

## Supplementary Material

### **Free-radical production after post-thaw incubation of ram spermatozoa is related to decreased *in vivo* fertility**

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Initially, we would like to suggest that long experience with our flow cytometer has allowed us to configure it so that no-DNA events could be correctly discriminated. We did not carry out any analysis with SYBR-14/Propidium Iodide (PI) in our experiment, since, during the set-up of the YO-PRO-1 staining, we performed the appropriate tests to check that no-DNA events were identified perfectly and they did not represent any problem for the YO-PRO-1/PI combination.

Here, we present the experimental development and results of the comparison of SYBR-14/PI and YO-PRO-1/PI stains, showing that under our conditions, both techniques are comparable. In addition, we corrected our YO-PRO-1/PI results as Petrunkina and Harrison (*Theriogenology* 73:839-47, 2010) propose and compared them with uncorrected results highlighting the high agreement between both methods.

In the test previously mentioned we used similar conditions to those described in this manuscript (incubation thawing samples 2h in the freezing extender and in SOF), and we analyzed many samples using both stains.

YO-PRO-1/PI and SYBR-14/PI stains were carried out as Martinez-Pastor et al. (2008) and Castro-González et al. (2010) suggest.

Next, we describe in more detail the *materials and methods on flow cytometry*, as suggested by the reviewers, thus removing this part of the original text. Table 1 shows probes, filters, lasers and voltages relative to flow cytometry, in order to reduce the main paper as reviewer 1 suggested. Table 1 showed voltages values during YO-PRO-1/PI sperm analysis; this value on FL1 was higher than in the rest of the analysis (714 mW). High voltage intensity induce a displacement to the right of the living spermatozoa subpopulation in a FL1/FL3 dot plot, separating live spermatozoa from no-DNA events.

All stained samples were assessed using a Cytomics FC500 flow cytometer (Beckman Coulter, Inc. USA). Excitation was provided by a 15 mW Argon-Ion laser which emits at 488 nm. The FSC (forward – scattered light) and SSC (side-scattered light) signals were used to gate out debris (non-sperm events). A region was drawn inside the FSC/SSC plot leaving debris out and sperm events inside. SSC light pass first through a 500LP filter and divided with a 600DLP filter. Ligth below 500 nm was used to analyze SSC. Regions were drawn inside the FSC/SSC and/or FL1/FL3 plots leaving debris out and sperm events inside (Fig.1 of supplementary data). Only events inside the region were showed and taking in consideration for the rest of the plots. Initially, sample events were acquired in a logarithmic FSC/SSC dot plot with an acquisition threshold in FSC photodetector over 6<sup>th</sup> channel. All events under this channel were ignored. That threshold helps cytometer technician to ignore electronic noise due the high voltage applied to FL1 photodetector. In the FL1/FL3 dot plot, a region was drawn trying to separate no-DNA events from spermatozoa. This region

was used to gate a second FL1/FL3 plot. Spermatozoa from this second FL1/FL3 plot were analyzed. Gate acquisition strategy is showed in Fig. 1.

Voltage intensities and non-DNA events exclusion strategy are included in supplementary data. Acquisition threshold were established in 6: all events below channel 6 in FSC in logarithmic scale during acquisition were ignored. Light lower than 550 nm is analyzed with FL1. Evaluation of green fluorescence, emitted by CM-H<sub>2</sub>DCFDA, CMA-3 and Acridine Orange (AO), was done using the FL-1 photodetector with a 530/28BP filter. This filter only permits the pass of fluorescence emissions with a 530±14 nm bandwidth.

Light spectrum with wavelength higher than 600nm is divided also with a 645DSP filter; afterwards light lower than 645 nm is analyzed in FL3. Light with a spectrum wavelength lower than 600nm is divided with a 550DLP filter.

Red fluorescences were measured using the FL3 photodetector with a 620SP filter that allow the fluorescence emission of AO (red) and PI events.

