Evaluating the role of long-term urine bio-banking on the stability of urine bio-markers in the diagnosis of pre-eclampsia

by
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Thesis presented in fulfilment of the requirements for the degree of
Masters of Pathology in the Faculty of Health and Medicine
at Stellenbosch University

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April 2019

DECLARATION

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Abstract

The aim of the pre-eclampsia (PE) and eclampsia monitoring, prevention and treatment consortium is to develop a rapid diagnostic test for PE in pregnant women because early identification of PE would decrease the likelihood of maternal and perinatal mortality and improve antenatal care, management and treatment. The identification of potential bio-markers is thus of great importance in PE because urine is a non-invasive bio-specimen and has the potential to help predict PE since proteinuria can be detected and quantified in urine. The purpose of this exploratory study was to evaluate the long-term stability of selected analytes within preservative-free urine in a pre-eclampsia cohort.

Calcium, Creatinine and total protein in long-term, stored urine samples were measured using both manual Siemens and Life Assay dipsticks and compared with high-throughput, laboratory measurements. Additionally, the diagnostic and prognostic potential of an Enzyme-linked immunosorbent assay (ELISA) -based, Adipsin or Complement Factor D (CFD) test for pre-eclampsia was evaluated. Furthermore, fresh urine samples were collected, and different processing and intermediate storage conditions were evaluated and compared to the medical research gold standard to determine to what extent pre-analytical variables could affect sample integrity. Albumin, Creatinine, Calcium, Urea and Total Protein were measured using high-throughput measurements.

The results of the study showed that the measurements for the Siemen dipstick and Life Assay dipstick were significantly similar. However, no agreement was found between the dipsticks and high-throughput laboratory measurements. Adipsin was measurable using the ELISA assay despite the assay not being validated for frozen urine samples. Our results also showed that the measurements for Creatinine, Protein, and Calcium were impacted after sample storage at room temperature for 48 hours, highlighting pre-analytical variable has a great influence on sample integrity.

This exploratory pilot study provided insight into the sample collection, handling, processing and long-term storage of urine bio-specimens and how each step of the process can have an impact. These insights led to an understanding of the limitations of this pilot study and can help to establish priorities for a larger study in terms of selected analytes to be measured, that could improve final research design and determine the best methods for data collection and analysis.

Opsomming

Die Pre-eklampsia (PE) en eklampsia monitering, voorkomings- en behandelings konsortium beoog om 'n vinnige diagnostiese toets vir PE in swanger vroue te ontwikkel, aangesien vroeë identifikasie van PE die waarskynlikheid van moeder- en perinatale sterftes verminder en voorgeboortelike sorg, bestuur en behandeling verbeter. Die identifisering van potensiële biomarkers is dus van groot belang in PE aangesien urine 'n nie-indringende bio-monster het die potensiaal om PE te help voorspel, aangesien proteïurie in urine opgespoor kan word en gekwantifiseer kan word. Die huidige loodsstudie was om die langtermyn stabiliteit van selekteerde metabolite in nie-gepreserveerde uriene in n PE kohort te evalueer.

Totale proteïen, kalsium en kreatinien in lang termyn gestoorde uriene was gemeet met die gebruik van die Siemens en Life Assay Dipstick en vergelyk met hoë-deurset laboratorium analise. Terselfdertyd, die diagnostiese en prognostiese potensiaal van n ELISA gebasseerde Adipsin/Komplement Faktor D (KFD) toets vir PE was ook geevalueer. Vars uriene was ook gekollekteer en op verskillende maniere geprosesseer en tydelik gestoor. Die verskillende kondisies was geevalueer en vergelyk met die goue standard om te bepaal tot watter punt voor –analitiese veranderinge monster intergiteit affekteer. Albumin, Kreatinien, Kalsium, Ureum en Totale Proteïen was gemeet deur hoë-deurset laboratorium metings.

Die studieuitslae toon dat die metings vir die Siemen en die LifeAssay dipstiek aansienlik gelyk is maar geen ooreenkoms was tussen die dipstieks en die hoë-deurvoer resultate gevind nie. Dus word verdere navorsing en valideringstudies voorgestel. Adipsin kon gemeet word deur die ELISA-toets te gebruik, ondanks die feit dat die toets nie vir urienmonsters valideer is nie. Ons resultate wys ook dat monsters wat by kamertemperatuur vir 48 uur gestoor is, het n invloed op die metings vir Kreatinien, Proteïen en Kalsium en beklemtoon dat voor –analitiese veranderinge n groot invloed het op monster integriteit.

Die verkennende studie het insig gegee in die versameling, hantering, verwerking en lang termyn storing van uriene monsters en hoe elke stap van die proses 'n impak kan hê. Hierdie insigte het ons begrip gegee van die beperkings van hierdie loodsstudie en sal help om prioriteite te vestig vir die groter studie in terme van die meting van selekteerde metaboliete, die verbetering van die finale navorsingsontwerp en die bepaling van die beste data-insameling en analise metodes

Dedication

I dedicate this Master's thesis to myself, because I finally stood on my own two feet and accomplished something on my own, with my own strength and mind.

Acknowledgements

I would like to acknowledge the following people and organisations for their support:

My supervisor, Dr Carmen Swanepoel for her dedication and sacrifice to make sure I graduated on time, and also teaching me everything I know. My co-supervisor, Shafieka Isaacs for always making sure I had everything I needed and teaching me how to be humble and kind. I would also like to thank my work colleague, Faghri February for always being there in a pinch when I needed help or advice. I would also thank the collaborators, James Roberts from Penn State University and Justus Hofmeyer at University of Frere and Wits, and the funders, LifeAssay Diagnostics, Medical Research Council and NSB, for their support of my project and teaching me work ethic and respect. The NRF is acknowledged for their contribution to my bursary for the two years of my Masters. I wish to thank my friends who endured my stress and anxiety and last, but not least, my family for their continued support and love throughout my education and career, especially my mother, Diane Bell, who dedicated her life and knowledge to the advancement of mine.

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List of Abbreviations

6-OHMS	6-hydroxy melatonin sulfate
Ab	Antibody
AMP	Adenosine monophosphate
B3Africa	Bridging Biobanking and Biomedical Research across Europe and Africa
BCNET	The Biobank and Cohort Building Network
Ca2+	Calcium
CAP	Calcium and Pre-eclampsia
CFD	Complement Factor D
CKD	Chronic Kidney Disease
crHTN	Chronic hypertension
ELISA	Enzyme-linked immunosorbent assay
fB	Factor B
fD	Factor D
fT3	Free triiodothyronine
fT4	Free thyroxine
GET	Global Emerging Pathogens Treatment Consortium
H3Africa	The Human Heredity and Health in Africa consortium
HDL-cholesterol	High-Density Lipoprotein Cholesterol
LIMS	Laboratory Information-Management System
MMP-9	Matrix Metalloproteinase-9
NHLS	National Health Laboratory Services
NM-BAPTA	5-nitro-5'-methyl-BAPTA
NMR	Nuclear Magnetic Resonance
NSB	National Health Laboratory Services (NHLS) in conjunction with Stellenbosch University (SU) Biobank

PE	Pre-Eclampsia
PIGF	Placental Growth Factor
Pr-Cr	Protein-Creatinine
PRE-EMPT	Pre-eclampsia and Eclampsia Monitoring, Prevention and Treatment
QC	Quality Control
SELDI-TOF–MS	Surface-enhanced laser desorption or ionization (SELDI) used with time-of-flight (TOF) is a soft ionization method in mass spectrometry (MS)
SCr	Serum Creatinine
sFlt-1	fms-like tyrosine kinase
SOP	Standard Operating Procedure
SU	Stellenbosch University
TPO	Thyrotropin
TSH	Thyroid Stimulating Hormone
UCP	Urinary C-peptide
Ucr	Urine Creatinine
VEGF	Vascular Endothelial Growth Factor
WHO	World Health Organisation

Chapter 1

Introduction and Literature Review

1.1. Introduction

A bio-bank is a research infrastructure that aids in research support, scientific advancement and innovation by providing a collection of high-quality, biological samples or bio-specimens that are stored temporarily or long term together with their associated data (Baker, 2012). Bio-specimens can be any type of biological sample, such as blood, eggs, sperm, hair, nucleic acid, urine, saliva, tissue etc. in which biological molecules or bio-markers can be found.

Today, bio-markers are becoming more important because they can serve as a measurable indicator to measure normal biological processes, the presence or progress of disease (pathogenesis), or the effects of treatment. However, bio-marker discovery and validation remain challenging owing to small sample sizes, low-quality bio-specimens and poorly captured clinical data. Access to high-quality bio-specimens with associated clinical data of high statistical reliability are required for validation purposes, and this is where a bio-bank is key as a tool. In a bio-bank it is possible to ensure strict application of standardised protocols and quality control (QC) and to address the effects of pre-analytical and storage variables.

There have been many studies focused on long-term storage of bio-specimens for use in bio-marker studies. Collectively, these studies highlight that bio-specimen type, storage temperature and the period being stored all affect the analytical results (Saude & Sykes, 2007).

Urine is one bio-specimen that is commonly and widely used as a non-invasive method to assess health, in both a clinical and epidemiological setting. It has advantages over blood as it enables the rapid development of assays, and is also very popular in metabolic investigations not only because of its non-invasive use, but also because of the complex metabolic nature of the fluid. Urine also gives the researcher the ability to collect multiple samples easily over a period of time (Saude & Sykes, 2007), thus, for the purpose of this literature review, the focus will be on urine as a substrate to be used in research.

For routine diagnostics and clinical purposes, the gold standard for urine collection, processing and analyses is within 24-hours. However, in addition, a few important factors needs to be considered include, collection procedures, the addition of preservatives, time taken between collection and storage, intermediate storage temperature, and number of freeze-thaw cycles (Remer, Montenegro-

Bethancourt & Shi, 2014). Therefore, studies of the long-term stability of urinary metabolites like creatinine, urea, osmolality, iodine, nitrogen, anions, cations, acid–base parameters, or organic acids following long-term storage are rare.

Saude and Sykes (2007) highlighted the importance of proper sample handling and storage techniques for urine samples for future metabolomic studies as it ensures that the sample reflects the original metabolic state it was in before the testing of metabolites. Thus, different methods of sample preparation, such as centrifugation and filtration, the absence or presence of a preservative, as well as sample temperature storage were described because these parameters had significant effects on urine metabolites over time (Saude & Sykes, 2007).

Urine, as mentioned before, is useful because it is a common and widely used non-invasive method to assess health, especially in a clinical setting. One example is pre-eclampsia (PE), which is a human-specific pregnancy disorder of which the origins are unknown, and which serves as the disease model on which one of the study's objectives was based (Buhimschi *et al.*, 2014). If PE is left untreated, the pregnant women might experience progressive clinical deterioration, which can result in seizures (eclampsia), stroke, haemorrhage, kidney damage, liver failure and death. New-onset hypertension and proteinuria after 20 weeks of pregnancy is the key presentation of the PE syndrome. (Buhimschi *et al.*, 2014).

Both hypertension and urine proteinuria are used as a diagnostic gold standard to predict PE, however these diagnostic measurements are compromised when the mother has other predisposing conditions, for example, chronic hypertension (crHTN) (Buhimschi *et al.*, 2014). Thus, there is a need to explore other urine bio-markers as potential diagnostic and or prognostic markers. But, if urine bio-markers are going to be considered as potential bio-markers then the stability of these metabolites needs to be considered when studying them, especially after storing them for long periods of time.

Altogether, PE, the bio-banking aspect which includes sample processing and handling, processing and storage conditions, as well as urine as an alternative non-invasive bio-specimen provide the context for subsequent discussions.

1.2. Fit for purpose: The importance of bio-specimen integrity

In the era of omics and bio-marker validation studies, the need for large cohorts of good quality bio-specimens is essential. In the past, researchers used to store samples with little consistency and forethought. Today, there is a collective effort to collect, store and distribute bio-specimens for

research and diagnostic purposes in a standardised and aligned manner. While storing samples is not new, the science of bio-banking and infrastructure, such as biobanks, have gained much attention lately as this complex science brings together multiple biological and social sciences and legislative disciplines. There are different types of bio-banks depending on where they are established, their size and range of bio-specimens collected. Bio-banks can either be based in an academic setting, hospitals, pharmaceutical or bio-technological companies or stand-alone organisations and there are bio-banks with specific focuses on what should be stored, i.e. tissue, population, twin or disease-specific bio-banks. Maintaining bio-specimen quality is critical for a bio-bank as it will influence downstream analytical applications. Thus any variable that might be introduced during processes from collection through to bio-specimen transportation, processing, storage and analysis could contribute to bio-specimen integrity and lead to distorted results if the bio-specimen is of bad or low quality (Hubel, Spindler & Skubitz, 2014).

Most bio-banks, whether small or large, should have a quality assurance and risk management plan in place to ensure the viability of the bio-specimens and to maintain bio-specimen integrity through facility and equipment monitoring and maintenance following best practices and guidelines. This is essential especially during continuous power failures, which happen quite frequently in developing countries and affect the stability of the bio-specimens. In addition, most bio-banks use a laboratory information management system (LIMS) to categorise, organise, track and manage storage of bio-specimens.

In summary, bio-banks are essential for future bio-marker discovery and validation studies as they can provide access to high-quality bio-specimens with associated clinical data with high statistical reliability and would also ensure strict application of standardised protocols and QC, and address the effects of pre-analytical and storage variables.

1.3. Infrastructure for bio-marker research

As new technologies develop over time, it becomes increasingly important to ensure that various samples are collected, handled correctly, and stored according to standard operating procedures (SOPs) in order to be fit for purpose, especially since the ideal sample type and volume for biomarker studies and research is unknown (Swanepoel, Snyders & Grewal, 2015).

The effect of storage conditions on the stability of bio-markers has been studied using various components such as purified nucleic acids, fluid bio-specimens such as serum, plasma, urine, cerebral spinal fluid and many more.

In pharmaceutical development, bio-markers have become important tools, serving as predisposition, predictive, prognostic and diagnostics companions to novel therapies which "aid in patient selection, treatment monitoring, adverse event risk assessment, and the extension of indications for established drugs" (Nolen *et al.*, 2013).

There are millions of bio-specimens currently stored around the world in bio-banks or as collections in academic settings containing bio-specimens that could be used for bio-marker validation studies. Since the storage conditions and processing conditions affect the suitability of these specimens for use in downstream processing, if not stored and processed according to standardised and aligned protocols, this could lead to uncertainty in the validity of the bio-marker studies and, subsequently, uncertainty in the interpretation of these studies.

The observation by Baker (2012) that "Our knowledge of the factors that impact a bio-specimen has not kept up, nor has the education of the users about how fragile a bio-specimen is" is crucial as we are constantly discovering new factors that influence samples and their stability.

There have been many studies focused on long-term storage of bio-specimens for use in bio-marker studies. From these studies, it is evident that bio-specimen type, storage temperature and the period of storage, all affect the analytical results after storage. For example, Kluger *et al.* (2011 demonstrated a 15% increase in the concentration of two, stored, serum cancer markers following a 10-year period. In another study by Kisand *et al.* (2010), a dramatic decrease in the vascular endothelial growth factor (VEGF) was observed after it was frozen and thawed repeatedly at either -20 ^{o}C or -75 ^{o}C. Similarly, another study by Rouy *et al.* (2005) showed that the matrix metalloproteinase-9 (MMP-9), a biomarker for cardiovascular risk detection in clinical studies, degraded over time even when stored at -80 ^{o}C. Interestingly, during low temperature storage, some bio-marker analytes would increase in concentration over time. For example, Männistö *et al.* (2007) showed that thyrotropin (TPO), thyroid hormones (TSH, fT4, fT3), and thyroid auto-antibody (Ab) concentrations (TPO-Ab, TG-Ab) in human serum increased in concentration when stored for 23 years at -25 ^{o}C. In a similar study, it was shown that thyroid stimulating hormone (TSH) decreased and free triiodothyronine (fT3) and free thyroxine (fT4) concentrations increased when stored at -80 ^{o}C storage for 8 to 11 years (Hillebrand, Heijboer & Endert, 2017). Thus the literature highlights that sample type, storage temperature, duration and how a bio-specimen is processed all affect the stability of the analytes that are examined. Which analyte is going to be studied also plays a role as certain analytes are more stable than others.

1.4. Urine as a bio-specimen for diagnostic purposes

The analysis of urine for any kind of diagnostic purpose dates well back to ancient times. Since then, urine and certain elements in the urine were used to diagnose renal diseases (Sharda *et al.*, 2014)

On average, urine contains up to 150mg/day of proteins and pathological proteinuria (>300mg/day), and how this occurs is divided into three categories (Figure 1.1 below). Firstly, Glomerula proteinuria (Glomerula disease) is the increase in the permeability of the glomerular capillary wall to macro-molecules, especially albumin. Secondly, Tubular proteinuria (Tubulointerstitium disease) is when there is reduced re-absorption of proteins, normally present in glomerular filtrate. Thirdly, overload proteinuria is the result of an excess or overload of low-molecular-weight proteins. These proteins are normally re-absorbed by the proximal tubules (Barratt & Topham, 2007).

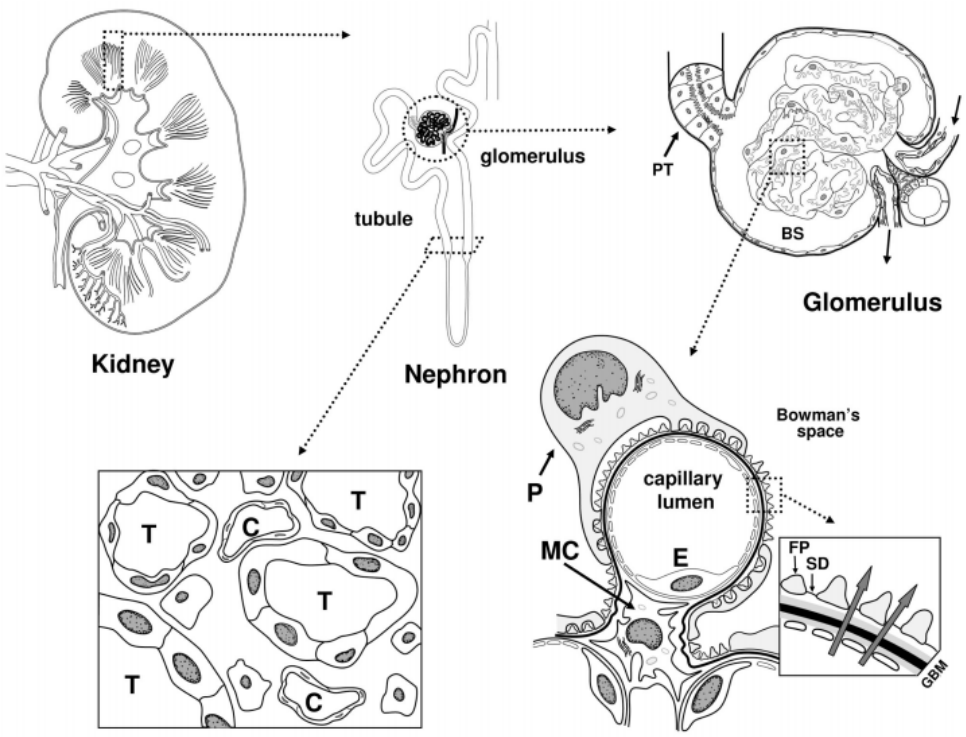


Figure 1-1: Kidney and its structural and functional components.

Extracted from (Julian et al., 2009). Each human kidney (left top) contains ~1,000,000 functional units or nephrons (middle, top) that span the two regions of the kidney: cortex and medulla. Each nephron is composed of a filtration structure (glomerulus) and a downstream tubule comprised of 11 functional segments. An afferent arteriole delivers blood to the encapsulated glomerulus (right, top) where it enters the capillary network, undergoes ultra-filtration and its residual volume exits by the efferent arteriole to return to the systemic circulation. The ultra-filtrate enters the proximal tubule (PT) as the primary urine and is progressively modified as it flows through the remaining segments of the tubule. Glomeruli are located in the cortex, while the tubular portions of the nephrons span the cortex and medulla. This structural feature creates the necessary concentration gradients for extraction of salts, water and various compounds from the urine in the tubular lumens. Endothelial cell (E) line the capillaries and contain openings (fenestrae) that permit water, salts, and small proteins and low-molecular-weight compounds to filter across the glomerular basement membrane (GBM); arrows in the enlarged inset on the right-hand side depict the flow of ultrafiltrate).

Preferable, urine protein concentrations are collected and analysed using the 24-hour gold standard, and are expressed as mg or g. On the other hand, in the untimed urine, spot collection method, the protein concentration is expressed as a ratio to compensate for variability of the urine volume and concentration between spots (Bertholf, 2014).

Urine proteins and metabolites are useful in research studies of renal diseases, but the process of sample handling, processing and storage also has a significant effect on the quality and integrity of the sample (Ferraz *et al.*, 2006). In urine, various metabolites can be assessed such as pH, citrate, uric acid, sulphate, sodium, potassium, cyclic adenosine monophosphate (AMP), calcium, magnesium, phosphorus, oxalate, ammonium and creatinine. Some important factors that can affect analysis include: if the urine is super-saturated with various solutes and contains bacteria if not collected

cleanly enough; if there is an error in sample handling then stone salt formation and precipitation can occur; also, oxidation of urinary ascorbic acid to oxalate can skew downstream analysis and subsequent results (Ferraz *et al.*, 2006). Therefore, proper sample handling is of outmost importance.

1.5. Urine metabolites and integrity

Since molecular weight proteins are not being filtered and passed through to the kidney into the urine, metabolomic studies use this characteristic of molecular weight protein differences to identify and profile unique metabolic differences to associate with a specific pathophysiology (Saude & Sykes, 2007). These profiles serve as urinary bio-markers which are used in practice to diagnose disease, thus a specific bio-marker can be used to diagnose a specific disease. One such example is PE, where the elevation of Adipsin (Complement Factor D) during pregnancy could be used as a bio-marker to test diagnostically for PE (Wang *et al.*, 2014,;2016). Another example includes the presence of albumin which is an indicator of renal disease. Tracking the fluctuations in the metabolic changes of a metabolite in response to a disease provides information about the disease which can then be used as a bio-marker, resulting in possible strategies for care and prevention. These metabolic changes as shown in human urine studies, indicate changes that can be a result of age and diet, but also relate to variations in populations and between individuals over time (Saude & Sykes, 2007).

The recent development of high-throughput, proteomic or metabolomic approaches has created new and expanded approaches to using bio-markers in urine for diagnostic purposes. This approach also enables the cataloguing of protein composition in fluids like plasma, serum, urine and saliva (Papale *et al.*, 2007). Thus, urine has many metabolites available to explore as a diagnostic tool. As the integrity of these metabolites over time is unknown, these metabolites need to be preserved in long-term storage at low temperatures. Therefore, prospective and longitudinal studies should pay extra attention to the collection and storage methodologies with regards to the stability of the metabolites in the urine because analysis cannot always occur immediately after collection. The stability of the metabolites in the urine is dependent on many factors and variables as mentioned earlier (Section 1.3). Other factors to consider include collection procedures, the addition of preservatives, time taken between collection and storage, intermediate storage temperature , and number of freeze-thaw cycles, (Remer, Montenegro-Bethancourt & Shi, 2014). The study of the long-term stability of urinary metabolites lacks, in particular, the influence of preservatives in long-term storage.

To date, there has been an increased reliance on the use of samples that have been stored for prolonged periods of time. One example involved patients with chronic kidney disease (CKD), where the impact

of sample handling, processing, duration of storage and other factors has become an area of significant interest (Liu *et al.*, 2016).

Saude and Sykes (2007) highlighted the importance of proper sample handling and storage techniques for urine samples to be used in future metabolomic studies because this ensures that the sample reflects the original metabolic state it was in before the testing of metabolites. Thus differences in sample preparation methods (centrifugation vs filtration), sample temperature storage (room temperature storage vs. ultra-low temperatures) are essential parameters that have significant effects on urine metabolites over time (Saude & Sykes, 2007).

If urine metabolites are considered to be potential bio-markers, the stability of these metabolites must considered when studying them in urine. Remer *et al.* (2014) investigated the stability of urinary analytes with preservatives, after 12 to 15 years of storage at -22 ^{o}C. The analytes examined included: creatinine, urea, osmolality, iodine, nitrogen, chloride, phosphate, sulphate, sodium, potassium, calcium, magnesium, components of renal net acid excretion, pH, titratable acidity, ammonium, bicarbonate, and the organic acids citrate, uric acid, oxalate, and total (titrated) organic acids. This study by Remer *et al.* (2014) provided valuable information especially for future epidemiological studies, or intervention studies such as pre-eclampsia.

Therefore, depending on the analyte that is being investigated, all the factors relating to laboratory techniques also need to be considered because the stability of the analyte depends on its metabolic structure as well as the matrix it is in, and the intra- and inter-individual, biological variabilities need to be remembered (Remer, Montenegro-Bethancourt & Shi, 2014).There have been several studies that evaluated certain urinary metabolites. Buyken *et al.* (2006) examined the stability of urinary C-peptide (UCP) over 12 years. Likewise, Griefahn *et al.* (2001) investigated and showed that the concentration of 6-hydroxy melatonin sulphate (6-OHMS) remained stable over a 15-year period.

Remer, Montenegro-Bethancourt and Shi (2014) noted and discussed a potential flaw in the literature where, for some research, no baseline measurements were taken before storage and after storage over a particular time. Liu *et al.* (2016) also discovered that the effect of long-term storage on bio-marker concentrations has not been studied well and that it is a really “methodological challenging problem to study”. Currently, there is no gold standard method by which to investigate the impact of storage time on bio-marker levels. Conducting repeated measurements from a single aliquot over the period of storage has been suggested but the study design can change and assays can change over time. Therefore, the methodology changes over time can invalidate the study (Liu *et al.*, 2016).

Interestingly, urinary creatinine seems to be regarded as one of the most stable metabolites as it is not easily affected by short- or long-term storage conditions as indicated by Spierto *et al.* (1997) and Parekh *et al.* (2007). Urinary creatinine is stable from 30 days to 2.5 years at various temperatures ranging from -4 ºC to -70 ºC.

Similarly, Remer, Montenegro-Bethancourt and Shi (2014) discovered and validated that urinary creatinine can also be stable at -22 ºC for 15 years.

On the other hand, oxalate is the exact opposite to creatinine as it is the least stable analyte (Ferraz *et al.*, 2006). Oxalate is the most variable urinary metabolite and there are several factors to consider when investigating it, such as analytical methods and interactions with other molecules. For these types of metabolites, the addition of preservatives to urine samples in order to stabilise certain molecules or to reduce bacterial degradation of certain molecules becomes essential. Interestingly, some studies have found no differences in the concentration of urinary metabolites, such as calcium, magnesium, oxalate, with and without preservatives (Ferraz *et al.*, 2006). By contrast, Yilmaz *et al.* (2008) discovered that calcium, magnesium, phosphate, and uric acid had a high level of stability when comparing measurement results of urine samples with and without preservatives.

Remer, Montenegro-Bethancourt and Shi (2014) demonstrated that urine samples stored at -20 ºC for up to 15 years have high stability for creatinine, urea, cations (sodium, potassium, calcium, and magnesium), ammonium, citrate, uric acid, iodine and nitrogen. Osmolality, anions (chloride, phosphate, sulphate), titratable acidity, bicarbonate, and total organic acids were found to be less stable or more prone to measurement errors (mostly small) over time. The study also highlighted that the addition of urine preservatives is not a requirement for long-term storage.

However it should be noted that, the metabolites that were tested in Remer, Montenegro-Bethancourt and Shi, 2014 were tested at -20 ºC, which raises the question of how they would have fared at -80 ºC. From the literature reviewed, there does not appear to be a collective study similar to study by Remer *et al.* (2014) but tested at -80 ºC.

However, in the study by Rist *et al.* (2013), urine aliquots were frozen either at −20 ºC, on dry ice, at −80 ºC or in liquid nitrogen and then stored at −20 ºC, −80 ºC or in liquid nitrogen vapour phase for 1 to 5 weeks before nuclear magnetic resonance (NMR) analysis. Results showed spectral changes depending on the freezing process. Samples frozen on dry ice showed the largest deviations which were found to be based on pH differences caused by variations in CO^2 concentrations that were

introduced by the freezing procedure. Therefore, the study recommended that urine samples should be frozen preferentially at -20 ^{o}C and then transferred to a lower temperature within one week. (Rist *et al.* (2013) also suggested that freezing procedures should be a part of all publication protocols or methodology.

Alternatively, Papale *et al.* (2007) assessed urine stored with and without the addition of protease inhibitors and left at room temperature from 0 to 48 hours. The study found that, when storing urine at room temperature, a progressive degradation of proteins occurs, which could have been prevented if protease inhibitors were added within 2 hours from collection time. Papale *et al.* (2007) also demonstrated that the timing of urine collection during the day only had a minor impact on the protein profile, but had an influence on the peak intensity following (Surface-enhanced laser desorption or ionization used with time-of-flight mass spectrometry (SELDI-TOF–MS analysis). Freeze or thaw cycles were also investigated, and it was concluded that 5 freeze or thaw cycles did not affect the intensity of the peaks. The study also considered the inter-subject variability, which was fairly consistent, but between-subject variability was noted, especially between the two sexes (Papale *et al.*, 2007).

1.6. The role and integrity of metabolites, albumin, creatinine, calcium, total protein and adipsin in urine

Currently, the options for the measurement of proteins are either the total level of protein in the urine or the measurement of single proteins. Considering total protein, the daily excretion is about 150mg/day. Ten mg of that 150mg is albumin (Barratt & Topham, 2007). Albumin is one of the urinary proteins that is measured to determine renal function because <100mg of albumin is filtered through the glomerulus per day when renal function is normal. Despite numerous proteins present in urine, albumin is the primary or first protein that will be observed thus making it a sensitive indicator for most renal diseases (Bertholf, 2014). Early renal disease is characterised by micro-albuminuria (30-300 mg/day) where the albumin is almost undetectable. Proteinuria or macro-albuminuria is characterised by >300mg/day.

Creatinine is also used in the studies of kidney disease. Serum creatinine (SCr), concentration in mg/dl, is used to estimate kidney function, screen for acute kidney injuries and also to determine disease progression. Urine creatinine (UCr), concentration in mg/dl, is also used to estimate kidney tubular function, determine disease progression, as well as accuracy of timed urine collection. As these measurements are key to diagnosing kidney disease, it is thus of utmost importance to

understand the different techniques and sample storage methods that affect the measurements of SCr and UCr. Askenazi *et al.* (2014) explored the effects of different methodologies, under different storage conditions, on SCr and UCr over time. It was observed that UCr measurements seem to vary according to methodology, whereas SCr was consistent; UCr declined over time, whereas SCr was stable over time (1 year) with no difference between UCr and SCr whether the sample was refrigerated or frozen (temperature did not make a difference) (Askenazi *et al.*, 2014). Creatinine can also be tested using the protein/creatinine ratio in UCr. To test the ratio, either the spot method can be used, which requires the collection of urine and drying it out on paper in a spot or the 24-hour urine collection method, which is the gold standard. Creatinine is also used in PE when combined with adipsin. The ratio between adipsin and creatinine can be used to assess PE diagnostically (Wang *et al.*, 2014; 2016).

Calcium (Ca^{2+}) is also an important metabolite in renal function and is used to diagnose hypercalciuria (elevated Ca^{2+} in the urine), a condition that indicates impairment of renal function, nephrocalcinosis (deposition of calcium salts in the renal parenchyma due to hyper-parathyroidism), and renal insufficiency (Ferraz *et al.*, 2006). Calcium plays a role in many cellular processes such as hormone secretion, muscle contraction, nerve conduction, and activation or inactivation of many enzymes. It is mostly absorbed in the gut, with only 30% absorbed from food. Other factors affect the absorption, such as the amount consumed and age or life stage. The amount absorbed will decrease as the amount of intake is increased; which is why infants or young children require more calcium than older adults. In addition, in some cases, an increase in calcium supplements has implications such as an increase of gall and renal stones, increased incidence of myocardial infarction and various fibrotic conditions. Similar complications such as increased heart attacks have also been observed in the elderly who take calcium supplements for the treatment of osteoporosis (Samozai & Kulkarni, 2015). In addition, Samozai and Kulkarni (2015) also described the effects of calcium supplements on menopausal women and concluded that giving calcium supplements does not change the concentration in calcium serum levels but did increase the urine calcium levels significantly. The gold standard would be 24-hour collections of urine but the random urine collections can also be used for calcium measurements and is expressed in relation to creatinine. The normal reference for the urine:creatinine ratio (mg/dL:mg/dL) is <0.14. Hypercalciuria occurs when the ratio is >0.20. Thus, if an elevation of calcium to >300mg/24hrs is observed, then it is indicative of an over-active parathyroid gland and is presented as hypercalciuria and hyperphosphaturia. However, only 1/3 of patients presenting with hyper-parathyroidism have normal urine calcium levels so calcium is not an ideal analyte for the measurement of parathyroidism. On the other hand, for pre-eclampsia, Imdad, Jabeen and Bhutta

(2011) discussed that many epidemiological and clinical studies have shown that there is an inversely proportional relationship between calcium uptake or supplementation and the development of hypertension during pregnancy. The effect of calcium supplementation varies according to baseline calcium intake and any pre-disposing factors. In their review of the literature, Imdad, Jabeen and Bhutta (2011) found that calcium supplementation was associated with a decrease in the risk of hypertension during pregnancy as well as pre-eclampsia, neonatal mortality, and pre-term birth.

Adipsin (also known as Complement Factor D, c3 convertase activator) is a serine protease secreted in adipose tissue that is expressed at high levels. Therefore, Adipsin forms part of the alternative pathway (Figure 1-2) for complement activation. Adipsin or factor D (fD) catalyses the formation of a C3-cleaving enzyme by interacting with factor B (fB) and C3b. During pregnancy, the complement system has an increased activation which has been suggested to offset the suppression of the adaptive immunity during normal pregnancy. There have also been indications where some complement complexes and proteins are deposited into both normal and pre-eclamptic placentas with the latter having more being deposited. The adipose tissue is therefore an important factor in PE. Especially in the production of pro-inflammatory cytokines that are involved in many endocrine functions. It is also noted that women with PE have decreased levels of high-density lipoprotein cholesterol (HDL-cholesterol) and increased levels of triglycerides and insulin levels. This relationship between PE and the complement activation pathway is important and measurements of complement activation products are shown to be increased in PE pregnancies in comparison with normal pregnancies (Poveda *et al.*, 2016). In the role of metabolic control, glucose transport systems and adipocyte triglyceride synthesis are stimulated by Adipsin through an insulin-dependent mechanism. Therefore, studies have shown that Adipsin is increased in obesity and type 2 diabetes. This raises the question of how to diagnose PE in pregnant patients who are either obese or diabetic, or both. Thus, Adipsin plays a role in fat metabolism and glucose homeostasis. In their research, Wang *et al.* (2014; 2016) describe a relationship between Adipsin and creatinine. The relationship between Adipsin and creatinine, measured by using a rapid test and expressed as a ratio, was closely correlated to the 24-hour urinary protein in patients with pre-eclampsia. The rapid test was then combined with the increased diastolic blood pressure levels ($\geq$ 90 mm Hg) to diagnose PE. The sensitivity was 90.3% and the specificity reached 100.0% for pre-eclampsia diagnosis.

From the above-mentioned studies for the different analytes, it is clear that protein, albumin, creatinine, calcium and Adipsin all play a role in PE and this makes each analyte an important bio-marker in the study of PE.

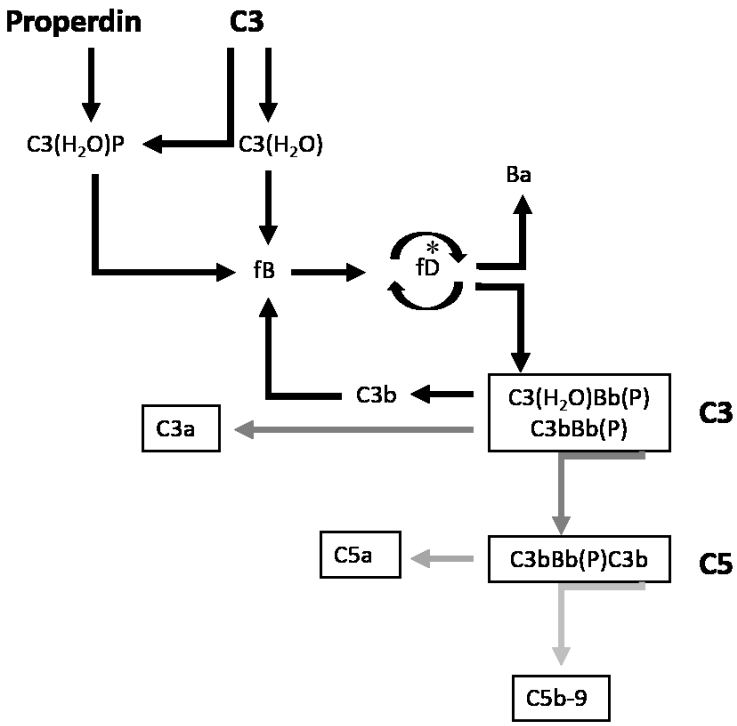


Figure 1-2: The Alternative Complement Pathway.

When a C3b fragment binds to a microbe, C3b changes shape which allows factor B (fB) to bind. This, in turn, allows Adipsin or factor D (fD) (referenced by * in the image) to bind to fB. This then cleaves fB to Ba and Bb. Bb will stay bound as C3(H2O)Bb(P) and Ba will become Ba on its own. C3 is the C3-convertase (C3 belongs to family of serine proteases and is necessary in innate immunity as a part of the complement system in the alternative pathway).

1.7. Urine stability challenges related to infrastructure

Urine metabolite stability is an important concern and there are standardised methods to ensure the stability of urine metabolites throughout handling and processing. Storing samples at the correct temperature is crucial to ensure urine metabolite stability. However, in practice, infrastructure challenges might influence the storage conditions that are considered. The decision also depends on the downstream applications for which the sample will be used and whether the required infrastructure is available or not. For example, one would use refrigeration (4ºC or -20ºC) as a short-term or temporary solution when high volumes of urine are submitted to a laboratory for processing, or long-term storage at -80 ºC for analysis at a later stage depending on whether the infrastructure is in place.

Froom *et al.* (2000) describes the use of refrigeration as a facility for overnight storage in order to complete all analysis without rushing which would introduce human error. In certain clinic settings, it can be quite difficult to ensure that samples arrive the same day as collection, which is why refrigeration is an important storage method as it partially stabilises the urine analytes for analysis

the next day (Froom *et al.*, 2000). It is also important to note that the degree of stability varies with the type of sample being used.

