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The implementation of laboratory investigations for diagnosing pyruvate kinase deficiency at the Johannesburg Hospital

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CHAPTER 1 INTRODUCTION

Numerous red cell enzyme disorders have been identified over the past few decades. However, very few diagnostic tests are routinely performed for these disorders due to technical difficulties of the assays and the heterogeneity of the genetic mutations. From a practical point of view, each enzyme assay generally requires different buffer systems, different concentrations of coenzymes / cofactors and can be labour intensive, which makes the analysis costly and logistically impractical. Fortunately many of these enzyme deficiencies are extremely rare and do not require routine testing. Glucose-6-phosphate dehydrogenase (G6PD) and pyruvate kinase (PK) deficiencies together constitute more than 90% of all red cell enzyme disorders (Eber, 2003) and it follows that initial investigations for this group of disorders should be directed at these two conditions. In South Africa, a screening test for G6PD deficiency is the only routinely available red cell enzyme assay for clinically suspicious cases. In this report the pathology related to PK deficiency is reviewed and the implementation of qualitative and quantitative assays for PK deficiency is described. In addition, mutation analysis of a PK deficient patient identified during the implementation of the PK assay is described.

1.1 The pathology of PK deficiency

Valentine et al (1961) are credited with identifying PK deficiency while investigating an apparently hereditary cause of non-spherocytic haemolytic anaemia. PK is a rate-controlling enzyme which catalyzes one of the reactions in the Embden-Meyerhof

pathway of anaerobic glycolysis (Figure 1). The reaction generates pyruvate from phosphoenolpyruvate (PEP) (equation 1) and is of critical importance in mature erythrocytes which lack mitochondria and therefore rely on anaerobic glycolysis for the generation of ATP. A PK deficiency is compensated for by oxidative phosphorylation in most cells, but in erythrocytes it results in a disruption of the membrane electrochemical gradients leading to cell water loss, cell shrinkage, membrane damage and ultimately premature destruction in the spleen.

$$\text{PEP} + \text{ADP} \xrightarrow{\text{PK } (+ K^{+} \text{ and } Mg^{2+})} \text{Pyruvate} + \text{ATP} \qquad \text{(equation 1)}$$

The degree of severity of PK deficiency is highly variable, depending on the mutant enzyme activity (Eber, 2003). Clinical presentations are therefore variable but always related to excessive haemolysis. Some patients are asymptomatic and reach adult life without being diagnosed and may be found to be PK deficient after investigations for a mild anaemia. Clinical symptoms include anaemia, jaundice, splenomegaly and pigmented gallstones. In more severely affected individuals anaemia and jaundice are present from birth and they may require regular blood transfusions. The haemolysis may be exacerbated during periods of infection or other stressors. In the most severe cases a hydrops foetalis picture may occur (Eber, 2003).

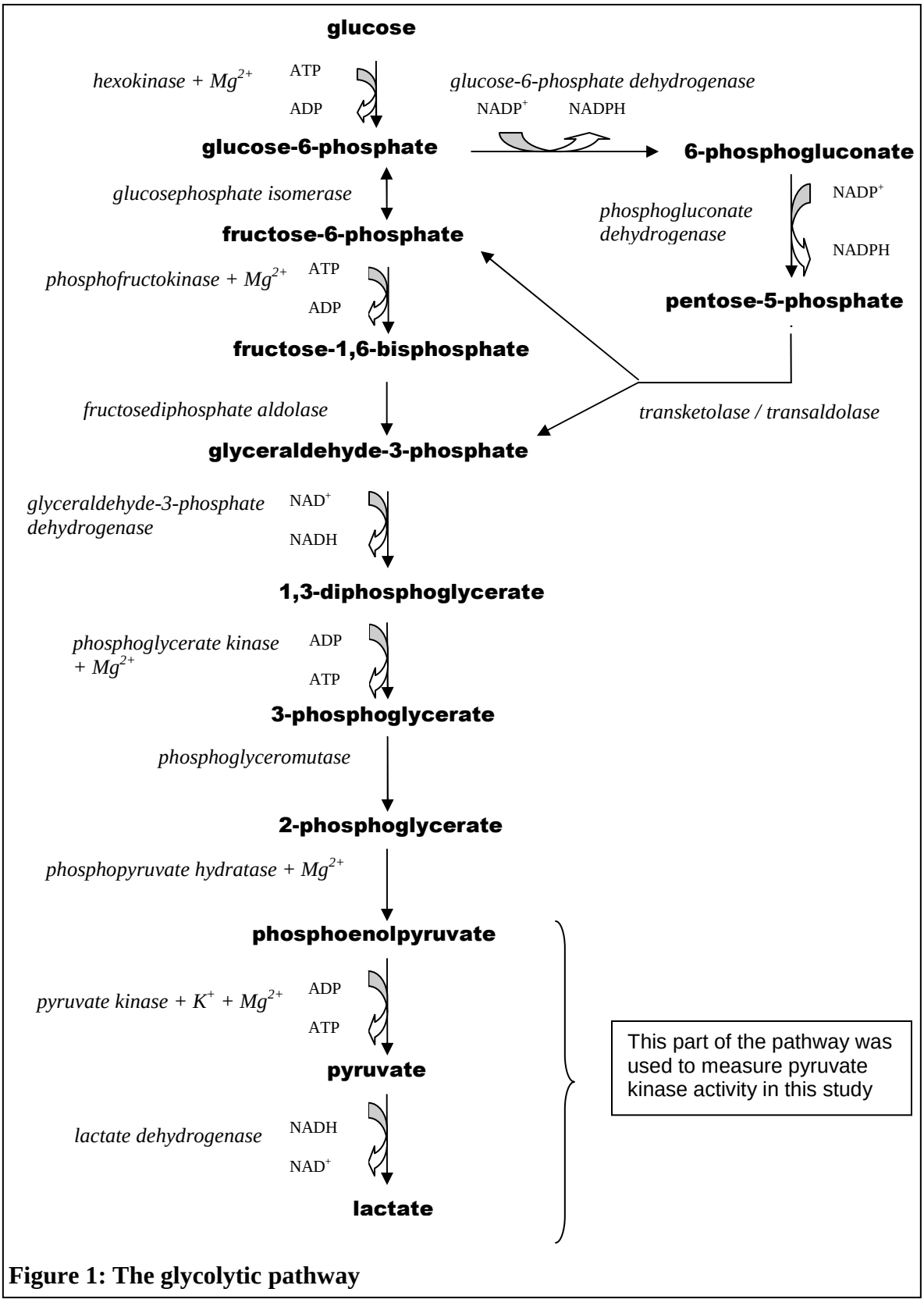


Figure 1: The glycolytic pathway

The morphological findings on peripheral blood smears are equally variable. Most cases exhibit a mild macrocytosis (as a result of a reticulocytosis and/or an associated megaloblastosis) with morphologically normal red cells. Occasionally erythrocytes develop irregular membrane projections (so-called “prickle cells”) and in severe cases there may be a marked anisopoikilocytosis. Pappenheimer bodies have been noted, particularly in patients that have undergone splenectomy.

1.2 Epidemiology

As is usual with relatively rare heterogeneous genetic diseases, estimating the prevalence of PK deficiency has been very difficult. Most cases of PK deficiency have been reported in Caucasians in North America, Europe, Australia, New Zealand, east Asia and near east Asia with the highest disease prevalence associated with populations that demonstrate a “founder effect” like the Amish in Pennsylvania, USA and European gypsies (reviewed in Zanella et al, 2005). One large scale study based on mutation analysis and frequently cited as a reference study, concluded that the prevalence of PK deficiency is 51 cases per million in the general Caucasian population (Beutler and Gelbart, 2000). For a long time it was assumed that the disease was rare in Africans but it became clear that the reason for this was that investigation and subsequent diagnosis of PK deficiency was seldom undertaken in developing countries. The findings that clinically significant PK mutations occur in African Americans (Beutler and Barancioni, 1996; Beutler and Gelbart, 2000) and that PK deficiency protects against malaria in the mouse model (Coburn, 2004; Min-Oo et al, 2007), suggests that PK deficiency is under-reported in Africa. One small study

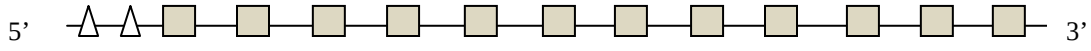
of African-Americans even estimated the heterozygote gene frequency to be 2.4 times the Caucasian frequency (Mohrenweiser, 1987). No other studies have been conducted to estimate disease prevalence in African populations.

1.3 Patterns of inheritance and molecular genetics

PK deficiency is a mono-allelic autosomal recessive condition. Carriers are therefore unaffected and the most common pattern of inheritance manifesting in disease is that of double heterozygosity, where two mutant variants of the PK enzyme are inherited (Eber, 2003; Zanella et al, 2007). Four isoforms of the enzyme exist: PK-M1 (skeletal muscle, heart and brain), PK-M2 (foetal tissue, kidney, leucocytes, platelets, pneumocytes, spleen and adipocytes), PK-L (liver) and PK-R (red cells). M1 and M2 are coded for by the PK-M gene, while the L and R isoforms are coded for by the PK-LR gene which is the focus of this report.

PK-LR is a ~9.5kb gene located on chromosome 1q21 (Genbank Accession number NM_181871) and transcriptional regulation of the L and R isoforms is under the control of alternate promoters (reviewed in Zanella, 2007). The gene contains 12 exons: the PK-R transcript contains exon 1, the PK-L transcript contains exon 2, and the remaining 10 exons are shared by both isoforms. Two CAC boxes and four GATA motifs have been identified within the promoter region of PK-R. Figure 2 is a schematic representation of the PK-LR gene and indicates the distribution of the 180 mutations, which produce varying degrees of chronic haemolytic anaemia (Beutler and Barancioni, 1996; Zanella et

al, 2005). Most mutations are missense (69%), splicing (13%) or stop codons (5%). Insertions, deletions and frameshift mutations are rare and most severe. The most frequently encountered mutations are located in exons 7 to 11 (Diez et al, 2005; Zanella et al, 2005; Pissard et al, 2006). The two most common mutations, 1529A and 1456T (located in exon 11), are distributed with strong ethnic and regional associations: 1529A is common in the USA and north-central Europe while 1456T is prevalent in southern Europe (Pissard et al, 2006). Interestingly, the 1456T mutation is also the most common mutation identified in African Americans (Beutler and Gelbart, 2000). The most frequent mutation in Asians is the 1468T mutation (Beutler and Barancioni, 1996).



exon	1(R)	2(L)	3	4	5	6	7	8	9	10	11	12
No. of mutations		2	9	11	14	9	26	30	27	15	24	9

Figure 2: Schematic representation of the PK-LR gene.
Exon 1 is specific to the red cell isoform and exon 2 to the liver isoform. The remaining 10 exons are common to both isoforms. Triangles depict the CAC and GATA promoter regions. The numbers of mutations in each exon are depicted and include point mutations, deletions and insertions of single nucleotides, and splice site mutations. The remaining mutations are in the promoter regions or are large deletions. These are not shown.

1.4 Enzyme structure and function

PK has been studied for over 50 years and the three-dimensional structures of a number of prokaryotic and eukaryotic PKs, including several disease-causing mutants, have been resolved (Valentini et al, 2002). The human red cell PK primary accession number in the Universal Knowledge Database (UniProt URL: http://www.ebi.uniprot.org) is P30613 and the Enzyme Catalogue number is EC 2.7.1.40. The functional enzyme is a highly conserved tetramer of approximately 240kDa. Each subunit has four domains: a small N-terminal helical domain; an A domain (comprising α helices and β sheets); a B domain (comprising β sheets) and a C domain (comprising α helices and β sheets) (Figure 3). The active site resides between the A and B domains. There is an allosteric site in a pocket in the C domain, which binds fructose 1,6-bisphosphate to enhance enzyme activity. The intersubunit contacts involve residues of the A and C domains. The multidomain architecture of the enzyme is important for regulation of activity due to different conformational states. Switching between a tight (T) inactive state and a relaxed (R) active state is brought about by domain and subunit rotations, which affect the active site geometry and depend upon interactions at the allosteric site. Two equivalents of bivalent cations Mn^{2+} or Mg^{2+}, and one equivalent of monovalent cation K^{+} are required for enzyme activity.

