


TECHNICAL ARTICLE

3R's applied to *in vivo* biological activity of recombinant human erythropoietin assay

Ana Paula Arevalo^{1#} , Tatiana Basika^{1,2,3#} , Sergio Ancheta¹, Karen Perelmutter², Alejandro Ricciardi[†], Mariela Bollati-Fogolin² , Martina Crispo^{1*} 

¹Institut Pasteur de Montevideo, Laboratory Animals Biotechnology Unit, Montevideo, Uruguay.

²Institut Pasteur de Montevideo, Cell Biology Unit, Montevideo, Uruguay.

³Institut Pasteur de Montevideo, Pasteur+INIA Joint Unit, Montevideo, Uruguay.

[#]These authors contributed equally to this work.

[†]Alejandro Ricciardi passed away on March 28, 2019

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Abstract

An enhanced protocol for the evaluation of *in vivo* biological activity of recombinant human erythropoietin (rhEPO) assay in mice is described, following European Pharmacopoeia (*Ph. Eur.*) version 10.0 guideline and optimized by applying the principles of Replacement, Reduction and Refinement (3Rs) in animal experimentation. In Laboratory Animal Science, one of the main principles is to optimize the experimental protocols in order to achieve the best results with the lowest number of animals and refine the procedures to avoid unnecessary suffering. The main objective of this protocol is to comply with international guidelines for rhEPO evaluation, applying refinement on procedures and reduction in animal use. Some of the features included in this protocol are the increase in number of rhEPO batches tested simultaneously against an international standard, leading to a substantial reduction in the number of animal's used, and refinement on animal handling techniques for subcutaneous drug administration and blood withdrawal. The implemented improvements were validated by reticulocyte estimation to ensure compliance with international criteria established for this trial and institutional quality management system.

Keywords: erythropoietin, biological activity, mice, 3R's, European *Pharmacopeia*.

INTRODUCTION

Erythropoietin (EPO) is a glycoprotein hormone that functions as the primary regulator of erythropoiesis rate. The recombinant human erythropoietin consists of 165 amino acid polypeptide chains heavily glycosylated, with a molecular mass of 30-34 kilodaltons¹. Therapeutically, rhEPO has strong implication in humans for treating anemia in chronic kidney diseases, as well as in the treatment of the anemia of cancer produced due to kidney damage or direct toxic effects on red bone marrow².

In the pharma industry, biological activity determination for batch release, stability or changes in the manufacturing process is a mandatory requirement for quality control of this therapeutic glycoprotein. In order to guarantee high quality and therapeutic efficacy, a specific biological response between produced batches versus an international standard reference is always compared³. The approved reference method to define rhEPO potency in laboratories around the world is based on the measurement of the *in vivo* stimulation of reticulocyte production in normo or polycythaemic mice after the administration of different doses of rhEPO⁴.

*Corresponding author: crispo@pasteur.edu.uy

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Despite several efforts being made to develop an alternative method to the *in vivo* assay^{5,6}, the use of laboratory animals is still the gold standard. Meanwhile, minimizing the number of animals used per experiment and refining the appropriate techniques is mandatory, always following an appropriately designed assay to ensure robustness and reproducibility of the method. Refinement of animal procedures such as housing, husbandry and experimental protocols leads to less animal distress and thus higher quality and more robust data. In that sense, any technical improvement must be explored and included. Focusing on promoting animal welfare and the 3R's principles, and while the method requested by the *Ph. Eur.* and the industry still demands the use of laboratory animals, this protocol describes some refinements in animal restraint, drug administration and blood sampling, as well as a substantial reduction in the number of used mice in the *in vivo* assay, aiming to follow and improve international guidelines when using laboratory animals.

The proposed experimental protocol was approved by the Institutional Animal Ethics Committee (CEUA IPMon 003-18), in accordance with Uruguay National Law 18.611 and international animal care guidelines⁷. Certified trained members carried out all procedures involving animals.