1.8. Overview of PE

PE is a human-specific pregnancy disorder of which the origins are unknown, and which serves as the disease model for this study. If PE is left untreated, pregnant women might experience progressive clinical deterioration which can result in seizures (eclampsia), stroke, haemorrhage, kidney damage, liver failure and death. New-onset hypertension and proteinuria after 20 weeks of pregnancy is the key symptom of PE syndrome. Table 1-1 below shows the diagnostic criteria and classification of hypertensive disorders during pregnancies (Williams, Pipkin & Fiona, 2011). Unfortunately, PE is not a predictable syndrome as its clinical presentation can be variable and the speed of progression can be slow or fast. It has been noted that altered concentrations of soluble fms-like tyrosine kinase 1 (sFlt-1) and placental growth factor (PIGF) can be used to predict if PE will occur, before the onset of the clinical symptoms. Unfortunately, these predictive indicators do not monitor the exact occurrence of PE (Wang *et al.*, 2014). Both hypertension and urine proteinuria are used as a diagnostic "gold standard" to predict PE but these diagnostic measurements are compromised when the mother has other predisposing conditions, for example, chronic hypertension (crHTN) (Buhimschi *et al.*, 2014). Hence the need to explore other urine bio-markers as potential diagnostic or prognostic markers.

Table 1-1: Diagnostic criteria and classification of hypertensive disorders of pregnancies.

Taken from Williams, Pipkin and Fiona, 2011.

Classification	Diagnostic criteria
Gestational hypertension	Hypertension: blood pressure ≥ 140/90 mmHg after 20th week of pregnancy in a previously normotensive woman.
Pre-eclampsia	Hypertension: blood pressure of ≥ 140/90 mmHg after 20th week of pregnancy in a woman who was previously normotensive. Proteinuria: urinary excretion ≥ 300 mg/L or 500 mg/24 hours in the absence of urinary tract infection.
Eclampsia	Occurs in a woman with pre-eclampsia. Characterised by seizures not attributed to other causes.
Super-imposed pre-eclampsia	Chronic hypertension with development of proteinuria during pregnancy.
Chronic hypertension	Hypertension present before 20th week of pregnancy, persistent for more than 6 weeks postpartum, or both.

1.9. Present Study

For the present study, a PE-based cohort which forms part of the Calcium and Pre-eclampsia (CAP) study was evaluated for one of the objectives. The CAP study was a World Health Organisation (WHO) collaboration that formed part of the pre-eclampsia and eclampsia monitoring, prevention and treatment (PRE-EMPT) consortium and was made up of collected urine bio-specimens from Argentina, Zimbabwe and South Africa. It aimed to develop a rapid diagnostic test for PE in pregnant women because early identification of PE would decrease the likelihood of maternal and perinatal mortality and improve antenatal care, management and treatment. Therefore, the identification of potential bio-markers is of importance in PE, and urine, being a non-invasive bio-specimen has the potential to help predict PE as proteinuria in the urine can be detected and quantified. Therefore, the

present study was an explorative study that took existing data from the larger mother study to conduct a pilot study into the different factors influencing metabolite stability in long-term storage at -80^{o}C.

The aim of this study was to investigate the effect of long-term urine bio-banking on the stability of urine bio-markers in the diagnosis of pre-eclampsia by understanding the variety of storage conditions and their effect on bio-markers.

To achieve the aim of this study, the objectives were:

1) Conduct a pilot study to compare high-throughput vs. dipstick measurements of Creatinine, Total Protein and Calcium in urine aliquots from the PE based CAP cohort stored long-term at -80^{o}C (henceforth shortened to "Objective 1: Dipsticks vs High-throughput").
2) Evaluate the Enzyme-linked immunosorbent assay (ELISA) validation of Adipsin or Complement Factor D measurements for long-term stored urine (henceforth shortened to "Objective 2: Adipsin").
3) Investigate the pre-analytic effect of pre- and post-storage conditions and processing of samples by measuring Total Protein, Urea, Albumin, Calcium and Creatinine after a set period of storage at -80^{o}C (henceforth shortened to "Objective 3: Storage").

Chapter 2

Methodology

2.1 Study Setting and Background

This explorative study was conducted in association with the National Health Laboratory (NHLS) in conjunction with Stellenbosch University (SU) Biobank, shortened to NSB, within the Division of Haematology based at Tygerberg Hospital and forms part of a larger study which was approved by the Health Research Ethics Committee of the Faculty of Medicine and Health Sciences of the University of Stellenbosch (Biobank ethics Ref number: N15/10/119 and University of Witwatersrand HREC Clearance Certificate: M10977; University of Cape Town: 457/2010; WHO trial reference number: A65750). NSB is a registered bio-bank/repository, and is affiliated with the NHLS and SU. NSB is also a partner/member of The Human Heredity and Health in Africa consortium (H3Africa), Bridging Biobanking and Biomedical Research across Europe and Africa Consortium (B3Africa), The Biobank and Cohort Building Network (BCNET), Global Emerging Pathogens Treatment Consortium (GET) and various other consortia. This specific study had 3 main objectives where three different *modus operandi* were employed. In this chapter, the target population of all three objectives has been discussed followed by discussion of separate methodologies in terms of sample collection, processing, and data analysis.

2.2 Cohort population background and pre-analytical variables and storage conditions

For objective 1, a PE-based cohort which formed part of the CAP study was evaluated. The CAP study is a WHO collaborative that forms part of the PRE-EMPT consortium and is made up of urine bio-specimens collected from Argentina, Zimbabwe and South Africa. The South African urine samples were collected since 2013 and stored onsite at -80^{o}C at Groote Schuur Hospital (Cape Town, Western Cape), Chris Hani Baragwanath Hospital (Johannesburg, Gauteng), Fort Hare (East London, Eastern Cape) and Cecilia Makiwane Hospital (East London, Eastern Cape) before it was consolidated and stored at NSB, a central repository facility for the CAP study in 2016. These urine samples were acquired from pre-pregnant, pregnant and post-pregnant women (labelled ADM, PPV, DPV8, DPV20, DPV32, POST-P and PE) as shown in Table 2-1.

2.2.1. Pre-analytical variables and storage conditions at collection sites

The WHO CAP SOP required samples to be stored immediately at -80ºC following collection; however, in cases where ultra-low temperature storage was not available, urine samples were allowed to be stored on ice for 24 hours or at -2 to -8ºC for 8 days or at -20ºC for 3-6 months before freezing at -80ºC (Appendix A). As samples were collected in busy, routine clinics, it is not known what the time frames and the short-term storage temperatures were after collections and whether these pre-analytical variables were captured. In addition, the prescribed SOP for collection of urine samples did not follow the gold standard method, which requires centrifugation of urine at low speed of 1600 x g /2500 rpm for 15 minutes and aliquoting the supernatant for long term storage at -80ºC.

Table 2-1: Labelling format of the weeks.

Abbreviation	Full
PPV	Pre-Pregnancy
ADM	Admission
DPV8	Week 8 of the pregnancy
DPV20	Week 20 of the pregnancy
DPV32	Week 32 of the pregnancy
POST-P	Post-Partum/Pregnancy
PE	Pre-eclampsia

2.2.2. Sample shipment to NSB central biorepository

Samples were shipped in batches from various sites on dry ice and packed according to NSB policies, guidelines and SOPs (Appendix A and B) as well as IATA regulations (IATA, 2018). The courier company, Marken, collected the Marken's BioSystem Boxes (BIOIII) filled with dry-iced from the sites at specific times for overnight delivery (Figure 2-1). Upon delivery, the samples were immediately transferred to freezers at -80ºC (Snijders Scientific, Tilburg, Netherlands). In order to

standardise and align procedures, NSB was given the responsibility to verify or cross-check the samples against the CAP compiled database, re-organise, re-label with both 1D and 2D unique barcodes and re-store and capture storage location sites because each site had its own guidelines for sample collection and data compilation. All data were captured using Excel.

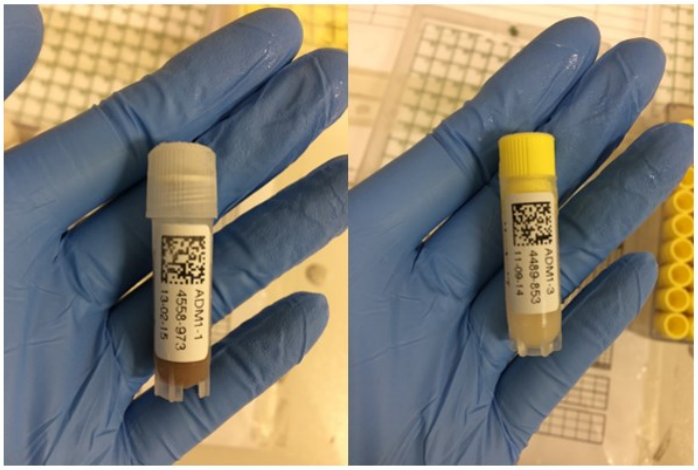
2.2.3. NSB sample verification, relabelling and re-storage

In 2016, it was requested that the urine bio-specimens be moved to NSB in Cape Town in order to relabel, re-organise and re-check them against the CAP compiled database. A number of issues were observed in the collections, of which labelling and overfilled tubes were major concerns. In order to standardise and align procedures, urine bio-specimens were re-labelled with both 1D and 2D unique barcodes, re-organised and re-checked (Figure 2-1 and 2-2). This resulted in improved ease of search and access to bio-specimens via a LIMS as well as an excellent cohort that would be of benefit to the wider PE research community. Figure 2-1 and 2-2 below show some of the issues and solutions.

The stored cohort were supposed to be used in a larger study which aims to determine the value of the Protein/Creatinine (Pr-Cr) strip for urinalysis developed by Life Assay Company in detection of early pre-eclampsia in pregnant women. The objectives were: (1) Value of the Pr-Cr strip vs standard protein only strip in identification of women with pre-eclampsia; (2) Compare the strip with a standard biochemical Pr-Cr assay and a more expensive Pr-Cr strip (Siemens) and with pregnancy outcome; (3) Further usefulness of the strip will be done by comparing readings by midwives with laboratory assay of protein and creatinine and the more expensive strip by Siemens.

A small pilot was performed first to measure the same analytes to determine whether the integrity of the samples was still intact and whether optimal results would be obtained despite the lack of knowledge regarding pre-variables prior to arrival at NSB.

A

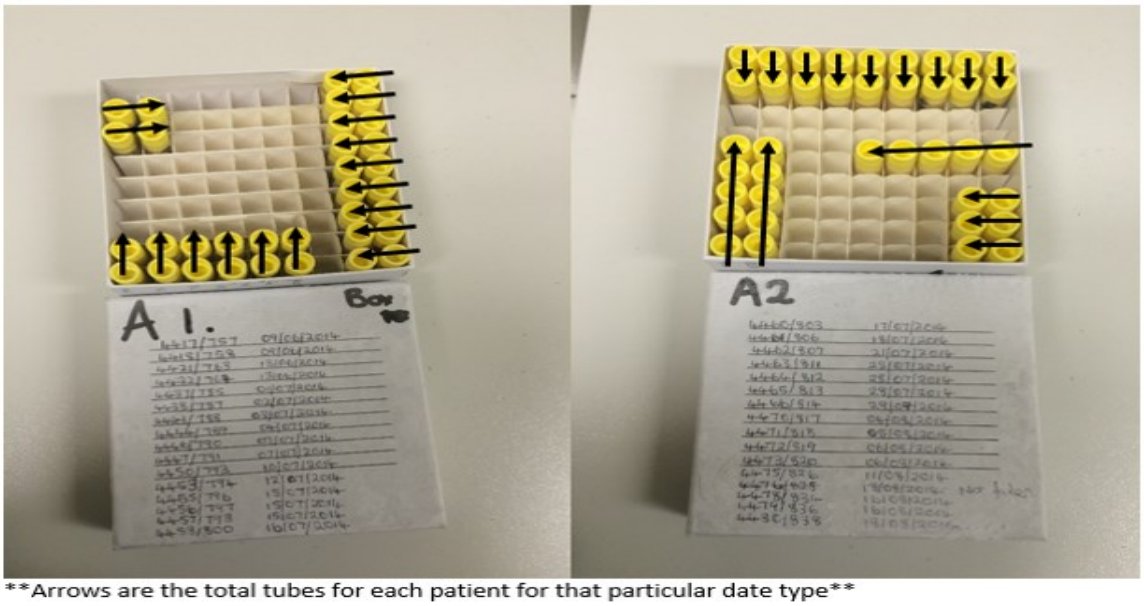


B

Figure 2-1: Shipments and Relabelling.

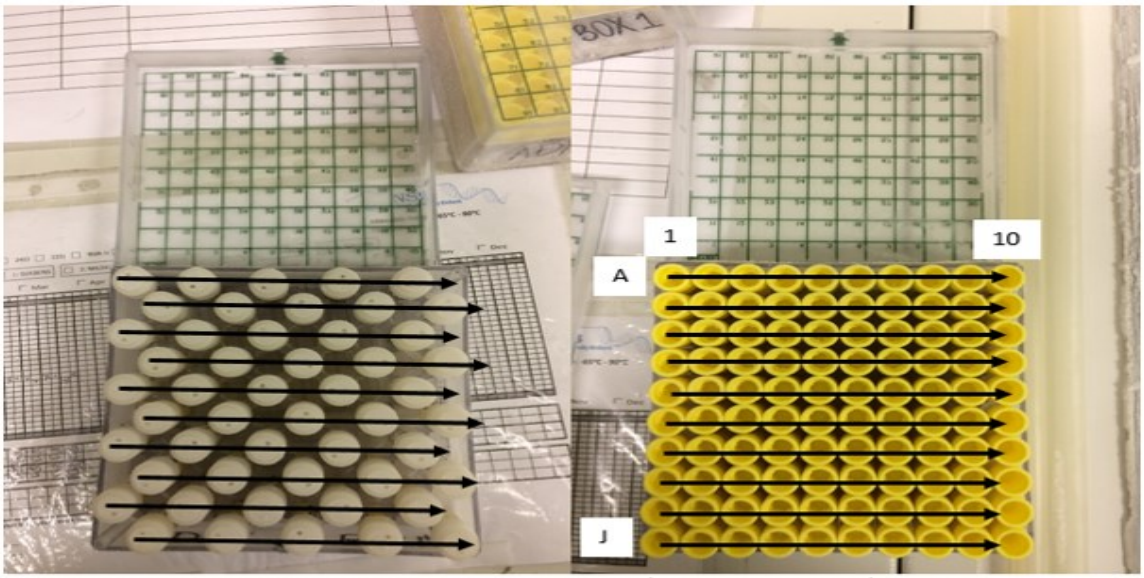
A indicates the arrival of the samples. They were packed according to IATA guidelines into a certified Marken box with dry ice for transport. B indicates the before and after labelling of tubes. Paper-based or permanent marker labels were replaced with both a 1D and 2D cryo-protecting barcode to allow for easy scanning and tracking.

Before:



Arrows are the total tubes for each patient for that particular date type

After:



Arrows indicate direction of tubes, starting at A1 to A9/A10, then B1 to B9/B10, etc
Continuous rounds
White tubes lids are bigger and therefore cannot store tubes next to each other

Figure 2-2: Re-organisation and storage.

Before and after photographs of the boxes showing open spaces. Following re-organisation, the 2ml tubes were re-organised from left to right (1-10) and placed left to right on each row (A-J) in order to use storage space appropriately and assign storage space location.

2.3 Target population and sample collection

2.3.1 Objective 1: Dipsticks vs high-throughput

For this part of the study (Objective 1), approximately 70 frozen urine samples were accessed from NSB per week. However, for this particular study, only 68 aliquots from Week 32 were used for the pilot and the subsequent data analysis. The samples were thawed overnight in the fridge at 4^{o}C before being processed the next morning.

Processing and analysis of the samples were two-fold: firstly, samples were aliquoted into the correct prescribed tubes for the diagnostic, clinical, high-throughput measurements and subsequently submitted to the Division of Chemical Pathology, an ISO 15189 accredited NHLS diagnostic laboratory at Tygerberg Hospital, to measure creatinine, calcium and total protein (See Section 2.4.1.2 for further details on test principles). High-throughput measurements for total protein were made via the automated system (Roche Cobas 6000 analyser, Risch-Rotkreuz, Switzerland) as well as the Siemens (protein) dipstick (Multistix 5, Siemens Healthcare Diagnostics Inc, Tarrytown, NY, USA), which is the gold standard used currently in South African clinics. In parallel, the same set of samples were used to test the newly developed LifeAssay dipstick which can measure both creatinine and protein.

2.3.2 Objective 2: Adipsin

For Objective 2, a small sub-set of the frozen urine samples from 14 patients over the weeks (ADM, DPV8, DPV20 and DPV32) were accessed from NSB. These samples were used for the analyte measurements described above as well as for the Adipsin validation study following overnight thawing at 4^{o}C. The Adipsin or Complement Factor D (CFD) measurement were determined via the Enzyme-linked Immunosorbent Assay Kit for CFD (Cloud-Clone Corp, Houston, USA) according to the manufacturer's protocol (See Section 2.4.2 for more details). The plates were then read on the ELISA plate reader (Bio-Rad, California, USA).

2.3.3 Objective 3: Storage

For Objective 3, fresh urine samples were acquired from 20 consenting volunteers (See Appendix C) using a 50 ml urine collection container (Tygerberg Hospital) according to the international best practices guidelines on urine sample collection (Appendix A). Subsequently, urine samples were

processed following the gold standard or according the workflow outlined in Section 2.4.3. The amount filled in the container was aliquoted equally over 12 urine tubes for storage.

2.4 Analyte measurements

The aim of the larger study was to compare the LifeAssay Dipstick with the Siemens gold standard Dipstick but also with high-throughput, clinical measurements obtained in an accredited, diagnostic laboratory's high-throughput instruments. The principles for each methodology employed for analyte measurement have been discussed in the following sections.

2.4.1 Objective 1: Dipsticks vs high-throughput

2.4.1.1. The newly developed LifeAssay vs Siemens Dipsticks

Both the Siemens Dipstick (performed as part of accredited, clinical, high-throughput) and LifeAssay Dipstick (performed in-house) were used to measure total protein, and both protein and creatinine in the case of the LifeAssay Dipstick. Prior to measurement, the thawed urine samples were gently mixed to ensure even distribution of particles. Following the mixing of urine, a small aliquot (1-2 drops) was dropped onto each section square of both dipsticks (LifeAssay and Siemens). After 60 seconds, both dipsticks were softly blotted onto a paper towel. Subsequently, a visual interpretation was done and a photograph was taken of the dipstick to ensure the colour change was recorded for each sample. See Figure 2-3 below.

A B

Figure 2-3: Representative images and visual interpretation of both the LifeAssay PrCr Dipstick (A) and the Siemens Protein Dipstick (B).

The yellow to green colours for the LifeAssay represents the presence and absence of total protein whereas white to brownish pink represents the presence or absence of creatinine.

2.4.1.2. High-throughput Clinical Lab Automated system

Clinical measurements for each analyte were taken according to validated SOPs (Appendix D, E and F).

For these clinical measurements, the thawed urine samples were mixed vigorously to ensure that precipitated calcium was dissolved adequately after long-term storage (See Appendix A). Precipitated Calcium would have skewed subsequent clinical Calcium measurements if urine samples were not properly mixed. Between 200ul to 1ml, depending on availability, were aliquoted into a blue-top tube provided by the Chemical Pathology diagnostic laboratory. Once aliquoted, the urine was analysed, and Creatinine, Calcium and Total Protein were measured using the Roche Cobas 6000 analyser (Roche, Risch-Rotkreuz, Switzerland) according to the laboratory's validated protocol. For the sake of context, the principle underlying each analyte measurement has been discussed briefly in the sub-sections below.

2.4.1.2.1. Clinical Creatinine measurement

An *in vitro* test for the quantitative determination of creatinine concentration in human serum, plasma and urine specimens carried out using the Roche/Hitachi Cobas C systems. This enzymatic method is based on the conversion of creatinine, with the aid of creatininase, creatinase, and sarcosine

oxidase, to glycine, formaldehyde and hydrogen peroxide. Catalysed by peroxidase, the liberated hydrogen peroxide reacts with 4-aminophenazone and (HTIBa) to form a quinone imine chromogen. The colour intensity of the quinone imine chromogen formed is directly proportional to the creatinine concentration in the reaction mixture as shown in Figure 2-4 (See Appendix D for more details on the test procedure).

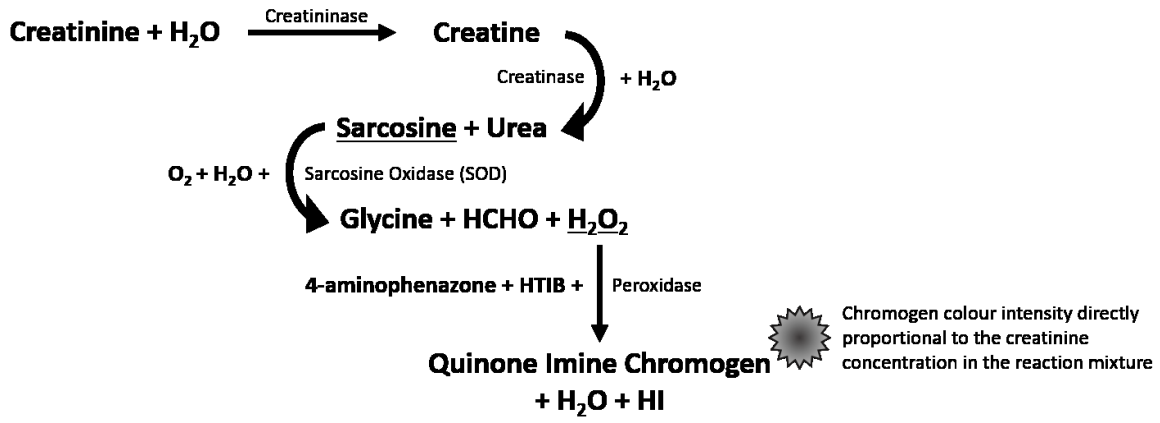


Figure 2-4: Test Principle for *in vitro* creatinine quantification.

2.4.1.2.2. Clinical Calcium measurements

An *in vitro* test for the quantitative determination of Calcium in human serum, plasma and urine was carried out using the Roche/Hitachi Cobas C systems. In this assay, Calcium ions reacted with 5-nitro-5'-methyl-BAPTA (NM-BAPTA) under alkaline conditions to form a complex. This complex reacts in the second step with EDTA. The subsequent changes in absorbance were directly proportional to the Calcium concentration and was measured photometrically as shown in Figure 2-5 (See Appendix E for more details on the test procedure).

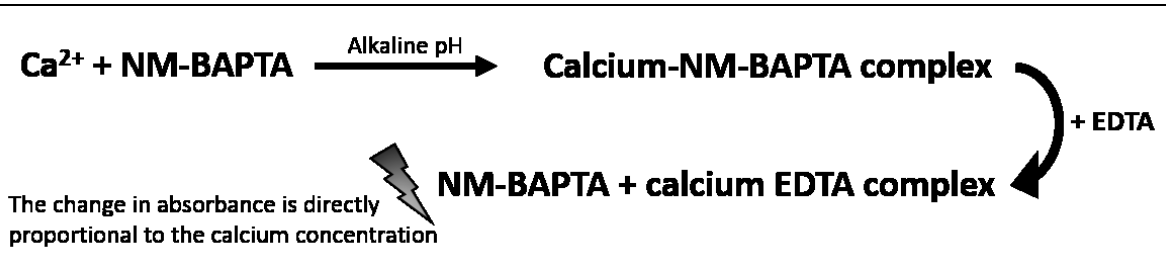


Figure 2-5: Test Principle for in vitro calcium quantification.

2.4.1.2.3. Clinical Total Protein measurements

An *in vitro* test for the quantitative determination of protein in human urine and cerebrospinal fluid was performed using the Roche/Hitachi Cobas C systems. An automated Turbidimetric method was employed. The sample was pre-incubated in an alkaline solution containing EDTA, which denatured the protein and eliminated interference from magnesium ions. Benzethonium Chloride was then added, producing turbidity as shown in Figure 2-6 (See Appendix F).

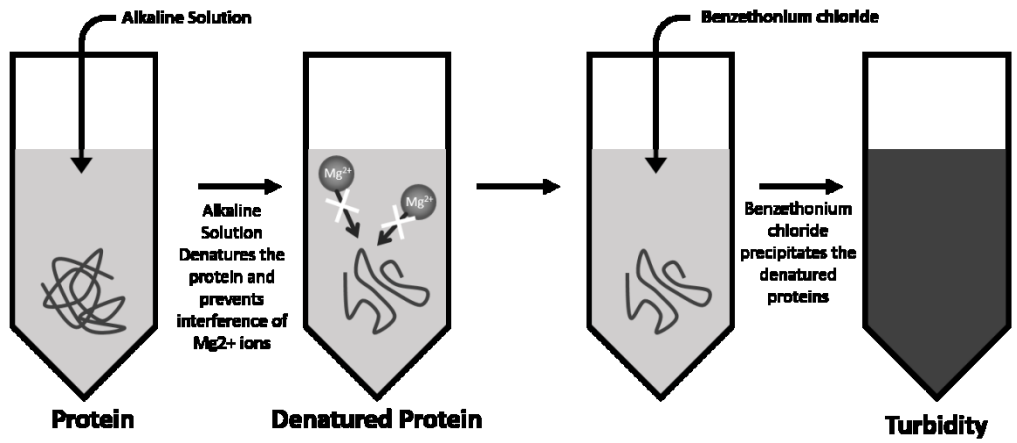


Figure 2-6: Test Principle of the Turbidimetric method for the quantitative protein in vitro test.

2.4.2 Objective 2: Adipsin

The commercial Enzyme-linked Immunosorbent Assay Kit for Complement Factor D (CFD) (Cloud-Clone Corp, Houston, USA) was used to measure Adipsin within the subset of 14urine samples chosen according to the manufacturer's protocol (Appendix G). The kit itself is a sandwich enzyme immune-assay that is used for the *in vitro* quantitative measurement of Adipsin or CFD in human serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids based on the Sandwich ELISA Concept (Figure 2-7). The microplate provided was pre-coated with an antibody specific to Adipsin or CFD. Standards and samples (100ul) were added, with the sample added in triplicate to the appropriate microplate wells with a biotin-conjugated antibody specific to CFD (Figure 2-8). Next, Avidin conjugated to Horseradish Peroxidase (HRP) (Detection Reagent B) was added to each microplate well and incubated. Subsequently, TMB substrate solution was added, and only those wells that contained CFD, biotin-conjugated antibody and enzyme-conjugated Avidin exhibited a change in colour. This enzyme-substrate reaction was terminated by the addition of sulphuric acid solution and the colour change was measured spectrophotometrically at a wavelength of 450nm ± 10nm on an ELISA plate reader (Bio-Rad, California, USA). The concentration of CFD in the samples was then determined by comparing the optical density (OD) of the samples to the standard curve.

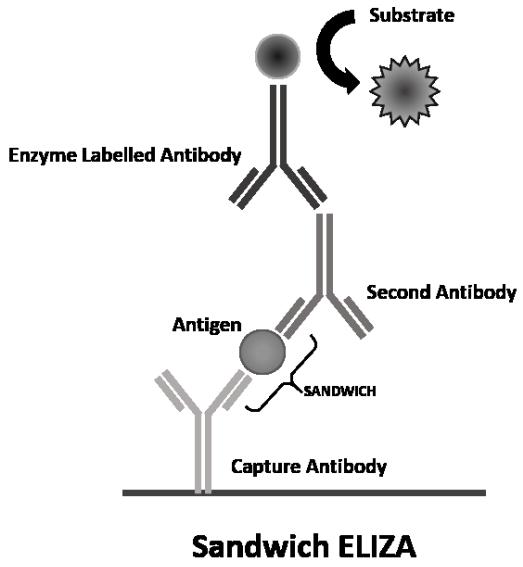


Figure 2-7: The Sandwich ELISA Concept.

The base/plate is coated with a capture antibody; following the addition of the test sample, the target antigen present will bind to the capture antibody. Subsequently, a second antibody is added, and binds to the antigen which creates a sandwich concept. Lastly an enzyme-labelled antibody is added, which binds to the second antibody. Following a specific substrate addition, enzyme conservation results in the measurement of a detectable form.

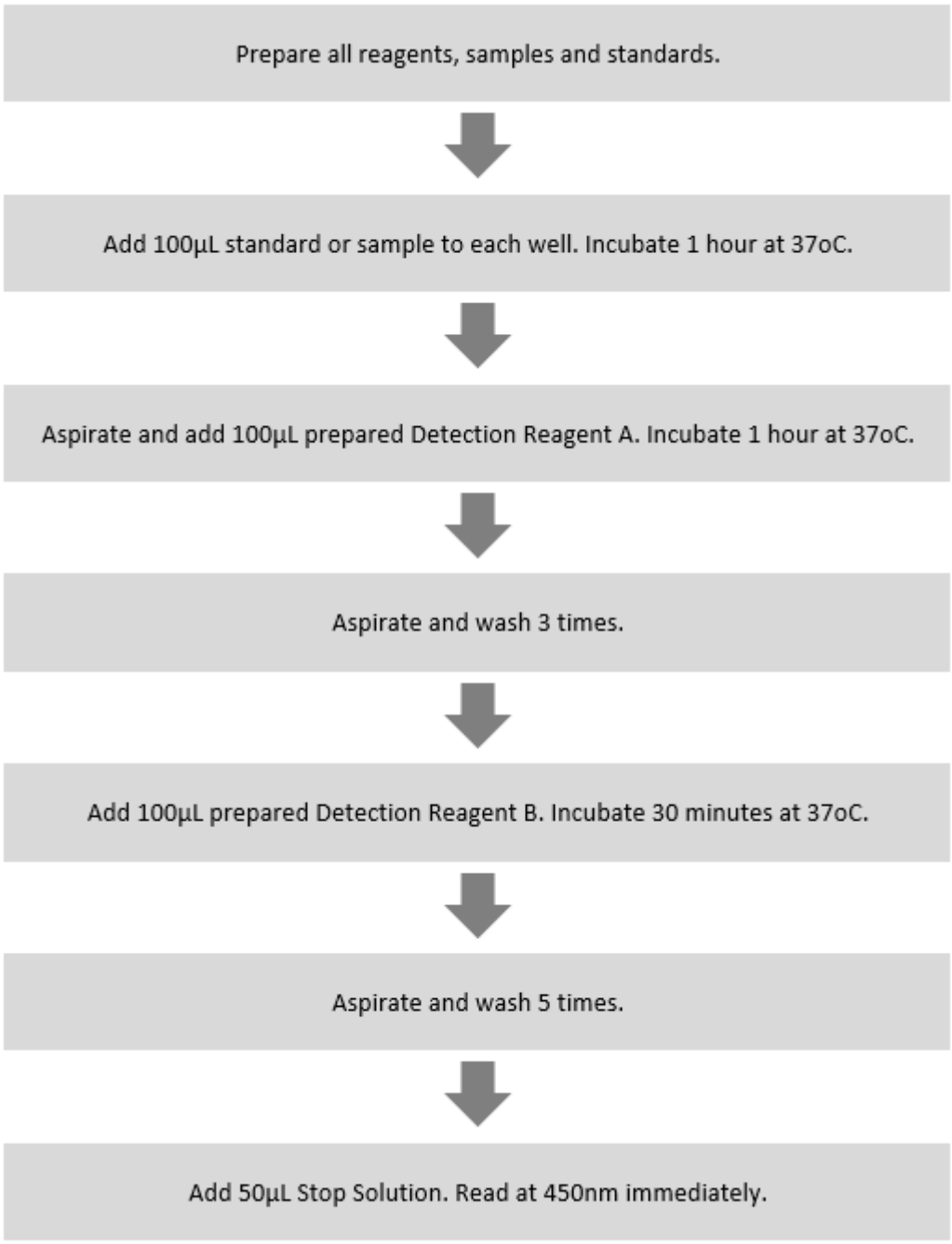


Figure 2-8: Summary flow diagram for the ELISA methodology.

2.4.3 Objective 3: Storage

Twenty participants provided urine in a 50ml container, which was subsequently aliquoted into 12 urine tubese. Therefore, there were 240 tubes after aliquoting. The 240 urine collection tubes were grouped into 4 groups in triplicate to account for human error and sample variation. Thus, each group had 60 tubes (in triplicate) as shown in Figure 2-9. For group 1, which served as the baseline and followed the gold standard, triplet urine tubes were centrifuged at 2500rpm for 10 minutes and then

immediately stored at -80°C. Group 2 were centrifuged at 2500rpm for 10 minutes and then stored at 4°C for 48 hours and subsequently stored at -80°C. Group 3 were centrifuged at 2500rpm for 10 minutes and then stored at room temperature (25°C) for 48 hours and subsequently stored at -80°C. Group 4 was stored only at -80°C without centrifugation. Each tube was then thawed after 3 months, according to guidelines (Appendix A). After thawing, each tube was transferred into a blue-top tube provided by the Chemical Pathology diagnostic laboratory. Once aliquoted, the urine was analysed, and Albumin, Creatinine, Calcium, Urea and Total Protein were measured using the Roche Cobas 6000 analyser (Roche, Risch-Rotkreuz, Switzerland) according to the laboratory's validated protocol (Appendices D, E, F, and H).

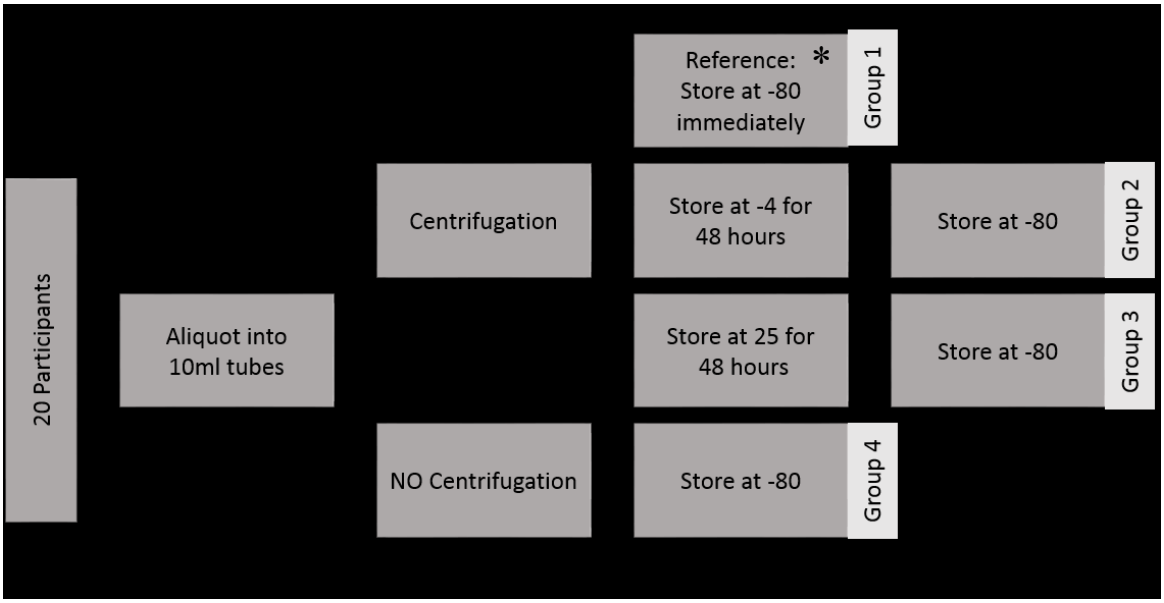


Figure 2-9: Flow diagram of the different types of processing being tested in comparison with the gold standard*.

N = 60 per group. Total = 240 tubes.

2.5 Data Analysis

Objectives 1 and 3 were achieved in conjunction with a statistician from the Statistics Department, Stellenbosch University. Objective 2 was achieved independently.

2.5.1 Objective 1: Dipsticks vs high-throughput

Agreement analysis was performed between the laboratory high-throughput and the dipsticks, using the concordance correlation co-efficient (Lin's coefficient). The concordance correlation co-efficient measures both precision and accuracy to determine how far the observed data deviate from the line of perfect concordance (y=0). Firstly, the Siemens dipstick results were compared with the Life Assay results, then both the Siemens and Life Assay results were compared respectively with the clinical high-throughput result.

Trend analysis was also calculated for each analyte (Calcium, Total Protein and Creatinine), for both the dipsticks and the high-throughput measurements, at each time point (ADM-1, DPV8-8, DPV20-20 and DPV32-32). In addition, the mean value and the corresponding 95% confidence interval were also calculated. Variance-weighted least-squares regression was used to test whether the linear trend was significant or not and to observe whether there was an increase over the weeks for each dipstick and laboratory result. Goodness-of-fit chi^2 was used to test whether the single population fitted the hypothesised distribution. Lastly, the model fit chi^2 was used to compare the weeks to each other (across categories).

2.5.2 Objective 2: Adipsin

For the ELISA-based results, the analysis was computed using Excel 2013 to create the standard curve for both the original and adjusted results, to acquire the R^2 value and equation to calculate the %CV values. Two measures of the Co-efficient of Variability (CV) should be reported: the Inter-Assay CV (plate-to-plate consistency) and the Intra-Assay CV (an average value calculated from the individual CVs) in order to express the precision, or repeatability. CV is a dimensionless number defined as the standard deviation of a set of measurements divided by the mean of the set.

2.5.3 Objective 3: Storage

Wilcoxon Signed-Rank Test was used for objective 3 Each group (Group 2, 3 & 4) were compared to the reference group (Group 1) using an average of the 20 patients for each analyte. The Wilcoxon Signed-Rank Test will indicate how different the groups' values are to the reference. The data will be

a matched data set (measurements were from the same individuals but under different conditions). Due to the small sample size, it will be assumed that the dataset will be skewed, however the Wilcoxon Sign-Rank Test used to account for the small sample size. Excel 2013 was then used to present the data in graphical format.

Chapter 3

Results

The aim of this study was to: investigate the role of long-term urine bio-banking on the stability of urine bio-markers in the diagnosis of pre-eclampsia by understanding the variety of storage conditions and their effects on bio-markers.

Therefore, to achieve the aim of this study, the objectives were:

1) Conduct a pilot study to compare high-throughput vs dipstick measurements of Creatinine, Total Protein and Calcium in urine aliquots from the PE based CAP cohort stored long-term at -80^{o}C (shortened to "Objective 1: Dipsticks vs High-throughput").
2) Evaluate the ELISA validation of Adipsin or Complement Factor D measurements for long-term stored urine (shortened to "Objective 2: Adipsin").
3) Investigate the pre-analytic effect of pre- and post-storage conditions and processing of samples by measuring Total Protein, Urea, Albumin, Calcium and Creatinine after a set period of storage at -80^{o}C (shortened to "Objective 3: Storage").

Thus, the results obtained have been presented in the above order.

3.1 Objective 1: Dipsticks vs high-throughput

3.1.1 Clinical high-throughput measurements verses dipsticks measurements comparisons – Concordance correlation co-efficient analysis

To compare the agreement between the laboratory high-throughput measurements with the dipsticks measurements, the concordance correlation co-efficient analysis (Lin's co-efficient) was used to measure both precision and accuracy by determining how far the observed data deviated from the line of perfect concordance.