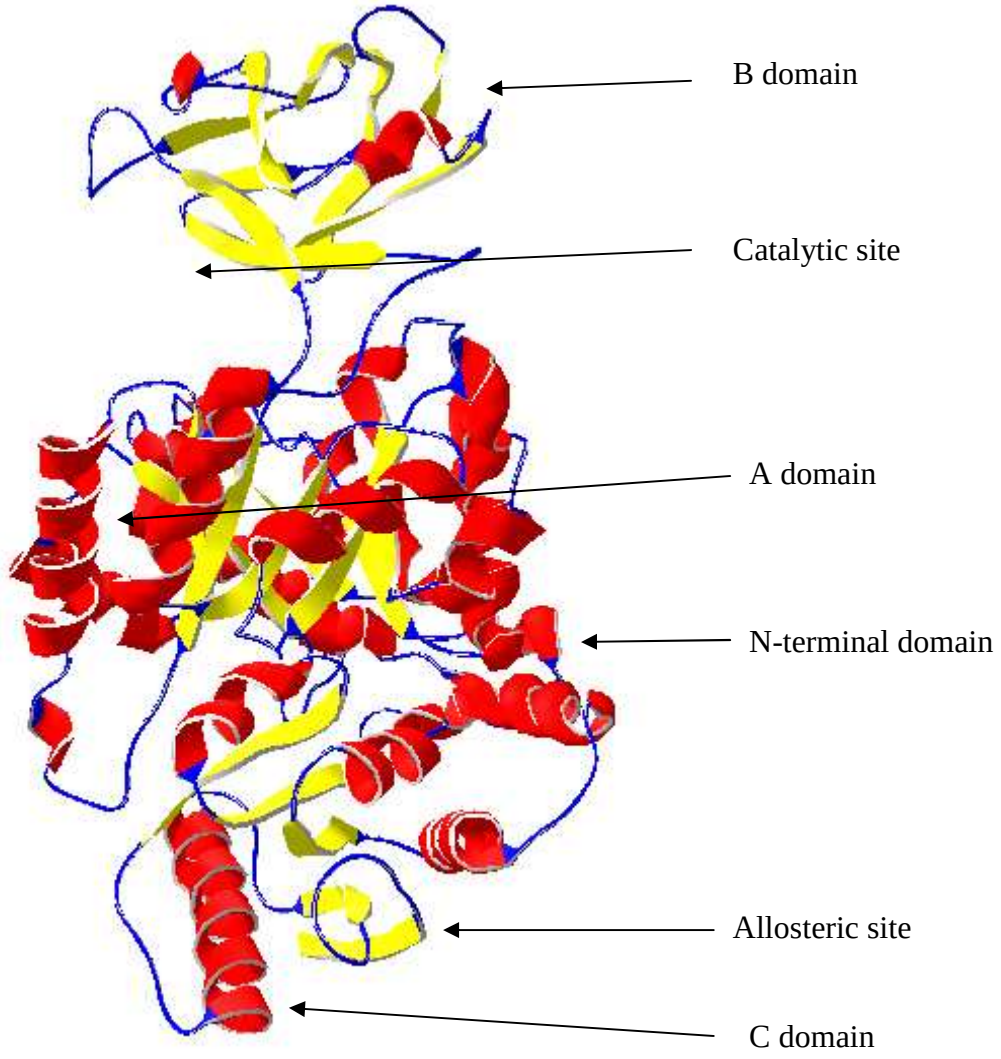


Figure 3: Molecular structure of PK-R.
The ribbon structure of a monomer is shown. The crystal structure data resolved to 2.72Å was imported from the Protein Data Bank (http://www.pdb.org) and modeled with SWISS-PDB Viewer (http://www.expasy.org/spdbv) (Schwede, 2003). The domains, catalytic site and allosteric binding site for fructose 1,6-bisphosphate are shown. α helices are in red, β sheets in yellow and turns and loops in blue.

1.5 Laboratory testing

A screening test based on the methods set out by Beutler (1975) may be used, coupling the PK reaction (equation 1) to lactate dehydrogenase (LDH), which converts pyruvate to lactate using NADH as a co-factor (equation 2). This takes advantage of the fact that NADH fluoresces under UV light, whereas NAD^+ does not. By providing an excess of LDH and PK substrate, phosphoenolpyruvate (PEP), PK activity is proportional to the rate of oxidation of NADH.

$$\text{Pyruvate} + \text{NADH} \xrightarrow{\text{LDH}} \text{lactate} + NAD^+ \qquad \text{(equation 2)}$$

Abnormalities detected by the screening test can be confirmed with a quantitative enzyme assay. This involves the same coupled reaction, but the oxidation of NADH is measured and quantified spectrophotometrically. The decrease in absorbance at 340nm is used to calculate enzyme activity. PK deficiency of clinical relevance is usually profound and manifests in individuals with $\leq$ 25% of normal enzyme activity (Beutler, 1975; Eber, 2003). Since there are at least 180 known mutations it is not technically feasible to employ genetic testing for the routine diagnosis of PK deficiency. The use of genetic testing lies in the identification of mutations in patients who have been diagnosed as PK deficient by the quantitative assay, and is initially directed at the most common mutations prevalent in the individual's race or ethnic group.

1.6 PK deficiency testing in South Africa

Nothing is known about the prevalence of PK deficiency in South Africa and currently no definitive laboratory investigation for the disorder is routinely conducted in the country. Commercial kits for assaying PK activity have been discontinued and all efforts by the author to source a commercial kit were unsuccessful. Requests for the investigation of PK activity are occasionally received by Prof. T. L. Coetzer, head of the Red Cell Membrane Unit, indicating that a need exists for an inexpensive PK assay which could be performed on an *ad hoc* basis. The capacity to diagnose PK deficiency would be useful in guiding physicians in patient management and avoiding unnecessary laboratory investigations.

1.7 Aims and objectives

The broad objectives of this project are to implement the qualitative and quantitative PK assays based on Beutler's published methods (1975), and to develop methods to investigate underlying mutations in confirmed cases of PK deficiency. The specific aims are:

1) To adapt and optimize the PK qualitative and quantitative assays and assess their use as a service offered by the Department of Molecular Medicine and Haematology at the University of the Witwatersrand Medical School / NHLS. To achieve this goal:
   - Twenty normal samples of each of two race groups (Africans and Caucasians) will be analysed and compared to the published reference range.

- The feasibility of offering a routine screening assay will be investigated by performing the assay on 10 samples received from laboratories around the country as part of a "haemolytic work-up".
- Quality control issues regarding the use of the assay in a routine laboratory will be examined. This will include a determination of the stability of PK activity at room temperature and at $4^{o}C$; the minimum volume of blood required to perform the assay; the reproducibility and precision of the quantitative assay; and the sensitivity of the qualitative assay.
- A costing analysis, SOP for the screening assay, and guidelines for referring physicians will be generated.

2) A PCR amplification protocol of exons 8 to 12 will be developed to allow for future investigation of the underlying mutations in confirmed cases of PK deficiency. To achieve this goal:
   - Primers will be designed to amplify exons 8 to 12 using PCR.
   - DNA sequencing of the amplified regions will be undertaken.

CHAPTER 2 MATERIALS AND METHODS

Ethics approval for the project was granted under the clearance number M070548. The methods described below for assaying PK activity are based on those published by Beutler (1975) and were slightly modified and optimized for the Red Cell Membrane Unit laboratory. Suppliers and reagents are listed in Appendices 1 and 2, respectively.

2.1 Blood collection and processing

Twenty samples were used from each of two race groups, Africans and Caucasians, to optimize and implement the PK assays and for comparison with the published reference range. Residual blood from samples submitted to the routine haematology laboratory was used. All samples had a normal haematological profile. Personal details such as age, race and gender were known. There was no history of haemolytic episodes, hepatosplenomegaly or blood transfusions. No other clinical history was available and the HIV status of the individuals was not known.

Volumes of at least 3ml of venous blood, collected in EDTA tubes, were used. Blood was centrifuged at 2500rpm for 10 min at 20^{o}C in a Beckman TJ-6 centrifuge. Plasma was aspirated and discarded. The buffy coat was removed using a pipette and stored at -70^{o}C. Red cells were washed three times with PBS or 0.9% saline to remove the residual buffy coat and finally resuspended in one volume of 0.9% saline and used within 1 hr. An aliquot (200µl) of the red cell suspension was analysed on a Beckman Coulter 750 LH

Analyzer to measure the Hb concentration and confirm that the white cells had been removed. A smear was made of the red cell suspension as a further check for white cell contamination. Leucocytes contain the PK-M1 isoform and it was therefore necessary to remove as many leucocytes as possible. A haemolysate was made to assess PK activity by adding 200µl resuspended red cells to 1.8ml stabilizing solution. To ensure complete red cell lysis, the suspension was frozen by immersion in acetone cooled to -70^{o}C. After freezing, the haemolysate was placed in a beaker of water at room temperature until completely thawed, kept at 4^{o}C and used within 2hrs. Leucocytes are resistant to lysis by this freeze-thaw process in stabilizing solution. This method is therefore used to achieve red cell lysis while allowing any leucocytes that may still be present to remain intact (Beutler, 1975).

2.2 PK Enzyme analysis

Table 1 lists the reagents used for each reaction. After the addition of PEP the total volume was 2ml. NADH was prepared weekly and stored at -20^{o}C. PEP and ADP were prepared monthly and stored at -20^{o}C. LDH was stored at 4^{o}C. Tris buffer, salt solutions and stabilizing solution were stored at 4^{o}C and used within 6 months. For each tube (blank, control, and sample) the reaction was started by adding 200µl 50mM PEP and the decrease in fluorescence (screening assay) or the decrease in absorbance at 340nm (quantitative assay) was monitored. LDH, PEP and ADP were provided in excess so that the activity of PK was measured by the rate of oxidation of NADH. Without ADP there is no PK activity (equation 1) and ADP was therefore excluded from the blank and from the

control and sample negatives. The blank did not contain any source of PK or ADP and measured the spontaneous oxidation of NADH as well as any inherent PK activity in LDH or any contaminating PK present in the commercial preparation of LDH. The control and sample negatives included the haemolysate and accounted for the absorbance due to Hb and the oxidation of NADH due to constituents present in the haemolysate.

Table 1: Reagents used for screening and quantitative assays.

Reagent	[Final reagent]	Blank	Sample negative	Sample
TE buffer	100mM Tris and 0.5mM EDTA	200	200	200
KCl (1M)	100mM KCl	200	200	200
$MgCl_2$ (0.1M)	10mM $MgCl_2$	200	200	200
NADH (2mM)	0.2mM NADH	200	200	200
ADP (30mM)	1.5mM ADP	-	-	100
LDH (60U/ml)	0.06U	1	1	1
Haemolysate	50x dilution	-	40	40
Stabilizing solution	50x dilution	40	-	-
Milli Q water	-	960	960	860
PEP (50mM)	5mM PEP	200	200	200

Volumes listed are in microlitres (µl). Each reaction was started by adding 200µl 50mM phosphoenolpyruvate (PEP), making a total volume of 2ml per reaction. TE buffer (pH=8 at 25^{o}C) contains 1M Tris and 5mM EDTA.

2.2.1 Terminology

In this report, the terms “qualitative” and “screening” refer to the same assay and are used interchangeably. Similarly, “quantitative” and “confirmatory” assays are the same.

"Blank" refers to the reaction without haemolysate and ADP. A blank was included only once each time an assay was performed. A "control" was included in each assay and refers to a healthy volunteer who is expected to have normal PK activity. For each control and sample, there was a negative, referred to as "control negative" or "sample negative", which excluded ADP in the reaction.

When reporting, the term "normal" refers to instances where normal PK activity was detected. The term "positive" is used here to report a result where decreased PK activity was detected. In the qualitative assay, a positive result may also be recorded as "decreased PK activity detected" so that the finding may be confirmed with the quantitative assay (see SOP in Appendix 3). The term "negative" is not used in reporting results to avoid confusion with the "control negative" and "sample negative" above.

2.2.2 Qualitative (screening) assay

Ten of the study samples were chosen at random and used for the screening assay. Samples were set up according to Table 1 and the reaction was started with PEP. Screening was done by spotting 5µl of the blank, sample negative and sample adjacent to each other on Whatman #1 filter paper at times 0, 5, 10 and 15 minutes and viewing under UV light. The test sample was compared to the sample negative. A loss of fluorescence within 15 minutes indicated PK activity and was recorded as a normal result. In individuals with ≥50% of normal enzyme activity (EA), a normal result should appear within 15 minutes (Beutler, 1975). Fluorescence beyond 15 minutes indicated

decreased PK activity and was recorded as “positive” or “decreased PK activity”. To demonstrate that a reduced level of PK activity could be detected by this screening test, 5x, 2.5x, 2x and 1.7x dilutions of normal haemolysate (20%, 40%, 50% and 60% of normal EA, respectively) were used. Images of the qualitative assays were captured with a SynGene Geldoc system.

2.2.3 Quantitative (confirmatory) assay

The quantitative assay for PK activity was performed using a Beckman DU65 spectrophotometer. 3ml glass cuvettes were used for measuring a decrease in absorbance at 340nm. Prior to the addition of PEP, the blank was used for calibration. Readings were taken at the following times after the addition of PEP: 30s, 1min, 2min, 3min, 4min, 5min, 10min, and 15min. During the first 5 minutes the reaction system was unstable and metabolites such as ADP, ATP, and NADH present in erythrocytes and therefore the haemolysate, contributed to the decrease in absorbance. After 5 minutes the system was stable, and erythrocyte PK activity was measured by the rate of change of absorbance. The difference between the sample and sample negative and blank was used to calculate the reaction rate per minute. Data points were plotted graphically and the linear portion (5min to 15min) selected for analysis. EA was calculated using equation 3 (Beutler, 1975) and expressed as U/g Hb, where 1U of enzyme oxidizes 1µmole NADH in 1min.

$$Enzyme\ activity = \frac{\frac{\Delta Abs}{min}}{\epsilon} \times \frac{Vc}{Vh} \times \frac{100}{[Hb]} \times 10$$

(equation 3)

ΔAbs_{340}/min = change in absorbance at 340nm per minute (5 to 15 mins)

Vc = sample volume in cuvette (2ml)

Vh = haemolysate volume in reaction system (0.04ml)

10 is the dilution factor in making up haemolysate

100 is the conversion factor for Hb measured in g/dl

ε = 6.22, the extinction coefficient of a 1mM NADH solution

[Hb] = haemoglobin concentration in g/dl

For the volumes used in this study, the equation can be simplified to:

$$Enzyme\ activity = \frac{\frac{\Delta Abs}{min}}{[Hb]} \times 8038.6$$

(equation 4)

EA was determined at room temperature which varied between 20°C and 25°C. EA decreases variably below 25°C due to enzyme instability and may lead to slight underestimations of EA (Beutler, 1975). The EA at 37°C was calculated by dividing the EA measured at room temperature by the conversion factor 0.432 (Beutler, 1975).

