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Re: Case Reference Name "Erythropoietin", Sample Code: 7006876

Dear Dr. Bentley:

I am providing to you this report based on my personal understanding of World Anti-Doping Agency (WADA) Technical Document TD2022EPO, on the Harmonization of Analysis and Reporting of Erythropoietin (EPO) and Other EPO-Receptor Agonists (ERAs) by Polyacrylamide Gel Electrophoretic (PAGE) Analytical Methods, and my reading of the two reports: (1) A SAMPLE LDOC 7006876 NA22-04962 TD2022LDOC, and (2) B SAMPLE LDOC - 7006876 NB23-20136 TD2022LDOC.

WADA TD2022EPO describes the procedure required for the discovery and reporting of ERAs, including external sourced EPO in urine and blood samples. "All Laboratories are required to apply the criteria established in this TD in the routine performance of PAGE Analytical Methods to identify ERAs in urine or plasma/serum Samples."

Because the results from the Initial Testing Procedure (ITP) was not provided, I cannot comment on this part, even though Mr. Philipp Reihlen and Prof. Dr. Mario Thevis referred to the ITP results in their Second Opinion Letter dated November 22nd, 2022.

My opinion will be focused on the results of the confirmation procedure (CP) as presented in the two reports for the A Sample and B Sample, respectively.

Executive Summary

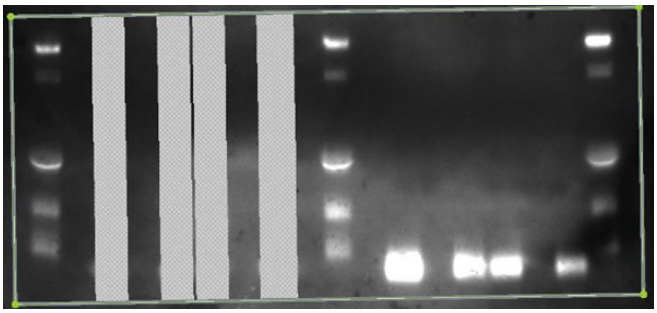
After a carefully examination of the reports for the A Sample and B Sample, I found that all the data as presented in the two reports showed negative results for recombinant erythropoietin (rEPO). The gel images are distorted, making some of the protein spots appear to have larger molecular weights that the identical protein standards run on a

separate lane. Even with the improperly drawn apex lines for Epoetin-δ, I estimated the diffusion overlap between the A sample and B Sample with Epoetin-δ, and the negative controls with Epoetin-δ. The numbers showed absolutely no evidence for the presence of any rEPO in the two samples tested.

The following are the more details observations.

1. Gel patterns obtained on 2022.10.26 showed a negative result

In "A SAMPLE LDOC 7006876 NA22-04962 TD2022LDOC", **Section 3.4.2**, it showed the following Gel Pattern:



An experienced lab personnel should have immediately recognized the overloading of the urine sample marked "04962", and followed the procedure described in the WADA Technical Document (TD2022EPO, hereafter referred as the WADA TD in this document).

The last paragraph in Page 5 of the WADA TD:

"*When needed and based on the results from the ITP,* ***the volume of the confirmation Aliquot taken from the "A" Sample or the volume of the eluate obtained after immunopurification of the Aliquot should be adjusted to ensure an appropriate ERA signal and facilitate the interpretation of results***".

The common practice is to adjust the test sample's intensity so that it is similar to the negative urine sample, by adjusting the amount loaded on to the gel, so that proper comparison can be made. This is because SAR-PAGE or SDS-PAGE is not a perfect technology in detecting rEPO, and diffusions occur often in the sample bands. Many research papers on SDS-PAGE or SAR-PAGE have shown that "**The presence of rhEPO α, β and δ along with huEPO can be difficult to detect by the current IEF and SDS-PAGE analyses**" (C. Ayotte *et al*., Drug Test Analysis 2013, 5, 870–876).

If one is to consecutively load more and more of the urine EPO (uEPO) negative control sample

in different lanes on the same gel, one would eventually observe the same gel pattern as for Sample 04962. On the other hand, if one is to dilute Sample 04962 by 5 or 10 time, to match the intensity of the uEPO negative control intensity, one would see a negative result right away.

If the same over loading resulted diffused pattern was observed in the ITP, the lab personnel should have adjusted the procedure before performing the CP.