Since the purpose was to assess the dipsticks compared with the high-throughput measurements, only the last of the cohort week (week 32) was selected for the agreement analysis. For agreement analysis using the protein measurements, the two dipsticks (Siemens vs LifeAssay) were compared with each other before the respective dipsticks was compared with the clinical high-throughput measurements. As creatinine was only measureable on the LifeAssay dipstick and not the Siemens dipstick, the LifeAssay results were compared with the clinical high-throughput measurements.

Data analysis was performed on both raw data and log transformed data where required, thus the data has been presented in that order.

3.1.1.1 Siemens Protein Dipstick (SP) vs Life Assay Protein Dipstick (DP)

For this analysis, the Lin's co-efficient graph generated (Figure 3-1) shows that some data points lay on the y=0 line, which indicates that the data set was correlating between the two dipsticks for the protein results (accuracy). The average agreement mean was 1.818. A data point outside of the 95% limits was also observed, which reduced the co-efficient value from 1. The rho_co-ef or concordance correlation co-efficient was 0.920 with a 95% confidence interval (0.879 to 0.960), p-value = 0.000, suggesting substantial agreement between the two dipsticks. The p-value obtained indicated that this observation was statistically significant. The slope was 0.996 with 1 being considered the ideal slope and the intercept was 1.838 with 0 being considered the ideal intercept. Unfortunately, due to some values being 0, log transformation couldn't be performed on this data set.

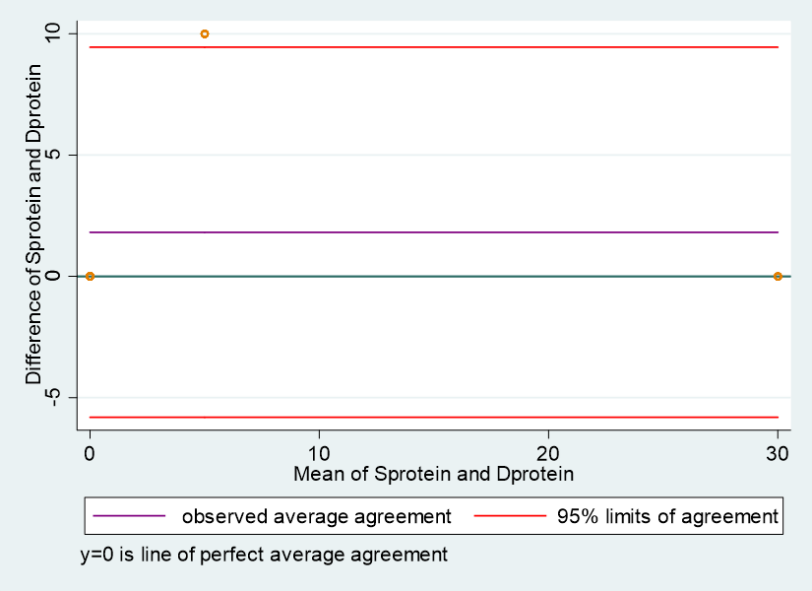


Figure 3-1: Concordance correlation co-efficient of Siemens protein dipstick (Sprotein) vs LifeAssay protein dipstick (Dprotein).

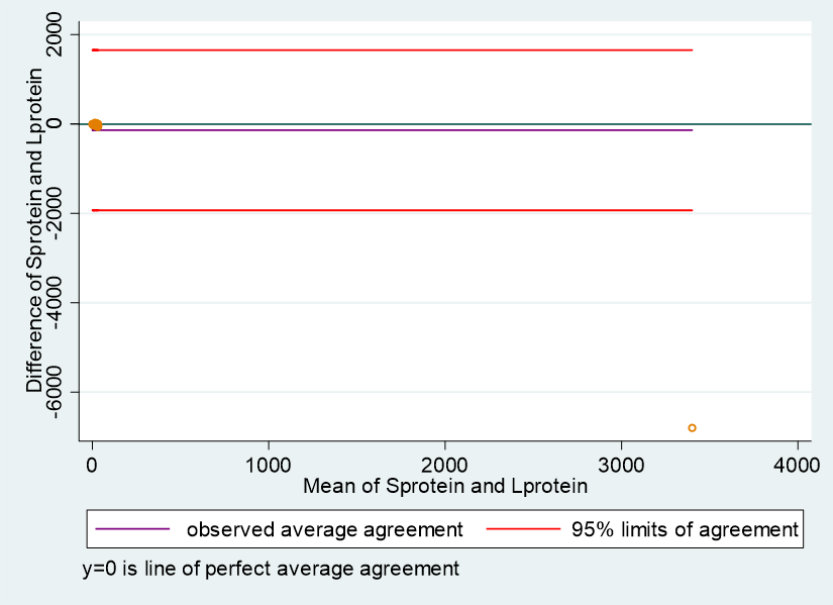
Substantial concordance was observed between the two dipsticks. Rho_Co-ef. = 0.920 (95%CI, 0.879 to 0.960), p-value = 0.000.

3.1.1.2 Siemens Protein Dipstick (SP) vs Laboratory High-throughput Protein (LP)

For this analysis, the Lin's co-efficient graph in Figure 3-2A below shows that the majority of the data points lay on the y=0 line. However, an observed data point was outside the 95% limits which reduced the co-efficient value from 1. The average agreement mean was -137.509. The rho_co-ef or concordance correlation co-efficient was -0.002 with a 95% confidence interval (-0.008-0.004), p-value = 0.564, suggesting poor agreement between the Siemens dipsticks and the clinical high-throughput protein measurements. The p-value obtained indicates that this observation was statistically non-significant. The slope was -0.012 with 1 being considered the ideal slope and the intercepts was 7.852 with 0 being considered the ideal intercept.

It was noted that the dipstick and the clinical high-throughput measurement ranges differed significantly from very low to very high between the two measurements. The minimum to maximum range for the Siemen's protein dipstick was 0-30 whereas the clinical high-throughput measurements minimum to maximum range was from 0-6800. Owing to these different measurement ranges, the data set was log transformed in order to normalise it to a specific range. This would make highly skewed data, as in this case, less skewed as a result of measurement range differences.

Thus, from this Lin's co-efficient plot, Figure 3-2B below shows that all data points lay between the 95% limits. The average agreement mean was -0.288. The rho_co-ef or concordance correlation co-efficient was 0.076 with a 95% confidence interval (-0.364 to -0.516), p-value = 0.735, suggesting poor agreement between the Siemen's dipsticks and the clinical high-throughput protein measurements. The p-value obtained indicated that this observation was statistically non-significant. The slope was 1.018 with 1 being considered the ideal slope and the intercept was -0.343 with 0 being considered the ideal intercept.



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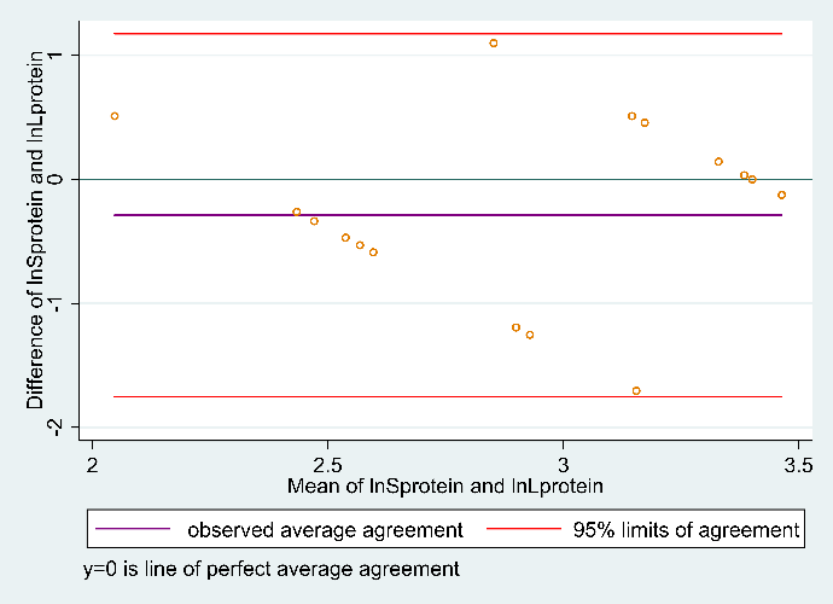


B

Figure 3-2: A: Concordance correlation co-efficient of Siemens protein dipstick (Sprotein) vs clinical high-throughput protein measurements (Lprotein). B: Concordance correlation co-efficient of Siemens protein dipstick (Sprotein) vs clinical high-throughput protein measurements (Lprotein) in log-transformed form.

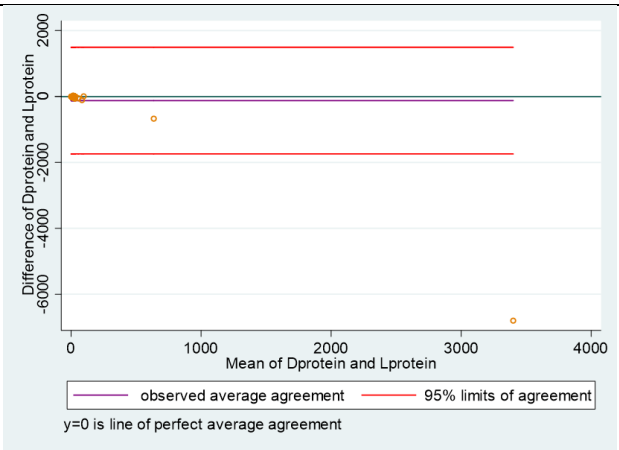
A - Poor concordance was observed between the Siemens dipstick and the clinical high-throughput measurements. Rho_Co-ef. = -0.002 (95% CI, -0.008 to 0.004), p-value = 0.564. B - Poor concordance was observed between the Siemens dipstick and the clinical high-throughput log transformed measurements. Rho_Co-ef. = -0.076 (95% CI, -0.364 to 0.516), p-value = 0.735.

3.1.1.3 Life Assay Protein Dipstick (DP) vs Laboratory High-throughput Protein (LP)

The Lin's co-efficient graph for the LifeAssay Dipstick compared with the clinical high-throughput measurements showed that some data points lay on the y=0 line (Figure 3-3A below). The average agreement mean observed was -24.985 which is not close to 0. An observed extreme data point was also outside the 95% limits which reduced the co-efficient value from 1. The rho_co-ef or concordance correlation co-efficient was 0.008 with a 95% confidence interval (-0.014 to 0.031), p-value = 0.456, suggesting poor agreement between the Life Assays dipsticks and the clinical high-throughput protein measurements. The p-value obtained indicate that this observation was statistically non-significant. The slope was 0.047 with 1 being considered the ideal slope and the intercept was 6.014 with 0 being considered the ideal intercept.

Owing to the extreme outlier value, the data set was adjusted by removing this outlier and the data were re-analysed to see how this influenced the results. As demonstrated before, the Lin's co-efficient graph in Figure 3-3B below showed that some data points lay on the y=0 line. The average agreement mean observed was -24.985 which is not close to 0. With the removal of the extreme data point a rho_coef or concordance correlation coefficient of 0.533 with a 95% confidence interval (0.0475 to 0.590), p-value = 0.000 was observed. Despite the removal of the outlier, the results still suggest poor agreement between the Life Assays dipsticks and the clinical high-throughput protein measurements. The p-value obtained indicates that this observation was statistically significant. The slope was 0.334 with 1 being considered the ideal slope and the intercepts was 0.090 with 0 being considered the ideal intercept.

Similarly, to the Siemens vs clinically laboratory results, the dataset was log transformed in order to normalise it to a specific range to account for the extreme differences in the measurements ranges between the two assays. Thus, for this Lin's coefficient plot, Figure 3-3C shows that the majority of the data points lies between the 95% limits. The average agreement mean was 0.061. The rho_coef or concordance correlation coefficient was 0.661 with a 95% confidence interval (0.460 to 0.861), p-value = 0.000. These results suggest poor agreement between the Life Assay dipsticks and the clinical high-throughput protein measurements despite log transformed. The p-value obtained indicates that this observation was statistically significant. The slope was 0.538 with 1 being considered the ideal slope and the intercepts was 1.705 with 0 being considered the ideal intercept.



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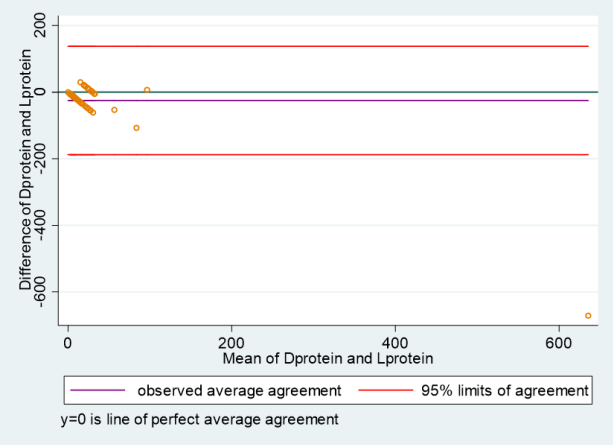

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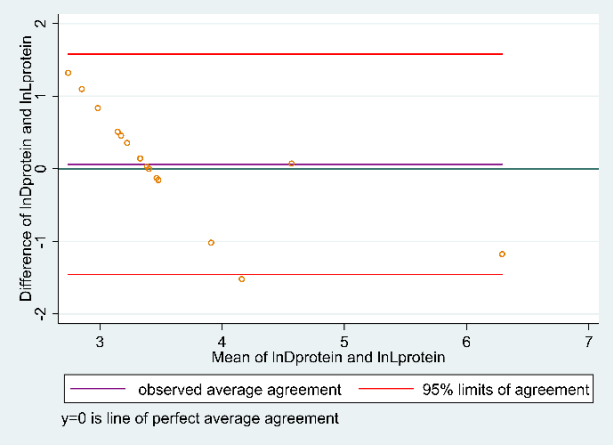


C

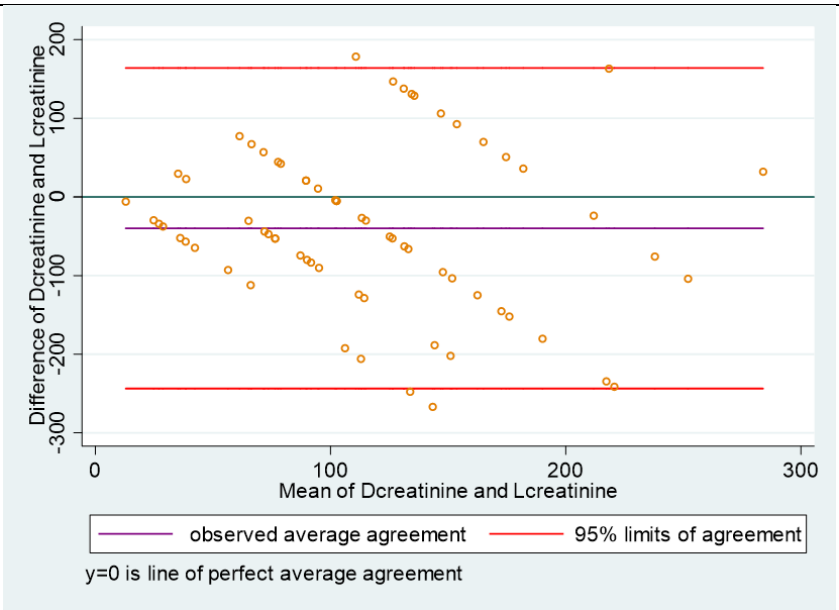
Figure 3-3: A: Concordance correlation co-efficient of LifeAssay protein dipstick (Dprotein) vs clinical high-throughput protein measurements (Lprotein). B: Concordance correlation co-efficient of LifeAssay protein dipstick (Dprotein) vs clinical high-throughput protein measurements (Lprotein) Adjusted. C: Concordance correlation co-efficient of LifeAssay protein dipstick (Dprotein) vs clinical high-throughput protein log-transformed measurements (Lprotein).

A - Poor concordance was observed between the LifeAssay dipstick and the clinical high-throughput measurements. Rho_Co-ef. = -0.08 (95% CI, -0.014 to 0.031), p-value = 0.456. B - Poor concordance was observed between the LifeAssay dipstick and the clinical high-throughput measurements. Rho_Co-ef. = 0.533 (95% CI, 0.475 to 0.590), p-value = 0.000. C - Poor concordance was observed between the LifeAssay dipstick and the clinical high-throughput measurements. Rho_Co-ef. = 0.661 (95% CI, 0.460 to 0.861), p-value = 0.000.

3.1.1.4 Life Assay Creatinine Dipstick (DC) vs Laboratory High-throughput Creatinine (LC)

The Lin's coefficient graph for the LifeAssay Dipstick creatinine measurements compared with the clinical high-throughput creatinine measurements showed that most data points fall within the 95% limits (Figure 3-4A below). The average agreement mean observed was -39.849 which is not close to 0. The rho_co-ef or concordance correlation co-efficient was 0.109 with a 95% confidence interval (-0.099 to 0.318), p-value = 0.304. These results suggested poor agreement between the life Assays dipstick's creatinine measurements and the clinical high-throughput's creatinine measurements. The p-value obtained indicated that this observation was statistically non-significant. The slope was 0.861 with 1 being considered the ideal slope and the intercept was -20.873 with 0 being considered the ideal intercept.

Similarly, to the Siemens vs clinically laboratory protein results, the creatinine data set was also log-transformed in order to normalise it to a specific range to account for the extreme differences in the measurement ranges between the two assays. The Lin's co-efficient graph for the LifeAssay dipstick's creatinine, log-transformed measurements compared with the clinical high-throughput creatinine, log-transformed measurements shows that most data points fell within the 95% limits (Figure 3-4B below). The average agreement mean observed was 0.514. The rho_co-ef or concordance correlation co-efficient was 0.128 with a 95% confidence interval (-0.065 to 0.320), p-value = 0.194. These results suggested poor agreement between the life Assays dipstick's creatinine measurements and the clinical high-throughput's creatinine measurements despite normalisation. The p-value obtained indicated that this observation was statistically non-significant. The slope was 0.709 with 1 being considered the ideal slope and the intercept was 1.731 with 0 being considered the ideal intercept.



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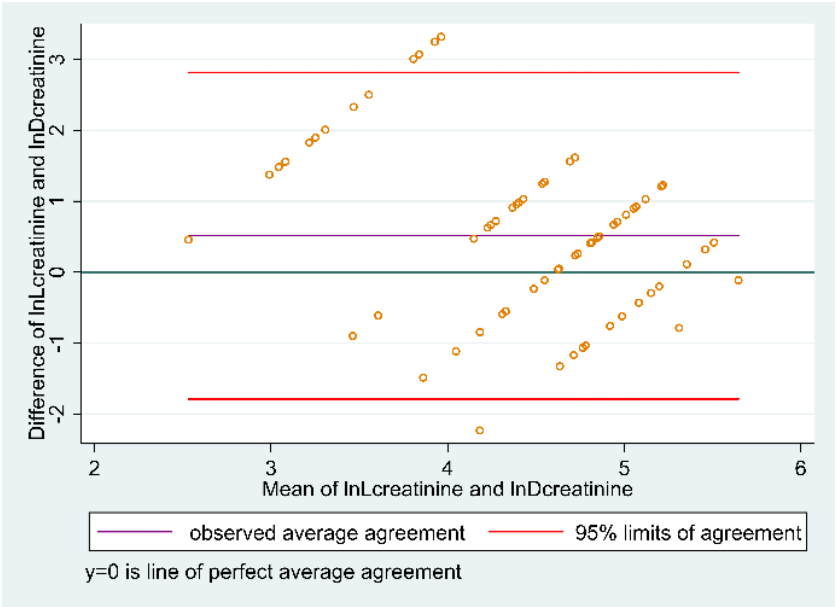


B

Figure 3-4: A: Concordance correlation co-efficient of LifeAssay creatinine dipstick (Dcreatinine) vs clinical high-throughput creatinine measurements (Lcreatinine). B: Concordance correlation co-efficient of LifeAssay creatinine dipstick (Dcreatinine) vs clinical high-throughput creatinine log transformed measurements (Lcreatinine).

A - Poor concordance was observed between the LifeAssay dipstick and the clinical high-throughput measurements. Rho_Co-ef. = 0.109 (95% CI, -0.099 to 0.318), p-value = 0.304. B - Poor concordance was observed between the LifeAssay dipstick and the clinical high-throughput measurements. Rho_Co-ef. = 0.128 (95% CI, -0.065 to 0.320), p-value = 0.194.

In summary, all the important agreement analysis parameters have been consolidated in Table 3.1 below.

Table 3-1: Summary of agreement analysis including the log-transformed results.

SP = Siemens Protein Dipstick. DP = LifeAssay Protein Dipstick. LP = Laboratory High-throughput Protein. DC= LifeAssay Creatinine Dipstick. LC = Laboratory High-throughput Creatinine. . lnSP = log of Siemens Protein Dipstick. lnDP = log of LifeAssay Protein Dipstick. lnLP = log of Laboratory High-throughput Protein. lnDC= log of LifeAssay Creatinine Dipstick. lnLC = log of Laboratory High-throughput Creatinine. *significant ($p < 0.001$).

	SP vs DP	SP vs LP	lnSP vs lnLP	DP vs LP	DP vs LP (adjusted)**	lnDP vs lnLP	DC vs LC	lnDC vs lnLC
Number (#) of observations	55	55	17	68	67	16	68	68
95% CI	0.879 to 0.960	-0.008-0.004	-0.364 to -0.516	-0.014 to 0.031	0.0475 to 0.590	0.460 to 0.861	-0.099 to 0.318	-0.065 to 0.320
Concordance correlation coefficient	0.920	-0.002	0.076	0.008	0.533	0.661	0.109	0.128
Average Agreement	1.818	-137.509	-0.288	-24.985	-24.985	0.061	-39.849	0.514
P-value	**0.000***	0.564	0.735	0.456	**0.000***	**0.000***	0.304	0.194
Slope	0.996	-0.012	1.018	0.047	0.334	0.538	0.861	0.709
Intercept	1.838	7.852	-0.343	6.014	0.090	1.705	-20.873	1.731

3.1.2 Trend analysis of each analyte over the different weeks

Since urine was collected from the same patients over different time points throughout their pregnancy, it was necessary to assess how the selected analytes (Calcium, Total Protein and Creatinine) performed at the different time points (ADM-1, DPV8-8, DPV20-20 and DPV32-32). Therefore, only a sub-set, where samples were available for all time points and dipsticks and on which clinical lab measurements were performed, was selected for statistical trend analysis for the dipsticks and the high-throughput measurements. The total number of patients analysed was 56, with 14 patients per week.

Variance-weighted least-squares regression analysis was used to test whether the linear trend was significant or not and to see if there was an increase over the weeks for each dipstick and laboratory result, respectively. Goodness-of-fit chi^2 was used to test whether the single population fitted the hypothesised distribution. Then the model fit chi^2 was used to compare the weeks to each other (across categories). All the important parameters observed have been summarised in Table 3-2 . In order to display the trends for the respective analytes per respective assay visually, the mean value and the corresponding 95% confidence interval was plotted in subsequent graphs (Figure 3-5 to 3-7). The dipstick assays and laboratory-based assays were also compared to determine whether the same trend was observed in both types of assays.

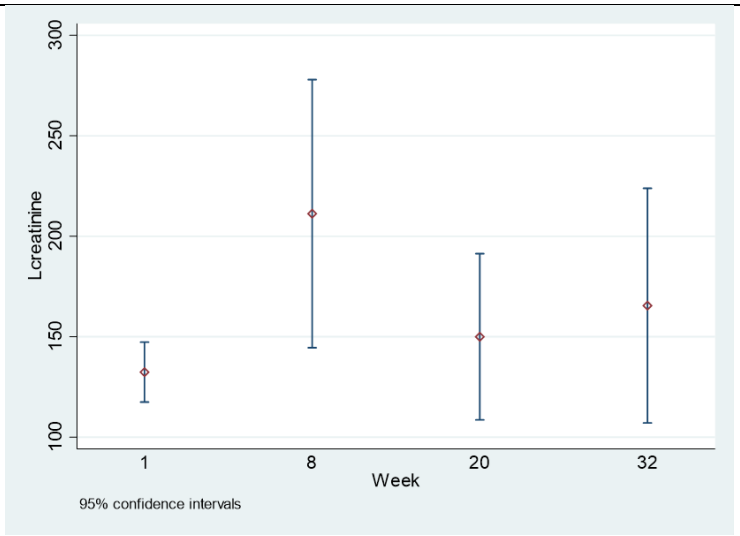
Table 3-2: Summary of Variance-weighted least-squares regression and chi² test over the weeks

DProtein = LifeAssay Protein Dipstick. LProtein = Laboratory High-throughput Protein. DCreatinine= LifeAssay Creatinine Dipstick. LCreatinine = Laboratory High-throughput Creatinine. LCalcium = Laboratory High-throughput Calcium. *significant ($p < 0.001$).

	LProtein	DProtein	LCreatinine	DCreatinine	LCalcium
# of observations	56	56	56	56	56
Mean	17.7	19	133.6	14.8	7.3
95% CI	12-23	12-25	119-147	1-27	4-10
Coefficient	0.3545141	0.2275635	1.12994	4.215532	0.3132418
P-value	0.236	0.407	0.114	**0.000***	0.022
Goodness-of-fit chi²	3.35	1.91	5.18	8.64	1.53
Model chi²	1.41	0.69	2.50	46.64	5.28

3.1.2.1 Laboratory High-throughput Creatinine (LC) vs Life Assay Creatinine measurements (DC)

The variance-weighted least-squares regression graph (Figure 3-5A) demonstrating clinical high-throughput creatinine measurements over the weeks showed a higher mean value for week 8 (DPV8) compared with the other weeks. In comparison, the variance-weighted least-squares regression graph (Figure 3-5B) displaying the dipstick-based creatinine measurements showed the highest value for week 20 (DPV20), followed by week 32 (DPV32).



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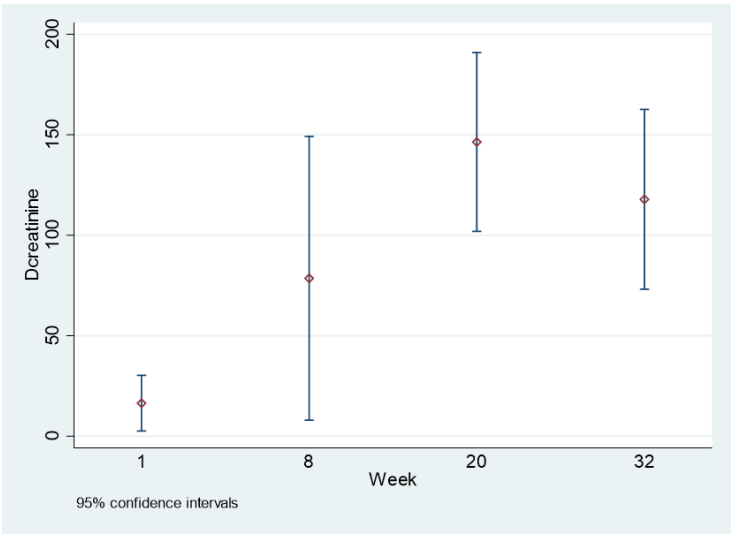


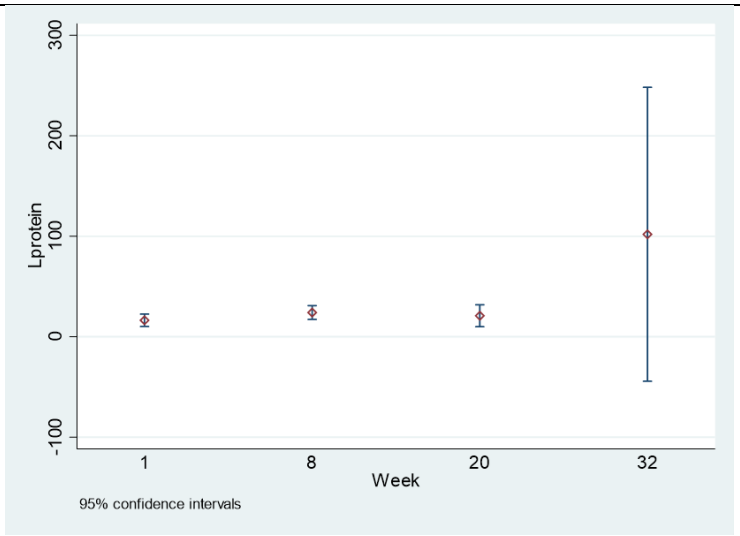
B*

Figure 3-5: A: Variance-weighted least-squares regression of Laboratory High-throughput Creatinine (LC) and B: LifeAssay Creatinine Dipstick (DC).

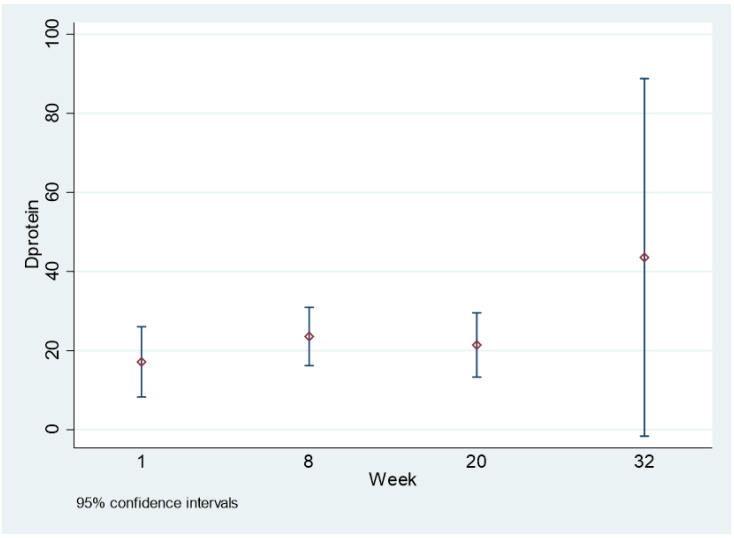
Total number of patients was 56, with 14 patients each week, A – co-efficient. = 1.12994 (increase per week), P value = 0.114; not significant, B – co-efficient. = 4.215532 (increase per week), P-value = 0.000. *Significant p<0.001.

3.1.2.2 Laboratory High-throughput Protein (LP) vs Siemens and LifeAssay Dipstick Protein measurements (DP)

For the protein trend analysis for the two assays, variance-weighted least-squares regression graph (Figure 3-6A) specifically for the laboratory protein measurements showed a higher value for week 32 (DPV32). The variance-weighted least-squares regression graph for the LifeAssay dipstick protein measurements (Figure 3-6B) also showed a higher value for week 32, similar to the laboratory-based protein trend analysis.



A



B

Figure 3-6: A: Variance-weighted least-squares regression of Laboratory High-throughput Protein (LP) and B: Life Assay Protein Dipstick (DP).

Total number of patients was 56, with 14 patients each week, A – co-efficient. = 0.3545141 (increase per week), P-value = 0.236, not significant; B. – co-efficient. = 0.2275635 (increase per week), P-value = 0.407, not significant.

3.1.2.3 Laboratory High-throughput Calcium (LCa) trend analysis

Calcium measurements were performed using only the laboratory-based assays. The Variance-weighted least-squares regression graph (Figure 3-7) showed an increasing trend, following the baseline week until a plateau was reached.

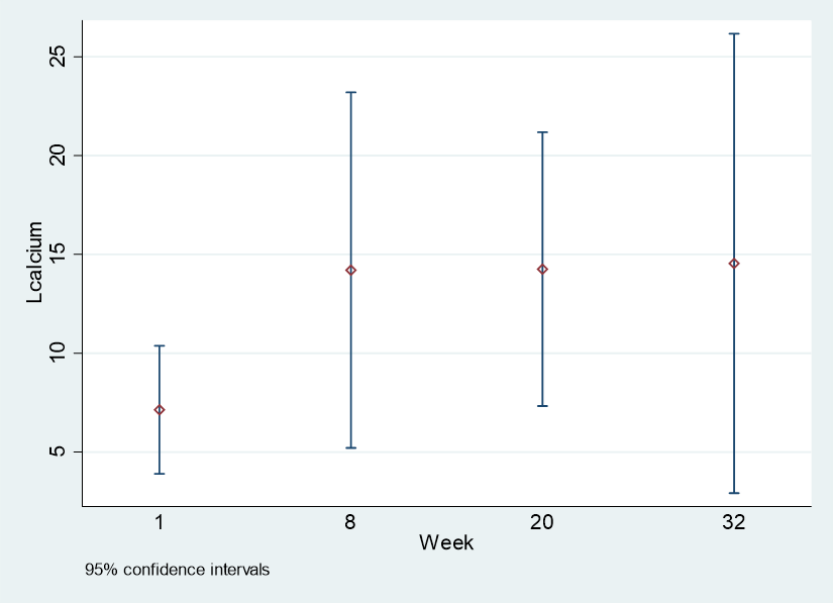


Figure 3-7: Variance-weighted least-squares regression of Laboratory High-throughput Calcium (LCa).

Total number of patients was 56, with 14 patients each week; co-efficient. = 0.3132418 (increase per week), P-value = 0.022, not significant.

3.2 Objective 2: Adipsin

For the ELISA-based results, the analysis was computed using Excel 2016 to create the standard curve for both the original and adjusted results, to acquire the R^2 value and equation to calculate the %CV value to measure intra- and inter-assay. The adjusted results came from the standard curve. When the original standard curve was calculated to get the R^2 value, it was observed to be low for both plates. Thus, after removing the 40ng/ul concentration and its associated OD value, the R^2 value improved. Therefore, both the original and the adjusted outcomes have been included.

3.2.1 Standard Curve

3.2.1.1 Standard Curve

The original standard curve including the 40ng/ul concentration and OD value is shown in Figure 3-8. Plate 1 generated an equation of y = 104.38x + 663.07 and a R^2 value of 0.8897. Plate 2 generated an equation of y = 86.92x + 639.47 and a R^2 value of 0.929. The original results for the calculated concentrations of each sample are in Section 3.2.3.

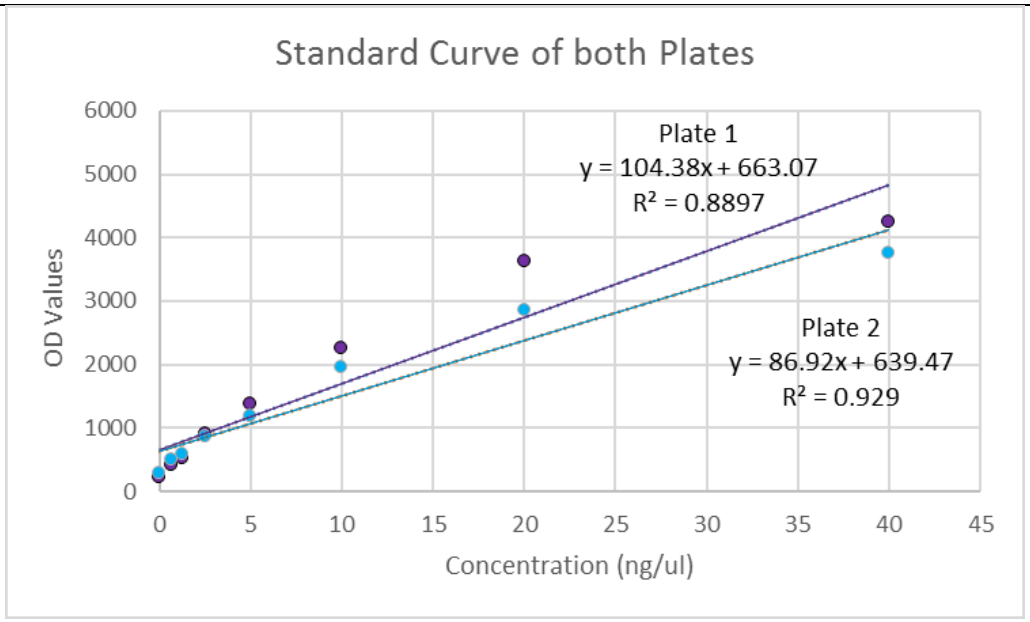


Figure 3-8: Standard curve calculated for each plate with their respective equations and R^2 values.

3.2.1.2 Adjusted Standard Curve

The adjusted standard curve excluding the 40ng/ul concentration and OD value is shown in Figure 3-9. Plate 1 generated an equation of y = 169.16x + 382.61 and a R^2 value of 0.9872. Plate 2 generated an equation of y = 127.09x + 465.55 and a R^2 value of 0.9763. The adjusted results for the calculated concentrations of each sample are in Section 3.2.3.

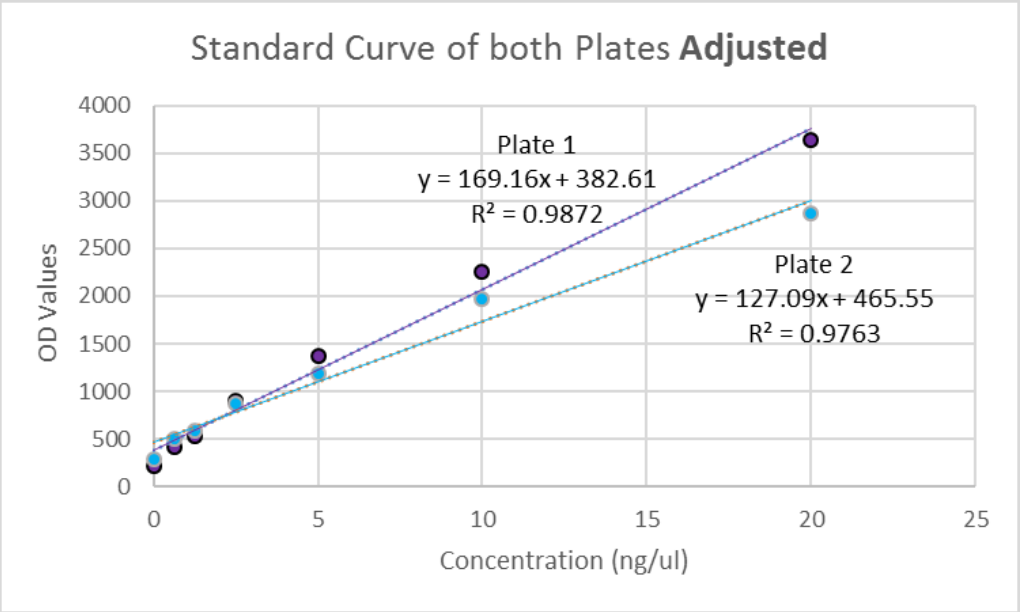


Figure 3-9: Standard curve calculated for each plate and adjusted, with their respective equations and R^2 values.

The 40ng/ul concentration and associated OD value were removed for both plates.

3.2.2 Intra-Assay of OD Values

When the ELISA-based process is used, two measures of the Co-efficient of Variability (CV) are reported. The Inter-Assay CV (plate-to-plate consistency) and the Intra-Assay CV (an average value calculated from the individual CVs) in order to express the precision or repeatability of the process. CV is a dimensionless number defined as the standard deviation of a set of measurements divided by the mean of the set. The %CV was calculated for the OD values and the concentrations (Section 3.2.3). The concentrations %CV has not been emphasised as they were negative values and are discussed in Chapter 4.

