot (Excel) created to compare correlation coefficient of apoA conc.between plasma & DBS* stored at standard condition

3.3B Evaluation & comparison of apolipoprotein-A (g/L) concentration between plasma and all DBS samples stored at 4^0C & -20^0C.

3.3B.1 Results: T-test, ANOVA, Scattered plot, Bland-Altman plot

Table 23[31]. T-test result, the mean comparison of apolipoprotein-A (apoA) concentration between plasma and DBS samples stored at 4^0C (DBS1, DBS3, DBS5)

Samples storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
Plasma (standard condition)	Immediate	40	8.061827	3.550	6.926 - 9.197	p > 0.388
DBS1 (4 Degree Celsius)	Between 0-24 hours	40	7.626474	2.115	6.949 - 8.303	
Plasma (standard condition)	Immediate	40	8.061827	3.550	6.926 - 9.197	p > 0.001
DBS3 (4 Degree Celsius)	Between 24-48 hours	40	3.344176	0.799	3.088 - 3.600	
Plasma (standard condition)	Immediate	40	8.061827	3.550	6.926 - 9.197	p > 0.001
DBS5 (4 Degree Celsius)	Between 48-72 hours	40	1.84873	0.466	1.699 - 1.998	

Table 24[32]. T-test result, the mean comparison of apolipoprotein-A (apoA) concentration between plasma and DBS samples stored at -20^0C (DBS2, DBS4, DBS6).

Blood samples storage condition	Transport duration	N	Mean	SD	95% CI	p - value
Plasma (standard condition)	Immediate	40	8.061	3.550	6.926 - 9.197	p > 0.001
DBS2 (-20 Degree Celsius)	Between 0-24 hours	40	3.232	0.855	2.958 - 3.506	
Plasma (standard condition)	Immediate	40	8.061	3.550	6.926 - 9.197	p > 0.001
DBS4 (-20 Degree Celsius)	Between 24-48 hours	40	2.640	0.703	2.415 - 2.865	
Plasma (standard condition)	Immediate	40	8.061	3.550	6.926 - 9.197	p > 0.001
DBS6 (-20 Degree Celsius)	Between 48-72 hours	40	3.230	0.775	2.982 - 3.478	

[31] Paired t-test (STATA) used to compare mean difference of apoA conc. between plasma and DBS stored at 4^0C

[32] Paired t-test (STATA) used to compare mean difference of apoA conc. between plasma and DBS stored at -20^0C

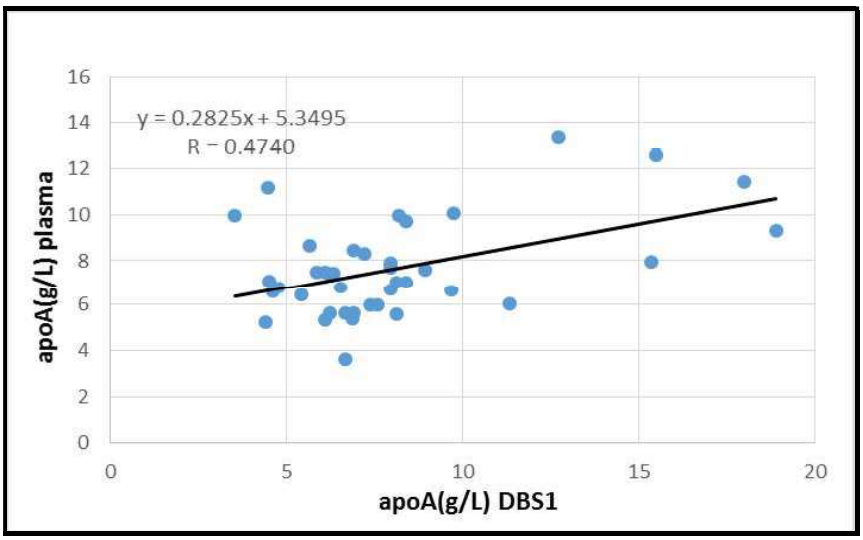
Table 25[33]. ANOVA-test result, multiple group mean comparison of apolipoprotein-A concentration between plasma and DBS samples stored at 4^0C & -20^0C irrespective of transport duration.

Plasma sample as Gold standard storage condition	**DBS samples stored condition irrespective of transport duration**	**F-value**	**p value**
Standard condition (n = 40)	4 degree Celsius (n = 120)	85.46	p>0.001
Standard condition (n = 40)	-20 degree Celsius (n = 120)	70.89	p>0.001

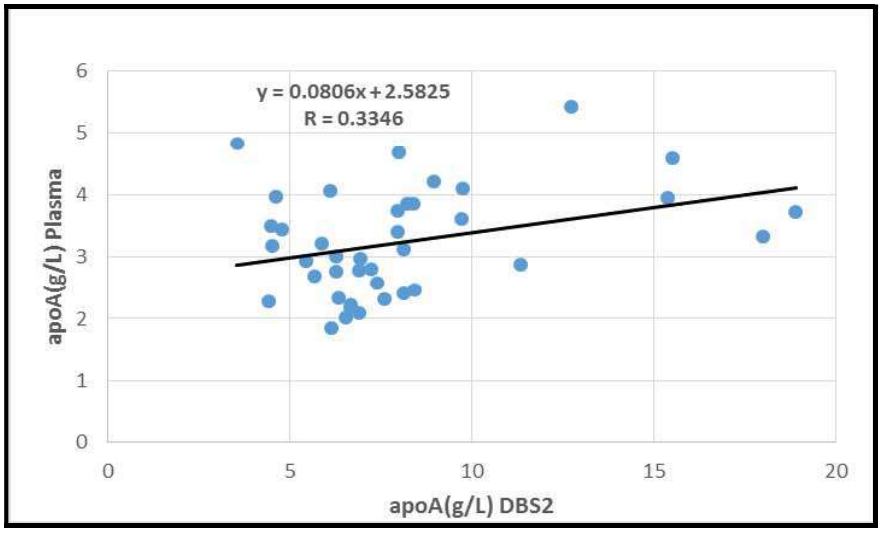
T-test has been conducted between all DBS and plasma samples. At 4^0C storage, statistically non-significant p value (p > 0.388) observed for DBS1, but for DBS3 (p > 0.001) & DBS5 significant p value (p > 0.001) observed. Whereas at -20^0C storage, there is statistically significant p-value observed for DBS2 (p > 0.001), DBS4 (p > 0.001), and DBS6 (p > 0.001). ANOVA test has been conducted to compare multiple group of all DBS samples stored at 4^0C (DBS1, DBS3 & DBS5) & -20^0C (DBS2, DBS4 and DBS6) with plasma irrespective of transport duration. Statistically significant (p>0.001) difference observed at both storage temperature with obtained F values (F = 85.46) at 4^0C and (F= 70.89) at -20^0C.

[33] Oneway ANOVA test (STATA) was conducted to compare mean concentration of apoA between plasma & DBS stored at 4^0C & -20^0C.

Figure 24[34]. Prepared scattered plot to compare correlation coefficients of apolipoprotein-AI concentration between plasma and all DBS samples stored at 4^0C & -20^0C.

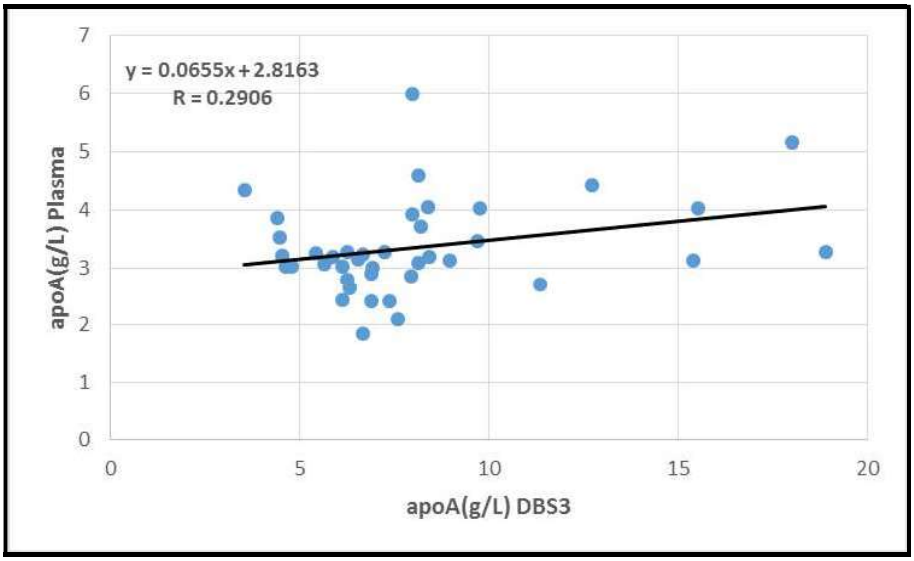


(Figure 24A) Scattered plot with correlation coefficient (r = 0.4740) between plasma & DBS1

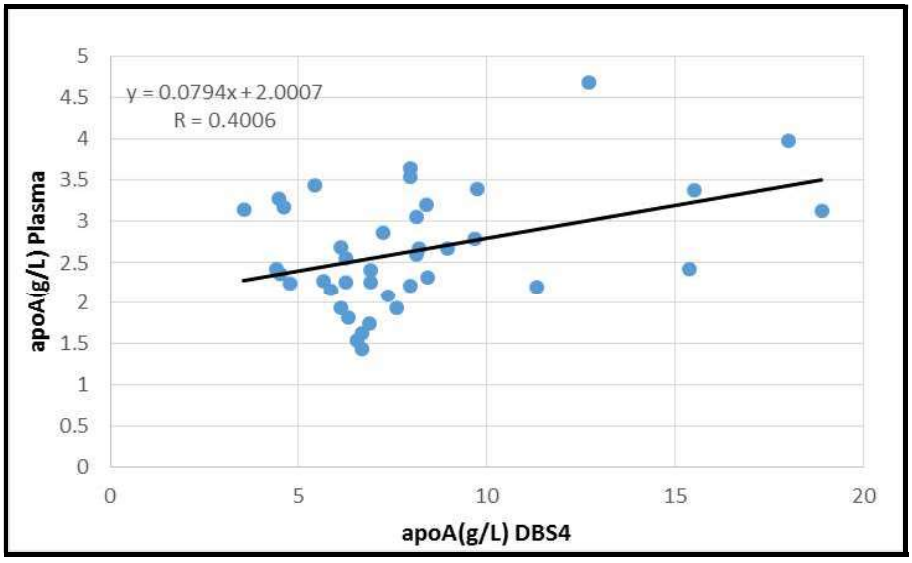


(Figure 24B) Scattered plot with correlation coefficient (r = 0.3346) between plasma & DBS2

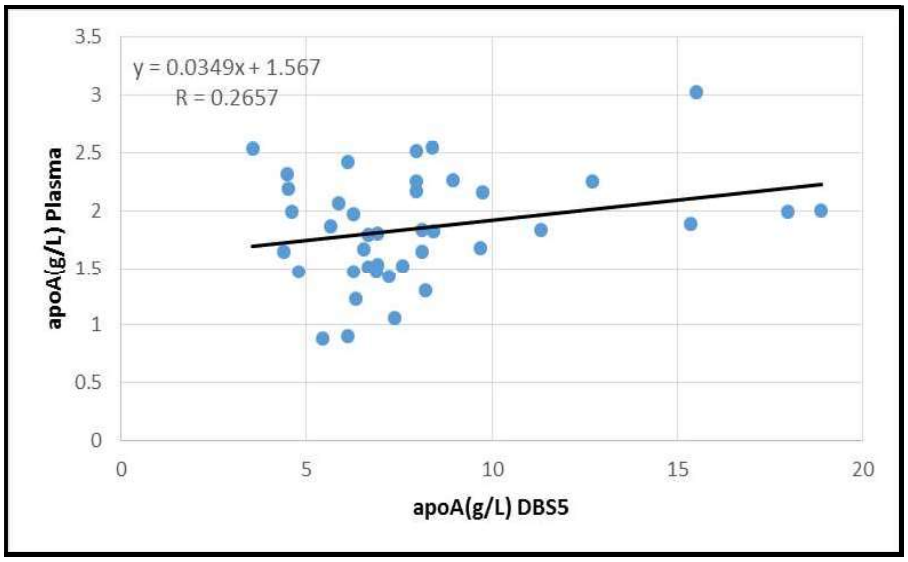
[34] Scattered plot (Excel) were used to compare correlation coefficient of apoA concentration between plasma & DBS1, DBS2, DBS3, DBS4, DBS5 & DBS6 separately.



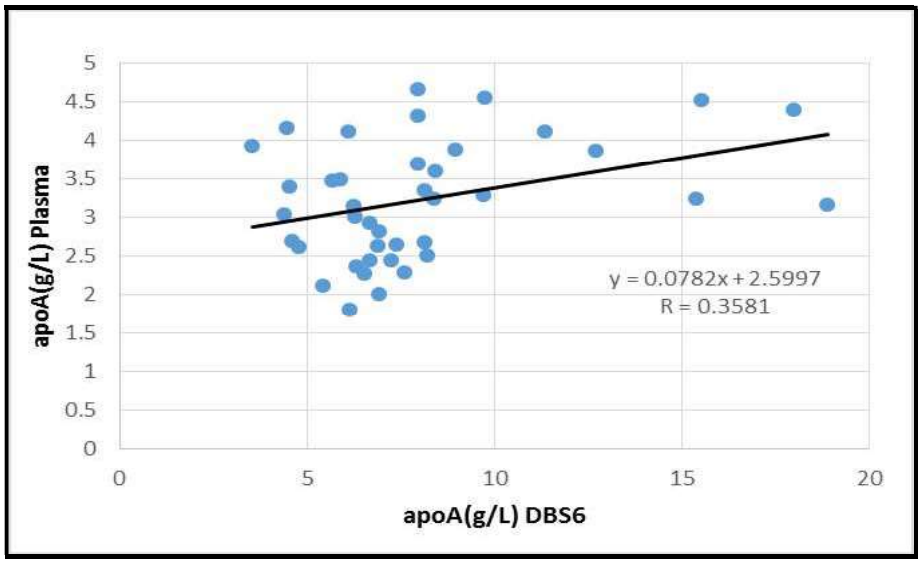
(Figure 24C) Scattered plot with correlation (r = 0.2906) between plasma & DBS3



(**Figure 24D)** Scattered plot with correlation (r = 0.4006) between plasma & DBS4

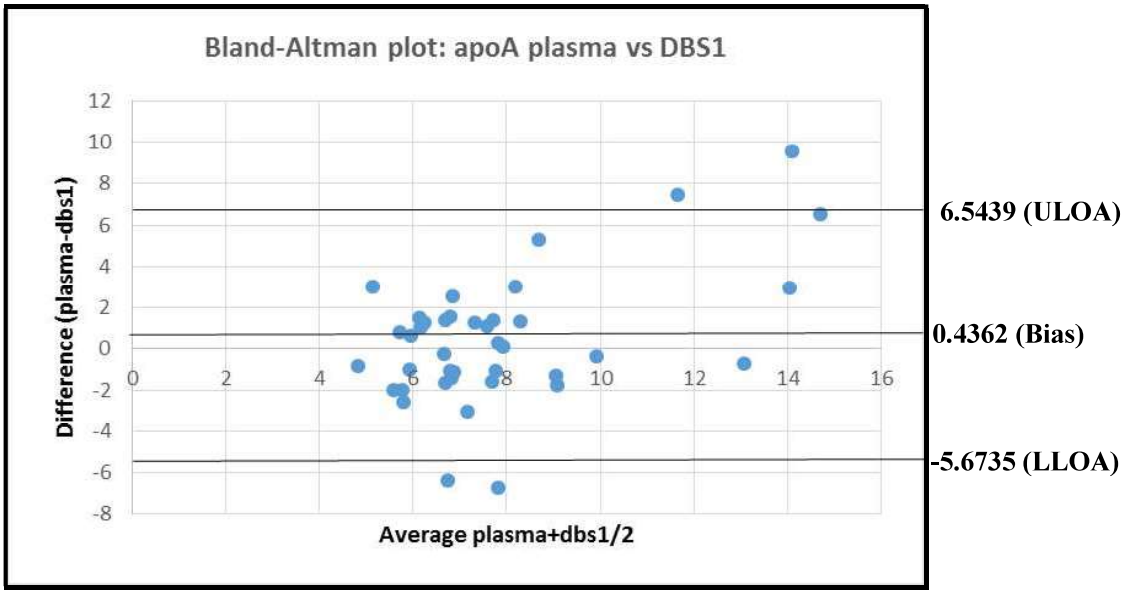


(**Figure 24E)** Scattered plot with correlation (r = 0.2657) between plasma & DBS5

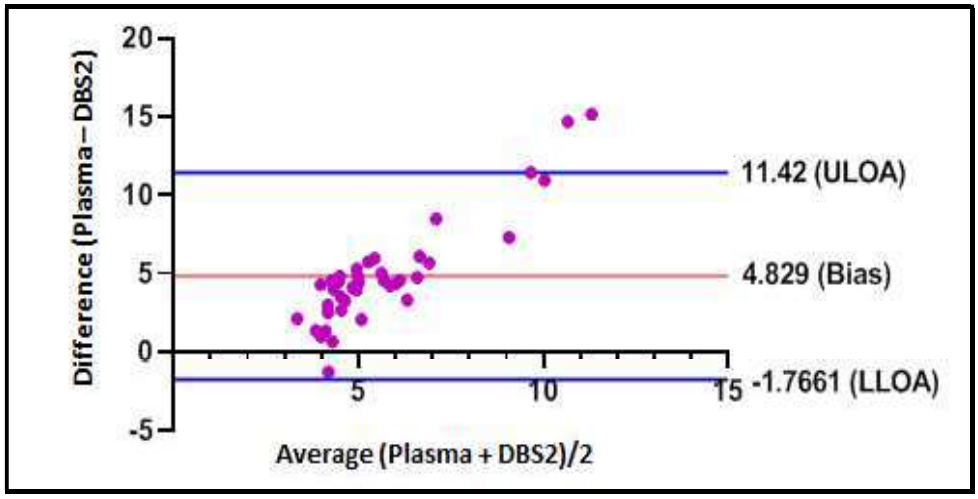


(Figure 24F) Scattered plot with correlation (r = 0.3581) between plasma & DBS6

Figure 25[35]**.** Prepared Bland-Altman graph, a plot of difference of apolipoprotein-AI concentration between plasma and DBS stored at 4^0C & -20^0C.

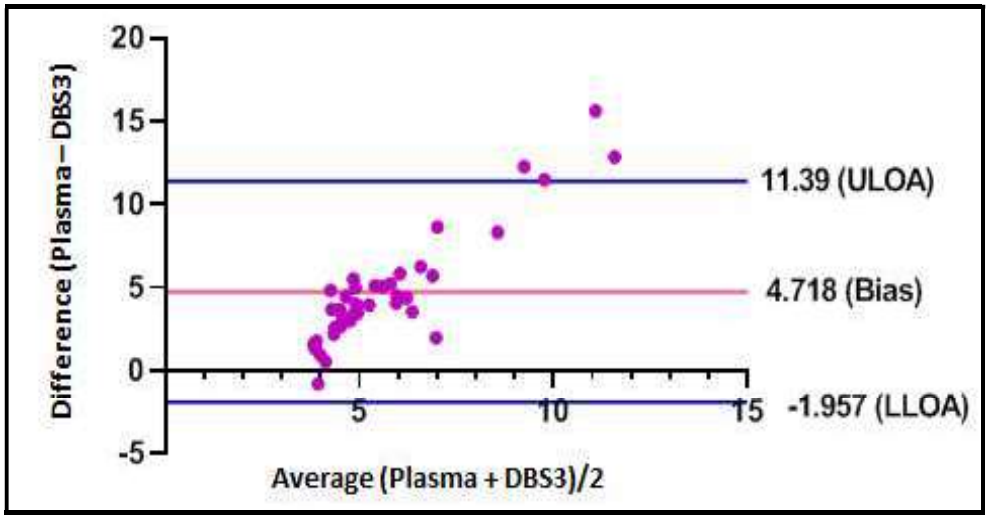


(Figure 25A) BA plot of apoA concentration between plasma and DBS1, with ULOA (6.5439), LLOA (-5.6735) and Bias (0.4362)

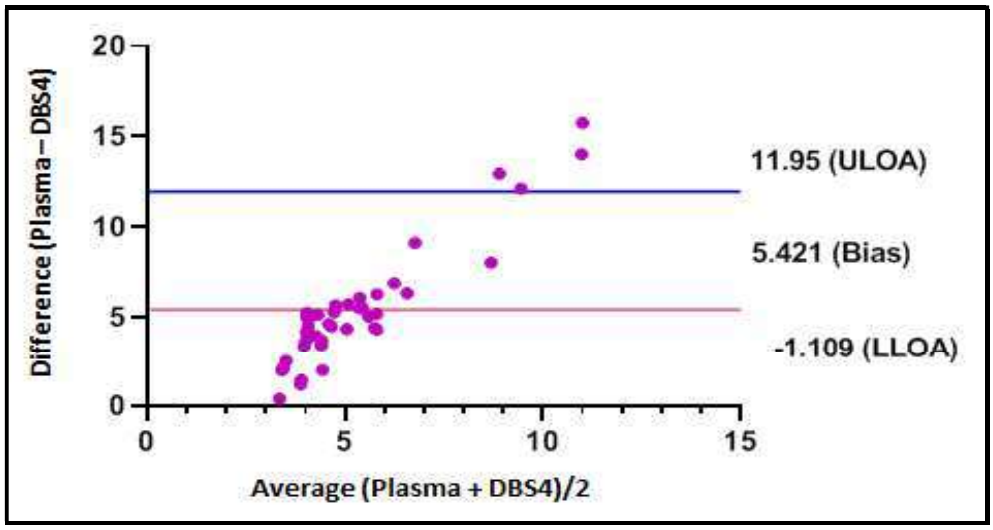


(**Figure 25B)** BA plot of apoA concentration between plasma and DBS2

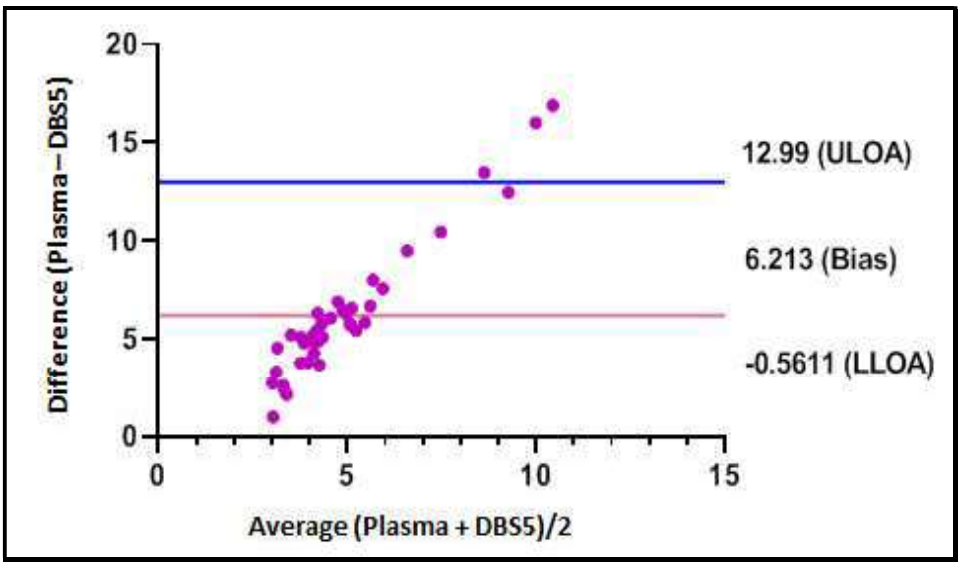
[35] Bland-Altman plot (GraphPad) of apoA lipoprotein created to observe the bias (mean difference) between plasma & DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately



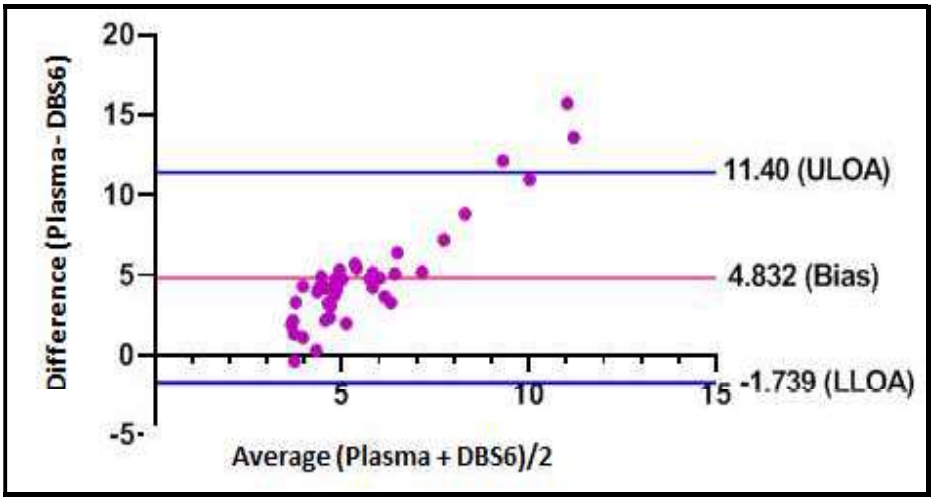
(**Figure 25C)** BA plot of apoA concentration between plasma and DBS3



(**Figure 25D)** BA plot of apoA concentration between plasma and DBS4



(Figure 25E) BA plot of apoA concentration between plasma and DBS5



(Figure 25F) BA plot of apoA concentration between plasma and DBS6

3.3C Evaluation & comparison of apolipoprotein-A (g/L) concentration between DBS* stored at standard condition and all DBS stored at 4^0C & -20^0C.

3.3C.1 Result: T-test, ANOVA, Bland-Altman plot, Scattered plot.

Table 26[36]. T-test result, the mean comparison of apolipoprotein-A (apoA) concentration between DBS* and DBS samples stored at 4^0C (DBS1, DBS3, DBS5)

Sample storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
DBS* (standard condition)	Immediate	40	7.366	2.179	6.669 - 8.063	p >0.2556
DBS1 (4 Degree Celsius)	Between 0-24 hours	40	7.626	2.115	6.949 - 8.303	
DBS (standard condition)	Immediate	40	7.366	2.179	6.669 - 8.063	p >0.001
DBS3 (4 Degree Celsius)	Between 24-48 hours	40	3.344	0.799	3.088 - 3.600	
DBS (standard condition)	Immediate	40	7.366	2.179	6.669 - 8.063	p >0.001
DBS5 (4 Degree Celsius)	Between 48-72 hours	40	1.84873	0.4669699	1.6993 - 1.9980	

Table 27[37]. T-test result, the mean comparison of apolipoprotein-A (apoA) concentration between DBS* and DBS samples stored at -20^0C (DBS2, DBS4, DBS6)

Sample storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
DBS (standard condition)	Immediate	40	7.3667	2.1790	6.6699 - 8.0636	p > 0.001
DBS2 (-20 Degree Celsius)	Between 0-24 hours	40	3.2325	0.85548	2.9589 - 3.5061	
DBS4 (-20 Degree Celsius)	Between 24-48 hours	40	2.6406	0.70346	2.4156 - 2.8655	p > 0.001
DBS6 (-20 Degree Celsius)	Between 48-72 hours	40	3.2301	0.77537	2.9821 - 3.4781	p > 0.001

[36] Paired t-test (STATA) used to compare mean difference of apoA lipoprotein between DBS* stored at standard condition & DBS stored at 4^0C.

[37] Paired t-test (STATA) used to compare mean difference of apoA lipoprotein between DBS* stored at standard condition & DBS stored at -20^0C

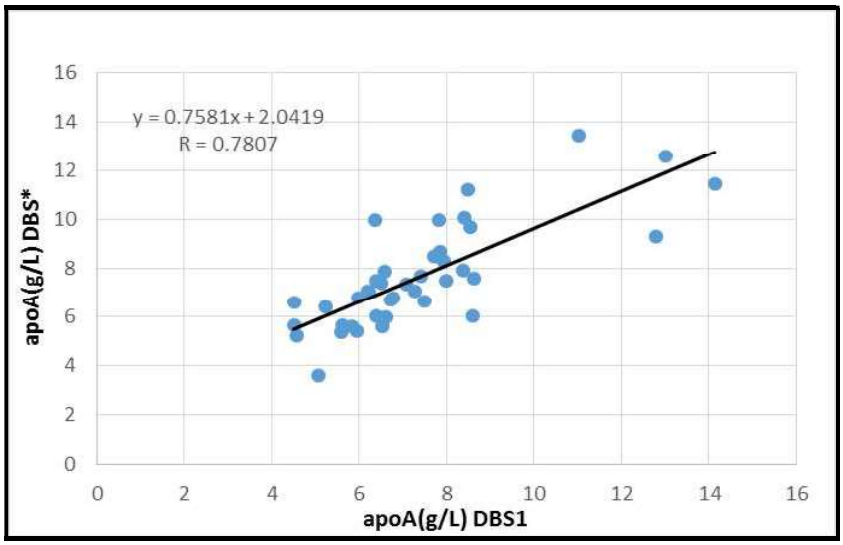
Table 28[38]. ANOVA-test result, multiple group mean comparison of apolipoprotein-A concentration between DBS* and DBS samples stored at 4^0C & -20^0C irrespective of all 3 transport duration.

DBS* sample storage condition	DBS samples stored condition irrespective of transport duration	F-value	p-value
Standard condition (n = 40)	4 degree Celsius (n = 120)	133.10	p>0.001
Standard condition (n = 40)	-20 degree Celsius (n = 120)	116.05	p>0.001

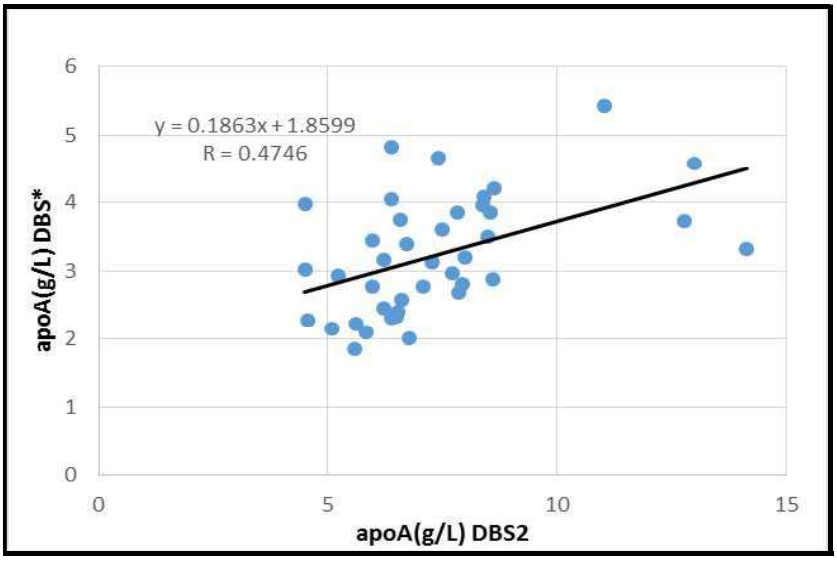
T-test has been conducted to compare group mean of apoA concentrations between all DBS samples and DBS* stored at standard condition. Statistically non-significant difference observed for DBS1 with p-value (p>0.2556), while significant difference observed for rest of the DBS samples such as DBS3 (p>0.001), DBS5 (p>0.001), DBS2 (p>0.001), DBS4 (p>0.001), and DBS6 (p>0.001). Similarly, ANOVA test conducted to compare group mean between all DBS samples stored at 4^0C & -20^0C and DBS* samples stored at standard condition. Significant difference (p>0.001) observed at 4^0C with F-value (F = 133.10) as well as at -20^0C with F-value (F = 116.05).

[38] Oneway ANOVA test (STATA) was conducted to compare mean concentration of apoA between DBS* stored at standard condition & DBS stored at 4^0C & -20^0C

Figure 26[39]. Prepared scattered plot to compare correlation coefficient of apoA concentration between DBS* and all DBS samples stored at 4^0C & -20^0C.

