

# Testing for recombinant erythropoietin

Joris R. Delanghe,<sup>1\*</sup> Mathieu Bollen,<sup>2</sup> and Monique Beullens<sup>2</sup>

**Erythropoietin (Epo) is a glycoprotein hormone that promotes the production of red blood cells. Recombinant human Epo (rhEpo) is illicitly used to improve performance in endurance sports. Doping in sports is discouraged by the screening of athletes for rhEpo. Both direct tests (indicating the presence of exogenous Epo isoforms) and indirect tests (indicating hematological changes induced by exogenous Epo administration) can be used for Epo detection. At present, the test adopted by the World Anti Doping Agency is based on a combination of isoelectric focusing and double immunoblotting, and distinguishes between endogenous and rhEpo. However, the adopted monoclonal anti-Epo antibodies are not monospecific. Therefore, the test can occasionally lead to the false-positive detection of rhEpo (epoetin- $\beta$ ) in post-exercise, protein-rich urine, or in case of contamination of the sample with microorganisms. An improved preanalytical care may counteract a lot of these problems. Adaptation of the criteria may be helpful to further refine direct Epo testing. Indirect tests have the disadvantage that they require blood instead of urine samples, but they can be applied to detect a broader range of performance improving techniques which are illicitly used in sports. *Am. J. Hematol.* 83:237–241, 2008. © 2007 Wiley-Liss, Inc.**

## Introduction

Erythropoietin (Epo) is a 34-kDa glycoprotein hormone that is primarily synthesized in the kidney and functions as a key stimulator of erythropoiesis [1–3]. Epo is the main regulator of erythropoiesis by stimulating the proliferation and differentiation of erythroid precursor cells in the bone marrow. The availability of recombinant human erythropoietin (rhEpo) has led to widespread therapeutic applications in anemia associated with, for example, chronic renal insufficiency, chemotherapy, and HIV infection. Since Epo increases the amount of oxygen that is carried to the muscles, rhEpo has also been embraced as an ergogenic aid in endurance sports [4]. However, apart from increasing the hematocrit, the excessive use of Epo can also result in serious side effects like hypertension, pure red cell aplasia, and heart failure [5,6]. Epo also appears to be implicated in angiogenesis [7]. Epo withdrawal may result in neocytolysis [8]. rhEpo abuse is a suspect in nearly 20 deaths in 4 years in European cyclists [9]. This, combined with the consideration that the use of rhEpo violates the athletic ethical standards, has led the International Olympic Committee to prohibit the use of rhEpo and to screen endurance athletes for rhEpo abuse.

Indirect Epo-tests disclose an abnormal stimulation of erythropoietic activity by comparing various hematological parameters of peripheral blood. In direct Epo-tests endogenous and recombinant forms of Epo can be differentiated by their distinct electrophoretic properties that result from distinct glycosylation patterns. In the present manuscript, we discuss the analytical possibilities, the limitations and pitfalls of both indirect and direct methods that have been developed to trace rhEpo abuse in endurance athletes.

## Indirect Methods for Epo Testing

Indirect methods for detecting rhEpo abuse are based on the analysis of a number of standard hematological parameters (e.g., hemoglobin (Hb), hematocrit, soluble transferrin receptors, serum Epo concentration, percentage reticulocytes, reticulocyte hematocrit, and hemoglobin content (CHr), percentage hypochromic red cells) that are indicative for an increased erythropoiesis [10,11]. A significant linear correlation was found between the relative amount of basic Epo variants and the relative levels of soluble transferrin

receptors, demonstrating that the relative levels of soluble transferrin receptor may be used as a marker to select urinary samples for further analysis of rhEpo by isoelectric focusing (IEF) in routine doping control [12].

Models incorporating increases in the hematocrit, serum Epo and soluble transferrin receptor concentrations, reticulocyte hematocrit, and % macrocytes have been successfully used for these purposes [13,14]. One model (ON-model) repeatedly identified 94–100% of rhEpo-treated athletes during the last 2 weeks of the administration phase. One false-positive result was recorded out of 189 cases. Another model (OFF-model) incorporating reticulocyte hematocrit, serum Epo, and hematocrit was applied during the wash-out phase and, during the period of 12 and 21 days after the last rhEpo injection, it repeatedly identified 67–72% of recent users, without false positives [13]. ON-models may fail to detect Epo abuse when blood sampling is performed 48 hr after injection of moderate or low doses of rhEPO. By contrast, an excellent rate of detection for OFF models after 14 days of wash-out has been observed [15]. Data on the effect of altitude training on blood model scores to detect Epo abuse are inconclusive [16].

