EXHIBIT 1

UNITED STATES DISTRICT COURT

DISTRICT OF MASSACHUSETTS

AMGEN INC., Plaintiff, v. F. HOFFMANN-LA ROCHE LTD., a Swiss Company, ROCHE DIAGNOSTICS GmbH, a German Company and HOFFMANN-LA ROCHE INC., a New Jersey Corporation, Defendants.	Civil Action No.: 05-12237 WGY

REBUTTAL EXPERT REPORT OF AJIT VARKI, MD

SUBJECT TO PROTECTIVE ORDER
CONTAINS BOTH ROCHE AND AMGEN CONFIDENTIAL MATERIAL
CONTAINS ROCHE BLA MATERIAL

sulfate residues on GalNAc residues on the N-glycans of pituitary glycoprotein hormones are recognized by specific receptors in the liver, altering their half-life and modifying their biological functions.[64]

2. The Clinical Test for EPO Doping with EPO Relies on the Difference Between Urinary and Recombinant EPO

a. Isoelectric focusing

99. One technique for characterizing glycoproteins is known as "isoelectric focusing" or "IEF." Isoelectric focusing is used to detect charge differences, such as those between some glycoprotein glycoforms. IEF works by separating glycoforms on the basis of their individual "isoeleisoelectric points" or "pI." A single protein can carry a net positive, negative, or zero charge depending on the pH of its environment, and for every protein there is a specific pH at which its net charge is zero; this is its pI. pIs of proteins generally fall in the range 3 - 12, with most being around 4 - 7. When a protein is placed in a medium with a pH gradient and subjected to an electric field, it will initially move towards the electrode with the opposite charge. During migration through the pH gradient, the protein will pick up or lose protons (charged hydrogen atoms). As it migrates, the net charge and the mobility will decrease and the protein will slow down. Eventually, the protein will arrive at the point in the pH gradient which is equal to its pI. Here, it will be uncharged and hence stop migrating. If the protein should happen to diffuse to a region outside its pI, it will pick up a charge and hence move back to the position where it is neutral. More negative (acidic) glycoforms will focus nearer to the anode

[64] Roseman, D.S., Baenziger, J.U., "The Man/GalNAc-4-SO4-receptor: relating specificity to function." Methods Enzymol. 363:121-33 (2003).

(positively charged electrode) and more positive (basic) isoforms will focus nearer to the cathode (negatively charged electrode). In this way, IEF condenses, or focuses, protein into sharp bands. Unlike SDS-PAGE electrophoresis, this technique does not separate proteins on the basis of their molecular weight. IEF is capable of extremely high resolution with proteins differing by a single charge being fractionated into separate bands. I explain the IEF technique in my demonstrative graphics.

100. It is important to note that a single band on an IEF gel may still not be a pure selection of one protein glycoform. It is possible — and for complex molecules like EPO, even likely — that a single band on an IEF gel is comprosed of two or more glycoforms with the different structures but approximately the same pI.

3. IEF Analysis of Erythropoietin

101. Illicit use of rEPO by athletes looking for a competitive edge is a well-known problem. The clinical test used to detect the illicit use of recombinant EPO is based on a difference in charge observed between uEPO and rEPO glycoforms which is detected using an IEF procedure.[65] The procedure is performed by analyzing individuals' urine. In normal (untreated) individuals, urinary EPO can be detected using an antibody[66] against EPO. If first applied to an IEF gel, many characteristic urinary EPO glycoforms (each differing in charge) can be detected. Using this technique, one can see that there is a population of EPO glycoforms in an

[65] http://www.wada-ama.org/rtecontent/document/td2004epo_en.pdf.

[66] Antibodies are a part of animal's immune systems. They are specialized proteins that can bind to another molecule very specifically and tightly. Anti-EPO antibodies bind EPO tightly, but do not bind other molecules. In the Erythropoietin Doping Assay anti-EPO antibodies are used to detect the tiny amounts of EPO molecules that are excreted into the urine.

untreated individuals' urine. If an individual who has recently received recombinant EPO is tested using this technique, a different pattern of glycoforms is revealed. Specifically, the glycoform population present in recombinant erythropoietin is, on average, less negatively charged than those observed in the population of urinary EPO glycoforms.

102. The EPO IEF test is widely used around the world to identify athletes' illicit use of recombinant EPO. Many major national and international sporting bodies, such as the International Olympic Committee routinely administer the IEF test to detect doping with recombinant EPO.[67]

103. The IEF technique for detecting recombinant EPO in urine absolutely depends on the differences between every individuals' natural, native urinary EPO and recombinant EPO. If the chemical structure of urinary and recombinant EPO were the same, the EPO doping assay simply could not work. Every natural urinary EPO tested, from many, many individuals, has been shown to be different from recombinant EPO:

Lasne, F. et al., "Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones."

- Although some differences were noted between individuals, ***all natural urinary EPO patterns were clearly different from those of the various recombinant patterns***. Some patterns comprised minor bands collocated with the recombinant isoforms, but in all cases, the major isoforms presented pIs that were more acidic and more basic than Epoetin and Darbepoetin, respectively.[68]

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[67] Cite WADA info. http://www.wada-ama.org/rtecontent/document/2005_Annual_Report_En.pdf.

[68] Lasne, *et al.* "Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones," *Anal. Biochem.* 311(2):119-26 at 122 (2002).