MATERIALS AND METHODS

Animal distribution and acclimatization

1. Allow mice to acclimatize in individually ventilated racks (IVC) one week prior to experimental procedure. In our conditions, mice are housed in IVC (Alesco, SP, Brazil) with wood chips bedding (SAFE, France) undergoing weekly cage changing. The housing environmental conditions during the protocol are as follows: 20 ± 1 °C temperature, 30–70% relative humidity, and a light/dark cycle of 14/10 h. Autoclaved standard mice diet (5K67, Labdiet, PMI Nutrition, MN, USA) and filtered and autoclaved water are administered *ad libitum*. Mice are specific pathogen free certified quarterly by IDEXX (USA) under FELASA standards.
2. Randomly distribute females in groups of six per cage. Prior to housing into the cages, identify each animal individually by ear punching (Figure 1A).
3. Identify each cage properly (Figure 1B).

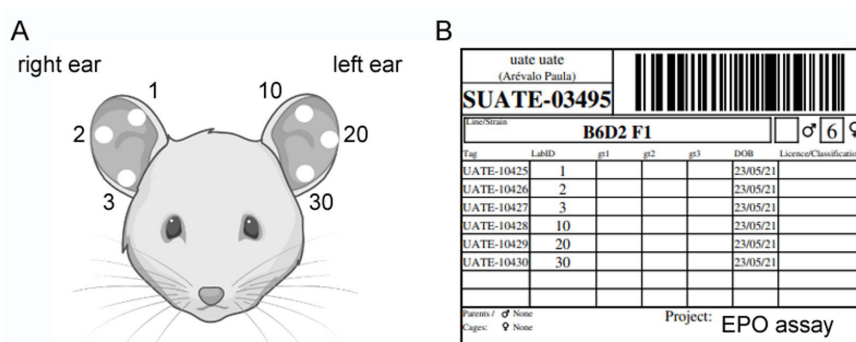


Figure 1. Animal identification code (A) and example of a cage label (B). The placement of the notch in the ear indicates the number that identifies the animal. Right ear: 1 on the top, 2 in the middle and 3 in the bottom. Left ear: 10 on top, 20 in the middle and 30 in the bottom. These numbers are used to identify each mouse and its corresponding tag on the cage label.

Subcutaneous compound administration

1. Receive samples ready to inject 30 minutes before the injection to allow reaching room temperature: three doses for each of the three batches tested (80; 40; 20 IU/mL), three doses of reference solution (80; 40; 20 IU/mL), and vehicle as control. Dilute each batch (#1, 2 and 3) and the reference

solution as follow: for Test solution (a) dilute the concentrated test solution in PBS to obtain a concentration of 80 IU/mL; for Test solution (b) mix equal volumes of Test solution (a) and PBS to obtain 40 IU/mL; for Test solution (c) mix equal volumes of Test solution (b) and PBS to obtain 20 IU/mL. Apply the same procedures to Reference solution (a): 80 IU/mL, Reference solution (b): 40 IU/mL and Reference solution (c): 20 IU/mL. Use PBS as Control solution.

2. Load 1 mL syringe with 0.5 mL of the appropriate solution avoiding bubble formation. Insert a 27-gauge needle in the syringe.
3. Place the mouse above the stainless-steel bar lid holding the tail back gently, the animal will grip to the bars with its front paws. Insert the needle with the bezel upwards, under the animal skin lining the upper thigh and lower part of the back and abdomen. Check that the needle is inserted subcutaneously (Figure 2A).
4. Inject the solution into the site; confirm a small bleb under the subcutaneous space (formation of fluid pocket under the skin). Remove the needle and put the mouse in a new cage containing the appropriate identification.

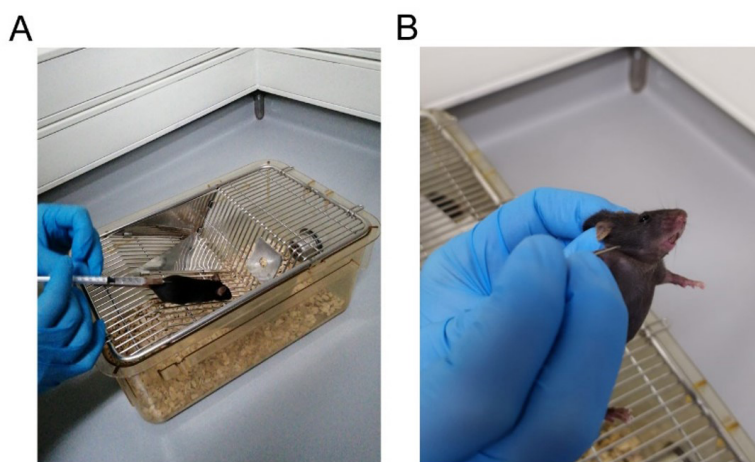


Figure 2. Subcutaneous injection (A) and submandibular blood extraction/bleeding (B). Representative images of mouse restraint with minimal stress and maximum outcomes.