Comparing an athlete's individual hematologic values against his or her own historical baseline rather than a population derived threshold value enhances the potency of indirect testing. An athlete's true baseline value can be estimated with just one prior blood test. Eliminating intraindividual variability by comparing new results against an historical baseline increased the ability to detect blood doping [17]. It was possible to delineate the longitudinal changes

<sup>1</sup>Department of Clinical Chemistry, University Hospital, Ghent, Belgium; <sup>2</sup>Laboratory of Biosignaling and Therapeutics, Department of Molecular Cell Biology, KULeuven, Leuven, Belgium

\*Correspondence to: Joris Delanghe M.D., Ph.D., Department of Clinical Chemistry, University Hospital Ghent, De Pintelaan 185, B 9000 Ghent, Belgium. E-mail: joris.delanghe@ugent.be

Received for publication 10 June 2007; Revised 17 August 2007; Accepted 27 August 2007

*Am. J. Hematol.* 83:237–241, 2008.

Published online 4 October 2007 in Wiley InterScience (www.interscience.wiley.com).

DOI: 10.1002/ajh.21081

in either Hb or OFF-rhEPO model score (an algorithm using both Hb and percent reticulocytes) caused by rhEpo treatment from the natural biological fluctuation in placebo-treated subjects. Longitudinal monitoring of athletes' blood profiles can help detect blood doping [17]. In the novel UCI anti-doping program, coined "100% Against Doping," an individual hematological profile for all professional cyclists will be created. Additionally, an international database will be set up, in cooperation with the World Anti-Doping Agency (WADA) and national anti-doping agencies ([www.uci.ch/includes/asp/getTarget.asp?type=FILE&id=MT10NDY](http://www.uci.ch/includes/asp/getTarget.asp?type=FILE&id=MT10NDY)).

Another model (based on hematocrit, reticulocyte count, soluble transferrin receptor, and beta-globine mRNA concentration), when used in a multiparametric formula, could detect rhEpo abuse in 57.5% of the investigated samples [18].

Indirect Epo detection methods require blood sampling, which is a practical disadvantage in doping control. To obtain optimal test efficiency, attention has to be paid to the preanalytical phase [19,20]. The combination of indirect and direct methods was implemented for the Sydney Olympics in 2000. In June 2003, the Executive Committee of the WADA decided that only urine tests can be used for detecting the presence of recombinant Epo in doping control.

On the other hand, indirect Epo tests offer the advantage that also other, related kinds of blood doping can be detected. The direct Epo test only detects the recent use of epoetin-alfa, epoetin-beta, and darbepoetin. However, the recently available dynepo/erythropoietin-delta, which is produced in cultured human cells [21], has no distinct glycosylation pattern that enables its detection by the direct Epo test. Furthermore, recent pharmacological progress in the stimulation of erythropoiesis by continuous erythropoietin receptor activator (CERA) [22] or by agents like K-11706 and K-7174, are potential dangers for direct Epo testing [23,24]. Also, other recent developments in blood doping [25], including (1) hypobaric tents recreating a high-altitude environment, (2) the administration of cobalt inducing a polycythemic response through a more efficient transcription of the Epo gene [26], (3) the use of blood transfusions, and (4) the threatening progress in the field of genetic doping [4], will stimulate indirect testing procedures. This will be further promoted by the increased availability of mobile hematological analysis equipment.

## Direct Testing

### Isoelectric focusing

From an analytical viewpoint, it was a difficult task to differentiate between endogenous and rhEpo since their amino acid sequence is identical. The Epo test that has eventually been adopted by WADA is based on differences in the extent of glycosylation, resulting in distinct sets of isoelectric points (pI) that can be detected by IEF [26]. Endogenous human Epo has a pI range of ~3.7–4.7. Epoetin  $\alpha$  and  $\beta$  are produced in Chinese Hamster cells and, probably as a result of hyposulfated sugar moieties, have a more basic pI range of 4.4–5.1 [27–30]. Aranesp, also known as darbepoetin-alfa, is a form of Epo that has been engineered to contain two extra *N*-glycosylation sites to increase its stability. These additional glycosylation sites account for the more acidic pI range (3.7–4) of Aranesp. Epoetin-omega and epoetin-delta, produced in human cells, do not differ from endogenous human Epo and can therefore not be detected by IEF.