2.3 PK activity of patient samples

To assess the feasibility of offering the assay as a countrywide service in the Red Cell Membrane Unit, samples were received from various laboratories around the country for PK testing. The assay was requested by the attending physician on patients who were undergoing investigations for haemolysis. Ten samples were used for this purpose: one from Port Elizabeth, two from Bloemfontein, two from Durban, two from Pretoria, and three from Johannesburg. The samples were sent in EDTA tubes at room temperature using the courier systems normally employed by the respective laboratories and received within two days of being taken. Both qualitative and quantitative assays were performed on these samples. In each case blood from a control (a volunteer known to have normal PK activity) was used to verify the assay on the day. The patient's EA was expressed as a percentage of the calculated mean derived in this study.

2.4 Quality control

The quantitative assay was performed on a normal volunteer to establish that the result was within the published reference range. Subsequently, quantitative and qualitative assays were performed on test samples. In addition to the sample, a control, control negative and sample negative were used for both screening and quantitative assays of the ten patient samples which were processed as part of a "haemolytic workup". To confirm

that a PK activity of ≤50% of normal is detected by the screening test, diluted haemolysates with 20%, 40%, 50% and 60% of normal EA were used.

The precision of the quantitative assay was confirmed by repeating the assay on 5 aliquots of the same sample on the same day. The reproducibility of the quantitative assay was determined by repeating the assay 5 times on the same individual, sampled at different times over a period of several weeks using freshly prepared NADH, ADP and PEP each time.

The stability of PK in whole blood stored at room temperature and at 4^{o}C was determined by measuring EA daily until it diminished. The stability of haemolysate stored at 4^{o}C was determined over one working day (~8hrs).

To determine whether the test could be offered for paediatric-sized samples, an aliquot of 500µl of whole blood was taken from one of the twenty Caucasian samples. The aliquot was processed and assayed as described for adult samples.

2.5 Enzyme analysis of mutant PK

Blood was obtained from a patient with PK deficiency (see Chapter 4) for verification of the PK assays.

2.5.1 Qualitative and quantitative assays

These assays were performed in the same way as described above (Section 2.2).

2.5.2 Thermal stability of mutant PK

Investigation of the thermal stability of the mutant PK was determined at 53^{o}C. The temperature of 53^{o}C was chosen as it has been previously used in thermal stability studies (Zanella et al, 2007). EA of the mutant PK was determined by heating 4 aliquots (100µl per aliquot) of haemolysate for durations of 0min, 1min 15sec, 2min 30sec and 3min. Each aliquot of haemolysate was heated just prior to performing the quantitative assay. A 1.5ml Eppendorf tube containing the haemolysate was placed in a water bath preheated to 53^{o}C. For comparison, the thermal stabilities of samples from a control and from the patient's mother were determined. In these cases, haemolysates were heated for durations of 0min and 3min.

2.6 PK mutation analysis

2.6.1 DNA extraction

A volume of 100µl of buffy coat stored at -70^{o}C was added to 400µl freshly-prepared 0.17M ammonium chloride in a 1.5ml Eppendorf tube. The ammonium chloride was used to selectively lyse erythrocytes. The sample was mixed and allowed to stand at room temperature for 20min. It was centrifuged for 1min in a Hägar HM2 microfuge at

14000rpm and the supernatant discarded. The white cell pellet was washed 3 times in 0.9% NaCl to remove residual Hb, resuspended in 200µl 0.05M NaOH and boiled for 10mins. The 0.05M NaOH solution was used to lyse white cells and proteins were denatured by boiling for 10 mins. The sample was then neutralized with 25µl 1M Tris HCl (pH=8 at room temperature) (Talmud et al, 1991). DNA was quantified spectrophotometrically in the following way:

DNA concentration (µg/µl) = (Abs_{260nm} x 50 x dilution factor)/1000

1 µg/ml of double stranded DNA has an absorbance at 260nm of 50.

2.6.2 Primer design

Primers were designed manually to amplify exons 8 to 10, exon 11 and exon 12 of the PK-LR gene. Due to the large intron sizes between exon 10 and 11 (~2000bp) and between exons 11 and 12 (~1000bp), and for ease of amplification, it was decided to amplify this part of the gene in three regions. The amplified regions included intron-exon boundaries to detect possible splice-site mutations (Figure 4).

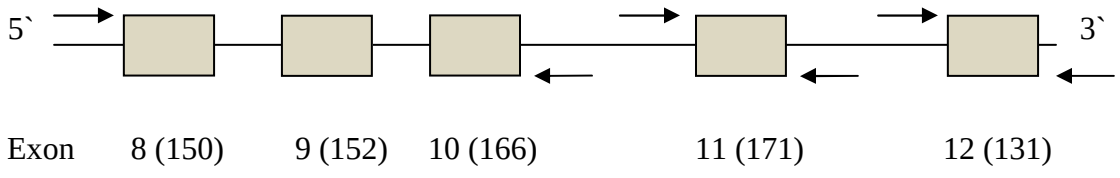


Figure 4: Regions of PK-LR gene amplified in this study.
Three regions of the PK-LR gene were amplified: exons 8 to 10, exon 11 and exon 12. Exons are indicated by shaded boxes. The bp number in each exon is indicated in parentheses. The positions and directions of the primers used to amplify the three regions are indicated by arrows and were designed to include intron-exon boundaries.

Oligonucleotides were analyzed with the Oligonucleotide Properties Calculator (Kibbe, 2007) to avoid complimentarity and 3' overlaps, and for estimating melting temperatures (Tm). For this reason primer lengths varied. Oligonucleotides were synthesized by Inqaba Biotech with a PolyGen 10 column High Throughput DNA synthesizer and were stored in Tris-EDTA buffer (10mM Tris-HCl; 1mM EDTA; pH=8) at -20^{o}C at a stock concentration of 100µM. Working concentrations of 10µM were used. Table 2 lists oligonucleotide details and expected amplicon sizes.

Table 2: Oligonucleotides for PK-LR gene amplification.

Exon(s)	Forward (F) and Reverse (R) Primers	Primer Tm (^{o}C)	Amplicon size (bp)
8 to 10	F: 5'CATCACCTTTCTTCTCCTGCC3'	61	1305
	R: 5'CTGACCCAAAGCTCCATCTGG3'	63	
11	F: 5'GTATGATGACTTACCCAGGGTCACA3'	65	396
	R: 5'GAGAGGCAAGGCCCTTTGAGTGG3'	66	
12	F: 5'CATCACCTTTCTTCTCCTGCC3'	61	943
	R: 5'GTTCTGGGGTTCTTGGTGTGTG3'	64	

2.6.3 PCR amplification

Thin-walled 0.5ml PCR tubes were used. The Roche Expand High Fidelity PCR System was used and a "hot start" method employed such that reactions were set up on ice and placed in the thermocycler (Eppendorf Mastercycler) set at 94^{o}C. Table 3 lists the reaction volumes and concentrations used under the following cycling conditions: 94^{o}C for 5min (1 cycle); 94^{o}C for 30s, 59^{o}C for 30s and 72^{o}C for 1min 30s (30 cycles); 72^{o}C for 5min. A volume of 5µl of each PCR product was electrophoresed at 100V through a 1% agarose gel containing 5µg ethidium bromide in TAE buffer (40mM Tris; 0.1% acetic acid; 1mM EDTA; pH=8 at room temperature) for 1 hour and visualized under UV light. Product sizes were determined by comparison to a Fermentas MassRuler DNA ladder Mix.

Table 3: Reagents used for PCR reactions

Reagent	**Concentrations**	**Volume (µl)**	**[Final reagent]**
Genomic DNA	(100-200ng/µl)	2	~300ng
Reaction buffer with Mg^{2+}	5x stock	4	1x
Forward primer	10µM	1	0.5µM
Reverse primer	10µM	1	0.5µM
Taq polymerase	5U/µl	1	5U
Nuclease free water	-	9.2	-
dNTPs	2.5mM	1.8	225µM
Final volume		20	

2.6.4 DNA analysis

DNA sequencing was performed by Inqaba Biotec on an ABI 3130XL DNA sequencer. Chromas software version 2.31 was used to view the raw sequence data. Sequence alignments were performed with ClustalW software (Thompson et al, 1994).

2.6.5 Computational analysis

The accession number for the PK-LR gene in Genbank (http://www.ncbi.nlm.nih.gov/entrez) is NM_181871. Protein sequence data were retrieved from the Protein Data Bank (http://www.pdb.org), UniProt reference P30613. Structural and mutation analysis and modeling was performed with SWISS-PDB Viewer (http://www.expasy.org/spdbv).

CHAPTER 3 PK ASSAY IMPLEMENTATION

3.1 Qualitative (screening) PK assay

In the presence of adequate controls, PK activity was demonstrated by the loss of fluorescence under UV light within 15 minutes. Figure 5 is a photograph of a typical result. Ten normal samples (confirmed by the quantitative assay, Section 3.2) all produced a normal qualitative result.

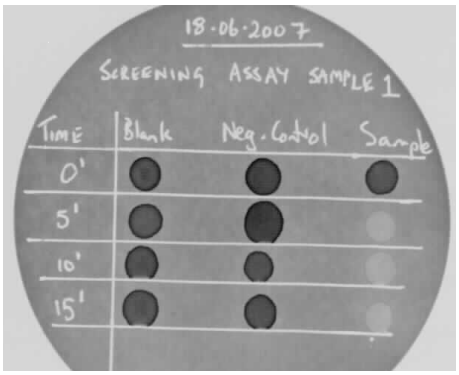


Figure 5: A normal result of a screening assay for PK deficiency.
Whatman #1 filter paper was spotted with 5µl of each reaction. The column labeled "Neg. Control" should read "Sample Negative" for consistency of terminology (Section 2.2.1). A loss of fluorescence was demonstrated after 5min in the sample indicating normal PK activity.

3.1.1 Sensitivity of screening PK assay

The haemolysate of one of the samples was diluted to 20%, 40%, 50% and 60% EA. EA (a loss of fluorescence) was detected with dilutions of 50% and 60% but was undetectable at dilutions of 20% and 40% (a persistence of fluorescence at 15 mins) (Figure 6). This confirmed the previous assertion that an EA of < 50% will be detected by the screening assay (Beutler, 1975). A Standard Operating Procedure (SOP) has been compiled for the screening assay in a routine laboratory (Appendix 3). A finding of decreased PK activity would require that a quantitative assay be performed.

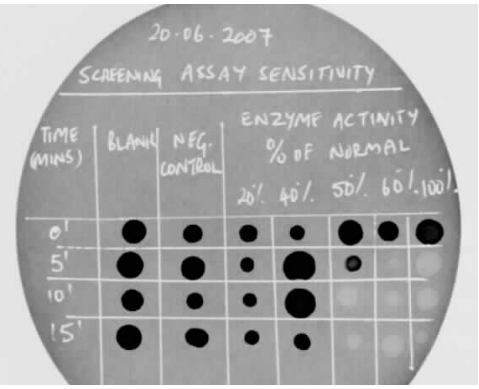


Figure 6: PK screening test sensitivity.
To determine the sensitivity of the screening assay, a normal sample was diluted 5x, 2.5x, 2x and 1.7x to give 20%, 40%, 50% and 60% respectively of normal EA. The blank and control negative demonstrated no PK activity. A minimum of 50% of normal PK activity was required for the screening assay to demonstrate loss of fluorescence.

3.2 Quantitative (confirmatory) PK assay

Table 4 gives two examples of typical data sets. These data were used to represent graphically the change in absorbance over time (Figure 7).

Table 4: Two data sets of quantitative PK assays

(a)

Time	Blank	Sample Neg.	Sample
No PEP	0.000	0.413	0.457
PEP+30 sec	-0.178	0.171	0.204
1 min	-0.187	0.159	0.197
2 min	-0.193	0.164	0.181
3 min	-0.192	0.157	0.174
4 min	-0.193	0.155	0.162
5 min	-0.191	0.157	0.152
10 min	-0.190	0.154	0.113
15 min	-0.190	0.151	0.074
ΔAbs (5-15min)	0.001	0.006	0.078
ΔAbs/min (5-15min)			0.007
Hb g/dl			10.3
EA U/g Hb at 25^{o}C			5.54
EA U/g Hb at 37^{o}C			12.83

(b)

Time	Blank	Sample Neg.	Sample
No PEP	0.000	1.999	1.983
PEP+30 sec	-0.171	1.77	1.703
1 min	-0.178	1.668	1.690
2 min	-0.180	1.665	1.679
3 min	-0.184	1.660	1.666
4 min	-0.187	1.657	1.658
5 min	-0.190	1.654	1.649
10 min	-0.191	1.645	1.593
15 min	-0.192	1.637	1.550
ΔAbs (5-15min)	0.002	0.017	0.099
ΔAbs/min (5-15min)			0.008
Hb g/dl			11.0
EA U/g Hb at 25^{o}C			5.85
EA U/g Hb at 37^{o}C			13.50

Two typical data sets recorded for a 38 year old white male (a) and a 31 year old black male (b). Absorbance readings at 340nm were taken at the times shown. PK activity was determined by the rate of change of absorbance from 5 mins to 15 mins. The EA at 25^{o}C was divided by the conversion factor 0.432 to determine the EA at 37^{o}C. ΔAbs = change in absorbance at 340nm; EA = enzyme activity in U/g Hb.

