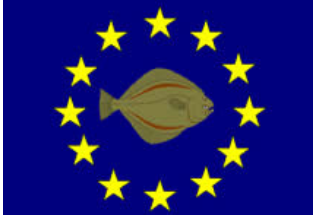


<p style="text-align: center;">FINAL REPORT 31/10/08</p>	<p style="text-align: center;">EROD Intercalibration 2008</p>	 <p style="text-align: center;">BEQUALM Biomarker Programme Office NIVA, Gaustadalléen 21, NO- 0349, Oslo, Norway</p>
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Participating laboratories

There were 10 participating laboratories including NIVA, all of which submitted EROD results for the microsomal fractions of fish liver homogenates.

Exposure study

The exposure study was carried out at the Marine research station in Solbergstrand, Norway. Adult turbot, *Scophthalmus maximus* (200-400 g) were exposed to a “high” (50 mg/kg) and “low” (5 mg/kg) dose of benzo[*a*]pyrene dissolved in corn oil, administered by intra-peritoneal injection. Turbot injected with corn oil only were used as a control group. The treatment groups were kept in separate tanks of clean seawater for one week prior to sampling.

After one week, the fish were killed humanely with a blow to the head and the liver removed. The liver samples were immediately placed in cryovials and snap frozen in liquid nitrogen. They were then refrigerated at -80°C prior to use.

Preparation and shipment of samples

Fish livers were pooled into three groups per treatment and liver samples prepared to produce both an S9 (microsome & cytosol) and microsome fraction. The homogeneity of the samples was checked internally prior to distribution. Each participating laboratory was sent 18 samples, consisting of 9 microsome fractions and 9 S9 fractions. These 9 fraction samples contained 3 control, 3 low dose and 3 high dose exposures.

All samples were distributed to each participating laboratory by courier on dry ice. Delivery time took approximately 48 h. In all cases, the samples remained frozen with plenty of dry ice when received by each laboratory. Samples were stored by each laboratory at -80°C prior to use.

Important note: an error in the preparation of the S9 fraction resulted in the S100 fraction being prepared and distributed to the participating laboratories. The S100 fraction contains the cytosol only and little microsomes, consequently no or very low activity was reported from these samples. For this reason, the EROD activities measured in the cytosol samples were not used in the inter-calibration. However, the protein concentration of the cytosol samples was used.

EROD assays

All samples were analysed for EROD and protein by the laboratories method of choice. For measurements of EROD, participants were recommended to use the Times 23 protocol based on Stagg & McIntosh, 1998, which can be obtained from the ICES website <http://www.ices.dk/products/techniques.asp>. However, each laboratory was asked to document which method was used, particularly in cases where their method deviated significantly from the Times 23 protocol.

In addition to the homogenated liver samples, resorufin standards were also sent to each participating laboratory. It was encouraged for each laboratory to use this resorufin standard and to compare with their own standards. Data for this comparison was provided by three laboratories.

Results and discussion

This inter-calibration exercise differs from those carried out for other parameters in that there is not necessarily a “correct” answer. The results may be used by the participating laboratories to modify their procedures if they produce a higher or lower EROD activity than other laboratories.

Data are presented as the reported value from each laboratory, which was normalised with respect to protein concentration (Table 1 and Figures 1 a-i). The inter-laboratory comparison of the measured protein concentration can be found in a parallel report (BEQUALM, 2008)

Table 1. EROD activity reported by all participating laboratories (pmol/min/mg protein)

B(a)P exposure	Sample code	Lab code									
		1	2	3	4	5	6	7	8	9	10
control	1	9.51	11.00	3.69	11.00	20.86	1.90	1.84	1.25	14.31	8.44
control	2	9.93	4.60	3.67	10.00	42.08	1.79	1.75	1.29	14.67	6.62
high dose	3	98.75	38.00	64.00	60.00	253.06	18.43	19.35	6.10	142.16	112.17
high dose	4	199.83	103.00	107.70	255.00	514.54	45.41	32.32	18.78	258.58	192.93
control	5	22.28	13.00	22.57	47.00	90.27	4.19	8.29	3.55	39.76	17.05
low dose	6	62.21	24.00	53.94	42.00	132.57	5.49	15.03	7.03	91.32	56.71
low dose	7	80.47	65.00	57.42	96.00	156.89	10.76	15.11	14.74	121.69	86.28
low dose	8	137.13	64.00	82.66	123.00	380.25	20.89	25.29	16.26	246.16	162.26
high dose	9	152.71	104.00	101.70	113.00	397.90	15.55	26.01	18.10	271.79	160.41

All laboratories were able to distinguish between EROD activity from control fish and those that were exposed to low and high doses of benzo[a]pyrene. An interesting observation was that sample 3, representing a high exposure to benzo[a]pyrene, exhibited a lower EROD activity than some of the low exposure samples (7 & 8), an observation that was detected by all laboratories.