Table 3-3 and Table 3-4 below show the results from the two plates, starting with the sample, each of which had a week attached (4 per sample), the plate on which it was processed, then the triplicate OD Value Results, then the Mean, Standard Deviation and finally the %CV which is Mean divided

by Standard Deviation. Plate 1 had a range of %CV values from 0.841% to 22.701%. Plate 2 had a range of %CV values from 1.797% to 48.951%.

Inter-assay %CVs of <15% are generally acceptable. Intra-assay %CVs should be <10%. These scores reflect the performance of the user who handled the assay. The %CVs that are >10% reflect poor pipetting technique of the user. It is the norm to use %CVs of the concentrations, but here the OD values %CVs have been emphasised.

Table 3-3: Intra-assay table of the OD Values for Plate 1.

Each sample had a week, then on which plate it was processed, then the triplicate OD Value Results, then the Mean, Standard Deviation and finally the %CV which is Mean divided by Standard Deviation.

Sample	Week	Plate	Result 1	Result 2	Result 3	Mean	Standard Dev	%CV
4438-787	1	1	504	584	632	573.33	52.797	9.209
4438-787	8	1	297	299	353	316.33	25.940	8.200
4438-787	20	1	142	148	119	136.33	12.499	9.168
4438-787	32	1	187	181	162	176.67	10.656	6.032
4455-796	1	1	428	419	403	416.67	10.339	2.481
4455-796	8	1	206	204	226	212.00	9.933	4.685
4455-796	20	1	310	306	319	311.67	5.437	1.744
4455-796	32	1	592	606	591	596.33	6.848	1.148
4456-797	1	1	139	122	168	143.00	18.991	13.281
4456-797	8	1	149	175	182	168.67	14.197	8.417
4456-797	20	1	167	185	176	176.00	7.348	4.175
4456-797	32	1	276	211	237	241.33	26.712	11.069
4462-807	1	1	200	179	175	184.67	10.965	5.938
4462-807	8	1	207	158	173	179.33	20.499	11.431
4462-807	20	1	179	174	193	182.00	8.042	4.418
4462-807	32	1	236	258	257	250.33	10.143	4.052
4466-814	1	1	181	171	189	180.33	7.364	4.083
4466-814	8	1	312	311	318	313.67	3.091	0.986
4466-814	20	1	211	216	125	184.00	41.769	22.701
4466-814	32	1	201	164	183	182.67	15.107	8.270
4470-817	1	1	323	322	296	313.67	12.499	3.985
4470-817	8	1	298	305	348	317.00	22.106	6.973
4470-817	20	1	210	220	220	216.67	4.714	2.176
4470-817	32	1	1821	1677	1754	1750.67	58.835	3.361
4471-818	1	1	963	965	947	958.33	8.055	0.841
4471-818	8	1	212	231	206	216.33	10.656	4.926
4471-818	20	1	171	190	152	171.00	15.513	9.072
4471-818	32	1	160	185	144	163.00	16.872	10.351
4473-820	1	1	192	172	209	191.00	15.122	7.917

Table 3-4: Intra-assay table of the OD Values for Plate 2.

Each sample had a week, then on which plate it was processed, then the triplicate OD Value Results, then the Mean, Standard Deviation and finally the %CV which is Mean divided by Standard Deviation. Highlighted grey area of value 473 is the known pipetting error, whereby too much substrate was added to the well.

Sample	Week	Plate	Result 1	Result 2	Result 3	Mean	Standard Dev	%CV
4473-820	8	2	210	201	203	204.67	3.859	1.885
4473-820	20	2	254	275	244	257.67	12.919	5.014
4473-820	32	2	286	298	286	290.00	5.657	1.951
4480-838	1	2	272	254	238	254.67	13.888	5.454
4480-838	8	2	254	242	257	251.00	6.481	2.582
4480-838	20	2	343	270	296	303.00	30.210	9.970
4480-838	32	2	257	274	283	271.33	10.781	3.973
4614-1033	1	2	274	245	271	263.33	13.021	4.945
4614-1033	8	2	143	152	142	145.67	4.497	3.087
4614-1033	20	2	181	177	217	191.67	17.988	9.385
4614-1033	32	2	364	369	300	344.33	31.415	9.123
4624-1045	1	2	200	199	217	205.33	8.260	4.023
4624-1045	8	2	207	237	200	214.67	16.049	7.476
4624-1045	20	2	376	286	327	329.67	36.791	11.160
4624-1045	32	2	261	254	274	263.00	8.287	3.151
4765-1199	1	2	226	228	242	232.00	7.118	3.068
4765-1199	8	2	195	186	211	197.33	10.339	5.239
4765-1199	20	2	150	214	262	208.67	45.879	21.987
4765-1199	32	2	209	459	151	273.00	133.636	48.951
4777-1209	1	2	213	236	209	219.33	11.898	5.424
4777-1209	8	2	473	236	268	325.67	104.996	32.240
4777-1209	20	2	236	187	189	204.00	22.642	11.099
4777-1209	32	2	196	231	255	227.33	24.226	10.657
4485-843	1	2	311	261	231	267.67	32.998	12.328
4485-843	8	2	201	193	200	198.00	3.559	1.797
4485-843	20	2	198	180	188	188.67	7.364	3.903
4485-843	32	2	230	227	186	214.33	20.072	9.365

Table 3-5 below shows the average intra-assay %CV for the two plates. Plate 1 had an average mean of 325, average standard deviation of 17 and an average %CV of 6.6%. Plate 2 had an average mean of 242, average standard deviation of 24 and an average %CV of 9.2%. Despite Plate 1 having a better %CV result, they were both below <10%. The average %CV across the plates is 7.9% which is also <10%.

Table 3-5: Average Mean, Standard Deviation and %CV for the OD Values.

	Average Mean	Average Standard Deviation	Average %CV	Average %CV across plates
Plate 1	325	17	6.6	7.9
Plate 2	242	24	9.2	

3.2.3 Concentration (ng/ul) results from OD Values

Table 3-6 and Table 3-7 below show the results using the original equation to calculate the concentrations from the OD values. The mean, standard deviation and %CV were also calculated from the concentrations. It was noted that most of the concentration results were negative and thus produced %CV values that were negative and could not be used.

Table 3-8 and Table 3-9 below show the t results using the adjusted equation to calculate the concentrations from the OD values. The mean, standard deviation and %CV were also calculated from the concentrations. It was noted that, despite the adjustment, most of the concentration results were negative and produced negative %CV values.

Plate 2 had a highlighted grey value of 473 which was a pipetting error. Too much substrate was added to the well by accident. The negative concentrations and %CV values have been discussed further in Chapter 4.

Table 3-6: Plate 1 Concentration values calculated from the OD Values using the original equation calculated.

Each sample had a week, then on which plate it was processed, then the triplicate OD Value Results, then the calculated concentration, then the Mean, Standard Deviation and finally the %CV which is Mean divided by Standard Deviation. The Mean, Standard Deviation and %CV were calculated from the concentration results.

Sample	Week	Plate	Result 1	Result 2	Result 3	[Result 1]	[Result 2]	[Result 3]	[Mean]	Standard Dev	%CV
4438-787	1	1	504	584	632	-2.68	-1.88	-1.40	-1.99	0.526	-26.468
4438-787	8	1	297	299	353	-4.74	-4.72	-4.19	-4.55	0.259	-5.683
4438-787	20	1	142	148	119	-6.29	-6.23	-6.52	-6.35	0.125	-1.964
4438-787	32	1	187	181	162	-5.84	-5.90	-6.09	-5.94	0.106	-1.788
4455-796	1	1	428	419	403	-3.44	-3.53	-3.69	-3.55	0.103	-2.903
4455-796	8	1	206	204	226	-5.65	-5.67	-5.45	-5.59	0.099	-1.771
4455-796	20	1	310	306	319	-4.61	-4.65	-4.52	-4.60	0.054	-1.179
4455-796	32	1	592	606	591	-1.80	-1.66	-1.81	-1.76	0.068	-3.880
4456-797	1	1	139	122	168	-6.32	-6.49	-6.03	-6.28	0.189	-3.015
4456-797	8	1	149	175	182	-6.22	-5.96	-5.89	-6.02	0.142	-2.350
4456-797	20	1	167	185	176	-6.04	-5.86	-5.95	-5.95	0.073	-1.231
4456-797	32	1	276	211	237	-4.95	-5.60	-5.34	-5.30	0.266	-5.026
4462-807	1	1	200	179	175	-5.71	-5.92	-5.96	-5.86	0.109	-1.864
4462-807	8	1	207	158	173	-5.64	-6.13	-5.98	-5.92	0.204	-3.454
4462-807	20	1	179	174	193	-5.92	-5.97	-5.78	-5.89	0.080	-1.361
4462-807	32	1	236	258	257	-5.35	-5.13	-5.14	-5.21	0.101	-1.941
4466-814	1	1	181	171	189	-5.90	-6.00	-5.82	-5.91	0.073	-1.243
4466-814	8	1	312	311	318	-4.59	-4.60	-4.53	-4.58	0.031	-0.673
4466-814	20	1	211	216	125	-5.60	-5.55	-6.46	-5.87	0.416	-7.094
4466-814	32	1	201	164	183	-5.70	-6.07	-5.88	-5.88	0.151	-2.560
4470-817	1	1	323	322	296	-4.48	-4.49	-4.75	-4.58	0.125	-2.722
4470-817	8	1	298	305	348	-4.73	-4.66	-4.24	-4.54	0.220	-4.850
4470-817	20	1	210	220	220	-5.61	-5.51	-5.51	-5.54	0.047	-0.848
4470-817	32	1	1821	1677	1754	10.45	9.01	9.78	9.75	0.587	6.017
4471-818	1	1	963	965	947	1.90	1.92	1.74	1.85	0.080	4.342
4471-818	8	1	212	231	206	-5.59	-5.40	-5.65	-5.55	0.106	-1.915
4471-818	20	1	171	190	152	-6.00	-5.81	-6.19	-6.00	0.155	-2.578
4471-818	32	1	160	185	144	-6.11	-5.86	-6.27	-6.08	0.168	-2.767
4473-820	1	1	192	172	209	-5.79	-5.99	-5.62	-5.80	0.151	-2.599

Table 3-7: Plate 2 Concentration values calculated from the OD Values using the original equation calculated.

Each sample had a week, then on which plate it was processed, then the triplicate OD Value Results, then the calculated concentration, then the Mean, Standard Deviation and finally the %CV which is Mean divided by Standard Deviation. The Mean, Standard Deviation and %CV were calculated from the concentration results. Highlighted grey area of value 473 is the known pipetting error, whereby too much substrate was added to the well.

Sample	Week	Plate	Result 1	Result 2	Result 3	[Result 1]	[Result 2]	[Result 3]	[Mean]	Standard Dev	%CV
4473-820	8	2	210	201	203	-6.16	-6.27	-6.25	-6.23	0.046	-0.740
4473-820	20	2	254	275	244	-5.64	-5.39	-5.76	-5.59	0.154	-2.759
4473-820	32	2	286	298	286	-5.25	-5.11	-5.25	-5.21	0.068	-1.298
4480-838	1	2	272	254	238	-5.42	-5.64	-5.83	-5.63	0.166	-2.948
4480-838	8	2	254	242	257	-5.64	-5.78	-5.60	-5.67	0.077	-1.365
4480-838	20	2	343	270	296	-4.57	-5.45	-5.13	-5.05	0.361	-7.145
4480-838	32	2	257	274	283	-5.60	-5.40	-5.29	-5.43	0.129	-2.372
4614-1033	1	2	274	245	271	-5.40	-5.74	-5.43	-5.53	0.156	-2.815
4614-1033	8	2	143	152	142	-6.96	-6.86	-6.97	-6.93	0.054	-0.775
4614-1033	20	2	181	177	217	-6.51	-6.56	-6.08	-6.38	0.215	-3.367
4614-1033	32	2	364	369	300	-4.32	-4.26	-5.09	-4.56	0.375	-8.235
4624-1045	1	2	200	199	217	-6.28	-6.29	-6.08	-6.22	0.099	-1.587
4624-1045	8	2	207	237	200	-6.20	-5.84	-6.28	-6.11	0.192	-3.140
4624-1045	20	2	376	286	327	-4.18	-5.25	-4.76	-4.73	0.440	-9.287
4624-1045	32	2	261	254	274	-5.55	-5.64	-5.40	-5.53	0.099	-1.790
4765-1199	1	2	226	228	242	-5.97	-5.95	-5.78	-5.90	0.085	-1.441
4765-1199	8	2	195	186	211	-6.34	-6.45	-6.15	-6.31	0.124	-1.956
4765-1199	20	2	150	214	262	-6.88	-6.11	-5.54	-6.18	0.548	-8.871
4765-1199	32	2	209	459	151	-6.17	-3.19	-6.87	-5.41	1.596	-29.511
4777-1209	1	2	213	236	209	-6.13	-5.85	-6.17	-6.05	0.142	-2.349
4777-1209	8	2	473	236	268	-3.02	-5.85	-5.47	-4.78	1.254	-26.238
4777-1209	20	2	236	187	189	-5.85	-6.44	-6.41	-6.23	0.270	-4.339
4777-1209	32	2	196	231	255	-6.33	-5.91	-5.62	-5.96	0.289	-4.860
4485-843	1	2	311	261	231	-4.96	-5.55	-5.91	-5.47	0.394	-7.202
4485-843	8	2	201	193	200	-6.27	-6.37	-6.28	-6.31	0.043	-0.674
4485-843	20	2	198	180	188	-6.31	-6.52	-6.43	-6.42	0.088	-1.371
4485-843	32	2	230	227	186	-5.92	-5.96	-6.45	-6.11	0.240	-3.924

Table 3-8: Plate 1 Concentration values calculated from the OD Values using the adjusted equation calculated.

Each sample had a week, then on which plate it was processed, then the triplicate OD Value Results, then the calculated concentration, then the Mean, Standard Deviation and finally the %CV which is Mean divided by Standard Deviation. The Mean, Standard Deviation and %CV were calculated from the concentration results.

Sample	Week	Plate	Result 1	Result 2	Result 3	[Result 1]	[Result 2]	[Result 3]	[Mean]	Standard Dev	%CV
4438-787	1	1	504	584	632	0.72	1.19	1.47	1.13	0.312	27.683
4438-787	8	1	297	299	353	-0.51	-0.49	-0.18	-0.39	0.153	-39.139
4438-787	20	1	142	148	119	-1.42	-1.39	-1.56	-1.46	0.074	-5.075
4438-787	32	1	187	181	162	-1.16	-1.19	-1.30	-1.22	0.063	-5.174
4455-796	1	1	428	419	403	0.27	0.22	0.12	0.20	0.061	30.357
4455-796	8	1	206	204	226	-1.04	-1.06	-0.93	-1.01	0.059	-5.822
4455-796	20	1	310	306	319	-0.43	-0.45	-0.38	-0.42	0.032	-7.663
4455-796	32	1	592	606	591	1.24	1.32	1.23	1.26	0.040	3.204
4456-797	1	1	139	122	168	-1.44	-1.54	-1.27	-1.42	0.112	-7.926
4456-797	8	1	149	175	182	-1.38	-1.23	-1.19	-1.26	0.084	-6.636
4456-797	20	1	167	185	176	-1.27	-1.17	-1.22	-1.22	0.043	-3.557
4456-797	32	1	276	211	237	-0.63	-1.01	-0.86	-0.84	0.158	-18.908
4462-807	1	1	200	179	175	-1.08	-1.20	-1.23	-1.17	0.065	-5.539
4462-807	8	1	207	158	173	-1.04	-1.33	-1.24	-1.20	0.121	-10.084
4462-807	20	1	179	174	193	-1.20	-1.23	-1.12	-1.19	0.048	-4.009
4462-807	32	1	236	258	257	-0.87	-0.74	-0.74	-0.78	0.060	-7.668
4466-814	1	1	181	171	189	-1.19	-1.25	-1.14	-1.20	0.044	-3.640
4466-814	8	1	312	311	318	-0.42	-0.42	-0.38	-0.41	0.018	-4.484
4466-814	20	1	211	216	125	-1.01	-0.98	-1.52	-1.17	0.247	-21.031
4466-814	32	1	201	164	183	-1.07	-1.29	-1.18	-1.18	0.089	-7.556
4470-817	1	1	323	322	296	-0.35	-0.36	-0.51	-0.41	0.074	-18.129
4470-817	8	1	298	305	348	-0.50	-0.46	-0.20	-0.39	0.131	-33.693
4470-817	20	1	210	220	220	-1.02	-0.96	-0.96	-0.98	0.028	-2.841
4470-817	32	1	1821	1677	1754	8.50	7.65	8.11	8.09	0.348	4.301
4471-818	1	1	963	965	947	3.43	3.44	3.34	3.40	0.048	1.399
4471-818	8	1	212	231	206	-1.01	-0.90	-1.04	-0.98	0.063	-6.409
4471-818	20	1	171	190	152	-1.25	-1.14	-1.36	-1.25	0.092	-7.331
4471-818	32	1	160	185	144	-1.32	-1.17	-1.41	-1.30	0.100	-7.683
4473-820	1	1	192	172	209	-1.13	-1.25	-1.03	-1.13	0.089	-7.892

Table 3-9: Plate 2 Concentration values calculated from the OD Values using the adjusted equation calculated.

Each sample had a week, then on which plate it was processed, then the triplicate OD Value Results, then the calculated concentration, then the Mean, Standard Deviation and finally the %CV which is Mean divided by Standard Deviation. The Mean, Standard Deviation and %CV were calculated from the concentration results. Highlighted grey area of value 473 is the known pipetting error, whereby too much substrate was added to the well.

Sample	Week	Plate	Result 1	Result 2	Result 3	[Result 1]	[Result 2]	[Result 3]	[Mean]	Standard Dev	%CV
4473-820	8	2	210	201	203	-2.01	-2.08	-2.07	-2.05	0.030	-1.479
4473-820	20	2	254	275	244	-1.66	-1.50	-1.74	-1.64	0.102	-6.214
4473-820	32	2	286	298	286	-1.41	-1.32	-1.41	-1.38	0.045	-3.222
4480-838	1	2	272	254	238	-1.52	-1.66	-1.79	-1.66	0.109	-6.586
4480-838	8	2	254	242	257	-1.66	-1.76	-1.64	-1.69	0.051	-3.021
4480-838	20	2	343	270	296	-0.96	-1.54	-1.33	-1.28	0.238	-18.585
4480-838	32	2	257	274	283	-1.64	-1.51	-1.44	-1.53	0.085	-5.551
4614-1033	1	2	274	245	271	-1.51	-1.74	-1.53	-1.59	0.102	-6.439
4614-1033	8	2	143	152	142	-2.54	-2.47	-2.55	-2.52	0.035	-1.406
4614-1033	20	2	181	177	217	-2.24	-2.27	-1.96	-2.16	0.142	-6.568
4614-1033	32	2	364	369	300	-0.80	-0.76	-1.30	-0.95	0.247	-25.916
4624-1045	1	2	200	199	217	-2.09	-2.10	-1.96	-2.05	0.065	-3.174
4624-1045	8	2	207	237	200	-2.03	-1.80	-2.09	-1.97	0.126	-6.397
4624-1045	20	2	376	286	327	-0.70	-1.41	-1.09	-1.07	0.289	-27.075
4624-1045	32	2	261	254	274	-1.61	-1.66	-1.51	-1.59	0.065	-4.091
4765-1199	1	2	226	228	242	-1.88	-1.87	-1.76	-1.84	0.056	-3.048
4765-1199	8	2	195	186	211	-2.13	-2.20	-2.00	-2.11	0.081	-3.855
4765-1199	20	2	150	214	262	-2.48	-1.98	-1.60	-2.02	0.361	-17.860
4765-1199	32	2	209	459	151	-2.02	-0.05	-2.48	-1.52	1.052	-69.403
4777-1209	1	2	213	236	209	-1.99	-1.81	-2.02	-1.94	0.094	-4.832
4777-1209	8	2	473	236	268	0.06	-1.81	-1.55	-1.10	0.826	-75.060
4777-1209	20	2	236	187	189	-1.81	-2.19	-2.18	-2.06	0.178	-8.657
4777-1209	32	2	196	231	255	-2.12	-1.85	-1.66	-1.87	0.191	-10.170
4485-843	1	2	311	261	231	-1.22	-1.61	-1.85	-1.56	0.260	-16.676
4485-843	8	2	201	193	200	-2.08	-2.14	-2.09	-2.11	0.028	-1.330
4485-843	20	2	198	180	188	-2.11	-2.25	-2.18	-2.18	0.058	-2.659
4485-843	32	2	230	227	186	-1.85	-1.88	-2.20	-1.98	0.158	-7.990

3.3 Objective 3: Storage

The majority of the sample cohort comprised archived samples with no baseline measurements and a lack of pre-variables data that could potentially influence analyte integrity. Therefore, it was decided to determine how an analysis of fresh urine samples, processed according to the gold standard method would compare with various scenarios that might have taken place in the clinic when urine samples was obtained in real life.

Thus, for this analysis, the Wilcoxon Signed-Rank Test was used and the analytes for each group (Group 2, 3 & 4) was compared with the reference group (Group 1) using an average of the 19 patients. The Wilcoxon Signed-Rank Test assesses how different the groups are from the reference

group. The Wilcoxon Sign-Rank Test was used as the data set was skewed. The data were a matched data set (measurements were from the same individuals but under different conditions).

Table 3-10, below summarises the Median, Mean and Standard Deviation for each analyte in each group. For some of the analytes, it was noticed that the Median and Mean were not similar, whereas others were very similar. Based on the Median versus Mean values for Urea, a large difference was observed between the Median = 366.4667 and Mean = 319.9263 in Group 1.

Table 3-10: Summary of results for each group, looking at the Median, Mean and Standard Deviation using the Wilcoxon Signed-Rank Test.

** Indicates the Medians that were significantly different from each other for each analyte.

		Urea	Creatinine	Calcium	Protein	Albumin
Group 1 (Reference)	**Median**	366.4667	**10.93333****	**2.733333****	**0.06****	3.466667
	Mean	319.9263	11.26491	3.37807	0.0677193	9.554386
	Standard Deviation	166.917	6.030811	2.837593	0.0437734	18.20232
Group 2 (4 °C)	**Median**	366.7333	10.93333	2.726667	0.06	3.633333
	Mean	321.1667	11.19474	3.364561	0.0638596	9.65614
	Standard Deviation	166.7052	5.944094	2.814591	0.0452328	18.03708
Group 3 (28 °C)	**Median**	368.2333	**10.73333****	**2.18****	**0.0733333****	3.4
	Mean	320.4202	11.14386	3.198596	0.0814035	9.563158
	Standard Deviation	166.8813	5.973026	2.872127	0.0497688	17.91448
Group 4 (no centrifugation)	**Median**	364.4333	10.9	2.853333	0.06	3.533333
	Mean	320.2193	11.22632	3.672807	0.0663158	9.580702
	Standard Deviation	166.1031	6.011795	3.007545	0.0424394	17.90039

Table 3-11 below indicates the different p-values for each group comparison to the reference. It was noted that for Creatinine, Calcium and Protein, in the comparison of Group 3 to the reference, it was highly significant ($p<0.05$) and therefore different to the reference.

Table 3-11: P-values for each group in comparison with the reference for each analyte.
*Significant ($p<0.05$).

	Urea	Creatinine	Calcium	Protein	Albumin
Group 1 vs Group 2	0.8721	0.0947	0.6148	0.1050	0.1473
Group 1 vs Group 3	0.9679	**0.0022***	**0.0079***	**0.0165***	0.4565
Group 1 vs Group 4	0.9359	0.1837	0.1416	0.5435	0.3236

Figure 3-8 below represents Table 3-11 as a bar chart depicting each of the p values; those closest to 0 (*Significant is $p<0.05$) were the creatinine, calcium and protein from the Group 3 versus Group 1 (reference).

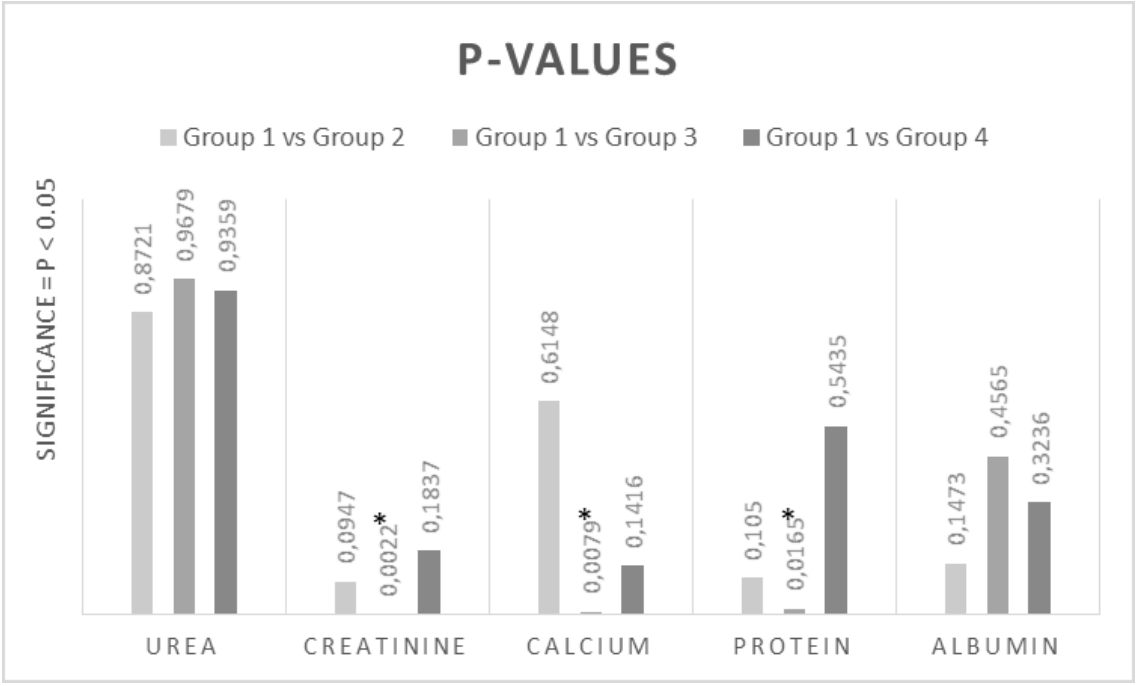


Figure 3-10: Table 3-11 depicted as a bar chart.
Group 1 vs 3 for creatinine, calcium and protein were significant. *Significant ($p<0.05$).

Chapter 4

Discussion

The effect of storage time and temperature on bio-marker levels in urine is not well understood as it is methodologically challenging to obtain measurements over various time points over extended time periods to study. Thus, in this exploratory study, the research question was to evaluate the long-term stability of select analytes in preservative-free urine in a pre-eclampsia cohort. Since this study formed part of a larger study titled: *Advancing a protein to creatinine rapid test for determining proteinuria status as an onset indicator of pre-eclampsia*, the present investigation would help to establish priorities for the larger study in terms of selected analytes to be measured, improve final research design and determine best data collection and analysis methods. Therefore, in order to provide context, the focus was on the bio-banking aspects, which included pre-analytical variables as well as sample processing and handling, and storage conditions, all of which could contribute to the effect on sample integrity. Therefore, for the present investigation, Calcium, Creatinine and Total Protein in long-term, stored, urine samples were measured using both manual dipstick as well as high-throughput laboratory measurements and these assays were compared using agreement analysis. Additionally, the diagnostic and prognostic potential of an ELISA-based Adipsin or Complement Factor D (CFD) test for pre-eclampsia was evaluated on long-term, stored, urine samples. Furthermore, because of the lack of baseline measurements for the larger study, fresh urine samples were collected to evaluate different processing and intermediate storage conditions compared with the gold standard to determine to what extent pre-analytical variables could affect sample integrity on selected analytes.

4.1 Comparison of clinically high-throughput measurements versus the newly developed LifeAssay Dipstick and the gold standard Siemens Dipstick

For objective 1, agreement analysis was performed on the laboratory high-throughput measurements and the dipsticks for creatinine and total protein, as both analytes are important bio-markers for pre-eclampsia in long-term, stored urine. In addition, calcium levels were obtained from high-throughput measurements with the underlying question of whether it would be measurable despite the long-term storage of the urine samples. Secondly, as the urine samples was obtained from the same people throughout their pregnancy over different time points, a trend analysis over the weeks was performed in order to determine whether there was a pattern of increase or decrease in selected analytes.

4.1.1 Agreement between laboratory high-throughput versus dipsticks

In the comparison to test the agreement between the laboratory high-throughput and the dipsticks, the concordance correlation co-efficient (Lin's co-efficient or CCC) was used (Lin, 1989; 2000), which has a scale ranging between -1 (perfect negative agreement) and 1 (perfect agreement)). The concordance correlation co-efficient combines measures of both precision and accuracy to determine how far the observed data deviate from the line of perfect concordance (y=0). Lin's co-efficient increases in value as a function of the nearness of the data's reduced major axis to the line of perfect concordance (the accuracy of the data) and of the tightness of the data about its reduced major axis (the precision of the data). Therefore, the closer the value of rho_co-ef is to 1, the more correlated the data is between the two data sets. CCC is a more appropriate statistic than the Pearson correlation co-efficient for assessing the level of agreement between two measurements of the same item. This is because the latter only quantifies the linear relationship whereas the former quantifies the linear relationship under the assumption that the slope equals 1 and the intercept equals 0. In addition to calculating estimates of the CCC, the 95% confidence intervals (CIs) were also calculated. The CCC was required to exceed 0.90 and 0.80 in order to claim that the paired measurements of a urinary bio-marker had excellent and good agreement, respectively, under the two conditions of each process.

It was assumed that the data set results would be the same for all the weeks and Week 32 was selected specifically for the agreement analysis (Lin's Co-efficient). In addition, for calcium measurement analysis, the last week was optimal to use because of the additional calcium supplements given to pregnant women in the last weeks of their pregnancy. For the protein measurements, the two dipsticks (Siemens - SP vs LifeAssay - DP) were compared with each other before the respective dipsticks were compared with the clinical laboratory measurements in the agreement analysis. In the age of point of care, dipsticks are used as a cheaper alternative to the high-throughput measurements in the case of developing countries.

In addition, it was noted that creatinine was only measureable on the LifeAssay Dipstick and not the Siemens Dipstick; therefore, the LifeAssay results were compared with the clinical laboratory measurement for agreement.

The Siemens Dipstick protein measurements (SP) were compared with the LifeAssay Dipstick protein measurements (DP). It was observed that the data correlated between the two dipsticks for the protein result. The rho_ co-efficient or concordance correlation coefficient was 0.920 which is close to one with a statistically significant p-value suggesting substantial agreement between the two dipsticks. As the results for protein analysis were congruent and similar, this indicated that the newly developed

LifeAssay Dipstick is on par with the diagnostic certified Siemens dipstick (Gold Standard) that is used in the laboratories globally. In order to implement the LifeAssay Dipstick as a more cost-effective assay than the Siemens in low- and middle-income countries (LIMC), this concordant result is of value to the larger study and is in line with what was expected. In turn, this study was also able to demonstrate that, despite the long-term storage of these urine samples, the specific analytes were still measurable in this specific cohort.

The gold standard, Siemens Dipstick protein results (SP) were compared with the laboratory protein result (LP), which was not significant, which meant the results were not similar and in agreement. The finding may be attributed most likely to the fact that a dipstick measurement will not always be exactly the same as a laboratory result. The high-throughput result uses specific equipment to determine the exact value (objective quantitative measurement), demonstrating that it is more sensitive whereas the dipstick is used for a more generalised view of what the possible value could be. The dipstick is also a subjective measurement relying on the user's perception of colour intensities which could also skew the results because of the big differences in measurement ranges. The data were log-transformed in order to normalise them to a specific range to account for the extreme differences in the measurements ranges between the two assays. However, despite the transformation, the agreement between the assay and the laboratory results was still poor.

Similarly, the LifeAssay Dipstick protein results (DP) were compared with the laboratory protein results (LP). In these protein measurements, an extreme outlier was observed which affected the initial calculations resulting in poor agreement and non-significance. It is very likely that the patient had an abnormally high value. The analysis was re-run excluding the outlier. In this case, the concordance correlation co-efficient improved from an extreme negative value to the value of 0.533 with a significant p-value demonstrating how the outlier skewed the results significantly. However, despite the significance, the agreement between the assay and the laboratory results was still poor. The data was log-transformed in order to normalise it to a specific range to account for the extreme differences in the measurement ranges between the two assays. However, despite the transformation, the agreement between the assay and the laboratory results was still poor with a concordance correlation co-efficient of 0.661 and a significant p-value of 0.000.observed.

For the subsequent creatinine results, the LifeAssay Dipstick results were compared with the laboratory creatinine results. Similar to the protein result, poor agreement and no significance was observed. Although the creatinine dipstick was newly developed, it was expected that there would, at least, be agreement between the values, even if it was not significant. In this case, it could be that the

sample size was too small or the values were over-estimated or under-estimated in the dipstick results. It is very easy to over or under-estimate subjective values and this could be the cause, but it was more likely a combination of subjective interpretation and a small sample size.

It is noted that, despite the non-significant values, this thesis was an exploratory pilot carried out on the existing data to determine whether there was an observable trend or pattern. Only one week was used for analysis which ranged between 55-68 observations that were included, thus the sample size was very small. This could have attributed to the non-significant values observed. However, the main factors contributing to non-significance can be attributed most likely to the subjective values of the dipsticks and, more importantly, the big difference in measurement ranges between the dipsticks and the laboratory results. This observation was confirmed by the significant values and the concordant agreement observed between the two dipsticks for the protein results, which was very informative. This agreement could also be attributable to the same measurement ranges for the two dipsticks compared with the laboratory measurements. This result was expected to be significant as the new dipstick was developed in line with known and certified values of the Siemens dipstick, which is the gold standard used.

With regards to the effect of long-term storage on sample integrity, owing to the lack of baseline measurements for this cohort, it would be difficult to infer differences related to storage time from differences related to biological or assay variability. Therefore, this matter for this objective has not been pursued further, which is a limitation of this study.

4.1.2 Trend analysis of measurements over various time points

For the trend analysis, at each time point (ADM-1, DPV8-8, DPV20-20 and DPV32-32), the mean value and the corresponding 95% confidence interval were plotted on each graph. Variance-weighted least-squares regression was used to test whether the linear trend was significant or not to determine whether there was an increase or decrease over the weeks for each dipstick and laboratory result for each analyte measured. Goodness-of-fit chi2 was used to test whether the single population fitted the hypothesised distribution. Then the model fit chi2 was used to compare the weeks to each other (across categories).

In summary, the Laboratory High-throughput Protein (LP), LifeAssay Protein Dipstick (DP), Laboratory High-throughput Creatinine (LC) and Laboratory High-throughput Calcium (LCa) trend showed no significant value for the co-efficient, goodness-of-fit chi^2, and model chi^2. It is possible that the small sample size of 14 patients over the weeks, resulting in a total of 56 samples analysed,

affected the results and a pattern could not be inferred. There was a high degree of variation in the standard deviations in each graph, which is mostly the result of a small sample size with large variations. Despite the lack of non-significance, it is noted that, both the laboratory and dipstick protein measurements showed a higher protein value for week 32 in both analyses compared with the other weeks. This observation is important and could be explained by the fact that pre-eclampsia affects pregnant women mostly in the 3rd trimester of their pregnancy. As the kidney and liver is most at risk during pre-eclampsia the high protein values observed in the latter weeks of gestation reflect kidney impairment.

It was noted for the Calcium measurements, that an increase of Calcium was observed following week 1 until a plateau is reached. This observation is consistent and could be justified through the fact that pregnant women receive a consistent dose of calcium supplement throughout their pregnancy.

However, the LifeAssay Creatinine Dipstick (DC) had a significant value for the co-efficient and model chi^2. It is possible that this was the result of subjective interpretation. For the creatinine dipstick, it was decided to over-estimate the value rather than under-estimate it, when it was unclear which colour it was. Owing to over-estimation, it was possible that the pattern formed as a result and it was indicated to be significant for a linear trend.

4.2 ELISA-based Adipsin evaluation

As mentioned in Chapter 1, Section 1.6, a relationship between the complement system and PE was demonstrated. As measurements of complement activation products were shown to be increased in PE pregnancies compared with normal pregnancies, this observation was explored further in this study. Thus, the objective was to determine whether Adipsin or Complement Factor D (CFD) could be measured via ELISA using urine samples that were frozen at -80^{o}C for 3+ years. A pilot study was carried out to evaluate Adipsin using an ELISA assay to discuss future implications and how the assay could be improved.

The standard curves that were generated for each plate had a relatively high but still low actual R^2 value. Certain data points were remove to test their effects. It was noticed that, when the 40ng/ul concentration and associated optical densities (OD) value were removed, the two standard curves improved considerably. It was therefore assumed that, when the stock solution was used to create the 160ng/ul and then the 40ng/ul, the values were not pipetted correctly or the bottle was not shaken enough. Evidently, a few errors occurred at this stage and, because they was consistent across both

plates, it was decided to remove them and use the adjusted standard curve also to calculate the concentrations.

Using the first standard curve, the concentrations were calculated from the OD values. When the values came up negative, the adjusted standard curve was applied. A few values became positive but were low values for the concentration. Despite the high R^2 value for each standard curve, it was not possible to calculate positive concentrations for the OD values which were already low.

The %CVs should be calculated from the calculated concentrations. In this case, the calculated concentrations were mostly negative, from both the original results and the adjusted results. There were two reasons why the concentration values were negative in this scenario. The one reason was that, because the urine samples were stored for so long, it was possible that the Adipsin had degraded over time. The other reason was that the ELISA assay was not sensitive enough for small concentrations of Adipsin, especially in urine. In the protocol (Appendix G) under the sample collection and storage section, there was not an exclusive section on urine samples. They did however mention that for samples not listed in the manual, a pilot study into the validity of the assay was required which we decided to do for the long term stored urine samples. This therefore emphasises that there should be more research studies carried out on the impact of sample handling, processing and storage conditions on urine samples for the measurement of bio-marker levels because there is no gold standard to examine the impact of storage time on bio-marker levels. Following from this limitation, a future study re-measuring Adipsin over time from aliquots of a single sample would provide a lot more information about why the value is so low. In addition, comparing the measurement of Adipsin in fresh samples as a baseline or reference is also essential. Since this study not have a baseline measurement because existing data and samples were used, the reason why the concentration values were low could not be assumed correctly. However, OD readings were obtained, which indicates that the ELISA assay can be used to measure Adipsin in urine but the assay needs to be validated further to account for the low concentrations.