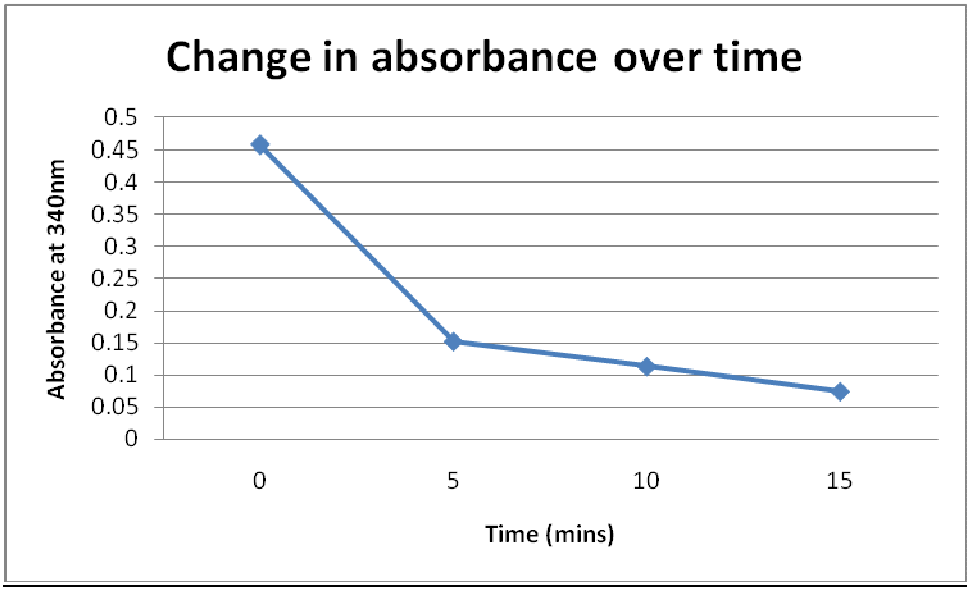


Figure 7: Rate of change of absorbance at 340nm as a measure of PK activity.
The data in Table 4 (a) were used to construct the graph. After the addition of substrate at time 0 mins, the system was left to stabilize for 5 minutes. Erythrocyte PK activity was measured by the rate of change of absorbance. PK activity was 12.83 U/g Hb at 37^{o}C.

The data from the two race groups (20 Caucasians and 20 Africans) were compiled and analyzed statistically and are summarized in Table 5. A two sample t-test using GraphPad Prism statistical software (Motulsky, 1999) showed there was no significant difference between the means of the two race groups (p value = 0.29). The combined mean (n=40) and standard deviation (SD) were 13.01 U/g Hb and 2.25, respectively. The mean falls within 1 SD of the published reference range of 15.00 ± 1.98 (mean ± SD) (Beutler, 1975; Eber, 2003).

Table 5: Quantitative PK assay data from 20 Caucasians and 20 Africans.

Sample	Caucasian samples		African samples	
	EA (U/g Hb)	Age (yrs) and gender	EA (U/g Hb)	Age (yrs) and gender
1	16.90	60F	13.50	31M
2	12.83	38M	17.30	42M
3	16.06	18M	11.40	30M
4	13.78	32M	11.31	16M
5	11.80	34F	14.70	21F
6	10.20	26M	9.81	33F
7	17.90	24F	10.40	36F
8	12.03	40M	11.21	27M
9	12.01	43M	11.73	36F
10	14.70	39M	12.59	32M
11	9.70	67F	9.73	45M
12	12.05	50F	14.03	29M
13	15.40	46F	17.30	31M
14	12.59	33M	13.76	27M
15	14.11	20M	13.67	59F
16	10.76	27F	11.03	35F
17	13.00	43M	11.13	37M
18	14.44	21F	12.84	43F
19	14.00	67F	13.40	53F
20	13.72	29M	16.89	18M
Mean	13.41		12.64	
SD	2.15		2.34	
	Combined mean (n=40) of 13.01 U/g Hb			
	Combined SD of 2.25			
	EA ranges from 9.70 to 17.90 U/g Hb			

There was no significant difference between the means of the two groups (two sample t-test, p = 0.29). The combined mean (n=40) falls within 1 SD of the published range of 15.00 ± 1.98 U/g Hb (Beutler, 2003; Eber, 2003). The reference range determined in this study was 8.51 to 17.51 U/g Hb (mean ± 2SD). SD = standard deviation; M=male; F=female. EA=enzyme activity.

3.3 Quality control

3.3.1 Sensitivity of PK qualitative assay

Haemolysate diluted 2x to give an EA of 50% of normal demonstrated a loss of fluorescence (Figure 6) while decreased PK activity was detected in haemolysates with <50% of normal EA (persistent fluorescence after 15min). The assay is therefore sensitive enough to identify individuals with <50% of normal PK activity. In these cases a confirmatory assay is indicated to quantify EA. Individuals with clinical symptoms of PK deficiency have EA of ≤25% of normal (Eber, 2003).

3.3.2 Paediatric size samples

Paediatric size (500µl) and adult size (3ml) aliquots of the same sample were processed in parallel and demonstrated EAs of 10.95 U/g Hb and 11.31 U/g Hb, respectively. The sample processed in this case was from whole blood with a normal haematological profile and normal haematocrit.

3.3.3 Precision and reproducibility of the quantitative assay

The precision was determined by repeating the assay on one sample five times. The assays were repeated on the same day immediately after one another using the same

reagents each time. The calculated EAs were 10.2; 10.9; 9.9; 10.4 and 9.6 U/g Hb with a mean of 10.2 U/g Hb, SD of 0.49 and a CV of 5%. The reproducibility was determined by repeating the assay five times on fresh blood from one individual over a period of 10 weeks. The calculated EAs were 14.7; 15.3; 14.5; 14.1; and 16.0 U/g Hb with a mean of 14.9 U/g Hb, SD of 0.74 and a CV of 5%.

3.3.4 Enzyme stability in whole blood

The stability of PK in whole blood stored at room temperature and at 4^{o}C was determined by repeating the assay on one sample on days 1, 2, 3, 4, 5 and 8. The results are recorded in Table 6. The sample stored at room temperature demonstrated a decrease in EA of >2 SD on day 4. The sample stored at 4^{o}C was stable for 5 days but demonstrated a decrease in EA of >2 SD on day 8. This indicated that it would be feasible to process a sample within 5 days of drawing blood if it had been stored at 4^{o}C. However, the sample would have to be processed within 3 days if it had been stored at room temperature.

Table 6: PK stability in whole blood.

Time frame	Whole blood stored at 4^{o}C	Whole blood stored at RT (25^{o}C)
Day 1	14.1	14.9
Day 2	13.7	15.4
Day 3	14.6	11.8
Day 4	13.0	7.4
Day 5	12.8	3.2
Day 8	7.6	-

Results are recorded as EA in U/g Hb at 37^{o}C. Whole blood stored at 4^{o}C and at room temperature demonstrated PK stability for 5 days and 3 days, respectively, after which there was a decrease in EA of >2 SD.

3.3.5 Enzyme stability in haemolysate

The assay was repeated 4 times on the same haemolysate stored at 4^{o}C over 8 hours (Table 7). After 2hrs, EA was almost the same as at 0hrs. At 4hrs there was a decrease in EA of ~1.5 SD, indicating that the assay needs to be performed within 2 hrs of preparing the haemolysate.

Table 7: PK stability in haemolysate.

Time frame	Haemolysate stored at 4^{o}C
0 hrs	14.1
2 hrs	13.8
4 hrs	11.7
8 hrs	8.1

Results are recorded as EA in U/g Hb at 37^{o}C. At 4hrs there is a decrease in EA of ~1.5SD.

3.4 Feasibility of PK assay as a nationwide service

Qualitative and quantitative assays were performed on 10 samples from patients being investigated for haemolysis at various hospitals around the country (Section 2.3). The screening assay was normal in each case (data not shown) and this was confirmed with the quantitative assay (Table 8). The result for each sample was reported as a percentage of the mean determined in this study (13.01U/g Hb). The mean and SD were calculated as 13.16 ± 1.21 U/g Hb.

Table 8: Quantitative PK assay results for 10 patients investigated for haemolysis.

Sample No.	Control EA	Sample EA	Sample EA as % of mean
1	17.3	16.7	128
2	17.3	14.8	113
3	17.3	15.2	117
4	17.3	14.5	111
5	17.3	12.9	99
6	11.5	14.2	108
7	12.0	14.7	113
8	12.0	14.6	112
9	16.3	13.6	104
10	16.3	12.4	95

The patient's PK activity was expressed as a percentage of the mean determined in this study (13.01U/g Hb). The mean and SD of the 10 samples were 13.16 ± 1.21 U/g Hb. EA is enzyme activity in U/g Hb at 37^{o}C. Samples 1-5, 7-8, and 9-10 were processed in 3 batches. Each batch had a different control.

3.5 Validation of PK assay with PK deficient blood

A patient was referred to the Red Cell Membrane Unit, Wits Medical School for confirmation of suspected PK deficiency. The patient's enzyme analysis is reported here as validation of the qualitative and quantitative PK assays. The patient's history and further genetic analysis are reported in Chapter 4.

3.5.1 Qualitative PK assay

The patient's qualitative assay (Figure 8) showed no loss of fluorescence after 15mins. This was in keeping with the assertion that the qualitative screening assay detects

individuals with clinical manifestations of a PK deficiency (Beutler, 1975) and that the assay is sensitive enough to detect an EA ≤50% of normal (Section 3.3.1). A qualitative assay was also performed on the PK deficient patient's mother (data not shown). This did not demonstrate a loss of fluorescence, which concurs with the quantitative assay EA of 58% (Section 3.3.7.2).

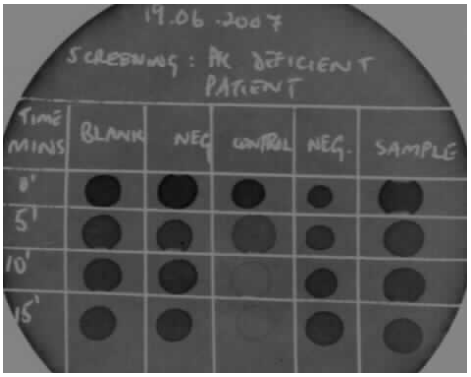


Figure 8: Screening assay for PK deficient patient.
The patient's sample remained fluorescent after 15min, indicating decreased PK activity. The columns labelled "Neg" correspond to the control negative and sample negative.

3.5.2 Quantitative PK assay

The patient's quantitative assay was performed 4 times to confirm the enzyme deficiency and EA was expressed as a percentage of the mean (13.01U/g Hb) as determined by this study. The EAs were 23%, 17%, 8%, and 13% with an average of 15% of normal. This concurs with the findings that an EA of <25% of normal is required to produce clinical disease (Beutler, 1975; Eber, 2003). Table 9 is a record of the quantitative assay readings the first time it was performed and the EA was calculated to be 23% of the mean on that occasion.

Table 9: Quantitative PK assay data of PK deficient patient.

Time	Blank	Control Negative	Control	Sample Negative	Sample
No PEP	0.000	3.094	3.017	2.984	3.010
PEP+30s	-0.245	2.874	2.794	2.733	2.748
1 min	-0.244	2.870	2.783	2.730	2.745
2 min	-0.244	2.868	2.777	2.729	2.740
3 min	-0.244	2.867	2.772	2.729	2.739
4 min	-0.243	2.866	2.768	2.728	2.737
5 min	-0.243	2.865	2.760	2.729	2.736
10 min	-0.242	2.863	2.728	2.727	2.729
15 min	-0.241	2.860	2.688	2.728	2.720
ΔAbs (5-15min)	0.002	0.005	0.072	0.001	0.016
ΔAbs/min (5-15mins)			0.0065		0.0013
Hb g/dl			8.6		7.9
EA in U/g Hb at 25^{o}C			6.1		1.3
EA in U/g Hb at 37^{o}C			14.2		3.0
EA as % of mean			**109%**		**23%**

The results are those of a 24 year old white female and confirm erythrocyte PK deficiency. Absorbance readings at 340nm were taken at the times shown. The patient's PK activity was 3.0 U/g Hb at 37^{o}C, which is 23% of the mean determined in this study (13.01U/g Hb). ΔAbs = change in absorbance at 340nm; EA = enzyme activity in U/g Hb.

Figure 9 is a graphical representation of the patient's PK activity over time using the readings in Table 9.

PK deficiency is an autosomal recessive disorder and the patient would have inherited an abnormal allele from each parent. The quantitative assay was also performed three times on the patient's mother and the average EA was 58%. The data recorded for the first assay are tabulated in Table 10. The patient's father was unavailable.

