UZ LEUVEN | LABORATORIUMGENEESKUNDE

**CAT**
**Critically Appraised Topic**

**Title: Red blood cell enzyme assays: (d)efficient?**

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Search/methodology verified by:
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## CLINICAL BOTTOM LINE

*Belangrijkste weerhouden bevindingen/ conclusies. Iemand die niet veel tijd heeft, moet hier de correcte weergave van de besluiten vinden..*

Red blood cell (RBC) enzyme assays were introduced by E. Beutler in the 1970's to diagnose enzymopathies associated with anemia. With regard to this work, a search of relevant literature as well as a small benchmarking study including a Belgian and a Dutch reference center showed that the original principles are still common practice.
Clear indications exist for glucose-6-phosphate dehydrogenase (G6PD), pyruvate kinase (PK) and hexokinase (HK), however, requests for more rare enzymes can be warranted in some specific cases. The above mentioned literature search and benchmarking study in combination with a LIS review of all our RBC enzymopathy requests suggests that the glutathione peroxidase assay can be abandoned. Implementation of GPI may be considered.
Although the analytical process is well established in our center, increasing internal and external quality demands offered an opportunity to evaluate the pre-analytical phase (preferred anticoagulant, stability), to look for optimization in quality control materials and to verify our reference values in our current population. Concerning the former, we will evaluate the use of healthy volunteer blood samples as internal control material. Additionally, a request has been made to set up proficiency testing for our G6PD assay with external control material from UK NEQAS. With regard to the reference-values verification, in-house experiments as well as the benchmarking study, supports adjustment of our reference-values for the G6PD assay to 4.6 – 13.5 U/g Hb.
Finally, the need or added value of molecular diagnostic tests was looked into. No straightforward guidelines were found and only the Dutch reference center offers genetic testing for this type of disorders. However, sufficient evidence is available to offer shipment of samples to this center for DNA analysis in case of anemia with unknow origin i.e. a negative direct Coombs test, a normal osmotic fragility/cryohemolysis test, no specific morphological abnormalities and no abnormal hemoglobin.

## CLINICAL/DIAGNOSTIC SCENARIO

*Hoe/ waarom is men tot de vraagstelling gekomen? Wat is eventueel de huidige praktijk en waarom wordt die nu in vraag gesteld?.*

a) Introduction

Hereditary RBC enzymopathies are genetic disorders arising from mutations in genes coding for RBC metabolic enzymes and cause a specific type of anemia designated hereditary non-spherocytic hemolytic anemia. Malfunctions of RBC enzymes increase the levels of oxidative stress and impair cellular energy balance. These increased levels of oxidative stress and impaired energy balance affect cellular integrity inducing the premature removal of the RBC in the spleen and thus a decreased RBC survival [1–3]. The extent of hemolysis varies from mild/no anemia to severe transfusion-dependent hemolysis and even fatal anemia at birth. The degree of hemolysis depends on the affected pathway, the role of the mutated enzyme in this pathway, the dysfunction induced by the mutation and the compensation of the cell for the loss of enzymatic activity [1,2]. The enzymopathy induced hemolysis includes the following typical clinical symptoms: splenomegaly, jaundice, gallstones and iron overload. Some enzymopathies are even associated with systemic manifestations such as neurological dysfunction, mental retardation, myopathy and increased susceptibility for infections.
Currently, no curative therapy is available for these enzyme defects. Patients with mild anemia do not need any treatment while for patients with severe anemia the treatment consists of supportive therapy (blood transfusions) and chelation therapy to reduce the iron overload. Patients with enzymopathies with reduced protection against oxidative stress should avoid the intake of oxidative substances [2,4].