The distinct glycosylation patterns of endogenous Epo and rhEpo have been used to develop a method based on IEF for the detection of rhEpo in urine [31–37]. The amount of endogenous Epo in urine varies from 0 to 3.7 IU/L in

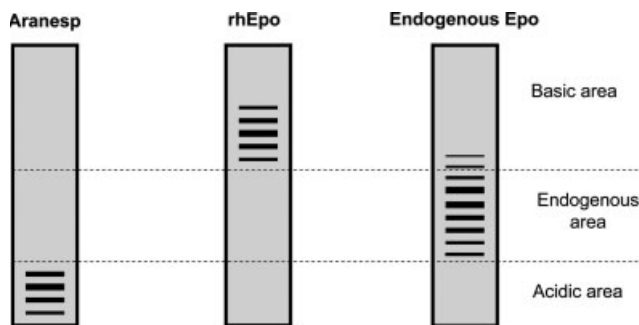


Figure 1. Interpretation of the direct Epo test. A comparative pherogram shows schematically the banding pattern of Aranesp, recombinant, and endogenous Epo.

normal subjects and from 7 to 20 IU/L in 102 professional cyclists [33]. In athletes, endogenous Epo levels of 0.25 IU/L for female and 1.28 IU/L for male were defined as median and 7.7 IU/L for female and 13.5 IU/L for male as higher levels [38].

Before analysis, the pH and the density of the urine specimen are determined, as an additional security measure against fraud. In view of the low Epo-concentration in urine, a large test volume (20 ml) and a strong concentration factor (700–1,000-fold concentration) of the urine specimen by ultrafiltration are required.

Subsequently, the proteins in the concentrated urine samples are separated in a pH-gradient by IEF and the different Epo isoforms are visualized by immunoblotting with monoclonal anti-Epo antibodies. Lasne [36] adapted the normal blotting procedure by a (patented) double-immunoblotting technique, to avoid the nonspecific interaction of the used secondary antibody with urinary proteins. The primary monoclonal antibody to Epo recommended by WADA (clone AE7A5, R&D Systems) is raised against the N-terminal 26 amino acids of human Epo [39]. Chemiluminescence is used for the visualization of Epo on the blot. The test can detect endogenous human Epo, epoetin-alpha, epoetin-beta, as well as Darbepoetin-alpha (aranesp). In Fig. 1, a schematic image of the result obtained in a direct Epo test is represented.

In rare cases an enzymatic activity can cause a shift in the electrophoretic banding pattern. Therefore, WADA recommends an additional stability test when a test is positive for rhEpo. For this test, the urine specimen is incubated overnight in an acetate buffer with rhEpo. In case of an "active sample", a shift of the banding pattern will be seen during the IEF and the sample will be declared negative for rhEpo [28,37].

This complex procedure has been accepted by the WADA to screen for rhEpo in urine samples. The test can be used both in competition and during training periods (off-competition testing). Because of the pharmacokinetics of rhEpo and the long acting biological effect of Epo administration, off-competition testing is a better setting for detecting Epo abuse.

### Interpretation

The interpretation rules of the Epo test applied by the accredited WADA laboratories have been fine-tuned over the years. They are described in a WADA technical document [37]. In accredited doping control laboratories, Epo images were originally evaluated by a combination of measurements on the immunoblot-image and the use of various

multipurpose software packages. However, these are not tailored to this specific task. Moreover, the use of different software tools does not guarantee a uniform interpretation of the data and has led to the development of a dedicated software program [40].

### Detection window

A single subcutaneous injection of Darbepoetin- $\alpha$  (40  $\mu\text{g}$  of ARANESP<sup>®</sup> injected) lead to a positive IEF-based urinary Epo test in all samples until 7 days after injection, which is approximately two times longer than for rhEpo- $\beta$  (4,000 IU of Recormon<sup>®</sup> injected). This indicates that Darbepoetin- $\alpha$  has a much longer detection window in urine than any other available Epo isoform, which is a major disadvantage for illegal use in sports. However, the detection window shows an interindividual variation because the actual positivity criteria take into consideration the endogenous Epo production rate, which varies enormously between individuals. By injecting microdoses of rhEpo, the window of detection can be reduced to as little as 12–18 hr postinjection [41].