Another obvious phenomenon that should have been noticed, but wasn't, is the intense band observed for Sample 04962. A person that recently doped with rEPO would not have such a high level of endogenous EPO. In "Effects of recombinant human erythropoietin in normal humans" (J Physiol 589.6 (2011) pp 1265–1271), it was found that treatment with rEPO may result in suppression of endogenous EPO production through a decrease in intrarenal oxygen consumption. This suppression of endogenous EPO expression was indeed observed even 10 days after micro dosing of rEPO (Drug Test. Analysis 2016, 8, 1119–1130). Therefore, positive samples often show a relatively low amount of uEPO compared to a normal person, making the presence of rEPO even more obvious. The gel pattern of Sample 04962 showed very strong uEPO intensity, indicating it did not come from a person recently used rEPO.

The suppression of endogenous EPO is well documented in the WADA TD. However, because not many people are informed of these physiological changes, these important factors are often overlooked. Specifically, in Page 16 of WADA TD, Figure 3, Lane 2, it shows the pattern from a volunteer's sample more than 4 days after a small dose of rEPO (50 IU/kg, please note 900 IU or less per injection is called micro dosing). The suppression of endogenous EPO is prominently shown. Figure 4 and 5 in the same document showed longer time patterns after dosing, endogenous suppression can also be easily observed. However, because Reference 24 in the TD is a conference presentation, the exact time after dosing cannot be found in the public domain. What is available, however, is both in the paper mentioned above (Drug Test. Analysis 2016, 8, 1119–1130), and a paper published in 2009, titled "SARCOSYL-PAGE: a new method for the detection of MIRCERA and EPO-doping in blood" (Christian Reichel, Friedrich Abzieher and Thomas Geisendorfer, Drug Test. Analysis 2009, 1, 494–504), the results showed that 10 days after dosing or microdosing, the suppression of endogenous EPO was still significant.

Had the lab personnel seen more samples of real doping, instead of only the man-made positive controls used in the assays presented, it would have been obvious that Sample 04962 is **NEGATIVE**. The lane labeled "Dynepo excretion" in the following figure from the WADA TD is a real positive sample, showing significant endogenous EPO suppression.

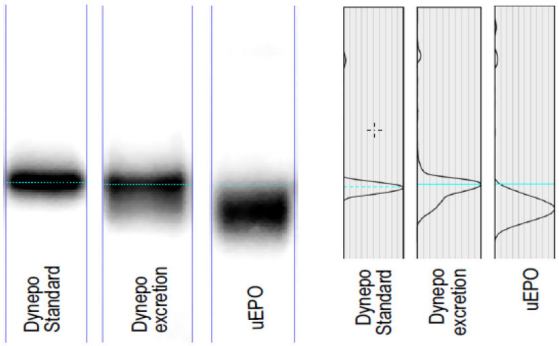


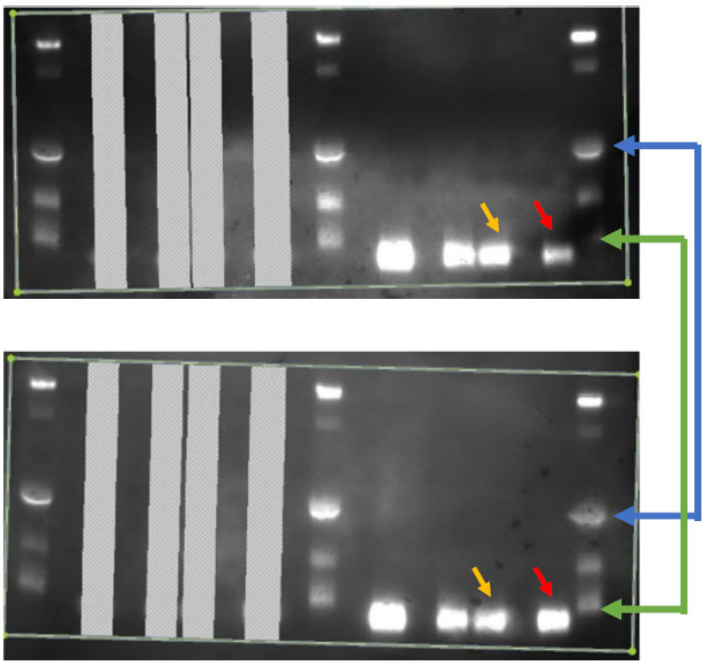
Figure 3. Immunoblot imaged obtained after SDS-PAGE separation of Dynepo reference standard, Dynepo excretion urine (100 h after subcutaneous application of 50 IU/kg Dynepo) and urinary reference standard (uEPO) and corresponding densitometric profiles (generated using GasEPO v2.1).