- The most striking feature is the clear difference observed from untreated subjects (natural urinary EPO) and those from the different recombinant hormones. In comparison with Epoetin α and β, ***natural urinary hormone is mainly composed of more acidic isoforms that are missing in the recombinant patterns***.[69]

Breidbach et al., "Detection of recombinant human erythropoietin in urine by isoelectric focusing."

- The patterns of urinary isoforms of rHuEPO differs from that of endogenous EPO. The former are clustered into four or five bands in the most basic portion of the gel, whereas the latter, which include as many as 14 bands, overlap with and are parallel to the rHuEPO bands in the basic region but are also found in the more acidic region.[70]
- Although endogenous HuEPO contains isoforms that focus in the same area as rHuEPO, there is significant difference between epoetin alfa and placebo groups in the urinary EPO isoform patterns with respect to the density of the band within one lane.[71]

Belalcazar et al., "Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine."

- IEF can be used to differentiate human urinary erythropoietin (uEPO), recombinant human erythropoietin or epoetin (rEPO) and darbepoetin (novel erythropoiesis stimulating protein (NESP)).[72]
- IEF analysis shows additional non-overlapping isoforms of uEPO appearing at more acidic pI values than those observed for rEPO…[73]
- The introduction of the IEF and the so-called double-blotting procedure for the detection of EPO in urine allowed the unambiguous detection of its abuse. The basis of this protocol is that the endogenous and exogenous

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[69] Lasne, *et al.*, "Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones," *Anal. Biochem.* 311(2):119-26 at 124 (2002).

[70] Breidbach *et al.*, "Detection of Recombinant Human Erythropoietin in Urine by Isoelectric Focusing," *Clin Chem.* 49(6 Pt 1):901-7 at 905 (2003).

[71] Breidbach *et al.*, "Detection of Recombinant Human Erythropoietin in Urine by Isoelectric Focusing," *Clin Chem.* 49(6 Pt 1):901-7 at 906 (2003).

[72] Belalcazar *et al.*, "Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine," *Electrophoresis* 27(22):4387-95 (2006).

[73] Belalcazar *et al.*, "Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine," *Electrophoresis* 27(22):4387-95 (2006).

substances (rEPO and NESP) have distinct pI values for some of their isoforms (partially overlapping).[74]

104. The following are figures from scientific research articles that illustrate the differences between urinary EPO and recombinant EPO as detected by the IEF technique. The first figure is from the 2000 article in the prestigious journal *Nature*. It shows an IEF gel with the anode at the bottom (so the most basic glycoforms are at the bottom of the gel) with comparisons between a (non-prior art) purified urinary EPO (lane a); Roche's recombinant epoetin beta (lane b); Johnson and Johnson's epoetin alfa (lane c); urine from a person who had not received recombinant EPO (lane d); urine from two patients who had been treated with recombinant epoetin beta (lanes e and f); and urine from two cyclists who competed in the 1998 Tour de France (lanes g and h):[75]

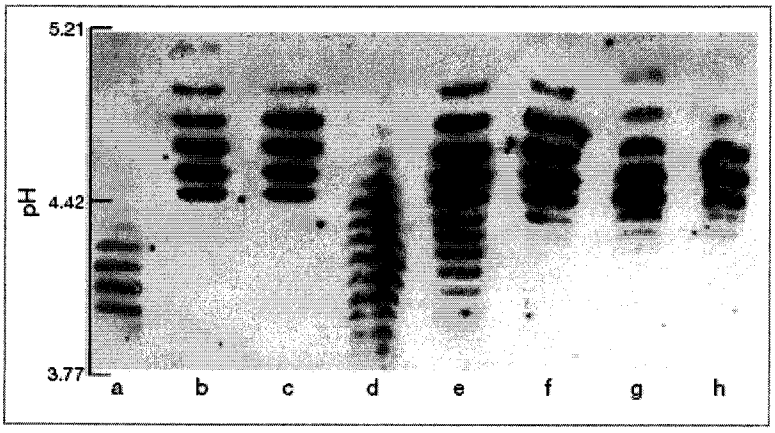

Figure 1 Autoradiograph of isoelectric patterns of exogenous and endogenous erythropoietin (EPO). Images were obtained by chemiluminescent immunodetection of blotted EPO after isoelectric focusing. **a,** Purified commercial human urinary natural EPO (Sigma); **b,** recombinant EPO-β (Neorecormon, France); **c,** recombinant EPO-α (Eprex, France); **d,** urine from a control subject; **e,f,** urine from two patients treated with Neorecormon EPO for post-haemorrhagic anaemia; **g,h,** urine from two cyclists from Tour de France 1998 (samples concentrated by ultrafiltration). Note the 'mixed' appearance of the pattern in **e**. The cathode is at the top; pH values are indicated on the left.

[74] Belalcazar *et al.*, "Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine," *Electrophoresis* 27(22):4387-95 (2006).

[75] Note that some IEF gels, like this one, are depicted with the anode (positive pole) down, whereas others shown below are depicted with the positive pole up. It makes no difference in the analysis, except one has to flip the gels over to compare.

49. Thus, one of ordinary skill in the art would have no difficulty determining whether or not a human EPO sample was non-naturally occurring — if the product was isolated from a natural source, such as urine or blood, it is not "non-naturally" occurring. If the product is derived from any other unnatural source, it is non-naturally occurring.