b) Specific characteristics and frequency

1) PK and G6PD deficiency: most common RBC enzymopathies

RBC PK deficiency is the most common glycolytic defect causing hereditary non-spherocytic hemolytic anemia with an estimated frequency of 1 : 20000 in the general Caucasian population and has a worldwide geographical distribution [5,6]. PK converts phosphoenolpyruvate to pyruvate generating 50% of total ATP in RBC. Thus RBC survival is dependent on the ATP produced during glycolysis. Less ATP results in a shortened lifespan for the cells by premature removal from the circulation [4,7]. More than 220 different mutations in the PK-LR (pyruvate kinase-liver and RBC) gene have been described in patients with PK deficiency and most of the mutations are missense mutations [2,4,8,9]. The clinical outcome of PK deficiency is ranging from mild anemia to severe transfusion-dependent hemolytic anemia. In most cases the anemia is stable but may become more severe due to physiological stress such as infections [2].

G6PD deficiency is an X-linked hereditary genetic disorder caused by mutations (mostly missense mutations) in the G6PD gene and it is the most frequent human enzyme defect (400 million cases worldwide). Because of the X-linked inheritance, this disorder is difficult to diagnose in heterozygous women using an enzyme assay. Therefore Prchal & Gregg [10] and Cappellini et al. [11] suggested DNA analysis for diagnosis in women (cfr. infra). The highest prevalence of G6PD deficiency is found in tropical Africa, the Middle East, tropical and subtropical Asia, Papua New Guinea and Mediterranean regions. In these areas, G6PD deficiency may represent a selective advantage due to increased resistance to severe *Plasmodium falciparum* infection [11,12].
G6PD provides reducing power to all cells in the form of NADPH (reduced state of nicotinamide adenine dinucleotide phosphate) [2]. The generated NADPH is required to preserve the reduced form of glutathione. When NADPH levels are not maintained, the glutathione levels decrease and oxidative damage occurs inducing acute hemolysis [12]. Most patients are asymptomatic but acute hemolysis may develop due to increased levels of oxidative stress such as the intake of specific drugs, beans or the occurrence of infections [11,13].

2) Other, more rare RBC enzymopathies

The characteristics of some more rare enzymopathies (for which enzyme assays are performed in UZ Leuven) are summarized in Table 1.

**Table 1: Summarized characteristics hexokinase (HK), glutathione reductase (GR), reduced glutathione (RD) and glutathione peroxidase (GPX) deficiency**

| *Enzyme* | *Description* | *Authors* |
|---|---|---|
| HK | • very rare red cell enzyme disorder<br>• associated with severe hemolytic anemia | Koralkova et al., 2014 [2]; Kanno et al., 2002 [14] |
| GR | • very rare red cell enzyme disorder<br>• regeneration of reduced glutathione<br>• deficiency characterized by increased susceptibility to oxidative stress<br>• generally asymptomatic<br>• acute hemolytic crisis by intake of drugs, fava beans | Van Zwieten et al., 2014 [15]; Kamerbeek et al., 2007 [16] |
| RG | • synthesized by glutamate cysteine ligase and glutathione synthetase<br>• deficiency characterized by increased susceptibility to oxidative stress<br>• rare disorder | Koralkova et al., 2014 [2] |
| GPX | • deficiency does not cause hemolysis<br>• must be regarded as a non-disease | Beutler 1979 [17]; Koralkova et al., 2014 [2] |

c) Laboratory diagnosis

The measurement of enzyme activity for diagnosis of the most enzymopathies is based on spectrophotometric analysis. These assays were described by E. Beutler in the 1970's [18]. In general, the activity of a specific enzyme is related to changes in absorbance over time. For example, for the determination of PK activity, phosphoenolpyruvate (PEP) and ADP (present in a reaction mixture) are converted to pyruvate and ATP by PK (extracted from the patient's blood and added to the reaction mixture). In a second step, pyruvate and NADH (present in a reaction mixture) converted to lactate and NAD. In this latter reaction, the decrease in absorbance due to conversion of NADH to NAD is monitored at 340 nm for a fixed amount of time (figure 1). For quantitative purposes, the sample hemoglobin is measured beforehand and the eventual enzymatic activity is expressed as IU/g Hb [18]. For the other RBC enzymes a similar procedure is used, the reactions are shown in Supplemental figure 1.