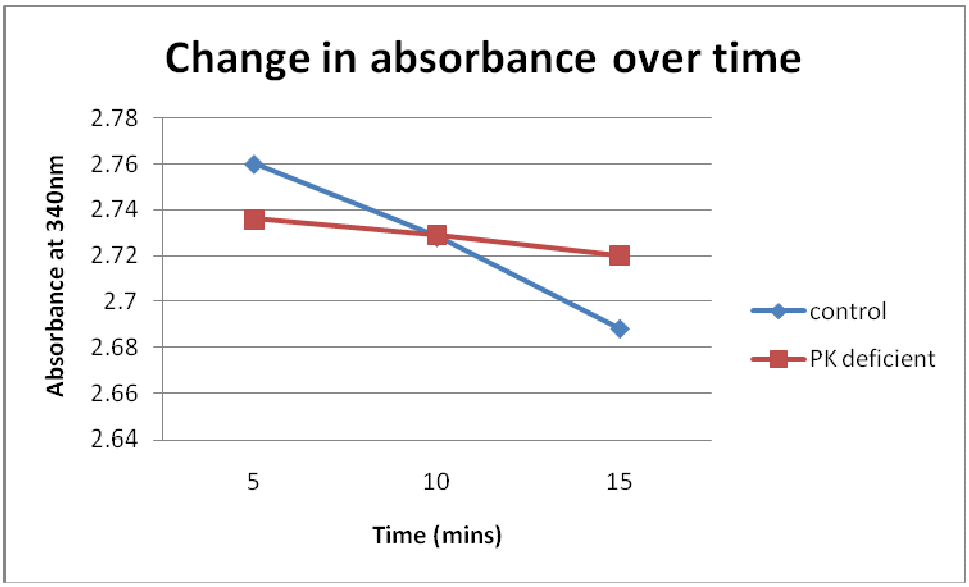


Figure 9: Quantitative assay of a PK deficient patient.
The data in Table 9 were used to plot the graph.

3.5.3 Thermal stability of mutant PK

There was an almost complete loss of EA in the patient's sample (homozygote) after 3min at 53^{o}C. Over the same time period, the patient's mother (heterozygote) demonstrated a loss in EA of 9%. The control was virtually unchanged. The thermal stabilities at 53^{o}C over 4 time periods are listed in Table 11.

Table 10: Quantitative PK assay of PK deficient patient's mother

Time	Blank	Control Negative	Control	Sample Negative	Sample
No PEP	0.000	0.882	0.879	0.807	0.765
PEP+30sec	-0.186	0.640	0.618	0.550	0.511
1 min	-0.187	0.639	0.610	0.544	0.504
2 min	-0.189	0.638	0.603	0.543	0.498
3 min	-0.191	0.636	0.597	0.543	0.493
4 min	-0.192	0.635	0.590	0.542	0.491
5 min	-0.192	0.634	0.575	0.542	0.489
10 min	-0.192	0.634	0.533	0.542	0.467
15 min	-0.192	0.634	0.491	0.541	0.445
ΔAbs (5-15min)	0.000	0.000	0.084	0.001	0.044
ΔAbs/min (5-15mins)			0.0084		0.0044
Hb g/dl			12.8		11.8
EA in U/g Hb at 25^{o}C			5.28		3.03
EA in U/g Hb at 37^{o}C			12.23		7.02
EA as % of mean			**94%**		**54%**

The results are those of a 55 year old white female. Absorbance readings at 340nm were taken at the times shown. After 5 mins, the system was stable and erythrocyte PK activity was determined by the rate of change of absorbance as 7.02 U/g Hb at 37^{o}C, which was 54% of the mean determined in this study (13.01 U/g Hb). ΔAbs = change in absorbance at 340nm; EA = enzyme activity in U/g Hb.

Table 11: Thermal stability of mutant PK at 53^{o}C

SAMPLE	TIME			
	0min	1min15sec	2min30sec	3min
Homozygote	17%	5%	2%	<1%
Heterozygote	69%	-	-	60%
Control	112%	-	-	108%

The homozygote represents the PK deficient patient's sample. The heterozygote represents the patient's mother's sample. Values listed are the EAs in U/g Hb as a percentage of the mean determined in this study (13.01 U/g Hb).

CHAPTER 4 MUTATION ANALYSIS OF A PK DEFICIENT PATIENT

After the implementation of the qualitative and quantitative PK assays and optimization of the PCR methods for mutation analysis, a patient with suspected PK deficiency was referred to the Red Cell Membrane Unit, Wits Medical School for investigation. The following details her history and an analysis of the genetic mutation. The results of the enzyme analysis may be found in Section 3.5.

4.1 Patient history

The patient is a 24 year old white female of north European ancestry, who had been diagnosed with a possible PK deficiency as an infant. A summary letter from Chris-Hani Baragwanath Hospital in June 1988 stated that she had severe haemolytic episodes during the first 6 months of life. The haemolytic episodes were severe enough to drop her Hb to 4g/dl and she required two blood transfusions during this period. The haematology laboratory at the hospital made a diagnosis of PK deficiency based on an enzyme assay that was used at the time, the details of which are unavailable. Following these events she had no other reported problems until this presentation.

Nine months prior to her referral to the Red Cell Membrane Unit, she had been assaulted, which necessitated her receiving a prophylactic antiretroviral (ARV) regimen of Crixivan, 3TC and AZT. Three days after starting the treatment she reported feeling ill, stopped the ARVs of her own accord and presented to her GP. Initial investigations

revealed that she had suffered a haemolytic episode: her Hb was 7.6 g/dl, haptoglobin was low, unconjugated bilirubin was high, LDH was high and she had a reticulocytosis. Her Hb was monitored but did not deteriorate further. She received folate, vitamin B12 and iron supplementation from her GP and after two months her Hb was being maintained at above 9g/dl. Nine months after the ARV-associated haemolytic episode, she attended a follow-up visit with her GP and was referred to the Red Cell Membrane Unit for the investigation of a possible PK deficiency and an explanation for the ARV-induced haemolysis. Her blood results were as follows: Hb 9.4 g/dl; reticulocytosis 19%; elevated unconjugated bilirubin and LDH; decreased haptoglobin; and normal liver enzymes.

4.1.1 Family and social history

Except for the episodes of haemolysis reported above, the patient had no other relevant medical history except that she occasionally suffered migraines. There was no history of hepatitis or other infections. She took folate supplements as advised by her mother and Sibelium for the migraines. No other medication was taken, nor is she on the oral contraceptive pill. She works as an agricultural trader, is a social drinker and smokes cigars. Her mother is of Irish descent and there is no available history regarding her father. She has one brother. No one else in her immediate family has suffered haemolytic episodes.

4.1.2 Physical findings

At the time of referral she was clinically well and there were no significant physical findings. In particular she was not clinically jaundiced or pale and there was no hepatosplenomegaly.

4.2 Routine haematology investigations

Nine months after the recent haemolytic episode, her GP reported that her haematological profile had returned to previous levels. Her unconjugated bilirubin and reticulocyte count remained elevated but the other parameters of haemolysis had normalized.

4.3 Qualitative and quantitative PK assays

The results of the PK assays are detailed in Section 3.5. In summary, the patient had an abnormal screening (qualitative) assay and the confirmatory (quantitative) assay demonstrated an average EA of 15% of the normal mean. The patient's mother had a normal screening assay and an average EA of 58% of the normal mean.

4.4 Mutation analysis

4.4.1 DNA extraction and PCR amplification

PCR amplification of selected exons of the PK-LR gene, harbouring the most common mutations was optimized on control DNA. The concentration of genomic DNA extracted from the patient's blood was measured spectrophotometrically and calculated to be 140ng/µl. Exons 8 to 10, exon 11 and exon 12 were amplified and amplicons were electrophoresed through a 1% agarose gel in TAE buffer and determined to be the expected size by comparison to a Fermentas DNA ladder mix (Figure 10).

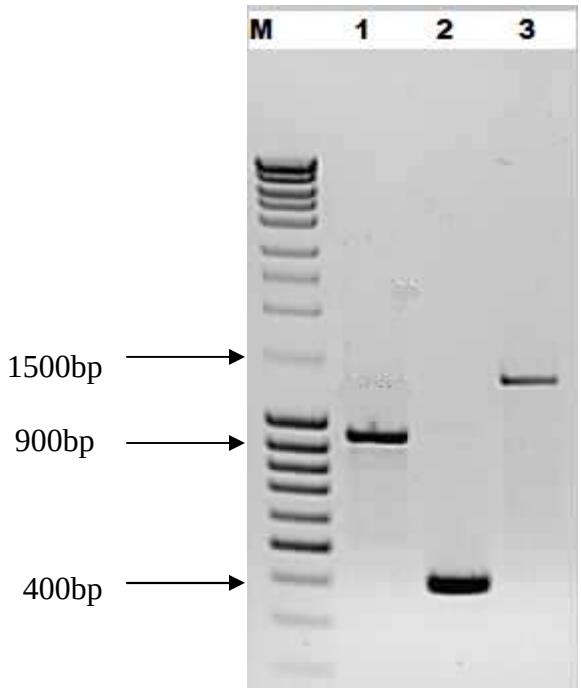


Figure 10: PCR amplification of three regions of the PK-LR gene.
Three regions of the gene which included the most common mutations were amplified using PCR: exon 12 (lane 1), exon 11 (lane 2) and exons 8 to 10 (lane 3). Amplicons were electrophoresed through a 1% agarose gel and sizes determined by comparison to a Fermentas MassRuler DNA ladder Mix (lane M). The expected sizes of the amplicons were 943bp (exon 12), 396bp (exon 11) and 1305bp (exons 8-10).

Once the mutation was identified in the patient (Section 4.4.2), genomic DNA was extracted from the patient's mother's blood. The genomic DNA concentration was

calculated to be 150ng/µl and used to amplify exon 11 for comparison to the patient's sequence.

4.4.2 DNA sequencing

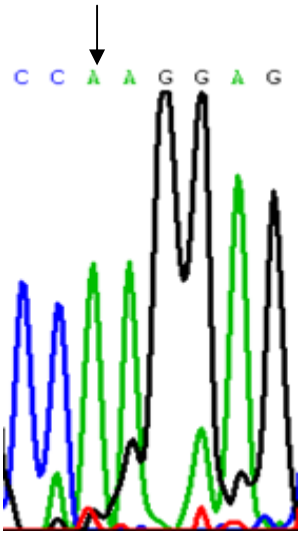
Sequence data analysis of the three PK-LR regions (exons 8 to 10, exon 11 and exon 12) amplified from the patient's genomic DNA revealed a homozygous point mutation at nucleotide 1529 in exon 11, where a guanine was replaced by an adenine (G1529A) (Figure 11a). There were no other mutations identified in the three regions. The amplification and sequencing were done on three occasions to verify results and exclude Taq polymerase errors or sequencing artifacts. The same results were obtained on all three occasions. Furthermore, since the patient was homozygous for the G1529A mutation, the patient's mother had to be heterozygous at the same residue, which was confirmed following sequencing (Figure 11b).

4.4.3 Structural analysis of the mutant PK

The G1529A point mutation results in an arginine to glutamine change at amino acid residue 510 (Arg510Gln). There is no crystal structure available for this particular mutation. The amino acid change was therefore mapped to the molecular structure using the SWISS-PDB Viewer (Schwede, 2003). From the model it appears that the mutation is not in direct contact with the catalytic site (Figure 12), however it is closer to the allosteric site where fructose 1,6-bisphosphate binding influences enzyme stability and

activity (Wang et al, 2001). Interactions between the mutant amino acid and neighbouring residues most likely cause conformational changes that result in the mutant's decreased EA.

(a) Homozygous A/A (residue 1529)



(b) Heteroygous A/G (residue 1529)

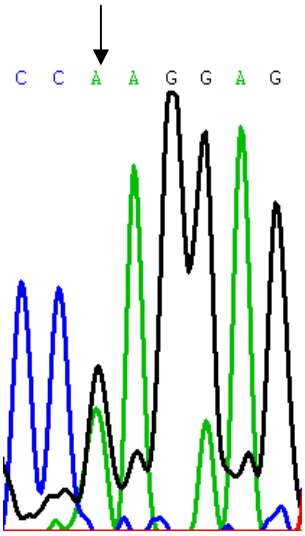


Figure 11: Sequence data of exon 11 of the PK-LR gene.
The PK deficient patient (a) is homozygous for the G1529A mutation. Her mother (b) is heterozygous for the same mutation as demonstrated by the similar heights of the A and G peaks at the same position.