In subjects with a naturally elevated or stimulated Epo production rate (altitude training, hypoxic tent, etc), there is a reduced detection window for bone marrow stimulators such as Darbepoetin- $\alpha$ . The positivity criteria used up to now by anti-doping laboratories for Darbepoetin- $\alpha$  are very strict and could be adapted by only taking into account the position and the specific distribution of the bands in the most acidic area of the gel and no longer the intensity of the bands. This would rule out the differences in interpretation of the test because of different endogenous Epo levels in individual athletes [42].

### Caveats of Direct Epo Testing

Although the direct method has been adopted in WADA-accredited laboratories, several problems were already identified in a WADA-commissioned reported by Peltre and Thormann in 2003 [43]. These include the lengthy procedure of urine sample preparation, the low sample load capacity, difficulties with interlaboratory standardization because of the use of irreproducible carrier ampholyte gels [44], the nonspecific binding of the secondary antibody to urinary proteins which requires a double blotting system to overcome [36], sensitivity issues and the high cost of the procedure.

With a 700–1,000-fold concentration, the concentrated urine is very viscous and forms a large pellet which is difficult to solubilize and apply on the gel [45]. Despite this enormous concentration, quite a large number of samples (up to 20%) do not show detectable Epo [43], indicating that the test sensitivity is rather low. The number of positive tests is relatively low. The American WADA-accredited laboratory has performed the direct test for rhEpo on more than 2,600 doping control samples. Of these, only nine positive cases for rhEpo have been reported. Only three of these have publicly confessed to using rhEpo and three others have accepted penalties. Given the low number of positive tests, it is not surprising that the frequency of false-positive tests is extremely low [46]. The issue of false-negative results is more difficult to deal with. Recently, some anti-doping scientists were concerned about protease-treated urine specimens which could mask Epo abuse [47].

The last two years the reliability of the direct Epo has been questioned by several authors [45,48–50]. The most worrisome problem is the proven nonspecific interaction of the used monoclonal Epo-antibody with non-Epo proteins. Khan et al. [45] identified cross-reacting urinary proteins in the pI range 3–5 by peptide mass fingerprinting as Tamm Horsfall glycoprotein, alpha-antichymotrypsin, alpha 2-thiol

proteinase inhibitor, and alpha-2-HS glycoprotein. Each of the cross-reacting abundant urinary proteins showed some homology with the Epo epitope, which probably explains the affinity of the Epo antibody for these proteins. The isoelectric points of these cross-reactive proteins overlapped with endogenous Epo and rhEpo, but they could be distinguished from Epo by 2D-electrophoresis (2DE).

The monoclonal Epo antibody AE7A5 also cross-reacts with a large number of bacterial proteins, such as *Escherichia coli* thioredoxin reductase (*E. coli* is known to occur in human urine and is widely adherent to human skin in the uro-ano-genital region). Furthermore, the AE7A5 antibody cross-reacts with extracellular proteins and with intracellular proteins from urothelial tissue that are released into urine upon tissue damage [48]. The antigenic peptide used for the generation of monoclonal Ab AE7A5 contained a central tetrapeptide with an amino acid sequence that occurs in more than 500 human and almost 185 *E. coli* proteins. Similarly, next to *E. Coli* proteins also a number of *Saccharomyces cerevisiae* proteins are cross-reacting with the primary antibody that is used for the Epo test [49]. As cross-reactivity with the primary antibody may result from the presence of some microorganisms that are commonly present in urine specimens, attention should be given to the preanalytical care after prelevation of urine specimens [50].