**Figure 1. Spectrophotometric analysis of PK deficiency. The reaction in green is monitored (**PK = pyruvate kinase; LDH = lactate dehydrogenase; PEP = phosphoenol pyruvate; ADP = adenosine diphosphate; ATP = adenosine triphosphate; NAD = nicotinamide adenine dinucleotide).

In UZ Leuven RBC enzyme assays are still performed according to the methods described by E. Beutler. In this regard, a review of methods currently used for diagnosis of RBC enzyme deficiencies, is an excellent project for the Critically Appraised Topic (CAT). On the one hand we reviewed scientific literature and on the other hand we visited two reference laboratories to learn how they manage the diagnosis of RBC enzyme deficiencies. Next to a review of the current UZ Leuven RBC enzyme assay portfolio, this information was used to examine optimization strategies to ensure up-to-date quality-assured reporting. More specifically, a thorough evaluation of the control (internal and external) materials, of the pre-analytical phase (preferred anticoagulant, stability) and of the reference values was conducted. Finally, the need for DNA diagnostic approaches was assessed.

**QUESTION(S)**

1) *Are RBC enzyme assays introduced by E. Beutler still the golden standard?*
    a. *Literature review*
    b. *Comparison with two reference centers*
    c. *Adjustments?*
2) *Is optimization from a pre-analytical and quality control standpoint warranted and achievable?*
3) *Should the UZ Leuven reference-values for the enzyme assays be updated?*
4) *Is characterization at DNA level always necessary?*

**SEARCH TERMS**

1) *MeSH Database (PubMed): MeSH term*
2) *PubMed Clinical Queries (from 1966; http://www.ncbi.nlm.nih.gov/entrez/query.fcgi): Systematic Reviews; Clinical Queries using Research Methodology Filters (diagnosis + specific, diagnosis + sensitive, prognosis + specific)*
3) *Pubmed (Medline; from 1966), SUMSearch (http://sumsearch.uthscsa.edu/), National Guideline Clearinghouse (http://www.ngc.org/), Institute for Clinical Systems Improvement (http://www.icsi.org), The National Institute for Clinical Excellence (http://www.nice.org.uk/), Cochrane (http://www.update-software.com/cochrane, Health Technology Assessment Database (http://www.york.ac.uk/inst/crd/htahp.htm)*
4) *National Committee for Clinical Laboratory Standards (NCCLS; http://www.nccls.org/), International Federation of Clinical Chemistry (IFCC; http://www.ifcc.org/ifcc.asp), American Diabetes Association (ADA; http://www.diabetes.org/home.jsp), National Diabetes Information Clearinghouse (NDIC; http://diabetes.niddk.nih.gov/), Westgard QC (http://www.westgard.com), Clinical Laboratory Improvement Amendments (CLIA; http://www.cms.hhs.gov/clia/)*
5) *UpToDate Online version 12.2 (2004)*

## 1. LITERATURE REVIEW

A recent review by Koralkova et al. [2] indicated that the enzyme assays introduced by Beutler are still the golden standard for diagnosis of enzymopathies. This was confirmed by several authors who in most cases to referred the book Red Cell Metabolism, A Manual of Biochemical Methods published by E. Beutler [1,6,7,10–13,17,19–23].