Unfortunately, much more work was carried out after the wrong conclusion was made. I will also comment on the data presented in the rest of the report.

2. Gel pattern obtained on 2022.10.28 showed a negative result

Because of the high background in the data obtained earlier, the same SDS-PAGE was re-probed. The results showed no difference. However, because there are more analyses done on this image, I will provide my observations.

2.1 First observation. The reprocessed gel showed some strange differences, not just the background. Please see the following figure:



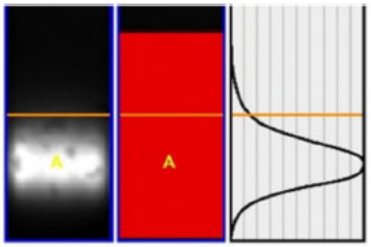
The bands pointed by the yellow arrows became weaker after the background reduction, the bands pointed by the red arrows became stronger after the background reduction, the band connected by blue arrows became smeared after the background reduction, and the bands connected by the green arrows are very different before and after the background reduction. The lab should provide plausible explanations to these highly unusual changes from the same gel.

2.2 A second observation is the poor quality of the standards. It is well known that Epoetin-δ should show a relatively sharp band when ran on a SAR-PAGE. This is shown in the WADA TD, and would be shown in any well-run SAR-PAGE gel. The sharp band of Epoetin-δ is important especially in this case because the Sample 04962 is alleged of doping with it. However, all three Epoetin-δ bands on the gel are smeared, making it difficult to tell where the

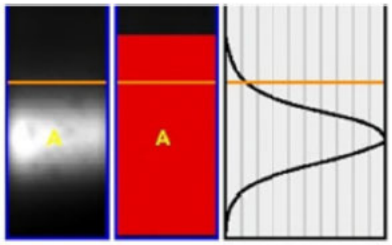
apex of Epoetin-δ is. More importantly, it suggests proteins with similar molecular weight, such as uEPO, may also diffuse more. This might cause artifacts that may result in false positives.

2.3 A third observation is the similarity between the Sample 04962 and the uEPO negative control is much higher than with any of the two positive controls.

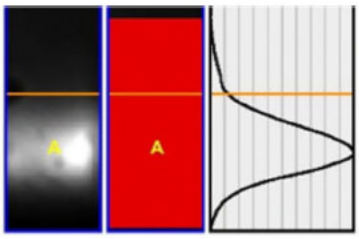
When the gel patterns were digitized by a densitometer, the obtained curves were shown on the right hand side of the gel patterns. Also shown are the relative intensities of the gel images. If one follows the brown lines showing the apex of Epoetin-δ, crossing with the digitized curve, one can easily see that the relative intensity of Sample 04962 is similar only to the negative control of uEPO. The positive control curve of U_100% MRPL Spike have much higher relative intensities when crossing with the apex line of Epoetin-δ. This further proves that Sample 04962 is negative. The U_50% MRPL wasn't a good positive control because the signal for rEPO was too low to be useful.