In case of pronounced postexercise proteinuria, false-positive results have been described [50]. Following strenuous physical exercise, a temporary mixed glomerular-tubular proteinuria can be found in certain athletes [51], which is characterized by a broad spectrum of urinary proteins. The false-positive detection of epoetin- $\beta$  may be restricted to (very) few athletes, as it may be linked to the extent and type of proteinuria. The extent of proteinuria correlates more with the intensity than the duration of exercise and has a half-time decay of about 1 hr. Some of the urinary proteins show some structural homology with Epo [45,48], which possibly accounts for their cross-reactivity with the anti-Epo antibodies. The double blotting technique used in the direct test does not protect against cross-reactivity against the primary antibody [50]. The false positive result appeared only shortly ( $\pm 1$  hr) after exercise. Since rhEpo has a half-life of more than 8 hr [1], this was an indication that the bands detected at 0 hr could not be explained by the presence of epoetin-beta. Immunoblotting with the Epo antibody after SDS-PAGE visualized several urinary proteins, with a major band of 42 kDa. None of the detected proteins correspond with the mass of the epoetin-isoforms (32–39 kDa). Removal of N-linked carbohydrates decreased the apparent mass of epoetin-isoforms from between 32 and 39 kDa to 18 kDa, but such a treatment did not cause a similar shift in the mass of the detected bands in a post-exercise urine sample. Immunoblotting before and after a N-glycosidase-F treatment confirmed that the major urinary protein that was visualized was not Epo. The visualized bands did not result from the interaction of urine proteins with the secondary antibodies but resulted from the interaction with the monoclonal anti-Epo antibodies (clone AE7A5). The cross-reactivity is not unexpected since this antibody is sold for research use and in proteinuria the concentration of numerous abundant specific urinary proteins exceeds that of Epo by several orders of magnitude.

Epo test results are clearly not always interpreted identically. For example, the athlete examined by Beullens et al. [50] was initially found guilty of rhEpo abuse by two WADA-accredited laboratories. During the ensuing appeal procedure, it was disclosed by a third WADA-accredited laboratory that the original data leading to the suspension of this athlete had been interpreted wrongly by the former two

**TABLE I. Overview of Epo-Abuse Detection Methods**

Method	Comment	Reference
<b>Direct methods</b>		
IEF/immunoblots	Standard method used by WADA accredited labs	33–37
Lectin-based IEF	Experimental	55
2D electrophoresis	May be used as a confirmatory test to rule out nonspecific reactions	45
Microscale sample purification combined with MALDI-TOF and IT/RTOF mass spectrometry	Experimental	56
<b>Indirect methods</b>		
Soluble transferrin receptor assay	Useful as a first screening	12
Combination of hematocrit, serum Epo, soluble transferrin receptor concentrations, reticulocyte hematocrit, and % macrocytes	Can be improved when comparing an athlete's individual hematologic values against his or her own historical baseline	13,14,17
Combination of hematocrit, reticulocyte count, soluble transferrin receptor, and beta-globine mRNA	Not very sensitive	18

WADA laboratories, and that the tests were actually negative for rhEPO. Catlin et al. [46] argued that the electropherogram that was termed “false-positive” by Beullens et al. [50,52], based on the published WADA-interpretation rules, should actually be interpreted as a negative result. The existence of a 42-kDa band following strenuous exercise was however confirmed [53]. To further add to the confusion, Lasne et al. reported that “false positive” or “atypical negative” blots can be explained by the aberrant migration of endogenous Epo [54]. Discrimination between atypical negative and positive Epo-tests relied on a statistical approach.

The use of the GAS-Epo software for quantitative analysis of Epo blots [40] was recommended by WADA. However, this software processing has been severely criticized by Franke and Heid [48].

**Alternative Direct Tests**

A 2DE method for the detection of rhEpo in urine and its separation from endogenous Epo has been described [45]. The 2DE method separates endogenous Epo and rhEpo isoforms by both isoelectric point and molecular mass. This method involves an acetonitrile based precipitation of the proteins, the addition of an internal standard, two-dimensional gel electrophoresis, a single immunoblot, and chemiluminescent immunodetection. The 2DE method has dealt with most of the WADA recommendations for a new Epo test [43] and provides a sensitive, reproducible and accurate detection of the Epo drug in urine. However, the 2D-test is time-consuming and is not easily adaptable for large-scale rhEpo screenings.

Nagano et al. [55] compared the binding behavior of lectins for endogenous and recombinant Epo, following IEF according to the WADA-accredited method. For the visualization of Epo, lectins were used instead of antibodies. *Lens culinaris* agglutinin and *Pisum sativum* lectin showed a clear preference for rhEpo isoforms located in the more basic region of the electropherogram. Since endogenous and recombinant Epo only differ in the composition of their *N*-glycan and *O*-glycan moieties, lectins might therefore be useful for affinity-enrichment/purification of rhEPO in doping control.

Stubiger et al. [56] could identify *N*- and *O*-glycopeptides of rhEpo as potential biomarkers for doping analysis by means of microscale sample purification combined with matrix-assisted laser desorption/ionization time of flight

(MALDI-TOF) and quadrupole ion trap/reflectron time-of-flight IT/RTOF mass spectrometry.