## 2. COMPARISON OF RBC ENZYME ASSAYS IN THREE CENTERS

Table 2 provides an overview of the RBC enzyme assays performed at two benchmarked reference centers as well as UZ Leuven. Evidently, the most common enzymopathies G6PD and PK are assayed in all three centers (Table 2-3). The same is true for HK. Although deficiency of hexokinase is not that common, it is associated with severe and potentially lethal hemolytic [2]. Because of this and because of the fact that both reference centers perform HK analysis, we will keep the HK assay running in UZ Leuven. Another interesting observation is that GPX is only analyzed in UZ Leuven. According to E. Beutler deficiency of this enzyme must be regarded as a non-disease [17]. Together with the fact that in the last 5 years no GPX enzyme deficiency was diagnosed in UZ Leuven, it can be concluded that it is unnecessary to keep an assay running for this enzyme (Table 3). Glutathione reductase (GR) and methemoglobine reductase (MetR) will, however, be kept in our portfolio because of the significant number (respectively 4 and 15) of diagnosed deficiencies in our center over the last five years (Table 3). Glucosephosphate isomerase (GPI) enzyme deficiency is analysed in the two visited centers but not in UZ Leuven. This enzyme is the second most frequent glycolytic enzyme with more than 50 families affected and 31 known mutations [Koralkova et al., 2014). We will evaluate the implementation of the GPI enzyme assay in consultation with clinicians. Of course if a clinician has strong suspicion for a specific RBC enzyme disorder for which we do not have an assay running in UZ Leuven, we can offer shipment to a specialized center.

**Table 2: Overview of RBC enzyme assays performed in 3 different centers (√ = performed; x = not performed)** [2]

| *Enzymes* | *UZ Leuven* | *Other Belgian Laboratory* | *Dutch Laboratory* | *Reported cases* |
|---|---|---|---|---|
| Glucose-6-phosphate dehydrogenase | √ | √ | √ | >400 x $10^6$ worldwide |
| Pyruvate kinase | √ | √ | √ | >500 families (1/20 000) |
| Hexokinase | √ | √ | √ | 20 cases |
| Adenosine deaminase | x | x | √ | 3 families |
| Adenylate kinase | x | x | √ | 12 families |
| phosphoglycerate mutase | x | x | √ | Not associated with hemolysis |
| FBP-aldolase | x | x | √ | 6 cases |
| Phosphofructokinase | x | x | √ | 50-100 cases |
| Phosphoglycerate kinase | x | x | √ | 40 cases |
| Glucosephosphate isomerase | x | √ | √ | >50 families |
| Glutathione peroxidase | √ | x | x | Not associated with hemolysis |
| Glutathione reductase | √ | x | √ | 2 families |
| Gereduceerd glutathione | √ | x | x | >62 families |
| Methemoglobine reductase | √ | x | x | >40 mutaties |
| Triosephospate isomerase | x | x | √ | 50-100 cases |

**Table 3: Reported cases versus cases in UZ Leuven** [2,24,25]

| *Enzymes* | *Number of reported cases/mutations* | *Number of cases in UZ Leuven last 5 years* | *% positive cases (%)* |
|---|---|---|---|
| Glucose-6-phosphate dehydrogenase | >400 million people worldwide, >160 mutations | 66 | 5.5 |
| Pyruvate kinase | >500 families, >200 mutations | 21 | 6.6 |
| Hexokinase | 20 cases, 5 mutations | 0 | 0 |
| Methemoglobine reductase | >40 mutations | 15 | 75 |
| Glutathione reductase | 2 families, 3 mutations | 4 | 3.5 |
| Reduced glutathione | >62 families | 25 | 25.3 |
| Glutathione peroxidase | ~ 17 cases* | 0 | 0 |

*17 cases were found via Pubmed search but not all articles were available in English