It was decided still to calculate the %CV from the OD values to assess how well the assay was processed. Plate 1 had a range of %CV values from 0.841% to 22.701%. Plate 2 had a range of %CV values from 1.797% to 48.951. Intra-assay %CVs (an average value calculated from the individual CVs) should be <10%. The scores obtained reflected the performance of the user who was handling the assay. The %CVs that are >10% reflect poor pipetting technique of the user. It is the norm to use %CVs of the concentrations, but here the OD values %CVs have been emphasised. The average %CV for plate 1 was 6.6% and plate 2 was 9.2%. They were both <10% which indicates good pipetting

technique. The average %CV across the plates was 7.9% which indicates consistent pipetting across both plates.

The Inter-Assay (plate-to-plate consistency) %CV of <15% is generally acceptable. In this scenario, inter-assay must be calculated using 8 repeats across 3 plates of high and low values. This was not included as the aim was to assess whether Adipsin could be measured and what further measures can be considered, not whether the plate-to-plate consistency was correct. The next step for validation would be to account for the small values and the plate-to-plate consistency once the small values have been accounted for.

4.3 Evaluating the pre-analytic effect of pre- and post-storage conditions and processing of fresh urine samples

Important limitations for this study were the lack of baseline measurements as well as lack of data relating to the pre-analytical variables, which made it difficult to infer whether differences observed because of storage or differences in assay methodologies and processing. Also, when it comes to urine analysis, a gold standard for processing needs to be followed, which includes centrifugation prior to storage of aliquots. The urine samples that formed part of this cohort had been collected in busy clinics in hospitals following a standardised protocol but it was not possible to account for the pre-analytical variables and the time for which the samples were stored because they were stored for 3+ years prior to long-term storage at NSB and the centrifugation steps that form part of the gold standard method were lacking. Therefore, it was not possible to determine whether these types of pre-analytical variables have an effect of analytes over time. Furthermore, it was not known whether the different sites that collected the urine samples adhered to the standardised protocol because of contributing factors such as availability of the -80 ^{o}C freezer, transport and access to fridges. It was necessary to gain some insights into how different, possible conditions could affect analytes in urine samples over time. In this case, it was decided to use the gold standard as a reference and test conditions where the samples were stored at 4^{o}C in a fridge, stored at room temperature (24^{o}C) or were centrifuged according to the gold standard and how this could affect the outcome on selected analytes.

Accordingly, the Wilcoxon Signed-Rank Test was used for this investigation. Each group (Group 2, 3 & 4) was compared with the reference group (Group 1 – gold standard) using an average of the 19 patients for each analyte. Twenty consenting patients participated but the clinical laboratory did not measure all of the analytes for one of the patients, therefore that patient was excluded. The Wilcoxon Signed-Rank Test examines how different the groups are in relation to the reference group and

accounts for skewed data sets. The data were a matched data set (measurements were from the same individuals but under different conditions).

Using the Wilcoxon Signed-Rank Test, the median for Creatinine, Calcium and Protein for both Group 1 and 3 were significantly different from each other using Group 1 as the reference. Therefore, Group 3 was significantly different from Group 1.

Group 1 was centrifuged, then stored at -80^{o}C for 3 months as the gold standard. Group 3 was centrifuged, then was stored at room temperature (24^{o}C) for 48 hours and then stored at -80^{o}C for 3 months. The fact that the samples were left at room temperature for 48 hours was most likely the contributing factor for their Creatinine, Calcium, and Protein measurements being significantly different from the gold standard. It is known that Albumin and Urea are quite consistent or stable as analytes, which is probably why their measurements for Group 3 were not significantly different from the gold standard.

4.4 Summary

Exploratory pilot studies were used for all three objectives to understand different aspects of urine sample collection, handling, and storage. Objective 1 was to consider how the possible use of dipsticks to diagnose PE would compare with laboratory measurements and whether, although the samples had been frozen for 3+ years, measurements could still be reliable despite the fact that dipsticks are generally assumed to be not as good as high-throughput measurements. In the case of comparing the Siemens protein dipstick to the newly developed LifeAssay protein dipstick, the measurements were significantly similar, which was expected because the process was under the researcher's control. The LifeAssay protein dipstick results were not similar to the laboratory results. Similar results were found for the creatinine measurements between the LifeAssay dipstick and the laboratory measurement. Thus, more research and evaluation studies are required for the newly-developed creatinine LifeAssay Protein Dipstick, which was the essential point of this pilot: to determined how this study's observations could help improve gaps and issues in the larger study.

Objective 2 was to consider the bio-marker, Adipsin. The aim was to determine whether Adipsin could be measured using the ELISA assay despite the assay not being validated for urine samples, especially urine samples that were frozen for 3+ years. The Adipsin could be measured, but the OD values were very low, which resulted in negative concentration values. There were many reasons why the values could have been negative as explained in Section 4.2. The most important finding from

this objective was that, despite the samples being stored for 3+ years, Adipsin could be measured using the ELISA assay.

Objective 3 was to consider how sample collection and handling can affect the results of the measurements. It was noted that Group 3, which was stored at room temperature, was the most affected and therefore deviated from the gold standard for Creatinine, Protein and Calcium.

Each objective of this study provided some insight into sample collection, handling, processing and storage and how each step of the process can have an impact. These insights also led to understanding of the limitations of this pilot study and possible future studies.

4.5 Limitations and Future Studies

In addressing Objective 1, because of the small sample size, it was not possible to infer correctly whether the dipsticks were similar to the laboratory measurements or not. A larger sample size would have given a better understanding of the results. Thus, in a future study, a larger amount of data would be the most beneficial. One patient that had results from every week was needed to obtain a consistent result for the trend analysis, but only 14 patients were found that had results from each week. This was a limitation because if there were more patients across the weeks, maybe 100 patients, then it would have been possible to see a clearer pattern. Another potential limitation was the possibility that some of the LifeAssay Dipsticks could be faulty since it was newly-developed and under evaluation. If so this could have had a major effect on the results.

In objective 2, the small sample size was also a limitation. Another limitation was that a measurement at different time intervals was not available. Therefore, a future study include re-measuring Adipsin over time from aliquots of a single sample. This would provide a lot more information about why the values measured were so low. A future study should also compare the measurements of Adipsin over time intervals with the measurement of fresh samples as a baseline or reference and also include a high and low control which this study lacked.

Objective 3 was to consider how sample collection and handling can impact the results of the measurements. The main limitations were that there was no baseline measurement against which to comparisons, and there were no interval measurements over the 3 months. Both of these limitations could be used in a future study as this would give more insights into the impact of sample collection and handling.

4.6 Conclusion

In conclusion, the results demonstrated that when studying bio-markers in the context of long-term stored samples, the impact of urine processing and duration of storage on bio-marker levels is essential. It is also methodologically challenging because of various measurements over various time points over an extended time period. Limitations such as sample size, lack of baseline measurements, pre-analytical variables, and assay variation or drift could affect results, which makes it difficult to infer differences related to storage time from differences related to biological or assay variability and requires further investigation. Despite these limitations, this exploratory study did help to establish priorities for the larger study in terms of the analytes selected to be measured, in improving the final research design, and determining the best data collection and analysis methods for future studies.

References

Askenazi, D. J. *et al.* (2014) 'Comparison of Methods, Storage Conditions, and Time to Analysis of Serum and Urine Creatinine Measured from Microsamples by Liquid Chromatography Mass Spectrometery (LC/MS) vs. Jaffe', *Journal of Clinical Laboratory Analysis*, 28(5), pp. 405–408. doi: 10.1002/jcla.21701.

Baker, M. (2012) 'Building better biobanks', *Nature*, 486, pp. 141–146. doi: 10.1038/486141a.

Barratt, J. and Topham, P. (2007) 'Urine proteomics: the present and future of measuring urinary protein components in disease', *Canadian Medical Association*, 177(4), pp. 361–368.

Bertholf, R. L. (2014) 'Proteins and Albumin', *Laboratory Medicine*, 45(1), pp. e25–e41. doi: 10.1309/LMKRNRGW5J03APZQ.

Buhimschi, I. a. *et al.* (2014) 'Protein misfolding, congophilia, oligomerization, and defective amyloid processing in pre-eclampsia', *Buhimschi, I. a., Nayeri, U. a., Zhao, G., Shook, L. L., Pensalfini, a., Funai, E. F., ...Buhimschi, C. S. (2014). Protein misfolding, congophilia, oligomerization, and defective amyloid processing in pre-eclampsia. Science Translational Medicine, 6(245), 24*, 6(245), p. 245ra92-245ra92. doi: 10.1126/scitranslmed.3008808.

Buyken, A. E. *et al.* (2006) 'Urinary C-Peptide Excretion in Free-Living Healthy Children Is Related to Dietary Carbohydrate Intake but Not to the Dietary Glycemic Index', *The Journal of Nutrition*, 136(7), pp. 1828–1833.

Ferraz, R. R. N. *et al.* (2006) 'Preservation of urine samples for metabolic evaluation of stone-forming patients', *Urological Research*, 34(5), pp. 329–337. doi: 10.1007/s00240-006-0064-2.

Froom, P. *et al.* (2000) 'Stability of common analytes in urine refrigerated for 24 h before automated analysis by test strips', *Clinical Chemistry*, 46(9), pp. 1384–1386.

Griefahn, B. *et al.* (2001) 'Long-term stability of 6-hydroxymelatonin sulfate in 24-h urine samples stored at −20°C', *Endocrine*, 15(2), pp. 199–202. doi: 10.1385/ENDO:15:2:199.

Hillebrand, J., Heijboer, A. and Endert, E. (2017) 'Effects of repeated freeze–thaw cycles on endocrine parameters in plasma and serum', *Annals of Clinical Biochemistry*, 54(2), pp. 289–292. doi: 10.1177/0004563216657361.

Hubel, A., Spindler, R. and Skubitz, A. P. N. (2014) 'Storage of human biospecimens: selection of the optimal storage temperature.', *Biopreservation and biobanking*, 12(3), pp. 165–75. doi: 10.1089/bio.2013.0084.

IATA (no date). Available at: https://www.iata.org/pages/default.aspx (Accessed: 30 November 2018).

Imdad, A., Jabeen, A. and Bhutta, Z. A. (2011) 'Role of calcium supplementation during pregnancy in reducing risk of developing gestational hypertensive disorders: A meta-analysis of studies from developing countries', *BMC Public Health*. BioMed Central Ltd, 11(SUPPL. 3), p. S18. doi: 10.1186/1471-2458-11-S3-S18.

Kisand, K. *et al.* (2010) 'Impact of cryopreservation on serum concentration of matrix metalloproteinases (MMP)-7, TIMP-1, vascular growth factors (VEGF) and VEGF-R2 in Biobank samples', *Clinical Chemistry and Laboratory Medicine*, 49(2), pp. 229–235. doi: 10.1515/CCLM.2011.049.

Kluger, H. M. *et al.* (2011) 'Plasma markers for identifying patients with metastatic melanoma',

Clinical cancer research : an official journal of the American Association for Cancer Research, 17(8), pp. 2417–2425. doi: 10.1158/1078-0432.CCR-10-2402.

Liu, K. D. *et al.* (2016) 'Storage Time and Urine Biomarker Levels in the ASSESS-AKI Study', *PloS one*, 11(10), p. e0164832.

Männistö, T. *et al.* (2007) 'The Effect of Freezing, Thawing, and Short- and Long-Term Storage on Serum Thyrotropin, Thyroid Hormones, and Thyroid Autoantibodies: Implications for Analyzing Samples Stored in Serum Banks', *Clinical Chemistry*, 53(11), pp. 1986–1987. doi: 10.1373/clinchem.2007.091371.

Nolen, B. M. *et al.* (2013) 'An Extensive Targeted Proteomic Analysis of Disease-Related Protein Biomarkers in Urine from Healthy Donors', *PLoS ONE*, 8(5). doi: 10.1371/journal.pone.0063368.

Papale, M. *et al.* (2007) 'Urine profiling by SELDI-TOF/MS: Monitoring of the critical steps in sample collection, handling and analysis', *Journal of Chromatography B: Analytical Technologies in the Biomedical and Life Sciences*, 856(1–2), pp. 205–213. doi: 10.1016/j.jchromb.2007.06.001.

Parekh, R. S. *et al.* (2007) 'Reliability of Urinary Albumin, Total Protein, and Creatinine Assays after Prolonged Storage: The Family Investigation of Nephropathy and Diabetes', *Clinical Journal of the American Society of Nephrology*, 2(6), pp. 1156–1162. doi: 10.2215/CJN.01030207.

Poveda, N. E. *et al.* (2016) 'Serum Adipsin Levels throughout Normal Pregnancy and Pre-eclampsia', *Scientific Reports*. Nature Publishing Group, 6, pp. 1–11. doi: 10.1038/srep20073.

Remer, T., Montenegro-Bethancourt, G. and Shi, L. (2014) 'Long-term urine biobanking: Storage stability of clinical chemical parameters under moderate freezing conditions without use of preservatives', *Clinical Biochemistry*. The Canadian Society of Clinical Chemists, 47(18), pp. 307–311. doi: 10.1016/j.clinbiochem.2014.09.009.

Rist, M. J. *et al.* (2013) 'Influence of freezing and storage procedure on human urine samples in NMR-based metabolomics', *Metabolites*. doi: 10.3390/metabo3020243.

Rouy, D. *et al.* (2005) 'Plasma storage at −80°C does not protect matrix metalloproteinase-9 from degradation', *Analytical Biochemistry*, 338(2), pp. 294–298. doi: 10.1016/j.ab.2004.10.052.

Samozai, M. N. and Kulkarni, A. K. (2015) 'Do calcium supplements increase serum and urine calcium levels in post-menopausal women?', *The journal of nutrition, health & aging*, 19(5), pp. 537–541. doi: 10.1007/s12603-014-0532-2.

Saude, E. J. and Sykes, B. D. (2007) 'Urine stability for metabolomic studies: Effects of preparation and storage', *Metabolomics*, 3(1), pp. 19–27. doi: 10.1007/s11306-006-0042-2.

Sharda, N. *et al.* (2014) 'Manual Urine Microscopy Versus Automated Urine Analyzer Microscopy in Patients with Acute Kidney Injury', *Laboratory Medicine*, 45(4), pp. e152–e155. doi: 10.1309/LMVJK6W4KQL1ZHKS.

Spierto, F. W. *et al.* (1997) 'Stability of urine creatinine1Use of trade names is for identification purposes only and does not constitute endorsement by the Public Health Service, or by the U.S. Department of Health and Human Services.1', *Clinica Chimica Acta*, 264(2), pp. 227–232. doi: 10.1016/S0009-8981(97)00080-6.

Swanepoel, C. C., Snyders, C. I. and Grewal, R. (2015) 'Biomarker discovery for diagnosis and treatment of tuberculosis : a role for biobanking ?', pp. 47–56.

Wang, T. *et al.* (2014) 'Elevation of urinary adipsin in pre-eclampsia correlation with urine protein concentration and the potential use for a rapid diagnostic test', *Hypertension*, 64(4), pp. 846–851.

doi: 10.1161/HYPERTENSIONAHA.113.02688.

Wang, T. *et al.* (2016) 'Clinical assessment of the specificity of an adipsin rapid test for the diagnosis of pre-eclampsia', *Hypertension in Pregnancy*. Taylor & Francis, 35(3), pp. 420–425. doi: 10.1080/10641955.2016.1178773.

Williams, P. J., Pipkin, B. and Fiona (2011) 'The genetics of pre-eclampsia and other hypertensive disorders of pregnancy . Best Practice & Research Clinical Obstetrics and Gynaecology', *The University of Nottingham*, 25(4), pp. 405–417. doi: 10.1016/j.bpobgyn.2011.02.007.

Yilmaz, G. *et al.* (2008) 'Are preservatives necessary in 24-hour urine measurements?', *Clinical Biochemistry*, 41(10), pp. 899–901. doi: 10.1016/j.clinbiochem.2008.03.002.

Appendices

List of appendices attached:

A: WHO CAP SOP

B: H3A SOP Sample transport and shipping

C: Project Info and Consent

D: SOP Urine CREATININE ChemPath

E: SOP Urine CALCIUM ChemPath

F: SOP Urine TOTAL PROTEIN ChemPath

G: ELISA Kit for Complement Factor D (CFD)

H: SOP Urine ALBUMIN ChemPath

Appendix A

A65750: Long-term calcium supplementation in women at high risk of pre-eclampsia: A randomized placebo controlled trial

CAP trial

SOP URINE COLLECTION AND PROCEDURES

1. When to collect urine samples

Urine will be collected on all randomized women at the following periods:

- As per protocol: at randomization visit, 20 weeks, 32 weeks to measure calcium and creatinine
- If possible, for pregnant women, collect during visit at 8 weeks and at onset of pre-eclampsia (hospitalized and accessible women)
- If possible, at the end of the study regardless of gestation,for all women (to capture early pregnancy loss women before 8 weeks gestation)

2. What materials are needed for urine collection

- Clean urine collection cup
- Five aliquot vials of 1.5 or 2ml per collection (if not possible to aliquot on site, use one 10 ml vial; however, 1.5- 2 ml aliquots are preferable)
- Micropipettes to aliquot the samples
- Labels (unless bar coded tubes are available) for each collection

3. How to collect, label and store the urine

- Ask the woman to void (midstream urine preferable) into the urine collection cup at least half and close the lid tightly
- Record the date and time of urine collection in the logbook (see below) as well as the specimen cup
- If it is not possible to aliquot, initially place in 10 ml vial and freeze in upright position
- For 1.5-2 ml vials, use a different storage tray for each type of visit (ADM tray, DPV 8 tray, DPV20 tray, DPV32 tray). Once a specific visit tray is full, take a new one and number the trays in sequential order AMD 1, ADM 2, etc.

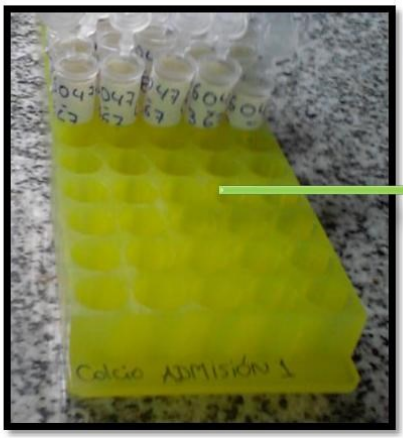


Figure 1: Tray for urine collected at ADM visit (tray number 1)

One line per participant and per urine collection

- *Samples at admission*: for ease of reference and track, ADM samples should preferably be stored following the screening/subject number order. Leave a line in the tray empty if the sample for a particular woman was not taken at ADM and try to get a sample in the next pre-pregnancy visit.

Figure 2: Note the vials stored in screening/subject order

- *Samples during pregnancy*: use a different tray for each type of pregnancy visit: DPV8, DPV20, DPV32. For ease of reference and tracking, samples should preferable be stored in the order that a woman falls pregnant.

- Before aliquoting, clearly label each tube with the screening and subject number (e.g.: 6001-504), the type of visit (e.g.: ADM, DPV20 or DPV32) and date of collection, (this will constitute the identifier number for the specimen).

  Labels should have this format: **6001-504 ADM1 DD-MM-YY**

- As we are collecting 5 x 2ml tubes, the label should also have the tube sequence (e.g.: 6001-504 ADM**1** DD-MM-YY tube1; 6001-504 ADM1 DD-MM-YY tube2; 6001-504 ADM1 DD-MM-YY tube3, etc.).
- If a sample was not collected at ADM, identify the samples by labeling with PPV and the date of the visit.
- For centers with bar coded aliquot specimen vials, ensure the system clearly records date of collection (not just date of storage in freezer), gestation and subject number in addition to the bar code.
- If possible, centers can colour code the specimen bottles to identify the randomization, 8 weeks, 20 weeks, 32 weeks and onset of preeclampsia or end of study specimen in addition to date of collection, screening and subject number and gestation at collection
- Use the micropipette to aliquot 2 ml in each of the five tubes already labeled.
- All specimens to be stored in the freezer in an upright position.
- The recommendation is to freeze the five 2 ml aliquots in -70°/-80°C freezer. If this is not possible immediately, see table below with the maximum time that samples can be stored other than in-70°/-80° C freezer. The sooner they are moved into -70°/-80° C freezer the better.

Storage type	Maximum time the samples can be stored
Ice	24 hours
-2ºC to -8 ºC	8 days
-20 ºC	3-6 months

4. Urine logbook

All centers should keep a clear and easily identifiable logbook (or computer based database with backup) of all specimens stored in the freezer. A logbook has been prepared for this purpose. The information in the logbook includes: screening and subject number, location (in freezer), date and time acquired, date and time moved into -70/-80°C freezer.

Instructions for completing the URINE logbook:

- Every time you store or move a sample you should register it in the URINE logbook.
- Excel columns A, B and C: The first time you register a sample you should complete the SCR number, the Subject Number and the ADM date. These numbers have already been entered for those women that already existed in OC before June 2014.
- Excel column D is for written comments. E.g.: Sample was not taken at ADM but at PPV, whether the participant is pregnant or not and if she has finished the study, or if the sample was not taken.
- The order of samples in this logbook should preferably be in the same order for storing samples in the trays.

The logbook allows recording for each urine sample:

- **Storage Tray (**Excel column E**):** complete the label of the tray where you are storing the sample i.e.: ADM1, ADM2, etc.

- **Urine collection date (**Excel column F**):**Complete with the date the sample was collected, if the first sample was not taken during admission complete with the PPV date when the sample was taken.
- **Date of moving the sample to a -70º/-80ºC freezer**(Excel columns G):In case of storing the samples in other than -70º/-80ºC freezer, please indicate in this column the date you moved the sample to the final -80 or -70 ºC. If you are storing samples directly into a -80º or -70ºC freezer, copy the urine collection date into this column.
- **Number of sample stored (**Excel column H)**:**complete withthe number of samples collected for that visit. Complete with 5 if you are taking 5 x 2 ml samples or with 1 or 2 if you are taking 10 ml samples.

5. Laboratory testing of urine specimen

In orderto maintain the quality of sample it is not advisable to thaw and re-freeze urine specimens.

If the sample is frozen in 1.5 or 2 ml aliquots, one aliquot is taken for creatinine and calcium determinations. Prior to assay, samples should be thawed and vigorously shaken (calcium is not very soluble and some of the precipitate may not dissolve) and then aliquoted including the particles in solution for the calcium assay. The material will dissolve when acidified for the calcium assay. The remainder of the original aliquot can be centrifuged if desired prior to creatinine assay.

If sample is in a 10ml aliquot, at the first thaw the samples should be stirred vigorously and placed in 1.5 to 2ml tubes with precipitate in solution, as above. One 1-5-2 mls aliquot is used for the calcium and creatinine assay and the others labeled and entered into the log and refrozen at -70°/-80°C for future use.

NOTE: We have agreed to collect urine five times for each woman who reaches full term:

- Randomization
- 8 weeks (if possible)
- 20 weeks
- 32 weeks
- Onset of pre-eclampsia (any gestation If possible)
- End of Study/postpartum (if possible)

The additional urine collections will be for the urine biomarkers research as well as storage in the biobank for Collaboratory.

Appendix B



SOP 03.003, v 1.0

SAMPLE SHIPPING & TRANSPORTATION

H3A Standard operating procedures SOP on sample shipping and transportation			
SOP Number:	03.003	Version:	1
Supersedes:		Category	Transport of biospecimens

Prepared by:	H3A Biorepository Working Group	Date Prepared:	August 2013
Signature/s:			
Date Adopted:			
Reviewed Period			

Approved by:	H3A Steering Committee Group	Date #
	Per:	Date #
	Signature	

1.0 PURPOSE

Biospecimens collected by H3Africa research groups will be transported to the processing/storage site (submission Site) and/or an H3Africa biorepository.
When approval by the DBAC has been obtained, Biorepositories will also distribute samples to approved secondary users. Human Biospecimens are a precious resource and therefore care should be taken to preserve and maintain the integrity of these samples during the transport process.

2.0 SCOPE

This SOP outlines processes for shipping biospecimens within the H3Africa consortium, including but not limited to processing/storage/submission site and ultimately an H3Africa biorepository. While some of the referred documents are specific to the H3Africa consortium, the document can also serve as a reference for biological shipments in general, as needed. The SOP specifies considerations that should be followed to ensure appropriate packaging and shipping of the samples.

3.0 LIMITATIONS

The procedures outlined in this SOP may be subject to change depending on variations in specific laboratory procedures and they do not override national guidelines.

4.0 RESPONSIBILITIES

This SOP applies to all personnel from H3Africa research groups and biorepositories who are involved in the shipping or receiving of samples.

Personnel	Responsibility/Role
Site coordinator/manager	Inform biorepository of samples to be sent. Ensure that samples are packaged correctly for shipping.
Shipper/Biobank Coordinator/Manager "when shipping to H3A biorepositories"	Initiate contact with courier to set up shipment. Coordinate sample receipt and storage

Note: It's the responsibility of the shipper to ensure proper labeling and packaging of the consignment

5.0 SPECIFIC NEEDS & REQUIREMENTS (Documents, forms & supplies)

The documents and equipment listed below are required by the submission site to ensure IATA requirementsare adhered to.

Materials and Equipment	Materials and Equipment (Site Specific)
MTA	
Valid Import/Export Permit (if required)	
Shipping waybill	
Temperature Loggers/monitor	
Labelled vials containing biospecimen	
Corrugated shipping carton containing Styrofoam container or Credo Shipper	
Lab tape (to secure tube in kit)	
Absorbent material	
Waterproof tape	
Sealable/Press-lock bag or Reusable ????	
Dry Ice or Ice Packs (if required)	
Shipping Category Labels (if required)	
Contact labels/markings	

6.0 PROCEDURE

An established and tested shipping procedure is essential, as inadequate shipping procedures may lead to the loss of the samples and additional costs for repeat shipments.

6.1 The safe and legal transport of biospecimens is based on the following mandated activities:

a. Classification and naming of the material to be shipped
b. Selection of packaging that will contain and protect the contents if the package is damaged
c. Packing the shipment correctly
d. Placing appropriate markings and labels onto the outer package
e. Documenting relevant aspects of each package and its contents
f. Training individuals about the requirements for appropriate packaging and shipping of biological substances and associated dangerous goods (dry ice).

6.2 Ethical & Regulatory Considerations (At least one month prior to shipment)

6.2.1 The shipper and/or recipient must contact the courier to establish what supporting documentation is needed to ship the sample to the specified destination. Note: When shipping to an H3A biorepository, the host biorepository will contact the courier.

- For international shipments, research any new regulations that may have been adopted or special permits that are needed for that destination.
- Determine whether "Exempt human specimen", Category A , or Category B requirements is appropriate and attain or request the associated packaging materials. Examples of labels required for Category B and Dry Ice are included below for reference as these are most applicable to H3Africa.

6.2.2 Ensure that the Material Transfer Agreement, Import/Export Permit, project Informed Consent document, IRB/ethical approval and requisition for requested samples are attained as required and that intended storage/use is consistent with associated Informed Consent, prior to proceeding with the remaining procedures. Note: Ethical and regulatory documents noted below, which may take weeks to several months to process after initiation.

6.3 Shipping Procedure

6.3.1 Two weeks prior to shipment:

a. Contact the recipient to inform them of the shipment details. Note: When shipping to an H3A biorepository a shipment notification form is required. Document/confirm telephone communication via email. Refer to H3A Shipment checklist for detailed description of requirements prior to shipping day and on the day of shipment. Please refer to the bio specimen guideline

6.3.2 At least one week before the shipment

a. Verify that required supplies are available using the H3A Shipment Checklist: such as packaging, refrigerant, and MTA (Import/Export) as required. Prior arrangements can be made with certain couriers (E.g. World Courier and Marken) to provide shipping materials such as shipping box, dry ice, ice packs, sealable bags and waybill (not including ethical and regulatory documents). This arrangement must be planned in advance.

- If temperature indicators and/or loggers will be included to monitor shipping temperature, ensure that the device is charged in advanced according to the associated protocol.
- If Credo (http://www.pelicanbiothermal.com/products/credo) or other shipper containing internal refrigerant is used, please ensure the shipper is charged in advance according to the associated procedures. Credo shippers eliminate the need to purchase/use refrigerant such as dry ice and ice packs due to self-contained refrigerant. They are available for controlled ambient, refrigerated and frozen shipment (-20°C and -50°C) for durations ranging up to seven days. See appendix for picture of Credo.

b. Prepare the H3A Shipment Manifest. The biospecimen list and quality control portions of the manifest may be either printed directly from the LIMS system or by creating the table in Microsoft Excel or Word. Ensure that all elements listed in the template are included as required. It also acceptable to complete c (below) first and create the manifest according to the order samples were pulled and arranged.

c. Pull the samples intended to be shipped and arrange them according to the H3A Shipment Manifest created above in the appropriate sample box, etc.

d. Replace the sample box/container back to storage until shipment as appropriate.

e. Replace any remaining samples, not being shipped, to storage.

6.3.3 At least one day before the shipment

a. Complete H3A Shipment Checklist Form, Shippers Waybill and Customs invoice (to provide contact information and to declare nature of contents to customs and regulatory agencies).

b. Prepare Shippers declaration as required. Dry ice is a Class 9 dangerous good, and requires completion of a shipper's declaration.

c. Print at least three copies of the H3A Shipment Manifest Form (one for your records, one to place inside the shipping box and one to place in the pouch attached to the outer shipping box).

d. Physically verify that the samples match the request and that arrangement and associated elements in the Shipment Manifest match the pulled vials. Where barcodes and scanner are available samples may be verified by scanning. If discrepancies are found correct them and ensure manifest is updated as required.

e. Verify that all shipping information, contacts and required documents are accurate and complete. Note: It is optimal to specify to whose attention the shipment is being delivered to. This measure should prevent the shipment from arriving and being delayed in the receiving department.

6.3.4 Shipment Day

a. Retrieve samples from storage and keep frozen on dry ice, cold on gel packs or at room temperature on the bench until packaged according to the appropriate shipment temperature

b. Use appropriate safety procedures when handling dry ice or when retrieving samples from liquid nitrogen containers.

c. Document sample retrieval in database and complete shipping log or equivalent document where incoming and outgoing shipments are recorded according to established procedure. The following information should be included:

- Waybill number for tracking package
- Address and contact information for the Recipient / source
- Date received or shipped
- Courier name and contact information
- Sample description/manifest
- Quantity shipped
- Researchers name
- Study name
- Confirmation of delivery

d. Package samples according to IATA shipping regulations.

- Contact labels for both shipper and consignee are required. Please ensure that the person indicated as the consignee provides a

telephone number (to be included in the label) that is reachable 24 hours per day until delivery. If phone service is unreliable provide an alternative number in case the primary number is inoperable.

- Ensure shipping category labels are attached as required. Note DNA and dried blood spots are not considered dangerous goods and do not have required markings except for contact details unless shipped in a box with other biospecimen, dry ice or liquid nitrogen. (See Appendix for examples of labels/markings)
- Triple packaging system required for Category and Category B biospecimen are referred to below (ex STP…) and pictures are also included in the appendix. Triple packaging is not required for DNA or dried blood spots; although, it is recommended if available. For more details see https://www.saftpak.com/ . STP and similar products are also available through international vendors such as www.fishersci.com and www.vwr.com

e. If microtubes are being shipped, place each freezer box to be shipped into a separate sealable bag/Tyvek envelop packaging system (shown below as STP 710 & STP 711 or equivalent).

- Include a white absorbent strip, (ex. STP 152 or equivalent) in each polybag (ex. STP 711 or equivalent) containing a freezer box.
- Try to squeeze out all access air before removing the tape and sealing the bag because if there is too much air, this pouch won't fit properly into the outer pouch (STP 710 or equivalent).
- Place each plastic biohazard pouch containing a freezer box into a white pouch, STP 710 or equivalent (shown below underneath the clear pouch). Again, try to remove all access air before sealing the pouch.
- In the space indicated on the white pouch, STP 710 or equivalent, please write the plate number, freezer box letter or number (Box A or Box 1…) and a detailed list of what's included in that particular freezer box. For instance 36 tubes containing ~2ml of HIV positive plasma)

f. If vacutainers or serologic tubes are being sent, Place the blood tubes in the slots of the bubble pouch (STP 600 or equivalent, STP 109 or equivalent, or STP 110 or equivalent).

- Roll each pouch separately and place it in the Air or Sea BioJar, STP-104/ STP-104R or equivalent reusable secondary vessels, or polybag/Tyvek envelope system as described above (ex. STP 710/711 or equivalent according to number of samples).

g. Place temperature indicators and/or in the area where biospecimen are stored according to the corresponding protocol. Note: Please ensure the monitoring device has been properly activated prior to sealing the shipping box.

6.3.5 Contact (call or e-mail) consignee to provide them with Waybill number and inform them that package has been shipped. Give them an estimated delivery time so that they can anticipate arrival of the sample. Also, attach an electronic copy of the Shipment Manifest in the email unless there is an automated system of data transfer.

6.3.6 Both parties should Track delivery (using the online tracking capability of the courier) to monitor shipment and expedite sample if delayed by Customs or regulatory agencies.

6.3.7 Timing of shipping (to prevent delays in-transit):

a. Schedule pick-up early in the day so that the package goes out on the earliest flight available.

b. Schedule pick-up for early in the week (Monday or Tuesday) to prevent delays in shipment or delivery due to the weekend schedules. Early pick up schedule will vary according to country guidelines.

c. Do not ship just before a holiday long weekend as it usually translates into delays in transit.

d. Be aware of public holidays in the province or country of destination to plan for optimal shipping dates.

6.4 Test Shipment

In some situations, especially for extremely precious samples or when shipping to a new destination, sites may choose to send a test shipment with approximate characteristics of the actual shipment. This process may identify potential obstacles that could arise. It allows for corrective actions to be implemented, thus ensuring more successful shipment.

8.0 APPLICABLE REFERENCES, REGULATIONS AND GUIDELINES

8.1 Canadian Tumour Repository Network: SOP on Sample Shipping and Transportation (09.001, ve2.0)
http://www.ctrnet.ca/operating-procedures
8.2 Declaration of Helsinki
http://www.wma.net/en/30publications/10policies/b3/index.html
8.3 Tri-Council Policy Statement 2; Ethical Conduct for Research Involving Humans; Medical Research Council of Canada; Natural Sciences and Engineering Council of Canada; Social Sciences and Humanities Research Council of Canada, December 2010.
http://www.pre.ethics.gc.ca/eng/policy-politique/initiatives/tcps2-eptc2/Default/
8.4 Human Tissue and Biological Samples for use in Research. Operational and Ethical Guidelines. Medical Research Council Ethics
http://www.mrc.ac.uk/Utilities/Documentrecord/index.htm?d=MRC002420
8.5 International Air Transport Association (IATA)
http://www.iata.org/Pages/default.aspx

8.6 Best Practices for Repositories I. Collection, Storage and Retrieval of Human Biological Materials for Research. International Society for Biological and Environmental Repositories (ISBER).
http://www.isber.org/Search/search.asp?zoom_query=best+practices+for+repositories
8.7 US National Biospecimen Network Blueprint
http://biospecimens.cancer.gov/resources/publications/reports/nbn.asp
8.8 National Bioethics Advisory Commission: Research involving human biological materials: Ethical issues and policy guidance, Vol. I: Report and recommendations of the National Bioethics Advisory Committee. August 1999.
http://bioethics.georgetown.edu/nbac/hbm.pdf
8.9 Qualman, SJ. et al. Establishing a tumour bank: banking, informatics and ethics. Br. J. Cancer (2004). 90-1115-1119.
8.10 L.D. Gray and J.W. Snyder, (2006) Practical guidance to facilitate compliance with current international regulations that govern the packing and shipping of dangerous goods. Chapter 21 in Biological Safety, Principles and Practice, 4th edition, ed. D.O. Fleming and D.L. Hunt.

9.0 MISCELANEOUS

9.1 Depiction of Shipping Supplies

250mL Absorbant (STP-152)

Polybag and Tyvek envelope (STP-710 & STP-711)

STP-600

STP-109 & 110

STP-104

STP-104R

Labels

STP-106

UN3373 (category B)

Category B

Class 9 Hazard (Dry Ice)

Dry Ice

Credo Shipper

10.0 REVISION HISTORY

SOP number	Date revised	Author	Summary of Revisions

11.0 APPENDECIS

A. Biospecimen Shipping & Transport Appendix A: Shipping Roadmap
B. Biospecimen Shipping & Transport Appendix B: Shipment Notification Instructions

Appendix C

PARTICIPANT INFORMATION LEAFLET AND CONSENT FORM

TITLE OF THE RESEARCH PROJECT:
Long-term storage of Urine at -80 degrees

Study Title for Study Participants: Collecting Urine Sample Donations for Research

PRINCIPAL INVESTIGATOR: Miss Jody Bell/Dr. Carmen Swanepoel

ADDRESS:
Division of Haematology, Faculty of Health Sciences and NHLS Tygerberg, Tyerberg Hospital

CONTACT NUMBER: [redacted]

You are being asked to donate urine for future research. Please take some time to read the information presented here, which will explain the details of this project. Please ask the study staff or doctor any questions about any part of this project that you do not fully understand. It is very important that you are fully satisfied that you clearly understand what this research entails and how you could be involved. Also, your participation is **entirely voluntary** and you are free to decline to participate. If you say no, this will not affect you negatively in any way whatsoever. You are also free to withdraw from the study at any point, even if you do agree to take part.

This project has been approved by the **Health Research Ethics Committee at Stellenbosch University** and will be conducted according to the ethical guidelines and principles of the international Declaration of Helsinki, South African Guidelines for Good Clinical Practice and the Medical Research Council (MRC) Ethical Guidelines for Research.

What is the National Health Laboratory Services/Stellenbosch University Biobank (NSB)

The NSB is a biorepository (biobank) that collects human biological specimens (samples) from persons. The NSB stores the samples and some of the donor's medical information for use by researchers in future research studies.

The NSB has an independent research panel that approves researchers' requests to use the NSB's stored samples for research studies. The NSB only gives samples and medical information to researchers after their projects have been approved. This information may help us learn more about the causes of disease and to develop better ways to screen, diagnose, and treat them.

What is this research project all about?

- The purpose of this study is to collect samples for the NSB for future research studies. Researchers may study samples from the NSB in combination with hundreds or thousands of other samples to explore how biologic or genetic factors may be related to diseases. The information might help doctors in the future to identify who will or will not benefit from treatment. The samples may be used to learn more about how HIV-related diseases and cancers develop. The samples may also lead to new tests or discoveries. Finally, researchers may use the samples to study the genetic material from your biospecimen and compare it to the material from your normal tissue (blood) to try to find the differences that exist. These studies could make it possible to identify many of the changes that are associated with diseases such as cancers. It may also help us tailor treatments to a patient's unique genetic make-up and/or to the genetic markers of the tumors.
- Your participation in this study is voluntary and not participating will not prevent you from care to which you are otherwise entitled.
- Samples can be obtained from patients in South Africa and from other Africa countries. A larger number of specimens available to researchers will help them to do better and more research, and there is not a limit to the total number of participants and specimens that can be collected.

What tests and procedures will I have if I take part in this project?