Table I summarizes the current direct and indirect assays which can be used for detecting Epo abuse.

**Conclusions**

The direct Epo test has been successfully used in anti-doping laboratories. However, the use of a primary antibody that is not monospecific and the evident use of ad hoc interpretation criteria by the WADA-accredited laboratories are worrisome. Improvement of the current test is therefore necessary. WADA has evaluated the current urinary Epo test [42] and recommended some new guidelines for improvement, related to new interpretation rules and the introduction of a stability test [36]. WADA also stimulates and supports research for alternative methods, e.g., methods based on mass-spectrometric analysis of Epo isoforms (for an overview see WADA Sponsered Research Projects at [www.wada-ama.org](http://www.wada-ama.org)).

Under exceptional circumstances (e.g., extreme exercise), the direct test occasionally appears to yield a false positive result in predisposed individuals. The false-positive detection of epoetin-β may be prevented by sampling before or at least 1 hr after exercise, which is particularly important for athletes presenting with pronounced exercise-induced proteinuria. The use of more specific antibodies could be helpful. Alternative tests can be performed to identify false-positive test results, such as 2DE [44], deglycosylation assays [50], or indirect assays [13–17,18].

Blood-based indirect Epo tests offer an interesting alternative. The rapidly changing landscape will definitely encourage the use of indirect Epo testing with a much broader application field.

**References**

1. Fisher JW. Erythropoietin: Physiological and pharmacological aspects. *Proc Soc Exp Biol Med* 1997;216:358–369.
2. Cheung JY, Miller BA. Molecular mechanism of erythropoietin signaling. *Nephron* 2001;87:215–222.
3. Richmond TD, Chohan M, Barber DL. Turning cells red: Signal transduction mediated by erythropoietin. *Trends Cell Biol* 2005;15:146–155.
4. Diamanti-Kandarakis E, Konstantinopoulou PA, Papailiou J, et al. Erythropoietin abuse and erythropoietin gene doping. *Sports Med* 2005;35:831–840.
5. Lappin TR, Maxwell AP, Johnston PG. EPO's alter ego: Erythropoietin has multiple actions. *Stem Cells* 2002;20:485–492.
6. Locatelli F, Del Vecchio L. Pure red cell aplasia secondary treatment with erythropoietin. *J Nephrol* 2003;16:461–466.