Anticoagulant preparation

1. Prepare anticoagulant solution for blood collection, diluting 360 μL of ethylenedinitrilo-tetraacetic acid (EDTA.Na_3) in 840 μL PBS containing 2 mM EDTA.
2. Dispense 10 μL of anticoagulant solution into 0.5 mL safe-lock plastic microtubes.
3. Label each tube according to the sample to be collected.
4. Place the microtubes in a rack near the animal cages and prepare the other materials (needles, sterile paper towels, vortex, etc.).

Blood collection

1. Ninety-six hours after injection, collect blood from the mice. Restrain each mouse with the non-dominant hand by grasping the loose skin over the shoulders and behind the ears; the skin over the mandible should be tight (Figure 2B).
2. Puncture the submandibular sinus with a 25-gauge needle slightly behind the mandible, in front of the ear canal. Use enough force to create a small stick hole, so that drops of blood exude from the point of penetration. Discard the needle in a sharp container.

3. Collect 70-100 μL of blood into the corresponding safe-lock plastic tube with anticoagulant. Immediately stop the bleeding by applying soft pressure with sterile paper towels at the puncture site. Put the mouse back into its cage and monitor the animal for a minute.
4. Mix the blood by vortexing at low speed. Refrigerate until use. Maximum time of storage is 2-3 hours post-collection.
5. As soon as the blood collection is finished euthanize animals using an automatic CO_2 chamber with a displacement rate of 30 to 70% of the chamber volume with CO_2 per minute⁸.

Sample processing for reticulocyte determination

1. Prepare the number of 96-well flat-bottom plates needed according to the total number of blood samples to be stained with 180 μL of PBS/2mM EDTA per well (this plate is referred to as "plate A").
2. Prepare the amount of 2.1 mL 96-well plates needed according to the number of samples with 1470 μL of PBS/2mM EDTA (this plate is called "plate B").
3. Prepare thiazole orange (THO) working solution composed of 30 mL PBS/2 mM EDTA and 6 μL stock solution of THO (1 mg/mL) in a 50 mL Falcon-type tube protected from light. This solution is stable for a period of 4 hours.
4. Homogenize blood sample with a gentle vortex and load 20 μL of blood, dry the tip with absorbent paper (dry point method) and discharge into the corresponding well of plate A. Homogenize 5 times or until no traces of blood are observed on the tip.
5. Load 20 μL of blood from each sample in an eppendorf-type tube to prepare a pool for unstained controls (16 samples per pool). Take 20 μL of blood from each pool made and place it in the corresponding well using the dry point method. Homogenize pipetting up and down 5 times and transfer 30 μL from plate A to plate B.
6. In a new 2.1 mL 96-well plate add 200 μL of THO working solution except in those wells for unstained control. Add 200 μL PBS/EDTA in tubes for unstained controls.
7. Homogenize diluted samples from plate B and transfer 200 μL to plate containing THO. Incubate at room temperature for 30 min⁹.

Flow cytometry determination of reticulocytes

1. Open the flow cytometer software and start the cytometer. Before samples are acquired, verify the analytical performance of the flow cytometer according to manufacturer instructions. Analyze samples exciting THO with a 488 nm laser and detecting emission using 530/30 bandpass filters.
2. Create two bivariate plots, FSC-A (forward scatter channel) vs SSC-A (side scatter channel); and THO channel vs FSC-A (Figure 3-A). Select the scale of the mentioned parameters in log scale. Acquire one unstained sample and establish a gate on the FSC-A vs SSC-A for the red blood cells (RBC) based on morphological aspect. Set the threshold to eliminate debris and noise from cell samples. Apply these settings for all samples in the experiment.
3. Apply RBC gate onto the THO vs FSC-A dot plot. Define the THO⁺ cells gate based on unstained red blood cells acquisition. Adjust flow rate in order to acquire all samples at a rate between 800 and 2000 events/second.
4. For each sample to be collected, acquire 10,000 events gated on a FSC versus SSC. Report results as the percentage of reticulocytes on total red blood cells obtained from the THO vs FSC-A dot plot.
5. Calculate the potency by the statistical method for a parallel line assay