VII. PRIOR ART URINARY EPO PREPARATIONS DO NOT RENDER ANY OF DR. LIN'S PRODUCT OR METHOD OF TREATMENT CLAIMS ANTICIPATED OR OBVIOUS

A. THE TESTS FOR NOVELTY AND NON-OBVIOUSNESS OF DR. LIN'S PRODUCT INVENTIONS

50. I have been informed that in order to anticipate a product patent claim, a single prior art reference must embody all of the limitations of the claim.

51. I have been informed that in order for a patent claim to be obvious, one or more prior art references must have (1) suggested that claimed invention and (2) provided a reasonable expectation of success in practicing the claimed invention to an ordinarily skilled artisan at the same time.

52. I have also been informed that claim terms directed to the process or source of a claimed product can serve to define the structure of that product.

53. I have also been informed that after-arising scientific literature can be relevant to whether Lin's EPO products claimed in part by their source or process of production, were novel and non-obvious at the time of Lin's invention.

54. I have also been informed that to show novelty and non-obviousness of Lin's EPO at the time of the invention, the proper comparison is with embodiments of EPO then in

such may distort any comparison to recombinant EPO.

108. The next figure is Figure 4 from Lasne *et al.,* "Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones."[76]

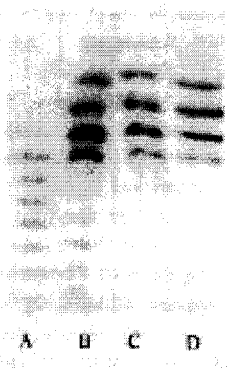

Fig. 4. IEF patterns of urinary EPO: natural EPO (A), 24 h after a first injection of Eprex (B), 24 h after a seventh injection of Eprex (2-week treatment) (C). For comparison, the IEF pattern of pure rHuEPO (Epoetin α) is shown in D. Anode is at the bottom of the figure.

109. This article by Lasne and colleagues confirms the results in the 2000 Nature paper. It also shows that urinary EPO has glycoforms not present in recombinant EPO and that recombinant EPO has glycoforms not present in urinary EPO.

110. Lastly, Don Catlin's laboratory at UCLA has also published on the EPO doping IEF assay. Figure 3 from Breidbach *et al.,* again demonstrates the difference between recombinant and urinary EPO:[77]

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[76] *Anal. Biochem.* 311(2):119-26 (2002) at 123.

[77] "Detection of Recombinant Human Erythropoietin in Urine by Isoelectric Focusing," *Clin. Chem.* 49(6 Pt. 1):901-7 (2003) at 905. Note that unlike the IEF gels shown above, this figure shows the anode (positive pole) at the top, instead of the bottom, so the urinary EPO glycoforms are seen below the recombinant EPO isoforms.

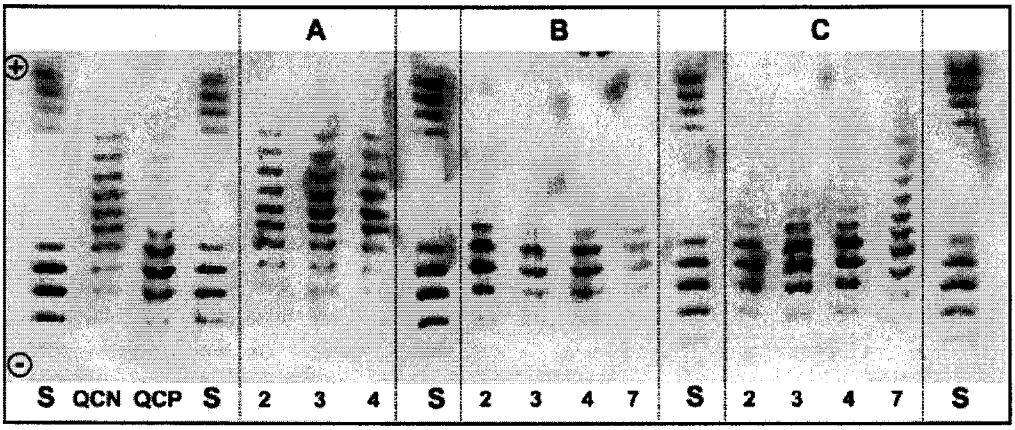


Fig. 3. Representative image of urinary EPO isoforms obtained by IEF analysis of urines from individuals treated with epoetin alfa and placebo.

The five lanes containing markers (*lanes S*) were spotted with 2 fmoles each of rHuEPO and darbepoetin. *Lanes QCP* and *QCN* represent urines from individuals known to be receiving rHuEPO and not to be receiving rHuEPO, respectively. The *lanes* in *section A* were obtained from a placebo-treated individual on postadministration days 2, 3, and 4. The *lanes* in *sections B* and *C* were obtained from epoetin alfa-treated individuals on days 2, 3, 4, and 7.

111. As can be seen by comparing the first lane (S), which has a mixture of recombinant EPO and Aranesp® product and the second lane (QCN), which is normal urine, it is clear that Catlin's group confirmed Lasne's conclusions that urinary and recombinant EPO each have some glycoforms in common and some glycoforms that are different. The rest of the gel demonstrates the suitability of the IEF test for identifying individuals who are doping with recombinant EPO.

