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# High myoglobin plasma samples risk being reported as falsely low due to antigen excess – follow up after a 2-year period of using a mitigating procedure

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## Abstract

**Objectives:** Antigen excess (AE) or high dose hook-effect can seriously affect the reported concentration of an analyte, which may impact clinical decisions. This study analyze to what extent the Roche Elecsys® Myoglobin assay is affected by antigen excess and how this can affect the reported myoglobin concentration.

**Methods:** A dilution series experiment was conducted on a patient sample with a very high myoglobin concentration ( $>150,000 \mu\text{g/L}$ ). Based on this, an AE mitigation procedure based on sample dilutions was developed and implemented. A retrospective analysis of reported patient results was performed to assess the potential frequency of samples that could be affected by AE.

**Results:** In the dilution experiment the Roche Elecsys® Myoglobin assay was susceptible to AE, where samples with concentrations  $>115,000 \mu\text{g/L}$  may be reported as  $<3,000 \mu\text{g/L}$ . Dilution series from patient samples ( $n=20$ ) subjected to the AE mitigation procedure showed the same pattern as the dilution experiment. The percentage of samples  $>115,000 \mu\text{g/L}$  increased significantly after the AE mitigation procedure had been implemented. After implementation of the AE mitigation procedure, the maximum myoglobin concentration observed was  $780,000 \mu\text{g/L}$ .

**Conclusions:** If the Roche Elecsys® Myoglobin assay is used according to the manufacturer, extremely high myoglobin

samples risk being reported as only mildly elevated or near normal. This may affect clinical decisions. Thus, we suggest an AE mitigation procedure with automatic dilution of samples where myoglobin concentrations exceed  $500 \mu\text{g/L}$  to mitigate this risk. Roche needs to take further actions to eliminate the consequences of the AE interference.

**Keywords:** high dose hook-effect; mitigating antigen excess; Cobas; Elecsys

## Introduction

Myoglobin is released during skeletal and cardiac muscle damage. It has a direct role in the development of acute kidney injury upon severe skeletal muscle damage (rhabdomyolysis). In severe rhabdomyolysis, defined as myoglobin  $>10,000 \mu\text{g/L}$ , extracorporeal elimination of circulating myoglobin resulted in more frequent recovery of kidney function [1]. Consequently, in a recent consensus statement from the rhabdomyolysis task force, it was suggested that patients with myoglobin  $>10,000 \mu\text{g/L}$  should be considered as a high-risk group for secondary organ damage. In these patients, removal of myoglobin from the blood by for example hemoadsorption should be considered. Furthermore, it was recommended that hemoadsorption should be continued until myoglobin concentrations were consistently  $<5,000 \mu\text{g/L}$  [2]. Thus, accurate monitoring of plasma myoglobin levels is of great importance for guiding treatment, to minimize the risk of sustained renal damage.

The Roche Elecsys® Myoglobin is a sandwich-type assay that have a measuring range in undiluted samples of up to  $3,000 \mu\text{g/L}$  [3, 4]. During routine validation when transferring the assay from a Cobas® e602 to a Cobas® e801 instrument, samples analyzed on the Cobas e602® were used to determine the method commutability. One of the samples had a concentration of  $158,000 \mu\text{g/L}$  on the e602, but the result was only  $2,500 \mu\text{g/L}$  on the e801 instrument. On the e602 instrument the initial concentration of the undiluted sample was  $>3,000 \mu\text{g/L}$  resulting in further dilutions, whereas the  $2,500 \mu\text{g/L}$  result on the e801 did not motivate

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any further automatic dilutions. When dilutions were manually ordered on the e801, the final concentration was determined to 147,000 µg/L. High dose hook-effect or more specific antigen excess (AE) interference was suspected.

Antigen excess (AE) is a well-known phenomenon hampering some immunoassays and is the depletion of signal with higher concentration (instead of an expected increased signal) when presented with very high concentrations of analyte antigen [5]. This is an interference observed mostly in Sandwich based immunoassays and turbidimetric methods, where both the capture and detection antibodies are added to the sample without an intermediate wash step (one-step). The interference is caused by a saturation of both capture and detection antibodies and results in reduced formation of Sandwich complexes, with a decrease in analytical signal as a result. AE is a potential problem when the expected analyte concentration range is wide. The Roche Elecsys® assay is a one-step Sandwich based assay [3, 4].