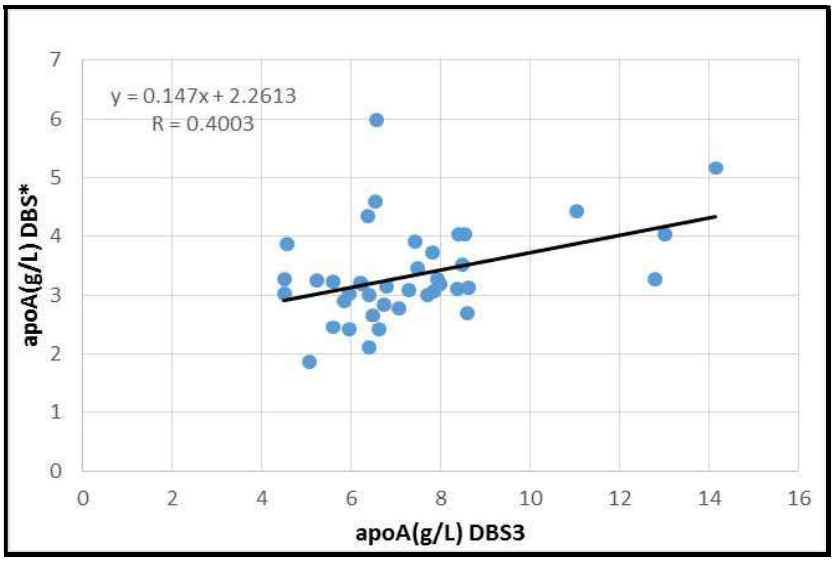


(Figure 26A) Scattered plot with correlation (r = 0.7807) between DBS* & DBS1

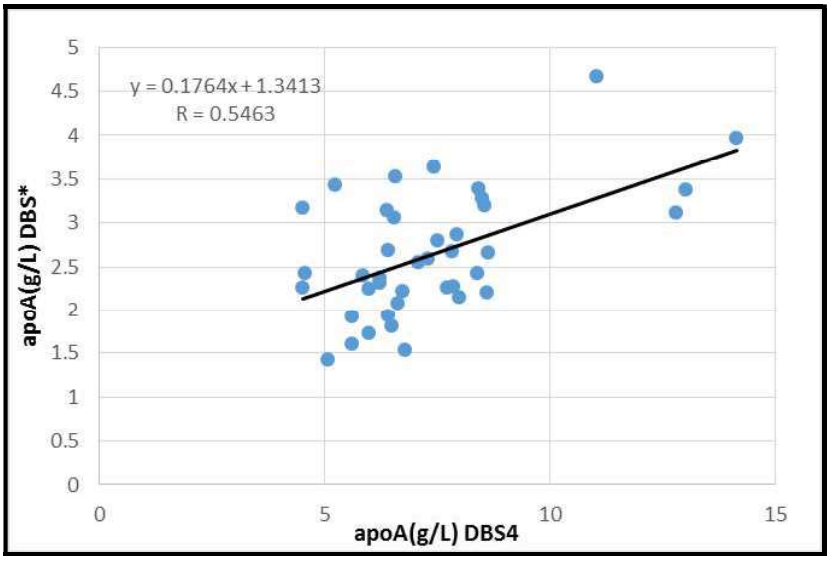


(Figure 26B) Scattered plot with correlation (r = 0.4746) between DBS* & DBS2

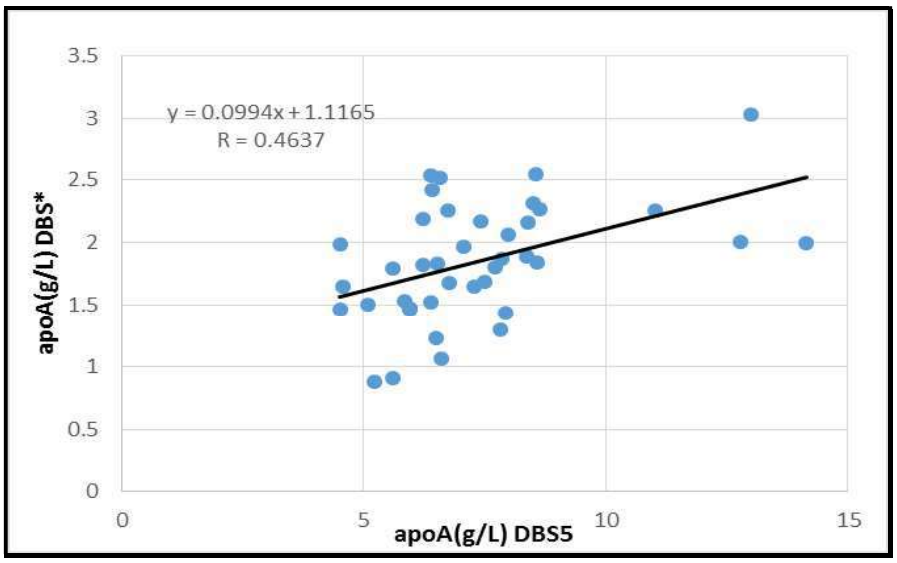
[39] Scattered plot (Excel) created of apoA lipoprotein to compare correlation coefficient between DBS* stored at standard condition & all DBS groups (DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6)



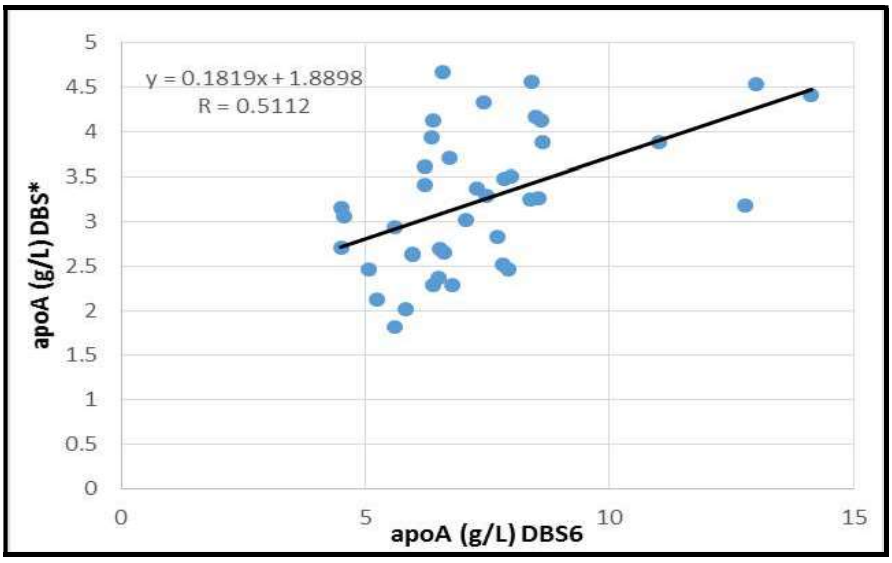
(Figure 26C) Scattered plot with correlation (r = 0.4003) between DBS* & DBS3



(Figure 26D) Scattered plot with correlation (r = 0.5463) between DBS* & DBS4

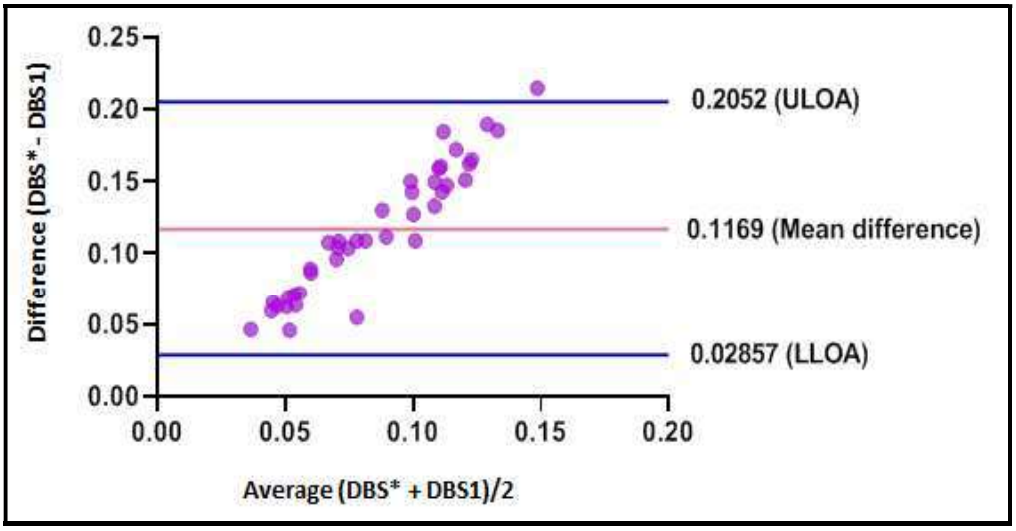


(Figure 26E) Scattered plot with correlation (r = 0.4637) between DBS* & DBS5

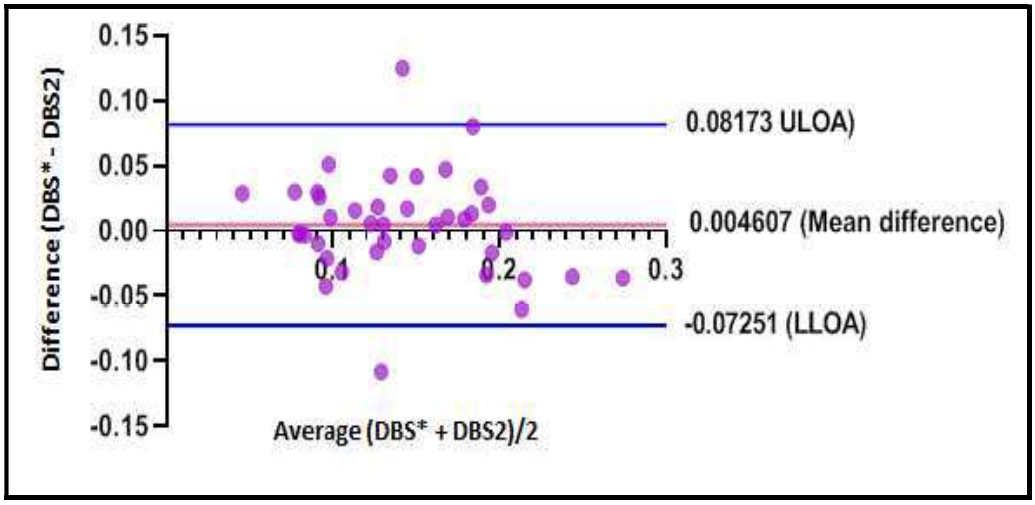


(Figure 26F) Scattered plot with correlation (r = 0.5112) between DBS* & DBS6

Figure 27[40]**.** Prepared Bland-Altman graph, a plot of difference of apolipoprotein-AI concentration between DBS* and all DBS stored at 4^0C & -20^0C.

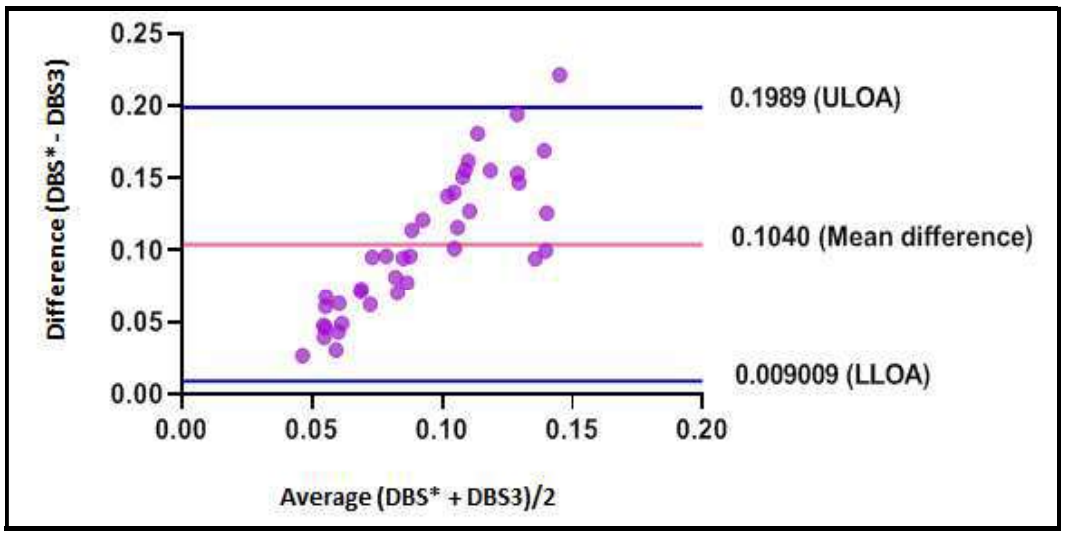


(Figure 27A) BA plot of apoA concentration difference between DBS* and DBS1

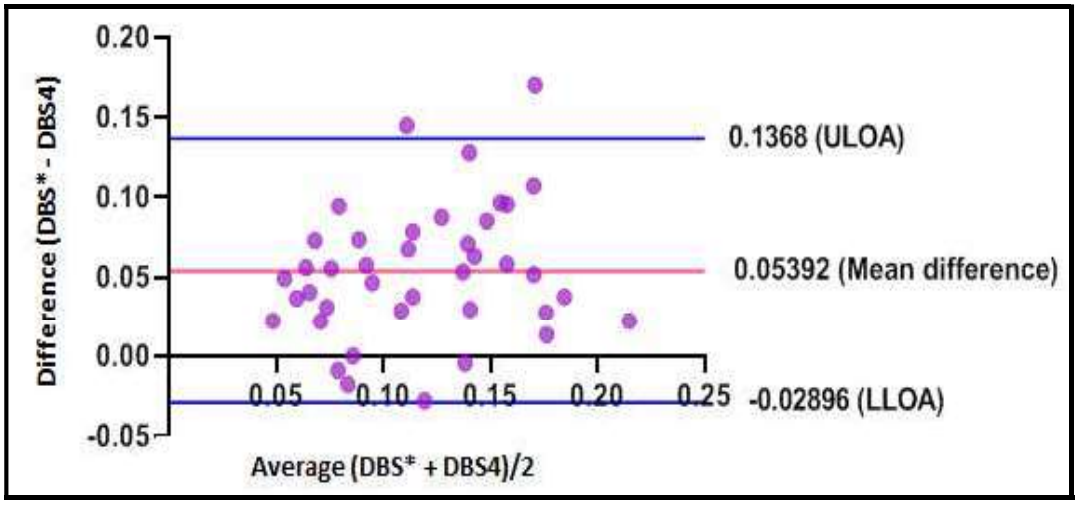


(**Figure 27B)** BA plot of apoA concentration difference between DBS* and DBS2

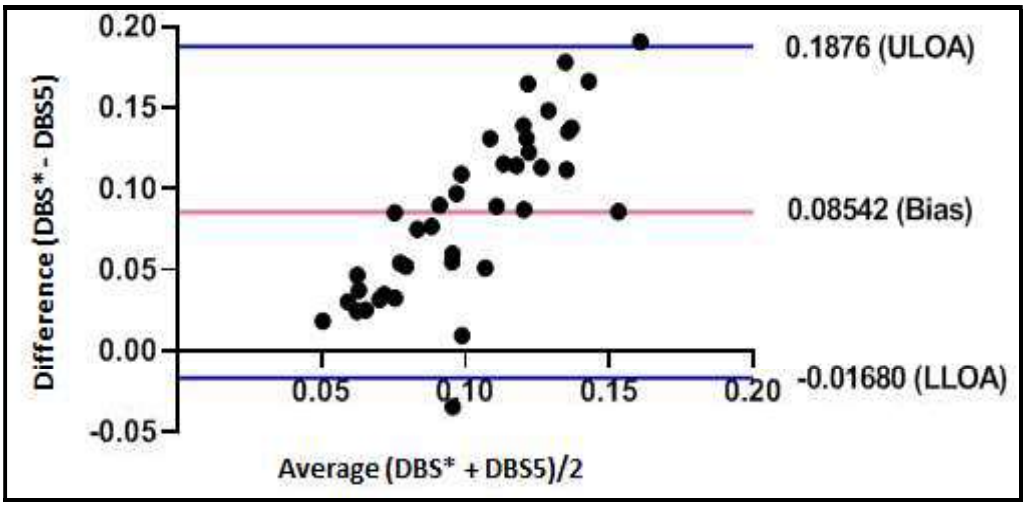
[40] Bland-Altman plot (GraphPad) of apoA lipoprotein created to observe the bias (mean difference) between DBS* stored at standard condition and DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately



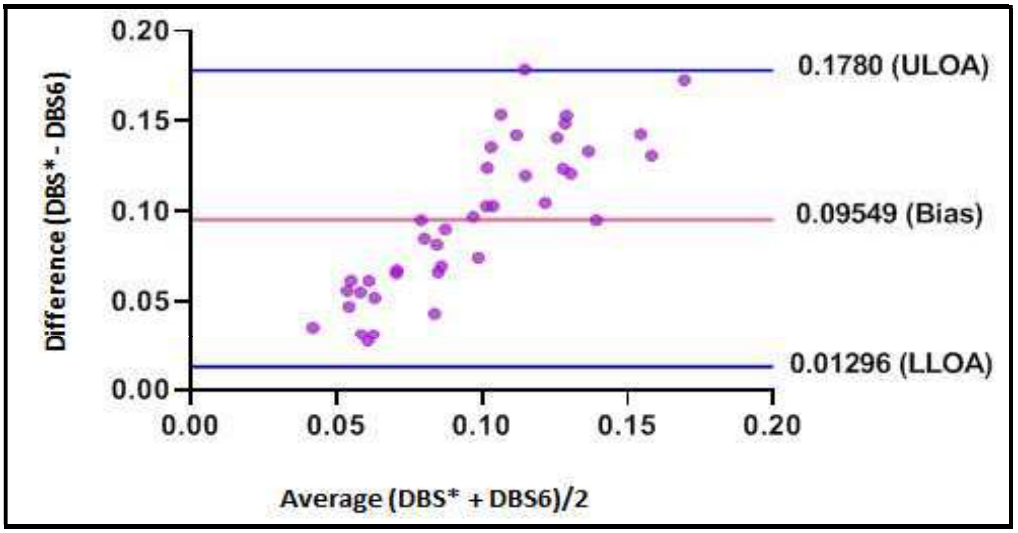
(Figure 27C) BA plot of apoA concentration difference between DBS* and DBS3



(Figure 27D) BA plot of apoA concentration difference between DBS* and DBS4



(Figure 27E) BA plot of apoA concentration difference between DBS* and DBS5



(Figure 27F) BA plot of apoA concentration difference between DBS* and DBS6

Table 29. Compare percentage change in apoAI concentration between plasma and all DBS samples stored at 4^0C & -20^0C.

Samples at standard condition	Percentage change in DBS* as compared to plasma		Percentage change in DBS as compared with samples stored at standard condition					
	-80^0C		**4^0C**				**-20^0C**	
	Plasma/WB	DBS*	DBS1	DBS3	DBS5	DBS2	DBS4	DBS6
Plasma/WB	0%	8%	5%	58%	77%	59%	67%	59%
DBS*	8%	0%	3%	54%	74%	56%	64%	56%

3.3C.2 Discussion: apolipoprotein-AI evaluation from DBS

Apolipoprotein-AI is a constituent part of high-density Lipoprotein (HDL), which is also called as good cholesterol and it is a mediator of lipid metabolism. apoA-I is a 243-amino acid molecule that contains a 28-kDa single polypeptide chain with lacks glycosylation or disulfide linkages. Aside from the N-terminal 44 amino acids, the apoA-I sequence appears to be organized into eight _-helical segments of 22 amino acids and two 11-mer repeats that are frequently separated by proline residues (Brouillette, Anantharamaiah, Engler, & Borhani, 2001). One lipoprotein class, high-density lipoproteins (HDL), has been intensively studied due to its association with various cardioprotective functions (Meurs, Van Eck, & Van Berkel, 2010). HDL proteins, called apolipoproteins, include (apo)A-I, apoA-II, apoA-IV, and much less abundant species (Gordon, Durairaj, Lu, & Davidson, 2010; Vaisar et al., 2007).

The primary aim of this study was to observe the impact of transport duration and storage condition on integrity of apolipoprotein-A biomarker. We have estimated the apoA concentration from paired DBS samples and compared it with gold standard (plasma & DBS*) samples stored at standard condition. A good correlation coefficient (r = 0.8435) observed between DBS* & plasma samples stored at standard condition but positive & satisfactory correlation coefficient observed between DBS1 & plasma (r = 0.4740), DBS2 & plasma (r= 0.3346), DBS3 & plasma (r = 0.2906), DBS4 & plasma (r = 0.4006), DBS5 & plasma (r =

0.2657) and DBS6 & plasma (r = 0.3581). Similarly, positive correlation also observed between DBS1 & DBS* (r= 0.7807), DBS2 & DBS* (r= 0.4746), DBS3 & DBS* (r= 0.4003), DBS4 & DBS* (r= 0.5463), DBS5 & DBS* (R= 0.4637) and DBS6 & DBS* (r= 0.5112). In some previous studies, variability have been seen in correlation coefficients such as, a study conducted by Sheerwod et.al where they have found good correlation (r=0.70) between DBS & plasma whereas (r=0.85) correation founded by Hirst & Beswick. We have concluded that if we transport DBS before 24 hours and stored it at 4^0C then we can get good recovery of analyte from DBS rather than stored at -20^0C. But storage at 4^0C for short time period (2-3 months) is permissible but for longer time period it should be stored at either -20^0C or -80^0C.

We have also compared all the stored DBS with DBS* stored at standard conditions. A good correlation coefficient (r = 0.7807) observed between DBS1 & DBS* and a satisfactory correlation for the rest of the DBS samples. This correlation shows, DBS should be transported before 24 hours of transport duration and store it at 4^0C for a short period or at -80^0C for a longer period. For apolipoproteins, transport duration beyond 24 hours is not feasible and recommended because with time their concentration decreases. In field epidemiological studies, after the preparation of DBS, it should be immediate transport to the laboratory to maintain the integrity and stability of apoA in dry conditions.

We have also calculated the percentage change (Table 29) of apoA concentration between DBS & plasma, here we have found only 8% mean difference between DBS* & plasma stored at standard condition, whereas 5% mean difference found between plasma & DBS1, and 3% mean difference found between DBS* & DBS1, for rest of the DBS this difference is >50%. With these differences, we have finally concluded that DBS should be stored at standard conditions before 24 hours of transport duration.

Further study warrants to investigate why the stability of apolipoproteins decreases with time and storage conditions, is there is any effect of detergents used for the elution process of DBS or there is any other physical or chemical effect on the linkage of apolipoproteins structures.

3.4 Evaluation of apolipoprotein-B (apoB) from Dried Blood Spots

3.4A Standardization and validation of estimation of apolipoprotein-B (apoB) from DBS stored at standard condition.

3.4A.1 Introduction

The principal protein component of low-density lipoprotein (LDL) is apolipoprotein B (ApoB) (LDL). Although the amount of cholesterol in LDL varies, each LDL carries exactly one ApoB protein. Therefore, ApoB is a superior indicator of circulating LDL compared to LDL cholesterol (LDL-C). ApoB has been shown to be equally effective in the presence of LDL particles as evaluated by nuclear magnetic resonance spectroscopy (Cole et al., 2013). ApoB is highly linked to an elevated risk of cardiovascular disease (CVD) and often exceeds LDL-C in terms of predicting coronary heart disease risk (Sierra-Johnson et al., 2009; Thompson & Danesh, 2006). Patient with acceptable non-HDL-C (or LDL-C) but raised ApoB are more likely to experience CVD; while patients with acceptable lower ApoB, but significant elevations non-HDL-C or LDL-C had a lesser chance of developing CVD (Benz, 2014; Pencina et al., 2015) Finally, in 7 different placebo-controlled randomized clinical trials, on-statin reduction of ApoB was more closely related to CVD risk reduction than non-HDL-C or LDL-C (Thanassoulis et al., 2014)

3.4A.2 Materials & methods

Commercial available Human Apo-B ELISA kit (Assay pro, Catalog Number EA7001-1, Lot Number: 01981806) is used.

3.4A.3 Principle of the Assay

The Assaymax Human Apolipoprotein B ELISA (Enzyme Linked Immunosorbent Assay) kit is design for detection of Apo-B in human serum, cell culture and cell lysate samples. This is a quantitative sandwich enzyme immunoassay technique which measures human Apo-B in less than 4 hours.

3.4A.4 Reagent preparation

Prepared all the reagent, standard solution, and samples as per kit protocol. The assay performed at room temperature (20-25^{0}C)

3.4A.5 Dilution of samples

All the DBS and plasma samples diluted in ratio (1:20000) as per suggested in kit protocol.

3.4A.6 Elution of plasma from DBS

We have excise 6mm single DBS with the help of punch plier and place it into 24 well not treated cell culture plates (Nunc). Add diluent (provided in the kit) into each well and put the plate on the shaker for 2 hours at room temperature.

3.4A.7 Assay procedure

1. Add 50µl of human ApoB standard or samples to each well, break the bubbles if any. Cover the plate with sealing tape and incubate it for 2 hours.
2. Wash plate 6 times with 300µl 1X wash buffer with the help of ELISA plate washer (TECAN) then invert the plate and decant the contents. Hit 4-5 times on absorbent material to completely remove the liquid.
3. Add 50µl of Biotinylated human ApoB antibody to each well, cover wells with a sealing tape and incubate for 1 hour
4. Wash plate with same procedure as describe above
5. Add 50µl of SP Conjugate to each well. Cover well with sealing tape and incubate it for 30 min.
6. Wash plate with same procedure as describe above
7. Add 50µl Chromogen Substrate to each well. Incubate for 10 min or until the optimal blue color density develops.
8. Pour 50µl of stop solution into each well. The hue will shift from blue to yellow. Before taking a reading, gently tap the plate to remove any bubbles.

9. Read the absorbance on microplate reader at wavelength 450nm immediately.

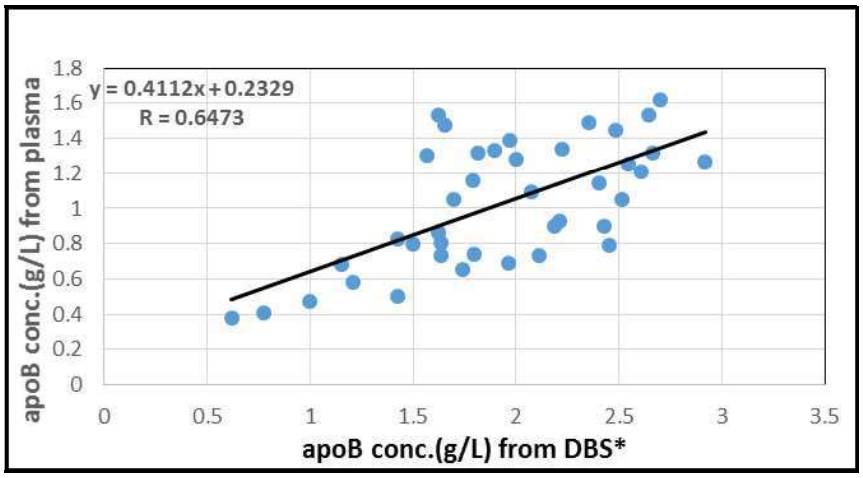
3.4A.8 Results: T-test, Scattered plot, Bland-Altman plot

T-test conducted to compare the mean of apolipoprotein B (apoB) between plasma and DBS* stored at standard condition. There is a statistically significant difference observed with p-value (p>0.001) (Table 30), with a moderate positive correlation coefficient (r = 0.6473) (Figure 28).

Table 30[41]. T-test result, the mean comparison of apolipoprotein-B (apoB) between plasma and DBS* stored at standard condition.

Samples with stored standard condition	Number of Observations	Mean	Standard Deviation	95% CI	p - value
Plasma	40	1.9254	0.5499	1.7495 - 2.1013	p > 0.001
DBS*	40	1.0246	0.3493	0.9129 - 1.1363	

Figure 28[42]. Prepared scattered plot to compare correlation coefficient of apoB concentration between plasma and DBS* stored at standard condition.

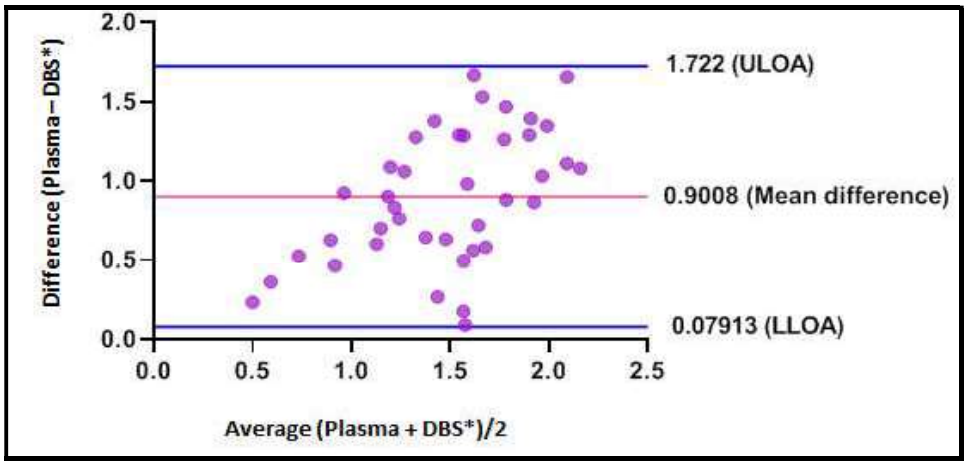


Correlation coefficient (r= 0.6473) of apoB concentration between plasma & DBS* stored at standard condition.

[41] Paired t-test (STATA) used to compare mean difference of apoB conc. between plasma and DBS* stored at standard condition

[42] Scattered plot (Excel) created to compare correlation coefficient of apoB conc. between plasma & DBS* stored at standard condition

Figure 29[43]. Prepared Bland-Altman graph, a plot of difference between apoB concentration between plasma & DBS* stored at standard condition.



3.4B Evaluation and comparison of apolipoprotein-B (g/L) concentration between plasma and DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6)

3.4B.1 Results: T-test, ANOVA, Scattered plot and Bland-Altman plot

Table 31[44]. T-test result, the mean comparison of apolipoprotein-B (apoB) concentration plasma and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5)

Samples storage condition	Transport duration	N	Mean (g/L)	Standard Deviation	95% CI	p - value
Plasma (standard condition)	Immediate	40	1.9254	0.5499	1.7495 - 2.1013	p > 0.001
DBS1 (4 Degree Celsius)	Between 0-24 hours	40	0.2063	0.0845	0.1792 - 0.2333	
Plasma (standard condition)	Immediate	40	1.9254	0.5499	1.7495 - 2.1013	p > 0.001
DBS3 (4 Degree Celsius)	Between 24-48 hours	40	0.1325	0.0510	0.1161 - 0.1488	
Plasma (standard condition)	Immediate	40	1.9254	0.5499	1.7495 - 2.1013	p > 0.001
DBS5 (4 Degree Celsius)	Between 48-72 hours	40	0.1024	0.0157	0.0974 - 0.1075	

[43] Bland-Altman plot (GraphPad) of apoB lipoprotein created to observe the bias (mean difference) between plasma & DBS* stored at standard condition.

[44] Paired t-test (STATA) used to compare mean difference of apoB conc. between plasma and DBS stored at 4^0C

Table 32[45]. T-test result, the mean comparison of apolipoprotein-B (apoB) concentration between plasma and paired DBS samples stored at -20^{0}C (DBS2, DBS4, DBS6).

Blood samples storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
Plasma (standard condition)	Immediate	40	1.9254	0.5499	1.7495 - 2.1013	p > 0.001
DBS2 (-20 Degree Celsius)	Between 0-24 hours	40	0.4276	0.1443	0.3814 - 0.4737	
Plasma (standard condition)	Immediate	40	1.9254	0.5499	1.7495 - 2.1013	p > 0.001
DBS4 (-20 Degree Celsius)	Between 24-48 hours	40	0.2295	0.1082	0.1949 - 0.2642	
Plasma (standard condition)	Immediate	40	1.9254	0.5499	1.7495 - 2.1013	p > 0.001
DBS6 (-20 Degree Celsius)	Between 48-72 hours	40	0.1570	0.06957	0.1348 - 0.1793	

T-test conducted to compare group mean between all DBS samples and gold standard (plasma & DBS*) samples. There is a statistically significant difference observed in means of all DBS samples stored at 4^{0}C & -20^{0}C as compared to plasma samples with p-value (p>0.001).

Table 33[46]. ANOVA-test result, multiple group mean comparison of apolipoprotein-B concentration between plasma and all DBS samples stored at 4^{0}C & -20^{0}C irrespective of all 3 transport duration.

Plasma sample as Gold standard storage condition	DBS samples stored condition irrespective of transport duration	F-value	p value
Standard condition (n = 40)	4 degree Celsius (n = 120)	405.85	p>0.001
Standard condition (n = 40)	-20 degree Celsius (n = 120)	328.17	p>0.001

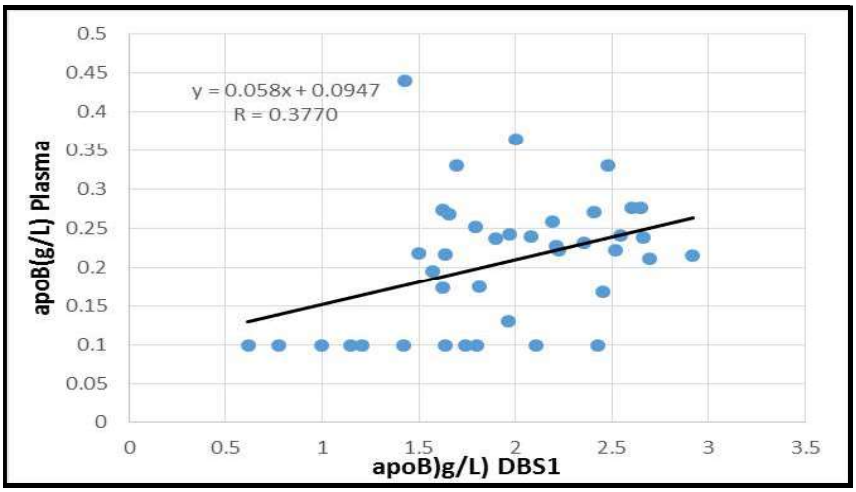
ANOVA test conducted to compare DBS samples stored at 4^{0}C and -20^{0}C with the gold standard (plasma & DBS*) separately irrespective of transport duration. Statistically

[45] Paired t-test (STATA) used to compare mean difference of apoB conc. between plasma and DBS stored at -20^{0}C

[46] Oneway ANOVA test was conducted to compare mean concentration of apoB between plasma & DBS groups stored at 4^{0}C & -20^{0}C

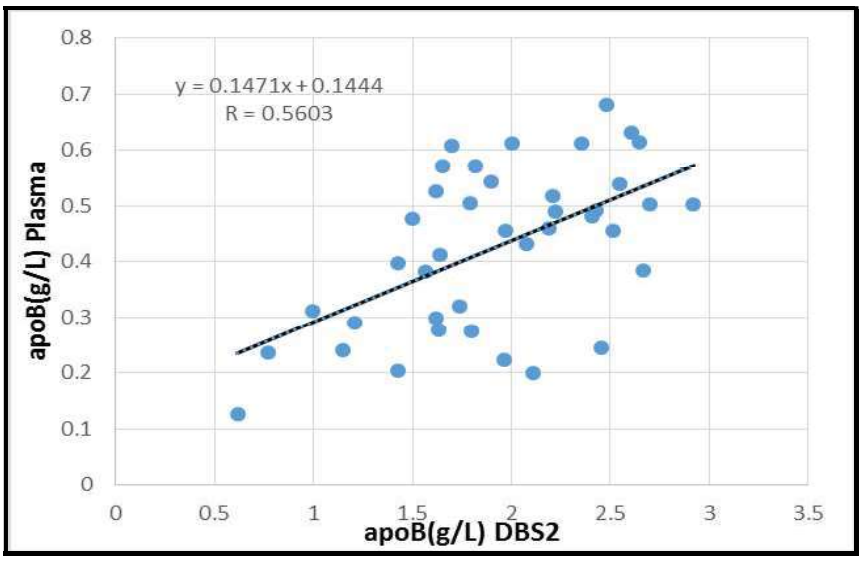
significant difference observed between DBS & plasma samples with p-value ($p>0.001$) and large F-value at 4^0C (F = 405.85) and F-value at -20^0C (F= 328.17). A large F-value indicates that there is a huge variation occurred in means, between and within groups in DBS samples.

