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# Technical improvement for the detection of erythropoietins in biological matrices: An update

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### Abstract

The initial version of SNAP i.d. system had the drawback of the limited number of samples (8-10) which can be simultaneously run, adequate for confirmation purposes, but that is usually too limited for the IEF initial testing procedure. The upgraded model of the device (SNAP i.d. version 2.0) consists in two "Midi" blot holders 8.5 x 13.5 which almost doubles the capacity of the initial system. This size permits to load almost 15 samples and so apply the system also for the initial testing procedure.

In preliminary tests we have verified the suitability of the system for both IEF and Sarcosyl-PAGE approaches, analyzing standard reference samples of human recombinant erythropoietin (BRP), Darbeboetin  $\alpha$  (NESP), pegylated erythropoietin (CERA), epoetin  $\delta$  (DYNEPO) and some others biosimilars, together with spiked urine samples at our current validated LODs. The system has demonstrated to fulfill WADA criteria as established in the current technical document (TD2013EPO), confirming previous observations. Finally, the robustness of the procedure also appears to have remarkably improved by this new version of the vacuum-based blotting system.

## Introduction

Erythropoietins (EPOs) are included in the WADA list [1]. The widely used method, based on isoelectrofocusing (IEF) and chemioluminiscent detection [2,3] for the research of human recombinant erythropoietin (hrEPO) presents some complexity and is time consuming. In normal working time conditions, the detection is achieved after three working days, a limiting factor, especially when narrow time constraints have to be met.

More recently the use of SDS-PAGE or Sarcosyl-PAGE electrophoresis has been added as a complementary method to the IEF procedure. This approach, which is the current "routine" analytical strategy, is still time consuming. In order to improve and accelerate the procedure for the detection of erythropoietin and its analogues, we have experienced a novel blotting system based on vacuum technology: the SNAP i.d. system from Millipore. The first version of this device consists of two "Mini" blot holders 8.5 x 7 cm. After the DDT incubation, the Immobilon membranes are positioned in a blot holders and vacuum is applied. All reagents (blocking, antibody and washing solutions) are forced to pass through the membranes actively, making possible to complete the entire analytical process in less than 12 hours from the beginning without any relevant loss of sensitivity [4]. We have now expanded the originally IEF-PAGE based vacuum driven blotting protocol for application with SDS-PAGE/Sarcosyl-PAGE. Following DDT incubation, the Immobilon membranes are positioned in the blot holders and vacuum is applied. All reagents (blocking, antibody and washing solutions) are forced to pass through the membranes actively, making solution with SDS-PAGE/Sarcosyl-PAGE. Following DDT incubation, the Immobilon membranes are positioned in the blot holders and vacuum is applied. All reagents (blocking, antibody and washing solutions) are forced to pass through the membranes actively, making possible to complete the entire analytical process in less than 12 hours without any relevant loss of sensitively, making possible to complete the entire analytical process in less than 12 hours without any relevant loss of sensitively, making possible to complete the entire analytical process in less than 12 hours without any relevant loss of sensitively. Making possible to complete the entire analytical process in less than 12 hours without any relevant loss of sensitivity [4]. We are now extending the above approach also to the SDS-PAGE/Sarcosyl-PAGE pr

### Experimental

The vacuum driven blotting system (SNAP i.d. 2.0) consists of two blot holders of different size, the "Mini" blot holder 8,5 x 7 cm suitable for SDS-PAGE or SAR-PAGE analysis, and the "Midi" 13,5 x 7 cm suitable for IEF-PAGE (Fig. 1). Any combination of both sizes can be done at any time. The procedure is basically the same already shown in a previous work [4] and briefly described below:



- after the first blotting and DTT incubation the Immobilon membrane is positioned in a pre-wet blot holder, being careful to remove any air bubbles;
- vacuum is applied to the system;
- 30 mL of a 0.25% in PBS low fat milk (LFM) blocking solution are added and then the membrane is washed only once with PBS (the same for the second blotting).
- the membrane is incubated out of the SNAP system with primary antibody for 1 hour in 0.5% LFM (the same for the incubation with secondary antibody and streptavidin);
- all washing steps are finally performed adding 30 mL of PBS four times in the SNAP system.

For best result the low fat milk solutions, both 0.25% and 0.5%, have to be well dissolved and filtered 2-3 times to avoid spots.



Figure 1: SNAP i.d. old version (on the left) and new 2.0 version (on the right) with the "Mini" and the "Midi" blot holders.

#### **Results and Discussion**

To verify the robustness of the new system we analyzed standard reference samples of recombinant EPO (BRP), Darbeboetin  $\alpha$  (NESP), pegylated EPO (CERA), epoetin  $\delta$  (Dynepo) and other biosimilars (Fig. 2). Two fortified urines for each erythropoietin formulation (BRP, NESP, CERA and Dynepo) and two urines from excretion study were assayed in a gel after immunopurification (Fig. 3 and 4). We applied the system both to IEF and to SAR-PAGE procedures. The system has shown no relevant loss of sensitivity compared to our current validated LODs for all tested EPOs. The acceptance and identification criteria established in the current WADA technical document (TD2013EPO) were fulfilled; finally, no false negative nor false positive results were reported.

The SNAP i.d. system should be also suitable for future applications requiring IEF-, SDS- and SAR-PAGE methods.





Figure 2: IEF and SARCOSYL-PAGE gel images results of different reference standards: recombinant erythropoietin BRP and NESP (lane 1 and 9 in IEF); CERA (lane 2); endogenous erythropoietin NIBSC (lane 3); EPOFIT (lane 4); CERITON (lane 5); EPOSIS (lane 6); ERYPRO (lane 7); Dynepo (lane 8).

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Figure 3: IEF-PAGE results of urines fortified with different erythropoietins: BRP and NESP, CERA, NIBSC and Dynepo reference standards (lane 1 and 15, 2, 3, 14 respectively), NESP positive urinary samples from WAADS QA2011 (lane 4), recombinant erythropoietin positive urine from excretion study (lane 5), NESP spiked urines (lanes 6-7); Dynepo spiked urines (lanes 8-9); CERA spiked urines (lanes 10-11); BRP spiked urines (lanes 12-13). All urine amples were immunopurified in the EPO ELISA-wells (Quantikine IV Epo kit from R &D) before loading the gel.





Figure 4: SAR-PAGE gel image of spiked urines: BRP and NESP, NIBSC, CERA and Dynepo reference standards (lanes 1, 4, 9 and 10 respectively), BRP and NESP spiked urine (lanes 2-3); CERA spiked urine (lanes 5-6); Dynepo spiked urine(lanes 7-8).

### References

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