Previous case reports indicated that the Roche Elecsys® Myoglobin assay may give false low myoglobin concentrations in plasma [6] and urine [7]. Furthermore, there are also earlier reports of AE problem [8, 9] with other myoglobin assays, indicating a common problem with myoglobin assays in general due to the potential wide concentration range in patient samples. However, there are no data on the prevalence of patient samples with very high myoglobin concentrations that could risk getting a false result due to AE. In the current study, we aim to determine above which myoglobin concentration a patient sample result might be adversely affected by AE. Furthermore, an AE mitigation procedure was implemented and the prevalence of samples identified as affected by AE are presented. Finally, data from two example patient cases are shown, illustrating the potential severe consequences of unidentified AE in myoglobin measurements.

## Materials and methods

### Determination of myoglobin in plasma samples

During the period 2016 to 2022 myoglobin was analyzed on a Cobas® e602 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using the Elecsys® Myoglobin assay (Catalog # 12178214122)[4]. With start in January 2023, myoglobin was analyzed on a Cobas® e801 instrument (Roche Diagnostics GmbH, Mannheim, Germany) using the Elecsys® Myoglobin assay (Catalog # 07027583190)[3]. The assays are essentially the same (same antibodies and incubation and detection principles), but the sample volume

has been reduced in the later version to fit the new platform.

### Assessment of antigen excess

To investigate the assay sensitivity to AE, a sample with a high myoglobin concentration (originally assessed to 158,000 µg/L on a e602 instrument not part of this study) was used to prepare dilution series. The dilution series was analyzed on two instruments, a Cobas® e801 and a Cobas® e602 at different occasions and with different reagent lots. The series consisted of at least 6 different diluted aliquots that were analyzed undiluted, after 1:10 automatic dilution, and when needed with a 1:100 manually ordered dilution. A new dilution series was prepared for each occasion. The “true” concentration of the undiluted sample was assessed based on the least diluted sample giving a measurable concentration without any additional dilution. To determine the onset of the hook effect and at what concentration the AE would give falsely low results, the analyte signal in different aliquots and subsequent dilutions was compared to the calculated analyte concentration. Calculation of analyte concentrations at different dilutions was based on the assessed true concentration of the sample and the applied dilution factor.

This procedure was then performed on the results of all patient samples from the period 2023–2024 with myoglobin concentrations >100,000 µg/L (n=20). During this period, all myoglobin results >500 µg/L were routinely diluted. Multiple reagent and calibrator lots were used during this period.

### Antigen excess mitigating procedure

According to the assay package insert, the upper limit of the measuring range without dilution is 3,000 µg/L. Concentrations above 3,000 µg/L are automatically diluted 1:10. Instead of using 3,000 µg/L as upper limit we applied an upper measuring limit of 500 µg/L, with a subsequent 1:10 dilution of samples above 500 µg/L. We chose 500 µg/L as a conservative limit, based on the results of the dilution experiments and taking into consideration the uncertainty of what the actual highest possible concentration of myoglobin could be. Factored in was also the practical aspect of limiting the number of samples that need to be diluted.

### Patient result review

All reported plasma myoglobin results for the period of 2016-06-01 – 2024-12-31 were reviewed. Only patient results were

included in the final analysis, i.e. myoglobin results of study subjects and technical tests were excluded.

The fraction of samples  $>30,000 \mu\text{g/L}$  and  $>115,000 \mu\text{g/L}$  were calculated for time periods 2016-06-01 - 2022-12-31, and 2023-01-01 - 2024-12-31, respectively. For time period 2016-06-01 - 2022-12-31, myoglobin results were obtained on the original Cobas® e602 without measures to mitigate AE.

Since 2023-01-01 steps to detect AE have been in place in the laboratory. For the time period 2023-01-01 to 2024-12-31 the proportion of samples analyzed that would have been reported with a false low concentration (if not the lower upper measuring range had been applied), was calculated in addition to proportion of samples above threshold levels. Two case examples (Patient A and B) from this time period were selected.

