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# DEVELOPMENT OF A HIGH SENSITIVE SANDWICH ELISA FOR MEASURING ERYTHROPOIETIN IN HUMAN SERUM

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## ABSTRACT

**The present paper describes a sandwich non-competitive enzyme-linked immunosorbant assay (ELISA) for erythropoietin (EPO). The ELISA utilizes a specific polyclonal antibody raised in rabbit against human recombinant EPO (rhu EPO). Anti-EPO was coated onto 96-well micro-titer plate, standard EPO or samples were added and left to bind to this catching antibody. This was followed by the addition of same antibody, which was conjugated with horse-radish peroxidase (HRP); and the enzyme reaction developed was measured at 450 nm. Recombinant human EPO was standardized against WHO 2$^{nd}$ International Reference Preparation for Erythropoietin. The minimal detectable concentration of rhu EPO was 20 pg mL$^{-1}$, which corresponded to 80 pg mL$^{-1}$ EPO in serum (serum diluted 1:4). The assay is easy to use, rapid, reproducible and quantitative, specific and sensitive to measure EPO content in serum samples.**

**Key words:** Conjugate, ELISA, immunization, horse radish peroxidase, polyclonal antibody, **r**ecombinant human erythropoietin

## INTRODUCTION

The production of erythrocytes is dependent on the continuous presence of glycoprotein erythropoietin (EPO) which acts on erythroid progenitor cells. The production of erythropoietin is usually dependent on the prevailing oxygen tension and, therefore, the haematocrit. The use of EPO assays has revealed that the situations may occasionally arise when EPO production does not correlate with haematocrit e.g. anemia of end-stage renal failure. A special aspect of EPO assay is to determine which disease state could benefit from substitution therapy with recombinant human EPO (rhuEPO) and to monitor the circulating EPO levels during that therapy. The International Olympic Committee (IOC), the World Anti-Doping Agency (WADA) and many major sports authorities have banned the use of EPO by athletes. Different methods to detect rhuEPO misuse have been described for monitoring recombinant human erythropoietin abuse among athletes (Marimuthu *et al.*, 2015) which include direct measurement in urine (de Ceaurriz and Lanse, 2000; Crepin *et al.*, 2002), and indirect measurements based on blood markers of altered erythropoiesis (Ashenden et *al.*, 2000; Ashenden *et al.*, 2003). The direct identification method is based on the analysis by isoelectric focusing, double blotting and chemiluminescent detection of erythropoietin (EPO) present in urine. Both exo- and endo-genous EPO have identical amino acid sequences but

show variable glycosilation pattern giving different isoelectric profile (de Ceaurriz and Lanse, 2000; Crepin *et al.*, 2002). However, the application of this method is limited by time and work load required to conduct such assays. The major drawback of urine-based tests is the disappearance of measurable levels of rhEPO from urine soon after administration (Berglund *et al*., 1995). Despite this, the athletes can retain physiologic benefits associated with an elevated red cell mass for a longer time (Audran *et al*., 1996; Breidbach *et al*., 2003). EPO concentrations in urine returns to base line value 4 days after the last subcutaneous rhEPO administration (Audran *et al*., 1996).

There are three categories of EPO assays available worldwide. The first is variation in pI values for different electrophoretic mobilities of EPOs which can be analyzed via IEF double immune-blotting (Lasne and de Ceaurriz., 2000). This technique is used to detect rhuEPO in urine but the method is compromised because of the requirement of a large volume of urine. The second category is *in vitro* bioassay using different target cells and end points (Rich *et al*., 1976; Krystal, 1983). These are usually more sensitive than *in vivo* bioassay, more rapid and less expensive, but disadvantageously are prone to extraneous (matrix) effects leading to non-parallelism of standard and sample dose response curves (Cotes, 1987). The third category consists of immunological assays. With the introduction of rhuEPO as reagent, it is possible to replace pure native EPO in RIA (Egrie ***et*** *al.*, 1987). The RIA is sensitive and quantitative but the primary disadvantage in it is the use of radioactivity labeled EPO. The combined availability of rhuEPO and mono- or poly-clonal antibodies has recently lead to the development of an enzyme-linked immunosorbent assay (ELISA) based on a preformed anti-EPO and anti-EPO HRP conjugated antibody. The present report describes the development and characteristics of a sandwich ELISA for EPO based on specific polyclonal IgGs.