Figure 30[47]. Prepared scattered plot to compare correlation coefficient of apolipoproteinB concentration between plasma and all DBS stored at 4^0C & -20^0C.

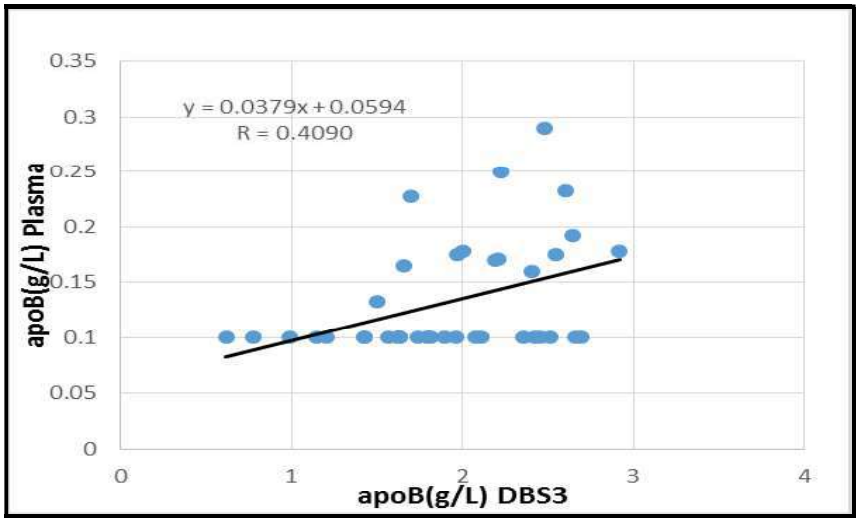


(Figure 30A) Correlation coefficient (r= 0.3770) between plasma & DBS1

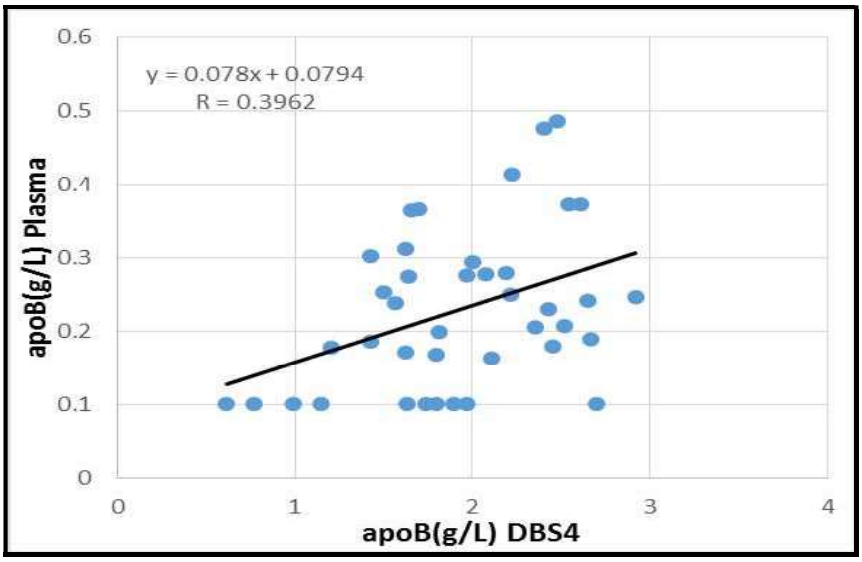
[47] Scattered plot (Excel) were used to compare correlation coefficient of apoB concentration between plasma & DBS1, DBS2, DBS3, DBS4, DBS5 & DBS6 separately



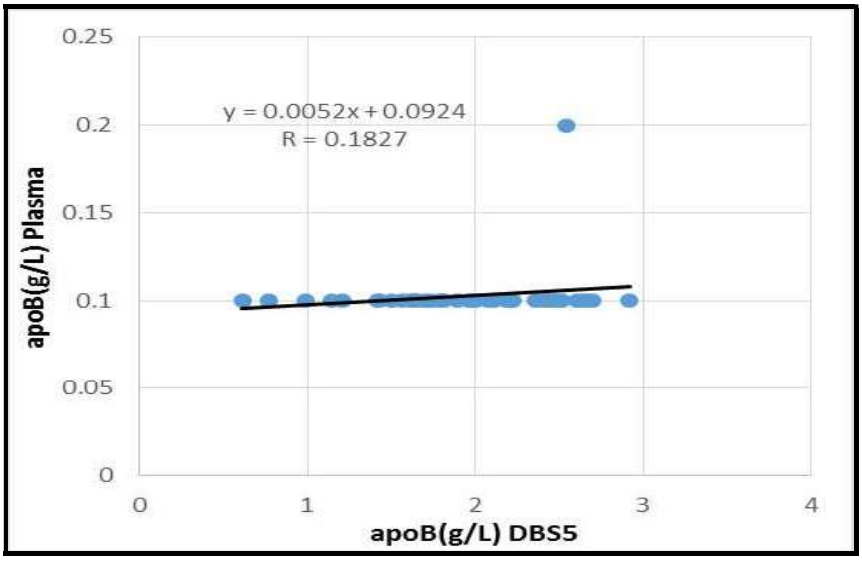
(Figure 30B) Correlation coefficient (r= 0.5603) between plasma & DBS2



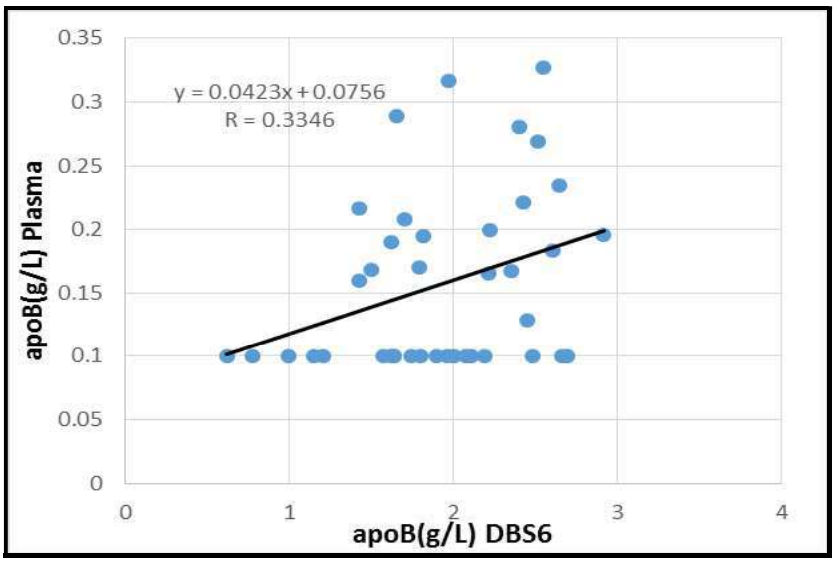
(Figure 30C) Correlation coefficient (r= 0.4090) between plasma & DBS3



(Figure 30D) Correlation coefficient (r= 0.3962) between plasma & DBS4

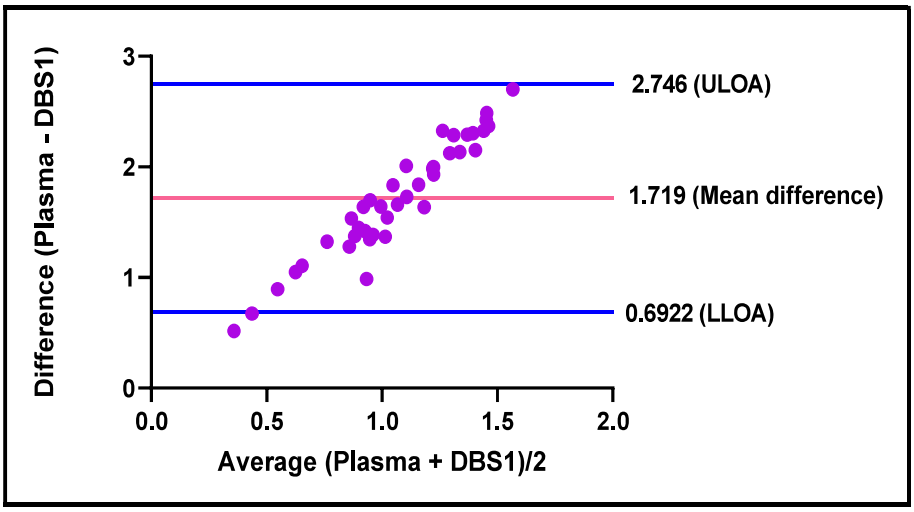


(Figure 30E) Correlation coefficient (r= 0.1827) between Plasma & DBS5



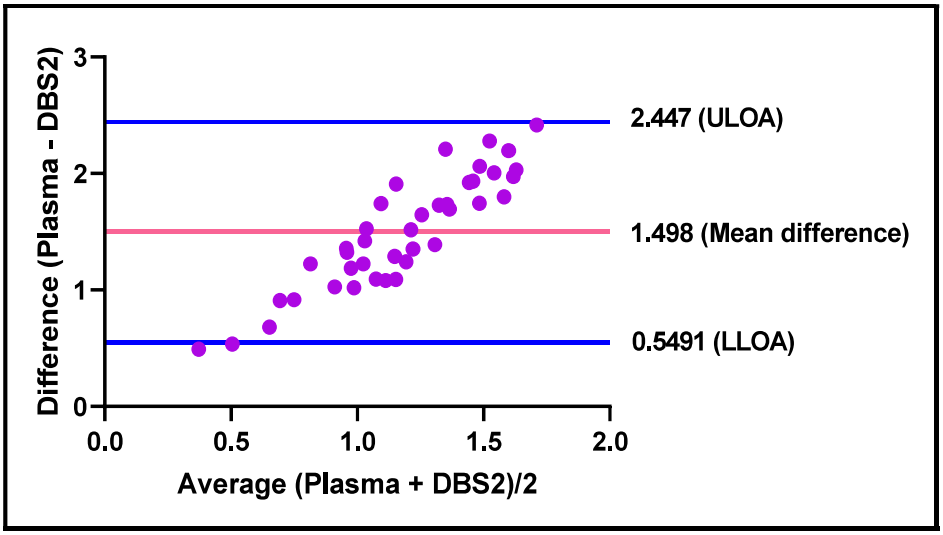
(Figure 30F) Correlation coefficient (r= 0.3346) between plasma & DBS6

Figure 31 [48]**.** Prepared Bland-Altman graph, a plot of difference of apolipoprotein-B concentration between plasma and all DBS samples stored at 4^0C & -20^0C.

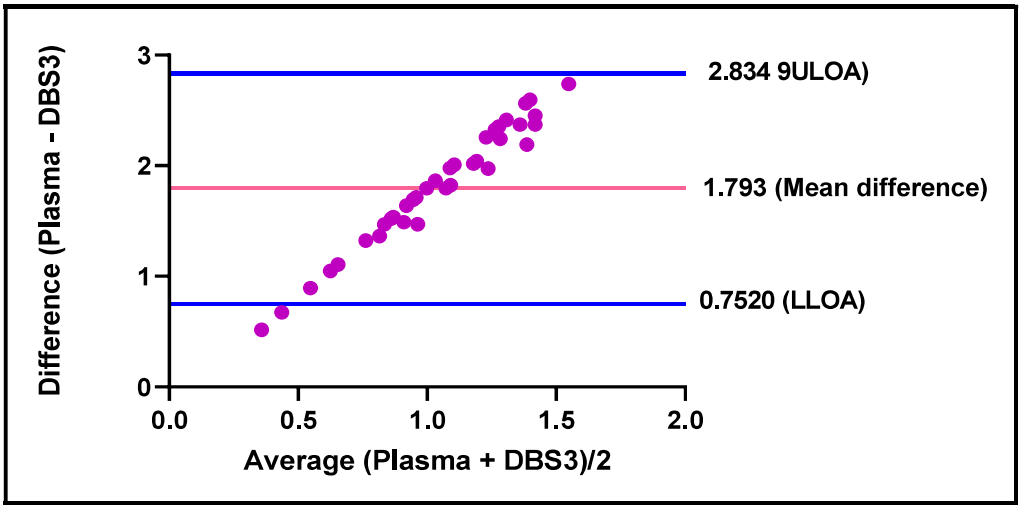


(Figure 31A) BA plot of apoB conc. between Plasma and DBS1

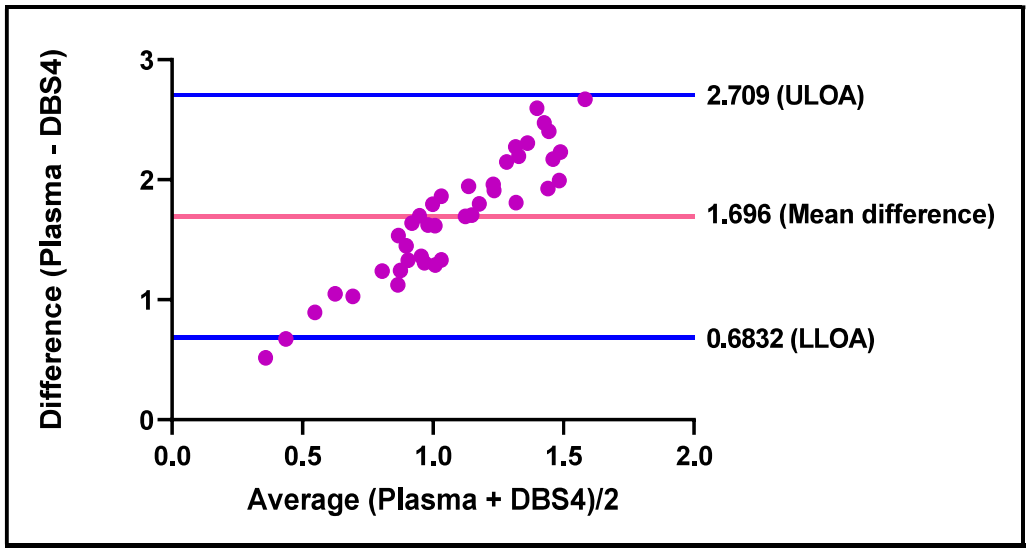
[48] Bland-Altman plot (GraphPad) of apoB lipoprotein created to observe the bias (mean difference) between plasma & DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately



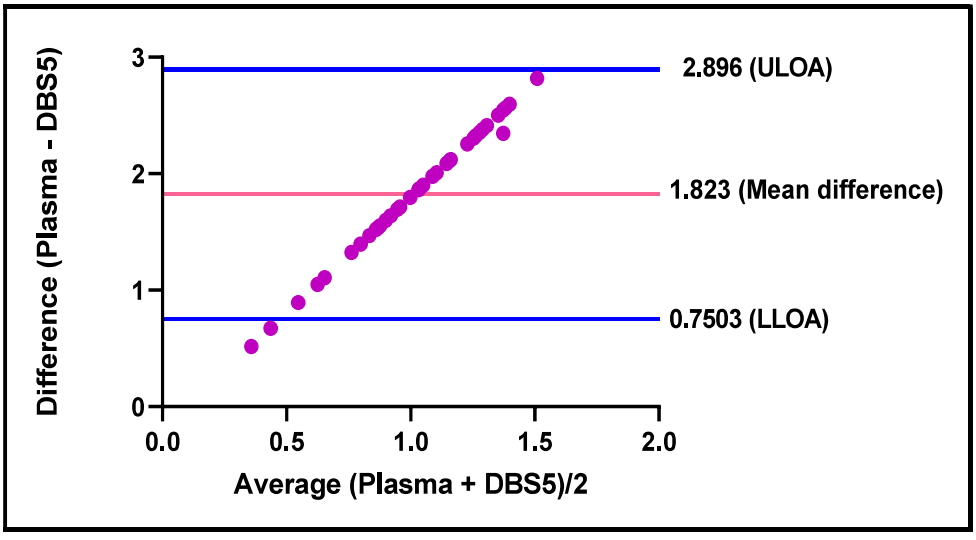
(**Figure 31B)** BA plot of apoB conc. between Plasma and DBS2



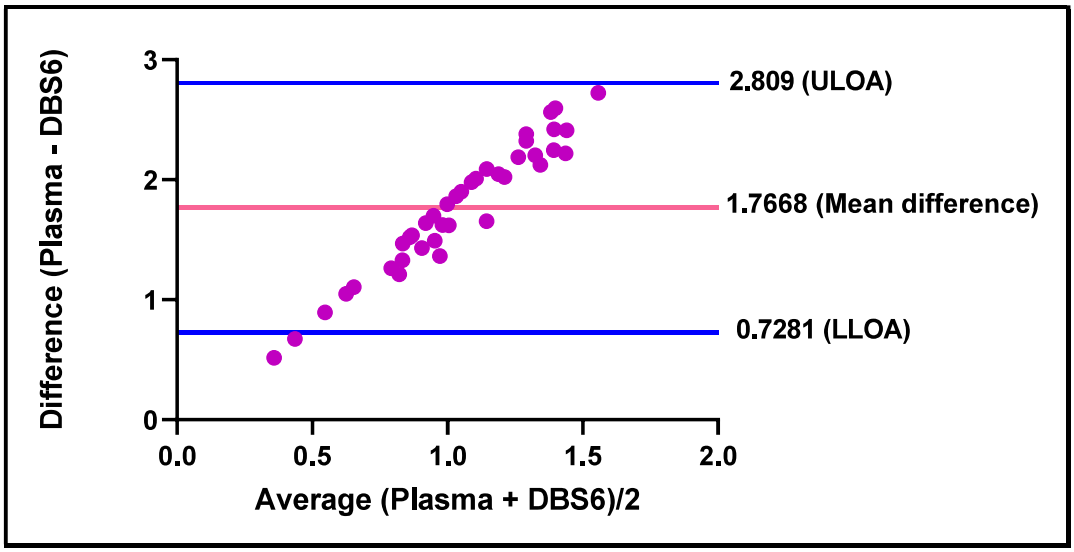
(Figure 31C) BA plot of apoB conc. between Plasma and DBS3



(Figure 31D) BA plot of apoB conc. between plasma and DBS4



(Figure 31E) BA plot of apoB conc. between plasma and DBS5



(Figure 31F) BA plot of apoB conc. between plasma and DBS6

3.4C Evaluation & comparison of apolipoprotein-B (g/L) concentration between DBS* stored at standard condition and all DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

3.4C.1 Result: T-test, ANOVA, Scattered plot and Bland-Altman plot.

Table 34[49]. T-test result, the mean comparison of apolipoprotein-B (apoB) concentration between DBS* and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5)

Sample storage condition	Transport duration	N	Mean (g/L)	SD	95% CI	p - value
DBS* (standard condition)	Immediate	40	1.0246	0.3493	0.9129 - 1.1363	p > 0.001
DBS1 (4 Degree Celsius)	Between 0-24 hours	40	0.2063	0.0845	0.1792 - 0.2333	
DBS* (standard condition)	Immediate	40	1.0246	0.3493	0.9129 - 1.1363	

[49] Paired t-test (STATA) used to compare mean difference of apoB lipoprotein between DBS* stored at standard condition & DBS stored at 4^0C

DBS3 (4 Degree Celsius)	Between 24-48 hours	40	0.1325	0.0510	0.1161 - 0.1488	p > 0.001
DBS* (standard condition)	Immediate	40	1.0246	0.3493	0.9129 - 1.1363	p > 0.001
DBS5 (4 Degree Celsius)	Between 48-72 hours	40	0.1024	0.0157	0.0974 - 0.1075	

Table 35[50]. T-test result, the mean comparison of apolipoprotein-B (apoB) concentration between DBS* and paired DBS samples stored -20^{0}C (DBS2, DBS4, DBS6).

Sample with storage condition	**Transport duration**	**N**	**Mean**	**SD**	**95% CI**	**p - value**
DBS* (standard condition)	Immediate	40	1.0246	0.3493	0.9129 - 1.1363	p > 0.001
DBS2 (-20 Degree Celsius)	Between 0-24 hours	40	0.4276	0.1443	0.3814 - 0.4737	
DBS* (standard condition)	Immediate	40	1.0246	0.3493	0.9129 - 1.1363	p > 0.001
DBS4 (-20 Degree Celsius)	Between 24-48 hours	40	0.2295	0.1082	0.1949 - 0.2642	
DBS* (standard condition)	Immediate	40	1.0246	0.3493	0.9129 - 1.1363	p > 0.001
DBS6 (-20 Degree Celsius)	Between 48-72 hours	40	0.1570	0.06957	0.1348 - 0.1793	

T-test has been conducted to compare group means of apolipoproteinB (apoB) concentration (g/L) between all DBS samples & DBS* sample stored at standard conditions. Statistically significant difference observed with p-value (p>0.001) between all DBS samples (stored at 4^{0}C & -20^{0}C) and DBS* sample stored at standard conditions.

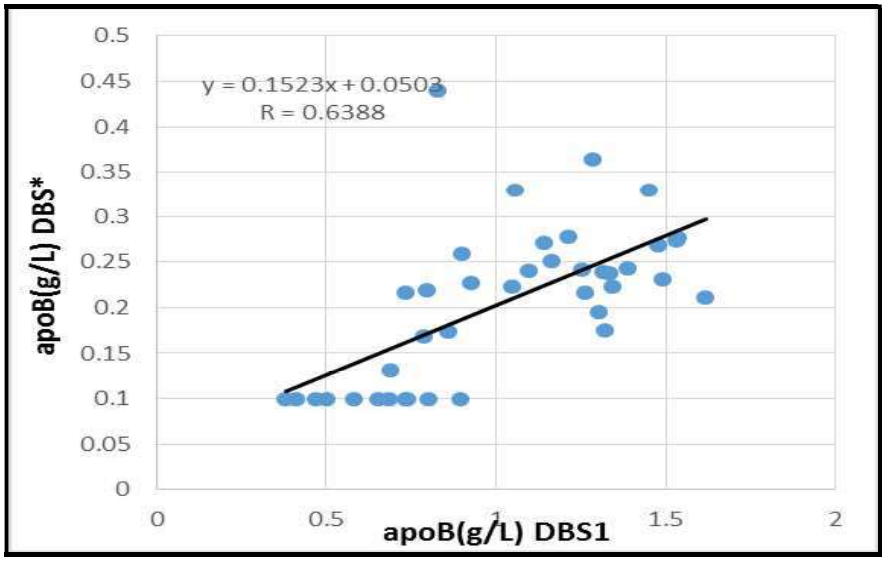
ANOVA test conducted for multiple comparisons of group means (apoB) separately between all DBS samples stored at 4^{0}C & all DBS samples stored at -20^{0}C with DBS* sample stored at standard condition irrespective of transport duration. Statically significant difference observed with p-value (p>0.001) at both stored temperature with large F value (F = 235.61) at 4^{0}C & F-value (F = 155.47) at -20^{0}C.

[50] Paired t-test (STATA) used to compare mean difference of apoB lipoprotein between DBS* stored at standard condition & DBS stored at -20^{0}C

Table 36[51]. ANOVA test result, multiple group mean comparison of apolipoprotein-B concentration between DBS* and all paired DBS samples stored at 4^0C & -20^0C irrespective of all 3 transport duration.

DBS* sample storage condition	DBS samples stored condition irrespective of transport duration	F-value	p value
Standard condition (-80^0C)	4 degree Celsius (n = 120)	235.61	p>0.001
Standard condition (-80^0C)	-20 degree Celsius (n = 120)	155.47	p>0.001

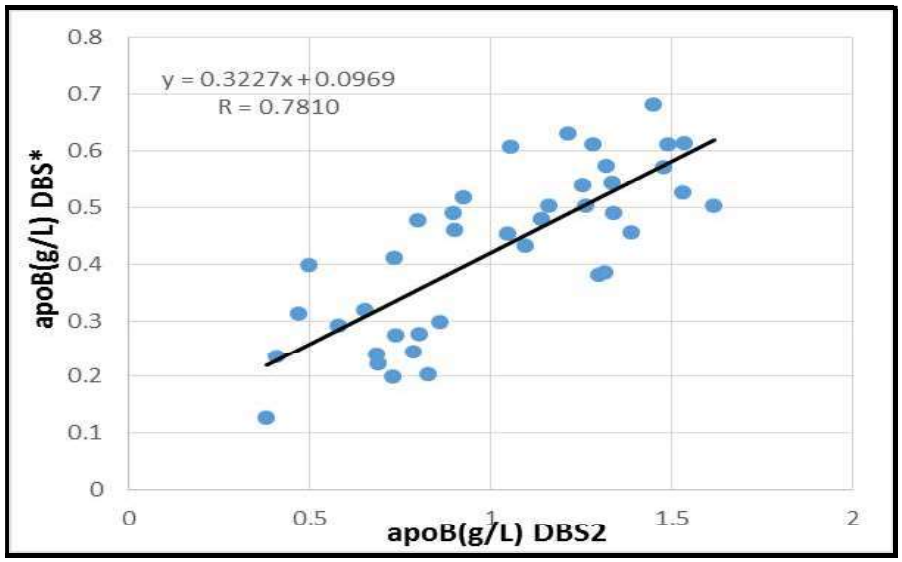
Figure 32[52]. Prepared scattered plot to compare correlation coefficient of apolipoprotein-B concentration between DBS* and all paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5) and -20^0C (DBS2, DBS4, DBS6).



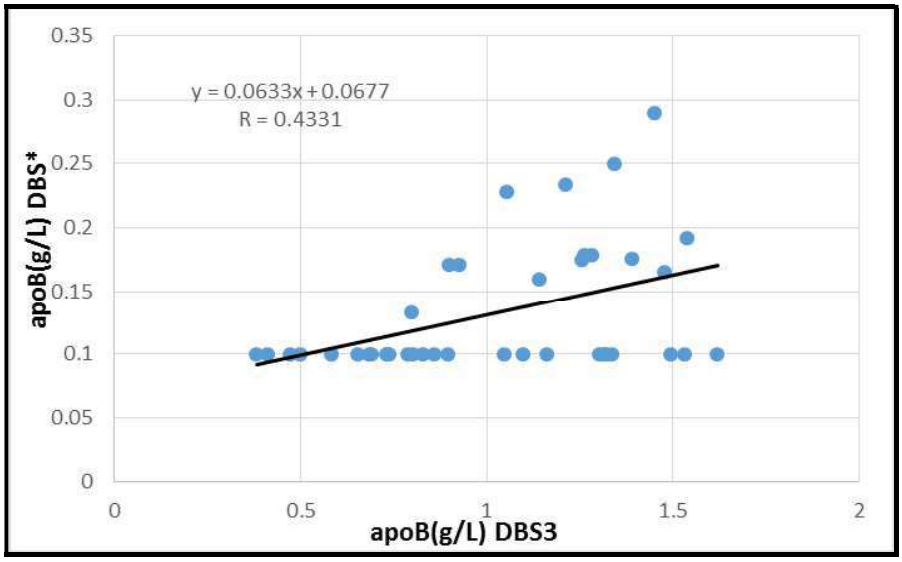
(Figure 32A) apoB conc. **c**orrelation coefficient (r= 0.6388) between DBS* & DBS1

[51] Oneway ANOVA test (STATA) was conducted to compare mean concentration of apoB lipoprotein between plasma & DBS stored at 4^0C & -20^0C

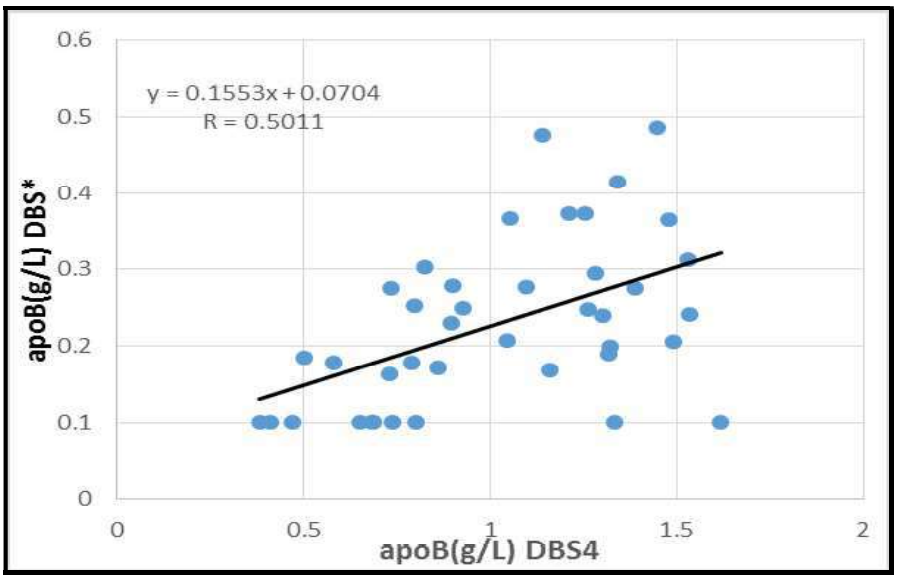
[52] Scattered plot (Excel) created of apoB lipoprotein to compare correlation coefficient between DBS* stored at standard condition & all DBS groups (DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6).