112. Even before the anti-EPO doping test was perfected, Wide and his colleagues very clearly identified the charge differences between urinary and recombinant EPO. In particular the authors measured the median charge for urinary and recombinant EPO and found them to be significantly different:

> The recombinant Epo preparations had a median charge which was much less negative than that of the 2nd IRP for Epo and of the Epo in serum in healthy individuals. As the polypeptide chain of recombinant Epo is claimed to be identical with that of human Epo, it seems likely that the differences are due to

different degrees in glycosylation." [78]

113. The authors showed a clear difference in median charge between rEPO and the 2nd International Reference Preparation of urinary EPO:

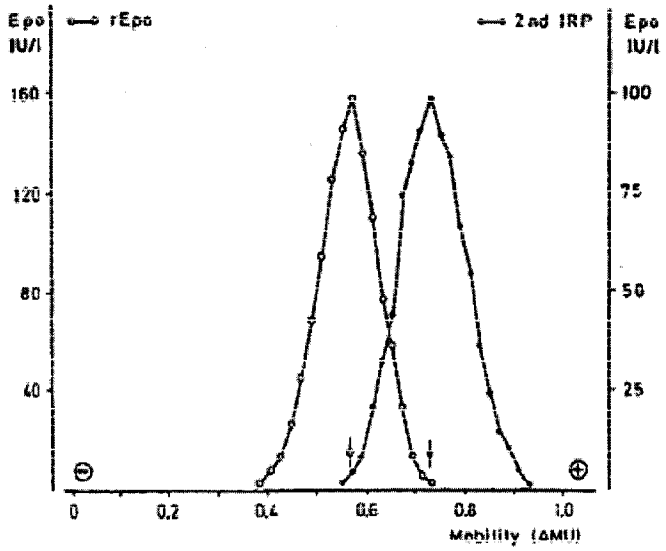


Fig 5. Elution patterns of Epo activity in a recombinant Epo (rEpo) preparation (Boehringer (○) and in the 2nd International Reference Preparation (2nd IRP) for Epo (●), by electrophoresis using a 1·5 x 67 cm column. The Epo activity in the eluates was plotted on scales which made the heights of the Epo peaks of the two preparations identical. The vertical lines indicate the median electrophoretic mobilities. AMU, albumin mobility unit; ⊕, anode; ⊖, cathode.

114. The authors further stated:

> The method used in this study to discriminate the differences between rhEpo and endogenous Epo is based upon the observation that the electric charge of the two Epo forms are different. ***The rhEpo is less negatively charged and has a lower mobility at electrophoresis than the endogenous Epo in healthy individuals.*** The rhEpo preparation used in the current study was from Boehringer Mannheim GmbH in collaboration with the Genetic Institute, and has a median mobility of 594 mAMU. Preparations from this manufacturer and from Amgen, Integrated Genetics Inc, in collaboration with Behringwerke AG and the Snow Brand Milk Products Co. Ltd. had similar mobilities with this electrophoretic technique. The manufacturer used three different types of cells for synthesis of rhEpo: Chinese hamster ovary cells, baby hamster kidney cells and the C127 mouse fibroblast cell line. It is interesting to note that the charge of the rhEpo preparations is similar to that of human liver Epo forms and forms produced by tumors in human tissue. ***It***

[78] Wide *et al.*, "Molecular charge heterogeneity of human serum erythropoietin," *Br. J. Haematol.* 76(1):121-7 at 126 (1990).

> ***seems that the human kidney has a unique capacity to produce the more acidic isoforms of Epo.***[79]

115. The authors also demonstrated a clear difference between rEPO in urine and in the EPO glycoforms found in the urine of the same patient before treatment:

[79] Wide *et al.*, "Detection in blood and urine of recombinant erythropoietin administered to healthy men," *Med. Sci. Sports Exerc.* 27(11):1569-76 at 1574-5 (1995) (emphasis added).

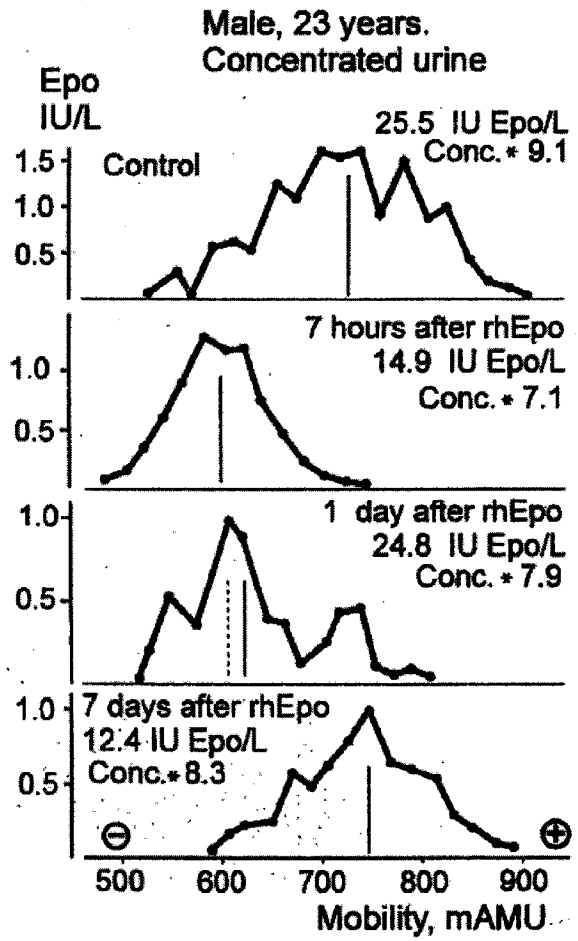


Figure 2—The elution patterns of Epo by electrophoresis of 1 ml of concentrated urine from a 23-yr-old man given one rhEpo injection (20 IU·kg^{-1} bw) three times a week. The urine samples were obtained 7 h, 1 d, and 7 d after the last (the 20th) subcutaneous injection of rhEpo. The control sample was obtained 3 wk after the last injection. The urine samples were concentrated 7.1–9.1 times. A vertical solid line indicates the median mobility of all Epo isoforms and a dashed vertical line the median mobility of isoforms in the basic peak.