a. Quality control

Optimization in quality control materials is essential, especially because of the increasing internal and external quality demands. Currently, commercial control materials are available and used for the G6PD enzyme assay (also a commercially available kit from TrinityBiotech). For the other enzyme assays no quality control materials are available. In the benchmarked reference centers, quality control material was prepared from healthy volunteer blood samples. The evaluation of this procedure in UZ Leuven is ongoing. Briefly, quality control material will be prepared and stored at -80 °C (SOP in preparation). These aliquots will be used in all enzymatic assays for one year and the outcome will be evaluated for every enzyme. Obviously, this material can only be used as a negative internal control. However, the same experiment can be performed for deficient samples, if sufficient material is available. The latter approach is also used in both reference centers.
Another interesting observation from our visits to both reference centers was that for every enzymatic assay performed, the enzymatic activity of HK was determined in parallel and the enzyme activities were reported as a ratio to HK activity (e.g. G6PD/HK) in which HK activity serves as an extra internal control. However, this requires the determination of reference-values for this ratio and it increases the cost for the enzymatic assay (Table 5). The implementation of this ratio with hexokinase is under investigation in UZ Leuven.
Finally, both benchmarked reference centers always analyze in duplicate to reduce the inherently high CV's of these assays (CVs of in house experiments ranged from 10-18%, for both EDTA and ACD). Evidently, this results in a higher cost (Table 5) and thus implementation is also under investigation in UZ Leuven.

b. Collection tube: ACD versus EDTA

In UZ Leuven, ACD is the preferred anticoagulant to collect blood for RBC enzyme assays but EDTA is also acceptable (same policy as Mayo Medical Laboratories). In order to make an adequate comparison between ACD and EDTA collection materials, blood was taken from 10 healthy volunteers using both types. Enzymatic activity was determined within a few hours of withdrawal for G6PD, PK, GR and RG (in duplo). For G6PD and GR no significant difference was found between ACD and G6PD. Only for PK and RG a statistically significant result was observed ($p<0.05$, two-tailed, paired t-test for PK, G6PD, RG and $p<0.05$, two-tailed, Wilcoxon for GR); PK: higher values for ACD – RG: higher values for EDTA; Bland-Altman plots are provided in Supplemental Figure 2.). Hence, the type of collection material (ACD versus EDTA) can influence the result of the enzyme assays for PK and RG. However, the values obtained for the enzymatic activities of PK and RG were all within the respective reference values. In addition, scientific literature indicated both tubes can be used for enzymatic assays [22,26]. Therefore the current procedures for the collection tubes will not be adjusted, however, a more detailed description will be provided in the laboratory guide. Similar experiments are planned for HX and MetR.

c. Stability

The current turnaround time in UZ Leuven is less than 3 days for all RBC enzyme assays and is in accordance with the turnaround of the benchmarked reference centers and also Mayo Medical Laboratories and Arup Laboratories have this same turnaround time. In this section the stability of the enzymes is more thoroughly evaluated.
To verify the stability of the RBC enzymes, the experiment in 2b. was elaborated and starting from blood sampled from 10 healthy volunteers (10 ACD and 10 EDTA tubes), the enzymatic assays were repeatedly performed at day 0-1-2-7-14-21, either at room temperature or at 4-8°C. We accepted an error of ± 15%. Initially we accepted an error of 3SD but this was not achievable due to the high CV's. The results are summarized in Table 4. Based on these results the necessary adjustments will be made in the laboratory guide.

**Table 4: Stability of red blood cell enzymes** [19,20,27]

| *Enzymes* | ***Recommended**** *Tube* | *Temperature (°C)* | *Storage time (days)* | *Stability experiment UZ Leuven*** |
|---|---|---|---|---|
| Pyruvate kinase | ACD/EDTA | 4-8 | 20 | EDTA – 4-8 °C – 21 days |
| G6PD | ACD/EDTA | 4-8 | 7 | EDTA/ACD – 4-8 °C – 7 days |
| Hexokinase | EDTA | 4-8 | 4 | experiment ongoing |
| Methemoglobine reductase | ACD/EDTA | 4-8 | 22 | experiment ongoing |
| Glutathione reductase | | No data available | | EDTA – 4-8 °C – 14 days |
| Glutathione peroxidase | | No data available | | experiment ongoing |
| Reduced glutathione | | No data available | | ACD – 4-8 °C – 14 days |

*data from Mayo Medical Laboratories and Arup Laboratories
**the optimal combination is mentioned in this column: EDTA or/and ACD; room temperature or 4-8 °C

d. Cost

An example of the cost to perform a RBC enzyme assay is shown in Table 5 (cost is representative for all other enzyme assays).