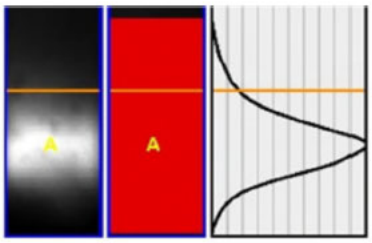
U_04962



uEPO, Negative control



U_50% MRPL Spike



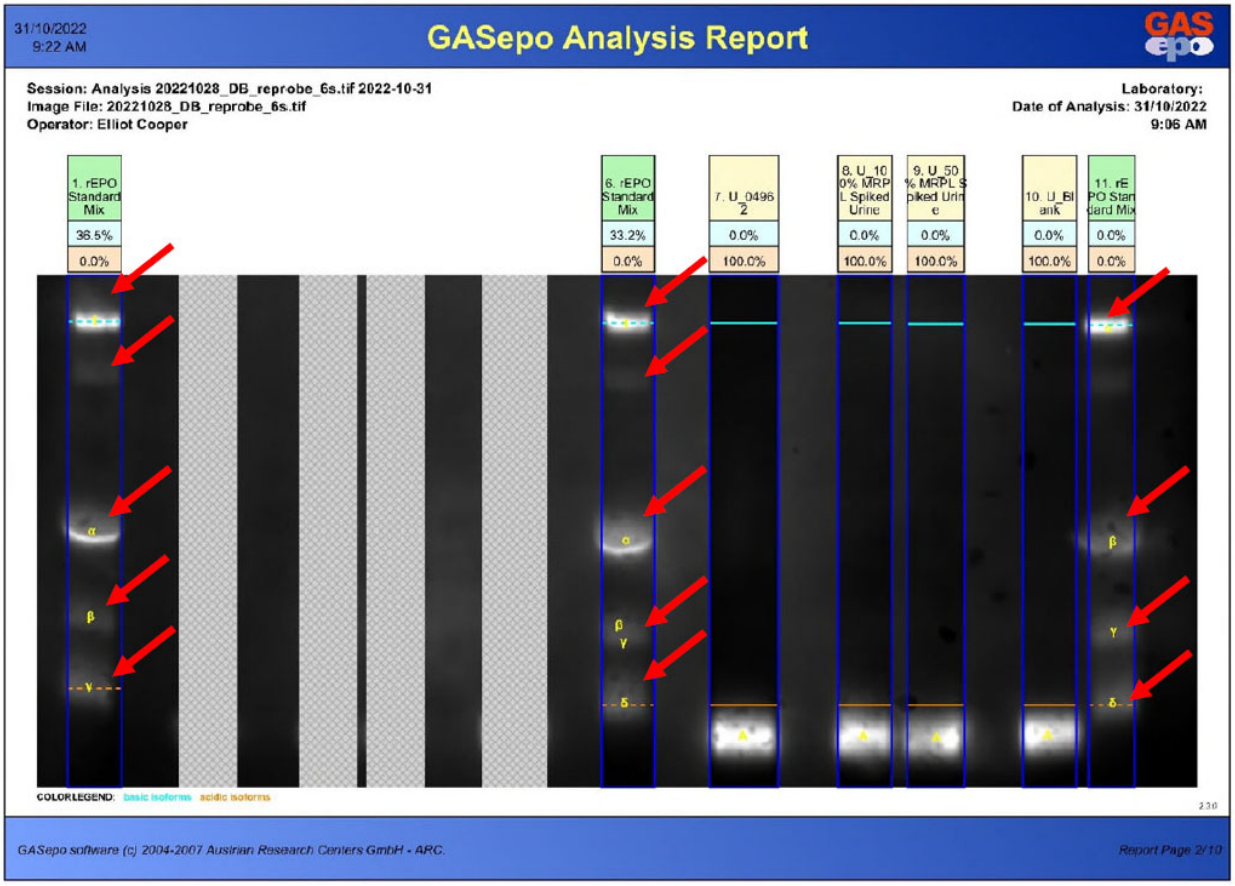
U_100% MRPL Spike

Upon further examining how the brown lines were drawn, one can see on Page 27 of the report, that the position of "apex" of Epoetin-δ, the "brown lines", was chosen based on the image of Line 11, titled "11. rEPO standard mix". The problem is that the gel is severely tilted towards the right-hand side. This makes the same sample mixture in Lane 6 to appear above the Apex in Lane 11, and the same sample mixture in Lane 1 to appear even higher on the gel, as if they have larger molecular weights than the identical standards in Lane 11. If one was to draw the apex line of Epoetin-δ in Lane 1 and use it as the brown line, no one would even think about anything but negative. The brown line in Lane 6 was forced through with reference to that of Lane 11,

making the U_04962 image appear artificially to have more diffusion than it really has.

My conclusion on this image is:

The electrophoresis is not performed with an acceptable quality, having proteins run significantly faster on the right-hand side than those on the left-hand side. Anything ran on the left-hand side will appear to have a larger molecular weight than those identical molecules run on the right-hand side of the gel. Diffusion is absolute with the SAR-PAGE technology. Please see the red arrows in the following image, which is the image shown on page 27 of the report. The only difference is how much the diffusion is relative to a negative control. When the standard was not chosen properly, any conclusion drown from such data will not be reliable.



(2) The gel patterns of the standard Epoetin-δ used in this work are not typical. The Epoetin-δ bands are usually relatively sharp, and the apex of this rEPO are usually easy to see. This observation is frequently referred in the WADA TD.