## MATERIALS AND METHODS

### *Immunization*

Anti-EPO serum was raised in two rabbits (Raaj Farms, Chennai) by intradermal immunization with 20-40 µg recombinant human (rhuEPO) [KMS Health center Pvt. Ltd] per immunization, emulsified in either complete (first immunization) or incomplete (boosts) Freund's adjuvant. The antibody used was obtained from a rabbit which received three immunizations each with 30 µg rhu EPO 14 days after the 1$^{st}$ immunization or the boosters. Blood was removed from the ear vein and the haematocrit, reticulocyte count and antiserum titre determined (Robert *et al.*, 1995). The antibody response was measured by an ELISA in which anti-EPO antibody was bound by recombinant EPO coated to wells and detected by goat-anti-rabbit IgG (KMS-DA-004) conjugated to HRP. The antisera with best antibody titers were pooled and affinity purified as described. The study was done in Micro-Therapeutics Research Lab Pvt. Ltd., Chennai from March to October 2014.

### *Depletion of endogenous EPO from human serum*

The serum used in this experiment was obtained from Micro-Therapeutic Research Lab Pvt. Ltd., Chennai, India. The wells of microtitre plate were coated with affinity purified anti-EPO antibody (1 µg mL$^{-1}$). After incubation of wells with PBS containing 0.1% tween-80 for 30 min., the undiluted human serum was added and allowed to react for 1 h. The serum was then removed and transferred to a new well coated with anti-EPO antibody. This procedure (binding and transfer) was repeated three times. An aliquot of serum after each step was assayed for the remaining EPO by Human Erythropoietin Quantikine IVD ELISA kit (R&D Systems). After first incubation of serum with bound anti-EPO antibody, no EPO was detectable in EPO-ELISA. After fourth adsorption the serum was used as ligand for an affinity chromatography column.

***Purification of anti-erythropoietin antibodies***

The antisera obtained at various time interval (14 days after 1st & 2nd booster) were pooled and subjected to the following purification protocol. Anti-EPO immunoglobulins were first chromatographed over protein A-sepharose Cl- 4B column (GE Healthcare). One volume of 0.5 M Na-phosphate [pH 8.0] was added to 4 volumes of rabbit antiserum and applied to the column equilibrated with 0.1 M Na-phosphate [pH 8.0]. After washing with the same buffer, the IgGs were eluted with 0.1 M acetic acid in 0.15 M NaCl, immediately neutralized with 1 M tris [pH 8.0] and dialyzed against phosphate buffered saline (PBS). Further purification of antibody preparation was performed by affinity chromatography using fast protein liquid chromatography (FPLC). Recombinant human EPO (0.5 mg) was coupled to tresyl-activated sepharose 4B (Pharmacia, Uppsala) according to the manufacturer's instructions. The IgGs were loaded onto the column, washed with PBS, and the bound IgG eluted with 0.1M glycine-HC1, 0.15M NaCl [pH 2.8] and immediately neutralized with 1 M tris [pH 8.0]. Five to ten runs were carried out automatically and the eluted IgGs fractionated into the same tubes to minimize unspecific protein binding to the tube walls (Sawatzki, 1987). Fractions containing protein were pooled and dialyzed against PBS. The last purification step of antibodies was performed by affinity chromatography over a Sepharose 4B column coupled with human serum depleted of endogenous EPO. The anti-EPO antibodies were loaded onto the column, followed by washing with PBS. In this case, the unbound anti-EPO antibodies were collected, dialyzed against 50 mM ammonium acetate buffer [pH 7.4] and lyophilized. To elute bound antibody fraction, 0.1 M glycine-HCI containing 0.15 M NaCl [pH 2.8] was used. The binding of purified anti-EPO antibodies to rhu EPO was demonstrated using Western blotting as shown in Fig. 1.