## Statistical analysis

Differences in proportions of samples were assessed using Fisher's exact test.  $p < 0.05$  were considered significant. All statistical analyses were carried out using R V4.4.1 (R Core Team (2024), <https://www.R-project.org/>).

## Results

The onset of high-dose hook effect and the concentration at which AE affects the reported myoglobin result were tested on two different instruments, a Cobas® e801 and a Cobas® e602, respectively. The myoglobin signal in respect to concentration in the two dilution series are presented in Figure 1. Both instruments show obvious signs of AE, where concentrations above approximately  $30,000 \mu\text{g/L}$  have reduced signals with increasing concentrations. However, AE becomes a problem only when the signal drops below the signal of the upper limit of the measuring range (slightly different on the two instruments), and in these experiments this corresponds to approximately  $115,000 \mu\text{g/L}$  and  $135,000 \mu\text{g/L}$ , on the Cobas® e801 and the Cobas® e602, respectively.

Response signal as a function of concentration for diluted samples with myoglobin  $>100,000 \mu\text{g/L}$  ( $n=20$ ) from the last 2-year period is presented in Figure 2. The results show the same pattern as in the structured dilution experiment. However, there are clear indications that individual samples may deviate from the general pattern (Figure 2, circled results).

The fraction of myoglobin results  $>30,000 \mu\text{g/L}$  and  $>115,000 \mu\text{g/L}$  in samples analyzed for myoglobin between June 2016 to December 2022 are presented in Table 1.

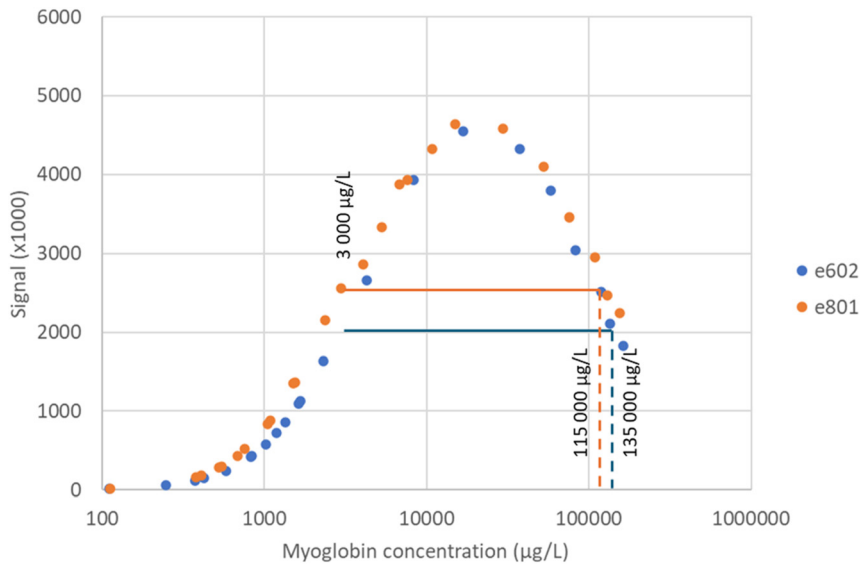
There were in total 32 samples with reported myoglobin concentrations  $>115,000 \mu\text{g/L}$  (0.14 % of the total number of myoglobin samples). Over the same period the percentage of samples  $>115,000 \mu\text{g/L}$  from the Intensive Care Unit (ICU) or Emergency Department (ED) of the hospital was 0.45 % (Table 1). Due to the variable nature of the AE it is highly likely that these numbers are underestimated. The highest concentration recorded during this period was  $240,000 \mu\text{g/L}$ .

The same retrospective analysis was performed for the period 2023 to 2024, during which a 1:10 dilution was applied to all samples with myoglobin  $>500 \mu\text{g/L}$  (Table 1). Over this period the number of samples with concentration  $>115,000 \mu\text{g/L}$  was 22 and the prevalence was approximately double compared to the earlier time period, 0.31 % and 0.88 %, of all samples and the ICU/ED samples, respectively. The increase in proportion of samples  $>115,000 \mu\text{g/L}$  was statistically significant ( $p < 0.01$ ). Seven samples had received falsely low myoglobin concentrations if antigen excess mitigating procedures had not been in place. The highest recorded concentration was  $780,000 \mu\text{g/L}$ .