Suspect outliers for each sample were identified and assessed using the Dixon Q-test. This is a simple test that enables the detection of outliers to be carried out in statistically robust manner. From ranked values for each separate sample the experimental Q-value (Q_{exp}) was calculated using the formula:

$$Q_{exp} = (X_N - X_{N-1}) / (X_N - X_1)$$

where X_1 is the lowest value and X_N is the highest value of the ranked sample measurements. The obtained Q_{exp} was compared to a critical Q-value (Q_{crit}) at 95% confidence limits (CL). In cases where the $Q_{exp} > Q_{crit}$ then the extreme measurement for the particular sample was treated as an outlier and was excluded from the calculation of the Z scores. The Dixon Q-test detected 3 outliers for samples 2, 4 and 5, which were excluded from the Z score calculation. All three measurements were submitted by the same laboratory (laboratory 5).

For a clearer comparison of the EROD activity measured between the participating laboratories, individual Z scores were calculated. The Z scores were calculated using the formula:

$$Z \text{ score} = (\text{measured value} - \text{mean value from all laboratories}) / \text{standard deviation from all laboratories}$$

An assessment criterion for each Z score was carried out based on the ISO 43 guidelines:

Z score < 2	satisfactory
2 < Z score < 3	questionable
Z score > 3	unsatisfactory
Z score > 6	extreme

EROD measurements submitted by Laboratory 5 were consistently higher than all other laboratories. This was particularly the case for samples 2, 4 and 5, which were found to be significantly higher at 95% CL and were excluded from the Z score calculation. Four of the six remaining values submitted by laboratory 5 were calculated with a Z score between 2 and 3 suggesting questionable results.

Consistently lower than average EROD activity was reported for laboratories 6, 7 and 8 for all samples, although the Z scores for these laboratories were often above or around -1 and considered as satisfactory.

With the exception of laboratory 5, all laboratories showed reasonable agreement for each of the samples, with satisfactory Z scores achieved by all laboratories. Although differences between laboratory methods exist for the EROD protocol no consistent relationship was identified between the protocol used and the relatively high or low values reported.

Additional comment: It was brought to our attention by one of the participating laboratories that an error in the Times 23 protocol exists. An error in Table 2 (page 7) of the official ICES Times 23 protocol. When reporting “Reagent volumes and concentrations used” for NADPH 10 µl of a 100 mM solution in 2 ml final volume, gives a final assay concentration of 0.5 mM (not 0.25 mM as indicated in Table 2).

Comparison of resorufin standards

Three laboratories submitted two sets of EROD activity data, one that was calculated using their own resorufin standard and a second calculated based on resorufin supplied by the coordinating laboratory. The data for each laboratory is shown in figures 2 (a-c).

For laboratory 3, similar values were reported for many of the samples. However, for some of the samples (samples 6 and 8) large differences in EROD activity were reported, with higher values calculated when the resorufin standard supplied by the co-ordinating laboratory was used. A similar pattern but with much larger differences were reported by laboratory 5 when using the different resorufin standards. Differences in the purity of the resorufin standards used

were likely to have resulted in the reported differences, with a relatively lower purity of the resorufin standard supplied by the co-ordinating laboratory.

In contrast, the data reported by laboratory 6 showed almost identical EROD measurements for the two resorufin standards used, reflecting the similarity of the purity of the resorufin standards used.