For example, in Page 16 of the WADA TD alone, it states:

"**Epoetin-δ (Dynepo) has a characteristic band shape ("sharp band")** and higher apparent molecular mass than endogenous uEPO/bEPO".

"due to the **sharper band** (albeit a faint smear may also be present in both the Dynepo standard and Dynepo administration samples, representing glycoforms of higher mass), Epoetin-δ can be also differentiated from other rEPOs (-α and -β as well as the biosimilars)".
The bands representing Epoetin-δ in this work are not sharp. The diffusions far exceeded those normally seen in similar experiments from other labs. These images are either resulted from the poor quality of the gels or the poor quality of the standards used.
The quality of Epoetin-δ is important because one needs to draw the apex lines of this rEPO from these images. And the apex lines are not always in the middle of the diffused pattern, as shown in the above image: the third red arrows in Lane 1, 6 and 11, pointed to such non-symmetrical patterns.

3. The lab personnel, including people providing the second opinion, didn't follow Wada TD Section 2.4, regarding how to evaluate and interpret the results.

In Page 16 of WADA TD, it specifically stated that to **consider an adverse analytical finding (AAF) for rEPO**, a second criterion has to be met:

"- Epoetin-δ (Dynepo) has a characteristic band shape ("sharp band") and higher apparent molecular mass than endogenous uEPO/bEPO. Due to the sharper band (albeit a faint smear may also be present in both the Dynepo standard and Dynepo administration samples, representing glycoforms of higher mass), Epoetin-δ can be also differentiated from other rEPOs (-α and -β as well as the biosimilars) (Fig. 2). **To consider an AAF for Epoetin-δ, the band apex line of the ERA in the Sample shall coincide with the corresponding apex line in the Epoetin-δ reference preparation (Fig. 3).**"

There is no Band apex line of ERA in the sample! Only a mere diffusion, that is characteristic of sample overload, is present.

Interestingly, even though the positive control of "U_50% MRPL Spike" sample has a very low amount of rEPO, it showed an apex line of the rEPO in the above figure. Another way to look at this data is to take a second derivative of the density curve. One will see a positive peak at the apex of rEPO. Even then, I will still use another method such as IEF-PAGE to confirm this "positive" finding because it is just too faint to be anything abnormal. Neither the Sample 04962 nor the uEPO negative control has an apex line for Epoetin-δ.

4. Section 3.6 of "A SAMPLE LDOC 7006876 NA22-04962 TD2022LDOC"

"As requested by expert", the sample was re-run with SINGLE BLOT (SB). Page 38 of the report shows gel images of white gel spots on the black background.

The data was analyzed in Section 3.6.2 of the report.

4.1 Page 40, upper image shows the densitometry curve of U_04962 crossing the rEPO apex at **15% relative intensity**.

4.2 Page 40, lower image shows the densitometry curve of U_Blank crossing the rEPO apex at **20% relative intensity**.

4.3 Page 41, upper image shows the densitometry curve of U_100% MRPL Spike crossing the rEPO apex at **30% relative intensity**.

4.4 Page 41, lower image shows the densitometry curve of U_50% MRPL Spike crossing the rEPO apex at **20% relative intensity**.

The only conclusion one can get from this set of data is that U_04962 is negative! Even more negative than the negative control!

5. Section 3.6.3. of the Report. The same data as shown in the Section 4 of my report above, except this version shows black spots on a white background.

5.1 Page 46, upper image shows the densitometry curve of U_04962 crossing the rEPO apex at **15% relative intensity**.

5.2 Page 46, lower image shows the densitometry curve of U_Blank crossing the rEPO apex at **20% relative intensity**.

5.3 Page 47, upper image shows the densitometry curve of U_100% MRPL Spike crossing the rEPO apex at **30% relative intensity**.

5.4 Page 47, lower image shows the densitometry curve of U_50% MRPL Spike crossing the rEPO apex at **20% relative intensity**.

Again, the only conclusion one can get from this set of data is that U_04962 is negative!

6. Section 3.7 of "A SAMPLE LDOC 7006876 NA22-04962 TD2022LDOC"

"As requested by expert", the sample was re-run with DOUBLE BLOT (DB).