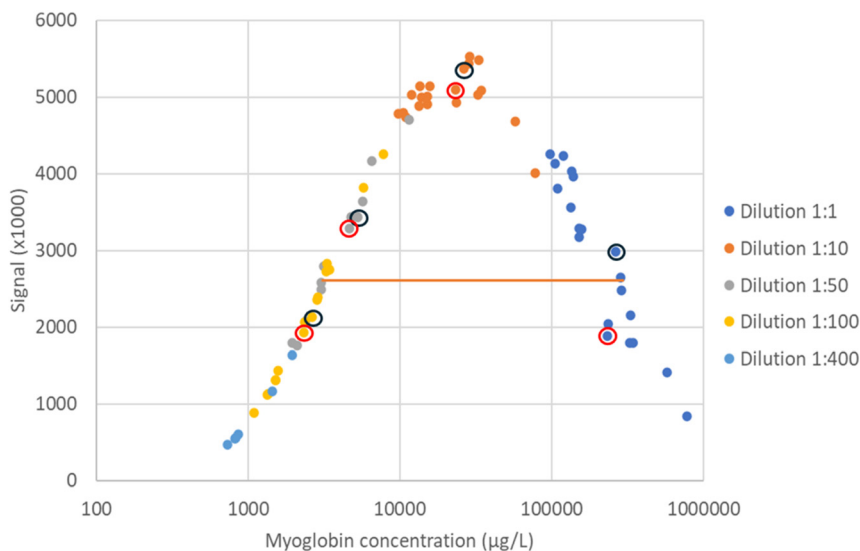
To illustrate the potential clinical impact of the antigen excess, the myoglobin concentrations in samples of two example patients collected over consecutive days are shown in Figure 3. Myoglobin was analyzed on admission and followed over a period of several days. In addition to the reported concentrations determined when antigen excess mitigating measures were applied, the concentrations that would have been reported if no AE mitigating procedure had been applied (i.e. using the measuring interval according to manufacturer), are shown. Finally, the estimated concentrations in a “worst case scenario” are visualized. This scenario represents concentrations assuming conditions similar to those at the time when the dilution experiment generating an antigen excess cut off of  $115,000 \mu\text{g/L}$  was performed.

## Discussion

Up until recently (June 2024) the Roche package insert regarding both the e602 and e801 platform only stated, “There is no high-dose hook effect at myoglobin concentrations up to  $30,000 \text{ ng/mL}$  [ $\mu\text{g/L}$ ]”. This statement alone was dubious, since it was unclear if this meant that Roche had only tested up to  $30,000 \mu\text{g/L}$  and not observed any hook effect or if a hook effect had been observed in samples with  $>30,000 \mu\text{g/L}$ . In an updated version of the insert (June 2024) it has been added “Above this concentration, the hook effect can be observed. In case of an unexpected low result, the sample should be diluted 1:10 (...) and retested.”. However, it is still unclear if Roche has data indicating that there



**Figure 1:** Response signal of different dilutions of a sample with myoglobin concentration of approx. 150,000 µg/L. The experiment was performed on two different instruments (blue e602 and orange e801). Horizontal line indicate signal at 3,000 µg/L (upper limit of measuring range) for both instruments at the time of the experiment. Vertical lines indicate AE cut off.



**Figure 2:** Response signal in relation to myoglobin concentration (logarithmic scale) in different dilutions of patient samples (n=20). Orange line represents average signal of the upper measuring limit (3,000 µg/L). Dilutions of two samples from the same patient (Patient A) are illustrated by circles. The reported concentration of the sample with black circles was 260,000 µg/L and the sample with red circles was 230,000 µg/L.

is a risk of falsely low results already at concentrations as low as >30,000 µg/L or that this is the onset of the “hook” and that AE is expected to create falsely low results only at substantially higher concentrations. Our data suggests that AE can give falsely low results in samples with myoglobin concentrations at least as low as 115,000 µg/L under certain conditions (Figure 1).