- If you agree to donate blood, the medical team will draw about 2 tablespoons of blood to give to the NSB. This takes about 10 minutes.
- If you agree to donate tissue, your leftover tissue biopsy material will be donated to and stored by the NSB.
- If you agree to donate urine, a collection container will be given to you with instructions on how to collect. The container will be donated to and stored by the NSB.
- Some of your clinical information will be released to the NSB and entered into their database. The information given to the NSB will not include your name or any information that could personally identify you.
- Normally no extra biopsies will be collected for the NSB. We will only give the NSB tissue that is left over after making decisions about your treatment or diagnosis.
- You may donate an optional biopsy for the NSB if you voluntarily agree to do so. It is your choice whether you would like to donate additional biospecimen to the NSB. You can still donate other samples to the NSB if you do not agree to this optional tissue donation.

Why have you been invited to participate?

- You are being asked to donate your urine to the NSB as the research study would like to see what freezing effects have on the sample. The NSB collects donated samples from any persons who are willing to donate their biospecimens for research studies.

What will your responsibilities be?

- You will not have any further responsibilities following the donation of urine.

Will you benefit from taking part in this research?

- This study is unlikely to help you. This study may help us learn things that may help people in the future.
- The information may help to identify those who are at increased risk and those who may benefit from targeted treatment and screening. In turn, these studies could help find ways to prevent or improve treatments for other diseases.
- We cannot tell you right now what future research these samples would be used for. Instead, we are asking that you give approval to give your samples for future testing without contacting you again. The results of whatever research is done on your samples will *not* be told to you or your doctor.

Are there in risks involved in your taking part in this research?

- Urine collection: The risks of donating urine comes with no pain or risk.
- Confidentiality: The NSB will receive study samples with code numbers. There will be no personal identifiers on the samples. The samples will be re-labeled with a barcode and stored for future testing. While the NSB and researchers who study NSB samples will have no information that could identify you, there is a risk that someone could use information from genetic studies to trace your samples back to you. The researchers believe the chance that someone will identify you is very small, but the risk may change in the future as people come up with new ways of tracing information. In some cases, this information could be used to make it harder for you to get or keep a job. There are laws against misuse of genetic information, but they may not give full protection. The researchers believe the chance these things will happen is very small, but cannot promise that they will not occur.
- Let your doctor know of any questions you have about these possible risks. You can ask your doctor questions about side effects at any time or you can contact the NSB to answer any questions that you may have.

If you do not agree to take part, what alternatives do you have?

- It is your choice to donate or not donate your urine. You will still receive the same clinical care and treatment if you choose not to donate urine samples to the NSB. You may also choose to donate:
  - Blood and or other biospecimens type

How long will the NSB keep my samples?

Your urine sample will be stored until it is used for research. The samples may be stored indefinitely.

Who will have access to your medical records?

- Your privacy is very important to us and the researchers will make every effort to protect it. Your information may be given out if required by law. For example, certain diseases require doctors to report to health authorities if they find a disease like tuberculosis (notifiable diseases). However, the researchers will do their best to make sure that any information that is released will not identify you.
- Some of your health information, and/or information about your specimen that you have donated to the NSB will be kept in a central database for research. Your name or contact information will not be put in the database.
- Only qualified researchers who participate in ethical approved research projects that meet the requirements of the NSB will be allowed to access your information and specimens.
- To protect your privacy, NSB do not keep identifying information that links participants to specific samples. As a result, the NSB will not be able to link the results from studies that use your samples back to you. Thus, information, including genetic information, that researchers may obtain in studies that use your samples may not be directly linked to you and will not be placed in your medical record.
- Your identity will never be published or used in any publications or research assignments or theses.

Consent to disclose medical conditions:

- Your privacy is very important to us and the researchers will make every effort to protect it.
- For this research study, we are looking at the effects of freezing on certain compounds in the urine. It would be beneficial for us to understand what is happening if you can disclose some information about yourself.
- This information will be kept in a central database for research. Your name or contact information associated will not be put in the database

What will happen in the unlikely event of some form injury occurring as a direct result of your taking part in this research study?

- There is no significant risk for injury in donating specimens to the NSB.

Will you be paid to take part in this study and are there any costs involved?

- There will be no cost to you for donating your samples to the NSB. You will not be paid for donating any specimens.

What are my rights in this study?

- Taking part in this study is your choice. No matter what decision you make, and even if your decision changes, there will be no penalty to you. You will not lose medical care or any legal rights.
- For questions about your rights while in this study, call the Stellenbosch University Health Research Ethics Committee at +27 21 938 9677 or the NSB at (telephone number and contact detail to be inserted).
- You are free to withdraw from the study at any point, even if you do agree to take part .If you decide that you no longer want your sample stored in the biobank, let us know. We can remove and destroy your sample. A proof of destruction certification can be requested. Alternatively it could remain in the biobank in a completely non-identifiable form. However it should be note that data already obtained from your samples might not be withdrawn since it may already have been used.

Is there anything else that you should know or do?

- You can contact [redacted] if you have any further queries or encounter any problems.
- You can contact the Health Research Ethics Committee at 021-938 9207 if you have any concerns or complaints that have not been adequately addressed by your doctor.
- You will receive a copy of this information and consent form for your own records.

Declaration by participant

By signing below, I …….. agree to take part in a research project entitled **Collecting Urine Sample Donations for Research**

Please circle your answer to show whether or not you would like to take part in the research study:

1. **I agree to donate my urine to the NSB for future research that may be used to learn about, prevent, and diagnose diseases**

   YES NO

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ……. on (*date*) ……20.....

…… ……

Signature of participant **Signature of witness**

Declaration by investigator

I *(name)* ……..…… declare that:

- I explained the information in this document to ……..
- I encouraged him/her to ask questions and took adequate time to answer them.
- I am satisfied that he/she adequately understands all aspects of the research, as discussed above
- I did/did not use a interpreter. (*If a interpreter is used then the interpreter must sign the declaration below*.

Signed at (*place*) ……..…… on (*date*) ……..……..20…..

…… ……

Signature of investigator **Signature of witness**

Declaration by interpreter

I *(name)* ……..…… declare that:

- I assisted the investigator (*name*) …… to explain the information in this document to (*name of participant*) ……..…….. using the language medium of Afrikaans/Xhosa/…….. (enter appropriate language).

- We encouraged him/her to ask questions and took adequate time to answer them.
- I conveyed a factually correct version of what was related to me.
- I am satisfied that the participant fully understands the content of this informed consent document and has had all his/her question satisfactorily answered.

Signed at (*place*) ……..…… on (*date*) ……..……..

…… ……

Signature of interpreter **Signature of witness**

CONSENT FORM FOR MEDICAL CONDITIONS

TITLE OF THE RESEARCH PROJECT:
Long-term storage of Urine at -80 degrees

Study Title for Study Participants: Collecting Urine Sample Donations for Research

Consent to disclose medical conditions:

- Your privacy is very important to us and the researchers will make every effort to protect it.
- For this research study, we are looking at the effects of freezing on certain compounds in the urine. It would be beneficial for us to understand what is happening if you can disclose some information about yourself.
- This information will be kept in a central database for research. Your name or contact information associated will not be put in the database

Declaration by participant

By signing below, I …… agree to take part in a research project entitled **Collecting Urine Sample Donations for Research**

1. I agree to disclose my medical conditions:

YES NO

Please circle your answer to show whether or not you have a certain medical condition:

1.	Hypertension/High Blood Pressure……	YES	NO
2.	Diabetes……	YES	NO
3.	I am currently pregnant……	YES	NO
4.	Kidney Disease……	YES	NO
5.	Heart Disease……	YES	NO

I declare that:

- I have read or had read to me this information and consent form and it is written in a language with which I am fluent and comfortable.
- I have had a chance to ask questions and all my questions have been adequately answered.
- I understand that taking part in this study is **voluntary** and I have not been pressurised to take part.
- I may choose to leave the study at any time and will not be penalised or prejudiced in any way.
- I may be asked to leave the study before it has finished, if the study doctor or researcher feels it is in my best interests, or if I do not follow the study plan, as agreed to.

Signed at (*place*) ……. on (*date*) ……20…..

…… ……
Signature of participant **Signature of witness**

Appendix D

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Creatinine plus ver.2

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
03263991 190	Creatinine plus ver.2 250 tests	System-ID 07 6612 7	Roche/Hitachi **cobas c** 311, **cobas c** 501/502
10759350 190	Calibrator f.a.s. (12 x 3 mL)	Code 401	
10759350 360	Calibrator f.a.s. (12 x 3 mL, for USA)	Code 401	
12149435 122	Precinorm U plus (10 x 3 mL)	Code 300	
12149435 160	Precinorm U plus (10 x 3 mL, for USA)	Code 300	
12149443 122	Precipath U plus (10 x 3 mL)	Code 301	
12149443 160	Precipath U plus (10 x 3 mL, for USA)	Code 301	
10171743 122	Precinorm U (20 x 5 mL)	Code 300	
10171735 122	Precinorm U (4 x 5 mL)	Code 300	
10171778 122	Precipath U (20 x 5 mL)	Code 301	
10171760 122	Precipath U (4 x 5 mL)	Code 301	
03121313 122	Precinorm PUC (4 x 3 mL)	Code 240	
03121291 122	Precipath PUC (4 x 3 mL)	Code 241	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05947626 160	PreciControl ClinChem Multi 1 (4 x 5 mL, for USA)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
05947774 160	PreciControl ClinChem Multi 2 (4 x 5 mL, for USA)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

cobas c 311/501 analyzers

CREA2: ACN 452 (serum, plasma, urine)

cobas c 502 analyzer

CREA2: ACN 8452 (serum, plasma)

CRE2U: ACN 8152 (urine)

Intended use

In vitro test for the quantitative determination of creatinine concentration in human serum, plasma and urine on Roche/Hitachi **cobas c** systems.

Summary[1,2,3,4,5]

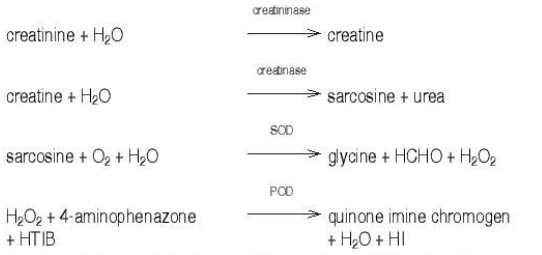
Chronic kidney disease is a worldwide problem that carries a substantial risk for cardiovascular morbidity and death. Current guidelines define chronic kidney disease as kidney damage or glomerular filtration rate (GFR) less than 60 mL/min per 1.73 m^2 for three months or more, regardless of cause. The assay of creatinine in serum or plasma is the most commonly used test to assess renal function. Creatinine is a break-down product of creatine phosphate in muscle, and is usually produced at a fairly constant rate by the body (depending on muscle mass). It is freely filtered by the glomeruli and, under normal conditions, is not re-absorbed by the tubules to any appreciable extent. A small but significant amount is also actively secreted.

Since a rise in blood creatinine is observed only with marked damage of the nephrons, it is not suited to detect early stage kidney disease. A considerably more sensitive test and better estimation of glomerular filtration rate (GFR) is given by the creatinine clearance test based on creatinine's concentration in urine and serum or plasma, and urine flow rate. For this test a precisely timed urine collection (usually 24 hours) and a blood sample are needed. However, since this test is prone to error due to the inconvenient collection of timed urine, mathematical attempts to estimate GFR based only on the creatinine concentration in serum or plasma have been made. Among the various approaches suggested, two have found wide recognition: that of Cockroft and Gault and that based on the results of the MDRD trial. While the first equation was derived from data obtained with the conventional Jaffé method, a newer version of the second is usable for IDMS-traceable creatinine methods. Both are applicable for adults. In children, the Bedside Schwartz formula should be used.[6,7,8,9]

In addition to the diagnosis and treatment of renal disease, the monitoring of renal dialysis, creatinine measurements are used for the calculation of the fractional excretion of other urine analytes (e. g., albumin, α-amylase). Numerous methods were described for determining creatinine. Automated assays established in the routine laboratory include the Jaffé alkaline picrate method in various modifications, as well as enzymatic tests.

Test principle

This enzymatic method is based on the conversion of creatinine with the aid of creatininase, creatinase, and sarcosine oxidase to glycine, formaldehyde and hydrogen peroxide. Catalyzed by peroxidase the liberated hydrogen peroxide reacts with 4-aminophenazone and HTIB[a] to form a quinone imine chromogen. The color intensity of the quinone imine chromogen formed is directly proportional to the creatinine concentration in the reaction mixture.



Creatine of the sample is destroyed by creatinase, SOD and catalase during incubation in R1.

a) 2,4,6-triiodo-3-hydroxybenzoic acid

Reagents - working solutions

R1 TAPS buffer (N-Tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid): 30 mmol/L, pH 8.1; creatinase (microorganisms): ≥ 332 µkat/L; sarcosine oxidase (microorganisms): ≥ 132 µkat/L; ascorbate oxidase (microorganisms): ≥ 33 µkat/L; catalase (microorganisms): ≥ 1.67 µkat/L; HTIB: 1.2 g/L; detergents; preservative

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R3 TAPS buffer: 50 mmol/L, pH 8.0; creatininase (microorganisms): ≥ 498 µkat/L; peroxidase (horseradish): ≥ 16.6 µkat/L; 4-aminophenazone: 0.5 g/L; potassium hexacyanoferrate (II): 60 mg/L; detergent; preservative

R1 is in position B and R3 is in position C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

For USA: For prescription use only.

Reagent handling

Ready for use

Storage and stability

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Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	8 weeks

Diluent NaCl 9 %

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.
Serum.
Plasma: Li-heparin and K_2-EDTA plasma

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Urine: Collect urine without using additives. If urine must be collected with a preservative for other analytes, only hydrochloric acid (14 to 47 mmol/L urine, e.g. 5 mL 10 % HCl or 5 mL 30 % HCl per liter urine) or boric acid (81 mmol/L, e.g. 5 g per liter urine) may be used.

Stability in ***serum/plasma***:[10]	7 days at 15-25 °C
	7 days at 2-8 °C
	3 months at (-15)-(-25) °C
Stability in ***urine*** (without preservative):[10]	2 days at 15-25 °C
	6 days at 2-8 °C
	6 months at (-15)-(-25) °C
Stability in ***urine*** (with preservative):[11]	3 days at 15-25 °C
	8 days at 2-8 °C
	3 weeks at (-15)-(-25) °C

Centrifuge samples containing precipitates before performing the assay.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- See "Order information" section
- General laboratory equipment

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma

cobas c 311 test definition

Assay type	2-Point End		
Reaction time / Assay points	10 / 25-57		
Wavelength (sub/main)	700/546 nm		
Reaction direction	Increase		
Units	µmol/L (mg/dL, mmol/L)		
Reagent pipetting		Diluent (H_2O)	
R1	77 µL	–	
R3	38 µL	–	
Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	5 µL	15 µL	135 µL
Increased	2 µL	–	–

cobas c 501 test definition

Assay type	2-Point End		
Reaction time / Assay points	10 / 37-70		
Wavelength (sub/main)	700/546 nm		
Reaction direction	Increase		
Units	µmol/L (mg/dL, mmol/L)		
Reagent pipetting		Diluent (H_2O)	
R1	77 µL	–	
R3	38 µL	–	

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	5 µL	15 µL	135 µL
Increased	2 µL	–	–

cobas c 502 test definition

Assay type	2-Point End		
Reaction time / Assay points	10 / 37-70		
Wavelength (sub/main)	700/546 nm		
Reaction direction	Increase		
Units	µmol/L (mg/dL, mmol/L)		
Reagent pipetting		Diluent (H_2O)	
R1	77 µL	–	
R3	38 µL	–	

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	5 µL	15 µL	135 µL

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Increased	4 µL	–	–

Application for urine

cobas c 311 test definition

Assay type	2-Point End
Reaction time / Assay points	10/25-57
Wavelength (sub/main)	700/546 nm
Reaction direction	Increase
Units	µmol/L (mg/dL, mmol/L)
Reagent pipetting	Diluent (H_2O)
R1	77 µL –
R3	38 µL –

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	5 µL	3 µL	147 µL
Decreased	2 µL	3 µL	147 µL
Increased	5 µL	3 µL	147 µL

cobas c 501 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 37-70
Wavelength (sub/main)	700/546 nm
Reaction direction	Increase
Units	µmol/L (mg/dL, mmol/L)
Reagent pipetting	Diluent (H_2O)
R1	77 µL –
R3	38 µL –

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	5 µL	3 µL	147 µL
Decreased	2 µL	3 µL	147 µL
Increased	5 µL	3 µL	147 µL

cobas c 502 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 37-70
Wavelength (sub/main)	700/546 nm
Reaction direction	Increase
Units	µmol/L (mg/dL, mmol/L)
Reagent pipetting	Diluent (H_2O)
R1	77 µL –
R3	38 µL –

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	5 µL	3 µL	147 µL
Decreased	2 µL	3 µL	147 µL
Increased	10 µL	3 µL	147 µL

Calibration

Calibrators	S1: H_2O S2: C.f.a.s.
Calibration mode	Linear
Calibration frequency	Blank calibration • after 4 weeks during shelf life 2-point calibration • after reagent lot change • as required following quality control procedures

Traceability: This method has been standardized against ID/MS.

Quality control

Serum/plasma

For quality control use undiluted Precinorm U, Precipath U, PreciControl ClinChem Multi 1 and PreciControl ClinChem Multi 2 as listed above.

In addition, other suitable control material can be used.

Urine

For quality control use Precinorm PUC and Precipath PUC as listed above.

In addition, other suitable control material can be used.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors:	µmol/L **x** 0.0113 = mg/dL
	µmol/L **x** 0.001 = mmol/L

Limitations - interference

Criterion: Recovery within ± 10 % of initial values at creatinine concentrations of 80 µmol/L (0.9 mg/dL) in serum and 2500 µmol/L (28.3 mg/dL) in urine.

Serum/plasma

Icterus:[12] No significant interference up to an I index of 15 for conjugated bilirubin and 20 for unconjugated bilirubin (approximate conjugated bilirubin concentration: 257 µmol/L or 15 mg/dL; approximate unconjugated bilirubin concentration: 342 µmol/L or 20 mg/dL).

Hemolysis:[12] No significant interference up to an H index of 800 (approximate hemoglobin concentration: 497 µmol/L or 800 mg/dL).

Lipemia (Intralipid):[12] No significant interference up to an L index of 2000. There is a poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Ascorbic acid: < 1.70 mmol/L or < 300 mg/L does not interfere.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[13,14]

Exceptions: Rifampicin, Levodopa and Calcium dobesilate (e.g. Dexium) cause artificially low creatinine results. Dicynone (Etamsylate) at therapeutic concentrations may lead to falsely low results.[15]

N-ethylglycine at therapeutic concentrations and DL-proline at concentrations ≥ 1 mmol/L (≥ 115 mg/L) give falsely high results.

No significant interference up to a creatine level of 4 mmol/L (524 mg/L).

Hemolyzed samples from neonates, infants or adults with HbF values ≥ 600 mg/dL interfere with the test.[16]

2-Phenyl-1,3-indandion (Phenindion) at therapeutic concentrations interferes with the assay.

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.[17]

Estimation of the glomerular filtration rate (GFR) on the basis of the Schwartz formula can lead to an overestimation.[18]

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Acetaminophen intoxications are frequently treated with N-Acetylcysteine. N-Acetylcysteine at a plasma concentration above 333 mg/L and the Acetaminophen metabolite N-acetyl-p-benzoquinone imine (NAPQI) independently may cause falsely low results.

Venipuncture should be performed prior to the administration of Metamizole. Venipuncture immediately after or during the administration of Metamizole may lead to falsely low results. A significant interference may occur at any plasma Metamizole concentration.

Urine

Icterus: No significant interference up to an approximate conjugated bilirubin concentration of 1197 µmol/L (70 mg/dL).

Hemolysis: No significant interference up to an approximate hemoglobin concentration of 621 µmol/L (1000 mg/dL).

Ascorbic acid < 22.7 mmol/L (< 4000 mg/L), glucose < 120 mmol/L (< 2162 mg/dL) and urobilinogen < 676 µmol/L (< 40 mg/dL) do not interfere.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[14]

Exceptions: Calcium dobesilate (e.g. Dexium), Levodopa and α-methyldopa cause artificially low creatinine results. Dicynone (Etamsylate) at therapeutic concentrations may lead to falsely low results.

High homogentisic acid concentrations in urine samples lead to false results.

Acetaminophen, Acetylcysteine and Metamizole are metabolized quickly. Therefore, interference from these substances is unlikely but cannot be excluded.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED
Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. The latest version of the carry-over evasion list can be found with the NaOHD-SMS-SmpCln1+2-SCCS Method Sheets. For further instructions refer to the operator's manual. **cobas c** 502 analyzer: All special wash programming necessary for avoiding carry-over is available via the **cobas** link, manual input is not required.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range

Serum/plasma

5-2700 µmol/L (0.06-30.5 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:4 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 4.

Urine

100-54000 µmol/L (1.1-610 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:2.5 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 2.5.

Lower limits of measurement

Lower detection limit of the test

Serum/plasma

5 µmol/L (0.06 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying 3 standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Urine

100 µmol/L (1.1 mg/dL)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying 3 standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Expected values

Serum/plasma

Adults[19]		
Females	45-84 µmol/L	(0.51-0.95 mg/dL)
Males	59-104 µmol/L	(0.67-1.17 mg/dL)
Children[20]		
Neonates (premature)	29-87 µmol/L	(0.33-0.98 mg/dL)
Neonates (full term)	27-77 µmol/L	(0.31-0.88 mg/dL)
2-12 m	14-34 µmol/L	(0.16-0.39 mg/dL)
1-< 3 y	15-31 µmol/L	(0.18-0.35 mg/dL)
3-< 5 y	23-37 µmol/L	(0.26-0.42 mg/dL)
5-< 7 y	25-42 µmol/L	(0.29-0.47 mg/dL)
7-< 9 y	30-47 µmol/L	(0.34-0.53 mg/dL)
9-< 11 y	29-56 µmol/L	(0.33-0.64 mg/dL)
11-< 13 y	39-60 µmol/L	(0.44-0.68 mg/dL)
13-< 15 y	40-68 µmol/L	(0.46-0.77 mg/dL)

Urine

1st morning urine[19]		
Females	2.55-20.0 mmol/L	(29-226 mg/dL)
Males	3.54-24.6 mmol/L	(40-278 mg/dL)
24-hour urine[21]		
Females	6-13 mmol/24 h	(720-1510 mg/24 h)
Males	9-19 mmol/24 h	(980-2200 mg/24 h)
Creatinine clearance[21]	66-143 mL/min	

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Roche has not evaluated reference ranges in a pediatric population.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol. ***Serum/plasma:*** repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). ***Urine:*** repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 10 days). The following results were obtained:

Serum/plasma

Repeatability	*Mean*	*SD*	*CV*
	µmol/L (mg/dL)	*µmol/L (mg/dL)*	*%*
Precinorm U	96.1 (1.08)	0.9 (0.01)	0.9
Precipath U	341 (3.85)	2 (0.02)	0.6
Human serum 1	191 (2.16)	2 (0.02)	1.1
Human serum 2	398 (4.50)	4 (0.05)	1.0
Intermediate precision	*Mean*	*SD*	*CV*
	µmol/L (mg/dL)	*µmol/L (mg/dL)*	*%*
Precinorm U	94.9 (1.07)	1.4 (0.02)	1.4
Precipath U	338 (3.82)	4 (0.05)	1.1
Human serum 3	190 (2.15)	2 (0.02)	1.1
Human serum 4	395 (4.46)	5 (0.06)	1.2

Urine

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Repeatability	*Mean*	*SD*	*CV*
	µmol/L (mg/dL)	*µmol/L (mg/dL)*	*%*
Control Level 1	7280 (82.3)	92 (1.0)	1.3
Control Level 2	14031 (159)	179 (2)	1.3
Human urine 1	17289 (195)	237 (3)	1.4
Human urine 2	7035 (79.5)	68 (0.8)	1.0

Intermediate precision	*Mean*	*SD*	*CV*
	µmol/L (mg/dL)	*µmol/L (mg/dL)*	*%*
Control Level 1	7219 (81.6)	112 (1.3)	1.5
Control Level 2	14018 (158)	212 (2)	1.5
Human urine 3	17326 (196)	244 (3)	1.4
Human urine 4	7008 (79.2)	104 (1.2)	1.5

Method comparison

Creatinine values for human serum, plasma and urine samples obtained on a Roche/Hitachi **cobas c** 501 analyzer (y) were compared with those determined using the corresponding reagent on a Roche/Hitachi 917 analyzer (x).

Serum/plasma

Sample size (n) = 63

Passing/Bablok[22]	Linear regression
y = 1.002x - 0.434 µmol/L	y = 0.991x + 2.94 µmol/L
τ = 0.978	r = 1.000

The sample concentrations were between 49 and 1891 µmol/L (0.55 and 21.4 mg/dL).

Urine

Sample size (n) = 75

Passing/Bablok[22]	Linear regression
y = 0.985x + 21.3 µmol/L	y = 0.977x + 80.0 µmol/L
τ = 0.990	r = 1.000

The sample concentrations were between 438 and 52577 µmol/L (4.95 and 594 mg/dL).

References

1 Thomas C, Thomas L. Labordiagnostik von Erkrankungen der Nieren und ableitenden Harnwege. In: Thomas L, ed. Labor und Diagnose, 6th ed. Frankfurt/Main: TH-Books 2005;520-585.

2 Lamb E, Newman DJ, Price CP. Kidney function tests In: Burtis CA, Ashwood ER, Bruns DE. Tietz textbook of clinical chemistry and molecular diagnostics. 4th ed. St.Louis, MO: Elsevier Saunders 2006;797-835.

3 http://www.kidney.org/

4 http://www.nkdep.nih.gov/

5 Lamb EJ, Tomson CRV, Roderick PJ. Estimating kidney function in adults using formulae. Ann Clin Biochem 2005;42:321-345.

6 Miller WG. Editorial on Estimating glomerular filtration rate. Clin Chem Lab Med 2009;47(9):1017-1019.

7 Schwartz GJ, Muñoz A, Schneider MF, et al. New Equations to Estimate GFR in Children with CKD. J Am Soc Nephrol 2009;20:629-637.

8 Schwartz GJ, Work DF. Measurement and Estimation of GFR in Children and Adolescents. Clin J Am Soc Nephrol 2009;4:1832–1843.

9 Staples A, LeBlond R, Watkins S, et al. Validation of the revised Schwartz estimating equation in a predominantly non-CKD population. Pediatr Nephrol 2010 Jul 22;25:2321-2326.

10 Guder W, Fonseca-Wollheim W, Ehret W, et al. Die Qualität Diagnostischer Proben, 6. Aufl. Heidelberg: BD Diagnostics, 2009.

11 Data on file at Roche Diagnostics.

12 Glick MR, Ryder KW, Jackson SA. Graphical Comparisons of Interferences in Clinical Chemistry Instrumentation. Clin Chem 1986;32:470-475.

13 Breuer J. Report on the Symposium "Drug effects in Clinical Chemistry Methods". Eur J Clin Chem Clin Biochem 1996;34:385-386.

14 Sonntag O, Scholer A. Drug interference in clinical chemistry: recommendation of drugs and their concentrations to be used in drug interference studies. Ann Clin Biochem 2001;38:376-385.

15 Dastych M, Wiewiorka O, Benovska M. Ethamsylate (Dicynone) Interference in Determination of Serum Creatinine, Uric Acid, Triglycerides, and Cholesterol in Assays Involving the Trinder Reaction; In Vivo and In Vitro. Clin Lab 2014;60:1373-1376.

16 Mazzachi BC, Phillips JW, Peake MJ. Is the Jaffe creatinine assay suitable for neonates? Clin Biochem Revs 1998;19:82.

17 Bakker AJ, Mücke M. Gammopathy interference in clinical chemistry assays: mechanisms, detection and prevention. Clin Chem Lab Med 2007;45(9):1240-1243.

18 Filler G, Priem F, Lepage N, et al. β-Trace Protein, Cystatin C, β2-Microglobulin, and Creatinine Compared for Detecting Impaired Glomerular Filtration Rates in Children. Clin Chem 2002;48:729-736.

19 Mazzachi BC, Peake MJ, Ehrhardt V. Reference Range and Method Comparison Studies for Enzymatic and Jaffé Creatinine Assays in Plasma and Serum and Early Morning Urine. Clin Lab 2000;53-55.

20 Schlebusch H, Liappis N, Kalina E, et al. High Sensitive CRP and Creatinine: Reference Intervals from Infancy to Childhood. J Lab Med 2002;26:341-346.

21 Junge W, Wilke B, Halabi A, et al. Determination of reference intervals for serum creatinine, creatinine excretion and creatinine clearance with an enzymatic and a modified Jaffé method. Clin Chim Acta 2004;344:137-148.

22 Bablok W, Passing H, Bender R, et al. A general regression procedure for method transformation. Application of linear regression procedures for method comparison studies in clinical chemistry, Part III. J Clin Chem Clin Biochem 1988 Nov;26(11):783-790.

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard.

CONTENT	Contents of kit
→	Volume after reconstitution or mixing
GTIN	Global Trade Item Number

FOR US CUSTOMERS ONLY: LIMITED WARRANTY

Roche Diagnostics warrants that this product will meet the specifications stated in the labeling when used in accordance with such labeling and will be free from defects in material and workmanship until the expiration date printed on the label. THIS LIMITED WARRANTY IS IN LIEU OF ANY OTHER WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR PARTICULAR PURPOSE. IN NO EVENT SHALL ROCHE DIAGNOSTICS BE LIABLE FOR INCIDENTAL, INDIRECT, SPECIAL OR CONSEQUENTIAL DAMAGES.

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www.roche.com

Distribution in USA by:
Roche Diagnostics, Indianapolis, IN
US Customer Technical Support 1-800-428-2336

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Appendix E

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CA2

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Calcium Gen.2

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
05061482 190	Calcium Gen.2 300 tests	System-ID 07 7476 6	Roche/Hitachi **cobas c** 311, **cobas c** 501/502
10759350 190	Calibrator f.a.s. (12 x 3 mL)	Code 401	
10759350 360	Calibrator f.a.s. (12 x 3 mL, for USA)	Code 401	
12149435 122	Precinorm U plus (10 x 3 mL)	Code 300	
12149435 160	Precinorm U plus (10 x 3 mL, for USA)	Code 300	
12149443 122	Precipath U plus (10 x 3 mL)	Code 301	
12149443 160	Precipath U plus (10 x 3 mL, for USA)	Code 301	
10171743 122	Precinorm U (20 x 5 mL)	Code 300	
10171735 122	Precinorm U (4 x 5 mL)	Code 300	
10171778 122	Precipath U (20 x 5 mL)	Code 301	
10171760 122	Precipath U (4 x 5 mL)	Code 301	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05947626 160	PreciControl ClinChem Multi 1 (4 x 5 mL, for USA)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
05947774 160	PreciControl ClinChem Multi 2 (4 x 5 mL, for USA)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:

CA2: ACN 698

S-CA2: ACN 699 (STAT, reaction time: 3)

For **cobas c** 502 analyzer:

CA2: ACN 8698

S-CA2: ACN 8699 (STAT, reaction time: 3)

Intended use

In vitro test for the quantitative determination of calcium in human serum, plasma and urine on Roche/Hitachi **cobas c** systems.

Summary[1]

Calcium is the most abundant mineral element in the body with about 99 percent in the bones primarily as hydroxyapatite. The remaining calcium is distributed between the various tissues and the extracellular fluids where it performs a vital role for many life sustaining processes. Among the extra skeletal functions of calcium are involvement in blood coagulation, neuromuscular conduction, excitability of skeletal and cardiac muscle, enzyme activation, and the preservation of cell membrane integrity and permeability.

Serum calcium levels and hence the body content are controlled by parathyroid hormone (PTH), calcitonin, and vitamin D. An imbalance in any of these modulators leads to alterations of the body and serum calcium levels. Increases in serum PTH or vitamin D are usually associated with hypercalcemia. Increased serum calcium levels may also be observed in multiple myeloma and other neoplastic diseases. Hypocalcemia may be observed e.g. in hypoparathyroidism, nephrosis, and pancreatitis.

Test principle

Calcium ions react with 5-nitro-5'-methyl-BAPTA (NM-BAPTA) under alkaline conditions to form a complex. This complex reacts in the second step with EDTA.

$$Ca^{2+} + \text{NM-BAPTA} \xrightarrow{\text{alkaline pH}} \text{calcium-NM-BAPTA complex}$$

$$\text{calcium-NM-BAPTA complex} + \text{EDTA} \longrightarrow \text{NM-BAPTA} + \text{calcium EDTA complex}$$

The change in absorbance is directly proportional to the calcium concentration and is measured photometrically.

Reagents - working solutions

R1 CAPSO:[a] 557 mmol/L; NM-BAPTA: 2 mmol/L; pH 10.0; non-reactive surfactant and stabilizer

R2 EDTA: 7.5 mmol/L; pH 7.3; non-reactive surfactant, preservative

a) 3-[cyclohexylamino]-2-hydroxy-1-propanesulfonic acid

R1 is in position B and R2 is in position C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

Reagent handling

Ready for use

Storage and stability

CA2

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	6 weeks

Diluent NaCl 9 %

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.
Serum: Fresh serum collected in the fasting state is the preferred specimen.
Plasma: Li-heparin plasma.

Serum or plasma should be separated from blood cells as soon as possible, because prolonged contact with the clot may cause lower calcium values.[2] Sera from patients receiving EDTA (treatment of hypercalcemia) are unsuitable for analysis, since EDTA will chelate the calcium and render it unavailable for reaction with NM-BAPTA. Co-precipitation of calcium with

CA2

Calcium Gen.2

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fibrin (i.e. heparin plasma), lipids, or denatured protein has been reported with storage or freezing.[1,3]

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Urine

Urine specimens should be collected in acid-washed bottles. 24-hour specimens should be collected in containers containing 20-30 mL of 6 mol/L HCl to prevent calcium salt precipitation. Precipitated calcium salts may not be completely dissolved by the addition of HCl following urine collection.[4]

Stability in *serum/plasma*:[5]	7 days at 15-25 °C
	3 weeks at 2-8 °C
	8 months at (-15)-(-25) °C
Stability in *urine*:[5]	2 days at 15-25 °C
	4 days at 2-8 °C
	3 weeks at (-15)-(-25) °C

Stored serum or urine specimens must be mixed well prior to analysis.

Centrifuge samples containing precipitates before performing the assay.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- See "Order information" section

General laboratory equipment

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for serum and plasma

cobas c 311 test definition

Assay type	2-Point End	
Reaction time / Assay points	10 / 6-8 (STAT 3 / 6-8)	
Wavelength (sub/main)	376/340 nm	
Reaction direction	Decrease	
Units	mmol/L (mg/dL)	
Reagent pipetting		Diluent (H_2O)
R1	20 µL	160 µL
R2	20 µL	-

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	3 µL	–	–
Decreased	3 µL	–	–
Increased	3 µL	–	–

cobas c 501/502 test definition

Assay type	2-Point End	
Reaction time / Assay points	10 / 10-13 (STAT 3 / 10-13)	
Wavelength (sub/main)	376/340 nm	
Reaction direction	Decrease	
Units	mmol/L (mg/dL)	
Reagent pipetting		Diluent (H_2O)
R1	20 µL	160 µL
R2	20 µL	-

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	3 µL	–	–
Decreased	3 µL	–	–
Increased	3 µL	–	–

Application for urine

cobas c 311 test definition

Assay type	2-Point End	
Reaction time / Assay points	10 / 6-8 (STAT 3 / 6-8)	
Wavelength (sub/main)	376/340 nm	
Reaction direction	Decrease	
Units	mmol/L (mg/dL)	
Reagent pipetting		Diluent (H_2O)
R1	20 µL	160 µL
R2	20 µL	-

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	4 µL	15 µL	135 µL
Increased	2 µL	–	–

cobas c 501/502 test definition

Assay type	2-Point End	
Reaction time / Assay points	10 / 10-13 (STAT 3 / 10-13)	
Wavelength (sub/main)	376/340 nm	
Reaction direction	Decrease	
Units	mmol/L (mg/dL)	
Reagent pipetting		Diluent (H_2O)
R1	20 µL	160 µL
R2	20 µL	-

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2 µL	–	–
Decreased	4 µL	15	135
Increased	2 µL	–	–

Calibration

Calibrators	S1: H_2O
	S2: C.f.a.s.

CA2

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Calibration mode	Linear
Calibration frequency	2-point calibration • after reagent lot change • as required following quality control procedures

Traceability: This method has been standardized against the SRM 956 c Level 2 reference material.

Quality control

Serum/plasma

For quality control, use control materials as listed in the "Order information" section.

In addition, other suitable control material can be used.

Urine

Quantitative urine controls are recommended for routine quality control.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors:	mmol/L x 4.01 = mg/dL

In studies with 24-hour urine, multiply the value obtained by the 24-hour volume in order to obtain a measurement in mg/24 h or mmol/24 h.

Limitations - interference

Criterion: Recovery within ± 0.22 mmol/L (0.9 mg/dL) of initial value of samples ≤ 2.2 mmol/L (8.8 mg/dL) and within ± 10 % for samples > 2.2 mmol/L.

Serum/plasma

Icterus:[6] No significant interference up to an I index of 60 for conjugated and unconjugated bilirubin (approximate conjugated and unconjugated bilirubin concentration: 1026 µmol/L or 60 mg/dL).

Hemolysis:[6] No significant interference up to an H index of 1000 (approximate hemoglobin concentration: 621 µmol/L or 1000 mg/dL).

Lipemia (Intralipid):[6] No significant interference up to an L index of 1000. There is a poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Magnesium: No significant interference up to a concentration of 15 mmol/L.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[7,8]

The interference of intravenously administered gadolinium containing MRI (magnetic resonance imaging) contrast media was tested (Omniscan®, Optimark®) but no interference was found at the therapeutic concentration. Interferences at higher concentrations were observed.

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.[9]

Urine

Icterus: No significant interference up to a conjugated bilirubin concentration of 1026 µmol/L or 60 mg/dL.

Hemolysis: No significant interference up to a hemoglobin concentration of 621 µmol/L or 1000 mg/dL.

Magnesium: No significant interference up to a concentration of 60 mmol/L.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[8]

The interference of intravenously administered gadolinium containing MRI (magnetic resonance imaging) contrast media was tested (Omniscan®, Optimark®). For Omniscan® no interference was observed at the therapeutic concentration, but there was interference at higher concentrations. For Optimark® interference was observed at therapeutic and higher concentrations.

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED

Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. The latest version of the carry-over evasion list can be found with the NaOHD/SMS/Multiclean/SCCS or the NaOHD/SMS/SmpCln1+2/SCCS Method Sheets. For further instructions refer to the operator's manual. **cobas c** 502 analyzer: All special wash programming necessary for avoiding carry-over is available via the **cobas** link, manual input is not required.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range

Serum/plasma

0.20-5.0 mmol/L (0.8-20.1 mg/dL)

Urine

0.20-7.5 mmol/L (0.8-30.1 mg/dL)

Determine urine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:5 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 5.