The 620SP filter only permits the pass of fluorescences emissions ~~from 0 to~~ below 620 nm. To avoid interferences of lower emissions it is preceded with a 600LP filter that only allows wavelengths higher than 600 nm through The acquisition was controlled using the MXP software (Beckman Coulter, Inc. USA). All the parameters were read using logarithmic amplification, except AO that was read in linear mode. We acquired 10,000 spermatozoa from each sample. Analysis and compensation were realized with WEASEL software v.3 (WEHI, Melbourne, Australia)

*Sperm viability assessment* is shown in Fig. 1. The percentage of YO-PRO-1 positive non-DNA particles ranged from 15.7% (freezing extender) to 17.9% (SOF). This figure is too high to be negligible, therefore an approach for excluding non-DNA containing particles as described in Petrunkina et al. (2010) (or one alternative

approaches described therein) should be used. The voltage intensity allow us to discriminate no-DNA events achieving similar results to those obtained with the mathematical estimation for the magnitude of overestimation propose by Petrunkina and Harrison (2010) and Petrunkina et al. (2010), considering therefore, the percentage of non-DNA events negligible. As Fig. 1 showed, live cell subpopulations and no-DNA events with a high voltage in the green photodetector (FL-1 in our equipment) are perfectly discriminable in the staining YO-PRO-1/PI. This is possible by the autofluorescence that most cells have in the green range of the spectrum, probably due to intracellular flavins (Shapiro 2004, page 35.). Thus, as Petrunkina and Harrison (2010) suggest, we have identified the alien particles and exclude them for the analysis. Differences in software and hardware between different cytometers could be the key to make gating debris exclusion a technique of common use. Each laboratory would make a previous experiment set-up and be consequent with found results.

We have used the procedure suggested by Blant and Altman (1986), a method of data plotting used in analyzing the agreement between two different assays. We performed the comparison for both amount of removed no-DNA events and the proportion of PI-impermeable spermatozoa ([YO-PRO-1-/PI-]+[YO-PRO-1+/PI-] and SYBR-14+/PI-, respectively). Live cells subpopulation and no-DNA events isolation of each stain method were considered.

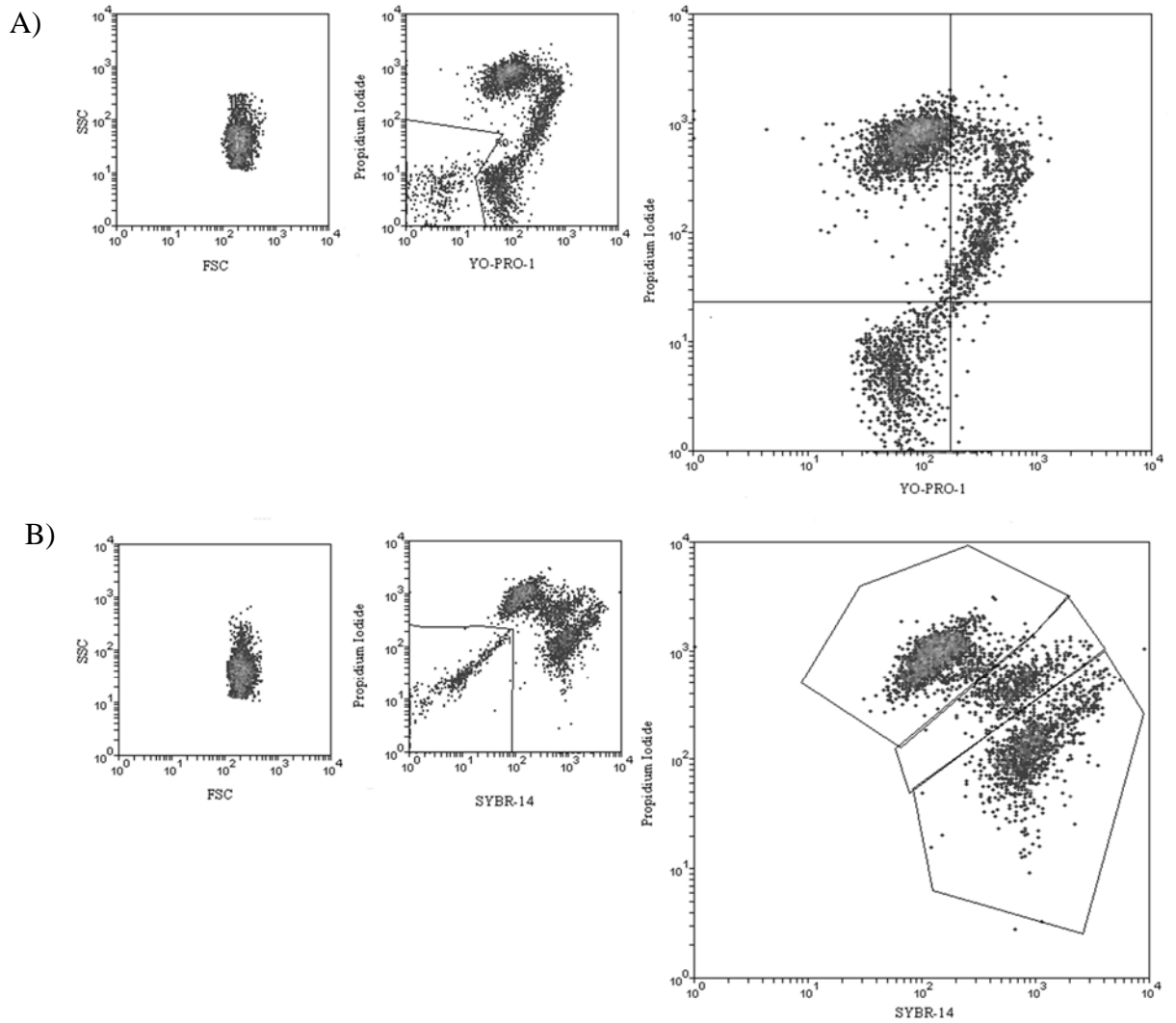
The comparison between YO-PRO-1/PI and SYBR-14/PI techniques show a good agreement for no-DNA events isolation and live cells subpopulation identification (Table 2) (Fig. 2 and 3). The mean of the differences with all the data analyzed together was of -2.34% for live cells and 2.07% for no-DNA events exclusion. When data were analyzed, treatment by treatment, sperm samples