7. Hardee ME. Erythropoietin blockade inhibits the induction of tumor angiogenesis and progression. *PLoS ONE* 2007;2:e549.
8. Trial J, Rice L, Alfrey CP. Erythropoietin withdrawal alters interactions between young red blood cells, splenic endothelial cells, and macrophages: An in vitro model of neocytolysis. *Investig Med* 2001;49:335–345.
9. Eichner ER. Blood doping: Infusions, erythropoietin and artificial blood. *Sports Med* 2007;37:389–391.
10. Breyman C. Erythropoietin test methods. *Baillieres Best Pract Res Clin Endocrinol Metab* 2000;14:135–145.
11. Robinson N, Giraud S, Saudan C, et al. Erythropoietin and blood doping. *Br J Sports Med* 2006;40 (Suppl 1):i30–i34.
12. Nissen-Lie G, Birkeland K, Hemmersbach P, Skibeli V. Serum sTfR levels may indicate charge profiling of urinary r-hEPO in doping control. *Med Sci Sports Exerc* 2004;36:588–593.
13. Parisotto R, Gore CJ, Emslie KR, et al. A novel method utilising markers of altered erythropoiesis for the detection of recombinant human erythropoietin abuse in athletes: *Haematologica* 2000;85:564–572.
14. Parisotto R, Wu M, Ashenden MJ, et al. Detection of recombinant human erythropoietin abuse in athletes utilizing markers of altered erythropoiesis. *Haematologica* 2001;86:128–137.
15. Connes P, Caillaud C, Simar D, et al. Strengths and weaknesses of established indirect models to detect recombinant human erythropoietin abuse on blood samples collected 48-hr post administration. *Haematologica* 2004;89: 891–892.
16. Ashenden MJ, Sharpe K, Schoch C, Schumacher YO. Effect of pre-competition and altitude training on blood models used to detect erythropoietin abuse by athletes. *Haematologica*. 2004;89:1019–1020.
17. Sharpe K, Ashenden MJ, Schumacher YO. A third generation approach to detect erythropoietin abuse in athletes. *Haematologica* 2006;91:356–363.
18. Magnani M, Corsi D, Bianchi M, et al. Identification of blood erythroid markers useful in revealing erythropoietin abuse in athletes. *Blood Cells Mol Dis* 2001;27:559–571.
19. Banfi G, Dolci A. Preanalytical phase of sport biochemistry and haematology. *J Sports Med Phys Fitness* 2003;43:223–230.
20. Robinson N, Saugy M, Mangin P. Effects of exercise on the secondary blood markers commonly used to suspect erythropoietin doping *Clin Lab* 2003;49: 57–62.
21. Kwan JT, Pratt RD. The Epoetin Delta Study Group. Epoetin delta, erythropoietin produced in a human cell line, in the management of anaemia in predialysis chronic kidney disease patients. *Curr Med Res Opin* 2007;23:307–311.
22. Macdougall IC. CERA (continuous erythropoietin receptor activator): A new erythropoiesis-stimulating agent for the treatment of anemia. *Curr Hematol Rep* 2005;4:436–440.
23. Nakano Y, Imagawa S, Matsumoto K, et al. Oral administration of K-11706 inhibits GATA binding activity, enhances hypoxia-inducible factor 1 binding activity, and restores indicators in an in vivo mouse model of anemia of chronic disease. *Blood* 2004;104:4300–4307.
24. Imagawa S, Nakano Y, Obara N, et al. A GATA-specific inhibitor (K-7174) rescues anemia induced by IL-1, TNF- or L-NMMA. *FASEB J* 2003;17:1742–1744.
25. Lippi G, Franchini M, Salvagno GL, Guidi GC. Biochemistry, physiology, and complications of blood doping: Facts and speculation. *Crit Rev Clin Lab Sci* 2006;43:349–391.
26. Lippi G, Franchini M, Guidi GC. Blood doping by cobalt. Should we measure cobalt in athletes? *J Occup Med Toxicol* 2006;1:18.
27. Wide L, Bengtsson C. Molecular charge heterogeneity of human serum erythropoietin. *Br J Haematol* 1990;76:121–127.
28. Belalcázar V, Gutierrez Gallego R, Liop E, et al. Assessing the instability of the isoelectric focusing pattern of erythropoietin in urine. *Electrophoresis* 2006;27:4387–4395.
29. Rice KG, Takahashi N, Namiki Y, et al. Quantitative mapping of the N-linked sialyloligosaccharides of recombinant erythropoietin: Combination of direct high-performance anion-exchange chromatography and 2-aminopyridine derivatization. *Anal Biochem* 1992;206:278–287.
30. Sasaki H, Bothner B, Dell A, Fukuda M. Carbohydrate structure of erythropoietin expressed in Chinese hamster ovary cells by human erythropoietin cDNA. *J Biol Chem* 1987;262:12059–12076.
31. Wide L, Bengtsson C, Berglund B, Ekblom B. Detection in blood and urine of recombinant erythropoietin administered to healthy men. *Med Sci Sports Exerc* 1995;27:1569–1576.
32. Breidbach A, Catlin DH, Green GA, et al. Detection of recombinant human erythropoietin in urine by isoelectric focusing. *Clin Chem* 2003;49:901–907.
33. Lasne F, de Ceaurriz J. Recombinant erythropoietin in urine. *Nature* 2000; 405:635.