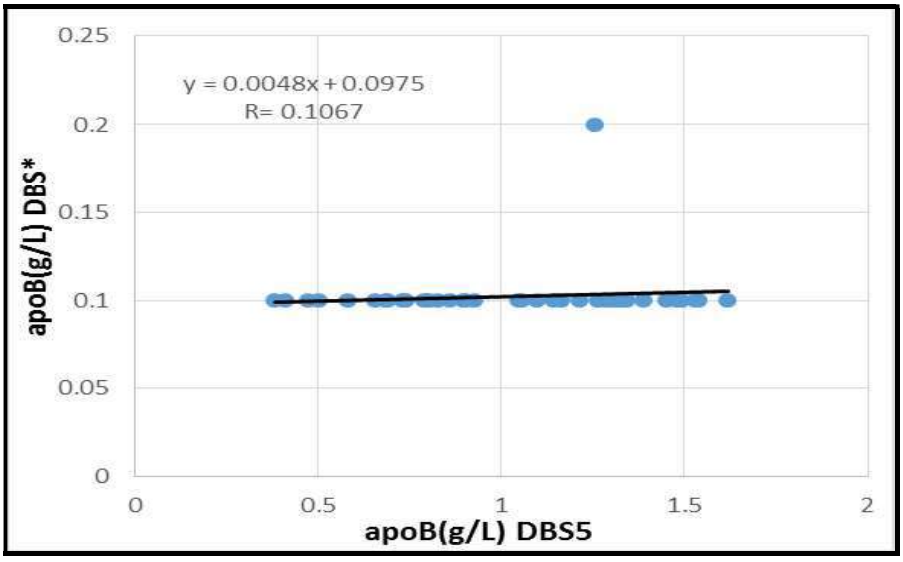
(Figure 32B) apoB conc. correlation coefficient (r= 0.7810) between DBS* & DBS2



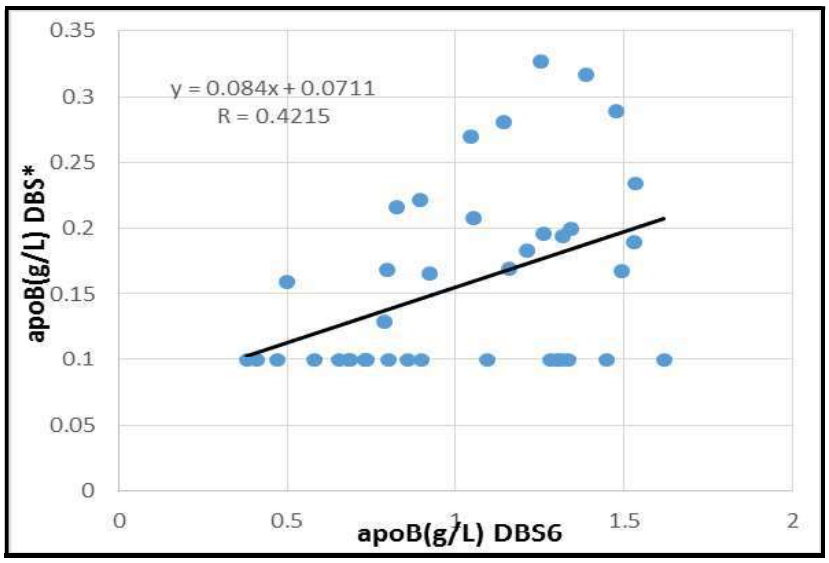
(Figure 32C) apoB conc. correlation coefficient (r= 0.4331) between DBS* & DBS3



(Figure 32D) apoB conc. correlation coefficient (r= 0.5011) between DBS* & DBS4

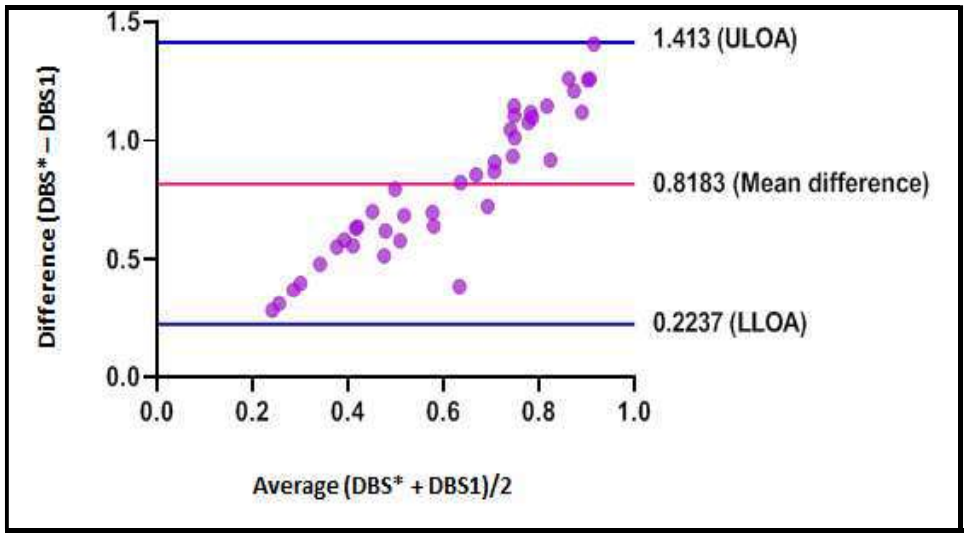


(**Figure 32E**) apoB conc. correlation coefficient (r= 0.1067) between DBS* & DBS5



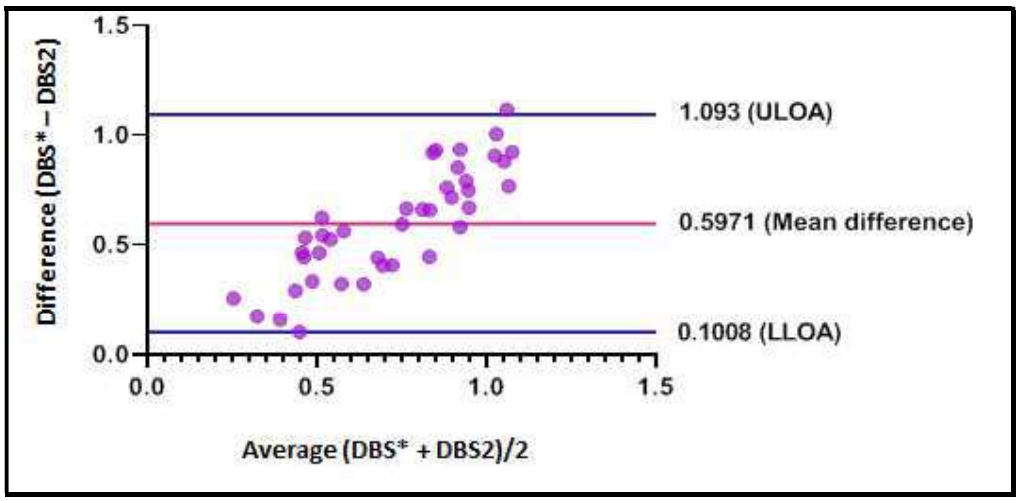
(Figure 32F) apoB conc. correlation coefficient (r= 0.4215) between DBS* & DBS6

Figure 33[53]**.** Prepared Bland-Altman graph, a plot of difference of apolipoprotein-B concentration between DBS* and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

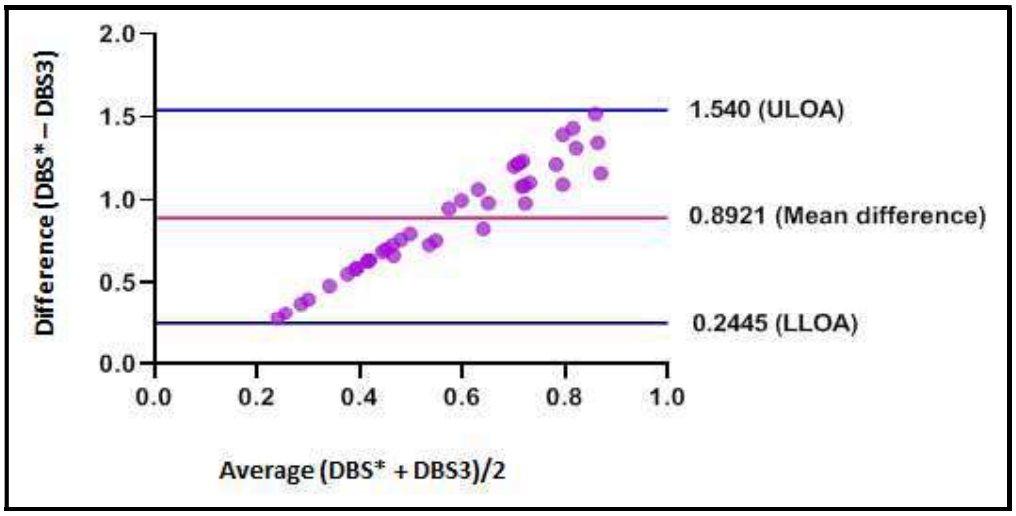


(Figure 33A) apoB conc., BA plot difference between DBS* & DBS1

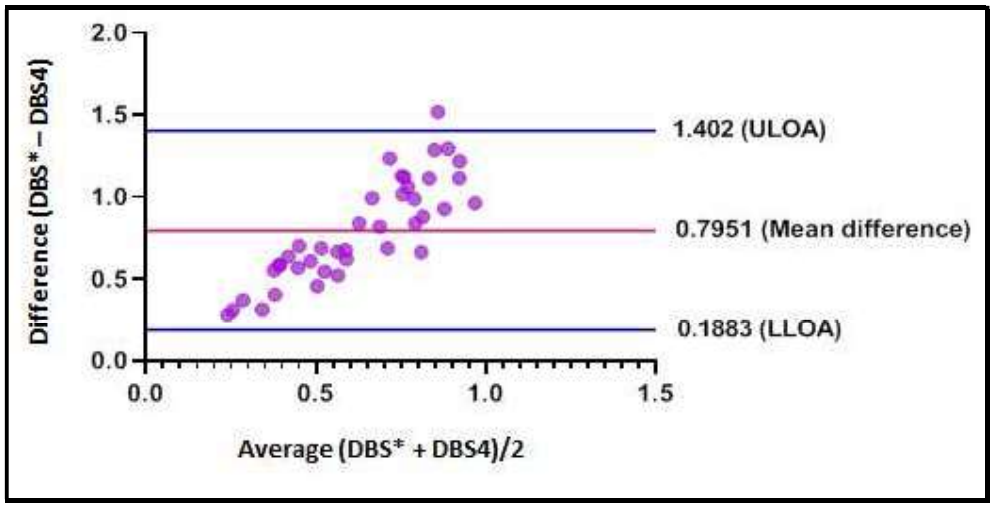
[53] Bland-Altman plot (GraphPad) of apoB lipoprotein created to observe the bias (mean difference) between DBS* stored at standard condition and DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately



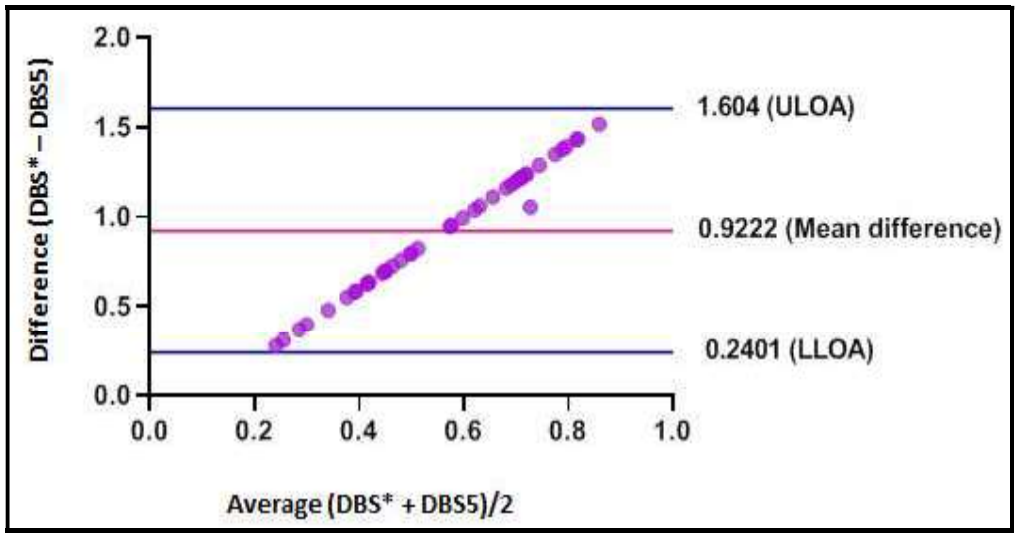
(Figure 33B) apoB conc., BA plot difference between DBS* & DBS2



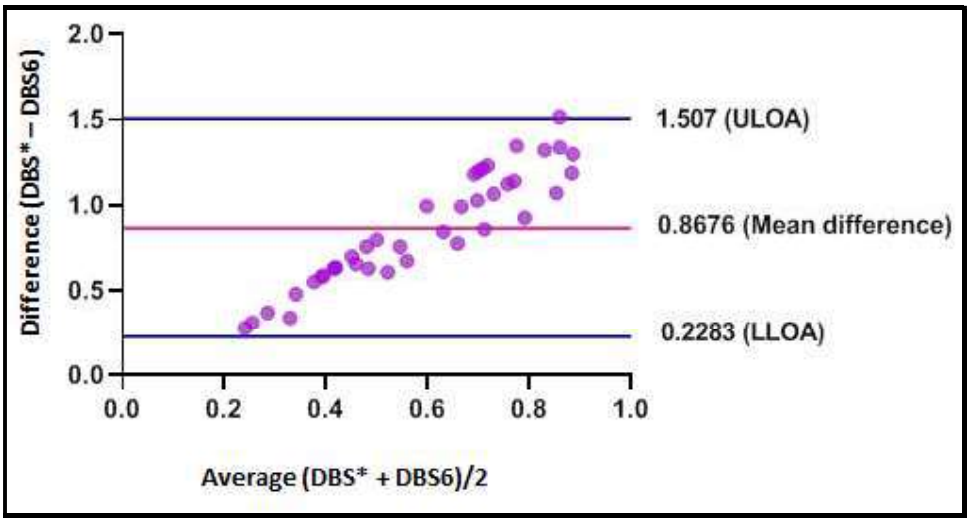
(Figure 33C) apoB conc., BA plot difference between DBS* & DBS3



(Figure 33D) apoB conc., BA plot difference between DBS* & DBS4



(Figure 33E) apoB conc., BA plot difference between DBS* & DBS5



(Figure 33F) apoB conc., BA plot difference between DBS* & DBS6

Table 37. Compare percentage change in apolipoprotein-B concentration between gold-standard (plasma & DBS*) samples and all DBS samples stored at 4^0C & -20^0C.

Samples at standard condition	Percentage change in DBS* as compared to plasma		Percentage change in DBS as compared with samples stored at standard condition					
	-80^0C		**4^0C**				**-20^0C**	
	Plasma/WB	DBS*	DBS1	DBS3	DBS5	DBS2	DBS4	DBS6
Plasma/WB	0%	46%	89%	93%	94%	77%	88%	91%
DBS*	46%	0%	79%	87%	90%	58%	77%	84%

3.4C.2 Discussion: apolipoprotein-B evaluation from DBS

Apolipoprotein-B is the sole protein component of low-density lipoprotein (LDL) and encompasses a variety of functions in lipid metabolism. LDL is a globular lipoprotein with a non-polar lipid core surrounding by an amphiphilic covering of protein, phospholipid, and cholesterol, similar to all other plasma lipoproteins (Laggner, Kostner, Degovics, & Worcester, 2006; MÜLLER, LAGGNER, GLATTER, & KOSTNER, 1978). Human apoB is a 4536-amino-acid-residue glycoprotein with a molecular mass of roughly 550 kDa. It's a

single chain protein that is associated non-covalently with hydrophobic molecules for easy transportation and hydrophilic targeting. Apolipoprotein B (apo B) is the structural protein for atherogenic lipoproteins and facilitates the transporting of lipids from the liver to peripheral tissues (Davidson, 2009; Marcovina & Packard, 2006). This study aims to compare the apoB concentration extracted from all DBS samples with gold standard (plasma & DBS*) samples stored at the standard condition to evaluate if there is any variation in the concentration of apoB lipoprotein while stored at different temperatures after 2- 3 days of transportation. There is no such study conducted on multiple comparisons of the same samples stored at different temperatures with different time points. We have also calculated how much percentage change occurred in the apoB concentration between DBS and gold standard samples.

At standard conditions, a good correlation coefficient (r = 0.6473) observed between DBS* & plasma samples. In some previously published articles, there is a strong correlation observed between plasma and DBS with little bias (Eick, Kowal, Barrett, Thiele, & Snodgrass, 2017). But in this study, 64.73% correlation observed after 4-5 months of storage. The literature shows, >85% correlation coefficient observed between plasma and DBS for apolipoproteins, but this concentration decreases with time (Reference). In a study, researchers measured apoB concentrations from plasma and DBS on the 20th day of storage & found (r = 0.87) correlation coefficient (Wang et al., 1989).

Furthermore, the immunoreactivity of apoB declined with the time of storage at 4^0C (Blades, Dudman, & Wilcken, 1987b). In a recent study, the researcher shows that proteins (apoB, apoA) can be stable on DBS for 7 days below 25^0C storage condition and there was a good correlation between plasma and DBS (r= 0.78) (Hoofnagle, 2017). Concerning published articles and results from this study, for getting good results, DBS should be stored at 4^0C or -

20^0C for a shorter period and -80^0C for longer-period but it should be transported within 24 hours of transport duration.

At standard condition, we have observed a 46% difference in the means concentration of apoB lipoprotein between DBS* & plasma, while there is a large percentage difference (58% - 94%) observed for all DBS samples stored at 4^0C & -20^0C.

Interestingly, we have observed low stability of apolipoprotein B as compared to apolipoprotein A due to a large percentage difference. In epidemiological studies, DBS can be exploited to predict CVD risk by measuring of apoB/apoA ratio, precaution needed for the DBS preparation time, its transportation, and their storage. After the complete analysis, we have focused on some key point which could be the reason for the lower concentrations or moderate correlations of apolipoproteins.

3.5 Evaluation of (apoB/apoA) ratio and its comparison between plasma and DBS samples

3.5A Evaluation & comparison of apoB/apoA ratio between plasma and DBS* stored at standard condition

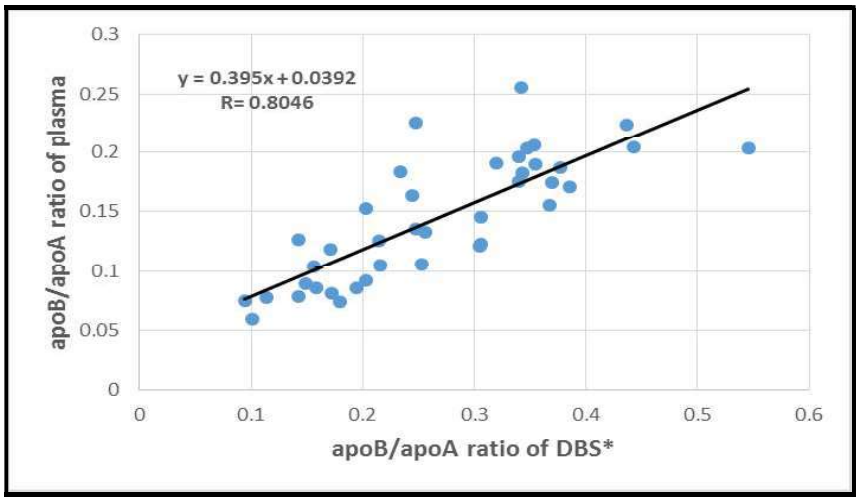
3.5A.1 Result: T test, Scattered Plot, Bland-Altman plot

Table 38[54]**.** T-test result, the mean comparison of apolipoprotein ratio (apoB/apoA) concentration between plasma and DBS* stored at standard condition.

Samples with stored condition	Number of Observations	Mean	Standard Deviation	95% CI	p - value
Plasma (Standard condition)	40	0.2667	0.1052	0.2330 - 0.3003	p >0.001
DBS (Standard condition)	40	0.1445	0.05166	0.1280 - 0.1610	

T-test has been conducted to compare mean of ratio (apoB/apoA) between paired DBS samples and gold standard (plasma & DBS*). At standard condition, there is statistically significant difference observed with p-value (p>0.001) between DBS* & plasma.

Figure 34[55]**.** Prepared scattered plot to compare correlation coefficient apoB/apoA ratio between plasma & DBS* stored at standard condition.

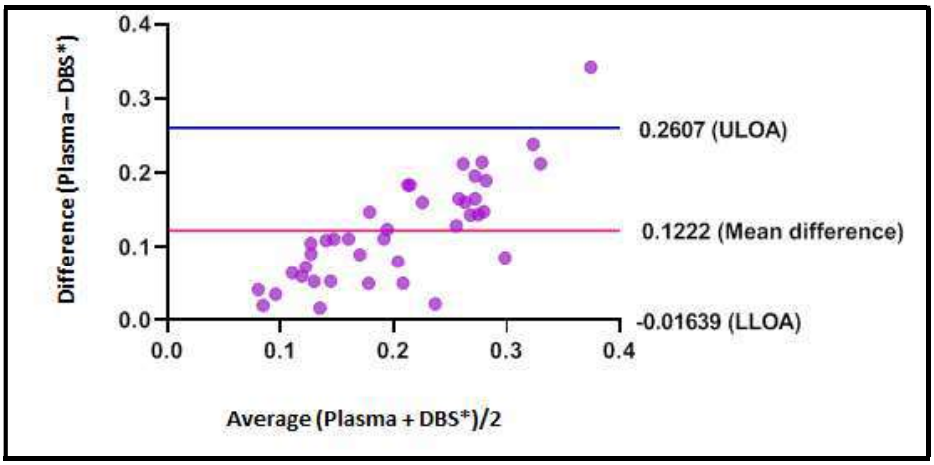


Correlation coefficient (r = 0.8046) between plasma & DBS* of apoB/apoA ratio

[54] Paired t-test (STATA) used to compare mean difference of apoB/apoA ratio between plasma and DBS* stored at standard condition.

[55] Scattered plot (Excel) created to compare correlation coefficient of apoB/apoA ratio between plasma & DBS* stored at standard condition

Figure 35[56]. Prepare Bland-Altman graph, a plot of difference of (apoB/apoA) concentration between plasma & DBS* stored at standard condition.



Evaluation and comparison of ratio of apoB/apoA concentration between plasma and DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6)

Result: T-test, ANOVA, Scattered plot, Bland-Altman plot

Table 39[57]. T-test result, the mean comparison of apolipoprotein (apoB/apoA) concentration between plasma and paired DBS samples stored at 4^0C (DBS1, DBS3, DBS5)

Samples storage condition	Transport duration	N	Mean	SD	95% CI	p - value
Plasma (standard condition)	Immediate	40	0.266	0.105	0.233 - 0.300	p > 0.001
DBS1 (4 Degree Celsius)	Between 0-24 hours	40	0.027	0.010	0.024 - 0.031	
Plasma (standard condition)	Immediate	40	0.266	0.105	0.233 - 0.300	p > 0.001
DBS3 (4 Degree Celsius)	Between 24-48 hours	40	0.040	0.015	0.035 - 0.045	
Plasma (standard condition)	Immediate	40	0.266	0.105	0.233 - 0.300	p > 0.001
DBS5 (4 Degree Celsius)	Between 48-72 hours	40	0.059	0.017	0.053 - 0.064	

[56] Bland-Altman plot (GraphPad) of apoB/apoA ratio created to observe the bias (mean difference) between plasma & DBS* stored at standard condition.

[57] Paired t-test (STATA) used to compare mean difference of apoB/apoA ratio between plasma and DBS stored at 4^0C

Table 40[58]. T-test, the mean comparison of apolipoprotein (apoB/apoA) concentration between plasma and paired DBS samples stored at -20^{0}C (DBS2, DBS4, DBS6)

Blood samples storage condition	Transport duration	N	Mean	Standard Deviation	95% CI	p - value
Plasma (standard condition)	Immediate	40	0.2667	0.1052	0.2330 - 0.3003	p > 0.001
DBS2 (-20 Degree Celsius)	Between 0-24 hours	40	0.1399	0.0582	0.1213 - 0.1585	
Plasma (standard condition)	Immediate	40	0.2667	0.1052	0.2330 - 0.3003	p > 0.001
DBS4 (-20 Degree Celsius)	Between 24-48 hours	40	0.0906	0.0433	0.0767 - 0.1045	
Plasma (standard condition)	Immediate	40	0.2667	0.1052	0.2330 - 0.3003	p > 0.001
DBS6 (-20 Degree Celsius)	Between 48-72 hours	40	0.0490	0.0183	0.0432 - 0.0549	

T-test has conducted to compare group mean ratio (apoB/apoA) between all stored (4^{0}C & -20^{0}C) DBS samples and gold standard samples (plasma & DBS*). Statistically significant difference observed between all DBS samples & plasma with p-value (p>0.001), while positive correlation coefficient observed between plasma & all paired DBS samples.

Table 41[59]. ANOVA-test result, multiple group mean comparison of apolipoprotein ratio (apoB/apoA) between plasma and paired DBS samples stored at 4^{0}C & -20^{0}C irrespective of all 3 transport duration.

Plasma sample as Gold standard storage condition	DBS samples stored condition irrespective of transport duration	F-value	p value
Standard condition (n = 40)	4 degree Celsius (n = 120)	173.42	p>0.001
Standard condition (n = 40)	-20 degree Celsius (n = 120)	85.39	p>0.001

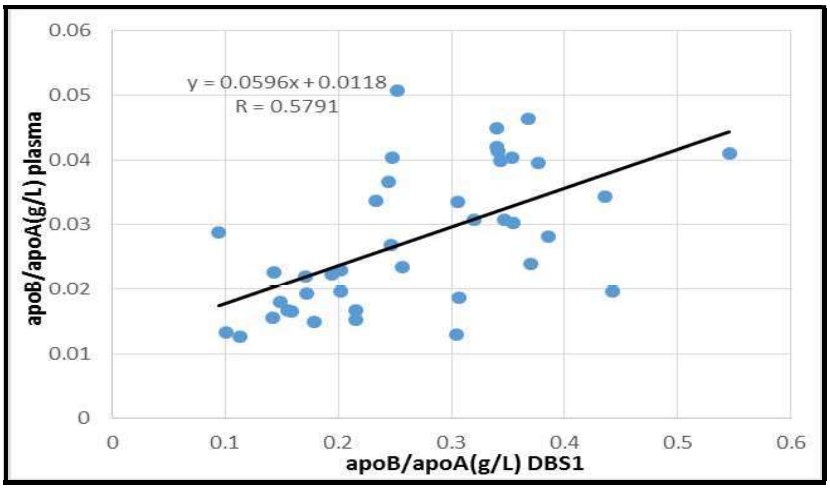
ANOVA test has been conducted to compare the apoB/apoA ratio between plasma with all 3 groups of paired DBS stored at 4^{0}C & -20^{0}C irrespective of their transport duration.

[58] Paired t-test (STATA) used to compare mean difference of apoB/apoA ratio between plasma and DBS stored at -20^{0}C

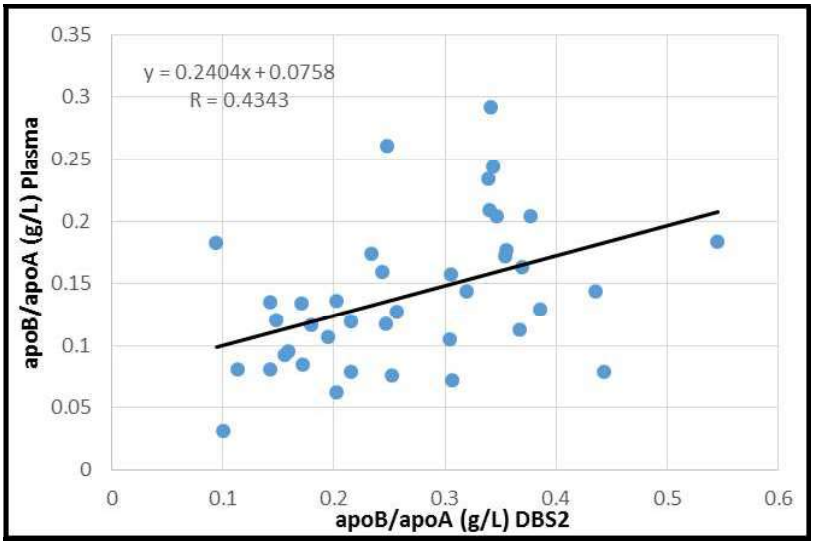
[59] Oneway ANOVA test (STATA) was conducted to compare mean concentration of apoB/apoA ratio between plasma & DBS stored at 4^{0}C & -20^{0}C

Statistically significant difference observed with p-value ($p>0.001$) at both stored temperature with large F value ($F = 173.42$) at 4^0C, & F value ($F = 85.39$) at -20^0C.

Figure 36[60]. Prepared scattered plot to compare correlation coefficient of apoB/apoA ratio between Plasma and DBS stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

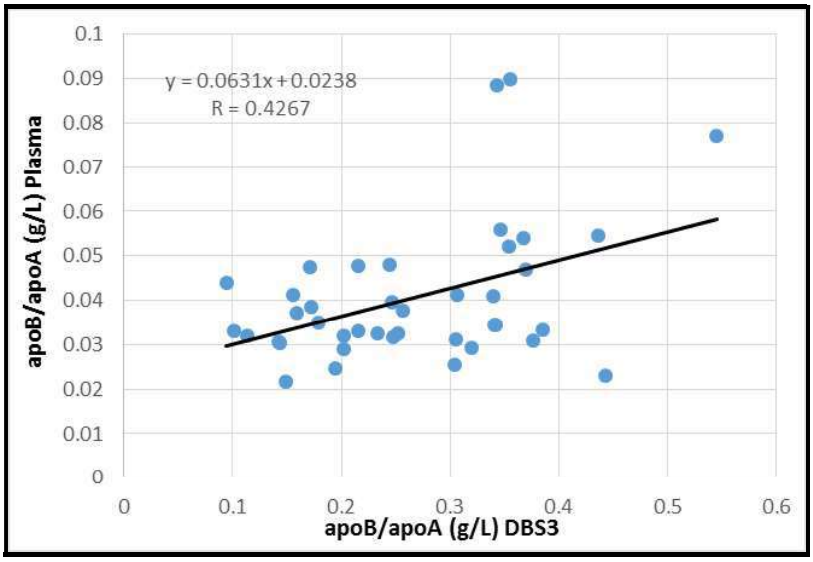


(Figure 36A) apoB/apoA conc. correlation coefficient ($r = 0.5791$) between plasma & DBS1

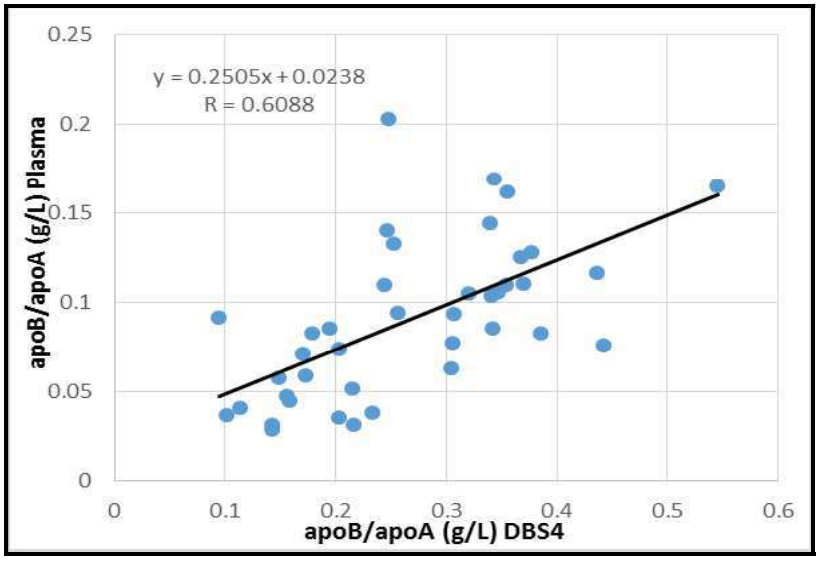


(Figure 36B) apoB/apoA conc. correlation coefficient ($r = 0.4343$) between plasma & DBS2

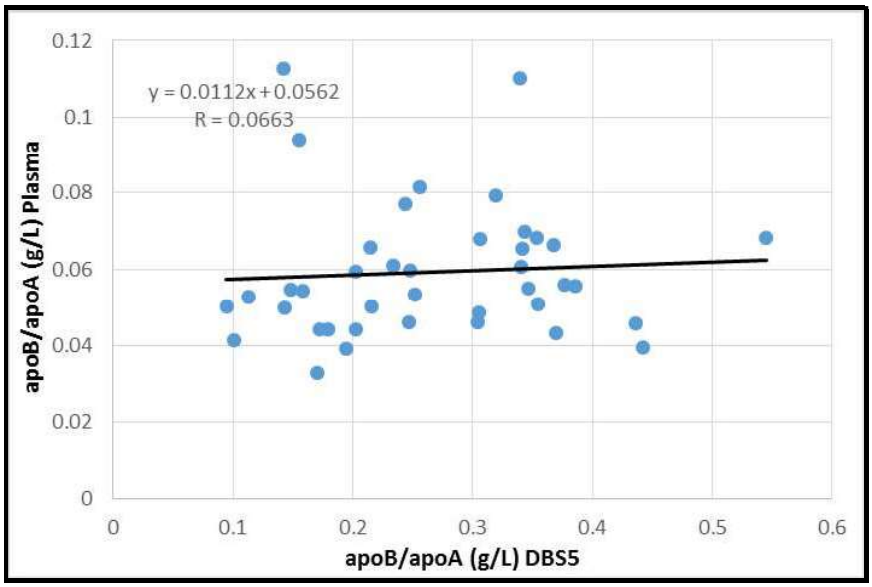
[60] Scattered plot (Excel) were used to compare correlation coefficient of apoB/apoA ratio between plasma & DBS1, DBS2, DBS3, DBS4, DBS5 & DBS6 separately



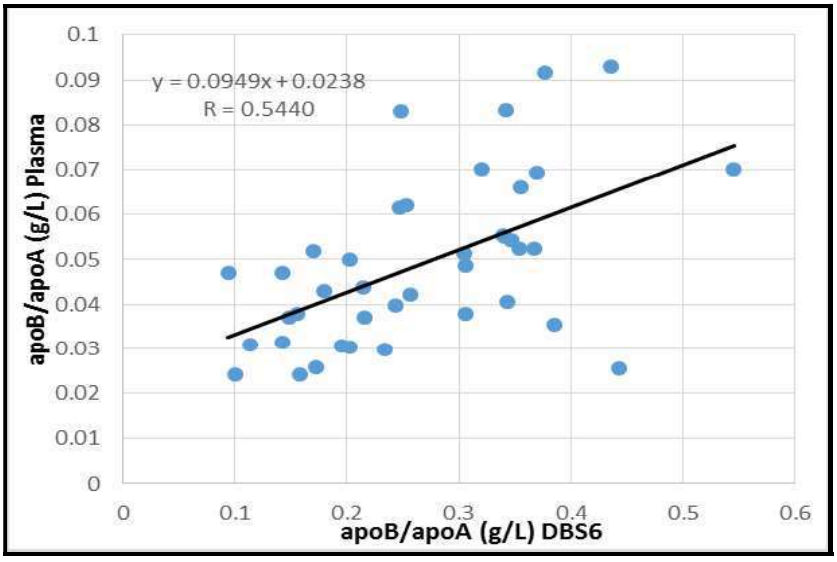
(Figure 36C) apoB/apoA conc. correlation coefficient (r = 0.4267) between plasma & DBS3



(Figure 36D) apoB/apoA conc. correlation coefficient (r = 0.6088) between plasma & DBS4

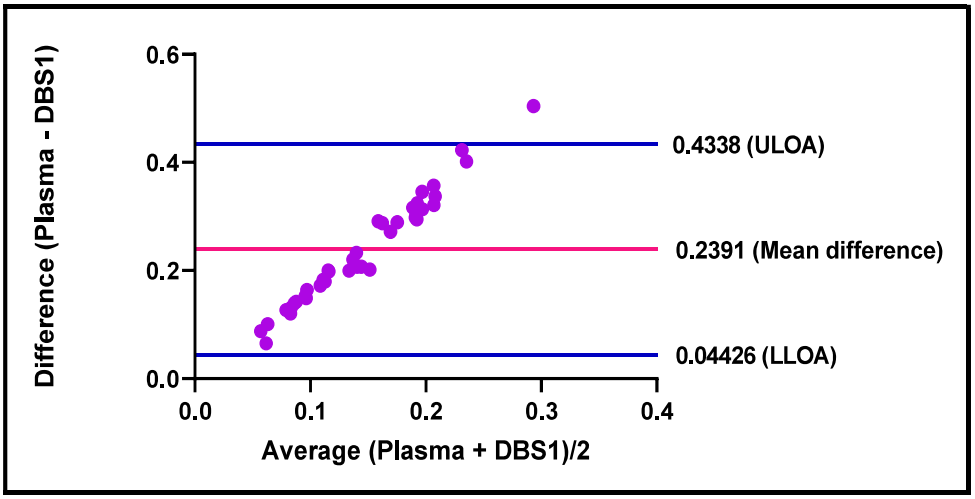


(Figure 36E) apoB/apoA conc. correlation coefficient (r = 0.0663) between plasma & DBS5

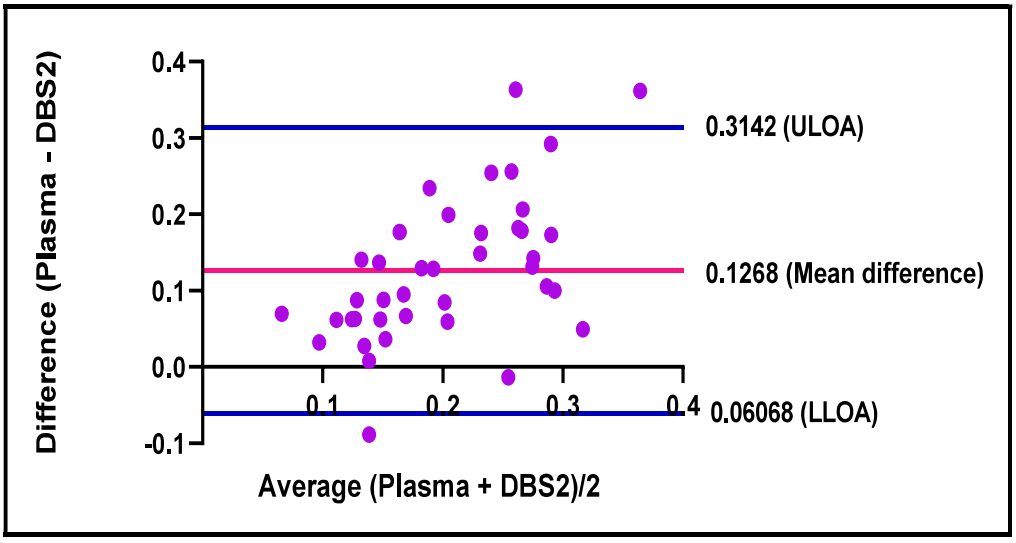


(Figure 36F) apoB/apoA conc. correlation coefficient (r = 0.5440) between plasma & DBS6