incubated with SOF showed a mean of -1.6% for live cells subpopulation and 0.84% for no-DNA events. Samples incubated with freezing extender showed higher means (-3.08% and 3.29% for live subpopulation and no-DNA events exclusion, respectively), the latter may be due to the lack of dilution step and that sperm samples incubated with SOF undergo. Standard deviation of the differences was always lower than 5% except for total and SOF treatment data that were closer (5.01% and 5.79%).

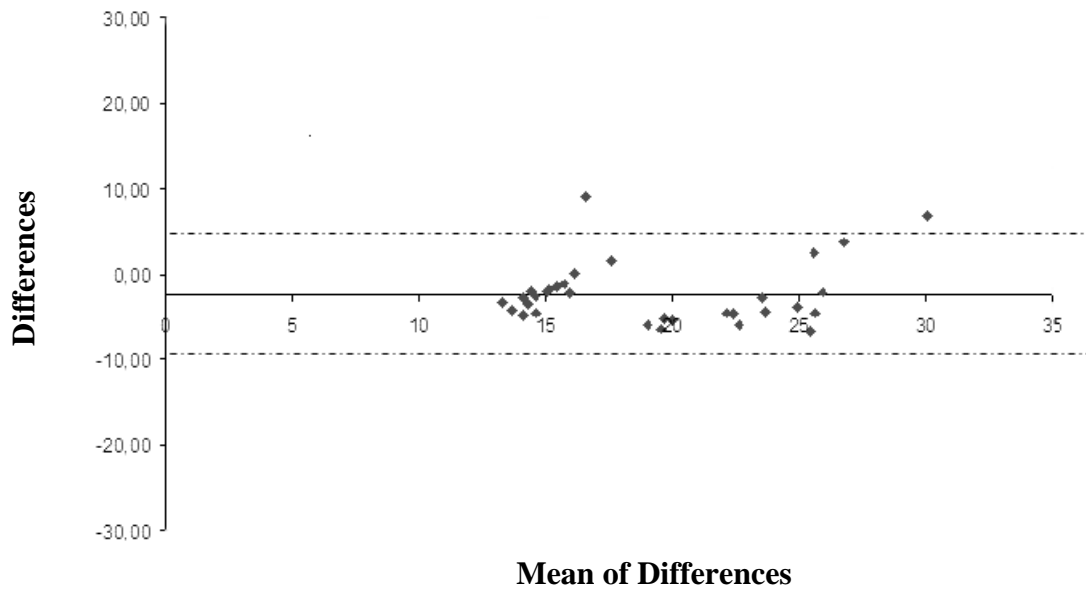
Furthermore, we have corrected our data following the suggestion of Petrunkina and Harrison (2010), using an original mathematical research on the rationale behind the overestimation. We compared results from the numeric no-DNA events exclusion method (Petrunkina and Harrison, 2010) with our gating exclusion method (Table 3)(Fig. 3 and 4). The mean of the differences of total data or for each treatment alone were always lower than 1%. Standard deviation was closer or lower than 5% (highest was 5.05% in SOF treatment). Our results showed that both staining techniques, YO-PRO-1/PI and SYBR-14/PI, detected the same percentages of non-DNA events and living cells.