116. The IEF test for recombinant EPO in urine has been shown to be effective for every recombinant EPO tested. For example, it was recently shown by the World Anti-Doping Agency ("WADA") that epoetin delta, which is produced in recombinant human cells, can be differentiated from urinary EPO by the IEF test:

A new version of EPO, the anemia treatment that sports cheats use to boost their stamina, is traceable in drugs tests after anti-doping enforcers got early access to the product.

"Athletes who use it will be in for a nasty surprise," Olivier Rabin, science director of the Montreal-based World Anti-Doping Agency, or WADA, said in an interview. "People thought it would be impossible to detect but we've carried out research and it is detectable."

Shire Plc, the U.K.'s third-largest drugmaker, last week started selling epoetin delta under the Dynepo brand in Germany and said it expects to begin marketing in other European countries "in the coming months." Epoetin delta is made from human cells. Previous derivatives of erythropoietin, known as EPO, came from animals such as hamsters and showed up in urine testing because they differ from naturally produced human cells.

Anti-doping agencies, aided by drug manufacturers, are tracking a "new generation" of EPO products, according to Patrick Schamasch, the International Olympic Committee's medical director. WADA has a budget this year of $23 million to combat a doping industry that Spanish Secretary of State for Sport Jaime Lissavetzky, citing Interpol figures, said was more than $19 billion in 2005, bigger than the global trade of social drugs like cocaine and marijuana.

Developed to treat anemia in cancer and kidney-disease patients, EPO drugs stimulate the production of red blood cells. Athletes have illegally used EPO to increase stamina since the 1980s -- red blood cells carry oxygen to the body, thereby increasing its ability to sustain aerobic activity for longer periods. Sports authorities didn't introduce a test for EPO until 2000.

Tainted Medals

Cross-country skiers Johann Muehlegg and Larissa Lazutina gave back their Olympic gold medals at the 2002 Winter Games after testing positive for EPO drug darbepoetin. Cyclist Roberto Heras was stripped of his record fourth Tour of Spain title after testing positive for EPO in 2005. Eight of 96 blood samples examined last year in a Spanish judicial probe into a suspected doping ring contained EPO.

Cyclists get EPO on the black market -- it costs 600 euros ($797) for six vials -- injecting small amounts over a long period to boost fitness, according to former rider Jesus Manzano, who has confessed to doping.

In October, Mario Zorzoli, chief medical officer of cycling ruling body Union Cycliste Internationale, said that "in theory" epoetin delta would be impossible to differentiate from naturally produced human cells in urine samples, cyclingnews.com reported at the time.

> Final Tests
>
> WADA contacted Basingstoke, England-based Shire about the product in 2005, when the company acquired the European rights to it as part of the purchase of Transkaryotic Therapies Inc.
>
> "We were happy to cooperate," company spokeswoman Jessica Mann said. "We have not paid them anything. We have provided them with the product to conduct trials."
>
> WADA, which says it's been tracking the development of epoetin delta since 2003, is now "fine-tuning" research on the drug to find out how long it stays in the body and is detectable in samples, Rabin said.
>
> "Generally speaking, we've got very, very good relationships" with pharmaceutical companies, Rabin said. "We're actually working on drugs that will be on the market in five years' time or more."
>
> Other companies that make EPO drugs include Basel, Switzerland-based Roche AG and Amgen Inc., which has its headquarters in Thousand Oaks, California.
>
> "There has been collaboration for a long time with pharmaceutical companies but it has accelerated the last four or five years," the IOC's Schamasch said. "The gap between the cheats and ourselves is getting smaller and smaller." [80]

117. Every individual's uEPO is more negative than recombinant EPO — otherwise there would be false positives in the IEF doping test. Likewise, every rEPO is less negative than uEPO, otherwise the highly sulfated EPO would be the doper's drug of choice.

118. Because the set of glycoforms in Lin's recombinant EPO is different from the set of glycoforms in *every* individual's urinary EPO, it must have been different from Goldwasser's uEPO preparation.

119. Dr. Bertozzi mentions in several places in her report that the IEF technique is

[80] *EPO From Human Cells Can Be Traced in Doping Tests, WADA Says*, By Alex Duff, March 21, 2007 (Bloomberg).

not mentioned in Dr. Lin's specification.[81] I find this observation to be of no moment. First, the differences between recombinant EPO and the prior art urinary EPO existed in 1984, whether or not there was an analytical technique available to detect them. Second, IEF was well-known and in wide use in 1984. I understand the Court made a factual finding to this effect in the *Amgen v. Hoechst* case: "Another technique employed by those skilled in the art in 1983 was isolectric focusing ('IEF')."[82] I also note that the Ikegami reference cited by Bertozzi used an electrofocusing technique to purportedly purify urinary EPO in 1977.