***HRP conjugation of anti EPO antibodies***

The HRP conjugation of the affinity purified antibodies was carried out by a procedure described in kits (Pierce Biotechnology Inc., Rockford, USA)

***ELISA for measurement of antiserum titre***

The wells of microtitre plates (MaxiSorp, Greiner bio-one, Germany) were coated for overnight at 4ºC with 100 $\mu L^{-1}$ rhu-EPO at a concentration of 1µg $mL^{-1}$ in PBS. After blocking nonspecific protein adsorption sites with PBS containing 0.05% tween-20 and 1% BSA, 100 $\mu L^{-1}$ of varying antiserum dilutions were added to the wells and incubated for 2 h at room temperature. The plates were washed again with PBS containing 0.1%, tween-20 and incubated with 100 $\mu L^{-1}$ of 1/8000 dilution of goat-anti-rabbit IgG-HRP conjugate (KMS-DA-004) [from KMS Health Center Pvt. Ltd., Chennai, India] for 2 h. The bound conjugate was detected by adding tetramethyl benzidine (TMB) (GeNei™ 62161018010A) and reading the extinction at 450 nm (BioTek Microplate Readers, USA).

***Erythropoietin ELISA protocol***

One microtitre plate (MaxiSorp, Greiner bio-one, Germany) were coated by incubating affinity purified anti-EPO antibodies with 1 µg $mL^{-1}$ (100 $\mu L^{-1}$ well) in PBS, for overnight at 4ºC. After washing the wells 3 times with 300 $\mu L^{-1}$ PBS containing 0.1% tween-20 (wash buffer), the plate was blocked with 300 µL blocking solution and incubated for 1 h at room temperature. Blocking solution contained 1% BSA and 0.05% tween-20 in 1X PBS. The plate were washed 3 times with wash buffer and then 100 $\mu L^{-1}$ rhu EPO standard (20-1600 pg $mL^{-1}$) or serum sample (diluted 1:4 or 1:8) added and allowed to react with coated antibody for 2 h. All dilutions were made in PBS containing 0.5 M NaCl and 0.1% tween-20 supplemented with 0.1% casein hydrolysate (HiMedia). After washing the plates 5 times with PBS containing 0.1% tween-20 and 100 $\mu L^{-1}$ anti-EPO-HRP, conjugate were incubated with bound antigen for 2 h at 37°C. The wells were washed as above and bound conjugate detected by adding 150 $\mu L^{-1}$ TMB solution (GeNei™, Merck). After incubation at

room temperature for 15 min. the reaction was stopped by adding 100 $\mu L^{-1}$ stop solution. The absorbance of enzyme reaction product was read with an automatic ELISA reader with at 450 nm.

***Determination of ELISA specificity***

Since it is possible that various substances in serum could adversely affect the assay, several of these were tested which included serum component and cytokine. The serum components and cytokines tested were human serum albumin, human IgG, human lactoferrin [prepared in our own laboratory according to the method of Sawatzki and Kubanek (1983), recombinant human interleukin 1$\alpha$ (IL-1$\alpha$) and interleukin 1β (IL-lβ) (National Biological Standards Board, Hertfordshire, UK).

***Human serum samples***

Sera from normal men and women blood donors were obtained from healthy volunteers of Micro-Therapeutics Research Labs Pvt. Ltd., Tamil Nadu, India. Samples were routinely stored at -20°C, although immunological EPO activity remained stable for at least 7 days at 4°C.