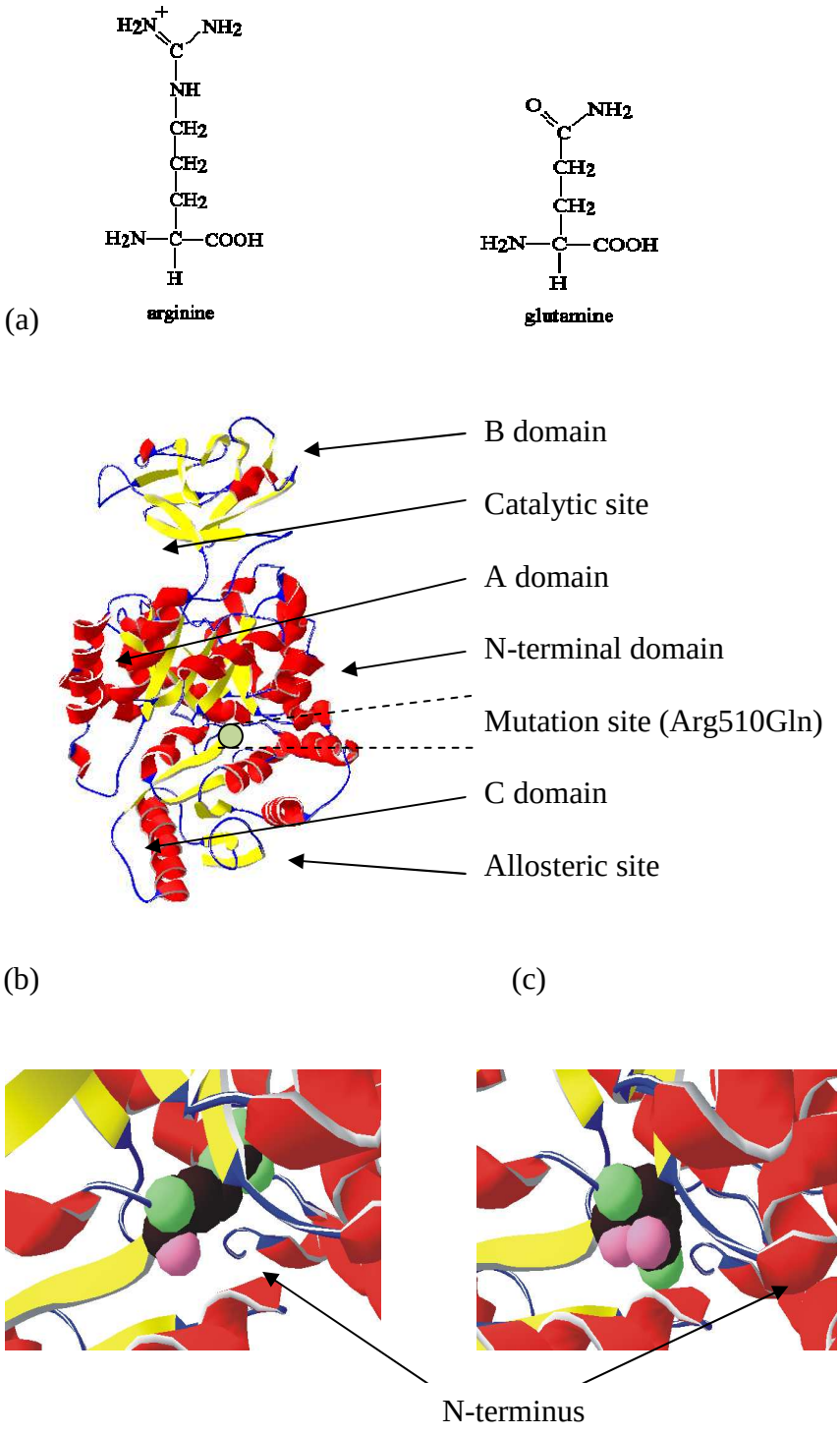


Figure 12: Modeling of the mutant Arg510Gln PK protein.
The chemical structures of arginine and glutamine and the site of the mutation in the 3D structure are shown (a). Space filling models were generated for the wildtype (b) and mutant (c) amino acid sites. Colour code: α helices – red; β sheets – yellow; turns – blue; carbon atoms – brown; nitrogen atoms – green; oxygen atoms – purple.

CHAPTER 5 DISCUSSION

Inherited red cell disorders comprise the haemoglobinopathies, membrane abnormalities and enzyme deficiencies. In South Africa, haemoglobinopathies are diagnosed in routine haematology laboratories and investigation of red cell membrane disorders is possible at the Red Cell Membrane Unit at the University of the Witwatersrand / NHLS. Routine investigation of the enzyme disorders is currently limited to a screening test for G6PD deficiency. The worldwide prevalence of PK deficiency suggests that this disorder may be under-diagnosed in Africa. Firstly, G6PD and PK deficiencies comprise the bulk of red cell enzyme disorders (>90% worldwide) and although G6PD is widely accepted as being the most common red cell enzyme disorder globally (Eber, 2003), the frequency of PK deficiency rivals that of G6PD deficiency in some areas eg, Northern Europe, China, regions in the Middle East, parts of North America and many equatorial malaria-endemic regions (Beutler and Barancioni, 1996; Beutler and Gelbart, 2000; Fermo et al, 2005; Zanella et al, 2005). Secondly, one of the few studies on Africans found a higher prevalence of PK deficient heterozygotes in African Americans than in Caucasian Americans. For over a decade the laboratory diagnosis of PK deficiency in South Africa has been unavailable due to the discontinuation of commercial kits. The objective of this report was therefore to implement a laboratory test for the diagnosis of PK deficiency at the Johannesburg Hospital, which may serve as a reference centre for this investigation. In addition, a PK deficient patient and her mother were evaluated and the causative mutation in the PK-LR gene was identified.

5.1 Qualitative PK assay

The qualitative assay demonstrated that an individual with an EA of <50% of normal will be detected as having decreased PK activity. Signs and symptoms of PK deficiency manifest when the EA is ≤25% (Eber, 2003) and the screening assay will therefore detect (with few exceptions, see next paragraph) clinically relevant cases. Heterozygotes will not be detected by the screening assay since these individuals will have one normal allele and therefore at least 50% EA. This should be borne in mind when family studies are undertaken. In this situation, the quantitative assay is necessary to detect carrier status.

5.2 Quantitative PK assay

There was no difference between the means of PK activity in the 20 Caucasians and 20 Africans. The combined mean (n=40) was therefore calculated as 13.01 U/g Hb ± 2.25 and is within 1 SD of the published mean (Eber, 2003). The reason for the slightly lower value is uncertain and may be due to slight differences in the way in which samples have been processed eg, the method for removing leucocytes differs between this study and Beutler's original method (Beutler, 1975). As more samples are processed in the Red Cell Membrane Unit, the values will be added to the 40 samples already processed to generate a new mean EA. The PK activity will be reported as a percentage of the mean determined in this study, and any slight differences between the findings of this study and the reported reference range will therefore not affect the accuracy of the results.

It was noted that the mean of the 40 samples processed to implement the assay and the mean of the 10 samples processed as part of a haemolytic workup were almost identical (13.01 and 13.16 U/g Hb, respectively).

The absolute values for the absorbance readings varied between quantitative assays as reflected in Tables 4, 8, 9 and 10. This can be accounted for by the slight differences in NADH and Hb concentrations. Variations in NADH concentration are the result of several factors. NADH needs to be prepared weekly and small differences in weighing NADH and instability of the reconstituted reagent, as well as differences in intra-erythrocytic NADH are all contributory. The absolute values are not important however, since the EA calculations are based on the rate of change in absorbance from 5min to 15min. EA was therefore determined from the rate of oxidation of NADH and was not affected by the initial NADH concentration (provided absorbance readings fall within the detection limits of the spectrophotometer). EA is also expressed as U/g Hb, which takes into account variations in Hb concentration.

5.3 PK assay limitations

The usefulness of the assay is limited in patients who have a marked reticulocytosis or who have a history of recent transfusions. In these instances, the reticulocytes or transfused cells will mask an underlying PK deficiency. In cases with a high index of suspicion, it may be necessary to consult with the attending physician and perform the

assay at an appropriate time. Alternatively, family studies or genetic studies may be beneficial.

Occasionally, kinetically abnormal mutant PKs are encountered (Zanella and Bianchi, 2000). Although the enzyme is ineffective *in vivo*, it displays normal or increased EA *in vitro*. This possibility should be considered in cases which are strongly suggestive of a PK deficiency but have a normal PK assay. These mutants can be identified by assaying EA at both high and low PEP concentrations with fructose 1,6-bisphosphate added at the low concentration (Beutler, 1975). This was not implemented in this study and will be considered when clinically appropriate.

5.4 Quality assurance

5.4.1 Sample handling and processing

EDTA tubes are generally used in a "haemolytic work-up". ACD or heparin tubes have also been reported as being suitable (Beutler, 1975). One tube should be submitted to the laboratory and 1ml of whole blood removed for performing the screening assay. The remaining blood should be stored at $4^{o}C$ for future use as necessary.

This study found that samples may be stored at room temperature for a maximum of 3 days, and at $4^{o}C$ for a maximum of 5 days prior to performing the assay. This was not known before this study and provides useful information to clinicians and pathologists. If

this time-frame is adhered to, it is feasible to receive samples from around the country and from other southern African states for processing. These results also indicated that it is possible to batch samples and process them every 3 to 5 days depending on the storage conditions and on the time lapsed since venipuncture. The minimum volume of blood required to perform the PK assay is 0.5ml and it is therefore possible to perform the assay on paediatric patients (guidelines for requesting physicians are included in Appendix 5). The results indicated that once a haemolysate is made it should be stored at $4^{o}C$ and processed within 2hrs.

An important step in sample processing is the removal of leucocytes. Inadequate leucocyte depletion is a recognized cause of an erroneous result despite the principle of the assay method to inhibit white cell lysis (Beutler, 1975; Zanella et al, 2007). There are no reported critical levels in the literature but leucocyte contamination should be considered when highly suspicious cases are found to have normal PK activity. As with all laboratory investigations, PK assay results must be interpreted in conjunction with the clinical scenario and if necessary, repeated.

5.4.2 Reagents

The salt solutions and buffer were stable at room temperature for the 6 month period over which the study was conducted. ADP and PEP were stable at $-20^{o}C$ and were prepared monthly. LDH was stable at $4^{o}C$ and used over 6 months. NADH is the most unstable reagent in the assay. In lyophylised form it may be stored according to the manufacturer's

instructions and expiry date. For use in the PK assay, NADH needs to be reconstituted weekly and kept at -20^{o}C. Reagents will be included in the kit supplied to the Haematology laboratory at the Johannesburg Hospital for use in the screening assay as required (see Section 5.4.5).

5.4.3 Accuracy, precision and reproducibility

There is no recognized gold standard for measuring PK activity and accuracy by comparison with a definitive method was therefore not possible. Commercial kits are also unavailable. However, the method employed in this study is the recommended method by the International Committee for Standardization in Hematology (Miwa et al, 1979). The precision and reproducibility of the quantitative method were measured by the CV and were both calculated as 5%. According to the Clinical and Laboratory Standards Institute (www.ncds.org), a CV of <10% is considered adequate.

5.4.4 Controls

For screening and confirmatory assays several controls were incorporated (Sections 2.2 and 2.2.1). A blank (no haemolysate and no ADP) was included once for each assay. For each control and sample, a control negative and sample negative (no ADP) were included. The difference between the sample and the sum of the blank and sample negative is a measure of PK activity and eliminates the oxidation of NADH due to other factors (see section 2.2). Similarly, the EA of the control is calculated as the difference

between the control and the sum of the blank and control negative. The control provides proof that the assay is working on the day.

5.4.5 Skills, training and equipment

The skills necessary to perform the qualitative assay fall within the scope of an experienced medical technologist. From the candidate's experience the qualitative assay can be performed within 90 minutes and it is envisaged that this test be offered as a screen for PK deficiency together with the G6PD screen in the routine laboratory. A SOP has been compiled for this purpose (Appendix 3). To facilitate the smooth implementation of the screening assay in the Haemtology laboratory, Johannesburg hospital, a kit containing aliquots of freshly prepared reagents may be supplied by the Red Cell Membrane Unit. This can be collected from the Unit as needed. Blood samples that are PK deficient on the screening assay should be referred for a confirmatory quantitative assay, which will be performed by the author of this work. In the event that the routine Haematology laboratory at the Johannesburg Hospital, NHLS wished to implement the screening PK assay, training can be provided by the author of this work.

All the necessary equipment for the screening test (eg centrifuge, freezer, pipettes, etc) is available in any routine haematology laboratory. In this study a -70^{o}C freezer was used in the red cell lysis step, but no difference was found when a -20^{o}C freezer was subsequently used. The projected costs of reagents may be found in Appendix 4. This excludes costs for labour, equipment and miscellaneous items such as pipette tips.

5.4.6 Reporting

5.4.6.1 Qualitative assay

The screening test should be reported as either “PK activity detected” or “decreased PK activity detected”. A comment should be included by the authorizing pathologist when PK activity is detected in clinically suspicious cases to alert the physician that a PK deficiency may be masked by a reticulocytosis or a recent transfusion. In cases where decreased PK activity is detected a confirmatory test should be recommended.

5.4.6.2 Quantitative assay

The quantitative assay will be reported by the Red Cell Membrane Unit as a percentage EA of the mean, determined in this study to be 13.01U/g Hb. Comments will be added to the report as appropriate.