120. Another conclusion that follows from the success of the clinical test for recombinant EPO is that the addition of the excess negative charge observed in uEPO must occur in the kidney cells that normally make EPO. If the negative charge were added after the EPO is secreted from the EPO-producing cells, then the negative charge would also be added to recombinant EPO in the serum or urine.

121. Likewise, Amgen and others have tested many other recombinant EPOs on the market around the world and shown that each of them has approximately the same IEF pattern as Amgen's Epogen®, and therefore clearly different than urinary EPO. More specifically, none have been found to be significantly more negatively charged than Epogen. For example, Schellekens tested eight recombinant EPOs from around the world and found that the differences between these samples predominantly consistent of additional more-positively charged bands

[81] *See*, e.g., ¶¶ 43, 104, 120.

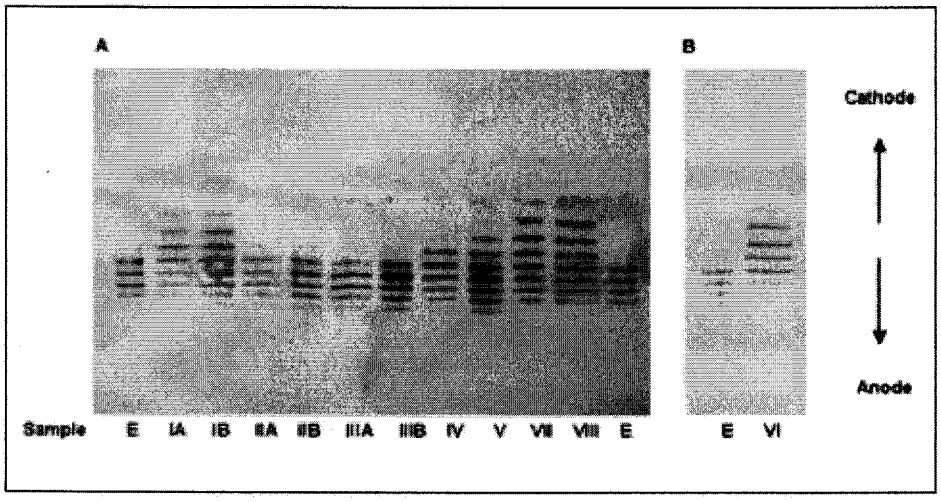
[82] *Amgen, Inc. v. Hoechst Marion Roussel, Inc.*, 126 F. Supp. 2d 69, 125 (D. Mass. 2001).

than found in the recombinant EPO standard, Epoetin alfa ("E" in the figure below).[83]

Table 1. Epoetin alfa products

Sample	Expiration Date	Concentration (IU/ml)	Country*
IA	April 2004	2,000	Korea
IB	April 2004	4,000	Korea
IIA	August 2003	2,000	Korea
IIB	November 2003	10,000	Korea
IIIA	January 2004	2,000	Korea
IIIB	January 2004	10,000	Korea
IV	April 2004	2,000	Argentina
V	July 2003	10,000	Argentina
VI	March 2004	4,000	India
VII	July 2004	10,000	China
VIII	August 2003		China

*Location where the marketed samples were obtained.



122. At most, a single additional acidic isoform was observed, not the more basic

[83] Schellekens., "Biosimilar epoetins: how similar are they?" *EJHP* 3:43-47 (2004). "This study shows that epoetin products from manufacturer outside Europe and the USA differ widely in composition. Although this does not necessarily mean that these products are clinically inferior to the innovator product, some of the products failed to meet their own specifications, indicating that some of the manufacturers do not have adequate control over their production process." At 46.

forms observed in urinary EPO:

> IEF was used to identify the isoelectric point of each sample, along with its unique isoform protein pattern. The number of isoforms visualized, relative abundance, and position within the pH gradient provide information about the tested protein. Isoform pattern comparisons are used to indicate uniformity and consistency within a production batch and to compare protein from different sources. The isoform patterns for epoetin alfa and the biosimilar epoetin samples are shown in Figure 1. Four major and two minor isoforms were identified in the epoetin alfa control. Two additional basic isoforms were identified in samples IA and B,V,VII, and VIII, and three additional basic isoforms were identified in sample VI. An additional acidic isoform was identified in samples IIB, IIIB, IV, and V. Variation in the intensity of isoform bands in comparison to epoetin alfa was noted for samples IV,V,VI,VII, and VIII.[84]

123. Since the glycoforms of urinary EPO are largely more negative than those in epoetin alfa, this result further supports the observed differences between urinary and recombinant EPO.

124. When it tested three other recombinant EPOs from Korea, Amgen observed very similar results to Schellekens:[85]

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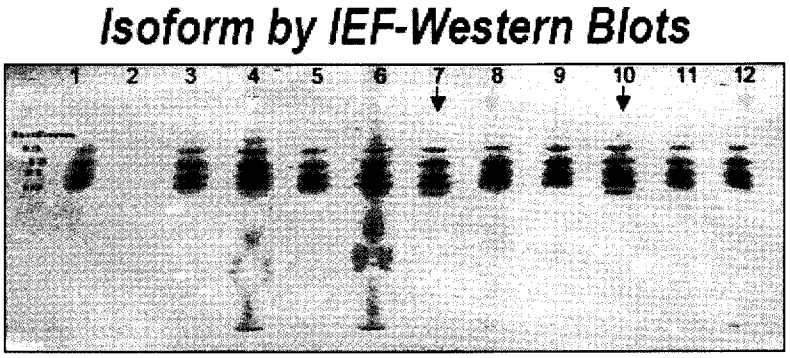
[84] Schellekens, "Biosimilar epoetins: how similar are they?" *EJHP* 3:43-47 at 46 (2004).