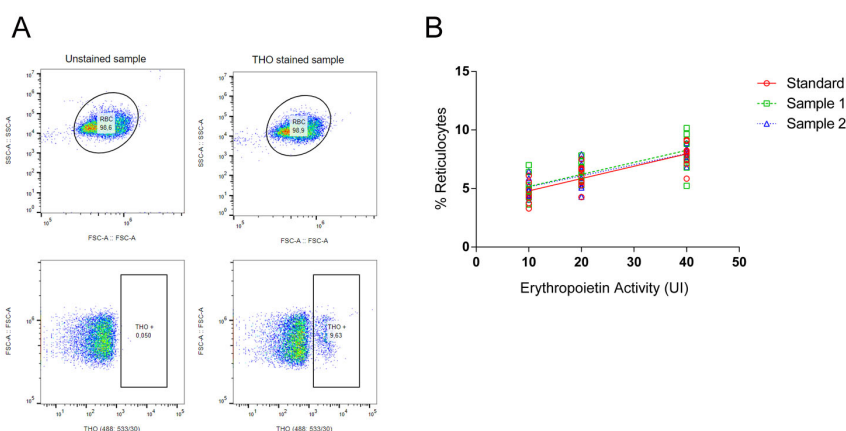


Figure 3. Representative results of flow cytometry reticulocyte determination (A) and dose response curves (B). Red Blood Cells (RBC) gate was defined based on morphological aspect in FSC-A vs. SSC-A plot (A), and THO⁺ gate was defined in THO channel (488:533/30) vs. FSC-A for an unstained (left) or stained sample (right). Numbers in the graphs indicate the frequency of events falling within each gate. Dose-response curve obtained with the European *Pharmacopeia* Biological Reference Preparation of erythropoietin (standard) and 2 different tested batches (B).

NOTES

1. For this protocol, specific pathogen free (under FELASA standards) normocythaemic hybrid B6D2F1/J female mice (8-weeks old) are used as specified by *Ph. Eur.* 10.0. Males can be used also, although dominance and fighting can pose a problem during the assay and interfere with the results.
2. Administer a total of nine mice within a given dose, and inject three animals with the control solution. The technician injecting animals should be blinded to the dose and type of sample (reference or test solution) administered. For each protocol to validate three test solution against the standard, a total of 108 mice are used (Table 1).
3. The technician performing blood processing and flow cytometry analysis should be blinded to the dose and type of sample (reference or test solution) analyzed.

Table 1. Animals used in the evaluation of one or more batches of rh-EPO test solution

N° batches tested	N° animals used		Animal reduction/ year (based on weekly testing)	Acceptance rate*** (accepted/ total batch)
	<i>Ph. Eur.</i> *	Improved protocol**		
1	48	Not performed	NA	NA
2	96	81	720	91% (50/55)
3	144	108	1728	81% (58/72)

* 8 animals per dilution. ** 9 animals per dilution; *** Acceptance criteria: linearity, parallelism and regression tests of samples against reference should be accomplished. Potency no less than 80% and no more than 125% of the declared potency. Confidence interval no less than 64% and no more than 156% of the declared potency. *Ph. Eur.*: European Pharmacopeia; NA: Not Applied.

4. Perform flow cytometry acquisition in a period no longer than 1 hour after conducting THO staining.
5. Criteria of acceptance:

The mean of the percentage of reticulocytes measured for the animals treated with the dilution (c) of the reference solution must be greater than or equal to the mean plus one standard deviation of the percentage of reticulocytes calculated for the animals in the control group.

For the standard curve, there must be a difference of at least 1% in the mean percentage of reticulocytes measured for animals treated with dilution (a) vs those treated with dilution (c).

6. Specifications:

The estimated potency should not be less than 80% nor more than 125% of the declared potency for the tested sample.

The confidence limit of error of the estimated potency ($P=0.95$) must not be less than 64% nor greater than 156% of the declared potency.