**Table 5: Cost for G6PD enzyme assay**

| *Enzyme* | *Reagents (€)* | *Lab technician (€)* | *Support (€)* | *Total (€)* | *Reimbursement (€)* | *Total-reimbursement (€)* |
|---|---|---|---|---|---|---|
| G6PD | 5.13 | 17.82 | 7.31 | 30.26 | 14.48 | 15.78 |
| HK | 5.13 | 21.75 | 7.31 | 34.20 | 14.48 | 19.72 |
| G6PD + HK | 10.26* | 21.75** | 7.31 | 39.33 | 28.96 | 10.37 |
| 2 x G6PD | 10.26 | 17.82 | 7.31 | 35.39 | 14.48 | 20.91 |
| 2 x HK | 10.26 | 21.75 | 7.31 | 39.33 | 14.48 | 24.85 |

Knowledge of the different components of the total cost allowed us to calculate the additional cost for implementing the ratio with HK for each enzyme. As mentioned above, in the benchmarked reference centers, the enzymatic activity was analyzed in duplicate. For both procedures, an additional enzymatic assay needs to be performed. Merely considering the extra reagent cost, since minimal manipulation is necessary with in-batch

analysis, this would result in an extra cost of 5 euros per assay* (with the cost for lab technician from HK because for HK the cost is somewhat higher**).
The analysis of an extra enzyme activity (i.e. HK) and the respective reimbursement for this assay will compensate the additional cost (nomenclature B-500). This is not the case when performing the assay in duplicate.
Although quality improvement can be expected from these approaches, there are currently no official guidelines recommending to perform the assays in duplicate or to use hexokinase as an internal control.

e. Reporting of the results

The small bench marking study pointed out the importance of adding a short comment with every result of the RBC enzyme assays. This enables the clinical biologist to help the clinician in the differential diagnosis for the type of hemolytic anemia (f.e. testing for other enzymopathies, referral for DNA testing or testing for other types of defects). This will be looked at with the LIS-team.

## 3. REFERENCE-VALUES ENZYME ASSAYS

a. G6PD

The references-values for the enzymatic activity of G6PD will be discussed separately from the other enzymes because for G6PD a commercially available kit is used (Trinity Biotech). Currently, reference-values of the previous G6PD assay are used (4.1 – 7.9 U/g Hb). Since the last verification was done in 2002, a small evaluation was setup. Analysis of G6PD activity was determined in blood of 10 healthy volunteers (10 ACD and 10 EDTA tubes). In 13 out of the 20 tubes, values higher than 7.9 U/g Hb were observed indicating the upper limit is too low. The manual of the kit mentions expected values to be 4.6 – 13.5 U/g Hb. The enzymatic activity of all 10 healthy volunteers was situated within the range 4.6 – 13.5 U/g Hb for ACD and EDTA tubes. Based on this verifaction and based on the kit manual, the UZ Leuven reference-values for the G6PD assay will be adjusted to 4.6 – 13.5 U/g Hb.

b. PK, GR, RG

The enzymatic activity on blood samples of 10 healthy volunteers was also analyzed for PK, GR and RG to verify the currently used reference-values. For all 3 three enzymes, the obtained enzymatic activities (the mean of in duplo measurement) were situated within the respective reference-values (for both, ACD and EDTA tubes). Based on these experiments, no adjustments are made. Experiments are ongoing for HK and MetR.

## 4. GENETIC TESTING

Hereditary RBC enzymopathies are genetic disorders arising from mutations in genes coding for red cell metabolic enzymes. Although there are no guidelines (yet) prescribing genetic testing, molecular characterization is performed in some centers in addition to the enzymatic assays. Especially for G6PD and PK, extensive literature is available on the mutations causing the enzyme deficiency (Table 4). Unfortunately, there is no uniform consent and only a few specialized centers within Europe confirm diagnosis of RBC enzyme deficiencies using DNA analysis [28].