***Quantification of erythropoietin concentrations***

Human recombinant EPO was standardized against the WHO 2nd International Reference Preparation of Human Urinary Erythropoietin (National Biological Standards Board, Hertfordshire, UK). The dose-response curves for both the preparations were parallel to each other. Erythropoietin concentrations present in samples were calculated using either a four-parametric logistic or cubic spline curve fitting programme. The minimal detectable concentration was determined when 3 times the standard deviation of lowest EPO concentration was significantly different (t-test) from that of background under the same conditions, wherever appropriate. The data was analyzed to observe standard deviation.

## RESULTS AND DISCUSSION

***Determination of anti-erythropoietin IgG titer***

Out of the two rabbit immunized with EPO, one rabbit showed better response than other (Table 1).

***Optimization of assay conditions***

*a) Plates:* Five different types of ELISA 96-well plates were tested for optimal sensitivity of sandwich assay: NuncMaxisorp and Polysorp, SLT Microtest plate F, Falcon Probind assay plate and MaxiSorp Greiner bio-one, Germany. There were no significant variations in the plates used from various manufacturers. The Greiner bio-one Maxisorp plate was used to develop this assay.

**Table 1: Titer value in rabbits**

| Immunization and booster in rabbit | 14 days from immunization | 14 days after 1st booster | 14 days after 2nd booster |
|---|---|---|---|
| Time point (days) | 14 | 29 | 44 |
| 1st rabbit | 1/12800 | 1/102400 | 1/409600 |
| 2nd rabbit | 1/12800 | 1/51200 | 1/102400 |

Titer value was calculated after 14 days of immunization and before booster dose.

*b) Concentration* of *catching antibody:* The wells of plate were coated with different concentrations (0.1 to 2.0 µg $mL^{-1}$) of affinity purified antibody (Fig. 2). For all of the experiments, an optimal catching antibody concentration of 1.0 µg $mL^{-1}$ was chosen.

*c) Concentration of HRP conjugated antibody:* To determine the optimal concentration of HRP conjugated antibody, it was serially diluted and tested from 10 to 0.25 µg $mL^{-1}$. A 1µg $mL^{-1}$ dilution of HRP conjugated antibody was chosen to obtain a near optimal reaction.

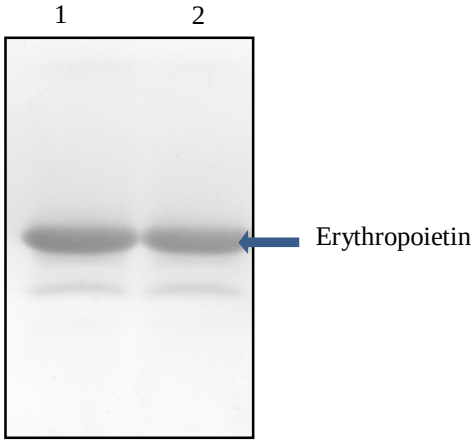


**Fig. 1: Western blot analysis of erythropoietin using in-house polyclonal antibody**

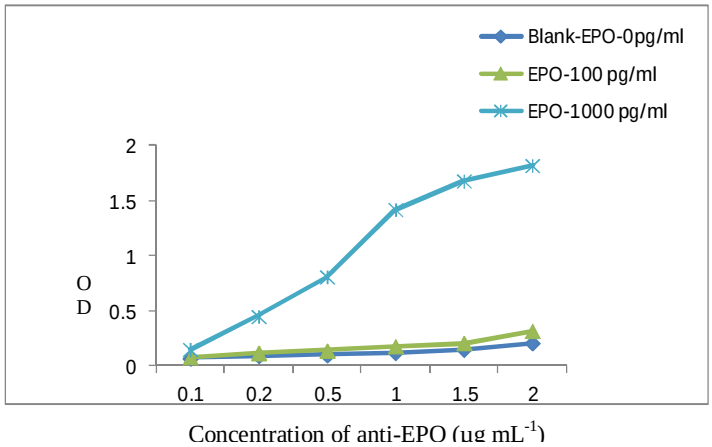


**Fig. 2: Concentration of catching antibody: The effect of different concentrations of solid-phase bound anti-EPO antibody tested with a low and a high EPO concentration. The background for every antibody concentration was determined in absence of EPO.**

*d) Reaction volume:* Three different volumes (50, 100 and 200 µL**)** for each reaction step were tested. Using 50 µL volume, the standard curve was too shallow while with 200 µL volume a steeper curve was obtained, but the background was too high. Therefore, an optimal reaction volume of 100 µL was employed producing a sufficiently steep dose-response curve with low background.