The voltage intensity could allow us discriminate no-DNA events achieving similar results to those obtained with DNA binding stain. As Fig. 4 showed, live cell subpopulations and no-DNA events with a high voltage in the green photodetector (FL-1 in our equipment) are perfectly discriminable in the staining YO-PRO-1/PI. This is possible by the autofluorescence that most cells have in the green range of the spectrum, probably due to intracellular flavins (Shapiro 2004, page 35.). But the differences in software and hardware between different cytometers could be the key to make gating debris exclusion a technique of common use. Each laboratory would make this experiment and be consequent with found results.

Finally, our results showed that the agreement between numeric no-DNA events exclusion method and gating no-DNA events exclusion method strengthens our hypothesis that increasing the voltage of the green photodetector enables us to exclude the main no-DNA events subpopulation. We hope that results presented here serve as a proof that our analysis has not resulted in excessive over-estimation of viable cells.

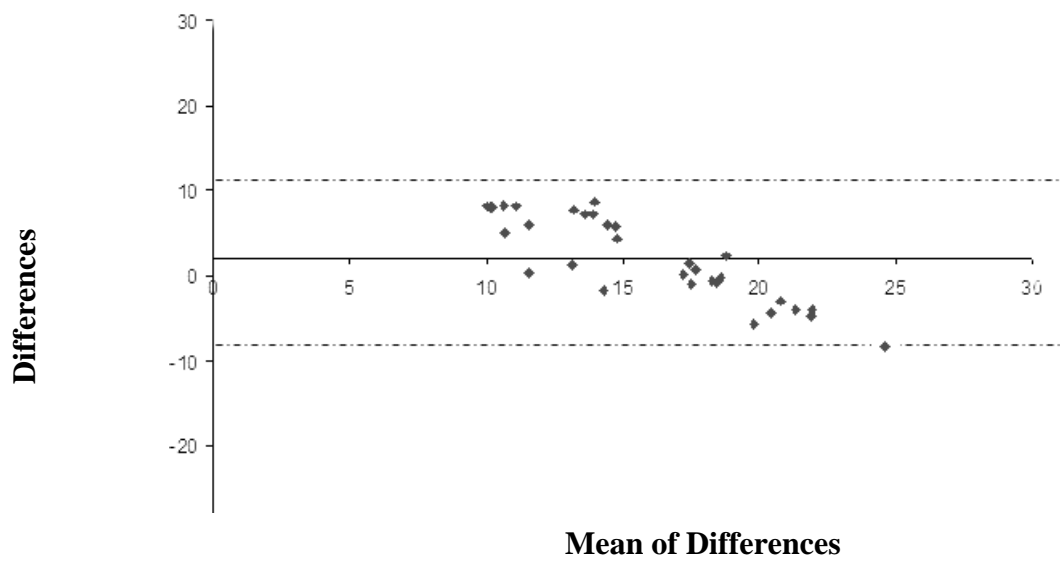


**Fig. S1.** Gating no-DNA events exclusion method performed in our laboratory. Upper plots belong to YO-PRO-1/PI stain technique (A). Lower plots belong to SYBR-14/PI stain technique (B). Excluded no-DNA events were annotated to compare with numeric exclusion no-DNA events comparison. Numeric no-DNA events exclusion method was performed with no gated data.