Figure 37 [61]**.** Prepare Bland–Altman graph, a plot of difference of apoB/apoA ratio concentration between plasma & DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

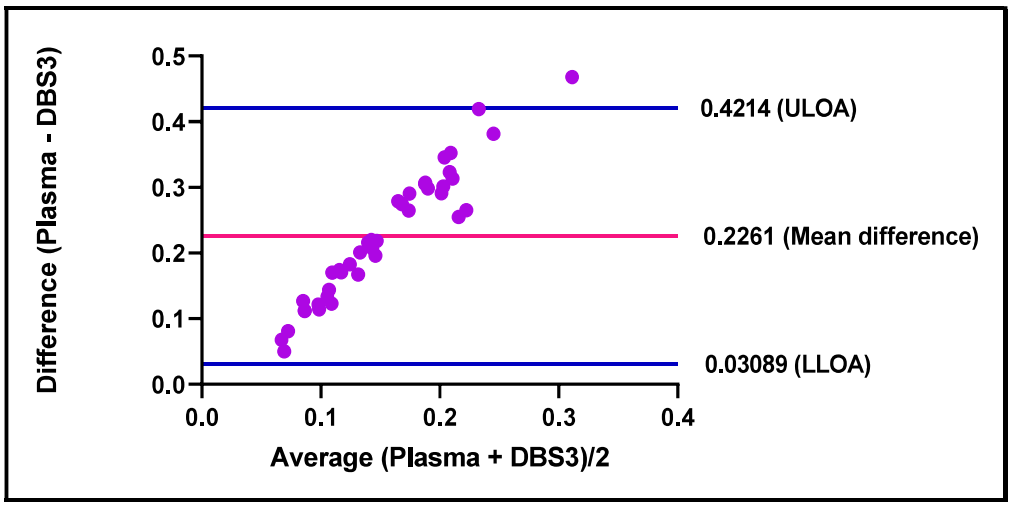


(Figure 37A) BA plot of apoB/apoA ratio between plasma & DBS1

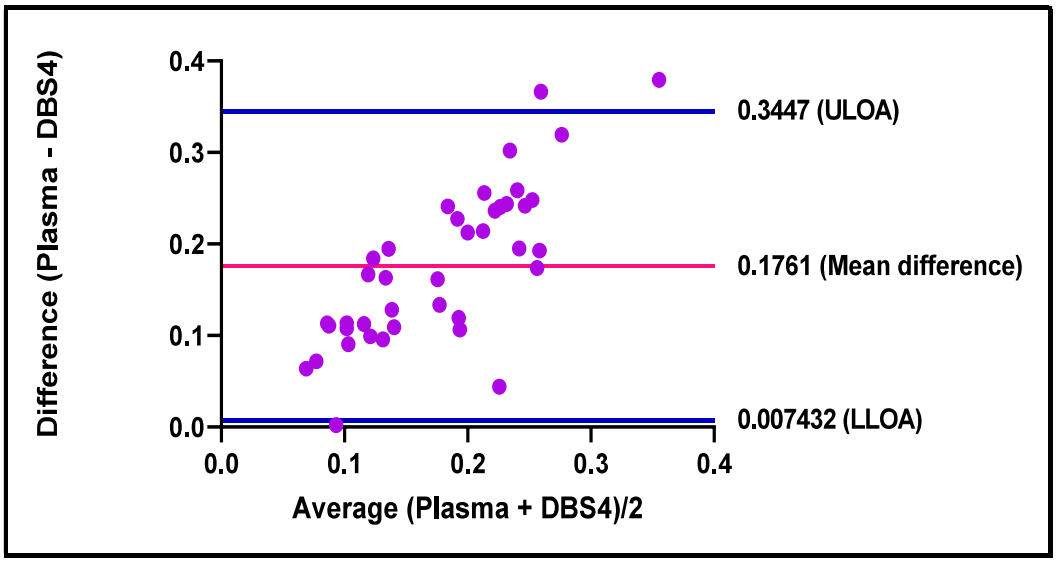


(Figure 37B) BA plot of apoB/apoA ratio between plasma & DBS2

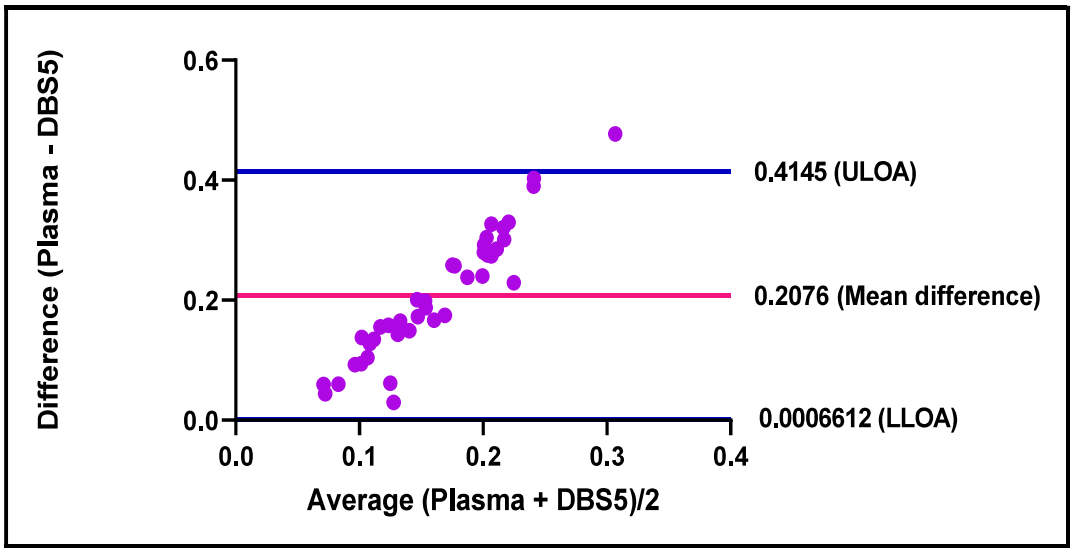
[61] Bland-Altman plot (GraphPad) of apoB/apoA ratio created to observe the bias (mean difference) between plasma & DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately



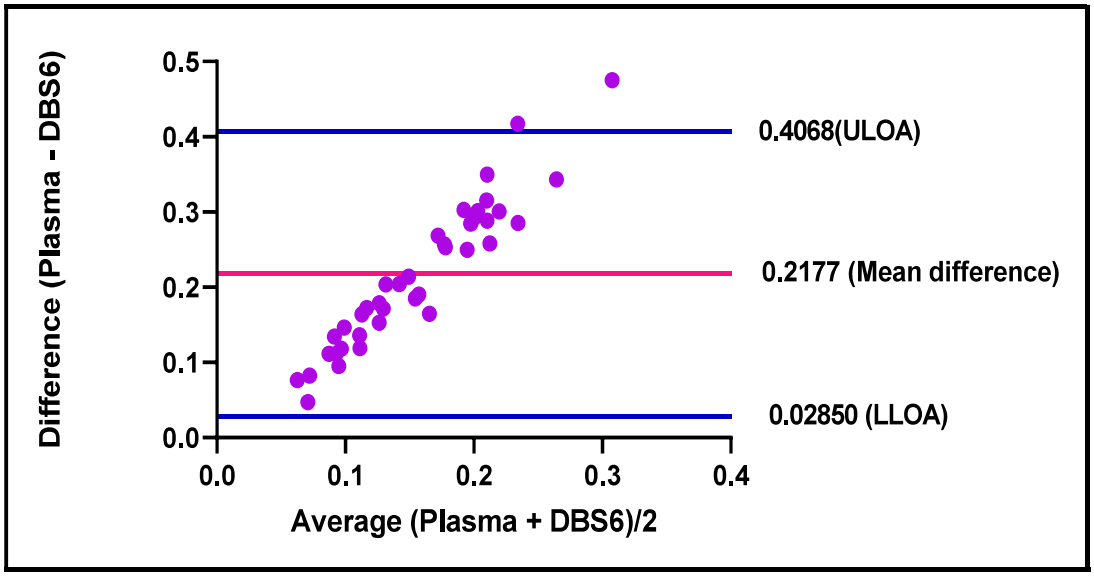
(Figure 37C) BA plot of apoB/apoA ratio between plasma & DBS3



(Figure 37D) BA plot of apoB/apoA ratio between plasma & DBS4



(Figure 37E) BA plot of apoB/apoA ratio between plasma & DBS5



(Figure 37F) BA plot of apoB/apoA ratio between plasma & DBS6

3.5B Evaluation & comparison of apoB/apoA ratio between DBS* stored at standard condition and DBS samples stored at 4^0C (DBS1, DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6).

3.5B.1 Result: T-test, ANOVA, Scattered plot, Bland-Altman plot

Table 42[62]. T-test result, the mean comparison of (apoB/apoA) ratio concentration between DBS* and all paired DBS samples stored 4^0C (DBS1, DBS3, DBS5).

Samples with storage condition	Transport duration	N	Mean	SD	95% CI	p - value
DBS* (standard condition)	Immediate	40	0.1445	0.05166	0.1280 - 0.1610	p > 0.001
DBS1 (4 Degree Celsius)	Between 0-24 hours	40	0.0276	0.0108	0.0242 - 0.0311	
DBS (standard condition)	Immediate	40	0.1445	0.05166	0.1280 - 0.1610	p > 0.001
DBS3 (4 Degree Celsius)	Between 24-48 hours	40	0.0405	0.0155	0.0356 - 0.0455	
DBS (standard condition)	Immediate	40	0.1445	0.05166	0.1280 - 0.1610	p > 0.001
DBS5 (4 Degree Celsius)	Between 48-72 hours	40	0.0591	0.0177	0.0534 - 0.0648	

Table 43[63]. T-test result, the mean comparison of (apoB/apoA) ratio concentration between DBS* and paired DBS samples stored at -20^0C (DBS2, DBS4, DBS6).

Sample with storage condition	Transport duration	N	Mean	SD	95% CI	p - value
DBS* (standard condition)	Immediate	40	0.144	0.0516	0.128 - 0.161	p > 0.463
DBS2 (-20 Degree Celsius)	Between 0-24 hours	40	0.139	0.0582	0.121 - 0.158	
DBS* (standard condition)	Immediate	40	0.144	0.0516	0.128 - 0.161	p > 0.001
DBS4 (-20 Degree Celsius)	Between 24-48 hours	40	0.090	0.0433	0.076 - 0.104	
DBS* (standard condition)	Immediate	40	0.144	0.0516	0.128 - 0.161	p > 0.001
DBS6 (-20 Degree Celsius)	Between 48-72 hours	40	0.049	0.0183	0.043 - 0.054	

[62] Paired t-test (STATA) used to compare mean difference of apoB/apoA between DBS* stored at standard condition & DBS stored at 4^0C

[63] Paired t-test (STATA) used to compare mean difference of apoB/apoA between DBS* stored at standard condition & DBS stored at -20^0C

T-test conducted to compare mean ratio (apoB/apoA) between all DBS samples and DBS* stored at standard condition. During the comparison, the statistically non-significant difference observed between DBS2 & DBS* with p-value (p>0.463), whereas significant difference observed for the rest of the DBS samples with p-value (p>0.001). Good correlation coefficient observed before 24 hours of transport duration.

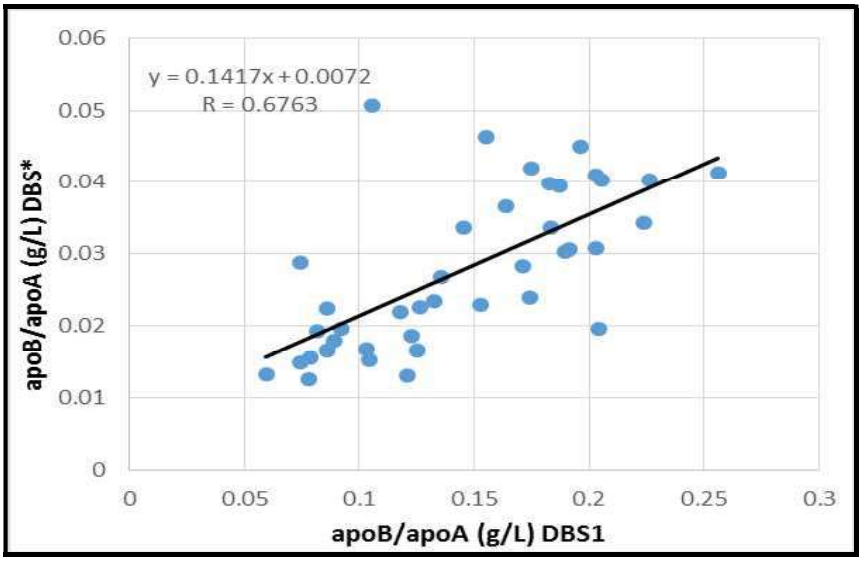
Table 44[64]. ANOVA test result, multiple group mean comparison of (apoB/apoA) ratio concentration between DBS* and all DBS samples stored at 4^0C & -20^0C irrespective of all 3 transport duration.

DBS* storage condition	DBS samples stored condition irrespective of transport duration	F-value	p value
Standard condition (n = 40)	4 degree Celsius (n = 120)	132.68	p>0.001
Standard condition (n = 40)	-20 degree Celsius (n = 120)	39.41	p>0.001

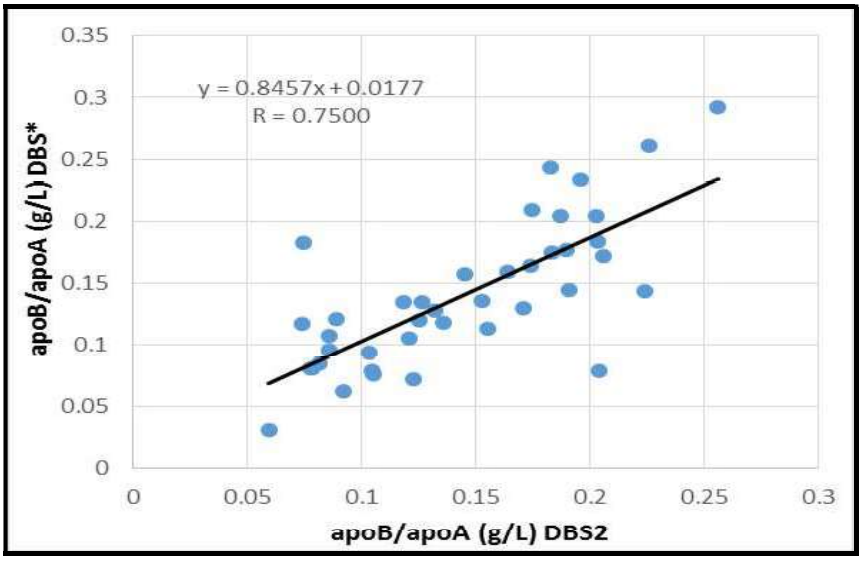
ANOVA test has been conducted for multiple comparisons between all DBS samples stored at 4^0C & -20^0C with DBS* sample stored at standard condition, irrespective of their transport duration. Statistically, significant difference observed with p-value (p>0.001) at both stored temperature with obtained F-value (F = 132.68) at 4^0C, and F-value (F = 39.41) at -20^0C.

[64] Oneway ANOVA test (STATA) was conducted to compare mean concentration of apoB/apoA ratio between plasma & DBS stored at 4^0C & -20^0C

Figure 38[65]. Prepare scattered plot to compare correlation coefficient of apoB/apoA ratio between DBS* and DBS samples stored at 4^0C (DBS1. DBS3, DBS5) & -20^0C (DBS2, DBS4, DBS6)

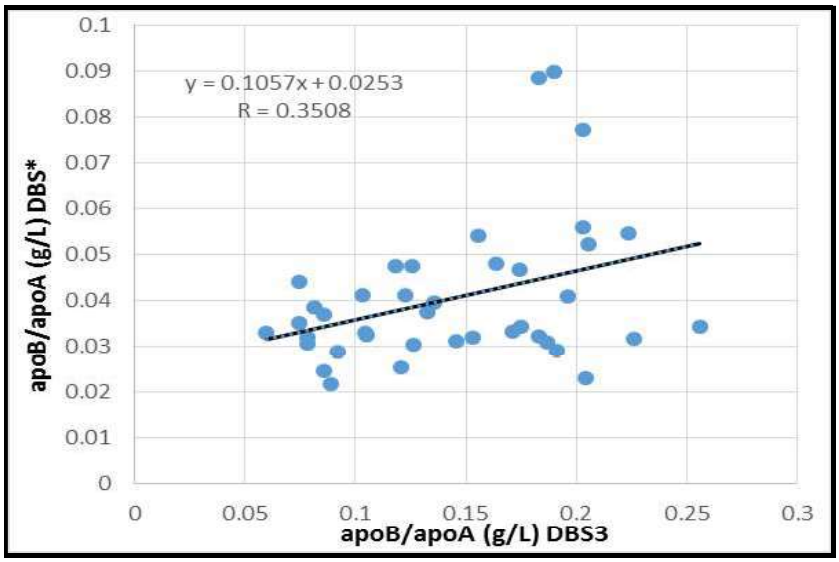


(Figure 38A) apoB/apoA conc. correlation coefficient (r = 0.6763) between DBS* & DBS1

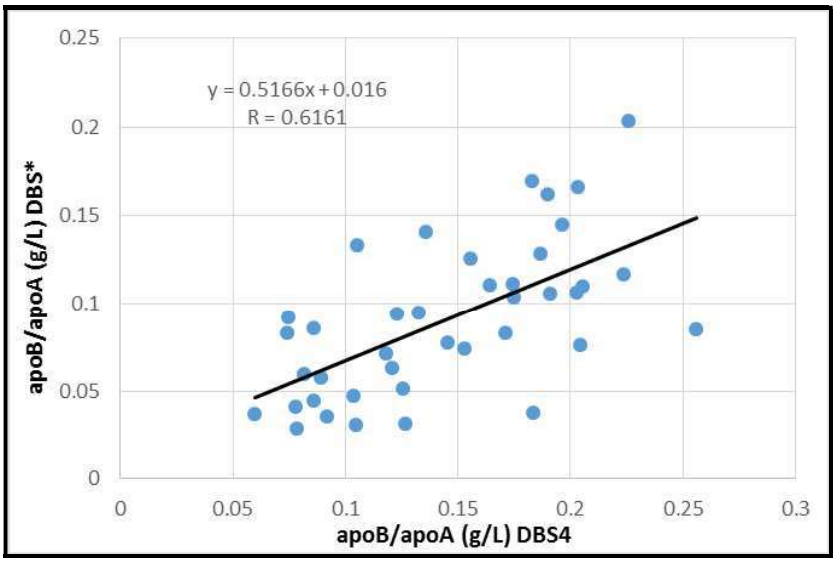


(Figure 38B) apoB/apoA conc. correlation coefficient (r = 0.7500) between DBS* & DBS2

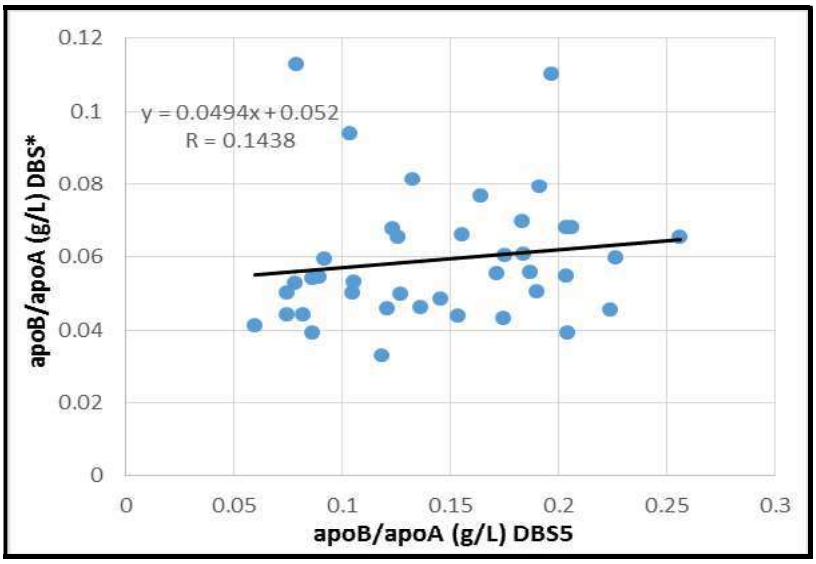
[65] Scattered plot (Excel) created of apoB/apoA ratio to compare correlation coefficient between DBS* stored at standard condition & all DBS groups (DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately)



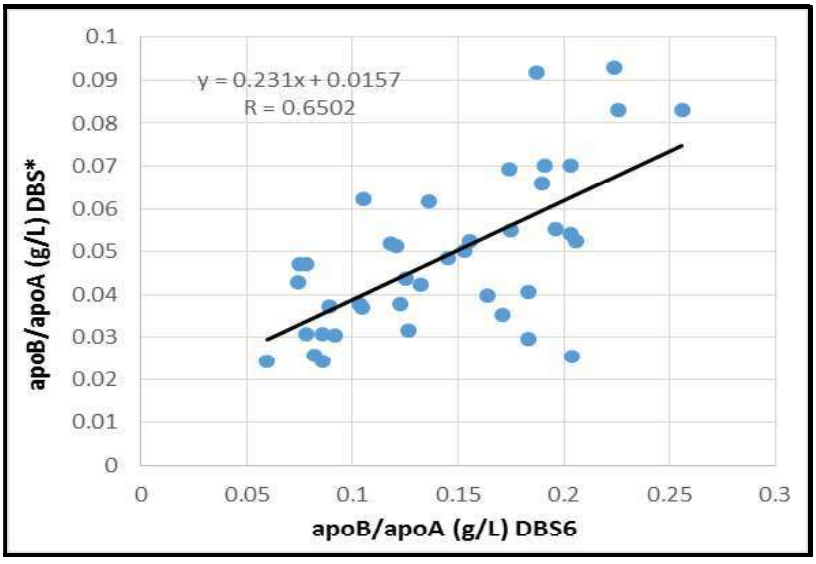
(Figure 38C) apoB/apoA conc. correlation coefficient (r = 0.3508) between DBS* & DBS3



(Figure 38D) apoB/apoA conc. correlation coefficient (r = 0.6161) between DBS* & DBS4

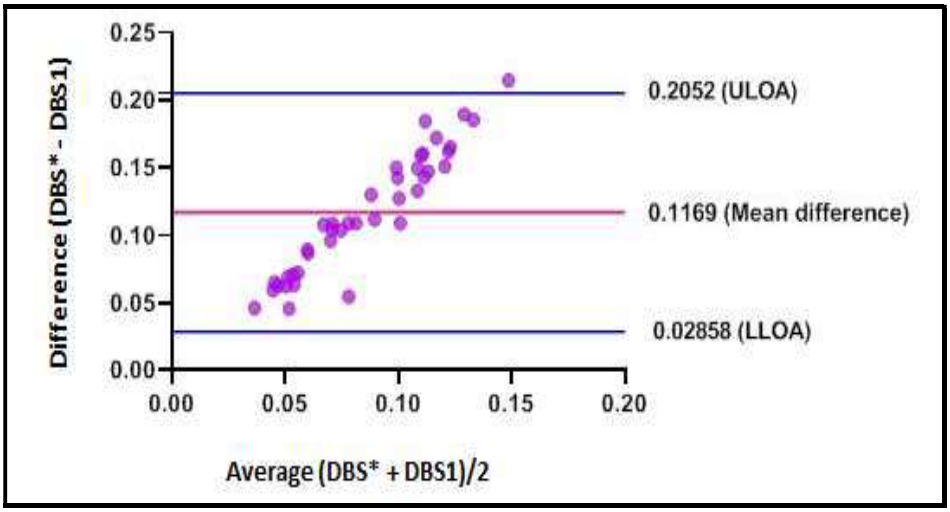


(Figure 38E) apoB/apoA conc. correlation coefficient (r = 0.1438) between DBS* & DBS5

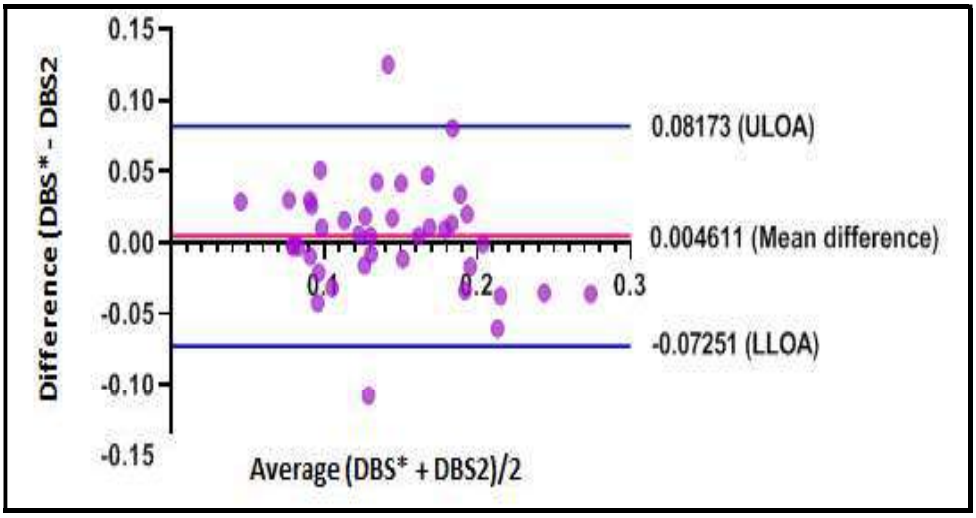


(Figure 38F) apoB/apoA conc. correlation coefficient (r = 0.6502) between DBS* & DBS6

Figure 39[66]. Prepare Bland-Altman graph, a plot of difference of apoB/apoA concentration between DBS* and all DBS samples stored at 4^0C & -20^0C.

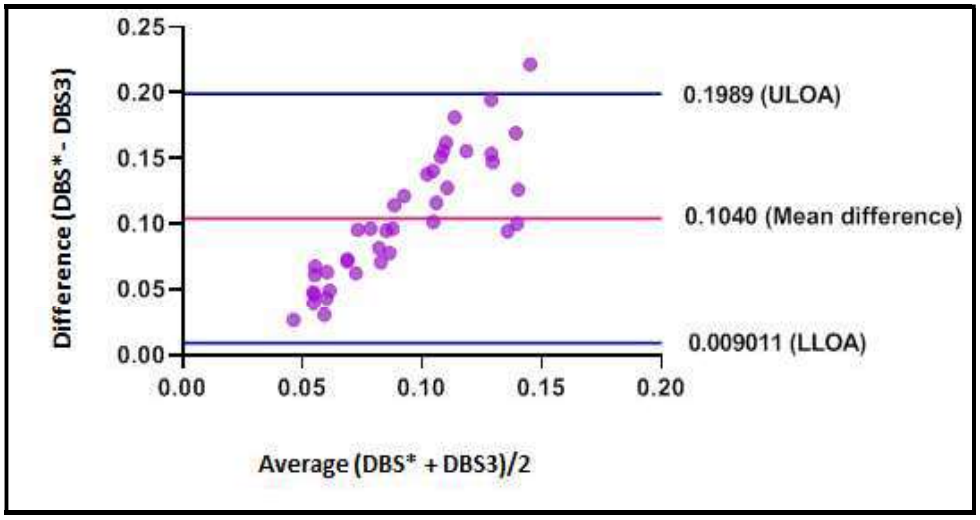


(Figure 39A) BA plot of apoB/apoA ratio between DBS* & DBS1

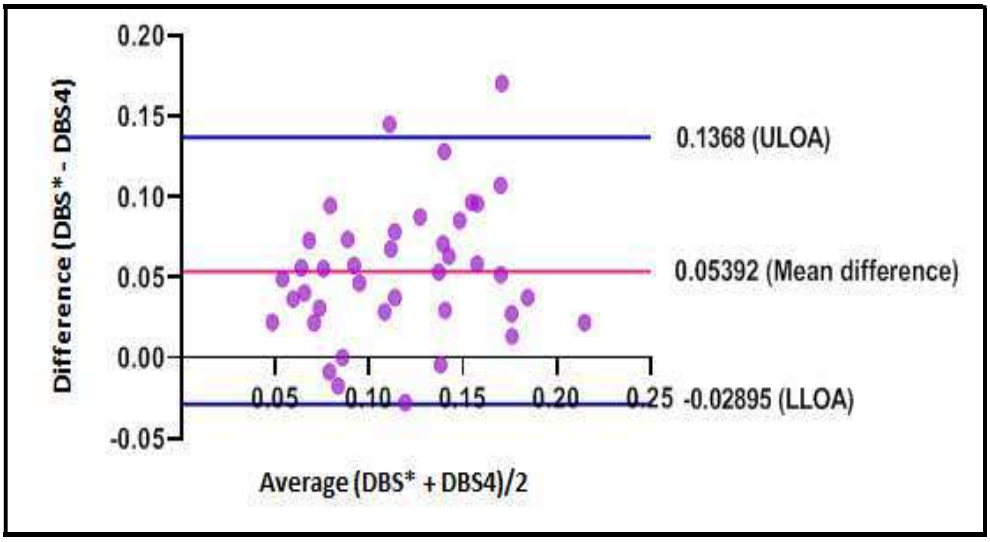


(Figure 39B) BA plot of apoB/apoA ratio between DBS* & DBS2

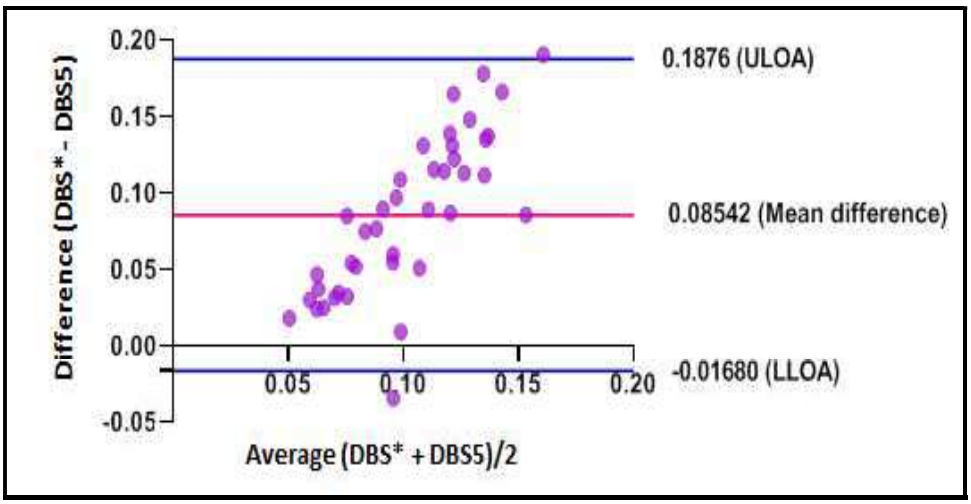
[66] Bland-Altman plot (GraphPad) of apoB/apoA ratio created to observe the bias (mean difference) between DBS* stored at standard condition and DBS1, DBS2, DBS3, DBS4, DBS5 and DBS6 separately



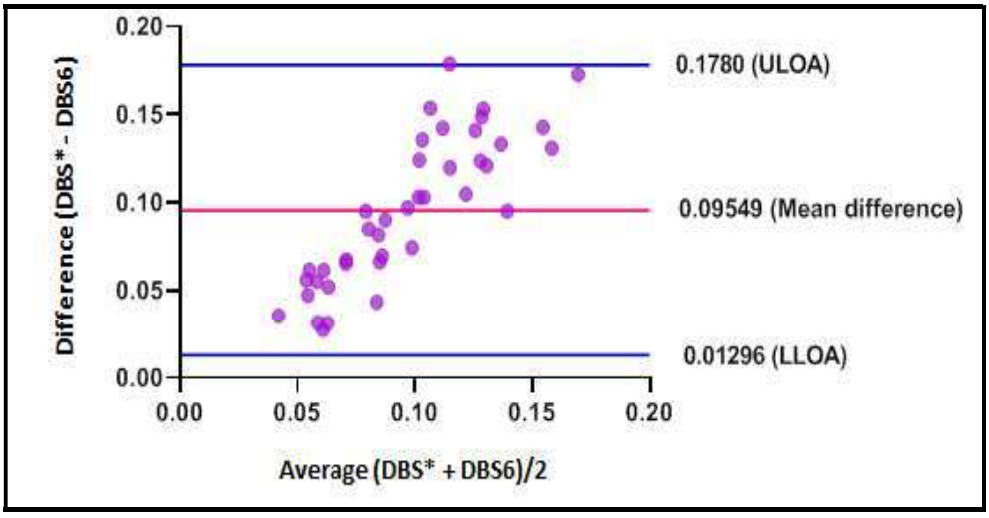
(Figure 39C) BA plot of apoB/apoA ratio between DBS* & DBS3



(Figure 39D) BA plot of apoB/apoA ratio between DBS* & DBS4



(Figure 39E) BA plot of apoB/apoA ratio between DBS* & DBS5



(Figure 39F) BA plot of apoB/apoA ratio between DBS* & DBS6

Table 45. Compare percentage change in apoB/apoA ratio between gold-standard (plasma & DBS*) and all DBS samples stored at 4^0C and -20^0C.