*e) Substrate incubation time*. A standard dose-response reaction was performed. The incubation time of TMB varied from 5 to 30 min. With increasing incubation times, the curves shifted to left corresponding to an increase in sensitivity. In order to measure the lowest concentrations in the standard dose-response curve, incubation times of 25 min were found necessary.

***Specificity arid sensitivity***

The main components of serum as well as important cytokines were tested for cross-reaction: h-serum albumin (2.5 mg $mL^{-1}$). h-IgG (2.5 mg $mL^{-1}$), normal human serum (diluted 1:4 with assay buffer), human lactoferrin (1 mg $mL^{-1}$), recombinant human interleukin 1$\alpha$ (0.5 mg $mL^{-1}$**)** and interleukin 1β (0.5 mg $mL^{-1}$**).** There was no reaction with any of the substances tested. Instead of anti-EPO ELISA plates were coated with the same concentration (0.4 pg $mL^{-1}$) of unspecific rabbit IgG, there was no reaction which demonstrated that there was no nonspecific binding of serum to anti-EPO and/or to the walls of the well. **A** typical standard curve for EPO is shown in Fig. 3**.**

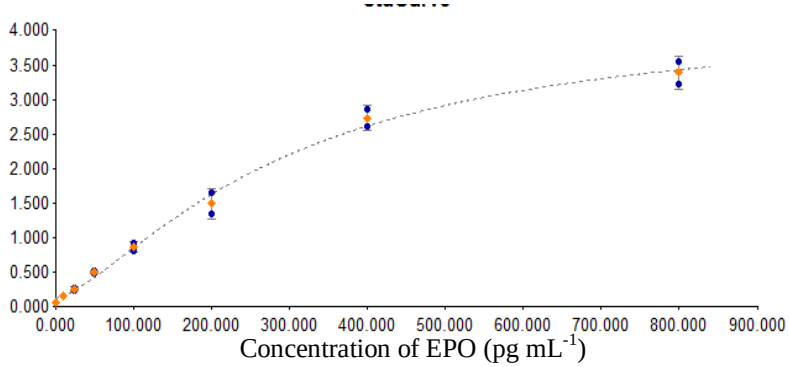


**Fig. 3: Standard curve with rhu EPO to determine the detection limit of the ELISA The curve represents the four-parameter logistic calculated from the standard curve.**

***Effect of serum on the recovery of EPO***

Known concentrations of standard-EPO were diluted in 25% human serum and observed concentrations measured and compared with those expected. An example of the recoveries of EPO in serum is shown in Table 2. In general, the recoveries ranged from 98 to 110%.

**Table 2: Recovery of rhu EPO in 25% human serum**

| Expected conc. (pg $mL^{-1}$) | Observed conc. (pg $mL^{-1}$) | Recovery (%) |
|---|---|---|
| 30 | 33 | 110 |
| 60 | 65 | 108 |
| 100 | 98 | 98 |
| 500 | 525 | 105 |
| 1000 | 1025 | 103 |
| 1500 | 1575 | 105 |

A normal human serum was spiked with known quantities of rhu EPO standard and the recovery of this exogenous rhu EPO was calculated by comparing the observed with the expected concentrations.