[85] Park *et al.*, "Analytical Comparisons Of Erythropoietin Products From Korea and US Epoetin alfa Manufactured By Amgen," poster presented at XLIII ERA-EDTA Congress — July 15-18, 2006. Again, this gel shows the positive pole at the top, not at the bottom.



Lane #	Company	Conc. (IU/mL)	Comments
#1	Ref std		
#2	Placebo	0	
#3	Amgen	2000	
#4	Dong-A (Eporon)	2000	Extra streaking, Additional isoforms
#5	Amgen	4000	
#6	Dong-A (Eporon)	4000	Extra streaking, Additional isoforms
#8	CJ (Epokine)	4000	Extra streaking
#9	Amgen	8000	
#11	Amgen	10,000	
#12	CJ (Epokine)	10,000	Extra streaking

4. The Literature Suggests Urinary EPO Is More Highly Sulfated Than Recombinant EPO

125. It is thought that the difference between recombinant EPO and urinary EPO on which the IEF test for recombinant EPO in urine is based must be due to negative charges on the sugars other than sialic acids. First, Takeuchi noted that some of the negative charges were resistant to sialidase (also known as "neuraminidase"), an enzyme which cleaves sialic acids off of glycans.[86] Belalcazar very clearly shows that some of the negative charge in urinary (the prior

[86] Takeuchi, *et al.*, "Comparative Study of the Asparagine-linked Sugar Chains of Human Erythropoietins Purified from Urine and the Culture Medium of Recombinant Chinese Hamster Ovary Cells," *J. Biol Chem.* 263(8):3657-63 at 3658 (1988).

art 2nd IRP reference standard), but not recombinant, EPO is resistant to sialidases:

> Incubations of the recombinant materials with a broad-specificity sialidase (Fig. 2) resulted in completely empty IEF gels indicating that complete desialylation yielded more basic molecules that migrated out of the pH range of the gel (2–6). In contrast, the resulting profile for the endogenous species still showed several isoforms within the range of the gel pointing towards charges different from sialic acid in uEPO only.[87]

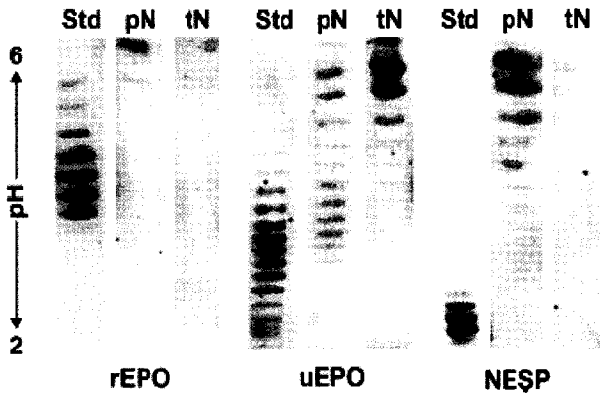


Figure 2. IEF profiles obtained for standard solutions of the reference compounds after partial and total neuraminidase digestions. Lane Std: reference compound. Lane pN: partial neuraminidase digestion. Lane tN: total neuraminidase digestion.

126. Belalcazar also confirmed that the charge differences between recombinant EPO and urinary EPO are restricted to the glycan portion of the molecules. The authors demonstrated this by removing all of the glycans from the two types of EPO and showing that after deglycosylation the samples were essentially identical when assayed by IEF:

> [T]hrough de-N-glycosylation the differences observed in the original IEF profiles of endogenous and exogenous EPOs can be eliminated. This certified that the charge differences between both are indeed contained in the N-linked

[87] Belalcazar *et al.*, "Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine," *Electrophoresis* 27(22):4387-95 (2006). Note this gel also analyses Amgen's NESP drug, which is not relevant to my analysis.

oligosaccharides and not localized in the polypeptide moiety.[88]

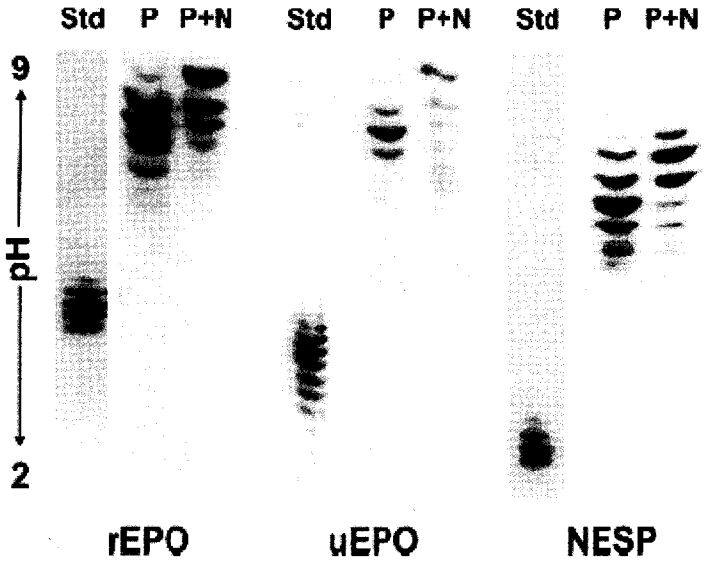


Figure 4. PNGase F digestions of rEPO, uEPO and NESP. Lane Std: profile of the corresponding standard solution. Lane P: profile obtained after PNGase F treatment. Lane P + N: profile obtained after PNGase F plus neuraminidase treatment.