Samples at standard condition	Percentage change in DBS* as compared to plasma		Percentage change in DBS as compared with samples stored at standard condition					
	-80^0C		**4^0C**				**-20^0C**	
	Plasma/WB	DBS*	DBS1	DBS3	DBS5	DBS2	DBS4	DBS6
Plasma/WB	0%	45%	89%	84%	77%	47%	66%	81%
DBS*	45%	0%	80%	71%	59%	3%	37%	66%

3.5B.2 Discussion: apoB/apoA ratio evaluation from DBS

Apolipoproteins are the proteins that bind with lipids and form lipoproteins. They transport lipids in the blood, cerebrospinal fluid and lymph. Atherosclerotic vascular disease is a major cause of morbidity and mortality in the Western world (Jialal, 1998). The ratio of apolipoproteins (apoB/apoA) is a potent & robust biological marker for the prediction of cardiovascular disease (CVD)(Eick et al., 2017; Ogedegbe et al., 2002; Tamang et al., 2014). Apolipoproteins are not only associated with the prediction of risk of CVD but apoB/apoAI ratio is significantly associated with insulin resistance in non-diabetic subjects (Sierra-Johnson et al., 2007). Apolipoprotein ratio (apoB/apoA) is a robust and potential biomarker for cardiovascular disease risk prediction (Ogedegbe et al., 2002). There is another study conducted in Nepal, apoB/apoA-I ratio is statistically a better predictor of cardiovascular disease (CVD) than conventional lipid profile (Tamang et al., 2014). In a study researchers have calculated correlation coefficient between plasma and DBS of apoB (r = 0.722), apoA (r = 0.851) and their ratio apoB/apoA (r= 0.953) (Hirst & Beswick, 1993).

Apo A-I is associated with high-density lipoprotein and its concentration in plasma is inversely related to a predisposition to coronary artery disease (CAD). Apo B is responsible

for the binding of low-density lipoprotein to the receptors and for transporting cholesterol to the cells. Apolipoprotein B (apoB100) is a protein that plays a role in moving cholesterol around your body. Increased apoB levels in the blood are linked to the development of CAD. A drop in the apoB/apoA ratio is linked to an increased risk of coronary heart disease. In this study, we have focused on the comparison of apoB/apoA ratio obtained from DBS with plasma. The stability of apolipoproteins on DBS is a major concern.

Correlations of apoB/apoA ratio between plasma and DBS

Good correlation coefficients (r = 0.8046) obtained between plasma and DBS* (apoB/apoA) at standard condition. But there is large variation obtained during comparison of all DBS transported at different time period & stored at 4^0C, and -20^0C. Generally, proteins becomes denatured when it is stored at RT or 4^0C or even -20^0C because it needs cold chains to maintain their intactness. In our study, we have obtained good apoB/apoA ratio correlation coefficient between DBS* & plasma (r = 0.8046) stored at standard condition, whereas moderate correlation observed at 4^0C in decreasing order DBS1 (r = 0.579), DBS3 (r = 0.4267), DBS5 (r = 0.0663), while at -20^0C it is DBS2 (r = 0.4343), DBS4 (r = 0.6088) and (r = 0.5440).

At standard condition we get good correlation with plasma, on other hand we have also measured correlation coefficients between all DBS with DBS* stored at standard condition. Correlation of apoB/apoA ratio at 4^0C storage between DBS* & DBS1 (r = 0.6763), DBS3 (r = 0.3509), DBS5 (r = 0.1440), whereas at -20^0C it is between DBS* & DBS2 (r = 0.7501), DBS4 (r = 0.6162) and DBS6 (r = 0.6502). It is clearly observed that at 4^0C correlation coefficient decrease as time period of transport duration increases but at -20^0C, correlation before 24 hours of transportation is good but after 24 hours of transportation is satisfactory.

The stability and intactness of apolipoproteins (apoB,apoA) decrease with time on dried blood spot samples. Results shows, apolipoproteins are stable at -20^0C for a shorter period

than 4^0C when DBS is transported within 24 hours of transport duration, but for longer period DBS should be stored at -80^0C. DBS can be utilized in large scale field epidemiological research to monitor the health status of the population by measuring the apolipoprotein (apoB/apoA) ratio to predict the risk of developing CVD.

The stability of apolipoproteins in dry form on filter paper is quite low as compared to *H. pylori* IgG antibodies. We have observed a very low concentration of apoB as compared to apoA, we have not studied the etiology of apoB for their low concentration, but the satisfactory result obtained in the correlation coefficient of apoB/apoA ratio. The dilution factor for the estimation apoA (1:100000) and apoB (1:20000) used as per the recommendation of kit manufacture. Positive good correlation coefficient observed between DBS & plasma samples, but this correlation can be enhanced if DBS stored at -80^0C within 24 hours of transport duration. We have standardized the apolipoprotein concentrations at these dilution factors, but further investigation needed to clarify the perfect dilution factor for these lipoproteins so that they can give the similar results as given by their corresponding gold standard samples (plasma). We have a focus on their ratio because apoB and apoA lipoproteins are robust biomarkers for cardiovascular disease and their ratio reveals the health condition at the population level. Therefore in epidemiological studies, it is a very important risk factor for non-communicable diseases.

Chapter 4
Guidelines: Dried Blood Spot

4.1 Development of guideline for preparation, transportation and storage of Dried Blood Spots

DBS act as an alternative source of blood collection in resource-limited settings & feasibility can be exploited for large scale epidemiological studies. It requires a very small amount of blood depends upon the diameter of the circle on the blood collection card. The volume of blood varies as per the diameter of the circle, approximately 30µl – 200µl blood spotted per collection area and overall 150µl - 1000µl per collection card. Here we have developed the guidelines for preparation, transportation, and storage of DBS cards based on results we have obtained.

We have previously described the preparation procedures of DBS. Such as DBS prepared from capillary blood and venous blood. But in this section, we will have a focus on what are the major challenges faced during the preparation of DBS and what precaution we must be taken before the start of DBS preparation.

4.2 Dried Blood Spot Preparation

Materials required for DBS preparation

1. Blood sampling cards: Many readymade biological specimen collection cards are available such as Whatman 903 protein saver cards, Alhstrom 226 card, FTA cardsm DMPK etc.
2. Gas-impermeable storage bags (ziplock)
3. 1-2 gm desiccant packs which is used as an adsorbent, which helps to remove complete moisture from blood spots on cards.
4. Whatman cards drying rack, used for air dry of wet blood spots on cards
5. Humidity and temperature indicator device
6. Powder free gloves
7. Waterproof marker

8. Vacutainer evacuated blood collection tube: EDTA blood collection tube, which contain anticoagulant which prevent blood from clot.
9. Tourniquet: A device which apply pressure on to limb or extremity to flow of blood, but not to stop blood.
10. Bandage/Plaster
11. Labelling of collection cards: DBS collection cards should be labelled with sample ID, gender, Date of blood collection, temperature, humidity, sometimes barcode is also used for complete information of blood sample collected on cards.

4.3 Collection of Venous blood using EDTA tube

1. The suction method is used to fill vacutainer blood collection tubes with a specified volume of blood. Rubber stoppers are color-coded to indicate which additives are present in that tube. A total of 4 mL of blood was taken. Approximately 50 µl of blood were required for each spot, with 250-280 µl required for a single card.
2. Label the tubes with the subject ID, Name, Gender, Age, Date of blood collection, Time of blood collection, Temperature, Humidity.
3. Fill the tube with venous blood, invert the tube with gentle mixing so that anticoagulant can work properly.
4. Apply 45 - 50µl blood over the circle of collection card with the help of pipette, and make sure the tip should not touch the substrate material onto the circle.
5. Dispense the blood in the center of circle, so that it can spread homogenously over the entire circle and it should not go outside the circle.
6. Make sure, blood should be spread on both side of collection card and do not touch the spot with naked finger or with tip.
7. Note time period gap between collection of blood in EDTA tube and preparation of DBS, because this time gap makes the difference in your final results.

8. Never overloaded the blood again on the same spot, otherwise it may be invalid blood samples
9. Always be careful for invalid samples, try to make valid DBS samples with correct measurements.

4.4 Drying of Blood Spot Specimen

1. After preparation of DBS cards, it should be air dry without covering flap of spot in a clean and dry place which is protected from insects, rodents.
2. Do not dry the spots with hot blower, dryer, directly exposed to sunlight, do not apply any heat.
3. In case of higher humidity, card should be dry overnight to prevent infection
4. The surface of blood spot should not touch with any other surfaces
5. Humidity and temperature must be recorded while drying process
6. We have to dry these blood spots at room temperature.

4.5 Transportation of Dried Blood Spots Specimen

1. Transportation of DBS is always challenging and is depend on your research objectives
2. Few studied have been done on transportation of DBS on an ambient temperature/room temperature
3. But transportation of DBS on room temperature can't give all the results correctly.
4. Transportation time of DBS is very important, in our study we have used transportation time for upto 3 days. Here we are interested to know how transportation time of DBS on room temperature will affect the stability of analyte on DBS.
5. Many studies have been done so far on dried blood spot to evaluate its utility in blood based research. But all the analyte will never be stable on single temperature, I have

seen from my results, there is large variation observed in results with long time period of transport duration.

6. DBS can be transported at all temperature such as at room temperature, 0^0C, 4^0C, -20^0C & -80^0C but the transportation of DBS always depend on which type of analyte you want to study from DBS.
7. After air drying of DBS, it should be packed in airtight ziplock bag with 1-2 gram desiccant sachets. Only single whatman card packed per bag. Ziplock bag also labelled with subject ID, Date of blood collection, Gender.
8. In our study we have found some variation in concentration of gDNA and IgG antibody but large variation observed in concentration of apolipoproteins.
9. As per result indicated, we can transport DBS at room temperature for gDNA and Infection study but not for apolipoprotein study. For protein study, we need to transport it in portable -20^0C device.

4.6 Storage of Dried Blood Spot Specimen

1. Even within 3 days of transport duration of DBS, we have not found such a huge difference in gDNA concentration. gDNA is stable and more robust on dried blood spots even on storage at either 4^0C or -20^0C for a shorter period.
2. The stability of IgG immunoglobulins is more robust on dried blood spot even after storage either at 4^0C or -20^0C within 3 days of transport duration. Excellent sensitivity and specificity observed at both the storage temperature. Stability of IgG is more in dry form on DBS & can be utilized to test infectious pathogens such as viruses or bacteria by testing IgG concentration against their infection. We have found a very good correlation (>85%) coefficient between plasma and DBS stored at 4^0C & -20^0C.
3. Estimation of apolipoproteins from DBS is very challenging, because their stability on DBS is very less. 84% correlation coefficient estimated for apoA at standard

condition between DBS* & plasma, while comparing at 4^0C with 3 transport duration we have obtained (r = 0.47, r = 0.29 & r = 0.26) and at -20^0C (r = 0.33, r = 0.40 & r = 0.35). But for apoB, 64% correlation coefficient at standard condition whereas at 4^0C (r= 0.37, r= 0.40 & r=0.18), and at -20^0C (r=0.56, r=0.39 & r=0.33). At -20^0C, the stability of apolipoproteins are higher than at 4^0C. We will highly recommend, for designing any epidemiological research we should store DBS cards either at -20^0C or at -80^0C before 24 hours of transport duration.

4. We have mentioned the transport duration and storage condition of DBS based on our study results. Similarly, today DBS is used in various fields to explore the utility of biomarkers such as molecular markers, drug discovery, cytokines measurements, heavy metals and many more but their stability depends on transport duration and storage conditions.

Figure 40. Package of Dried Blood Spots in Ziplock bag

4.7 Factors influence DBS characteristics

4.7A Temperature

Temperature play lead role during DBS preparation, transportation and storage. The integrity of blood-based biomarkers affected inside and outside the body by changing environmental conditions such as temperature, weather, and humidity. The stability of an analyte in dry form depends on how much we maintain a cold chain to resist the integrity of analyte. Some researcher's show it should be stored at ambient temperature to eliminate the need for freezing, resulting in a significant saving in shipping and storage cost (Xu et al., 2013).

4.7B Hematocrit effect

It is defined as the volume of red blood cells to the total volume of blood. Previously described hematocrit values differ as per age and gender (Table 1). During the preparation of DBS, percentage of hematocrit always interfere in preparation steps because if hematocrit volume is large then blood will not be continuously spread onto blood collection card and vice versa, which results in the formation of valid and invalid blood spots.

4.7C Humidity effect

Effect of humidity on DBS is another challenging risk because due to humid condition the DBS cards cannot dry properly and it could be infected or contaminated. To remove moisture on DBS cards, blood specimens packed with silica-based desiccant sachets to absorb complete moisture from blood spots, but sometimes due to improper handling and precaution, these DBS cards become invalid & we cannot use it further.

4.7D Quality assessment of DBS

The quality of DBS is based on its validity. It is a very important aspect that, DBS samples should be good in condition, if there are any incomplete blood cards then probably it should be rejected because due to incomplete recovery of eluate for our study and might be chance

we will not get valid results. Quality of DBS is a major before analyzing DBS cards for biomarker measurements.

Chapter 5
Conclusion and Future Perspective

5.1 Why Dried Blood Spot Methodology

Although the DBS methodology offers advantages over venipuncture, certain aspects of DBS should be considered before its implementation within a study. Due to their higher variability as compared to venipuncture, some DBS assays applicable at population-level, public health research, while venipuncture sampling remains the gold standard for blood-based, diagnostic testing of individuals.

As new biomarkers become available for clinical standard measurement, they will first need to be adapted to DBS assays before they can be reliably measured by DBS. However, this opportunity to expand biomarker DBS serum-equivalents illustrates the flexibility of this new methodology. In addition, it should be noted that some labs currently lack a DBS sample protocol or the capacity to analyze DBS samples while maintaining clinical applicability through validation and verification(Mcdade, Williams, & Snodgrass, 2007). Here are costs associated with the DBS methodology, including the purchase of laboratory grade freezers for storage and DBS protocol training for non-medically trained staff(Mcdade et al., 2007). Nonetheless, the total costs of DBS are generally lower than those of serum venipuncture methods.

5.2 Limitations to the Study

It is possible that increasing the number of sample set aside for quality control may have resulted in a more accurate study-specific DBS-to-serum equivalence relationship. In addition, although our current paired sample size (**n=80**) was of sufficiently good quality, additional samples used may have produced more strongly correlated equivalence relationships. We have performed apolipoprotein experiment serologically (ELISA) while other methods are also available (Wang, Wilcken, & Dudman, 1991; N. Wong, Beeso, Sherwood, & Peters, 1995). We have performed the experiment after 4-5 months of DBS collection. Detergents present in assay buffers attenuates the decrease in apolipoproteins concentration (Ojha et al).

5.3 Importance to the Biomarker Research

This study provides the validation for the use of DBS methodology for gDNA, Infectious pathogen and apolipoproteins (apoA &apoB). No doubt, DBS does exhibit greater variation and assay error for the tested analytes, suggesting that DBS may have greater utility in group or population studies but may be less useful in individual clinical decisions than standard venipuncture samples. A particular strength of the present study was the demonstration through the use of time controls that DBS samples remain fairly constant over time.

The DBS method allows for biomarker assays to be implemented in studies using larger, community-based samples and those that target more vulnerable populations, broadening the applicability and generalizability of biomarker use in CVD research. Over the past decades, the DBS technique has established as a significant tool in epidemiological research, new born screening and drug discovery & monitoring.

5.4 Future perspective

In coming years, future of using DBS will be increases in large scale field epidemiological studies at population level. We have to plan to increase our work further to elaborate the testing of biochemical parameters such as HbA1c, total cholesterol, heavy metals, other infectious pathogens, hormones etc with the same manner as we did in this study to find out how much difference we observed while multiple groups comparison at different transport duration to establish correct transport duration with their stability.

5.5 Conclusion of the study

DBS could be a remarkable tool to measure the analytes/biomarkers (genomic DNA, antibodies raised against infection, apolipoproteins) even if it is stored at different thermal condition (4^0C or -20^0C) with varying transport duration up to 72 hours. The positive result showed that, the stability of biomarkers & feasibility of DBS can be utilize to conduct large

scale field epidemiological studies to know the disease burden & health challenges in community.

5.6 Implications of the study with recommendation

This study was conducted to analyze the utility & feasibility of Dried Blood Spot (DBS) in field epidemiological studies when there is limited availability of infrastructure to collect & process the venous blood. DBS has been used in research for 2-3 decades, specially for testing of phenylketonuria in neonates. Validity of DBS samples, preparation methodology, temperature, humidity, transportation time from one place to other, storage thermal condition all these parameters have some impact on analyte stability in DBS samples. With address to above parameters, our major aim & objective is to whether DBS can be useful tool to detect important biomarkers if is stored at different thermal condition within 0-24, 24-48 & 48-72 hours of transport duration. Because there is no such study is conducted where they show how the stability of biomarkers affected when DBS stored between 0-72 hours after preparation. Single analyte (genomic DNA, IgG antibody and apolipoproteins) compared 6 times with the reference standard to evaluate how the variation observed in the concentration of analyte, probably focus on which storage thermal condition & transport duration will be best for the DBS to answer many research questions. In this study, we observed good result for extraction of gDNA from all DBS at all stored temperature & transport duration but statistically mean concentration of gDNA at 4^0C storage slightly greater than at -20^0C, similarly, excellent result observed for detection of IgG antibody concentration against H. *pylori* at all stored temperature with sensitivity >87% & specificity >90%. Whereas, there is more variation seen in stability of apoliproteins with transport duration, the concentration of apolipoproteins decreases gradually with increase in transport duration from DBS preparation site to thermal storage site. Final recommendation for those researchers who wish to work

with this DBS technology for these analytes, you must store the DBS within 24 hours of preparation for comparable results with your reference standard.

5.7 Funding Source

This research study was funded by Tata Memorial Centre, Mumbai.

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61st Annual National Conference of
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Optimization of Extraction of Human Genomic DNA from Dry Blood Spot & its Application in Large Scale Epidemiological Studies

during the **61st Annual National Conference of Indian Public Health Association (IPHA) & First State Conference of IPHA Rajasthan** held from **24th Feb, 2017** to **26th Feb, 2017** organized by **Department of Community Medicine and Family Medicine, AIIMS Jodhpur, Rajasthan**.

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S. Chiplunkar

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RESEARCH ARTICLE

REVISED Optimization of extraction of genomic DNA from archived dried blood spot (DBS): potential application in epidemiological research & bio banking [version 3; peer review: 2 approved, 1 approved with reservations]

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Abstract

Background: Limited infrastructure is available to collect, store and transport venous blood in field epidemiological studies. Dried blood spot (DBS) is a robust potential alternative sample source for epidemiological studies & bio banking. A stable source of genomic DNA (gDNA) is required for long term storage in bio bank for its downstream applications. Our objective is to optimize the methods of gDNA extraction from stored DBS and with the aim of revealing its utility in large scale epidemiological studies.
Methods: The purpose of this study was to extract the maximum amount of gDNA from DBS on Whatman 903 protein saver card. gDNA was extracted through column (Qiagen) & magnetic bead based (Invitrogen) methods. Quantification of extracted gDNA was performed with a spectrophotometer, fluorometer, and integrity analyzed by agarose gel electrophoresis.
Result: Large variation was observed in quantity & purity (260/280 ratio, 1.8-2.9) of the extracted gDNA. The intact gDNA bands on the electrophoresis gel reflect the robustness of DBS for gDNA even after prolonged storage time. The extracted gDNA amount 2.16 – 24 ng/µl is sufficient for its PCR based downstream application, but unfortunately it can't be used for whole genome sequencing or genotyping from extracted gDNA. Sequencing or genotyping can be achieved by after increasing template copy number through whole genome amplification of extracted gDNA. The obtained results create a base for future research to develop high-throughput research and extraction methods from blood samples.
Conclusion: The above results reveal, DBS can be utilized as a potential and robust sample source for bio banking in field epidemiological studies.

Open Peer Review

Reviewer Status ✓ ✓ ?

	Invited Reviewers		
	1	**2**	**3**
REVISED **version 3** published 14 Nov 2019	✓ report	✓ report	? report
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Keywords
Dried blood spot (DBS), Whatman 903 cards, FTA cards, Human genomic DNA, Bio-banking, Epidemiology

Any reports and responses or comments on the article can be found at the end of the article.

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REVISED **Amendments from Version 2**

Revised the article as per comments raised by 2 reviewers. Made some corrections in subject enrolment section, Table 1, Results section, Table 2, Table 3, Figure 3 & add statistical analysis section.

Any further responses from the reviewers can be found at the end of the article

Introduction

The concept of using dried blood spot (DBS) in new born screening was presented by Guthrie and Susie in 1963[1]. DBS has been used for the last 5 decades by researcher in medical research. In field epidemiological studies there is a need for robust sample sources so they can be stored for long periods without any damage or spoilage. DBS is a much better option as compared to venous blood in low resource field setups for large epidemiological studies. Biomarkers reveals biological information from normal to disease condition and provide information about the disease condition, as it also acts as a prognostic marker. The collection of DBS is simple compared to venous blood collection as it only requires a finger prick, compared to venous puncture via needle for venous blood collection. Today DBS samples are utilized to test for a variety of health related markers including; infectious pathogens, HbA1c, total cholesterol, creatinine, uric acid, low density lipoprotein (LDL), high density lipoprotein (HDL), very low density lipoprotein (VLDL), Triglyceride and many more[2–4]. DBS is prepared by spotting 40–50µl of whole blood on Whatman 903 protein saver cards, air dried for 2 hours by hanging or by placing in a rack, and then packed it in sealed ziplock bags with desiccant. Other cards available for blood sampling include Ahlstrom, Whatman No.6, DMPK, FTA etc. however the Whatman 903 card is US Food and Drug Administration (FDA) approved for medical research[5]. In epidemiological research the protein saver cards act as information storage devices in terms of blood based analytes and provide genetic, environmental, immunological information. Genomic DNA (gDNA) is a very robust and stable biological sample when stored on paper cards, and has been used for many decades[3,4]. RNA, which is less stable than gDNA in solution, appears to also be stable on DBS[6]. Due to the small amount of blood in DBS, the obtained concentration of genetic material is also low but this problem can be overcome by amplification of the whole genome, and yield high quality DNA for performing assays, such as sequencing and genotyping arrays, at low cost[7]. Limited studies are available regarding the use of DBS for downstream SNP genotyping following whole genome amplification[6,8].

This study was performed with the aim of extracting maximal gDNA using archived DBS cards obtained from the Centre for Global Health Research (CGHR) Bangalore unit to establish its feasibility for downstream applications and biobanking in large scale epidemiological studies.

Methods

Ethical considerations and consent

The study was ethically approved by Institutional Ethics Review Board (IERB) of St. John's Medical College and Hospital, Bangalore (India) with approval number IERB/1/77/05. After explaining the study to participants, informed written consent (as per norms of Indian Council of Medical Research (ICMR) Government of India) was obtained from volunteer participants.

Subjects enrollment

This was part of a multicenter study involving the Centre for Global Health Research (CGHR) Bangalore unit and Tata Memorial Centre, Mumbai unit, who worked together to conduct study DBS. DBS samples were collected at health checkup camps in rural and urban areas of Bangalore city through a sample registration system (SRS). 3000 DBS samples were prepared during health checkup at Bangalore Centre. DBS samples were collected between years 2005–2007 & stored at 4°C, but later on it transported to Mumbai at ambient temperature in year 2013 while laboratory experiments were conducted in year 2016. DBS samples were prepared through finger prick method by using lancet (Accu Chek Softclix Lancet, Roche), puncture the finger site using lancet, drop of blood form which is lightly touch the circle of filter paper cards (GE Health Care Life Science, Catalog no. 10534612) and form valid DBS during health checkup camp by CGHR at Bangalore unit and transported to Tata memorial Centre (TMC) Mumbai for further analysis. Samples were collected between the years 2005–2007, but samples transported to Mumbai from Bangalore at ambient temperature in year 2013 and laboratory experiments conducted in 2016. DBS samples were collected by trained staff. Systematic random samples (n=40) were selected from top to bottom order from collected DBS. The following anthropometric measurements were recorded; height, weight, waist-to-hip ratio, blood pressure with gender and age. The complete study was explained to the subjects, and only voluntary participants aged between 18–49 years were included in this study after obtaining written informed consent as per the norms of Indian Council of Medical Research (ICMR) Government of India.

Sample collection, transport and storage

Finger prick blood DBS Preparation. Finger prick blood was collected using a lancet to puncture the fingertip. Once a full small drop of blood is formed it was lightly touched to the center of the circle on the filter paper (GE Health Care Life Science, Catalog no. 10534612) to form valid DBS. These were collected from study participants during health checkup organized at government schools, and at the center of villages. Cards were dried for 2 hours in velcro rack and packed in sealed ziplock bag with 1–2gm desiccant sachet (Figure 1). Only valid DBS samples were used for gDNA extraction determined by the blood sample completely saturating each circle on the card, and not overlapping or merging with other blood circles. Prepared DBS samples were transported to the laboratory at the Centre for Cancer Epidemiology, Tata Memorial Centre, where they were stored in a -80°C refrigerator.

Sample quality & validity

We have used only good quality DBS samples for gDNA extraction, it is defined as the complete saturation of whole blood over the complete circle of blood collection card[9], blood card should be labelled and blood should adsorb on both side of the card. We have used only valid samples for gDNA extraction (Figure 2).

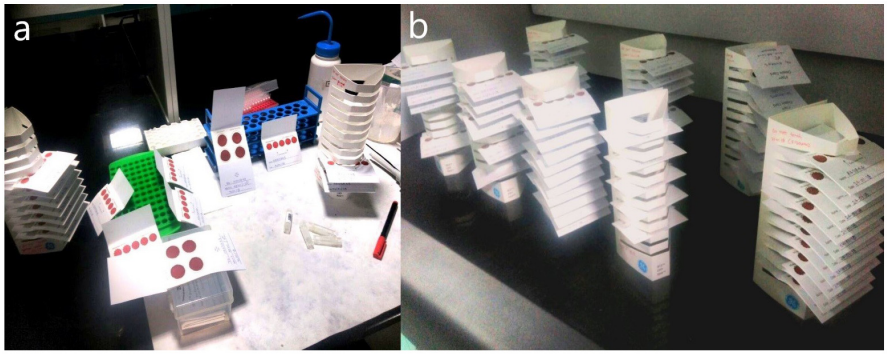

Figure 1. Preparation and drying of dried blood spot (DBS) cards. (**a**) Prepared DBS cards. (**b**) Drying of blood spots at room temperature.

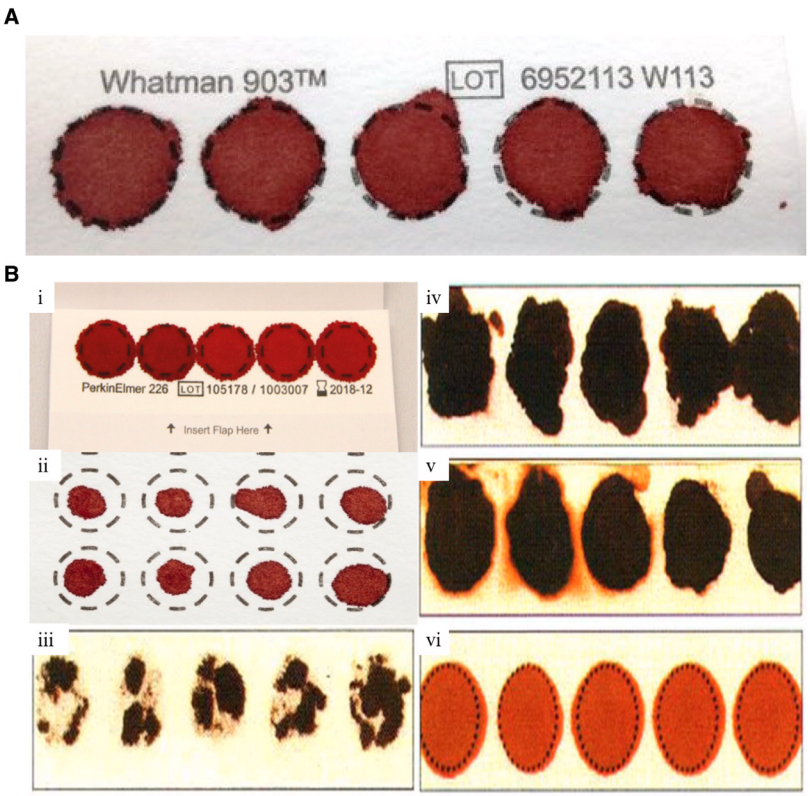

Figure 2. Valid / Invalid dried blood spot (DBS) specimens. (**A**) **Valid specimen**. DBS with complete filled circle with proper air dry with no hemolyzed blood or serum ring. (**B**) **Invalid DBS Specimen**. (**i**) DBS with overlapping of spotted blood. (**ii**) DBS with insufficient filled blood. (**iii**) DBS with incomplete absorption which reduces blood volume. (**iv**) DBS that is potentially rubbed and develop scratches. (**v**) DBS with hemolyzed or contaminated blood. (**vi**) DBS with improper air drying before packaging in ziplock bags.

DBS processing

A generic single hole 6mm punch plier was used to cut the blood spots from the Whatman 903 paper card. A punch of diameter 6mm represents approximately 8.7±1.9µl of blood spotted[10]. 1 – 4 blood spots of size 6 mm punch was added to an eppendorf tube and incubated with 200µl PBS (readymade PBS buffer used with pH 7.4 supplied by Gibco with Ref No. 10010-023) overnight at room temperature. Our major aim was to extract the maximum amount of gDNA, therefore we have used 6mm × 1 spot to 6mm × 4 spots for extracting gDNA from DBS (Table 1). We have used phosphate buffer saline (PBS) for extraction of completely dry blood matrix on Whatman 903 for easy gDNA extraction, because once the surface of blood spot becomes wet, it is easy to extract DNA from the adsorbed blood.

Table 1. Average genomic DNA (gDNA) concentration with different number of blood spots.

Number of blood spots used of size 6mm	Average gDNA concentration (ng/µl) + SE^{M}	Average 260/280 ratio	Total elution volume	Total gDNA Yield
6mm × 1 spot (n=10)	3.43 ± 0.2893	1.93	30µl	102.9 ng
6mm × 2 spots (n=10)	6.38 ± 0.3540	2.18	30µl	191.4 ng
6mm × 3 spots (n=10)	7.23 ± 0.2491	2.30	30µl	216.9 ng
6mm × 4 spots (n=10)	8.91 ± 1.6863	2.84	30µl	267.3 ng

Genomic DNA extraction methods

We have applied 2 methods for gDNA extraction from DBS. (**1**) Column based (QIAamp DNA kit, Qiagen) (**2**) Magnetic bead based (ChargeSwitch Forensic DNA Purification Kit, Invitrogen).

Column based gDNA extraction from DBS. We used the QIAamp DNA kit (Qiagen, Catalog no. 56304). 1 – 4 blood spots 6mm in size were added with 180 µL of cell lysis buffer ATL (Lysis buffer supplied with Qiagen kit), and incubated in a waterbath (Trishul Equipment, Sr. No. 5460311) at 85°C for 10 min. 20 µL Proteinase K was added and incubated it at 56°C for 1 hour to denature the proteins. 3–4 µL RNAse was added immediately after to degrade RNA, then 200 µL buffer AL (Lysis buffer supplied with Qiagen kit) was added & mixed thoroughly by vortexing and incubated at 70°C in a waterbath (Trishul Equipment with Sr. No. 5460311) for 10 min. Buffer AL helps in complete cell lysis and binding of gDNA with the silica gel of the column provided in the Qiagen kit. gDNA was then immediately precipitated by adding 200 µL of 70% v/v ethanol. The solution was then transferred into a spin column (supplied with Qiagen kit) and centrifuged (Eppendorf 5810R) at 8000 rpm for 1 min. The spin column has the capacity to load approximately 600 microliter sample at a time, but generally we had approximately 1.2 or 1.4 ml of solution, we therefore performed the process 2–3 times. In this process gDNA becomes bound with the column, impurities are then washed out with 700 µL buffer AW1 (Wash buffer with a low concentration of quinidine) followed by 700 µL of AW2 (Wash buffer with Tris based ethanol solution used for removal of salts) buffer. Buffers AW1 & AW2 remove unwanted impurities from the gDNA. The empty tube was then centrifuged at 14000 rpm for 3 min for complete removal of ethanol from the gDNA. Finally the gDNA was eluted with 30 µl of pre-incubated elution buffer (AE).

Magnetic bead based gDNA extraction from DBS. We used the ChargeSwitch Forensic DNA Purification Kit (Thermofisher, Catalog No. CS11200). Processed DBS samples were added to 1ml lysis buffer with 10 µL of Proteinase K in a tube, vortexed, and incubated at 55°C in a waterbath (Trishul Equipment Sr. No. 5460311) for 1 hour. Blood spots were removed after complete cell lysis and 200 µL of purification buffer added, followed by 20 µL magnetic beads. The solution was then gently mixed, left for 5 minutes, and then incubated on a Magna Rack (Thermofisher, catalog no. AM10027) for 1 minute. The supernatant was removed after complete binding of the pellet to the magnet of the Magna Rack. The pellet containing gDNA was then washed with 500 µL wash buffer (W12) 3 times and finally DNA eluted with 30–60 µL Elution Buffer (E5). We have followed the protocol as per manufacturer recommendation with some modifications; incubation time was increased from 4 hours to overnight for complete extraction of eluate from filter paper, and we also increased the time for proper binding of gDNA with the spin column.

Genomic DNA quantification

Concentration of extracted gDNA was measured using a Qubit 3.0 fluorometer and purity was measured by a spectrophotometer. Quality and integrity of gDNA was checked by performing a 0.8% Agarose gel electrophoresis.

Qubit 3.0 Fluorometer (Thermofisher, Catalog No. Q32850) was used to measure gDNA concentration by taking 1 µl gDNA sample in 0.2ml PCR tube with 200 µl buffer (199µl dsDNA BR buffer + 1 µl Ethidium Bromide dye) after proper mixing for 1min. Concentration was then measured with a fluorometer.

A spectrophotometer at wavelength 260/280 ratio (NanoDrop 2000 Thermofisher) was used to check purity of gDNA, by applying 1 µl gDNA sample directly to the device and measuring the 260/280 ratio.