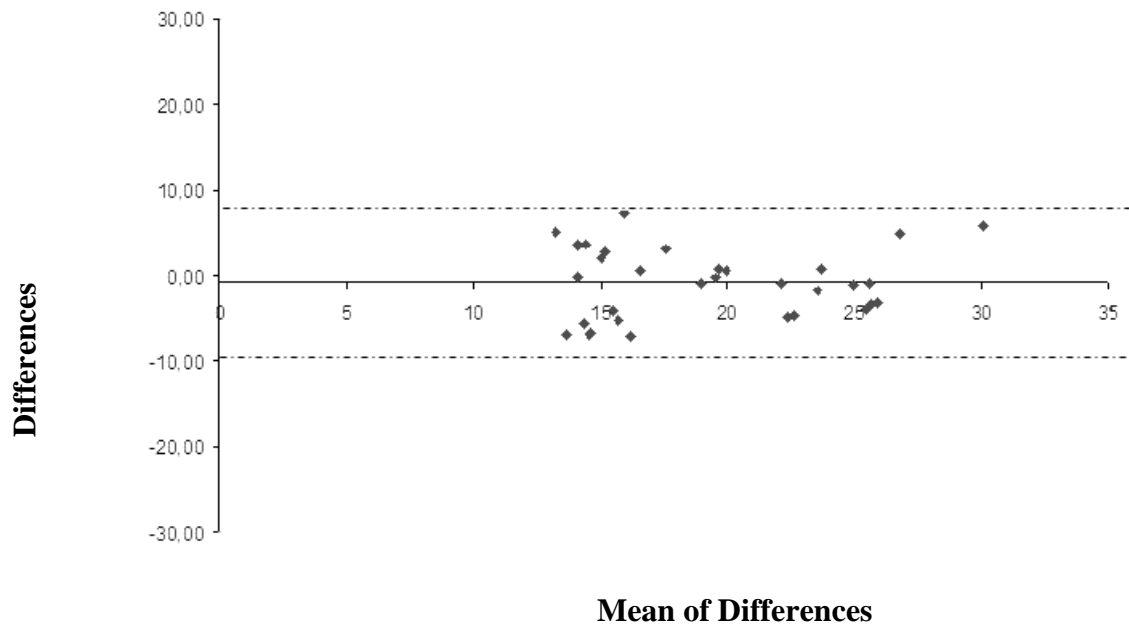


**Fig. S2.** Live cell subpopulation determination method agreement. Bland & Altman plot. Dotted line show the Mean $\pm$ 2SD. % YO-PRO-1 (+ and - events)/PI-. Differences= (%YO-PRO-1/ PI -) - (%SYBR 14 + / PI -). Mean of Differences = (%YO-PRO-1/PI- + %SYBR 14+/PI-)/2.





**Fig S3.** No-DNA events determination methods agreement. Bland & Altman plot. Dotted line show the Mean $\pm$ 2SD. % YO-PRO-1 (+ and – events)/PI-. Differences = [%No-DNA events from YO-PRO-1/PI - %No-DNA events from SYBR-14/PI]/2. Mean of Differences = [%No-DNA events from YO-PRO-1/PI + %No-DNA events from SYBR-14/PI]/2



**Fig. S4.** Comparative study between numeric no-DNA events adjustment and gating no-DNA events exclusion with YO-PRO-1/PI stain. Bland & Altman plot. Dotted line show the Mean $\pm$ 2SD. % YO-PRO-1 (+ and - events)/PI-. Differences = (% YO-PRO-1/PI No-DNA events) - (% YO-PRO-1/PI No DNA events corrected). Mean of differences = (% YO-PRO-1/PI No-DNA events + % YO-PRO-1/PI No-DNA events corrected)/2.

**Table S1. Voltage intensities used in each staining techniques (mV)**

Voltage intensities applied to FL1 and FL3 cytometer photodetectors. FL1 photodetector was used to detect green fluorescence emitted by YO-PRO-1, CMA3, CM-H<sub>2</sub>DCFDA (ROS), FITC antibody-Conjugated (TUNEL) and acridine orange (AO). FL3 Photodetector was used to detect red fluorescence emitted by PI and acridine orange (AO). Higher voltage implies higher sensitivity and electronic noise.

<b>Cytometer Staining Techniques Voltage (mV)</b>							
<b>Detector</b>	<b>YO-PRO-1</b>	<b>PI</b>	<b>CMA3</b>	<b>ROS</b>	<b>TUNEL (FITC)</b>	<b>TUNEL (PI)</b>	<b>AO</b>
<b>FL1</b>	714		585	413	460		483
<b>FL3</b>		606				597	597

**Table S2. Comparison between two viability test (SYBR-14/PI and YO-PRO-1/PI staining methods)**

Data are expressed as the mean and standard deviation (SD) of the differences between the two staining methods ( $n = 16$ ). Live cells: YO-PRO-1(+ or -)/[PI-] and SYBR-14+/PI- subpopulations. No-DNA events: gated events as non-DNA (gating exclusion technique) and SYBR-14-/PI-. FE (freezing extender), SOF (Synthetic Oviductal Fluid).

Treatment	Live cells (%)		No-DNA events (%)	
	Mean	SD	Mean	SD
<b>FE</b>	-3.08	3.95	3.29	3.89
<b>SOF</b>	-1.6	3.30	0.84	5.79
<b>Overall</b>	-2.34	3.66	2.07	5.01

**Table S3. Comparison between analysis methods of YO-PRO/PI, gating exclusion technique vs numeric exclusion technique (following the instructions of Petrunkina and Harrison (2010))**

Data are expressed as the mean and standard deviation (SD) of the differences between the two methods ( $n = 16$ ). Results express YO-PRO(+ or -)/PI-. FE (freezing extender), SOF (Synthetic Oviductal Fluid).

Treatment	YO-PRO-1/PI vs corrected YO-PRO-1/PI (%)	
	Mean	SD
<b>FE</b>	-0.8	3.04
<b>SOF</b>	-0.93	5.05
<b>Overall</b>	-0.87	4.10