34. Catlin DH, Breidbach A, Elliott S, Glaspy J. Comparison of the isoelectric focusing patterns of darbepoetin alfa, recombinant human erythropoietin, and endogenous erythropoietin from human urine. *Clin Chem* 2002;48:2057–2059.
35. Lasne F, Martin L, Crepin N, de Ceaurriz J. Detection of isoelectric profiles of erythropoietin in urine: Differentiation of natural and administered recombinant hormones. *Anal Biochem* 2002;311:119–126.
36. Lasne F. Double-blotting: A solution to the problem of non-specific binding of secondary antibodies in immunoblotting procedures. *J Immunol Methods* 2003; 276:223–226.
37. Catlin D, Nissen-Lie G, Howe C, et al. Harmonization of the method for the identification of epoetin alfa and beta (EPO) and darbepoetin alfa (NESP) by IEF, double blotting, and chemiluminescent detection. WADA technical document TD2004EPO. Accessed October 15, 2004. Available at [http://www.wada-ama.org/rtecontent/document/td2004epo\\_en.pdf](http://www.wada-ama.org/rtecontent/document/td2004epo_en.pdf)
38. Emslie KR, Howe C, Trout H. Measurement of urinary erythropoietin levels in athletes. In: Schänzer W, Geyer H, Gotzmann A, Mareck-Engelke U, editors. *Proceedings of the Manfred Donike Workshop, 17th Cologne Workshop on Dope Analysis, Recent advances in doping analysis, Vol. 7, Sport and Buch Strauß, Cologne; 1999.* pp 291–299.
39. Sue JM, Sytkowski AJ. Site-specific antibodies to human erythropoietin directed to the NH<sub>2</sub>-terminal region. *Proc Natl Acad Sci USA* 1983;80:3651–3655.
40. Bajla I, Holländer I, Minichmayr M, et al. Gasepo software GASepo—A software solution for quantitative analysis of digital images in Epo doping control. *Comput Methods Programs Biomed* 2005;80:246–270.
41. Ashenden M, Varlet-Marie E, Lasne F, Audran M. The effects of microdose recombinant human erythropoietin regimens in athletes. *Haematologica* 2006; 91:1143–1144.
42. Lamon S, Robinson N, Mangin P, Saugy M. Detection window of Darbepoetin-alpha following one single subcutaneous injection. *Clin Chim Acta* 2007; 379:145–149.
43. Peltre G, Thormann W. Evaluation Report of the Urine EPO Test. Paris, France, and Bern, Switzerland: Council of the World Anti-Doping Agency (WADA); 2003.
44. Righetti PG. Isoelectric focusing: Theory, methodology and applications. In: Burdon RH, van Krippenberg PH, editors. *Laboratory Techniques in Biochemistry and Molecular Biology, Vol. 11.* Amsterdam: Elsevier; 1990.
45. Khan A, Grinyer J, Truong ST, et al. New urinary EPO drug testing method using two-dimensional gel electrophoresis. *Clin Chim Acta* 2005;358:119–130.
46. Catlin D, Green G, Sekera M, et al. False-positive Epo test concerns unfounded. *Blood* 2006;108:1778.
47. Lamon S, Robinson N, Sottas PE, et al. Possible origins of undetectable EPO in urine samples. *Clin Chim Acta* 2007;385:61–66.
48. Franke WW, Heid H. Pitfalls, errors and risks of false-positive results in urinary EPO drug tests. *Clin Chim Acta* 2006;373:189–190.
49. Khan A, Baker MS. Non-specific binding of monoclonal human erythropoietin antibody AE7A5 to *Escherichia coli* and *Saccharomyces cerevisiae* proteins. *Clin Chim Acta* 2007;379:173–175.
50. Beullens M, Delanghe JR, Bollen M. False-positive detection of recombinant human erythropoietin in urine following strenuous physical exercise. *Blood* 2006;107:4711–4713.
51. Kouri TT, Gant VA, Fogazzi GB, et al. Towards European urinalysis guidelines. Introduction of a project under European Confederation of Laboratory Medicine. *Clin Chim Acta* 2000;297:305–311.
52. Beullens M, Delanghe JR, Bollen M. False-positive detection of rhEpo remains a real concern *Blood* 2006;108:1779–1780; author reply.
53. Lasne F. No doubt about the validity of the urine test for detection of recombinant human erythropoietin. *Blood* 2006;108:1778–1780.
54. Lasne F, Thioulouse J, Martin L, de Ceaurriz J. Detection of recombinant human erythropoietin in urine for doping analysis: Interpretation of isoelectric profiles by discriminant analysis. *Electrophoresis* 2007;28:1875–1881.
55. Nagano M, Stubiger G, Marchetti M, et al. Detection of isoforms of recombinant human erythropoietin by various plant lectins after isoelectric focusing. *Electrophoresis* 2005;26:1633–1645.
56. Stubiger G, Marchetti M, Nagano M, et al. Characterization of N- and O-glycopeptides of recombinant human erythropoietins as potential biomarkers for doping analysis by means of microscale sample purification combined with MALDI-TOF and quadrupole IT/RTOF mass spectrometry. *J Sep Sci* 2005;28: 1764–1778. Erratum in *J Sep Sci* 2005;28:2235.