**Table 3: Intra- and inter-assay variations**

| Erythropoietin (pg $mL^{-1}$) | | N | CV (%) |
|---|---|---|---|
| Intra-assay variation | | | |
| Sample 1 | 40 | 10 | 12.5 |
| Sample 2 | 400 | 10 | 8.0 |
| Sample 3 | 1000 | 12 | 5.5 |
| Inter-assay variation | | | |
| Sample 1 | 40 | 12 | 22.0 |
| Sample 2 | 400 | 9 | 12.5 |
| Sample 3 | 1000 | 15 | 8.0 |

The intra-assay variation was determined by performing 10 replicates of a serum sample in one 96-well plate. For the determination of the interassay variation the serum was repeatedly measured (nine or 15 times) on separate days.

***Intra- & inter-assay variation: Assay precision***

The intra-assay coefficient of variation ranged from about 5.5 to 12.5% depending upon the EPO concentration (Table 3). Inter-assay variability was tested in seven assays with three different sera performed on separate days. The coefficient of variation ranged from 8 to 22%**.** The coefficients of variation for the lowest EPO values appeared high, but such values are not uncommon in ELISA assays.

The present report demonstrates that an EPO ELISA based on polyclonal anti-EPO rather than monoclonal antibodies can be developed and optimized so that it is easy to use, rapid, reproducible, but above all quantitative, specific and sensitive. The results showed that every single component and reaction step has to be examined in order to optimize the assay. Our goal was not simply to measure normal and above normal EPO concentrations, but also to quantitatively and reliably measure EPO concentrations in lower than normal range. This is a prerequisite to distinguish between primary and secondary polycythaemia and is also necessary to determine which patients may benefit from EPO therapy. It should be noted, however, that the ELISA measures immunological and not the biological activity. From two immunized rabbits, one produced significantly higher titers of anti-EPO which could be used in the ELISA. Immunization with as little as 60 µg EPO (2 x 30 µg) was found successful for the production of antibody. The first rise in antibody titre was always accompanied by a decline in the haematocrit and reticulocyte count in the rabbits. This is probably caused by the binding of antibody to the rabbit's own EPO.

The use of a polyclonal antibody system required that the antibody be in the purest form possible in order to bind EPO specifically. This indicates that the impure antibody preparation can be fractionated over rhu EPO affinity column which, in turn, required sufficient recombinant human EPO-bound to the column matrix support i.e. the efficiency of the purification procedure depends on the amount of EPO bound to the matrix. The third step in the purification protocol, affinity chromatography on a column coupled with human serum freed of EPO, was performed to remove those antibodies which gave false positive reactions in ELISA due to cross reaction with serum components.

All the components and reaction steps were studied, including testing the different types of ELISA plates. Some normal sera gave false positive reactions when tested with unspecific coating antibodies. 'This problem could be overcome by adding unspecific rabbit IgGs to the dilution buffer, indicating that those sera had immunoglobulins which cross-reacted with rabbit IgGs.

The optimized procedure reported here is most sensitive immunological assay for erythropoietin reported measuring at least 20 pg $mL^{-1}$ of rhu EPO equivalent to 80 pg $mL^{-1}$ of a 1:4 dilution of serum. This, together with the specificity, parallelism between standard curve and serum dilution curve thereby demonstrating the almost total absence of matrix effects, revealed that the assay is well suited to quantitatively measure EPO in very low to high ranges. **A** frequent argument against the use of polyclonal antibodies (instead of monoclonals) in an ELISA is the instability of assay because of variations in antibody preparations. However, to date, we have not encountered any instability in ELISA due to different antibody preparations from the same rabbit. In general, the EPO values measured by ELISA for normal persons are relatively low but agree with values obtained by some RIAs (Wide *et al.,* 1989; Jelkmann and Wiedemann, 1990). However, by using ELISA**,** it is still possible to distinguish between normal EPO levels and most subnormal values, characteristic of polycythaemia Vera patients. We demonstrated that this assay is specific, sensitive and with a minimal detectable concentration of 20 pg $mL^{-1}$ for rhu EPO corresponding to 80 pg $mL^{-1}$ EPO in serum.