Lower limits of measurement

Limit of Blank (LoB), Limit of Detection (LoD) and Limit of Quantitation (LoQ)

Serum/plasma and urine

Serum/plasma

Limit of Blank:	= 0.10 mmol/L (0.4 mg/dL)
Limit of Detection:	= 0.20 mmol/L (0.8 mg/dL)
Limit of Quantitation	= 0.20 mmol/L (0.8 mg/dL)

The Limit of Blank, Limit of Detection and Limit of Quantitation were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP17-A2 requirements.

The Limit of Blank is the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.

The Limit of Detection is determined based on the Limit of Blank and the standard deviation of low concentration samples.

The Limit of Detection corresponds to the lowest analyte concentration which can be detected (value above the Limit of Blank with a probability of 95 %).

The Limit of Quantitation is the lowest analyte concentration that can be reproducibly measured with a total error of 30 %. It has been determined using low concentration calcium samples.

Expected values[10]

Serum/plasma

Children (0-10 days):	1.90-2.60 mmol/L (7.6-10.4 mg/dL)
Children (10 days-2 years):	2.25-2.75 mmol/L (9.0-11.0 mg/dL)
Children (2-12 years):	2.20-2.70 mmol/L (8.8-10.8 mg/dL)
Children (12-18 years):	2.10-2.55 mmol/L (8.4-10.2 mg/dL)
Adults (18-60 years):	2.15-2.50 mmol/L (8.6-10.0 mg/dL)
Adults (60-90 years):	2.20-2.55 mmol/L (8.8-10.2 mg/dL)
Adults (> 90 years):	2.05-2.40 mmol/L (8.2-9.6 mg/dL)

Urine

2.5-7.5 mmol/24 h (100-300 mg/24 h) with normal food intake.

Roche has not evaluated reference ranges in a pediatric population.

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Repeatability and intermediate precision were determined using human samples and controls in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP5 requirements (2 aliquots per run, 2 runs per day, 21 days). The following results were obtained:

The following results were obtained:

Serum/plasma

Repeatability	*Mean* *mmol/L (mg/dL)*	*SD* *mmol/L (mg/dL)*	*CV* *%*
Human serum 1	0.60 (2.4)	0.01 (0.0)	2.0
Human serum 2	2.55 (10.2)	0.02 (0.1)	0.8
Human serum 3	4.46 (17.9)	0.04 (0.2)	0.8
Precinorm U	2.25 (9.0)	0.02 (0.1)	0.8
Precipath U	3.51 (14.1)	0.03 (0.1)	0.8
Intermediate precision	*Mean* *mmol/L (mg/dL)*	*SD* *mmol/L (mg/dL)*	*CV* *%*
Human serum 1	0.60 (2.4)	0.02 (0.1)	2.5
Human serum 2	2.55 (10.2)	0.02 (0.1)	0.9
Human serum 3	4.46 (17.9)	0.04 (0.2)	0.9
Precinorm U	2.25 (9.0)	0.02 (0.1)	0.8
Precipath U	3.51 (14.1)	0.03 (0.1)	0.9

Urine

Repeatability	*Mean* *mmol/L (mg/dL)*	*SD* *mmol/L (mg/dL)*	*CV* *%*
Human urine 1	0.58 (2.3)	0.02 (0.1)	3.0
Human urine 2	3.92 (15.7)	0.04 (0.2)	1.1
Human urine 3	5.18 (20.8)	0.05 (0.2)	0.9
Human urine 4	6.09 (24.4)	0.08 (0.3)	1.3
Control Level 1	1.85 (7.4)	0.02 (0.1)	1.3
Control Level 2	2.72 (10.9)	0.03 (0.1)	1.1
Intermediate precision	*Mean* *mmol/L (mg/dL)*	*SD* *mmol/L (mg/dL)*	*CV* *%*
Human urine 1	0.58 (2.3)	0.02 (0.1)	3.1
Human urine 2	3.92 (15.7)	0.05 (0.2)	1.2
Human urine 3	5.18 (20.8)	0.06 (0.2)	1.1
Human urine 4	6.09 (24.4)	0.08 (0.3)	1.3
Control Level 1	1.85 (7.4)	0.03 (0.1)	1.5
Control Level 2	2.72 (10.9)	0.04 (0.2)	1.3

Method comparison

Calcium values for human serum, plasma and urine samples obtained on a Roche/Hitachi **cobas c** 501 analyzer using the Roche Calcium Gen.2 reagent (x) were compared to those determined on a Roche/Hitachi MODULAR P analyzer using the same reagent (y).

Serum/plasma

Sample size (n) = 69

Passing/Bablok[11]	Linear regression
y = 0.982x + 0.061 mmol/L	y = 0.982x + 0.059 mmol/L
τ = 0.979	r = 1.00

The sample concentrations were between 0.33 and 4.76 mmol/L (1.3 and 19.1 mg/dL).

Urine

Sample size (n) = 65

Passing/Bablok[11]	Linear regression
y = 0.989x + 0.064 mmol/L	y = 0.983x + 0.079 mmol/L
τ = 0.989	r = 1.00

The sample concentrations were between 0.28 and 7.47 mmol/L (1.1 and 30.0 mg/dL).

Calcium values for human serum, plasma and urine samples obtained on a Roche/Hitachi **cobas c** 501 analyzer using the Roche Calcium Gen.2 reagent (y) were compared with those determined using the Roche Calcium reagent on a Roche/Hitachi MODULAR P analyzer (x).

Serum/plasma

Sample size (n) = 69

Passing/Bablok[11]	Linear regression
y = 1.018x - 0.027 mmol/L	y = 1.023x - 0.036 mmol/L
τ = 0.976	r = 1.00

The sample concentrations were between 0.28 and 4.65 mmol/L (1.1 and 18.6 mg/dL).

Urine

Sample size (n) = 65

Passing/Bablok[11]	Linear regression
y = 1.024x + 0.018 mmol/L	y = 1.020x + 0.029 mmol/L
τ = 0.988	r = 1.00

The sample concentrations were between 0.30 and 7.25 mmol/L (1.2 and 29.1 mg/dL).

References

1 Endres DB, Rude RK. Mineral and Bone Metabolism. In: Burtis CA, Ashwood ER, Bruns ED, eds. Tietz Textbook of Clinical Chemistry and Molecular Diagnostics, 4th ed. St. Louis (MO): Saunders Elsevier 2006:1891-1965.

2 Heins M, Heil W, Withold W. Storage of Serum or Whole Blood Samples? Effect of Time and Temperature on 22 Serum Analytes. Eur J Clin Chem Clin Biochem 1995;33:231-238.

3 Wilding P, Zilva JF, Wilde CE. Transport of specimens for clinical chemistry analysis. Ann Clin Biochem 1977;14:301-306.

4 Burtis CA, Ashwood ER, Bruns DE, eds. Tietz Fundamentals of Clinical Chemistry, 6th ed.St. Louis (MO): Saunders Elsevier 2008:715.

5 Use of Anticoagulants in Diagnostic Laboratory Investigations. WHO Publication WHO/DIL/LAB/99.1 Rev. 2. Jan. 2002.

6 Glick MR, Ryder KW, Jackson SA. Graphical Comparisons of Interferences in Clinical Chemistry Instrumentation. Clin Chem 1986;32:470-475.

7 Breuer J. Report on the Symposium "Drug effects in Clinical Chemistry Methods". Eur J Clin Chem Clin Biochem 1996;34:385-386.

8 Sonntag O, Scholer A. Drug interference in clinical chemistry: recommendation of drugs and their concentrations to be used in drug interference studies. Ann Clin Biochem 2001;38:376-385.

9 Bakker AJ, Mücke M. Gammopathy interference in clinical chemistry assays: mechanisms, detection and prevention. Clin Chem Lab Med 2007;45(9):1240-1243.

10 Wu AHB, ed. Tietz Clinical Guide to Laboratory Tests, 4th ed. St. Louis (MO): Saunders Elsevier 2006:202-207.

11 Bablok W, Passing H, Bender R, et al. A general regression procedure for method transformation. Application of linear regression procedures for method comparison studies in clinical chemistry, Part III. J Clin Chem Clin Biochem 1988 Nov;26(11):783-790.

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard.

CONTENT	Contents of kit
→	Volume after reconstitution or mixing

FOR US CUSTOMERS ONLY: LIMITED WARRANTY

Roche Diagnostics warrants that this product will meet the specifications stated in the labeling when used in accordance with such labeling and will be free from defects in material and workmanship until the expiration date printed on the label. THIS LIMITED WARRANTY IS IN LIEU OF ANY OTHER WARRANTY, EXPRESS OR IMPLIED, INCLUDING ANY IMPLIED WARRANTY OF MERCHANTABILITY OR FITNESS FOR PARTICULAR PURPOSE. IN NO EVENT SHALL ROCHE DIAGNOSTICS BE LIABLE FOR INCIDENTAL, INDIRECT, SPECIAL OR CONSEQUENTIAL DAMAGES.

CE

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com

Distribution in USA by:
Roche Diagnostics, Indianapolis, IN
US Customer Technical Support 1-800-428-2336

Appendix F

0103333825190c501V12.0

TPUC3

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Total Protein Urine/CSF Gen. 3

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
03333825 190	Total Protein Urine/CSF Gen.3 150 tests	System-ID 07 6763 8	Roche/Hitachi **cobas c** 311, **cobasc** 501/502
03121305 122	C.f.a.s. PUC (5 x 1 mL)	Code 489	
03121313 122	Precinorm PUC (4 x 3 mL)	Code 240	
03121291 122	Precipath PUC (4 x 3 mL)	Code 241	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:

TPU3: ACN 708

TPC3: ACN 402

For **cobas c** 502 analyzer:

TPU3: ACN 8708

TPC3: ACN 8402

Intended use

In vitro test for the quantitative determination of protein in human urine and cerebrospinal fluid on Roche/Hitachi **cobas c** systems.

Summary

Protein measurements in urine are used in the diagnosis and treatment of disease conditions such as renal or heart diseases, or thyroid disorders, which are characterized by proteinuria or albuminuria. Cerebrospinal fluid (CSF) protein measurements are used in the diagnosis and treatment of conditions such as meningitis, brain tumors and infections of the central nervous system.[1]

Urine is formed by ultrafiltration of plasma across the glomerular capillary wall. Proteins with a relative molecular mass > 40000 are almost completely retained, while smaller substances easily enter the glomerular filtrate. Most CSF protein originates by diffusion from plasma across the blood-CSF barrier. Elevated levels occur as a result of increased permeability of the blood-CSF barrier or with increased local synthesis of immunoglobulins.

Turbidimetric methods using trichloroacetic acid (TCA) or sulfosalicylic acid (SSA) precipitate proteins in the sample depending on their size; the resulting turbidity may be unstable and flocculate. Reagents of dye-binding methods such as Coomassie blue and pyrogallol red-molybdate react with proteins depending on their amino acid composition, but may stain glass and plastic ware. Due to their reaction mechanisms all methods, turbidimetric and colorimetric, exhibit different sensitivities to various proteins, especially to protein fragments such as Bence Jones proteins[2] and small proteins such as α1-microglobulin.

The Roche Diagnostics Urinary/CSF Protein assay is based on the method described by Iwata and Nishikaze,[3] later modified by Luxton, Patel, Keir, and Thompson.[4] In this method, benzethonium chloride reacts with protein in a basic medium to produce a turbidity that is more stable and evenly distributed than that observed with the SSA or TCA methodologies. This assay shows an underrecovery of γ-globulin compared to albumin of about 30 %,[5] and no interference from magnesium ions due to the addition of EDTA.

Test principle

Turbidimetric method.

The sample is preincubated in an alkaline solution containing EDTA, which denatures the protein and eliminates interference from magnesium ions. Benzethonium chloride is then added, producing turbidity.

Reagents - working solutions

R1 Sodium hydroxide: 677 mmol/L; EDTA-Na: 74 mmol/L

R2 Benzethonium chloride: 32 mmol/L

R1 is in position B and R2 is in position C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

This kit contains components classified as follows in accordance with the Regulation (EC) No. 1272/2008:

Danger

H290	May be corrosive to metals.
H314	Causes severe skin burns and eye damage.
H412	Harmful to aquatic life with long lasting effects.

Prevention:

P234	Keep only in original container.
P264	Wash skin thoroughly after handling.
P273	Avoid release to the environment.
P280	Wear protective gloves/ protective clothing/ eye protection/ face protection.

Response:

P301 + P330 + P331	IF SWALLOWED: Rinse mouth. Do NOT induce vomiting.
P303 + P361 + P353	IF ON SKIN (or hair): Remove/Take off immediately all contaminated clothing. Rinse skin with water/shower.
P304 + P340	IF INHALED: Remove person to fresh air and keep comfortable for breathing.
P305 + P351 + P338	IF IN EYES: Rinse cautiously with water for several minutes. Remove contact lenses, if present and easy to do. Continue rinsing.
P310	Immediately call a POISON CENTER or doctor/physician.
P337 + P313	If eye irritation persists: Get medical advice/attention.
P363	Wash contaminated clothing before reuse.
P390	Absorb spillage to prevent material damage.

Storage:

P405	Store locked up.
P406	Store in corrosive resistant stainless steel container with a resistant inner liner.

Disposal:

P501	Dispose of contents/container to an approved waste disposal plant.

Product safety labeling primarily follows EU GHS guidance.
Contact phone: all countries: +49-621-7590

TPUC3

Total Protein Urine/CSF Gen. 3

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Reagent handling

Ready for use

Storage and stability

TPUC3

Shelf life at 15-25 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	6 weeks

Diluent NaCl 9 %

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.

Urine

Use random or 24-hour urine specimens. Use no preservatives. Refrigerate specimen during collection.

CSF

No special additives are required. Blood in a CSF specimen invalidates the protein value.[1]

Samples for urinary/CSF protein should be collected before fluorescein is given or at least 24 hours later.[6]

Note: Urine, CSF and control samples with a protein concentration above 7000 mg/L must not be measured with TPUC3 as this may clog the instrument lines.

Stability:[7]

Urine:	1 day at 15-25 °C
	7 days at 2-8 °C
	1 month at (-15)-(-25) °C
CSF:	1 day at 15-25 °C
	6 days at 2-8 °C
	> 1 year at (-15)-(-25) °C

Centrifuge samples containing precipitates before performing the assay.

Non centrifuged samples may produce elevated results.

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- See "Order information" section
- General laboratory equipment

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for urine and CSF

cobas c 311 **test definition**

Assay type	2-Point End
Reaction time / Assay points	10 / 6-14
Wavelength (sub/main)	700/505 nm
Reaction direction	Increase
Units	mg/L (mg/dL, g/L)

Reagent pipetting		Diluent (H_2O)	
R 1	100 µL	–	
R 2	40 µL	–	
Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluentt (NaCl)*
Normal	6 µL	–	–
Decreased	2 µL	–	–
Increased	6 µL	–	–

cobas c 501 **test definition**

Assay type	2-Point End
Reaction time / Assay points	10 / 10-30
Wavelength (sub/main)	700/505 nm
Reaction direction	Increase
Units	mg/L (mg/dL, g/L)

Reagent pipetting		Diluent (H_2O)	
R1	100 µL	–	
R2	40 µL	–	
Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	6 µL	–	–
Decreased	2 µL	–	–
Increased	6 µL	–	–

cobas c 502 **test definition**

Assay type	2-Point End
Reaction time / Assay points	10 / 10-30
Wavelength (sub/main)	700/505 nm
Reaction direction	Increase
Units	mg/L (mg/dL, g/L)

Reagent pipetting		Diluent (H_2O)	
R1	100 µL	–	
R2	40 µL	–	
Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	6 µL	–	–
Decreased	2 µL	–	–
Increased	12 µL	–	–

Calibration

Calibrators	S1: H_2O
	S2-S6: C.f.a.s. PUC

Multiply the lot-specific C.f.a.s. PUC calibrator values by the factors given below to determine the standard concentrations for the 6-point calibration curve.

TPUC3

Total Protein Urine/CSF Gen. 3

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	S2: 0.025	S5: 0.250
	S3: 0.050	S6: 1.0
	S4: 0.125	
Calibration mode	RCM	
Calibration frequency	Full calibration - after reagent lot change - as required following quality control procedures	

Traceability:[8] This method has been standardized against a primary standard traceable to NIST.

Quality control

For quality control, use control materials as listed in the "Order information" section.

In addition, other suitable control material can be used.

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors:	mg/L x 0.1 = mg/dL
	mg/L x 0.001 = g/L

To calculate 24-hour urine protein excretion:

mg/L x total volume (liters per 24 hours) = mg/day.

Limitations - interference

Criterion: Recovery within ± 10 % of initial value at a total protein concentration of 120 mg/L (12 mg/dL; 0.12 g/L).

High dose hook-effect: Sample results with high total protein concentrations above the measuring range up to 100000 mg/L will be flagged by the instrument with > TEST or > ABS.

Urine

Icterus: No significant interference up to a concentration of 342 µmol/L (20 mg/dL) for conjugated bilirubin.

Hemolysis: Hemoglobin interferes.[9]

Drugs: No interference was found at therapeutic concentrations using common drug panels.[10]

Exception: Levodopa, methyldopa and Na_2-cefoxitin cause artificially high total protein results and calcium dobesilate causes artificially low protein results.

Other: Patient samples containing > 8 g/L of organically bound iodine from Radiopaque media (e.g. Hexabrix) may have falsely elevated results.

High levels of homogentisic acid can be found in urine of patients with the rare genetic disorder Alkaptonuria.[11] Homogentisic acid in urine samples at concentrations > 0.6 mmol/L can cause false results.

The administration of gelatin-based plasma replacements can lead to increased urine protein values.

CSF

Hemolysis: Hemoglobin interferes.[9]

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED
Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. The latest version of the carry-over evasion list can be found with the NaOHD-SMS-SmpCln1+2-SCCS Method Sheets. For further instructions refer to the operator's manual. **cobas c** 502 analyzer: All special wash programming necessary for avoiding carry-over is available via the **cobas** link, manual input is not required.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range

40-2000 mg/L (4-200 mg/dL; 0.04-2 g/L)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:3 dilution. Results from samples diluted by the rerun function are automatically multiplied by a factor of 3.

Lower limits of measurement

Lower detection limit of the test

40 mg/L (4 mg/dL; 0.04 g/L)

The lower detection limit represents the lowest measurable analyte level that can be distinguished from zero. It is calculated as the value lying 3 standard deviations above that of the lowest standard (standard 1 + 3 SD, repeatability, n = 21).

Expected values

Urine:[12]	24 h:	< 140 mg/24 h*
	random:	< 150 mg/L*
	* Values obtained from centrifuged samples	
CSF:	reference range acc. to Tietz: 150-450 mg/L (15-45 mg/dL)[13]	
	reference range acc. to Thomas: 200-400 mg/L (20-40 mg/dL)[14]	

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol with repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). The following results were obtained:

Urine

Repeatability	*Mean*	*SD*	*CV*
	mg/L (mg/dL)	*mg/L (mg/dL)*	*%*
Precinorm PUC	159 (15.9)	1 (0.1)	0.7
Precipath PUC	1576 (158)	8 (0.8)	0.5
Human urine 1	101 (10.1)	1 (0.1)	1.0
Human urine 2	191 (19.1)	4 (0.4)	2.2
Intermediate precision	*Mean*	*SD*	*CV*
	mg/L (mg/dL)	*mg/L (mg/dL)*	*%*
Precinorm PUC	156 (15.6)	2 (0.2)	1.5
Precipath PUC	1482 (148)	8 (0.8)	0.5
Human urine 3	106 (10.6)	2 (0.2)	1.6
Human urine 4	154 (15.4)	1 (0.1)	0.9

CSF

Repeatability	*Mean*	*SD*	*CV*
	mg/L (mg/dL)	*mg/L (mg/dL)*	*%*
Control Level 1	281 (28.1)	4 (0.4)	1.5
Control Level 2	691 (69.1)	4 (0.4)	0.6
Human CSF 1	355 (35.5)	4 (0.4)	1.1

TPUC3

Total Protein Urine/CSF Gen. 3

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Human CSF 2	517 (51.7)	5 (0.5)	1.0
Intermediate precision	*Mean*	*SD*	*CV*
	mg/L (mg/dL)	*mg/L (mg/dL)*	*%*
Control Level 1	272 (27.2)	4 (0.4)	1.6
Control Level 2	660 (66.0)	6 (0.6)	0.9
Human CSF 3	349 (34.9)	4 (0.4)	1.2
Human CSF 4	501 (50.1)	7 (0.7)	1.5

Method comparison

Total protein values for human urine and CSF samples obtained on a Roche/Hitachi **cobas c** 501 analyzer (y) were compared with those determined with the corresponding reagent on a Roche/Hitachi 917 analyzer (x).

Urine

Sample size (n) = 70

Passing/Bablok[15]	Linear regression
y = 0.985x + 6.23 mg/L	y = 0.988x + 5.35 mg/L
τ = 0.970	r = 1.000

The sample concentrations were between 47.0 and 1887 mg/L (4.70 and 189 mg/dL).

CSF

Sample size (n) = 86

Passing/Bablok[15]	Linear regression
y = 1.015x - 7.51 mg/L	y = 1.010x - 5.23 mg/L
τ = 0.975	r = 0.999

The sample concentrations were between 53.0 and 1087 mg/L (5.30 and 109 mg/dL).

References

1 Tietz NW. Fundamentals of Clinical Chemistry, 3rd ed. Pa: WB Saunders Co 1987:336.

2 Boege F. Bence Jones-Proteine. J Lab Med 1999;23(9):477-482.

3 Iwata J, Nishikaze O. New micro-turbidimetric method for determination of protein in cerebrospinal fluid and urine. Clin Chem 1979;25(7):1317-1319.

4 Luxton RW, Patel P, Keir G, et al. A micro-method for measuring total protein in cerebrospinal fluid by using benzethonium chloride in microtiter plate wells. Clin Chem 1989;35(8):1731-1734.

5 Hohnadel DC, Koller A. Urine protein total. In: Pesce AJ, Kaplan LA, editors. Methods in clinical chemistry, St. Louis, Mosby 1987.

6 Koumantakis G. Fluorescein Interference with Urinary Creatinine and Protein Measurements. Clin Chem 1991;37/10:1799.

7 WHO Publication: Use of anticoagulants in diagnostic laboratory investigations, WHO/DIL/LAB/99.1 Rev.2 Jan 2002.

8 Standard Reference Materials from NERL, traceable to NIST (National Institute of Standards and Technology).

9 Yilmaz FM, Yücel D. Effect of Addition of Hemolysate on Urine and Cerebrospinal Fluid Assays for Protein. Clin Chem 2006;52:152-153.

10 Sonntag O, Scholer A. Drug interference in clinical chemistry: recommendation of drugs and their concentrations to be used in drug interference studies. Ann Clin Biochem 2001;38:376-385.

11 Phornphutkul C, Introne WJ, Perry MB, et al. Natural History of Alkaptonuria. N Engl J Med 2002;347(26):2111-2121.

12 Junge W, Wilke B, Halabi A, et al. Reference Intervals forTotal Protein in Collected and Random Urine using the Benzethonium Chloride Method [Abstract]. Clin Chem 2006;52:157.

13 Tietz NW, ed. Clinical Guide to Laboratory Tests, 3rd ed. Philadelphia, PA: WB Saunders Company 1995;518-523.

14 Thomas L. Labor und Diagnose, 6. Auflage, TH-Books Verlagsgesellschaft mbH, Frankfurt/Main 2005;930-934.

15 Bablok W, Passing H, Bender R, et al. A general regression procedure for method transformation. Application of linear regression procedures for method comparison studies in clinical chemistry, Part III. J Clin Chem Clin Biochem 1988 Nov;26(11):783-790.

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Symbols

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CONTENT	Contents of kit
→	Volume after reconstitution or mixing
GTIN	Global Trade Item Number

CE

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com

Roche

Appendix G

SEB833Hu 96 Tests
Enzyme-linked Immunosorbent Assay Kit
For Complement Factor D (CFD)
Organism Species: *Homo sapiens* (Human)
Instruction manual

FOR RESEARCH USE ONLY
NOT FOR USE IN CLINICAL DIAGNOSTIC PROCEDURES

12th Edition

[INTENDED USE]

The kit is a sandwich enzyme immunoassay for in vitro quantitative measurement of CFD in human serum, plasma, tissue homogenates, cell lysates, cell culture supernates and other biological fluids.

[REAGENTS AND MATERIALS PROVIDED]

Reagents	Quantity	Reagents	Quantity
Pre-coated, ready to use 96-well strip plate	1	Plate sealer for 96 wells	4
Standard	2	Standard Diluent	1×20mL
Detection Reagent A	1×120μL	Assay Diluent A	1×12mL
Detection Reagent B	1×120μL	Assay Diluent B	1×12mL
TMB Substrate	1×9mL	Stop Solution	1×6mL
Wash Buffer (30 × concentrate)	1×20mL	Instruction manual	1

[MATERIALS REQUIRED BUT NOT SUPPLIED]

1. Microplate reader with 450 ± 10nm filter.
2. Single or multi-channel pipettes with high precision and disposable tips.
3. Microcentrifuge Tubes.
4. Deionized or distilled water.
5. Absorbent paper for blotting the microplate.
6. Container for Wash Solution.
7. 0.01mol/L (or 1×) Phosphate Buffered Saline(PBS), pH7.0-7.2.

[STORAGE OF THE KITS]

1. **For unused kit:** All the reagents should be kept according to the labels on vials. The **Standard**, **Detection Reagent A, Detection Reagent B** and the **96-well strip plate** should be stored at -20°C upon receipt while the others should be at 4°C.
2. **For used kit:** When the kit is used, the remaining reagents need to be stored according to the above storage condition. Besides, please return the unused wells to the foil pouch containing the desiccant pack, and zip-seal the foil pouch.

Note:

It is highly recommended to use the remaining reagents within 1 month provided this is prior to the expiration date of the kit. For the expiration date of the kit, please refer to the label on the kit box. All components are stable up to the expiration date.

[SAMPLE COLLECTION AND STORAGE]

Serum - Use a serum separator tube and allow samples to clot for two hours at room temperature or overnight at 4°C before centrifugation for 20 minutes at approximately 1,000×g. Assay freshly prepared serum immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Plasma - Collect plasma using EDTA or heparin as an anticoagulant. Centrifuge samples for 15 minutes at 1,000×g at 2-8°C within 30 minutes of collection. Remove plasma and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Tissue homogenates - The preparation of tissue homogenates will vary depending upon tissue type.

1. Tissues were rinsed in ice-cold PBS to remove excess blood thoroughly and weighed before homogenization.
2. Minced the tissues to small pieces and homogenized them in fresh lysis buffer (catalog: IS007, different lysis buffer needs to be chosen based on subcellular location of the target protein) (w:v = 1:20-1:50, e.g. 1mL lysis buffer is added in 20-50mg tissue sample) with a glass homogenizer on ice (Micro Tissue Grinders woks, too).
3. The resulting suspension was sonicated with an ultrasonic cell disrupter till the solution is clarified.
4. Then, the homogenates were centrifuged for 5 minutes at 10,000×g. Collect the supernatant and assay immediately or aliquot and store at ≤-20°C.

Cell Lysates - Cells need to be lysed before assaying according to the following directions.

1. Adherent cells should be washed by cold PBS gently, and then detached with trypsin, and collected by centrifugation at 1,000×g for 5 minutes (suspension cells can be collected by centrifugation directly).
2. Wash cells three times in cold PBS.
3. Resuspend cells in fresh lysis buffer with concentration of 10^7 cells/mL. If it is necessary, the cells could be subjected to ultrasonication till the solution is clarified.
4. Centrifuge at 1,500×g for 10 minutes at 2-8°C to remove cellular debris. Assay immediately or aliquot and store at ≤-20°C.

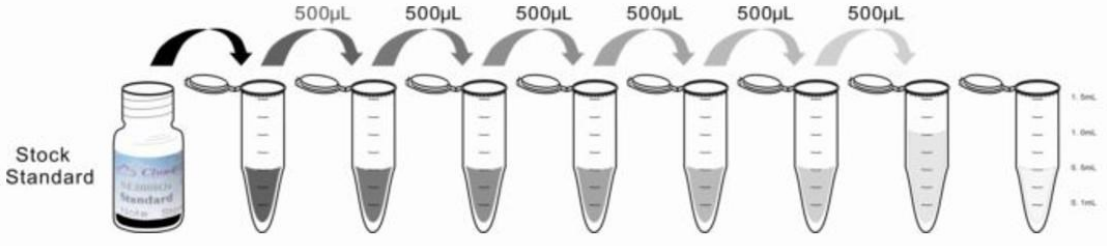
Cell culture supernatants and other biological fluids - Centrifuge samples for 20 minutes at 1,000×g. Collect the supernant and assay immediately or store samples in aliquot at -20°C or -80°C for later use. Avoid repeated freeze/thaw cycles.

Note:

1. Samples to be used within 5 days may be stored at 4°C, otherwise samples must be stored at -20°C (≤1 month) or -80°C (≤2 months) to avoid loss of bioactivity and contamination.
2. Sample hemolysis will influence the result, so hemolytic specimen should not be used.
3. When performing the assay, bring samples to room temperature.
4. It is highly recommended to use serum instead of plasma for the detection based on quantity of our in-house data.

[REAGENT PREPARATION]

1. Bring all kit components and samples to room temperature (18-25℃) before use. If the kit will not be used up in one time, please only take out strips and reagents for present experiment, and leave the remaining strips and reagents in required condition.
2. **Standard -** Reconstitute the **Standard** with 1.0mL of **Standard Diluent,** kept for 10 minutes at room temperature, shake gently(not to foam). The concentration of the standard in the stock solution is 160ng/mL. Please firstly dilute the stock solution to 40ng/mL and the diluted standard serves as the highest standard (40ng/mL). Then prepare 7 tubes containing 0.5mL Standard Diluent and use the diluted standard to produce a double dilution series according to the picture shown below. Mix each tube thoroughly before the next transfer. Set up 7 points of diluted standard such as 40ng/mL, 20ng/mL, 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL, and the last EP tubes with **Standard Diluent** is the blank as 0ng/mL.



Tube	1	2	3	4	5	6	7	8	9
ng/mL	160	40	20	10	5	2.5	1.25	0.625	0

3. **Detection Reagent A and Detection Reagent B -** Briefly spin or centrifuge the stock Detection A and Detection B before use. Dilute them to the working concentration 100-fold with **Assay Diluent A** and **B**, respectively.
4. **Wash Solution** - Dilute 20mL of Wash Solution concentrate (30×) with 580mL of deionized or distilled water to prepare 600mL of Wash Solution (1×).
5. **TMB substrate** - Aspirate the needed dosage of the solution with sterilized tips and do not dump the residual solution into the vial again.

Note:

1. Making serial dilution in the wells directly is not permitted.
2. Prepare standards within 15 minutes before assay. Please do not dissolve the reagents at 37℃ directly.
3. Please carefully reconstitute Standards or working Detection Reagent A and B according to the instruction, and avoid foaming and mix gently until the crystals are completely dissolved. To minimize imprecision caused by pipetting, use small volumes and ensure that pipettors are calibrated. It is recommended to suck more than 10μL for one pipetting.
4. The reconstituted Standards, Detection Reagent A and Detection Reagent B can be **used only once**.
5. If crystals have formed in the Wash Solution concentrate (30×), warm to room temperature and mix gently until the crystals are completely dissolved.
6. Contaminated water or container for reagent preparation will influence the detection result.

[SAMPLE PREPARATION]

1. We are only responsible for the kit itself, but not for the samples consumed during the assay. The user should calculate the possible amount of the samples used in the whole test. Please reserve sufficient samples in advance.
2. Please predict the concentration before assaying. If values for these are not within the range of the standard curve, users must determine the optimal sample dilutions for their particular experiments.
3. Serum/plasma samples require about a 500 fold dilution. For example, to prepare a 1:500 dilution of sample, transfer 20μL of sample to 180μL PBS. This yields a 1:10 dilution. Next, dilute the 1:10 sample by transferring 10μL to 490μL PBS. You now have a 1:500 dilution of your sample. Mix thoroughly at each stage. Sample should be diluted by 0.01mol/L PBS(PH=7.0-7.2).
4. If the samples are not indicated in the manual, a preliminary experiment to determine the validity of the kit is necessary.
5. Tissue or cell extraction samples prepared by chemical lysis buffer may cause unexpected ELISA results due to the impacts from certain chemicals.
6. Due to the possibility of mismatching between antigen from other origin and antibody used in our kits (e.g., antibody targets conformational epitope rather than linear epitope), some native or recombinant proteins from other manufacturers may not be recognized by our products.
7. Influenced by the factors including cell viability, cell number or sampling time, samples from cell culture supernatant may not be detected by the kit.
8. Fresh samples without long time storage is recommended for the test. Otherwise, protein degradation and denaturalization may occur in those samples and finally lead to wrong results.

[ASSAY PROCEDURE]

1. Determine wells for diluted standard, blank and sample. Prepare 7 wells for standard, 1 well for blank. Add 100μL each of dilutions of standard (read Reagent Preparation), blank and samples into the appropriate wells. Cover with the Plate sealer. Incubate for 1 hour at 37°C.
2. Remove the liquid of each well, don't wash.
3. Add 100μL of **Detection Reagent A** working solution to each well, cover the wells with the plate sealer and incubate for 1 hour at 37°C.
4. Aspirate the solution and wash with 350μL of 1× Wash Solution to each well using a squirt bottle, multi-channel pipette, manifold dispenser or autowasher, and let it sit for 1~2 minutes. Remove the remaining liquid from all wells completely by snapping the plate onto absorbent paper. Totally wash 3 times. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against absorbent paper.
5. Add 100μL of **Detection Reagent B** working solution to each well, cover the wells with the plate sealer and incubate for 30 minutes at 37°C.
6. Repeat the aspiration/wash process for total 5 times as conducted in step 4.
7. Add 90μL of **Substrate Solution** to each well. Cover with a new Plate sealer. Incubate for 10 - 20 minutes at 37°C (Don't exceed 30 minutes). Protect from light. The liquid will turn blue by the addition of Substrate Solution.

8. Add 50μL of **Stop Solution** to each well. The liquid will turn yellow by the addition of Stop solution. Mix the liquid by tapping the side of the plate. If color change does not appear uniform, gently tap the plate to ensure thorough mixing.
9. Remove any drop of water and fingerprint on the bottom of the plate and confirm there is no bubble on the surface of the liquid. Then, run the microplate reader and conduct measurement at 450nm immediately.

Note:

1. **Assay preparation:** Keep appropriate numbers of wells for each experiment and remove extra wells from microplate. Rest wells should be resealed and stored at -20°C.
2. **Samples or reagents addition：Please use the freshly prepared Standard.** Please carefully add samples to wells and mix gently to avoid foaming. Do not touch the well wall. For each step in the procedure, total dispensing time for addition of reagents or samples to the assay plate should not exceed 10 minutes. This will ensure equal elapsed time for each pipetting step, without interruption. Duplication of all standards and specimens, although not required, is recommended. To avoid cross-contamination, change pipette tips between additions of standards, samples, and reagents. Also, use separated reservoirs for each reagent.
3. **Incubation:** To ensure accurate results, proper adhesion of plate sealers during incubation steps is necessary. Do not allow wells to sit uncovered for extended periods between incubation steps. Once reagents are added to the well strips, DO NOT let the strips DRY at any time during the assay. Incubation time and temperature must be controlled.
4. **Washing:** The wash procedure is critical. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Solution by aspirating or decanting and remove any drop of water and fingerprint on the bottom of the plate. Insufficient washing will result in poor precision and false elevated absorbance reading.
5. **Controlling of reaction time:** Observe the change of color after adding **TMB Substrate** (e.g. observation once every 10 minutes), if the color is too deep, add **Stop Solution** in advance to avoid excessively strong reaction which will result in inaccurate absorbance reading.
6. **TMB Substrate** is easily contaminated. Please protect it from light.
7. The environment humidity which is less than 60% might have some effects on the final performance, therefore, a humidifier is recommended to be used at that condition.

[TEST PRINCIPLE]

The microplate provided in this kit has been pre-coated with an antibody specific to CFD. Standards or samples are then added to the appropriate microplate wells with a biotin-conjugated antibody specific to CFD. Next, Avidin conjugated to Horseradish Peroxidase (HRP) is added to each microplate well and incubated. After TMB substrate solution is added, only those wells that contain CFD, biotin-conjugated antibody and enzyme-conjugated Avidin will exhibit a change in color. The enzyme-substrate reaction is terminated by the addition of sulphuric acid solution and the color change is measured spectrophotometrically at a wavelength of 450nm ± 10nm. The concentration of CFD in the samples is then determined by comparing the O.D. of the samples to the standard curve.

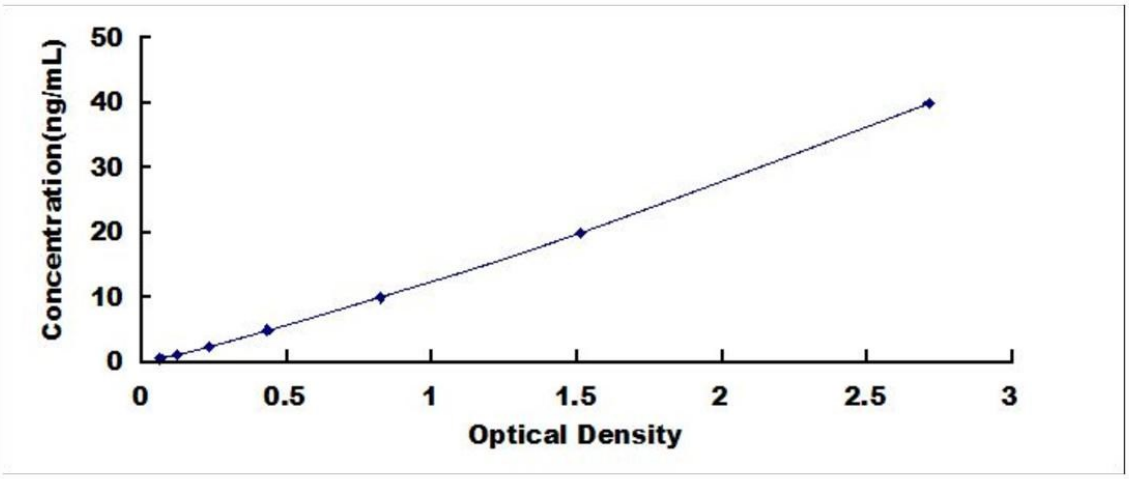
[CALCULATION OF RESULTS]

Average the duplicate readings for each standard, control, and samples and subtract the average zero standard optical density. Construct a standard curve by plotting the mean O.D. and concentration for each standard and

draw a best fit curve through the points on the graph or create a standard curve on log-log graph paper with CFD concentration on the y-axis and absorbance on the x-axis. Using some plot software, for instance, curve expert 1.30, is also recommended. If samples have been diluted, the concentration read from the standard curve must be multiplied by the dilution factor.