Due to the wide expected concentration range all myoglobin immunoassays can in theory be affected by AE to some extent based on the assay methodology. It should be mentioned that even though the information given by Roche is unclear it is still, at least in the updated insert, alerting the user of a potential issue. Notably, a different manufacturer (Abbott) has no comment regarding any potential risk of AE in their myoglobin assay package inserts. If this is because

they have validated that the assay do not suffer from AE or if they are unaware of the issue is not clear. Siemens on the other hand clearly states in their assay package insert that concentrations above 200,000 µg/L will give falsely low results. However, it is unclear how Roche and Siemens expect the customer to identify the samples that are giving “unexpected low results”.

Our results show that the AE varies. In the dilution experiments on the same patient sample performed on the two different models of Cobas® instruments (e602 and e801), both platforms were clearly suffering from AE (Figure 1), but to a different degree. This indicate that reagent lots and/or individual instruments (maintenance status or platforms) play a role in the variability of the AE. This is supported by historical data from the original Cobas® e602 instrument that

**Table 1:** Number of myoglobin samples analyzed at Sahlgrenska University Hospital during different time periods. Proportions of samples above threshold levels were compared between time periods using Fisher’s exact test.

Hospital unit	Number of samples			
	Total	>30,000 µg/L (% of total)	>115,000 µg/L (% of total)	Samples re- ported wrong if not diluted
2016-06-01 – 2022-12-31. No AE mitigation procedure				
All	22,905	247 (1.1 %)	32 (0.14 %)	?
ICU and ED	6,183	179 (1.3 %)	28 (0.45 %)	?
2023-01-01 – 2024-12-31. AE mitigation procedure implemented				
All	7,118	100 (1.4 % <sup>a</sup> )	22 (0.31 % <sup>b</sup> )	7 (0.098 %)
ICU and ED	2034	80 (3.9 % <sup>a</sup> )	18 (0.88 % <sup>a</sup> )	7 (0.34 %)

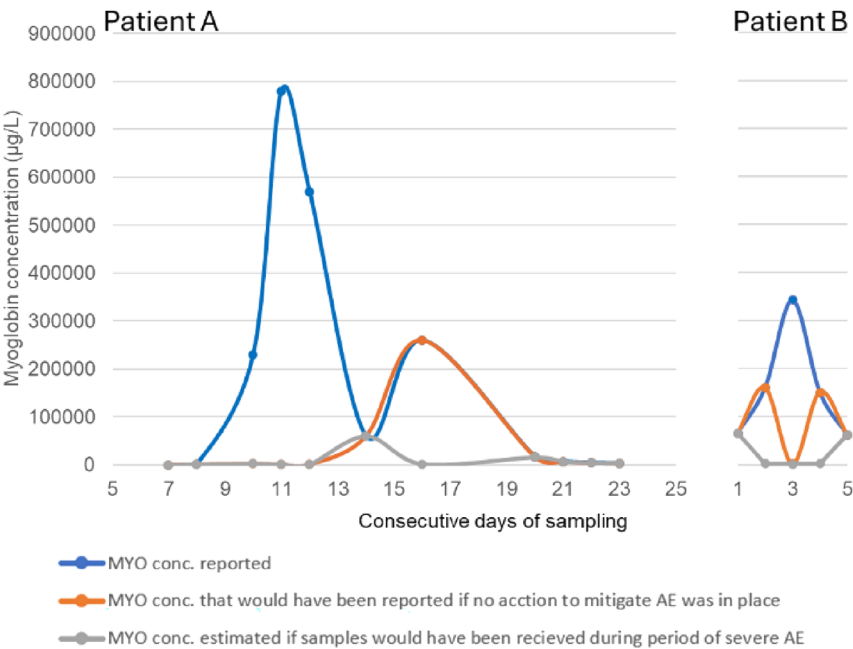
<sup>a</sup>p<0.05, <sup>b</sup>p<0.01.

have (at least in periods) been less susceptible to AE with reported myoglobin results up to 240,000 µg/L. Also, since the implementation of the new Cobas® e801 instrument, samples with concentrations up to 290,000 µg/L have been >3,000 µg/L as undiluted, thus, demonstrating that also the e801 instrument fluctuates in sensitivity for AE. This would indicate that reagent lots rather than instrument platforms are of more importance. However, based on the data from the last 2-year period when an AE mitigating

routine has been implemented, there is no clear correlation with antigen excess and specific reagent lots (data not shown). On the other hand, our results indicate that the degree of AE at least partly may be dependent on the plasma matrix, as a clear variation in AE sensitivity in different samples from the same individual (Figure 2, circled data) was noted. It can be speculated that this may be caused by differences in for example treatments or disease states. Further studies are, however, needed to elucidate this.