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## REFERENCES

Ashenden, M.J., Brugnara, C., Emslie, K.R., Gore, C.J., Hahn, A.G., Howe, C., Martin, D.T. and Parisotto, R. 2000. A novel method utilizing markers of altered erythropoiesis for the detection of recombinant human erythropoietin abuse in athletes. *Haematologica,* **85**: 564-572.

Ashenden, M.J., Emslie, K.R., Gore, C.J., Hahn, A.G., Hopkins, W., Howe, C., Kazlauskas, R., Parisotto, R., Sharpe, K., Stray-Gundersen, J. and Trout, G.J. 2003. Second-generation blood tests to detect erythropoietin abuse by athletes. *Haematologica*, **88**: 333-344.

Audran, M., Bressolle, F., Chana,l JL., Duvallet, A., Gareau, R. and Souillard, A. 1996. Pharmacokinetics and pharmacodynamics of recombinant human erythropoietin in athletes. Blood sampling and doping control. *British Journal of Clinical Pharmacology*, **42**: 355-364

Berglund, B., Bengtsson, C., Ekblom, B. and Wide, L. 1995. Detection in blood and urine of recombinant erythropoietin administered to healthy men. *Medicine and Science in Sports and Exercise*, **27**: 1569-1576.

Breidbach, A., Catlin, D.H., Green, G.A., Tregub, I., Truong, H. and Gorzek, J. 2003. Detection of recombinant human erythropoietin in urine by isoelectric focusing. *Clinical Chemistry*, **49:** 901-907.

Cotes, P.M. 1987. The estimation of erythropoietin (Epo): Principles, problems and progress. NATO ASI series. *Molecular and Cellular Aspects* of *Erythropoietin and Erythropoiesis*, **H8:** 377-387.

Crepin, N., de Ceaurriz, J., Lasne, F. and Martin, L. 2002. Detection of isoelectric profiles of erythropoietin in urine: Differentiation of natural and administered recombinant hormones. *Analytical Biochemistry*, **311**: 119-126.

De Ceaurriz, J. and Lasne, F. 2000. Recombinant erythropoietin in urine. *Nature*, **405**: 635-637.

Egrie, J.C., Cotes, P.M., Lane, J., Gaincs Das, R.E. and Tam, R.C. 1987. Development of radio-immunoassays for human erythropoietin using recombinant erythropoietin as tracer and immunogen. *Journal of Immunological Methods*, **99:** 235-241.

Krystal, G. 1983. A simple rnicroassay for erythropoietin based on $^{3}$H-thymidine incorporation into spleen cells from phenylhydrazine-treated mice. *Experimental Hematology*, **11:** 649-660.

Marimuthu, C., Subash, C.B.G., Thangavel, L., Thean, H.T. and Yeng, C. 2015. Monitoring recombinant human erythropoietin abuse among athletes. *Biosensors and Bioelectronics*, **63**: 86-98.

Rich, I.N. and Kubanck. B. 1976. Erythroid colony formation in foetal liver and adult bone marrow and spleen from the mouse. *Blut*, **33**: 171-180.

Robert, L.H., Samuel E.L. and Thomas P.S. 1995. *Blood Principles and Practice of Hematology* (2nd edn.), Lippincott Williams & Wilkins, Philadelphia, USA.

Sawatzki, C. and Kubanek, B. 1983. Isolation and ELISA of mouse and human lactoferrin. pp. 441-443. **In**: *Structure and Function of Iron Storage and Transport Proteins* (eds. I. Urushizaki *et al.)*, Elsevier Science Publishers B.V., New York, USA.

Sawatzki, G.1. 1987. Isolation of anti-erythropoietin by automated immuno-affinity FPLC using recombinant EPO. NATO ASI series, *Molecular and Cellular Aspects* of *Erythropoietin and Erythropoiesis*, **H8:** 409-416.

Wide, L., Bengtsson, C. and Birgegard, G. 1989. Circadian rhythm of erythropoietin in human serum. *British Journal of Haematology*, **72**: 85-90.