5.5 PK deficient patient

5.5.1 Clinical aspects

Zanella et al (2007) have described the PK deficient phenotype as mild, moderate or severe based on the number of times a patient has been transfused, age at time of

diagnosis, Hb concentration and mutation analysis. The moderate phenotype is characterized by a Hb of 9 to 10 g/dl and a history of haemolytic episodes that required one or more blood transfusions. The patient investigated here is typical of the moderate phenotype: she had neonatal jaundice that required blood transfusions and her Hb has been maintained at 9 to 10 g/dl. The moderate phenotype typically has some degree of ongoing haemolysis and this patient's GP reported that her serum unconjugated bilirubin was consistently above the upper limit of normal, a finding that together with the moderate anaemia indicated ongoing haemolysis. At the time of referral, her laboratory results confirmed the ongoing haemolysis: she had a low Hb and haptoglobin; the indirect bilirubin and serum LDH were elevated and there was a reticulocytosis. She had not been splenectomised, which was appropriate for her as splenectomy is usually reserved for the severe phenotype (Zanella et al, 2007). The patient had maintained her Hb at 9-10g/dl until the haemolytic episode associated with ARV treatment. There was nothing in the history to indicate another cause for the sudden haemolytic episode and her Hb once again stabilized after discontinuing the ARV treatment.

A search of the literature revealed no documented association between ARVs and the induction of haemolysis in PK deficiency. The package inserts of the three drugs that the patient received (Crixivan, 3TC and AZT) report no evidence of drug-associated haemolysis. However, 3TC and AZT are both nucleoside reverse transcriptase inhibitors (NRTIs), which are known to be toxic to mitochondria and several recent reviews have been published on the subject (eg Kohler and Lewis, 2007). PK deficient normoblasts rely on ATP generated from oxidative phosphorylation and mitochondrial toxicity will

therefore decrease intracellular ATP concentrations leading to intra-medullary cell death. This would explain the sudden decrease in Hb concentration and the haemolytic parameters in this patient. ARV treatment, per se, has also been implicated in haemolysis although the mechanism remains obscure (Diop et al, 2006).

5.5.2 PK assay results

The patient's screening assay demonstrated decreased PK activity and the quantitative assay confirmed the deficiency. The average of four assays demonstrated an EA of 15% of the normal mean. The EAs in the four assays ranged from 8% to 23%. The reason for this is uncertain but may be related to varying degrees of reticulocytosis. Her mother had a normal screening assay, which requires an EA of ≥50%. This was confirmed by her quantitative assay which demonstrated an EA of 58% of the normal mean. Previous estimates of the EA of the G1529A homozygous mutation have ranged from 1% to 10% (Lenzner et al, 1994; reviewed in Zanella et al, 2007), which is slightly less than the 15% calculated in this patient. However, the absolute value for EA varies in different individuals and is likely to be due to factors like intra- and inter-individual variation, differences in the assays, and the degree of the reticulocytosis in the affected patient.

5.5.3 Mutation analysis and thermal stability of the Arg510Gln mutant PK

The patient originates from north-central Europe where the G1529A mutation is the most common PK-LR mutation (41%) (Lenzner et al, 1997; Pissard et al, 2006). Due to the

high frequency of the G1529A mutation, PK deficient patients are often identified as being homozygous for this mutation. The thermodynamic stability of the mutant protein Arg510Gln has previously been investigated and demonstrated a decreased heat stability at 53^{o}C ($t_{1/2}$ = 3min) (Wang et al, 2001; Zanella et al, 2007). The results in this report (Section 3.5.3) corroborate the thermal instability although the loss in EA was more dramatic (EA was <1% after 3min). This difference may be explained by the fact that the experiments in this report were performed on the native mutant, whereas Wang et al (2001) analysed the thermal stability of the recombinant mutant enzyme.

To date 10 PK mutants have been produced as recombinant forms, including the Arg510Gln mutant (Wang et al, 2001; Valentini et al, 2002; Fermo et al, 2005). The crystallographic structure of this mutant has not been resolved, however, it has been suggested that the amino acid change disrupts hydrogen bonds between the Arg510 of the C domain and the Ala399 and Thr88 of the A domain resulting in protein instability (Wang et al, 2001). The mutant crystal structure is required, however, to investigate the conformational changes in more detail.

5.6 PK deficiency in South Africa

Very little is known about the prevalence of PK deficiency and nothing about the different mutations in South Africa. PK deficiency certainly occurs in this region and during the 1980s and early 1990s when the commercial PK assay was available at Chris Hani Baragwanath Hospital, several cases were identified in Caucasian and African

patients (Dr. N. Alli, Principal pathologist, Chris Hani Baragwanath hospital, personal communication). These cases were routinely diagnosed using a commercial kit but mutation analysis was not done. Investigation for PK deficiency was halted when the commercial kits became unavailable. Recently a case of a compound PK deficient, sickle cell trait patient was investigated at Chris Hani Baragwanath Hospital and the mutation analysis performed at King's Hospital, London (Alli et al, 2007).

5.7 PK deficiency and malaria

Epidemiological studies have estimated PK deficient alleles to occur at polymorphic frequencies (frequency >1%) in many temperate and equatorial regions eg, Central Europe, southeast Asia and parts of China (Mohrenweiser, 1987; Beutler and Gelbart, 2000; Fermo et al, 2005). This suggests there has been a driving force that maintained the PK deficiency alleles at these frequencies. One hypothesis is that PK deficiency is partially protective against malaria. Malaria was endemic in many temperate climates including North America and Europe until as recent as the end of the 19th century (Hay et al, 2004). The overlap between the historical geographic distribution of malaria and the polymorphic frequencies of PK deficiency supports the hypothesis that PK deficiency confers protection against malaria. Furthermore, PK deficiency is prevalent in many areas (eg, southeast Asia) where malaria is still endemic. Strong supportive laboratory evidence demonstrates a protective effect of PK deficiency against *Plasmodium* parasitism in the mouse model (Coburn, 2004; Min-Oo et al, 2007). The author of this work and Prof T. L. Coetzer in the Department of Molecular Medicine, University of the Witwatersrand and

NHLS, are currently investigating the potential protective effect in humans. A series of experiments has demonstrated repeatedly that the parasitaemia fails to increase as expected when *P. falciparum* is cultured with PK deficient erythrocytes. This provides the strongest support yet for the "malaria-protective hypothesis" and a manuscript describing these findings is currently being prepared.

5.8 Concluding remarks

The potential to perform assays for PK activity will enrich the capacity of the NHLS to investigate haemolytic anaemias in South Africa and will assist in avoiding unnecessary investigations in PK deficient patients once a diagnosis is made. A further positive spinoff is that valuable information will be gained in determining the prevalence of this disease in South Africa. In addition, analysis of the PK-LR gene in confirmed PK deficient patients will identify the underlying mutations in South African population groups.

REFERENCES

Alli NA, Coetzee MJ, J van Rensburg BW, et al. Homozygous sickle clinical phenotype in a sickle carrier with co-existing pyruvate kinase deficiency. The 47th annual congress of the Federation of South African Societies of Pathology. 2007, oral presentation HPO1.

Beutler E. Red Cell Metabolism. A Manual of Biochemical Methods. Second Edition. Grune and Stratton. New York 1975.

Beutler E and Barancioni L. Mutations in Pyruvate Kinase. Hum Mut 1996, 7: 1-6.

Beutler E and Gelbart T. Estimating the prevalence of pyruvate kinase deficiency from the gene frequency in the general white population. Blood 2000, 95: 3585-3588.

Coburn BA. Pyruvate Kinase Deficiency: Providing Protection from *Plasmodium* Parasitism. Clin Genet 2004, 65: 264-266.

Diez A, Gilsanz F, Martinez J, et al. Life-threatening nonspherocytic hemolytic anemia in a patient with a null mutation in the PKLR gene and no compensatory PKM gene expression. Blood 2005, 106: 1851-1856.

Diop M-E. Bastard J-P, Meunier N, et al. Inappropriately low glycated hemoglobin values and hemolysis in HIV-infected patients. AIDS Res and Human Retroviruses 2006, 22:1242-1247.

Eber SW. In Handin RI, Lux SE and Stossel TP, eds. Blood: Principles and Practice of Haematology. Second Edition. Lippincott, Williams and Wilkins, Philadelphia. 2003, Chapter 53: 1895-1901.

Fermo E, Bianchi P, Chiarelli LR, et al. Red cell pyruvate kinase deficiency: 17 new mutations of the PK-LR gene. Br J Haematol 2005, 129: 839-846.

Hay SI, Guerra CA, Tatern AJ, et al. The global distribution and population at risk of malaria: past, present and future. Lancet Infect Dis 2004, 4:327-336.

Kibbe WA. 'OligoCalc': an online oligonucleotide properties calculator. Nucl Acids Res 2007, Web Server Issue:1-4.

Kohler JJ and Lewis W. A brief overview of mechanisms of mitochondrial toxicity from NRTIs. Environ Mol Mutagen 2007, 48: 166-172.

Lenzner C, Nurnberg P, Thiele BJ et al. Mutations in the pyruvate kinase L gene in patients with hereditary haemolytic anaemia. Blood 1994, 83: 2817-2822.

Lenzner C, Nurnberg P, Jacobasch G et al. Molecular analysis of 29 pyruvate kinase-deficient patients from central Europe with hereditary haemolytic anaemia. Blood 1997, 89: 1793-1799.

Min-Oo G, Tam M, Stevenson MM, et al. Pyruvate kinase deficiency: correlation between enzyme activity, extent of hemolytic anemia and protection against malaria in independent mouse mutants. Blood Cells Mol Dis 2007, 39:63-69.

Miwa S, Boivin P, Blume KG, et al. Recommended methods for the characterization of red cell pyruvate kinase. Br J Haematol 1979, 43:275-282.

Mohrenweiser HW. Functional hemizygosity in the human genome: Direct estimate from twelve erythrocyte enzyme loci. Hum Genet 1987, 77:241-245.

Motulsky HJ. Analyzing data with GraphPad Prism. GraphPad Software Inc.1999, San Diego, www.graphpad.com

Pissard S, Maz-Audit I, Skopinski L, et al. Pyruvate kinase deficiency in France: a 3-year study reveals 27 new mutations. Br J Haematol 2006, 133:683-689.

Schwede T, Kopp J, Guex N, and Peitsch MC. SWISS-MODEL: an automated protein homology-modeling server. Nucl Acids Res 2003, 31:3381-3385.

Talmud P, Tybjaerg-Hansen A, Bhatnagar D, et al. Rapid screening for specific mutations in patients with a clinical diagnosis of familial hypercholesterolaemia. Atherosclerosis 1991, 89:137-141.

Thompson JD, Higgins DG and Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucl Acids Res 1994, 11:4673-4680.

Valentine WN, Tanaka KR and Miwa S. A specific glycolytic enzyme defect in three subjects with congenital non-spherocytic haemolytic anaemia. Trans Assoc Am Physicians 1961, 74: 100-110.

Valentini G, Chiarelli LR, Fortin R, et al. Structure and function of human erythrocyte pyruvate kinase. Molecular basis of nonspherocytic hemolytic anemia. J Biol Chem 2002, 277:23807-23814.

Wang C, Chiarelli LR, Bianchi P, et al. Human erythrocyte pyruvate kinase: characterization of the recombinant enzyme and a mutant form (R510Q) causing nonspherocytic haemolytic anaemia. Blood 2001, 98:3113-3120.

Zanella A, Bianchi P, Baronciani L, et al. Molecular characterization of the PK-LR gene in pyruvate kinase deficient patients. Blood 1997, 89:3847-3852.

Zanella A and Bianchi P. Red cell pyruvate kinase (PK) deficiency: from genetics to clinical manifestations. Baillieres Best Pract Res Clin Haematol 2000, 13:57-81.

Zanella A, Fermo E, Bianchi P, et al. Red cell pyruvate kinase deficiency: molecular and clinical aspects. Br J Haematol 2005, 130:11-25.

Zanella A, Fermo E, Bianchi P, et al. Pyruvate kinase deficiency: the genotype-phenotype association. Blood Rev 2007, 21:217-231.