REPRESENTATIVE RESULTS

In this report a refined protocol to validate rhEPO *in vivo* biological activity is described. The original protocol is published in *Ph. Eur.* 10.0⁴, and is the gold standard required by the pharma industry to release rhEPO batches to the market. Nevertheless, animal methods in *Ph. Eur.* are not described in detail and the number of animals used can be significantly reduced with acceptable results.

Correct handling of the animal during substance administration and blood extraction is critical to reduce the stress of the mouse. Figure 2 demonstrates how the animals are perfectly restrained and bleed for the procedures with minimum discomfort. It is of utmost relevance to perform these procedures with the highest wellbeing standards for the animals, procedures that are not described at all in the *Ph. Eur.*

Table 1 shows results for animal reduction when two or three batches are analyzed together. Our laboratory does not perform a single batch test any more to comply with animal reduction. When analyzing 2 or 3 batches simultaneously, 720 or 1728 mice per year can be reduced, respectively, based on a weekly testing and with a high acceptance rate. A global reduction of 15% or 25% is observed for two or three batches, respectively. Considering that the *in vivo* rhEPO assay can be performed weekly throughout several years in mice facilities, the animal reduction is largely satisfactory. These results comply with the 3Rs principles in animal experimentation and thus encourage the application of this protocol to perform the rhEPO assay.

The representative results of THO percentage determination by flow cytometry and representative dose-response curves are shown in Figure 3. In figure 3A FSC-A vs. SSC-A and FSC-A vs. THO channel plots for an unstained and stained sample are shown.

DISCUSSION

In this protocol we describe a refined version for the rhEPO *in vivo* biological activity assay, aiming to respect animal welfare and 3R's. The protocol presented here is based on the *Ph. Eur.* monography, with substantial improvements in animal handling during subcutaneous administration and blood extraction. During the solution administration, animal restriction is minimized, reducing anxiety and stress^{10,11}, thus contributing to increase the wellbeing of the animal in terms of stress due to restrain during the experiment. Specific technical training and technicians working in this field during more than 15 years, animal strain and species behavior knowledge¹² makes the procedure safe for the personnel and the animals, complying with international animal welfare guidelines⁷.

The *Ph. Eur.* does not recommend any specific method for blood extraction. However, the vast majority of publications regarding this assay perform blood extraction via retrobulbar bleeding¹³⁻¹⁶. In a recent study, retro orbital sinus puncture resulted in reduced activity and increased anxiety in mice, whereas submandibular bleeding had the lower adverse effects on welfare parameters¹⁷. Gjendal *et al.* compared three different bleeding techniques (sublingual, facial and retro-bulbar) in mice, reporting that none proved to be superior to the others in terms of nest building activity, level of fecal corticosterone metabolites, body weight, fur status, macroscopic changes or variation in blood quality¹⁸. Nevertheless, it has been reported that retro-bulbar technique leads to severe tissue damage when compared to sublingual vein in mice¹⁹. Additionally, submandibular blood collection has been largely refined²⁰ and is recommended over other methods by international scientific organizations such as NC3R²¹.

Furthermore, an increase in the number of batches tested against one international reference standard, rigorously performed and properly validated, decreased the total number of animals required in comparison to the standard method as detailed in Table 1. This again comply with the 3Rs principles in animal experimentation.

To test the biological activity of recombinant erythropoietin, few *in vitro* methods have been published with the aim of simplifying the assay and overcome ethical issues associated with animal experimentation. The evaluation of proliferative stimulation of an erythroid cell line called UT-7 cell bioassay or reporter UT-7 cell bioassay^{6,22}, or the development of an *in vitro* TF-1 cell proliferation assay for the determination of the content of sialic acids⁵ were reported with variable success. Nevertheless, despite all the efforts to apply alternative methods *in vitro*, the gold standard assay for *Ph. Eur.* to determine the biological activity of rhEPO is still the *in vivo* test⁴. Until *in vitro* tests and alternative methods are properly validated, thousands of mice are still being used every year worldwide in pharmaceutical and research fields to ensure safety, efficacy and quality of products for human and animal therapies²³. A reduction in the number of animals used and refinement in the experimental procedures can be achieved without any kind of technical limitation, supporting the international commitment regarding animal welfare standards and 3Rs principles. This protocol strongly encourages laboratories performing this test to use the methodology described here in mice.

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