An additional factor that could contribute to the observed AE variation is the manufacturers calibration method, i.e. applying two calibrators to adjust a general assay specific master curve. The high adjustment calibrator has the concentration of approx. 1,200 µg/L, thus quite far from the manufacturers upper measuring range. The shape of the master curve, as well as how the adjustment of the master curve at 1,200 µg/L adjust for actual changes in the response function towards 3,000 µg/L, are not provided by the manufacturer. The lack of transparency makes it hard to evaluate how much of the variability in calculated results from diluted samples (interpreted as AE variability) that can be attributed to different calibrations, but this could be a contributing factor.

To mitigate the observed risk of AE, a procedure was implemented where samples were automatically diluted >500 µg/L. In the present study, the maximum measured concentration was 780,000 µg/L after dilution. For this sample, the initially measured concentration prior to dilution was 1,120 µg/L. Thus, the >500 µg/L limit may seem conservative, but due to the observed variable and





unpredictable nature of sensitivity to AE, we considered this an acceptable precaution. Consequently, more than 25 % of all samples requires dilution instead of 6–7% using measuring range according to package insert.

If the AE mitigation procedure described in the present study is not used, there is a risk of not identifying the very high myoglobin samples. The fraction of samples with concentrations higher than 115,000 µg/L increased significantly, approximately 2-fold, after adapting the lower upper limit of the measuring range. Notably, after applying the AE mitigation procedure, we observed a plasma sample with a myoglobin concentration as high as 780,000 µg/L compared to the previous highest sample of 240,000 µg/L. Also, after introducing the AE mitigating procedure, there were 7 samples that would have been reported as <3,000 µg/L, where the actual concentration was >200,000 µg/L.

The number of samples exceeding 115,000 µg/L, almost 1 % from the ICU or ED units, cannot be regarded as neglectable when considering the potential clinical implications. Clinical decisions may be seriously misled if extremely high results are reported as near normal due to AE. In the present study, results from two patient cases are presented (Figure 3). In Patient A, the dramatic release of myoglobin into circulation starting at approximately day 9 would have gone completely unnoticed without actions to mitigate AE. Furthermore, if analyses had been carried out during a period of severe AE, the increase in myoglobin concentration would have gone almost completely unnoticed for the whole course of events. In Patient B, a 6-fold increase in the concentrations during the first two days followed by a decline over the next 2 days going back to the concentration at admission was observed. If no actions had been taken to mitigate AE, the observed myoglobin concentration over time would have taken the appearance of a sinus curve shape. If the analyses had been carried out during a period of severe AE, the concentration dynamics would have been completely inverted compared to the actual dynamic.

Although we did not have access to clinical data for the included patients, it is reasonable to assume that these two example patients may have received treatment with extracorporeal elimination of myoglobin due to the very high levels observed. If, on the other hand, concentration dynamics would have followed the AE susceptible pattern, treatment would perhaps not have been initiated at all or terminated prematurely, resulting in increased risk of permanent renal damage.

In conclusion, if the Roche® Elecsys myoglobin assay is performed according to package insert it is unsuitable for the intended use because of the risk of reporting falsely low

myoglobin concentrations due to AE. The assay sensitivity to AE depends on many factors making it hard to routinely assess. In order to mitigate the risk of AE, we suggest truncating the measuring range of the undiluted sample from <3,000 µg/L to <500 µg/L. When using this routine, we have recorded myoglobin concentrations up to 780,000 µg/L. Antigen excess may result in severely underestimated very high myoglobin levels, possibly affecting treatment decisions. If the assay is performed according to manufacturer instruction, almost 1 % of the total number of myoglobin samples from ED and ICU departments, risk being reported as falsely low.

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**Author contributions:** All authors have accepted responsibility for the entire content of this manuscript and approved its submission.

**Use of Large Language Models, AI and Machine Learning**

**Tools:** None declared.

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