Agarose gel electrophoresis was performed following preparation of a 0.8% agarose gel which was loaded with 5 µl gDNA in wells and run at 70–80 volts for 2 hours. A gel photograph was captured using a gel dock (Model: UVP EC3-Imaging System).

Statistical analysis

All the data analysis done by using excel to calculate average, total yield and standard error of mean (SE_M: SD/√N), where SD is standard deviation, N is total number of samples

Results

Genomic DNA extraction efficiency

Table 1 showed, average amount of gDNA extracted from DBS with their average yield. In our findings, we observed large variation in the concentration, from 2.16ng/µl to 24ng/µl, of the extracted gDNA (Table 2). The integrity of gDNA was checked using 0.8% agarose gel electrophoresis, and highly intense single bands were observed on the gel (Figure 3). The purity of gDNA was measured at an absorbance of 260/280nm (1.8–2.0) ratio. If it is less than 1.8 or greater than 2.0 it

Table 2. Genomic DNA quantification (gDNA) by Fluorometer on stored dried blood spot (DBS) at standard condition.

Type of filter paper	gDNA concentration range (ng/µl)	Elution volume	Total Yield (ng)
Whatman 903 card	2.16 ng/µl - 24 ng/µl	30 µl	64.8 ng – 720 ng

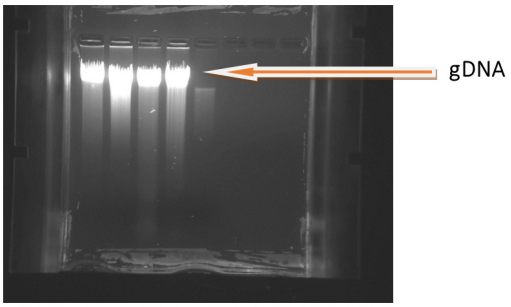

Figure 3. Agarose gel electrophoresis of extracted gDNA from dried blood spots. Figure shows highly intense bands which are mostly intact with little smear. 5µl DNA loaded in lane1 & lane3 with concentration 7.19 ng/µl & 5µl DNA loaded in lane2 & lane 4 with concentration 8.7 ng/µl.

indicates the presence of impurities in the genetic material. gDNA concentration of each DBS with different number of blood spots, with their purity and total gDNA yield are presented in Table 3.

This amount of gDNA extracted can be used for polymerase chain reaction (PCR), and PCR based molecular assays such as PCR based sequencing, PCR based genotyping, but can't be used for whole genome sequencing or genotyping[11].

Discussion

For the last 5 decades, DBS sample have been collected and stored in bio-banks to conduct field epidemiological studies worldwide. DBS collection on filter paper is more applicable and acceptable method in epidemiological research as compared with standard venous blood. The advantage of DBS over venous blood collection include less discomfort for the subject, especially if many samples are needed within a short period of time, only a small amount of blood is needed to perform the assay. Our findings shows that, we can extract the gDNA from dried blood spots. Previously, studies have been performed to compare genomic DNA extraction methods to examine its feasibility in genetic studies[12]. As per obtained results, we have found good concentration of total gDNA, In this study, our target was to improve the maximum extraction of gDNA from DBS. We followed 2 methods; one column based and one magnetic bead based. Before proceeding to cell lysis process, we had treated the blood spots with PBS (pH 7.4) & kept it overnight at 37°C to elute the complete matrix from the Whatman for efficient & complete cell lysis. DNA samples can be stable on filter paper for many years if it is stored in dry conditions[8]. Our main objective was to extract the maximum amount of gDNA from DBS irrespective of methodology use, therefore we have used 2 methods to extract gDNA to evaluate from which method we have got more gDNA but unfortunately, we have not found any difference in gDNA concentration with between both methods. We did assay randomly from both methods with full focus on maximum quantity of gDNA extraction from DBS. We have

Table 3. Genomic DNA (gDNA) concentration of each dried blood spot (DBS) from different number of blood spots.

Total number of blood spots used	DNA Yield (ng/µl)	260/280 ratio	Total Elution Volume	Total Yield
6mm × 1 spot	3.4	1.84	30	102
6mm × 1 spot	3.52	1.9	30	105.6
6mm × 1 spot	4.52	1.8	30	135.6
6mm × 1 spot	4.7	1.39	30	141
6mm × 1 spot	2.16	2.4	30	64.8
6mm × 1 spot	2.19	1.98	30	65.7
6mm × 1 spot	2.12	2.1	30	63.6
6mm × 1 spot	4.66	2.23	30	139.8
6mm × 1 spot	3.56	2.21	30	106.8
6mm × 1 spot	3.47	1.5	30	104.1
6mm × 2 spots	4.98	2.4	30	149.4
6mm × 2 spots	5.95	2.2	30	178.5
6mm × 2 spots	6.36	2.23	30	190.8
6mm × 2 spots	6.4	2.13	30	192
6mm × 2 spots	8.98	2.28	30	269.4
6mm × 2 spots	8.06	2.32	30	241.8
6mm × 2 spots	5.4	2.1	30	162
6mm × 2 spots	5.49	1.82	30	164.7
6mm × 2 spots	6.4	2.15	30	192
6mm × 2 spots	5.82	2.21	30	174.6
6mm × 3 spots	6.86	2.45	30	205.8
6mm × 3 spots	8.08	2.61	30	242.4
6mm × 3 spots	5.72	2.28	30	171.6
6mm × 3 spots	7.13	2.41	30	213.9
6mm × 3 spots	6.94	2.21	30	208.2
6mm × 3 spots	7.18	2.51	30	215.4
6mm × 3 spots	8.7	1.98	30	261
6mm × 3 spots	6.43	2.31	30	192.9
6mm × 3 spots	8.12	2.38	30	243.6
6mm × 3 spots	7.19	1.95	30	215.7
6mm × 4 spots	9.9	2.9	30	297
6mm × 4 spots	10.9	2.84	30	327
6mm × 4 spots	7.6	2.93	30	228
6mm × 4 spots	8.23	2.74	30	246.9
6mm × 4 spots	9.25	2.62	30	277.5
6mm × 4 spots	24	2.89	30	720
6mm × 4 spots	3.36	2.84	30	100.8
6mm × 4 spots	7.62	2.91	30	228.6
6mm × 4 spots	4.17	2.86	30	125.1
6mm × 4 spots	4.12	2.96	30	123.6

tried gDNA extraction with direct cell lysis of DBS by using lysis buffer and also blood spots treated with PBS overnight to complete elution of eluate. We have done these experiments to evaluate gDNA concentration but unfortunately there are no such yield increases with these modifications. As our results show, there is large variation in the concentration and purity (260/280) of extracted gDNA in both the methods. This variation might be due to the small volume of blood, long term storage, loss during assays, cell debris, cellulosic component of the Whatman card etc. In the case of column (QIAamp) based gDNA extraction, 5% loss is predicted by the manufacturer, where as in magnetic bead based DNA extraction, some 5–10% beads are lost during assay, results there is loss of gDNA. The obtained purity also shows variation, 260/280 ratio 1.8–2.9, this might be due to interference of cellulosic components of Whatman paper. It is true that a 260/280 ratio >2.0 indicates impurities. But due to limitation of blood spots we have not increased the number of spots beyond 4. As I have mentioned that we have got DNA concentration in a range 64.8ng – 720ng. This amount of DNA can be used in downstream applications and we can remove the impurities by gel purification method.

gDNA concentration depends on the blood matrix on spots. In a study using DBS stored for 6 years and they found reduced gDNA concentration in quality and quantity.[13], but some other studies reported that gDNA is stable for at least 11 years under ambient tropical conditions[8]. We have performed some modification to the protocol for the extraction, we increased the time for cell lysis, binding of DNA with column & beads, washing with buffer & final elution. Due to regular successive research on DBS, today DBS samples are used for genetic analysis, proteome research, vitamins estimation, infection agent, epigenetic research, nucleic acid research[14–17].

A punch of diameter 6mm represents approximately 8.7±1.9µl of blood spotted. This difference in blood volume from a single spot might be due to the presence of hematocrit, because due to increased percentage of hematocrit in blood, the blood becomes very viscous and it can't spread homogenously over the Whatman circle which results the concentration of DNA and blood on 6mm spot changes accordingly. Composition of hematocrit value influence the gDNA concentration with different number of blood spots. Filter paper contains cellulosic fibers, probably referred as cotton linters, while extracting gDNA from Whatman card, the cellulosic composition of filter paper interfere the concentration and purity of gDNA because during thermal agitation and vortex steps in protocol, these cotton linters are also present in supernatant and interfere with final DNA elution steps.

Our findings show, approximately 64.8ng – 720ng gDNA is extracted from Whatman 903 card >50 ng is sufficient for PCR based applications[18]. DBS extracted gDNA can be used in downstream applications such as polymerase chain reaction (PCR), PCR based sequencing, PCR based genotyping, disease diagnosis, molecular basis of disease etiology and study of genetic variants. the extracted amount of gDNA, however, cannot be used for whole genome sequencing, but this can be overcome by whole genome amplification of extracted gDNA, as this will increase the concentration of gDNA by increasing the copy number of templates. DBS is a suitable and applicable sample source in large scale epidemiological studies and biobanking. Further study is warranted to explore DBS efficiency in high throughput research to reveal other biochemical analytes stability on filter paper card to replace venous blood collection in future epidemiological studies.

Conclusion

Analyte stability on filter paper in dry form is a good biological sample source to perform molecular epidemiological based assays. Dried blood spot on paper card act as a potential and robust sample source for biobanking in large scale epidemiological studies.

Data availability

Data underlying this study is available from Open Science Framework. Dataset 1: Optimization of extraction of gDNA from DBS: Potential application in epidemiological research & biobanking. http://doi.org/10.17605/OSF.IO/FZYTM[19]

Acknowledgements

Authors are thankful to the Director, Tata Memorial Centre, Mumbai, for providing the necessary facilities, Centre for Global Health Research (CGHR) Canada for support to carry out the research on *Dried Blood Spots*. This work was supported by the Bill and Melinda Gates Foundation [OPP1148667].

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Open Peer Review

Current Peer Review Status: ✔ ✔ ?

Version 3

Reviewer Report 05 December 2019

https://doi.org/10.21956/gatesopenres.14229.r28253

?

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Department of Biomedicine, University of Aarhus, Aarhus, Denmark

Kumar and authors compare two gDNA extraction approaches from dried blood spots (DBSs) with the intent to optimize their usability in epidemiological studies.

I have the following comments:

- Description of biomarkers in the introduction is too simplistic. Biomarkers can both be of e.g. prognostic, diagnostic, or predictive value and they are not always associated with disease but e.g. exposure or any normal phenotype. Please revise.
- The statement that there is a limited knowledge concerning the use of DBSs for genotyping is not true. Several published works by the iPSYCH and PGC consortium proves otherwise. Please revise.
- In general references are often outdated, leading to false conclusions. The authors should revise their reference list.
- Describe the process of picking randomized samples in greater detail. Also, the wording “systematic random samples” is contradictive. Please revise.
- Why did the authors record meta-data (height etc.) on the participants? There appears to be no correction for any of these parameters.
- The paragraphs “subjects enrollment” and “sample transportation…” should be merged as essentially the same samples are being introduced twice, making it difficult to follow.
- There seems to be no actual comparison between the two extraction methods applied, which appears strange as this is the main aim? A t-test or ANOVA should be performed (depending on set-up) comparing the two methods.

- Where does the conc. range 64.8-720ng come from? How does it compare to table 1?
- How does the used methods and outcome compare to other used approaches, e.g. the one used by Hollegaard (ref 7 and 13)?
- Rephrase the last paragraph in results section. Sequencing e.g. MeDIP-seq and MBD-seq has been performed on unamplified DBS gDNA and genotyping (as the authors also write) can be done on amplified gDNA. So, both approaches are feasible.
- Removing impurities by gel purification is not an optimal approach as DNA is lost and impurities from the gel material will be present.
- The language in general requires improvement, there are many grammar and spelling mistakes.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
No

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Epigenetics, NGS, Array, Psychiatric Disorders

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 28 November 2019

https://doi.org/10.21956/gatesopenres.14229.r28232

Anubhav Tripathi
Center for Biomedical Engineering, School of Engineering, Brown University, Providence, RI, USA

The revised version addresses all the issues raised by the reviewers.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Applied Genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Reviewer Report 28 November 2019

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Lakshmy Ramakrishnan
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Grammar still needs to be corrected in several places.

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Partly

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry, diagnostics, dried blood technology, epidemiology

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard.

Version 2

Reviewer Report 15 October 2019

https://doi.org/10.21956/gatesopenres.14166.r27922

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Anubhav Tripathi
Center for Biomedical Engineering, School of Engineering, Brown University, Providence, RI, USA

The paper “Optimization of extraction of genomic DNA from archived Dried Blood Spot (DBs): potential application in epidemiological research and bio-banking” is a well characterized study of gDNA extraction from epidemiological samples. However, the reviewer still has some significant concerns with the material presented as outlined below:

Concerns:

- **Innovation:**

  - This paper deals specifically with gDNA extraction from DBS in biobanking and epidemiology. However, it remains unclear to the reviewer how this work significantly builds upon the substantive literature on DNA extraction and longevity in DBS that exists in the literature (some of which is nicely summarized within this work).

- The stated gap in knowledge the authors are looking to address is “the use of DBS for downstream SNP genotyping following whole genome amplification”. How does this differ from previous works that have investigated DBS quality in relation to SNP genotyping (such as Rajatileka *et al.*, 2013[1])?

- The reasons for needing a separate optimized process are not made clear within the paper.

- **Methods:**

- Please add a section outlining calculations and statistical methods.

- How was total yield calculated?

- How many blood spots were processed for each method? Replicates?

- The addition of statistical methods (e.g. Standard error of the mean) would greatly aid readers in interpretation of datasets.

- **Figures:**

- All figures and tables: The process of extraction used in each sample needs to be clearly represented on all figures and tables.

- Table 2: The purpose of table 2 is unclear as this information is already displayed in both tables 1 and 3.

- Figure 3:
- It is unclear if these four samples are different blood spots, different testing methods or just replicates. Please label on gel image.
- Lane 2 does look degraded – more so than the other lanes. Is there a reason for this degradation?

- **Minor comments:**

- Table 3 — The units on the column marked total yield are missing.

References

1. Rajatileka S, Luyt K, El-Bokle M, Williams M, Kemp H, Molnár E, Váradi A: Isolation of human genomic DNA for genetic analysis from premature neonates: a comparison between newborn dried blood spots, whole blood and umbilical cord tissue.*BMC Genet*. 2013; **14**: 105 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?

Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
No source data required

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Applied Genomics

I confirm that I have read this submission and believe that I have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however I have significant reservations, as outlined above.

Reviewer Report 26 September 2019

https://doi.org/10.21956/gatesopenres.14166.r27514

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The authors have attempted to answer the queries raised by us.

1. Since two methods of DNA extraction were employed we expected two sets of values in Table 3 (DNA yield both the methods separately).

2. It is not clear at what temperature the DBS samples were stored in Bangalore from 2006-2007 till 2013 when the samples were shifted to Mumbai.

3. The grammar and language still needs correction in many places, for instance it is not clear what the authors want to convey in the statement "We obtained average gDNA concentrations of 6mm × 1 spot to 6mm × 4 spots from DBS cards" under the results section or the sentence "3000 DBS samples were prepared through finger prick method by using lancet (Accu Chek Softclix Lancet, Roche), puncture the finger site using lancet, drop of blood form which is lightly touch the circle of filter paper cards (GE Health Care Life Science, Catalog no. 10534612) and form valid DBS during

health checkup camp by CGHR at Bangalore unit and transported to Tata memorial Centre (TMC) Mumbai for further analysis. Samples were collected between the years 2005–2007, but samples transported to Mumbai from Bangalore at ambient temperature in year 2013 and laboratory experiments conducted in 2016" under subject enrollment.

Is the work clearly and accurately presented and does it cite the current literature?
Yes

Is the study design appropriate and is the work technically sound?
Yes

Are sufficient details of methods and analysis provided to allow replication by others?
Yes

If applicable, is the statistical analysis and its interpretation appropriate?
Yes

Are all the source data underlying the results available to ensure full reproducibility?
Yes

Are the conclusions drawn adequately supported by the results?
Yes

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry, diagnostics, dried blood technology, epidemiology

We confirm that we have read this submission and believe that we have an appropriate level of expertise to confirm that it is of an acceptable scientific standard, however we have significant reservations, as outlined above.

Version 1

Reviewer Report 28 November 2018

https://doi.org/10.21956/gatesopenres.13936.r26761

Lakshmy Ramakrishnan
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Blood collected and stored as DBS is a very attractive and non-invasive alternative for measurement of several analytes. The only caveat is that the analyte of interest should be stable on drying and should be selectively eluted from blood with least interference from other contaminants.

The paper by Kumar et al. describes the utility of DBS as a source of gDNA for PCR based molecular assay. The authors have extracted DNA by two methods: column based gDNA extraction and magnetic based gDNA extraction. It is not clear as to why two methods of DNA extraction was employed. The results do not show comparison of yield or quality of DNA by the two methods, Was one method superior to other? If all the 40 samples were extracted by both the methods the result should indicate the 260/280 ratio and yield for both the methods. It is also not clear if the DBS sample processed by overnight incubation in PBS was used as starting material for both the methods. In the column based method it is mentioned that spots were added to lysis buffer whereas in the magnetic bead based method processed DNA samples were added.

A 260/280 ratio >2.0 is indicative of impurity as mentioned by the authors also. Most samples showed ratio >2.0 specially when the number of spots were increased. How is this likely to influence the downstream applications should be mentioned in the discussion. Since the authors have not demonstrated that the extracted DNA is suitable for downstream applications by actually performing some assays, this should be mentioned as limitation of the study.

Several papers have been published on extraction of DNA from DBS, some authors have described increasing the yield by employing various means [1-2-3]. A mention of these papers in the discussion would help the readers.

In the method section the following information would be useful - since the spotted blood samples were left at room temperature for drying, the ambient temperature during collection period should be mentioned. It is not clear as to how long the samples were kept at Bangalore before transportation to Mumbai and at what temperature? At what temperature were the DBS samples when they were transported to Mumbai?
The manuscript need to be edited to avoid repetitions of information and also language and grammar editing would improve the quality of the paper.

References

1. Choi EH, Lee SK, Ihm C, Sohn YH: Rapid DNA extraction from dried blood spots on filter paper: potential applications in biobanking.*Osong Public Health Res Perspect*. 2014; **5** (6): 351-7 PubMed Abstract | Publisher Full Text
2. Saavedra-Matiz CA, Isabelle JT, Biski CK, Duva SJ, Sweeney ML, Parker AL, Young AJ, Diantonio LL, Krein LM, Nichols MJ, Caggana M: Cost-effective and scalable DNA extraction method from dried blood spots.*Clin Chem*. 2013; **59** (7): 1045-51 PubMed Abstract | Publisher Full Text
3. Sirdah MM: Superparamagnetic-bead Based Method: An Effective DNA Extraction from Dried Blood Spots (DBS) for Diagnostic PCR.*J Clin Diagn Res*. 2014; **8** (4): FC01-4 PubMed Abstract | Publisher Full Text

Is the work clearly and accurately presented and does it cite the current literature?
Partly

Is the study design appropriate and is the work technically sound?
Partly

Are sufficient details of methods and analysis provided to allow replication by others?
Partly

If applicable, is the statistical analysis and its interpretation appropriate?
Not applicable

Are all the source data underlying the results available to ensure full reproducibility?
Partly

Are the conclusions drawn adequately supported by the results?
Partly

Competing Interests: No competing interests were disclosed.

Reviewer Expertise: Biochemistry, diagnostics, dried blood technology, epidemiology

We confirm that we have read this submission and believe that we have an appropriate level of expertise to state that we do not consider it to be of an acceptable scientific standard, for reasons outlined above.

Author Response 17 Jun 2019

Prabhat Jha, Tata Memorial Centre, Navi Mumbai, India

Our article 12855 – responses to comments by reviewers Dr. Lakshmy Ramakrishnan (AIIMS, New Delhi) and Dr. Ransi Ann Abraham (AIIMS, New Delhi)

Dr. Lakshmy and Dr. Ransi: Blood collected and stored as DBS is a very attractive and non-invasive alternative for measurement of several analytes. The only caveat is that the analyte of interest should be stable on drying and should be selectively eluted from blood with least interference from other contaminants.

Dr. Lakshmy and Dr. Ransi: The paper by Kumar et al. describes the utility of DBS as a source of gDNA for PCR based molecular assay. The authors have extracted DNA by two methods: column based gDNA extraction and magnetic based gDNA extraction. It is not clear as to why two methods of DNA extraction was employed. The results do not show comparison of yield or quality of DNA by the two methods, Was one method superior to other? If all the 40 samples were extracted by both the methods the result should indicate the 260/280 ratio and yield for both the methods. It is also not clear if the DBS sample processed by overnight incubation in PBS was used as starting material for both the methods. In the column based method it is mentioned that spots were added to lysis buffer whereas in the magnetic bead based method processed DNA samples were added.
Reply by Abhinendra: Our main objective was to extract the maximum amount of gDNA from DBS irrespective of methodology use, therefore we have used 2 methods to extract gDNA to evaluate from which method we have got more gDNA but unfortunately we have not found any difference in gDNA concentration with between both methods. We did assay randomly from both methods with full focus on maximum quantity of gDNA extraction from DBS. We have tried gDNA extraction with direct cell lysis of DBS by using lysis buffer and also blood spots treated with PBS overnight to complete elution of eluate. We have done these experiments to evaluate gDNA concentration but unfortunately there are no such yield increases with these modifications.

Dr. Lakshmy and Dr. Ransi: A 260/280 ratio >2.0 is indicative of impurity as mentioned by the authors also. Most samples showed ratio >2.0 specially when the number of spots were increased. How is this likely to influence the downstream applications should be mentioned in the discussion. Since the authors have not demonstrated that the extracted DNA is suitable for downstream applications by actually performing some assays, this should be mentioned as limitation of the study.
Reply by Abhinendra: Yes it is true that a 260/280 ratio >2.0 indicates impurities. But due to limitation of blood spots we have not increased the number of spots beyond 4. As I have mentioned that we have got DNA concentration in a range 64.8ng – 720ng. This amount of DNA can be used in downstream applications and we can remove the impurities by gel purification method.

Dr. Lakshmy and Dr. Ransi: Several papers have been published on extraction of DNA from DBS, some authors have described increasing the yield by employing various means [1-2-3]. A mention of these papers in the discussion would help the readers. In the method section the following information would be useful - since the spotted blood samples were left at room temperature for drying, the ambient temperature during collection period should be mentioned. It is not clear as to how long the samples were kept at Bangalore before transportation to Mumbai and at what temperature? At what temperature were the DBS samples when they were transported to Mumbai?
The manuscript need to be edited to avoid repetitions of information and also language and grammar editing would improve the quality of the paper.
Reply by Abhinendra: DBS samples were transferred from Bangalore to Mumbai at ambient temperature.

Competing Interests: No competing interests were disclosed.

Comments on this article

Version 2

Author Response 11 Nov 2019

Abhinendra Kumar, Homi Bhabha National Institute, Training School Complex, Anushaktinagar, Mumbai, India

We have done revisions to our published article version 2 (12855) based on reviewer comments, but some comments required only reply and not revision. We want to reply to those comments through this section.

Comment 1 (By Dr. Anubhav): This paper deals specifically with gDNA extraction from DBS in biobanking and epidemiology. However, it remains unclear to the reviewer how this work significantly builds upon the substantive literature on DNA extraction and longevity in DBS that exists in the literature (some of which is nicely summarized within this work).
Reply: Our major objective was to extract maximum amount of genomic gDNA from DBS, therefore we used 1 spot to 4 spots to evaluate how concentration changes while increasing the number of spots. As

confirmed with results, we can say that on an average how much gDNA can be extracted from single blood spot.

Comment 2 (By Dr Anubhav): The stated gap in knowledge the authors are looking to address is “the use of DBS for downstream SNP genotyping following whole genome amplification”. How does this differ from previous works that have investigated DBS quality in relation to SNP genotyping (such as Rajatileka *et al.*, 2013[1])?
Reply: We have evaluated the reference and found the difference between cited reference and this reference (Rajatikela *et al.* 2013). In cited reference, researchers used wgaDNA (whole genome amplified DNA) for genotyping & found it is reliable for genotyping of 610000 SNPs, while in Rajatileka *et al.* paper, they used genomic DNA (gDNA) for genotyping & found 6% and 14% was unsuccessful for detection of re1835740 & rs4354668.

Comment 3 (By Dr Anubhav): The reasons for needing a separate optimized process are not made clear within the paper.
Reply: We have optimized the methodology to extract maximum amount of genomic DNA, but unfortunately we have not found any difference in both the methodology.

Comment 4 (By Dr Anubhav): How many blood spots were processed for each method? Replicates?
Reply: We have processed 1 – 4 blood spots for each method & 2 spots for each replicate, but results are similar.

Comment 5 (By Dr Lakshmy): Grammar mistakes
Reply: Corrected some sentences grammatically.

Competing Interests: No competing interests were disclosed.

Original Article

Utility of Dried Blood Spots in Detecting *Helicobacter pylori* Infection

Abhinendra Kumar[1,2], Sharayu Mhatre[1,2], Rajesh Dikshit[1,2]

[1]Centre for Cancer Epidemiology, Advanced Centre for Treatment Research and Education in Cancer, Tata Memorial Centre, Navi Mumbai, [2]Homi Bhabha National Institute (HBNI), Training School Complex, Anushaktinagar, Mumbai, Maharashtra, India

Abstract

Purpose: Identifying infectious pathogens by collecting intravenous blood (IVB) is a well-established procedure, however, the collection of IVB in field epidemiological study is challenging. The dried blood spot (DBS) as an alternative to IVB has been introduced, although, there is a limited study to demonstrate the utility of DBS stored at various storage conditions and transported at different periods. This is an observational study, which evaluates the effectiveness of DBS in field epidemiological studies to identify infectious pathogens. **Materials and Methods:** A total of 264 paired DBS samples prepared from IVB, stored at 4°C, −20°C after period 24, 48 and 72 h. Serologically, enzyme-linked immunosorbent assay [ELISA] IgG antibody detected against *Helicobacter pylori* infection from DBS and compared with IVB. **Results:** Quantitatively, IgG antibody reactivity showed >87% correlation between IVB and DBS samples stored at 4°C or −20°C within 48 h of transport duration. DBS stored at 4°C shows, equal sensitivity 87.5% and specificity 95% before 48 h of transport duration, while at −20°C storage similar sensitivity 87.5% observed but slightly less specificity 86.36% observed as compared to 24 h of transport duration. One-way analysis of variance showed, nonsignificant difference at both (−20°C and 4°C) the stored condition with *P* value ($P > 0.851$) and ($P > 0.477$). Kappa values showed good inter-rater reliability between DBS and IVB in a range (0.77–0.81). **Conclusion:** No significant difference was observed in detecting *H. pylori* when ELISA was conducted using IVB or DBS stored at 4°C and transported even after 48 h. This confirms that DBS collected even in compromised conditions in the field can be used for detecting infection.

Keywords: Dried blood spot, epidemiology, gastric cancer, *Helicobacter pylori,* serology

Introduction

The idea of sampling biological fluids was given by Ivar Bang nearly a century ago.[1] The concept that capillary whole blood, obtained by heel or finger prick and blotted on to a filter paper (Guthrie card), could be used to screen for metabolic disease in large populations of neonates.[2] In preparation of Dried Blood Spot (DBS), few microliter bloods is required as compared to venous blood for testing of analytes and are generally nonhazardous and can be easily transported from remote area to laboratory site. However, DBS have not been used for regular serological testing as it is not been proven to be useful for all types of analytes. DBS has been used for many decades in the detection of infectious disease pathogens,[3-9] despite of this there are no guidelines reporting the impact of transport and storage condition of DBS on the stability of antibodies on filter paper.

DBS is widely used in epidemiological studies, diagnosis and monitoring the virus infection including HIV, *Cytomegalovirus*, hepatitis B virus, Epstein–Barr virus, Rubella and dengue virus.[4,10-12] Few studies analysed the correlation coefficient ($r = 0.92$) and observed no significant changes in DBS viral load even after storage at room temperature for 12 weeks[13] while high sensitivity and specificity (>98%)[14,15] observed for DBS hepatitis C virus serology. The previous published studies shows the etiology of *Helicobacter pylori* infection for the development of gastrointestinal disease.[16] *H. pylori* is a microaerophilic fastidious human pathogen. The bacterium has been implicated in acid

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peptic diseases of the stomach and duodenum and neoplasm of the stomach.[17-19] *H. pylori* have been detected in individuals of all ages throughout the world and its prevalence ranges between 20% and 80%.[20]

It is challenging to collect, transport and storage of intra-venous blood (IVB) in field settings due to limited infrastructure. The DBS as an alternative to IVB has been introduced, the objective of this study is to demonstrate the utility of DBS stored at various storage conditions and transported at different periods to identify the infectious pathogens (*H. pylori*) and compared the results with paired IVB used as gold standard. To evaluate the effectiveness of DBS cards, each paired DBS sample compared 6 times and results were correlated with IVB.

Materials and Methods

Subject enrollment and blood collection

An observational study has been conducted between January 2018 and May 2018 at Centre for Cancer Epidemiology, Tata Memorial Centre, Mumbai (Maharashtra). The study participants with age group between 18 and 69 years have been enrolled in this study after informed consent obtained from subjects. With 0.8 power, 90% correlation at standard condition and an estimated 70% correlation at stored condition, 88 paired sample sizes were obtained. This study was approved by the Institutional Ethical Committee of the organisation. A volume of 3 ml–4 ml venous blood has been collected by trained staff in ethylenediaminetetraacetic acid tubes and immediately transported to the laboratory for further processing.

Dried blood spot preparation and storage

The Whatman 903 (GE Health Care, US) blood collection card is labeled with sample ID, date of blood collection, *transport duration and storage temperature. To maintain the homogenous nature of blood spots, 50 µl of full blood was immediately applied to 5 spots on DBS filter paper cards (Whatman 903 Protein Saver Blood Collection Cards; Schleicher and Schuell).[9] Remaining IVB centrifuged at 3000 rpm/4°C for 15 min, plasma (gold standard) separated and stored at −80°C. Blood spots dried[21] at room temperature for 2–3 h with the help of the Whatman card rack [Supplementary 1] without applying any direct heat, blower, dryer, etc., While in the drying process, the surface of DBS have not to touch with any other surface to prevent it from any type of contamination. DBS cards packed in airtight ziplock bags [Supplementary 2] with 1–2 g desiccant sachets and stored at respective temperatures (4°C and −20°C). Desiccant sachets act as an adsorbent to remove any moisture from blood spots to prevent any type of contamination/fungal infection. Packed DBS cards stored at 4°C and −20°C within 24 h, 48 h and 72 h of transport duration.

Terminologies used for stored dried blood spot samples

- DBS1: DBS sample stored at 4°C within 24 h of transport duration
- DBS2: DBS sample stored at −20°C within 24 h of transport duration
- DBS3: DBS sample stored at 4°C within 48 h of transport duration
- DBS4: DBS sample stored at −20°C within 48 h of transport duration
- DBS5 DBS sample stored at 4°C within 72 h of transport duration
- DBS6: DBS sample stored at −20°C within 72 h of transport duration.

Validity and processing of dried blood spot samples

In the Valid DBS card, blood-filled completely in each circle of blood collection card and spread homogeneously, whereas invalid samples are scratched, half-filled, overlapped, merged with other circles and not dried completely.[22] Only valid DBS samples used for serology of *H. pylori* infection. Excise single 6 mm diameter blood spot from DBS card with the help of 6 mm steel punch plier, add 500 µl assay diluent buffer (provided in the kit) and shake at 200 rpm for 2–3 h at room temperature, the elute directly used as a sample for assay [Supplementary 3].