[TYPICAL DATA]

In order to make the calculation easier, we plot the O.D. value of the standard (X-axis) against the known concentration of the standard (Y-axis), although concentration is the independent variable and O.D. value is the dependent variable. However, the O.D. values of the standard curve may vary according to the conditions of assay performance (e.g. operator, pipetting technique, washing technique or temperature effects), plotting log of the data to establish standard curve for each test is recommended. Typical standard curve below is provided for reference only.



Typical Standard Curve for CFD, Human ELISA.

[DETECTION RANGE]

0.625-40ng/mL. The standard curve concentrations used for the ELISA's were 40ng/mL, 20ng/mL, 10ng/mL, 5ng/mL, 2.5ng/mL, 1.25ng/mL, 0.625ng/mL.

[SENSITIVITY]

The minimum detectable dose of CFD is typically less than 0.238ng/mL.
The sensitivity of this assay, or Lower Limit of Detection (LLD) was defined as the lowest protein concentration that could be differentiated from zero. It was determined by adding two standard deviations to the mean optical density value of twenty zero standard replicates and calculating the corresponding concentration.

[SPECIFICITY]

This assay has high sensitivity and excellent specificity for detection of CFD.
No significant cross-reactivity or interference between CFD and analogues was observed.

Note:

Limited by current skills and knowledge, it is impossible for us to complete the cross- reactivity detection between CFD and all the analogues, therefore, cross reaction may still exist.

[RECOVERY]

Matrices listed below were spiked with certain level of recombinant CFD and the recovery rates were calculated by comparing the measured value to the expected amount of CFD in samples.

Matrix	Recovery range (%)	Average(%)
serum(n=5)	80-94	87
EDTA plasma(n=5)	85-101	96
heparin plasma(n=5)	82-97	90

[LINEARITY]

The linearity of the kit was assayed by testing samples spiked with appropriate concentration of CFD and their serial dilutions. The results were demonstrated by the percentage of calculated concentration to the expected.

Sample	1：2	1：4	1：8	1：16
serum(n=5)	79-105%	89-97%	93-101%	84-98%
EDTA plasma(n=5)	78-90%	96-106%	85-103%	83-95%
heparin plasma(n=5)	80-96%	91-99%	81-91%	90-104%

Samples were diluted prior to assay as described in the SAMPLE PREPARATION section.

[PRECISION]

Intra-assay Precision (Precision within an assay): 3 samples with low, middle and high level CFD were tested 20 times on one plate, respectively.

Inter-assay Precision (Precision between assays): 3 samples with low, middle and high level CFD were tested on 3 different plates, 8 replicates in each plate.

CV(%) = SD/meanX100

Intra-Assay: CV<10%

Inter-Assay: CV<12%

[STABILITY]

The stability of ELISA kit is determined by the loss rate of activity. The loss rate of this kit is less than 5% prior to the expiration date under appropriate storage condition.

To minimize extra influence on the performance, operation procedures and lab conditions, especially room temperature, air humidity, incubator temperature should be strictly monitored. It is also strongly suggested that the assay is performed by the same operator from the beginning to the end.

[SAMPLE VALUE]

Serum/Plasma - Samples from apparently healthy volunteers were evaluated in this assay. No medical histories were available for the volunteers used in this assay.

Sample	Range(μg/mL)	Detectable(%)
Serum (n=28)	0.72-8.4	100
EDTA plasma (n=18)	0.68-8.1	100
Heparin plasma (n=18)	0.66-7.8	100

These data are our in-house data, only for reference.

[ASSAY PROCEDURE SUMMARY]

1. Prepare all reagents, samples and standards;
2. Add 100μL standard or sample to each well. Incubate 1 hour at 37°C;
3. Aspirate and add 100μL prepared Detection Reagent A. Incubate 1 hour at 37°C;
4. Aspirate and wash 3 times;
5. Add 100μL prepared Detection Reagent B. Incubate 30 minutes at 37°C;
6. Aspirate and wash 5 times;
7. Add 90μL Substrate Solution. Incubate 10-20 minutes at 37°C;
8. Add 50μL Stop Solution. Read at 450nm immediately.

[IMPORTANT NOTE]

1. Limited by the current conditions and scientific technology, we can't completely conduct the comprehensive identification and analysis on the raw material provided by suppliers. So there might be some qualitative and technical risks to use the kit.
2. The final experimental results will be closely related to validity of the products, so the kit should be used prior to the expiration date. And please store the kits exactly according to the instruction.
3. Kits from different batches may be a little different in detection range, sensitivity and color developing time. Please perform the experiment exactly according to the instruction attached in kit while electronic ones from our website is only for reference.
4. Do not mix or substitute reagents from one kit lot to another. Use only the reagents supplied by manufacturer.
5. Protect all reagents from strong light during storage and incubation. All the bottle caps of reagents should be covered tightly to prevent the evaporation and contamination of microorganism. TMB Substrate should remain colorless till it is reacted with the enzyme which binds to the microplate.
6. There may be some foggy substance in the wells when the plate is opened at the first time. It will not have any effect on the final assay results. Do not remove microplate from the storage bag until needed.
7. Wrong operations during the reagents preparation and loading, as well as incorrect parameter setting for the plate reader may lead to incorrect results. A microplate reader with a bandwidth of 10nm or less and an optical density range of 0-3 O.D. at 450 ± 10nm wavelength is acceptable for use in absorbance measurement. Please read the instruction carefully and adjust the instrument prior to the experiment.
8. Variation in sample preparation and each step of experimental operation may cause different results. In order to get better reproducible results, the operation of each step in the assay should be controlled.
9. Each kit has been strictly passed Q.C test. However, results from end users might be inconsistent with our in-house data due to some unexpected transportation conditions or different lab equipments. Intra-assay variance among kits from different batches might arise from above factors, too.

10. Kits from different manufacturers with the same item might produce different results, since we haven't compared our products with other manufacturers.
11. The standard of the kit and immunogen used for antibody preparation are commonly recombinant proteins, as different fragments, expression systems, purification methods might be used in recombinant protein preparation, we can not guarantee the kit could detect recombinant protein from other companies. So, it is not recommended to use the kit for the detection of recombinant protein.
12. Please predict the concentration of target molecules in samples, or arrange a preliminary experiment, it is a good way to solve specific problem, e.g. the concentration of samples are beyond the detection range of the kit.
13. The kit might not be suitable for detection of samples from some special experiment, for instance, knock-out experiments, due to their uncertainty of effectiveness.
14. The instruction manual is also for the kit of 48T, but all reagents of 48T kit are reduced by half.
15. The kit is designed for research use only, we will not be responsible for any issue if the kit was used in clinical diagnostic or any other procedures.

[PRECAUTION]

The Stop Solution suggested for use with this kit is an acid solution. Wear eye, hand, face, and clothing protection when using this material.

[TROUBLE SHOOTING]

Problem	Possible Source	Correction Action
Poor Standard Curve	Improper standard curve preparation	Ensure accurate operation of the dilution
	Incomplete washing and aspiration	Adequate washing and adequate aspiration
	Inaccurate Pipetting	Check and Calibrate pipettes
Poor Precision	Incomplete washing of wells	Ensure sufficient washing
	Inadequate mixing and aspiration reagents	Adequate aspiration and mixing reagents
	Reused pipette tips, containers and sealers	Change and use new pipette tips, containers and sealers
	Inaccurate Pipetting	Check and Calibrate pipettes
Low O.D Values	Inadequate reagent volumes added to wells	Calibrate pipettes and Add adequate reagents
	Incorrect incubation times	Ensure sufficient incubation times
	Incorrect incubation temperature	Reagents balanced to room temperature
	Conjugate or substrate reagent failure	Mix conjugate & substrate, color should develop immediately
	No stop solution added	Follow the assay protocol in the kit manual
	Read beyond suggested reading time	Read within the time recommended in the manual
Sample Values	Improper Sample Storage	Store the sample properly and use the fresh sample
	Improper sample collection and preparation	Take proper sample collection and preparation method
	Low quantity of analyte in samples	Use new sample and repeat assay

Appendix H

04469658190c501V11.0

ALBT2

cobas®

Tina-quant Albumin Gen.2

Order information

REF	CONTENT		Analyzer(s) on which **cobas c** pack(s) can be used
04469658 190	Tina-quant Albumin Gen.2 100 tests	System-ID 07 6743 3	Roche/Hitachi **cobas c** 311, **cobas c** 501/502
03121305 122	C.f.a.s. PUC (5 x 1 mL)	Code 489	
03121313 122	Precinorm PUC (4 x 3 mL)	Code 240	
03121291 122	Precipath PUC (4 x 3 mL)	Code 241	
10557897 122	Precinorm Protein (3 x 1 mL)	Code 302	
11333127 122	Precipath Protein (3 x 1 mL)	Code 303	
05117003 190	PreciControl ClinChem Multi 1 (20 x 5 mL)	Code 391	
05947626 190	PreciControl ClinChem Multi 1 (4 x 5 mL)	Code 391	
05117216 190	PreciControl ClinChem Multi 2 (20 x 5 mL)	Code 392	
05947774 190	PreciControl ClinChem Multi 2 (4 x 5 mL)	Code 392	
04489357 190	Diluent NaCl 9 % (50 mL)	System-ID 07 6869 3	

English

System information

For **cobas c** 311/501 analyzers:

ALBU2: ACN 253 (Albumin in urine)

ALBS2: ACN 128 (Albumin in serum)

ALBC2: ACN 412 (Albumin in CSF)

For **cobas c** 502 analyzer:

ALBU2: ACN 8253 (Albumin in urine)

ALBS2: ACN 8128 (Albumin in serum)

ALBC2: ACN 8412 (Albumin in CSF)

Intended use

In vitro test for the quantitative determination of albumin in human urine, serum, plasma and CSF (albumin CSF/serum ratio) on Roche/Hitachi **cobas c** systems.

Summary[1,2,3,4,5,6,7,8,9,10]

Albumin is a non-glycosylated protein with a molecular weight of 66000 daltons. It is synthesized in liver parenchymal cells at a rate of 14 g/day. Quantitatively, albumin is normally the most important protein component (> 50 %) in plasma, CSF and urine. A small, but abnormal albumin excretion in urine is known as microalbuminuria. Causes of microalbuminuria can be glomerular (e.g. due to diabetic microangiopathy, hypertension, minor glomerular lesion), tubular (inhibition of reabsorption) or postrenal. Albumin is also a marker protein for various forms of proteinuria.

In selective glomerular proteinuria, 100-3000 mg albumin/g creatinine are excreted in the urine. Non-selective glomerular proteinuria is characterized by elevated excretion of high-molecular weight proteins (IgG more than 10 % of the albumin value). Prerenal proteinuria is recognized by a discrepancy between albumin and total protein (albumin accounting for less than 30 %, with concurrent elevation of total protein). Simultaneous elevation of albumin and microproteins is found in glomerulotubular proteinuria occurring due to overloading of tubular reabsorption in glomerulopathy (e.g. nephrotic syndrome), combined glomerular tubulointerstitial nephropathy or in renal failure following diabetic nephropathy or other causes (overflow proteinuria). Albumin has two main functions in plasma: maintaining the oncotic pressure (80 % due to albumin in plasma) and transport. It is the most important transport protein for substances having low water solubility (such as free fatty acids, bilirubin, metal ions, hormones and pharmaceuticals).

Depressed albumin levels are caused by hyperhydration, hepatocellular synthesis insufficiency, secretion disorders in the intravascular space, abnormal distribution between the intravascular and extravascular space, catabolism and loss of albumin, acute phase reactions and congenital analbuminemia.

Blood brain barrier disorders can be reliably quantified with the aid of the albumin CSF/serum ratio. Elevated albumin ratios are indicative of a blood brain barrier disorder.

By simultaneously determining IgG in CSF and serum while taking into account the individual albumin ratios, it is possible to differentiate between IgG originating from the blood and CNS-synthesized immunoglobulin. IgG predominates in multiple sclerosis, chronic HIV encephalitis, neurosyphilis and herpes simplex encephalitis.

A variety of methods, such as radial immunodiffusion, nephelometry and turbidimetry, are available for the determination of albumin.

Test principle[1]

Immunoturbidimetric assay.
Anti-albumin antibodies react with the antigen in the sample to form antigen/antibody complexes which, following agglutination, are measured turbidimetrically.

Reagents - working solutions

R1 TRIS buffer: 50 mmol/L, pH 8.0; PEG: 4.2 %; EDTA: 2.0 mmol/L; preservative

R2 Polyclonal anti-human albumin antibodies (sheep): dependent on titer; TRIS buffer: 100 mmol/L, pH 7.2; preservative

R3 Reagent for antigen excess check.
Albumin in diluted serum (human); NaCl: 150 mmol/L; phosphate buffer: 50 mmol/L, pH 7.0; preservative

R1 is in position A, R2 is in position B and R3 is in position C.

Precautions and warnings

For in vitro diagnostic use.
Exercise the normal precautions required for handling all laboratory reagents.
Disposal of all waste material should be in accordance with local guidelines.
Safety data sheet available for professional user on request.

All human material should be considered potentially infectious. All products derived from human blood are prepared exclusively from the blood of donors tested individually and shown to be free from HBsAg and antibodies to HCV and HIV.
The testing methods applied were FDA-approved or cleared in compliance with the European Directive 98/79/EC, Annex II, List A.
However, as no testing method can rule out the potential risk of infection with absolute certainty, the material should be handled with the same level of care as a patient specimen. In the event of exposure, the directives of the responsible health authorities should be followed.[11,12]

Reagent handling

Ready for use

Storage and stability

ALBT2

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.
On-board in use and refrigerated on the analyzer:	12 weeks

Diluent NaCl 9 %

Shelf life at 2-8 °C:	See expiration date on **cobas c** pack label.

ALBT2

Tina-quant Albumin Gen.2

cobas®

On-board in use and refrigerated on the analyzer: 12 weeks

Specimen collection and preparation

For specimen collection and preparation only use suitable tubes or collection containers.

Only the specimens listed below were tested and found acceptable.
Urine
Serum
Plasma: Li-heparin and K_2-EDTA plasma
CSF

The sample types listed were tested with a selection of sample collection tubes that were commercially available at the time of testing, i.e. not all available tubes of all manufacturers were tested. Sample collection systems from various manufacturers may contain differing materials which could affect the test results in some cases. When processing samples in primary tubes (sample collection systems), follow the instructions of the tube manufacturer.

Centrifuge samples containing precipitates before performing the assay.

CSF

Stability:[13]	up to 3 days	at 2-8 °C
	6 months	at (-15)-(-25) °C
	indefinitely	at (-60)-(-80) °C

Serum, plasma

Stability:[14]	10 weeks	at 15-25 °C
	5 months	at 2-8 °C
	4 months	at (-15)-(-25) °C

Urine

Spontaneous, 24-hour urine or 2nd morning urine.

Stability:[14]	7 days	at 15-25 °C
	1 month	at 2-8 °C
	6 months	at (-15)-(-25) °C

Materials provided

See "Reagents – working solutions" section for reagents.

Materials required (but not provided)

- See "Order information" section

General laboratory equipment

Assay

For optimum performance of the assay follow the directions given in this document for the analyzer concerned. Refer to the appropriate operator's manual for analyzer-specific assay instructions.

The performance of applications not validated by Roche is not warranted and must be defined by the user.

Application for urine

cobas c 311 test definition

Assay type	2-Point End	
Reaction time / Assay points	10 / 6-15	
Wavelength (sub/main)	700/340 nm	
Reaction direction	Increase	
Units	mg/L (µmol/L, mg/dL)	
Reagent pipetting		Diluent (H_2O)
R1	100 µL	–
R2	20 µL	–
R3	6 µL	20 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	6.0 µL	–	–
Decreased	6.0 µL	15 µL	150 µL
Increased	6.0 µL	–	–

cobas c 501 test definition

Assay type	2-Point End	
Reaction time / Assay points	10 / 10-34	
Wavelength (sub/main)	700/340 nm	
Reaction direction	Increase	
Units	mg/L (µmol/L, mg/dL)	
Reagent pipetting		Diluent (H_2O)
R1	100 µL	–
R2	20 µL	–
R3	6 µL	20 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	6.0 µL	–	–
Decreased	6.0 µL	15 µL	150 µL
Increased	6.0 µL	–	–

cobas c 502 test definition

Assay type	2-Point End	
Reaction time / Assay points	10 / 10-34	
Wavelength (sub/main)	700/340 nm	
Reaction direction	Increase	
Units	mg/L (µmol/L, mg/dL)	
Reagent pipetting		Diluent (H_2O)
R1	100 µL	–
R2	20 µL	–
R3	6 µL	20 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	6.0 µL	–	–
Decreased	6.0 µL	15 µL	150 µL
Increased	12 µL	–	–

Application for serum and plasma

cobas c 311 test definition

Assay type	2-Point End	
Reaction time / Assay points	10 / 6-18	
Wavelength (sub/main)	700/340 nm	
Reaction direction	Increase	
Units	g/L (µmol/L, mg/dL)	

ALBT2

Tina-quant Albumin Gen.2

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Reagent pipetting		Diluent (H_2O)
R1	100 µL	–
R2	20 µL	–

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	1.5 µL	1.5 µL	180 µL
Decreased	1.5 µL	1.5 µL	180 µL
Increased	1.5 µL	1.5 µL	180 µL

cobas c 501 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 10-34
Wavelength (sub/main)	700/340 nm
Reaction direction	Increase
Units	g/L (µmol/L, mg/dL)
Reagent pipetting	Diluent (H_2O)
R1	100 µL –
R2	20 µL –

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2.0 µL	2.1 µL	175 µL
Decreased	2.0 µL	1.7 µL	180 µL
Increased	2.0 µL	2.1 µL	175 µL

cobas c 502 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 10-34
Wavelength (sub/main)	700/340 nm
Reaction direction	Increase
Units	g/L (µmol/L, mg/dL)
Reagent pipetting	Diluent (H_2O)
R1	100 µL –
R2	20 µL –

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	2.0 µL	2.1 µL	175 µL
Decreased	2.0 µL	1.7 µL	180 µL
Increased	4.0 µL	2.1 µL	175 µL

Application for CSF

cobas c 311 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 6-15
Wavelength (sub/main)	700/340 nm
Reaction direction	Increase
Units	mg/L (µmol/L, mg/dL)
Reagent pipetting	Diluent (H_2O)
R1	100 µL –
R2	20 µL –
R3	6 µL 20 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	6.0 µL	10 µL	110 µL
Decreased	3 µL	5 µL	180 µL
Increased	6.0 µL	10 µL	110 µL

cobas c 501 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 10-34
Wavelength (sub/main)	700/340 nm
Reaction direction	Increase
Units	mg/L (µmol/L, mg/dL)
Reagent pipetting	Diluent (H_2O)
R1	100 µL –
R2	20 µL –
R3	6 µL 20 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	6.0 µL	10 µL	110 µL
Decreased	3 µL	5 µL	180 µL
Increased	6.0 µL	10 µL	110 µL

cobas c 502 test definition

Assay type	2-Point End
Reaction time / Assay points	10 / 10-34
Wavelength (sub/main)	700/340 nm
Reaction direction	Increase
Units	mg/L (µmol/L, mg/dL)
Reagent pipetting	Diluent (H_2O)
R1	100 µL –
R2	20 µL –
R3	6 µL 20 µL

Sample volumes	*Sample*	*Sample dilution*	
		Sample	*Diluent (NaCl)*
Normal	6.0 µL	10 µL	110 µL
Decreased	3 µL	5 µL	180 µL
Increased	12.0 µL	10 µL	110 µL

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Calibration

Calibrators	S1: H_2O			
	S2-6: C.f.a.s. PUC			
	Multiply the lot-specific C.f.a.s. PUC calibrator value by the factors below to determine the standard concentrations for the 6-point calibration curve:			
cobas c 501/502	S2:	0.0138	S5:	0.467
	S3:	0.0228	S6:	1.00
	S4:	0.0455		
cobas c 311	S2:	0.0276	S5:	0.467
	S3:	0.0456	S6:	1.00
	S4:	0.0909		

Calibration mode: RCM

Calibration frequency:
Full calibration
- after reagent lot change
- and as required following quality control procedures

Traceability: This method has been standardized against the certified reference material in human serum of the IRMM (Institute for Reference Material and Measurements) ERM-DA470k/IFCC.

Quality control

For quality control, use control materials as listed in the "Order information" section.

In addition, other suitable control material can be used.

ALBU2: Precinorm PUC, Precipath PUC

ALBS2: Precinorm Protein, Precipath Protein, PreciControl ClinChem Multi 1, PreciControl ClinChem Multi 2

ALBC2: undiluted Precipath PUC

The control intervals and limits should be adapted to each laboratory's individual requirements. Values obtained should fall within the defined limits. Each laboratory should establish corrective measures to be taken if values fall outside the defined limits.

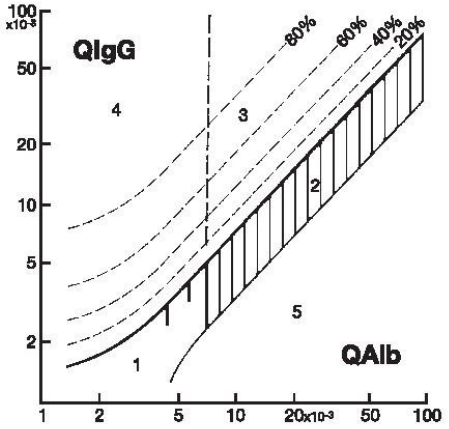
Follow the applicable government regulations and local guidelines for quality control.

Calculation

Roche/Hitachi **cobas c** systems automatically calculate the analyte concentration of each sample.

Conversion factors:
g/L x 100 = mg/dL
g/L x 15.2 = µmol/L
mg/L x 0.1 = mg/dL
mg/L x 0.0152 = µmol/L

The calculation employs a ratio diagram including hyperbolic functions as differential lines according to Reiber and Felgenhauer. Results from the determination of IgG and albumin in CSF and serum (IgG and albumin ratios)[15] are plotted.



1. Reference range. 2. Blood brain barrier functional disorder without local IgG synthesis. 3. Blood brain barrier functional disorder with concomitant IgG-synthesis in the CNS. 4. IgG synthesis in the CNS without blood brain barrier functional disorder. 5. As confirmed empirically, there are no values in this region (i.e. values here are due to errors introduced by blood sampling or analytical errors). Generally speaking, cases not associated with local IgG synthesis in the CNS lie below the bold line (hyperbolic function). The percentage values indicate what percentage of the total IgG in CSF (minimum) originates in the CNS relative to the statistically-defined 0 % differential lines.

Limitations - interference

Urine

Criterion: Recovery within ± 10 % of initial value at an albumin concentration of 20 mg/L (0.30 µmol/L, 2 mg/dL).

Icterus: No significant interference up to a conjugated bilirubin concentration of 855 µmol/L or 50 mg/dL.

Hemolysis: No significant interference up to a hemoglobin concentration of 248 µmol/L or 400 mg/dL.

No interference by acetone ≤ 60 mmol/L, ammonia chloride ≤ 0.11 mol/L, calcium ≤ 40 mmol/L, creatinine ≤ 0.18 mol/L, γ-globulin ≤ 500 mg/L, glucose ≤ 0.19 mol/L, urea ≤ 0.8 mol/L, uric acid ≤ 5.95 mmol/L and urobilinogen ≤ 378 µmol/L.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[16]

High dose hook-effect: Using the prozone check, no false result without a flag was observed up to an albumin concentration of 40000 mg/L (608 µmol/L, 4000 mg/dL).

Serum/plasma

Criterion: Recovery within ± 10 % of initial value at an albumin concentration of 35 g/L (532 µmol/L, 3500 mg/dL).

Icterus:[17] No significant interference up to an I index of 60 for conjugated and unconjugated bilirubin (approximate conjugated and unconjugated bilirubin concentration: 1026 µmol/L or 60 mg/dL).

Hemolysis:[17] No significant interference up to an H index of 1000 (approximate hemoglobin concentration: 621 µmol/L or 1000 mg/dL).

Lipemia (Intralipid):[17] No significant interference up to an L index of 1500. There is poor correlation between the L index (corresponds to turbidity) and triglycerides concentration.

Rheumatoid factors ≤ 1200 IU/mL do not interfere.

Drugs: No interference was found at therapeutic concentrations using common drug panels.[16,16]

In very rare cases, gammopathy, in particular type IgM (Waldenström's macroglobulinemia), may cause unreliable results.[19]

CSF

Criterion: Recovery within ± 10 % of initial value at an albumin concentration of 240 mg/L (3.65 µmol/L, 24 mg/dL).

Hemolysis: No significant interference up to a hemoglobin concentration of 620 µmol/L or 1000 mg/dL.

High dose hook-effect: Using the prozone check, no false result without a flag was observed up to an albumin concentration of 30000 mg/L (456 µmol/L, 3000 mg/dL).

For diagnostic purposes, the results should always be assessed in conjunction with the patient's medical history, clinical examination and other findings.

ACTION REQUIRED

Special Wash Programming: The use of special wash steps is mandatory when certain test combinations are run together on Roche/Hitachi **cobas c** systems. The latest version of the carry-over evasion list can be found with the NaOHD/SMS/Multiclean/SCCS or the NaOHD/SMS/SmpCln1+2/SCCS Method Sheets. For further instructions refer to the operator's manual. **cobas c** 502 analyzer: All special wash programming necessary for avoiding carry-over is available via the **cobas** link, manual input is not required.

Where required, special wash/carry-over evasion programming must be implemented prior to reporting results with this test.

Limits and ranges

Measuring range

Urine

cobas c 501/502: 3-400 mg/L (0.05-6.08 µmol/L, 0.3-40 mg/dL)

cobas c 311: 3-200 mg/L (0.05-3.04 µmol/L, 0.3-20 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:11 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 11.

Serum, plasma

cobas c 501/502: 3-101 g/L (46-1540 µmol/L, 300-10100 mg/dL)

cobas c 311: 3-96 g/L (46-1459 µmol/L, 300-9600 mg/dL)

cobas c 501/502: Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:1.27 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 1.27.

cobas c 311: Determine samples having higher concentrations by a manual predilution of 1:2. Calculate the final results by multiplying the measured value with a factor of 2.

CSF

cobas c 501/502: 36-4800 mg/L (0.55-73.0 µmol/L, 3.6-480 mg/dL)

cobas c 311: 36-2400 mg/L (0.55-36.5 µmol/L, 3.6-240 mg/dL)

Determine samples having higher concentrations via the rerun function. Dilution of samples via the rerun function is a 1:6.2 dilution. Results from samples diluted using the rerun function are automatically multiplied by a factor of 6.2.

Lower limits of measurement

Limit of Blank (LoB) and Limit of Detection (LoD)

Urine

LoB = 2 mg/L

LoD = 3 mg/L

Serum, plasma

LoB = 1 g/L

LoD = 2 g/L

CSF

LoB = 20 mg/L

LoD = 36 mg/L

The Limit of Blank and Limit of Detection were determined in accordance with the CLSI (Clinical and Laboratory Standards Institute) EP17-A requirements.

The Limit of Blank is the 95th percentile value from n ≥ 60 measurements of analyte-free samples over several independent series. The Limit of Blank corresponds to the concentration below which analyte-free samples are found with a probability of 95 %.

The Limit of Detection is determined based on the Limit of Blank and the standard deviation of low concentration samples.

The Limit of Detection corresponds to the lowest analyte concentration which can be detected (value above the Limit of Blank with a probability of 95 %).

Values below the Limit of Detection (≤ 3 mg/L (urine); ≤ 3 g/L (serum, plasma); ≤ 36 mg/L (CSF)) will not be flagged by the instrument.

Expected values

Urine

2nd morning urine:[5]

Adults:	< 20 mg albumin/g creatinine or < 2.26 g (34.35 µmol) albumin/mol creatinine
Children (3-5 years):[20]	< 20 mg/L (0.304 µmol/L, 2 mg/dL) albumin < 37 mg albumin/g creatinine
24-hour urine:[21]	< 20 mg/L (0.304 µmol/L, 2 mg/dL) < 30 mg/24 h (0.456 µmol/24 h)

Serum/plasma

Reference Range Study:[22]

Adults	3.56-4.61 g/dL (35.6-46.1 g/L; 541-701 µmol/L)

Consensus values:[23]

Adults	3.5-5.2 g/dL (35-52 g/L; 532-790 µmol/L)

Reference intervals according to Tietz:[24]

Newborns 0-4 d:	2.8-4.4 g/dL (28-44 g/L; 426-669 µmol/L)
Children 4 d-14 yr:	3.8-5.4 g/dL (38-54 g/L; 578-821 µmol/L)
Children 14-18 yr:	3.2-4.5 g/dL (32-45 g/L; 486-684 µmol/L)

Albumin CSF/serum ratio ($Q_{ALB} \times 10^3$)

Adults:[6]	up to 15 years	5.0
	up to 40 years	6.5
	up to 60 years	8.0

Each laboratory should investigate the transferability of the expected values to its own patient population and if necessary determine its own reference ranges.

Specific performance data

Representative performance data on the analyzers are given below. Results obtained in individual laboratories may differ.

Precision

Precision was determined using human samples and controls in an internal protocol with repeatability (n = 21) and intermediate precision (3 aliquots per run, 1 run per day, 21 days). The following results were obtained:

Urine

Repeatability	*Mean*	*SD*	*CV*
	mg/L (µmol/L, mg/dL)	*mg/L (µmol/L, mg/dL)*	*%*
Precinorm PUC	30.7 (0.467, 3.07)	0.2 (0.003, 0.02)	0.8
Precipath PUC	108 (1.64, 10.8)	1 (0.01, 0.1)	0.7
Human urine 1	14.3 (0.217, 1.43)	0.2 (0.003, 0.02)	1.6
Human urine 2	252 (3.83, 25.2)	4 (0.06, 0.4)	1.6

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Intermediate precision	*Mean*	*SD*	*CV*
	mg/L (µmol/L, mg/dL)	*mg/L (µmol/L, mg/dL)*	*%*
Precinorm PUC	31.2 (0.474, 3.12)	0.5 (0.008, 0.05)	1.7
Precipath PUC	105 (1.60, 10.5)	1 (0.02, 0.1)	1.2
Human urine 3	13.6 (0.207, 1.36)	0.4 (0.006, 0.04)	2.8
Human urine 4	60.6 (0.921, 6.06)	1.4 (0.021, 0.14)	2.3

Serum/plasma

Repeatability	*Mean*	*SD*	*CV*
	g/L (µmol/L, mg/dL)	*g/L (µmol/L, mg/dL)*	*%*
Precinorm Protein	39.9 (606, 3990)	0.5 (8, 50)	1.2
Precipath Protein	66.6 (1012, 6660)	1.4 (21, 140)	2.1
Human serum 1	27.6 (420, 2760)	0.3 (5, 40)	1.3
Human serum 2	62.5 (950, 6250)	0.9 (14, 90)	1.5

Intermediate precision	*Mean*	*SD*	*CV*
	g/L (µmol/L, mg/dL)	*g/L (µmol/L, mg/dL)*	*%*
Precinorm Protein	42.3 (643, 4230)	0.9 (14, 90)	2.0
Precipath Protein	70.5 (1072, 7050)	1.6 (24, 160)	2.2
Human serum 3	7.78 (118, 778)	0.74 (11, 74)	9.5
Human serum 4	36.2 (550, 3620)	0.7 (11, 70)	2.1

CSF

Repeatability	*Mean*	*SD*	*CV*
	mg/L (µmol/L, mg/dL)	*mg/L (µmol/L, mg/dL)*	*%*
Precipath PUC	99.2 (1.51, 9.92)	1.4 (0.02, 0.14)	1.4
Human CSF 1	174 (2.64, 17.4)	3 (0.05, 0.3)	1.7
Human CSF 2	383 (5.82, 38.3)	4 (0.06, 0.4)	1.0
C.f.a.s. PUC	454 (6.90, 45.4)	4 (0.06, 0.4)	0.8

Intermediate precision	*Mean*	*SD*	*CV*
	mg/L (µmol/L, mg/dL)	*mg/L (µmol/L, mg/dL)*	*%*
Precipath PUC	91.0 (1.38, 9.1)	2.9 (0.04, 0.29)	3.2
Control level 2	389 (5.91, 38.9)	7 (0.11, 0.7)	1.7
Human CSF 3	166 (2.53, 16.6)	4 (0.06, 0.4)	2.3
Human CSF 4	366 (5.56, 36.6)	5 (0.07, 0.5)	1.3

Method comparison

Urine

Albumin values for human urine samples obtained on a Roche/Hitachi **cobas c** 501 analyzer (**y**) were compared with those determined using the same reagent on a Roche/Hitachi 917 analyzer (**x**).

Sample size (n) = 129

Passing/Bablok[25]	Linear regression
y = 1.021**x** - 2.91 mg/L	y = 1.026**x** - 3.66 mg/L
τ = 0.984	r = 0.999

The sample concentrations were between 4.60 and 386 mg/L (0.070 and 5.87 µmol/L, 0.460 and 38.6 mg/dL).

Serum/plasma

Albumin values for human serum and plasma samples obtained on a Roche/Hitachi **cobas c** 501 analyzer (**y**) were compared with those determined with a nephelometric albumin test (**x**).

Sample size (n) = 80

Passing/Bablok[25]	Linear regression
y = 0.950**x** + 0.195 g/L	y = 0.941**x** + 0.581 g/L
τ = 0.923	r = 0.993

The sample concentrations were between 5.70 and 107 g/L (86.6 and 1626 µmol/L, 570 and 10700 mg/dL).

CSF

Albumin values for human CSF samples obtained on a Roche/Hitachi **cobas c** 501 analyzer (**y**) were compared with those determined with a nephelometric albumin test (**x**).

Sample size (n) = 85

Passing/Bablok[25]	Linear regression
y = 1.000**x** - 8.75 mg/L	y = 0.991**x** + 0.301 mg/L
τ = 0.936	r = 0.992

The sample concentrations were between 115 and 2640 mg/L (1.75 and 40.1 µmol/L, 11.5 and 264 mg/dL).

References

1 Multicenter study of Tina-quant Albumin in urine and β-N-acetyl-glucosaminidase (β-NAG) in urine. Workshop Munich, November 29-30, 1990 Wien Klin Wschr 1991;103 Suppl.189:1-64.

2 Greiling H, Gressner AM, eds. Lehrbuch der Klinischen Chemie und Pathobiochemie, 3rd ed. Stuttgart/New York: Schattauer Verlag 1995:749-750.

3 Rothschild MA, Oratz M, Schreiber SS. Serum albumin. Hepatology 1988;8(2):385-401.

4 Schaufelberger H, Caduff F, Engler F, et al. Evaluation eines Streifentests (Micral-Test) zur semiquantitativen Erfassung der Mikroalbuminurie in der Praxis. Schweiz Med Wschr 1992;122:576-581.

5 Hofmann W, Guder WG. A diagnostic program for quantitative analysis of proteinurea. J Clin Chem Clin Biochem 1989;27:589-600.

6 Reiber H. External Quality Assessment in Clinical Neurochemistry: Survey of Analysis for Cerebrospinal Fluid (CSF) Proteins based on CSF/Serum Quotients. Clin Chem 1995;41:256-263.

7 Reiber H, Felgenhauer K. Protein transfer of the blood cerebrospinal fluid barrier and the quantitation of the humoral immune response within the central nervous system. Clin Chim Acta 1987;163(3):319-328.

8 Reiber H. Die diagnostische Bedeutung neuroimmunologischer Reaktionsmuster im Liquor cerebrospinalis. Lab med 1995;19:444-462.

9 Zimmermann K, Marr U, Linke E. Liquordiagnostik, MTA 1996;11:258-260.

10 Reiber H. Flow rate of cerebrospinal fluid (CSF) – a concept common to normal blood-CSF barrier function and to dysfunction in neurological diseases. J Neurol Sci 1994;122:189-203.

11 Occupational Safety and Health Standards: bloodborne pathogens. (29 CFR Part 1910.1030). Fed. Register.

12 Directive 2000/54/EC of the European Parliament and Council of 18 September 2000 on the protection of workers from risks related to exposure to biological agents at work.

13 Tietz NW, ed. Clinical Guide to Laboratory Tests, 3rd ed. Philadelphia, PA: WB Saunders Company 1995;24.

14 Use of Anticoagulants in Diagnostic Laboratory Investigations. WHO Publication WHO/DIL/LAB/99.1 Rev. 2. Jan. 2002.

15 Reiber H. The hyperbolic function: a mathematical solution of the protein flux/CSF flow model for blood-CSF barrier function. J Neurol Sci 1994;126:243-245.

16 Sonntag O, Scholer A. Drug interference in clinical chemistry: recommendation of drugs and their concentrations to be used in drug interference studies. Ann Clin Biochem 2001;38:376-385.

17 Glick MR, Ryder KW, Jackson SA. Graphical Comparisons of Interferences in Clinical Chemistry Instrumentation. Clin Chem 1986;32:470-475.

18 Breuer J. Report on the Symposium "Drug effects in Clinical Chemistry Methods". Eur J Clin Chem Clin Biochem 1996;34:385-386.

19 Bakker AJ, Mücke M. Gammopathy interference in clinical chemistry assays: mechanisms, detection and prevention. Clin Chem Lab Med 2007;45(9):1240-1243.

20 Hubbuch A. Results of a multicenter study of provisional reference ranges for albumin in urine of children and adults. Roche publication.

21 Hasslacher C. Diagnostische Überwachung und Therapie in den Stadien der diabetischen Nierenerkrankung. Akt Endokr Stoffw 1989;10:60-63.

22 Junge W, Bossert-Reuther S, Klein G, et al. Reference Range Study for Serum Albumin using different methods. Clin Chem Lab Med (June 2007 Poster EUROMEDLAB) 2007;45 Suppl:194.

23 Dati F, Schumann G, Thomas L, et al. Consensus of a group of professional societies and diagnostic companies on guidelines for interim reference ranges for 14 proteins in serum based on the standardization against the IFCC/BCR/CAP reference material (CRM 470). Eur J Clin Chem Clin Biochem 1996;34:517-520.

24 Burtis CA, Ashwood ER, Bruns DE (eds.). Tietz Textbook of Clinical Chemistry and Molecular Diagnostics. 4th ed. St Louis, Missouri; Elsevier Saunders 2006;549.

25 Bablok W, Passing H, Bender R, et al. A general regression procedure for method transformation. Application of linear regression procedures for method comparison studies in clinical chemistry, Part III. J Clin Chem Clin Biochem 1988 Nov;26(11):783-790.

A point (period/stop) is always used in this Method Sheet as the decimal separator to mark the border between the integral and the fractional parts of a decimal numeral. Separators for thousands are not used.

Symbols

Roche Diagnostics uses the following symbols and signs in addition to those listed in the ISO 15223-1 standard.

CONTENT	Contents of kit
→	Volume after reconstitution or mixing

CE

Roche Diagnostics GmbH, Sandhofer Strasse 116, D-68305 Mannheim
www.roche.com