**Table 6: Genetic testing**

| *Enzyme* | *Genetic testing?* | *Authors* |
|---|---|---|
| Pyruvate kinase | Confirmation of the diagnosis on DNA level | Koralkova et al., 2014 [2] |
| | Due to large number of the mutations it is difficult to replace enzymatic assays with molecular diagnostic methods | Prchal & Gregg, 2005 [10] |
| | Care must be taken in interpreting in vitro PK assays | Zanella et al. 2005, 2007 [6,7] |
| | DNA testing enables prenatal diagnosis | Baronciani & E. Beutler, 1994 [29] |
| Glucose-6-fosfaat dehydrogenase | Confirmation of the diagnosis on DNA level | Koralkova et al. [2] |
| | Molecular diagnostic methods can be used for the diagnosis of females who are heterozygous for common variants | Prchal & Gregg, 2005 [10] |
| | DNA-based test for the screening of the most frequent mutations in a specific geographical area can be used as an alternative tool to the biochemical assay | Minucci et al., 2009 [12] |
| | Molecular analysis is the only method by which a definitive diagnosis can be made of a female's status | Cappellini et al., 2008 [11] |

In UZ Leuven, there is no intention (yet) to implement DNA analysis for diagnosis of RBC enzyme deficiencies. However, the possibility for referral to a specialized center for molecular characterization in order to confirm the diagnosis in case of female carriers or in case of doubt (borderline results and an unknown cause of anemia i.e. a negative direct Coombs test, a normal osmotic fragility/cryohemolysis test, no specific morphological abnormalities and no abnormal hemoglobin) or for heterozygous women (Table 6) will be offered to the clinicians.

## 5. CONCLUSION

- Methods introduced by E. Beutler are still common practice
- Clear indications to keep G6PD, PK and HK in our portfolio
- GPX can be abandoned
- Implementation of GPI may be considered
- The storage conditions are enzyme dependent (Table 4)
- Reference-values for the G6PD assay will be adjusted to 4.6 – 13.5 U/g Hb, no adjustments are necessary for the other enzyme assays
- Genetic testing will not be implemented in UZ Leuven, but possibility for molecular diagnostics in a reference center will be offered

## 6. REFERENCES


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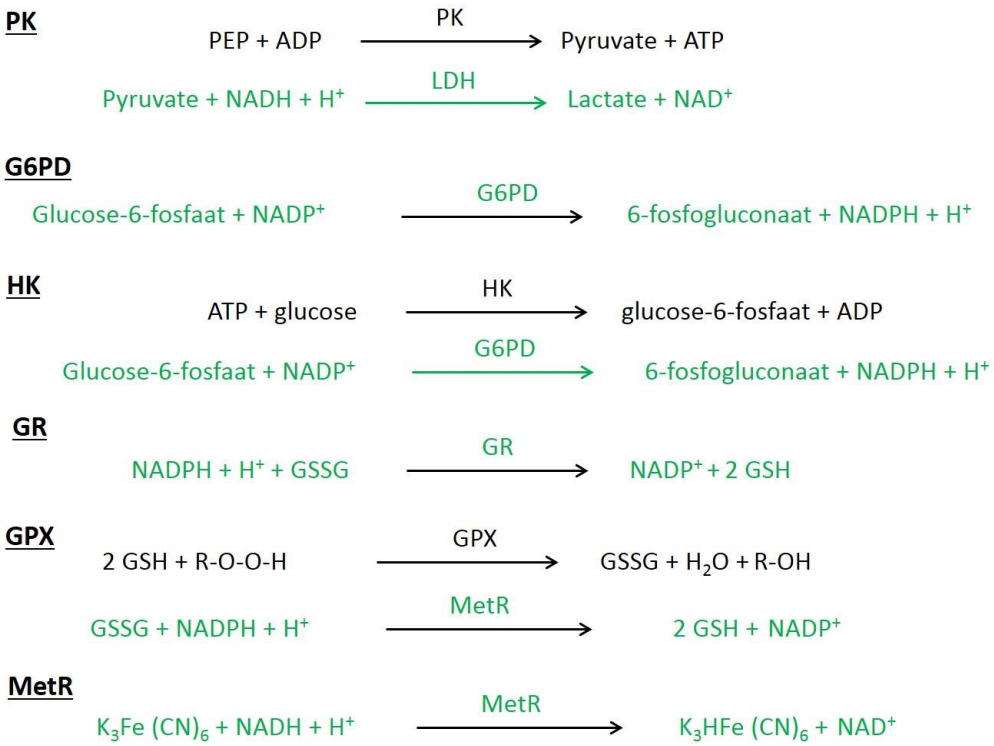
## TO DO/ACTIONS