Assay protocol and data analysis

Commercial available enzyme-linked immunosorbent assay ELISA kit (IBL International *H. pylori* IgG ELISA, RE56381) used for detection of IgG antibody against *H. pylori* infection. As per kit standards, the cut off valve for the seropositivity of *H. pylori* infection was >12 U/ml. Standard curve have been prepared from available standards in kit with their tested IgG antibody concentrations (A = 1U/ml, B = 10 U/ml, C = 25 U/ml and D = 150 U/ml) and quantifying sample result according to standard curve. The scattered plot was prepared to observe the correlation coefficient [Figure 1] at all transport duration and stored thermal conditions. Prepared the Bland-Altman (BA) plot [Figure 2] of the difference between DBS and IVB to evaluate bias in the mean concentration of antibody. *t*-test [Table 1] and one-way Analysis of Variance (ANOVA) [Table 2] test conducted to compare mean all DBS samples stored at 4°C and −20°C with IVB. All quantitative data analysed using

Table 1: *t*-test, immunoglobulin G antibody mean concentration comparison between intravenous blood and each group of dried blood spot stored at 4°C and −20°C

Comparison group of blood samples (plasma and DBS)	Mean±SD	95% CI	*P*
DBS1	30.29±30.29	18.408-42.187	>0.0637
DBS2	35.14±44.32	21.669-48.621	>0.620
DBS3	29.48±37.15	18.192-40.784	>0.0345
DBS4	31.88±40.76	19.494-44.282	>0.136
DBS5	26.74±31.35	17.208-36.275	>0.0095
DBS6	32.19±41.96	19.434-44.952	>0.245

*Plasma mean value 36.68±45.89 with 95% CI (22.728-50.633) compared with all dried blood spot group.[23] DBS: Dried blood spot, CI: Confidence interval, SD: Standard deviation

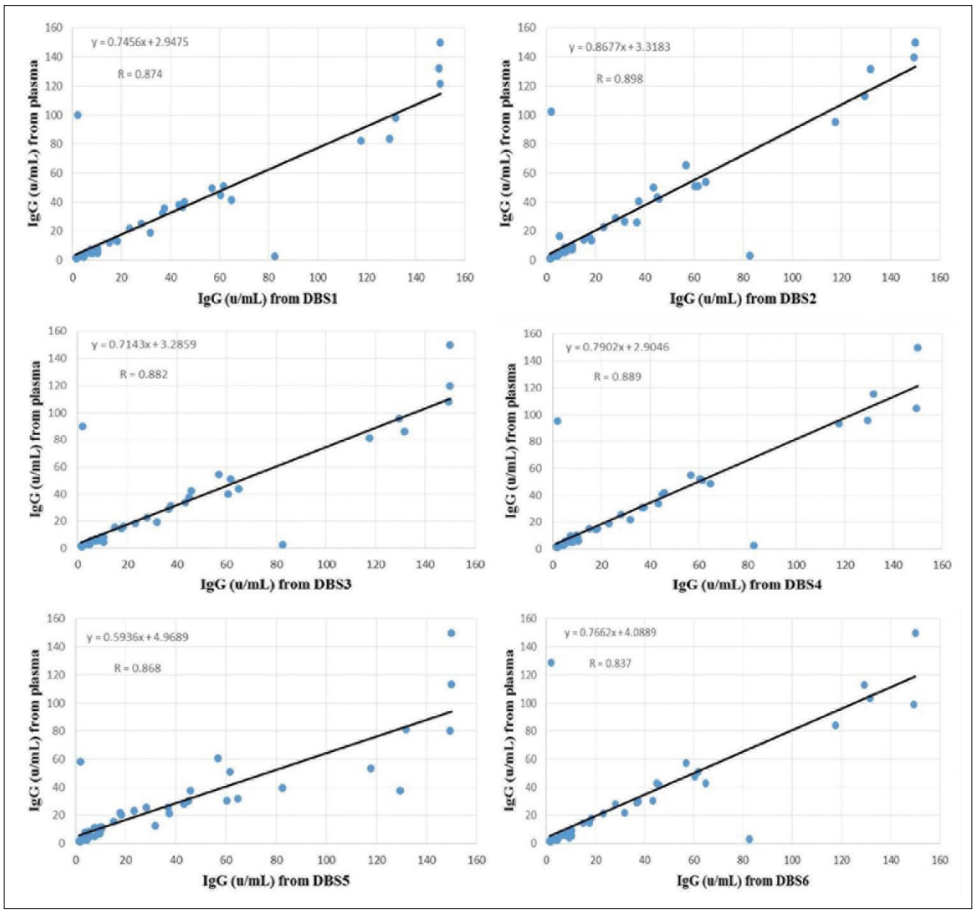

Figure 1: Scattered plot for comparison of correlation between plasma (intravenous blood) and dried blood spot stored at 4°C (dried blood spot 1, dried blood spot 3, dried blood spot 5) and −20°C (dried blood spot 2, dried blood spot 4, dried blood spot 6)

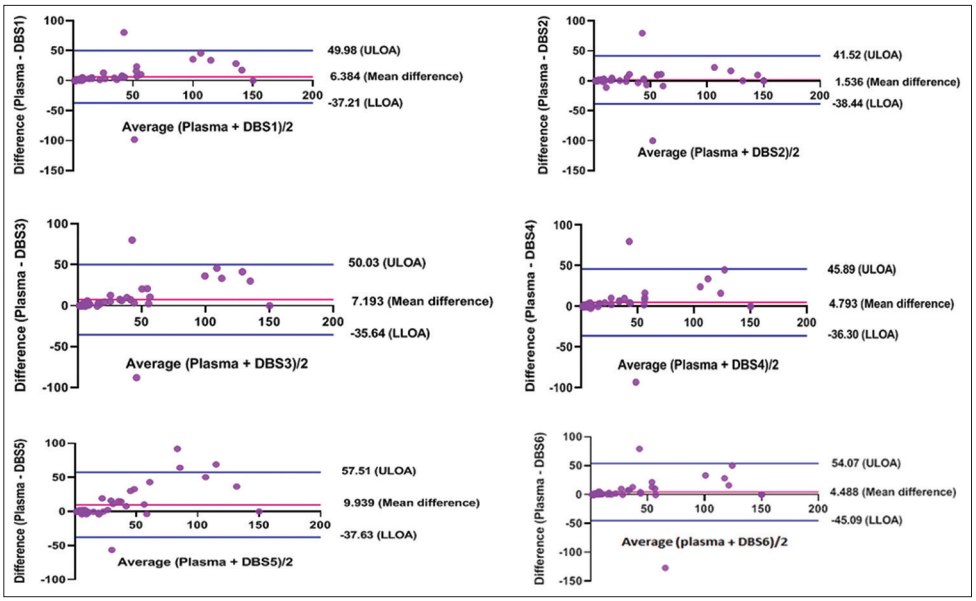

Figure 2: Bland-Altman Graph, a plot of difference of IgG antibody concentration against *Helicobacter pylori* infection between plasma and dried blood spot, plotted against average of plasma (intravenous blood) and dried blood spot stored at 4°C (dried blood spot 1, dried blood spot 3, dried blood spot 5) and −20°C (dried blood spot 2, dried blood spot 4, dried blood spot 6)

statistical software Excel, STATA 15.0 Details (StataCorp LLC, Texas, USA and Graph-Pad. *P* value ($P \leq 0.05$) at 95% confidence interval (CI) considered a statistically significant difference.

Results

DBSs prepared from IVB and stored at the different thermal conditions at varying time periods. DBS samples were stored for 5 months before the experiment was conducted. Serological experiments were performed in our laboratory at 22°C temperature with humidity range 75%–85%. The sandwich ELISA assay performed as per kit protocol and data were analysed quantitatively through the standard curve.

IgG Seropositivity against *Helicobacter pylori* infection

A total of six groups were created from DBS1 to DBS6 with 44 DBS samples in each group, overall 264 DBS replicates tested for the detection of IgG antibody titer against *H. pylori* infection and compared with IVB. The blood sample >12 U/ml IgG antibody titer consider as positive for *H. pylori* infection. Of the 44 IVB samples, 24 (54%) samples were detected IgG positive and 20 (46%) samples were IgG negative against *H. pylori* infection. All 264 DBS samples were tested for IgG seropositivity in each group. 50% (22/44) samples were detected IgG seropositivity for group DBS1, 52% (23/44) samples for DBS2, 50% (22/44) for DBS3, 50% (22/44) for DBS4, 65% (29/44) for DBS5 and 50% (22/44) for DBS6. On average, 50%–52% DBS samples detected seropositive for *H. pylori* infection as compared to IVB (54%) irrespective of transport duration and storage condition, but group DBS5 shows the highest seropositivity (65%), and it might be possible due to chance.

Dried blood spot sensitivity and specificity

Although our method is qualitative and quantitative, the specific aim of this essay is to identify *H. pylori* infection through qualitative assay but quantitative data analysed through statistical methods. To evaluate the performance of our assay, we used DBS replicate samples derived from IVB. DBS sensitivity and specificity for Whatman 903 blood collection card were defined for IgG-*H. pylori* ELISA only. IVB IgG was used as a reference to determine the positive and negative status of antibodies, as well as to define false positive (FP) and false negative (FN) for each DBS analyzed, whereas sensitivity and specificity of DBS calculated by 2 × 2 table below Table 3.

Similar sensitivity (87.5%) observed for all DBS groups except DBS5 (100%), there is no FN sample observed for DBS5. While there is slight variation observed for specificity in DBS2 (90%) and DBS5 (75%), rest of the DBS groups have similar highest specificity (95%) [Table 4].

The proportion of positive and negative infection also calculated with positive predictive values (PPV) and negative predictive values (NPV), and the strength of agreement between DBS and IVB was depicted by kappa statistics. At 4°C storage, excellent PPV reported (95.45%) for DBS at 48 h of transport duration, but slightly decreased in PPV (82.75%) at transport duration within 72 h. While at −20°C storage, good PPV value reported (91.30%) within 24 h of transport duration, but slightly increased in PPV value for DBS4, DBS6 (95.45%), and there is no specific difference observed at 48 and 72 h of transport duration. A large percentage of PPV and NPV values indicates that the small FP and FN detected through this testing procedure are true and correct. Kappa statistics are used to measure inter-rater reliability for a qualitative test. It is a more robust measure agreement between two raters. It is expressed as K = Po−Pe/1−Pe, where Po is a relative observed agreement among raters and Pe is the hypothetical probability of chance agreement. Overall good agreement (K = 0.77–0.81) observed between all the DBS groups (DBS1–DBS6) and IVB, respectively, indicating very strong correlation beyond chance [Table 5].

Evaluate correlation coefficient between dried blood spot and intravenous blood

Correlation is a statistical technique, that shows how strongly pairs of variables are related, and it is denoted as the correlation coefficient®. Correlation coefficient computed as the ratio of covariance between variables to the product of their standard deviation and its value ranges from −1 to +1. Our results indicate that overall quantitative DBS and intravenous results are well comparable. Overall the correlation ® between DBS and IVB were high, ranging from (R = 0.83 to 0.89) for all DBS groups (DBS1–DBS6). At 4°C storage of DBS, it showed small variation in correlation between DBS/IVB (R = 0.87 for DBS1, 0.88 for DBS3 and 0.86 for DBS5). These results reflect, quantitatively there is a moderate difference observed in IgG antibody titers between DBS/IVB irrespective of transport duration. While at −20°C storage, correlation decreases continuously at transport duration of 24, 48 and 72 h (R = 0.89 for DBS2, 0.88 for DBS4 and 0.83 for DBS6) [Figure 1]. The result showed, IgG antibody stability decrease with an increase in transport duration, but there is no such big difference observed when DBS transported before 48 h and −20°C storage.

Evaluate comparison between two groups: *t*-test

Quantitatively, the group mean of IVB (36.68 ± 45.89) at 95% CI (22.72–50.63) compared with mean of DBS1 (30.29 ± 39.10) at 95% CI (18.40–42.18), statistically non-significant difference observed with *P* value (*P* > 0.063),

Table 2: One-way analysis of variance test, immunoglobulin G antibody mean concentration comparison between intravenous blood and paired dried blood spot samples stored at 4° and 20°C

Plasma sample stored at -80°C	DBS samples stored condition irrespective of transport duration	Total DBS samples	*F*	*P*
Standard condition (*n*=44)	4°C	*n*=132	0.83	>0.477
Standard condition (*n*=44)	−20°C	*n*=132	0.26	>0.851

DBS: Dried blood spot

Table 3: 2 x 2 table to calculate sensitivity and specificity of diagnostic test

Outcome of diagnostic test	Intravenous blood (plasma) *H. pylori* infection		
	Positive	Negative	
DBS *H. pylori* infection			
Positive	a	b	PPV: a/a+b
Negative	c	d	NPV: d/c+d
	Sensitivity: a/a+c	Specificity: d/b+d	N: a+b+c+d

DBS: Dried blood spot, PPV: Positive predictive value, NPV: Negative predictive value, *H. pylori: Helicobacter pylori*

while significant difference observed for DBS3 (29.48 ± 37.15) at 95% CI (18.19–40.78) and DBS5 (26.74 ± 31.35) at 95% CI (17.20–36.27) with *P* value ($P > 0.034$) and ($P > 0.009$), respectively. Good result obtained for DBS groups stored at −20°C (DBS2, DBS4 and DBS6) as compared to 4°C, statistically non-significant difference observed for all three groups with *P* value ($P > 0.620$), ($P > 0.136$) and ($P > 0.245$), respectively. No difference observed in IgG concentration between DBS (DBS2, DBS4, DBS6) stored at −20°C and IVB. We can detect infection even if DBS transported within 72 h at ambient temperature and stored at −20°C.

Evaluate comparison between multiple groups: Analysis of variance

One-way ANOVA test has been conducted to compare IgG antibody concentration between IVB and DBS groups stored at 4°C ($n = 132$) and −20°C ($n = 132$) irrespective of transport duration. Statistically there is non-significant difference observed at both the stored temperature with their *P* values ($P > 0.477$) and ($P > 0.26$) and corresponding F-value ($F = 0.83$) and ($F = 0.26$). Result shows more similarity observed in IgG concentration between DBS/IVB at 4°C as compared to − 20°C storage, because F value 0.83 indicate that the within groups variance is 0.17 (1−0.83 = 0.17) times the size of the between group variance, whereas at −20°C storage it shows within group variance is 0.74 (1 − 0.26 = 0.74) times the size of the between group variance.

Evaluation of Bland-Altman plot

Altman plot is an alternative analysis, based on the quantification of the agreement between two quantitative measurements by studying the mean difference and constructing limits of agreement. It is a simple way to evaluate bias between mean differences and to estimate an agreement interval, within which 95% of the differences of the second method, compared to the first one, fall. The BA plot method only defines the intervals of agreements, it does not say whether those limits are acceptable or not. Acceptable limits must be defined *a priori*, based on clinical necessity, biological considerations, or other goals.[24]

Our result plotted the difference of IgG concentration between IVB and all DBS groups (DBS1–DBS6) separately. BA plot shows, more differences observed between DBS and IVB at 4°C storage as compared to −20°C storage.(6.384), (7.193) and (9.939) mean differences observed for DBS1, DBS3 and DBS5 while (1.536), (4.793) and (4.488) observed for DBS2, DBS4 and DBS6. Based on the BA plot results, DBS should be transported within 24 h and stored at −20°C rather than beyond 24 h. At 4°C storage, it is clear that with the increase in transport duration, the mean difference also increases between DBS and IVB.

Discussion

This study aimed to evaluate, whether the DBS is an alternative to IVB in the field setting, as DBS is an attractive tool for field studies as only finger prick is required for its collection and it can be easily transported from remote area to laboratory. However, its use in field studies is limited as detailed guidelines for storage and transportation are not standardised. Previous studies have demonstrated usefulness of DBS in performing HIV and other infections.[10,25,26] It has been argued that different downstream application requires different storage and transport conditions. For example, DNA can be extracted effectively from DBS stored at −20°C if transported before 24 h.[22] While different storage and transport conditions are required for analysis of biochemical parameters and infectious agents. In the present study, we discuss about conditions required for storage and transport of DBS to investigate *H. pylori* infection.

We demonstrated that for testing pathogens there is no effect of transport duration even if samples are transported after 48 h after proper drying. These findings are important for large scale epidemiological studies when carried out in remote areas with poor access to immediate transport and samples cannot be reached within 24 h. The study also recommends that once samples are transported to study center, the best way to store them is at −20°C before further testing is done on the samples. Many other studies have evaluated the usefulness of DBS in disease surveillance and testing for pathogens including HIV, Hepatitis B, H pylori; however, none of them have evaluated for transport duration and different storage conditions.[9]

Our results indicate that overall DBS and IVB result are well comparable; however, we have observed slight deviation in correlation coefficient. ANOVA result shows, there is no significant difference observed at both the storage temperature, while quantitatively less difference observed in IgG concentration at − 20°C rather than at 4°C. With qualitative ELISA, infectious agents can be detected at both the storage temperature due to observed higher sensitivity and specificity; however quantitatively, low IgG antibody titer detected at 4°C as compared to −20°C. With these results, we can justify that DBS can be transport within 48 h and must be stored at −20°C or −80°C to restore the stability of antibodies titers on DBS.

Conclusion

DBS offer an excellent method for blood collection to measure

Table 4: Sensitivity and specificity of dried blood spot compared to intravenous blood in immunoglobulin G *Helicobacter pylori* ELISA

Blood spots	Positive IgG*	Negative IgG**	FP	FN	Sensitivity (%)	Specificity (%)
DBS1	21	19	1	3	87.5	95
DBS2	21	18	2	3	87.5	90
DBS3	21	19	1	3	87.5	95
DBS4	21	19	1	3	87.5	95
DBS5	24	15	5	0	100	75
DBS6	21	19	1	3	87.5	95

*Positive IgG was defined for samples with concentration >12U/ml, **Negative IgG was defined for samples with concentration <8 U/ml. FP: False positive, FN: False negative, IgG: Immunoglobulin G, DBS: Dried blood spot

Table 5: Positive predictive value and negative predictive value of dried blood spot compared to intravenous blood in immunoglobulin G *Helicobacter pylori* ELISA

Blood spots	Positive IgG	Negative IgG	FP	FN	PPV (%)	NPV (%)	Kappa agreement
DBS1	21	19	1	3	95.45	86.36	0.80
DBS2	21	18	2	3	91.30	85.71	0.77
DBS3	21	19	1	3	95.45	86.36	0.81
DBS4	21	19	1	3	95.45	86.36	0.81
DBS5	24	15	5	0	82.75	100	0.77
DBS6	21	19	1	3	95.45	86.36	0.81

FP: False positive, FN: False negative, PPV: Positive predictive value, NPV: Negative predictive value, DBS: Dried blood spot, IgG: Immunoglobulin G

infectious pathogens. As stomach cancer is common cancer sites,[27] DBS can be used to understand burden of *H. pylori* infection in India and to conduct randomised trials to eradicate *H pylori* infection and reduce mortality from stomach cancer.

Acknowledgment

Authors are thankful to the Director, Tata Memorial Centre, Mumbai for providing the necessary facilities to carry out the research on DBS.

Financial support and sponsorship

This study was financially supported by Tata Memorial Centre, Mumbai.

Conflicts of interest

There are no conflicts of interest.

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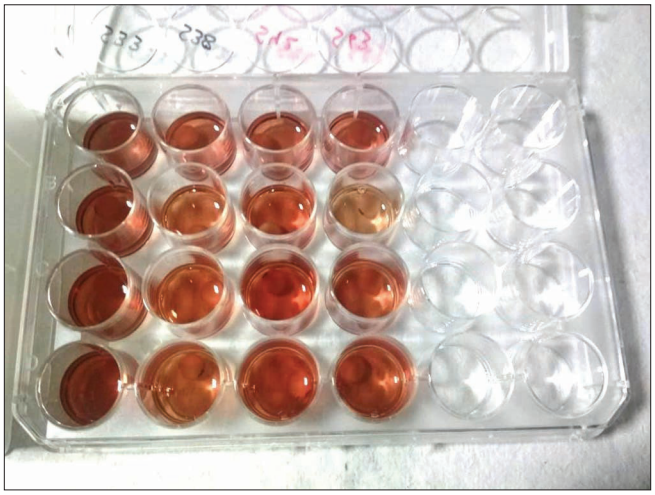
Supplementary 1: Drying of blood spots in Whatman 903 rack

Supplementary 2: Dried blood spot samples pack into tight sealed ziplock bags



Supplementary 3: Processing of dried blood spot spots in 24 well plate with assay diluent for serology of *Helicobacter pylori*

Homi Bhabha National Institute

Ph.D. Thesis Evaluation Report

1. **Name of the Constituent Institution:** Centre for Cancer Epidemiology, Tata Memorial Centre
2. **Name of the Student:** Abhinendra Kumar
3. **Enrolment No.:** HLTH09201304001
4. **Title of the Thesis:** Efficacy of Dried Blood Spot for Assessing Biomarkers in Field Epidemiological Studies
5. **Name of the Board of Studies:**

Recommendations

Tick one of the following:

1. The thesis in its present form is commended for the award of the Ph.D. Degree. ✓ ☐
2. The thesis is commended for the award of the Ph.D. degree. However, my suggestions for improving the thesis may be considered at the time of the viva voce examination and if the viva voce board deems it appropriate, the same may be incorporated in the thesis based on the discussions during the viva voce examination. The revised thesis need not be sent to me. ☐
3. The thesis should be revised as per the suggestions enclosed. I would like to see the revised thesis incorporating my suggestions before I give further recommendations. ☐
4. The thesis is not acceptable for the award of the Ph.D. degree. ☐

Date:
25
July,
2020

(Signature):

Name of Examiner:
Dr JS Thakur, Professor
of Community
Medicine, PGIMER,
Chandigarh

Please give your detailed report in the attached sheet. You may use additional sheets, if required.

1. **Name of the Student:** Abhinendra Kumar

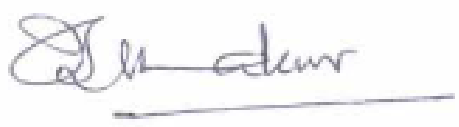
2. **Title of the Thesis:** Efficacy of Dried Blood Spot for Assessing Biomarkers in Field Epidemiological Studies

DETAILED REPORT

1. In methodology, study design, study setting, study duration should mentioned
2. Sample size calculation may be added
3. Appropriate tables and figures for test of significance and correlation coefficient should be used.
4. In standardization and validation of extraction of genomic DNA (gDNA) from DBS stored at standard condition, interpretation of Bland-Altman plot should be mentioned.
5. Conclusion of the study and recommendation may be added
6. Funding, if any should be mentioned

Name of Examiner: Dr JS Thakur

Signature and Date:



25th July, 2020

Homi Bhabha National Institute

Ph.D. Thesis Evaluation Report

1. **Name of the Constituent Institution:** Centre for Cancer Epidemiology, Tata Memorial Centre
2. **Name of the Student:** Abhinendra Kumar
3. **Enrolment No.:** HLTH09201304001
4. **Title of the Thesis:** Efficacy of Dried Blood Spot for Assessing Biomarkers in Field Epidemiological Studies
5. **Name of the Board of Studies:**

Recommendations

Tick one of the following:

1. The thesis in its present form is commended for the award of the Ph.D. Degree.	X
2. The thesis is commended for the award of the Ph.D. degree. However, my suggestions for improving the thesis may be considered at the time of the viva voce examination and if the viva voce board deems it appropriate, the same may be incorporated in the thesis based on the discussions during the viva voce examination. The revised thesis need not be sent to me.	
3. The thesis should be revised as per the suggestions enclosed. I would like to see the revised thesis incorporating my suggestions before I give further recommendations.	
4. The thesis is not acceptable for the award of the Ph.D. degree.	

Date:4 Aug 2020

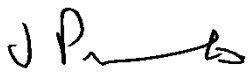
Name of Examiner:
Prabhat Jha, Professor of Global Health, University of Toronto

Please give your detailed report in the attached sheet. You may use additional

DETAILED REPORT

1. A clearer layout of the thesis scope is justified to outline key objectives and identify which sections of the thesis cover which topics.
2. Every statistical test used (except for very common ones like chi squared) should have footnotes explaining the test.
3. Add a section on implications of the study at the end.

Name of Examiner: Prof Prabhat Jha



Signature and Date:
Aug 4 2020

Homi Bhabha National Institute

Ph. D. PROGRAMME

ORAL GENERAL COMPREHENSIVE EXAMINATION REPORT (OGCE)

1. Name of the Student:	**Abhinendra Kumar**
2. Name of the Constituent Institution:	**Tata Memorial Centre**
3. Enrolment No:	**HLTH09201304001**
4. Board of Studies:	**Health Science**

The Oral General Comprehensive Examination of Shri / **Abhinendra Kumar** was conducted on **26/10/2015**. The monitoring / doctoral committee is fully satisfied with his performance and permits him to continue with his Ph.D. programme. The student has studied research methodology theory and on examination was found to be proficient in research methodology as applicable to his field of study. He fulfills the criterion 4 and criterion 6 of UGC 11 point criteria.

Monitoring / Doctoral Committee:

S.No.	Name	Designation	Signature	Date
1	**Dr. R A Badwe**	Chairman		26/10/2015
2	**Dr. R P Dikshit**	Convener		26/10/2015
3	**Dr. F Ram**	Member		26/10/2015
4	**Dr. C S Pramesh**	Member		26/10/2015
5	**Dr. S Godbole**	Special Invitee		26/10/2015

To
Dean-Academic
C.C. All doctoral committee members and reporting Student

Version approved in the meeting of Standing Committee of Deans held on September 05, 2014.

Homi Bhabha National Institute

Ph.D. PROGRAMME

PROGRESS REVIEW[1] REPORT

1. Name of the Student: **Abhinendra Kumar**
2. Name of the Constituent Institution : **Tata Memorial Centre**
3. Enrolment No. **HLTH09201304001** Date of Enrolment: **01/09/2013**
4. Title of the Thesis: **"Efficacy of Dried Blood Spot for Assessing Biomarkers in Field Epidemiological Studies"**
5. **OGCE:** Held on **26/10/2015**
6. Review Period: **2015-2016** Date of Meeting: **27/04/2017**

1. **Report of Doctoral Committee**

Suggestions are enclosed (Encl. 1)

2. **Recommendations**

Grade: Excellent/Very Good/~~Good~~/Satisfactory/Poor

Recommendation for continuation: ~~Yes~~/No

Likely year of finishing the programme (in case recommended to continue):

2018

[1] To be conducted only after a student has submitted a brief progress report to doctoral committee explaining progress made during the period in research, papers published, completed courses with marks obtained (taught courses, self-study courses, credit seminars) with appropriate certification thereof, pending courses and work planned in next year. Report from the student should be attached to the progress review report.
Version approved during the meeting of Standing Committee of Deans held during 29-30 Nov 2013

3. Future Guidelines

The Work may be continue as for recommendation.

4. Next[2] Progress Report due on: NOV. 2017

5. Doctoral Committee:

S. No.	Name	Designation	Signature	Date
1.	Dr. R A Badwe	Chairman		02/5/2017
2.	Dr. R P Dikshit	Guide & Convener		02/5/2017
3.	Dr. F Ram	Member		02/5/2017
4.	Dr. C S Pramesh	Member		02/5/2017
5.	Dr. S Godbole	Special Invitee		02/5/2017

To
Dean-Academic

CC. Dean, HBNI

Reporting Student

[2] One progress report per year is mandatory. Doctoral committee can ask more frequent progress reports.
Version approved during the meeting of Standing Committee of Deans held during 29-30 Nov 2013

Homi Bhabha National Institute

Ph.D. PROGRAMME

PROGRESS REVIEW[1] REPORT

1. Name of the Student : **Abhinendra Kumar**
2. Name of the Constituent Institution : **Tata Memorial Centre**
3. Enrolment No. and Date of Enrolment : **HLTH09201304001**
4. Title of the Thesis : **"Efficacy of Dried Blood Spot for Assessing Biomarkers in Field Epidemiological Studies"**
5. **OGCE:** Held on **26/10/2015**
6. Review Period and Date of Meeting : **2016-2017 and 22/03/2018**

1. **Report of Doctoral Committee**

Progress is Satisfactory. My present final results in August 2018.

Signature

2. **Recommendations**

Grade: Excellent/Very Good/Good/Satisfactory/Poor

Recommendation for continuation: Yes/No

Likely year of finishing the programme (in case recommended to continue):

[1] To be conducted only after a student has submitted a brief progress report to doctoral committee explaining progress made during the period in research, papers published, completed courses with marks obtained (taught courses, self-study courses, credit seminars) with appropriate certification thereof, pending courses and work planned in next year. Report from the student should be attached to the progress review report.
Version approved during the meeting of Standing Committee of Deans held during 29-30 Nov 2013

3. Future Guidelines

Validation of DBS result with Poll Stanjsmi test to be done

4. Next[2] Progress Report due on: August 2018

5. Doctoral Committee:

S. No.	Name	Designation	Signature	Date
1	Dr. R A Badwe	Chairman		23/3/18
2	Dr. Rajesh Dikshit	Guide & Convener		23/3/18
3	Dr. C S Pramesh	Member		23/3/18
4	Dr. F Ram	Member		23/3/18
5	Dr. Sheela Godbole	Special Invitee		23/3/18

To
Dean-Academic

CC Dean HBNI

Reporting Student

[2] One progress report per year is mandatory. Doctoral committee can ask more frequent progress reports [illegible]

Homi Bhabha National Institute

Ph.D. PROGRAMME

PROGRESS REVIEW[1] REPORT

1. **Name of the Student: Abhinendra Kumar**
2. **Name of the Constituent Institution : Tata Memorial Centre**
3. **Enrolment No. and Date of Enrolment: HLTH09201304001, 01/09/2013**
4. **Title of the Thesis: "Efficacy of dried blood spot for assessing biomarkers in field epidemiological studies"**
5. **OGCE: Held on 31/08/2018**
6. **Review Period and Date of Meeting : 2017 - 2018**

1. **Report of Doctoral Committee**

Synopsis to be submitted in March 2019

2. **Recommendations**

Grade: Excellent/Very Good/Good/Satisfactory/Poor

Recommendation for continuation: Yes/No

Likely year of finishing the programme (in case recommended to continue):

July 2019

[1] To be conducted only after a student has submitted a brief progress report to doctoral committee explaining progress made during the period in research, papers published, completed courses with marks obtained (taught courses, self-study courses, credit seminars) with appropriate certification thereof, pending courses and work planned in next year. Report from the student should be attached to the progress review report.
Version approved during the meeting of Standing Committee of Deans held during 29-30 Nov 2013

3. **Future Guidelines**

Nil

4. **Next[2] Progress Report due on:** NA

5. **Doctoral Committee:**

S. No.	Name	Designation	Signature	Date
1.	Dr. R.A. Badwe	Chairman		22/12/18
2.	Dr. Rajesh Dikshit	Guide & Convener		22/12/18
3.		Co-guide (if any) Member		
4.	Dr. C.S. Pramesh	Member		22/12/18
5.	Dr. Pankaj Chaturvedi	Member		22/12/18
6.	Dr. Sheela Godbole	Member		22/12/18
7.		Technology Adviser		

To
Dean-Academic

CC. Dean, HBNI

Reporting Student

[2] One progress report per year is mandatory. Doctoral committee can ask more frequent progress reports.
Version approved during the meeting of Standing Committee of Deans held during 29-30 Nov 2013

Homi Bhabha National Institute

CHECK LIST FOR 11 CRITERIA AS PER UGC (MINIMUM STANDARD AND PROCEDURE FOR AWARD OF PH.D. DEGREE) REGULATION 2009[1]

1. **Name of the Student:** Abhinendra Kumar
2. **Name of the Constituent Institution:** Tata Memorial Centre
3. **Enrolment No.:** HLTH09201304001
4. **Board of Studies:** Medical & Health Sciences

Sr. No.	Particular	**Remark** (Tick the appropriate one)
1.	Mode of selection for Ph.D. Programme	Written Test / Interview / Both
2.	Number of Ph.D. students under the guide ≤ 8 during the period	Yes / No
3.	Reservation Policy applied for selection	Yes / No
4.	Course work done	Yes / No
5.	Reviews of Annual Progress held	Yes / No
6.	Test on Research Methodology held	Yes / No
7.	Pre-synopsis presentation held	Yes / No
8.	At least 1 journal paper published	Yes / No
9.	At least of 2 papers in conference / seminar presented	Yes / No
10.	Evaluation reports of Ph.D. Thesis from guide and two experts (one expert is out of state) received	Yes / No
11.	Soft copy of Thesis submitted to HBNI	Yes / No

Dean-Academic

To
Dean, HBNI

[1]Ph.D. student has to fulfill all the 11 criteria laid by UGC to meet eligibility criteria for employment in Indian Universites. HBNI will issue required certificate only if a student meets all the eleven criteria.

Ph.D. Thesis Highlight

Name of the Student: Abhinendra Kumar **Enrolment No.:** HLTH09201304001

Discipline: Medical & Health Sciences **Sub-Area of Discipline:** Epidemiology

Name of the CI: Centre for Cancer Epidemiology, Tata Memorial Centre

Thesis Title: Efficacy of Dried Blood Spots for Assessing Biomarkers in Field Epidemiological Studies

Date of viva-voce: 15/02/2021

In this study design, dried blood spots (DBS) prepared from EDTA blood samples collected from healthy population. DBS samples stored at different thermal condition (4^0C & -20^0C) at varying transport duration (0-24h, 24-48h & 48-72h). DBS samples used to study the impact of storage condition and transport duration on stability of biomarkers (IgG antibody against *Helicobacter pylori*, genomic DNA & apolipoproteins: apoA & apoB). Commercial ELISA kit were used for testing IgG antibody against H.*pylori* & apolipoproteins, whereas column based method used for gDNA extraction. DBS samples processed as per kit manufactured protocol. All the data analysed through statistical software STATA (t-test, ANOVA) and Bland Altman graph were prepared from Graphpad software.

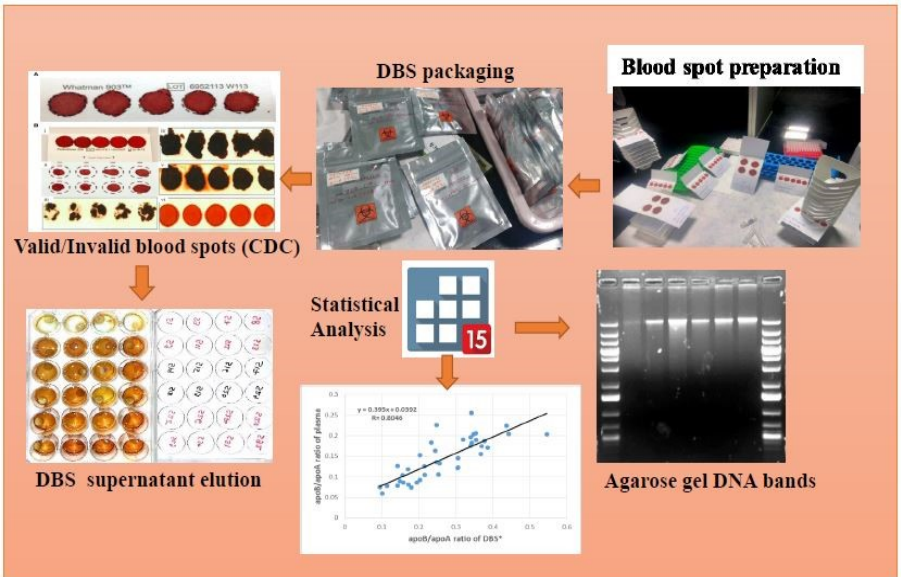


Figure 1. *An observational study to investigate the biomarkers assessment through Dried Blood Spots.*

Research finding reflect, DBS could be a remarkable tool to measure the analytes /biomarkers (genomic DNA, antibodies raised against infection, apolipoproteins) even if it is stored at different thermal condition (4^0C or -20^0C) with varying transport duration up to 72 hours. Excellent correlation observed for IgG antibody concentration against *H. pylori* with >85% sensitivity & specificity. Good concentration of gDNA obtained from DBS as compared to gold standard to conduct downstream applications, while stability of apolipoproteins decreases with increase in transport duration. Finally concluded, feasibility of DBS could be utilised to conduct field epidemiological studies.