APPENDICES

APPENDIX 1: SUPPLIERS

Amersham Pharmacia (www.amersham.org)
Power supply

Beckman (www.beckman.com)
Centrifuge; Haematology Analyzer; Spectrophotometer

Eppendorf (www.eppendorf.com)
Mastercycler gradient; Eppendorf tubes

Fermentas (http://www.fermentas.com)
Fermentas MassRuler DNA ladder Mix (Catalogue no. SM0403)

Inqaba Biotec (http://www.inqababiotec.co.za)
Oligonucleotide synthesis; DNA sequencing

Merck (www.merck.co.za)
EDTA (Catalogue no. 108415); β-mercaptoethanol (Catalogue no. 805740)

Mettler (http://us.mt.com/home)
Balance

Microsep (www.microsep.co.za)
Millipore MIlli-Q Water System

Roche (www.roche.com)
L-LDH (Catalogue no. 10127230001); Roche Expand High Fidelity PCR System (Catalogue no. 03300226001); Phosphoenolpyruvate (Catalogue no. 10108294001);

NADH (Catalogue no. 10107735001); ADP (Catalogue no. 10127507001); Tris (Catalogue no. 10708976001)

Syngene (www.syngene.com)
Geldoc imaging system

TaKaRa (www.takara-bio.com)
dNTPs 2.5mM

UVP, Inc. (www.UVP.com)
Transilluminator

Whatman International (www.whatman.com or www.merck.co.za)
Whatman no. 1 filter paper

Whitehead Scientific (www.whitesci.co.za)
PCR tubes (Catalogue no. 445-Mixed)

APPENDIX 2 REAGENTS

30mM ADP: (M_r = 471.2, disodium salt); 280mg/20ml; lyophylised form stored at -20^{o}C according to manufaturer's instructions, reconstituted form stored at -20^{o}C for 1 month

1M KCl: (M_r = 74.55); 7.5g/100ml; stored at room temperature for 6 months

L-LDH: specific activity of 60U/ml where 1 enzyme unit reduces 1µmole of pyruvate to L-lactate in 1 min at 25^{o}C, pH 8; stored at 4^{o}C according to manufacturer's instructions

0.1M $MgCl_2$: (M_r = 95.2, 6-hydrate); 0.95g/100ml; stored at room temperature for 6 months

0.9% NaCl: 0.9g/100ml; stored at room temperature for 6 months

2mM NADH: (M_r = 709.4, disodium salt); 28mg/20ml; lyophylised NADH stored at 4^{o}C according to manufacturer's instructions, reconstituted NADH stored at -20^{o}C for 1 week

PBS: 8g NaCl, 0.2g KCl, 1.78g $Na_2HPO_4.2H_2O$ and 0.2g KH_2PO_4 (anhydrous) made up to 1l with MilliQ water (pH=7.4); stored at room temperature for 6 months

50mM PEP: (M_r = 206.1, monopotassium salt); 206mg/20ml; lyophylised form stored at 4^{o}C according to manufacturer's instructions, reconstituted and stored at -20^{o}C for 1 month

Stabilizing solution: 2.5µl 100% β-mercaptoethanol, 250µl 0.5M EDTA (pH~8) made up to 50ml with MilliQ water; stored at 4^{o}C for 6 months

TE buffer: (M_r Tris = 121.1; M_r EDTA = 292.2, disodium salt); to make a 1M Tris, 5mM EDTA buffer, dissolve 12.1g Tris (base) and 1.46g EDTA (acid) in 1l MilliQ water; pH~8 with dilute HCl

APPENDIX 3: SOP: PK SCREENING ASSAY

Objective

To outline a simple screening test to determine pyruvate kinase status of red blood cells.

Clinical aspects

Pyruvate kinase (PK) deficiency is one of the commonest inherited red cell enzyme disorders. The clinical presentation is very variable, ranging from a mild anaemia to a severe chronic non-spherocytic haemolytic anaemia. For this reason it is important to screen for this disorder in clinically suspicious cases so that appropriate management can be advised.

Test principle

PK catalyzes the formation of pyruvate and ATP from PEP and ADP using Mg^{2+} as a cofactor. This is coupled to a second reaction whereby LDH (lactate dehydrogenase) uses pyruvate as a substrate to form lactate. In this second reaction, NADH is a cofactor and is oxidized to NAD^{+}. PK activity is the rate limiting step in the assay and the test makes use of the fact that NADH fluoresces at 340nm whereas NAD^{+} does not. This test is useful for screening clinically relevant cases where PK activity is less that 50% of normal. ADP is essential for the first reaction and is omitted in the control negative and sample negative.

Responsibility

All staff working on the “specials” bench must be familiar with the principles and procedures outlined in this document.

Frequency of testing

Samples are batched and processed within 3 days (if sample was stored at room temperature) or within 5 days (if sample was stored at $4^{o}C$).

Specimens

Venous blood collected in an EDTA tube is used.

Quality control

1. Venous blood in EDTA tubes is stable for 5 days if kept at 4^{o}C or for 3 days if kept at room temperature.
2. Each assay includes a blank, a control and control negative, and for each patient, a sample and sample negative (see Methods below). The control is collected from the QC officer and is set up to ensure the assay is working.
3. Reagents (except for SABAX or MilliQ water, and 0.9% saline) used in the assay can be collected in the form of a kit from the Red Cell Membrane Unit, Dept. of Molecular Medicine and Haematology, 7^{th} floor, Medical School on the day the assay is performed. When the kit is collected, the number of samples to be processed must be known so that adequate volumes of reagents are collected. The reagents in the kit are listed below.

Reagents

1. TE buffer (pH~8) comprising 1M Tris and 5mM EDTA
2. 1M KCl
3. 0.1M $MgCl_2$
4. 2mM NADH
5. 30mM ADP
6. LDH (60U/ml)
7. Stabilizing solution comprising 2.5µl 100% β-mercaptoethanol, 250µl 0.5M EDTA (pH~8) made up to 50ml with Milli-Q or SABAX water

Equipment

1. Routine Haematology Centrifuge
2. Beckman Coulter Auto-Analyzer
3. -20^{o}C freezer
4. 4^{o}C fridge
5. 5ml test tubes
6. 2ml Eppendorf tubes

7. Pipettes: 10µl, 100µl and 200µl
8. Whatman no. 1 filter paper (11cm diameter)
9. Stopwatch
10. UV lightbox

Method

1. Thaw out reagents and keep at room temperature, except for the LDH, which should be kept in the 4^{o}C fridge.
2. Centrifuge blood at 2500rpm for 10min at room temperature in the routine haematology centrifuge.
3. Aspirate and discard plasma. Aspirate the buffy coat and keep in a labeled 1.5ml Eppendorf tube at 4^{o}C (this will be stored at -20^{o}C if the assay detects a decreased PK activity, see "Interpretation of results" below; if a normal PK activity is found it may be discarded).
4. Wash the red cells three more times by resuspending in an equal volume 0.9% saline and centrifuging as above, removing any residual buffy coat each time. It is not important if some red cells are lost in the process. NOTE: It is important to remove all the white cells of the buffy coat because they interfere with the assay and give incorrect results!
5. The final resuspension of washed cells is in an equal volume 0.9% saline. Measure the Hb concentration on the Beckman Coulter auto-analyzer (it uses ~200µl of your suspension). The Hb measurement is required if a decreased PK activity is detected.
6. Keep the remaining red cell suspension on ice and use within 1hr.
7. Make a haemolysate by adding 200µl resuspended cells to 1.8ml stabilizing solution in a 2ml Eppendorf tube.
8. To ensure complete haemolysis, place the haemolysate in -20^{o}C acetone and leave in the freezer for 10 mins (acetone or another organic solvent such as ethanol can be used and is left in the -20^{o}C freezer for at least 1 hr before using).
9. Place the haemolysate in a beaker containing water at room temperature until completely thawed and keep on ice. The haemolysate must be used within 1hr. It

is important to keep the haemolysate since this will be used to perform a confirmatory test if a decreased PK activity is detected (see "interpretation of results" below).

10. Label 5 x 5ml reaction tubes (or more depending on how many samples there are to process) appropriately and add the reagents as listed in the table below. All reagents must be at room temperature before aliquoting. NOTE: for each assay there is one blank; there is a control and control negative; there is a sample and sample negative. For each patient, a sample and sample negative are required.

Reagent	Blank	Control Neg.	Control	Sample Neg.	Sample
TE buffer (1M Tris, 5mM EDTA)	100	100	100	100	100
KCl (1M)	100	100	100	100	100
$MgCl_2$ (0.1M)	100	100	100	100	100
NADH (2mM)	100	100	100	100	100
ADP (30mM)	-	-	50	-	50
LDH (60U/ml)	1	1	1	1	1
Haemolysate	-	20	20	20	20
Stabilizing solution	20	-	-	-	-
SABAX or MilliQ water	480	480	430	480	430

11. Using Whatman no. 1 filter paper, draw out a grid as indicated below.

Time	Blank	Control Neg	Control	Sample Neg	Sample
0min					
5min					
10min					
15min					
Result					

12. Before adding substrate, spot 5µl of each reaction on the paper (0min). Check that all the spots fluoresce under UV light. If any of the spots do not fluoresce the assay will not work and advice needs to be sought from the registrar on specials.
13. Begin the reactions by adding 100µl PEP.
14. Blot 5µl onto filter paper every 5 minutes for 15 minutes.
15. Check for fluorescence under UV light after each 5min interval.
16. Interpret and record the result as below and sign and date the filter paper.

Interpretation of results

After 15min, check that the controls have worked: fluorescence should persist in the blank, control negative and in each sample negative. Fluorescence should have disappeared in the control. If all the controls have worked, examine the sample(s) and compare with the corresponding sample negative(s) (If any of the controls are not working, see "troubleshooting" below). A loss of fluorescence in the sample(s) after 15min is recorded as "PK activity detected". A persistence of fluorescence after 15 minutes indicates decreased PK activity and should be recorded as "decreased PK activity detected". A confirmatory assay will be performed at this point. Immediately contact Dr. P. Durand in Dept. Molecular Medicine and Hematology, 7^{th} floor, (Tel. 0117172418 or 011 7172188), to perform a confirmatory test. The resuspended red cells, haemolysate and buffy coat which have been stored at 4^{o}C will be collected for this purpose.

Troubleshooting

1. No fluorescence in one or more spots at time 0min, before PEP substrate has been added: the assay will not work; repeat the setting up of those reactions that do not work or contact the registrar on specials for advice.
2. Loss of fluorescence in blank, or control negative, or sample negative: the reaction is contaminated with ADP (and in the case of the blank with haemolysate or another source of PK as well); set up all reactions again.
3. Persistence of fluorescence in control: the PK reaction is not working; one of the reagents is missing from the reaction; set up all reactions again.

Reporting

Results are reported as:

1) PK activity detected, or

2) Decreased PK activity detected - confirmatory test to be performed.

Safety

Refer to the NHLS Safety Manual, Sections B, C and E.

Reference

Beutler, E. Red Cell Metabolism A Manual of Biochemical Methods. Second Edition. Grune and Stratton. New York. 1975.

APPENDIX 4: PROJECTED PK QUALITATIVE ASSAY COSTS

Each time the assay is performed on one sample there are at least 5 reactions (blank, control and control negative, sample and sample negative). For each additional sample that is analysed when the assay is performed, there will be 2 more reactions (sample and sample negative). The final costs are quoted for performing the PK assay on one sample. An additional quote is supplied for any additional sample that may be processed at the same time.

A. Reagents and other consumables

Costs per reagent are calculated for processing one sample and then for every additional sample. Prices were taken from the Sigma, Roche and Merck 2006-2007 catalogues.

1. NADH: R42.20 / 250mg (R2.40 for 1 sample, 50c for each additional sample)
2. ADP: R73.30 / 1g (R3 for 1 sample and 60c for each additional sample)
3. PEP: R66.60 / 0.5g (65c for 1 sample and 26c for each additional sample)
4. LDH: R43 / 60U (20c for 1 sample and 8c for each additional sample)
5. KCl: R46 / 500g (5c for 1 sample and 2c for each additional sample)
6. $MgCl_2$: R47 / 100g (5c for 1 sample and 2c for each additional sample)
7. Tris-EDTA (1M, 5mM) R76/500mg Tris and R44/100g EDTA: (5c for 1 sample and 2c for each additional sample)
8. Filter paper R12 / 100 sheets (50c for 1 sample and no cost for additional samples)
9. beta-mercaptoethanol (molecular grade): R243/250ml (1c for 1 sample and 0.2c for each additional sample)

B. Cost estimates

The cost for processing one sample is R6.91. For each additional sample processed at the same time the cost is R1.50. This excludes pipette tips, MilliQ (or SABAX) water, Eppendorf tubes, test tubes and the cost of measuring Hb concentration on the auto-analyser. It also excludes labour costs, which are calculated depending upon the technologist performing the assay.

C. Labour

The PK screening assay may be batched 3-5 days depending on sample storage and time elapsed since venipuncture. Batched samples will take <2hrs to process. Depending upon the level of expertise of the medical technologist performing the assay, calculations may be based on 2-4 hrs labour time per week. The SOP stipulates that when the screening assay detects deceased PK activity, DR. P. Durand should be contacted within an hour to perform a quantitative assay using the stored haemolysate from the screening assay. If this is done the PK quantitative assay will take 1.5hrs. If there is a delay in performing the quantitative assay, a fresh haemolysate needs to be prepared and the labour time increases to 2.5hrs.

APPENDIX 5: PK ASSAY: PHYSICIAN GUIDELINES

1. Epidemiology and patient history

PK deficiency is well-documented in Caucasians and Asians. The prevalence in Africans is unknown.

There is often no family history of haemolytic disease.

The patient may have a history of haemolysis or blood transfusions.

The haemolysis may be chronic and ongoing or acute and episodic.

The anaemia varies in severity depending on the underlying mutation.

There may be associated complications due long-term haemolysis (eg, gallstones, folate or B12 deficiency).

The peripheral slide red blood cell morphology is usually normal and is always non-spherocytic.

Signs and symptoms of PK deficiency are clinically apparent when PK activity is ≤25% of normal.

2. Sample submission

One EDTA tubes sent at room temperature. A minimum volume of 0.5ml is required. The PK assay can be performed on blood stored at $4^{o}C$ for 5 days, or stored at room temperature for 3 days.

3. Reporting

Results will be reported as “PK activity detected” or “decreased PK activity detected”. The screening assay is designed to detect PK deficient patients with clinical disease (PK activity of ≤25%). Heterozygotes (PK activity ≥50%) will not be detected. A marked reticulocytosis or a recent blood transfusion will mask a PK deficiency. Some rare forms of PK deficiency are not detected by the routine PK assay and normal results in highly suspicious cases should be discussed with a haematologist. If a “decreased PK activity” is detected, a confirmatory assay will be performed.

4. Turn-around time

Samples are batched and processed every 3-5 days.