1) Possible abandonment of the GPX assay
2) Evaluation of internal quality material based on blood samples of healthy volunteers for all RBC enzyme assays
3) Set up proficiency testing for our G6PD assay with external control material from UK NEQAS
4) Adjustment of reference-values for the G6PD enzyme assay to 4.6 – 13.5 U/g Hb
5) Offer shipment of samples for DNA analysis
6) Addition of supervision interpretation with every result of the RBC enzyme assays, this will be looked at with the LIS-team.
7) Evaluation of the possible implementation of the ratio with hexokinase
8) Stability experiments for HK and MetR
9) Verification of reference-values for HK and MetR
10) In laboratory guide: preferable anticoagulant/storage will be adapted in function of the data obtained in this project

## ATTACHMENTS

Attachment 1
RBC enzymatic reactions




**Supplemental figure 1: Overview RBC enzymatic reactions [18].** The monitored reactions are indicated in green.

Attachment 2

Bland-Altman plots ACD versus EDTA

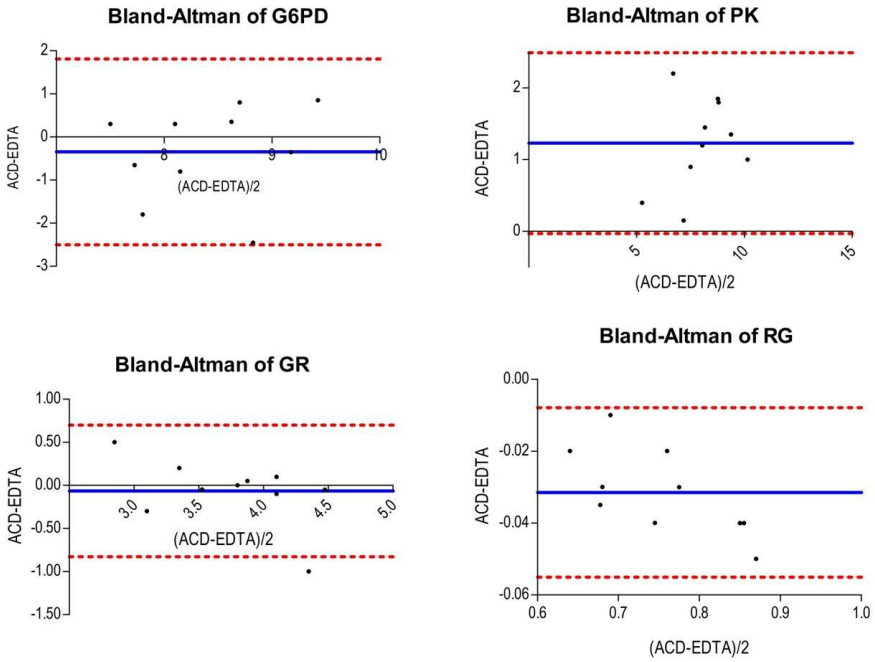



**Figure 2: Bland-Altman plots for comparison of collection tubes: ACD versus EDTA.**