In Section 3.7.2. Version 1 of this gel image is shown with white spots and black background. Despite severely over loading the U_04962 sample, the following are the densitometry readings:

6.1 Page 59, lower image shows the densitometry curve of U_04962 crossing the rEPO apex at **15% relative intensity**.

6.2 Page 59, upper image shows the densitometry curve of U_Blank crossing the rEPO apex at **20% relative intensity**.

6.3 Page 58, lower image shows the densitometry curve of U_100% MRPL Spike crossing the rEPO apex at **30% relative intensity**.

6.4 Page 58, upper image shows the densitometry curve of U_50% MRPL Spike crossing the rEPO apex at **25% relative intensity**.

Yet again, the only conclusion one can get from this set of data is that U_04962 is negative!

7. In Section 3.7.3. Version 2 of this gel image is shown with black spots and white background. Despite severely over loading the U_04962 sample, the following are the densitometry readings:

7.1 Page 65, lower image shows the densitometry curve of U_04962 crossing the rEPO apex at **25% relative intensity**.

7.2 Page 65, upper image shows the densitometry curve of U_Blank crossing the rEPO apex at **20% relative intensity**.

7.3 Page 64, lower image shows the densitometry curve of U_100% MRPL Spike crossing the rEPO apex at **35% relative intensity**.

7.4 Page 64, upper image shows the densitometry curve of U_50% MRPL Spike crossing the rEPO apex at **30% relative intensity**.

Yet again, the only conclusion one can get from this set of data is that U_04962 is negative!

8. On Section 3.8, Quality Control

In my opinion, the Epoetin-δ band in the mixed standards showed uncharacteristically wide smear. In my opinion, new standards should have been purchased.

9. Second Opinion

I disagree with the opinion expressed by Mr. Philipp Reihlen and Prof. Dr. Mario Thevis. The clearly described procedure, that must be used to identify an AAF for rEPO in Page 16 of WADA TD, was not followed.

All of the experiments, including re-probing, and re-analyzing with different versions of the data of single and double blotted gels, have consistently shown that U_04962 is negative. If the experts still had doubts, WADA TD clearly states that IEF-PAGE should have been performed. However, this was not done.

10. CP Requirement

As described in the WADA TD, simply repeating ITP may not be enough to be considered a successful CP. It is stated in the TD:

"The "A" CP should differ, where necessary, from the ITP. This difference may apply, for

example (but not limited), to any of the following:

-Application of a different Sample preparation procedure, including use of a different antibody or combination of antibodies for immune purification;

- Use of a different electrophoretic separation technique (IEF-PAGE vs. SDS-PAGE vs. SAR-PAGE, where applicable);

- Performance of double-blotting where single blotting using a biotinylated antibody is applied for the ITP;

- Use of a different detection antibody."

It is noted that further to the above instruction, it was stated that specifically for rEPOs,

"• The CP of rEPOs shall be performed by SAR-PAGE or SDS-PAGE.

[Comment: The Laboratory may decide to apply a second, complementary confirmation PAGE Analytical Method, such as IEF-PAGE, as additional scientific evidence of the presence or absence of rEPO in the Sample (see also Article 3.0).]

• The same PAGE Analytical Method (SAR-PAGE or SDS-PAGE) may be applied for both the ITP and the CP."

My understanding regarding this last point is that if the SAR-PAGE or SDS-PAGE results are so convincing, other methods may not be needed. However, the "Comment" above instructed that when in doubt, a complementary method should be used.

From what was provided in Report A, SAR-PAGE was used for both ITP and AP. Although technically this practice is allowed by the WADA TD, when the results were not conclusive in the eyes of a reasonably trained person, methods such as IEF-PAGE should have been used to confirm the PAAFs.

11. Sample B, on "B SAMPLE LDOC - 7006876 NB23-20136 TD2022LDOC"

The digitized data presented in sample B showed the same numbers as in Sample A, in my opinion, if one cares to estimate the percentage overlap. It only confirmed that the sample was negative for rEPO. The data are summarized as follows:

For Single blotted data:

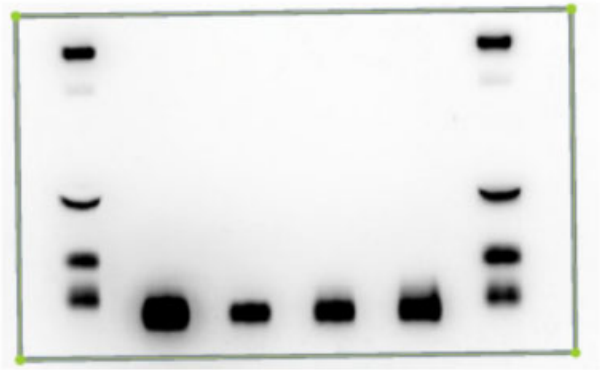
Sample 20136: **15%**, Sample NQC: **12%**, Sample SPK 50%, **25%**, and Sample SPK 100%, **40%. Considering how much the Sample 20136 is over loaded, the only conclusion is that Sample 20136 is negative!**