127. Second, since it is known that the glycoforms found in recombinant EPOs are very nearly fully sialylated,[89] it logically follows that the excess negative charges found in urinary EPO glycoforms must be due to some other structure present on the urinary EPO glycans.

> The origin of the differences between natural urinary EPO and Epoetin α and β, however, is not clear…the tetrasialylated oligosaccharides have been shown to be the prevalent forms in recombinant CHO EPO. Thus, the more acidic isoforms of natural urinary EPO cannot be imputed to supplementary sialic acid residues….[90]

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[88] Belalcazar *et al.*, "Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine," *Electrophoresis* 27(22):4387-95 (2006).

[89] Strickland, U.S. Patent No. 5,856,298 (filed Nov. 3, 1994).

[90] Lasne *et al.*, "Detection of isoelectric profiles of erythropoietin in urine: differentiation of natural and administered recombinant hormones," *Anal. Biochem.* 311(2):119-26 at 124-25

128. The literature suggests that the excess negative charges present in uEPO are due to sulfation:

- Thus, the results suggested the presence of small amounts of oligosaccharides containing both sialic acid residues and ***possibly sulfate group***...however, no detailed analysis of them to assign the location of sialic acid and sulfate residues was performed because of the limited amounts of samples. [91]
- Thus, this migration behavior cannot be explained in terms of sialic acid residues only and indicates that uEPO contains additional charges that have not been addressed thus far (*e.g.* sulfation, phosphorylation, *etc.*)...however, those features are essential since they are employed to discriminate between endogenous and exogenous EPO.[92]
- In this paper we have addressed the interesting observation that endogenous urinary EPO contains glycoforms with more acidic pI values than its recombinant counterpart. These additional charges have been proven to be different from sialic acids but localized within the carbohydrate moiety of the hormone. ***At present, the most likely explanation includes the occurrence of sulfate groups on either N-acetyl glucosamine or galactose residues of the N-glycans.*** This phenomenon could be somehow exploited in the field of doping control, as a sialidase digestion of suspicious specimens would yield empty lanes in the case of recombinant EPO only.[93]

129. Recombinant EPO is known to have a small amount of sulfated glycans. See for example, Kawasaki *et al.*:

- [W]e found that some major N-linked oligosaccharides are partly sulfated in EPO expressed in BHK cells. Some of these sulfated oligosaccharides are

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(2002).

[91] Takeuchi *et al.*, "Comparative Study of the Asparagine-linked Sugar Chains of Human Erythropoietins Purified from Urine and the Culture Medium of Recombinant Chinese Hamster Ovary Cells," *J. Biol. Chem.* 263(8):3657-63 (1988) at 3658 (emphasis added).

[92] Belalcazar *et al.*, "Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine," *Electrophoresis* 27(22):4387-95 (2006).

[93] Belalcazar *et al.*, "Assessing the instability of the isoelectric focusing patterns of erythropoietin in urine," *Electrophoresis* 27(22):4387-95 (2006) (emphasis added).

also found in EPO expressed in CHO cells (details to be published elsewhere).[94]

- [I]t was indicated that the sulfation occurs on a GlcNAc on the nonreducing side in EPO. Sulfation of GlcNAc is facilitated on the C-6 position… [95]
- In our study, NMR spectroscopy suggested that the sulfate group in the sulfated tetraantennary is located on GlcNAc-7 in the GlcNAcβ1-4Manα1-3 branch.[96]

130. I agree that additional sulfation in the glycans of urinary EPO as compared to recombinant EPO is the most likely explanation for the differences observed in the IEF test.

5. Amgen's Internal Research On Urinary Epo Charge And Sulfation

a. Amgen tested Goldwasser's urinary EPO and found it to have different charge and sulfation than recombinant EPO

131. Between 1987 and 1994, scientists at Amgen, principally Drs. Thomas Strickland and Gary Rogers, pursued a very comprehensive study comparing recombinant EPO to urinary EPO. Importantly, unlike any of the published studies concerning the glycosylation of urinary EPO, Amgen actually used Goldwasser's original preparations of α and β urinary EPO.[97] Amgen's internal research has consistently shown the difference between Goldwasser's uEPO and rEPO by IEF as well as other more sensitive analytical techniques.

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[94] Kawasaki *et al.*, "Structural analysis of sulfated N-linked oligosaccharides in erythropoietin," *Glycobiology* 11(12):1043-9 (2001) at 1046.

[95] Kawasaki *et al.*, "Structural analysis of sulfated N-linked oligosaccharides in erythropoietin," *Glycobiology* 11(12):1043-9 (2001) at 1047.

[96] Kawasaki *et al.*, "Structural analysis of sulfated N-linked oligosaccharides in erythropoietin," *Glycobiology* 11(12):1043-9 (2001) at 1047.

[97] *See* Strickland LNB 3435, page 2, AM-ITC 00188002-019; Strickland LNB 4790, page 79, AM-ITC 01078985-9086; Strickland LNB 5668, page 36, AM-ITC 01078664-738.