For Double blotted data:

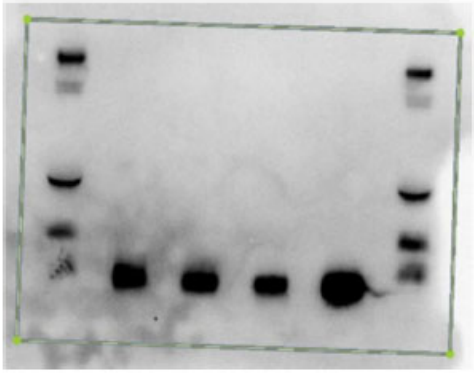
Sample 20136: **17%**, Sample NQC: **15%**, Sample SPK 50%, **37%**, and Sample SPK 100%, **50%.**

Again, considering how much Sample 20136 is over loaded, the only conclusion one can make is that Sample 20136 is negative.

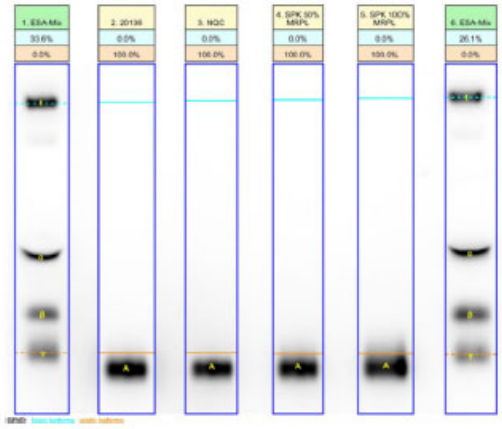
A disturbing observation was made while examining the data presented for Sample B as demonstrated in the following figure:



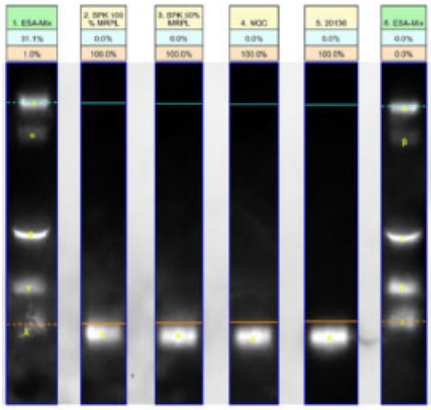
(a)



(c)



(b)



(d)

It is noted that (a) and (b) are from the same SB gel image, and (c) and (d) are from the same DB gel image. First of all, the quality of the gels was worrisome to me. Secondly the spot shapes and sizes for Sample 20136 are alarmingly different from (a) to (b) and from

(c) to (d). Some of the gel images seems to be overly processed.

12. Conclusion

The results as presented for both the A Sample and B Sample showed negative results for rEPO.

The faint diffusions above the uEPO bands for these samples are the results of overloading. The WADA TD stipulates that the maximum amount of urine sample used in a test is 15 ml. All tests performed for this individual used 15 ml of urine sample, which was the maximum allowed. An experienced lab person should have understood that this was the upper limit for affinity purification of urine samples. For many athletes, this amount is too high, causing diffusion of bands from sample overloading.

With the understanding of human physiology, a high intensity of uEPO band in these samples showed that there was no suppression of endogenous EPO. The side effect of rEPO usage was **NOT** present. Further, since repeated SDS-PAGE or SAR-PAGE could not determine the presence of rEPO in the sample, an orthogonal analytical procedure such as IEF-PAGE should have been used to provide additional scientific. No such confirmatory tests were performed.

In summary, neither of this person's urine tests (A Sample or B Sample) showed the presence of recombinant erythropoietin. Only naturally occurring endogenous erythropoietin was observed

The reported finding of rEPO AAF was improper and did not meet the criteria required by the WADA TD.

If you have any questions regarding this report, please feel free to contact me.

Yours sincerely,

David D. Y. Chen
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Associate Member
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Faculty of Medicine
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