Preliminary conclusions and the way forward

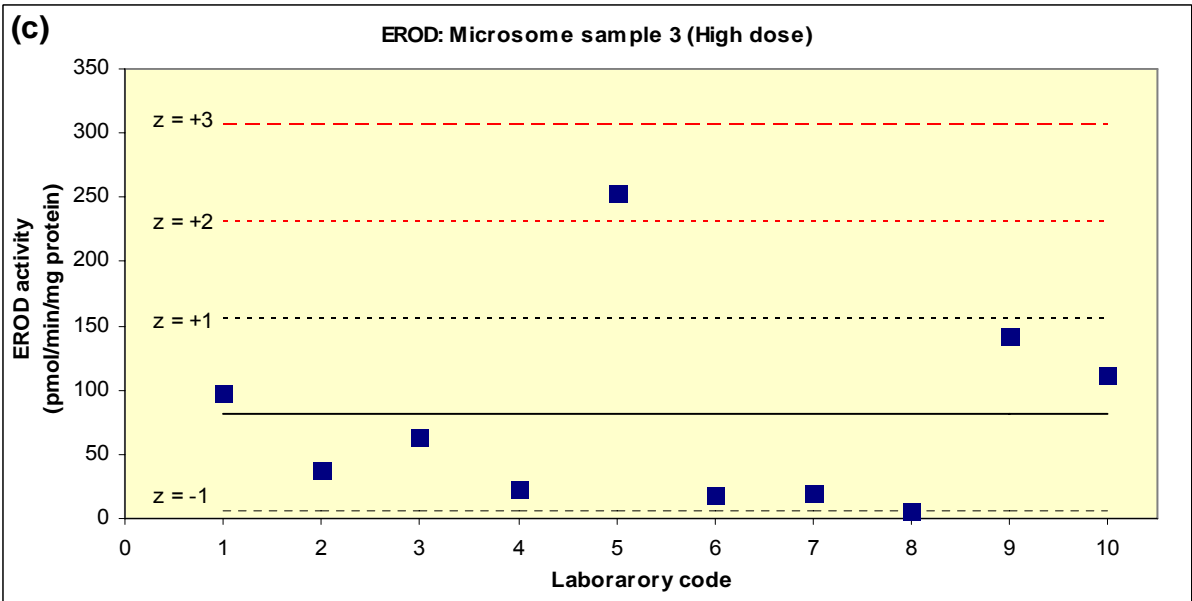
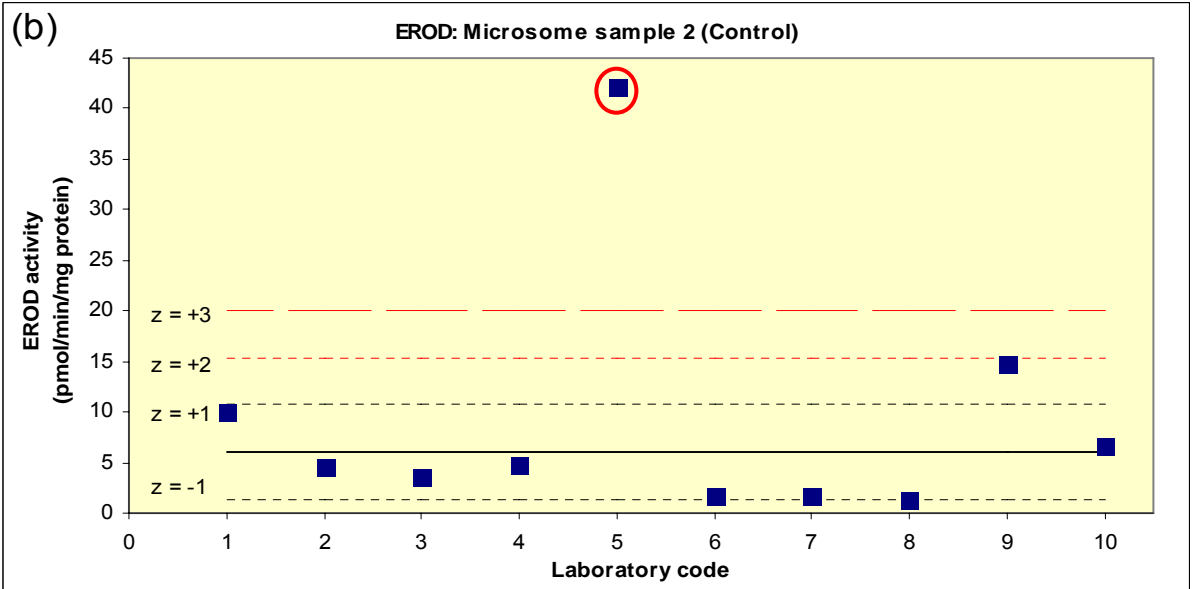
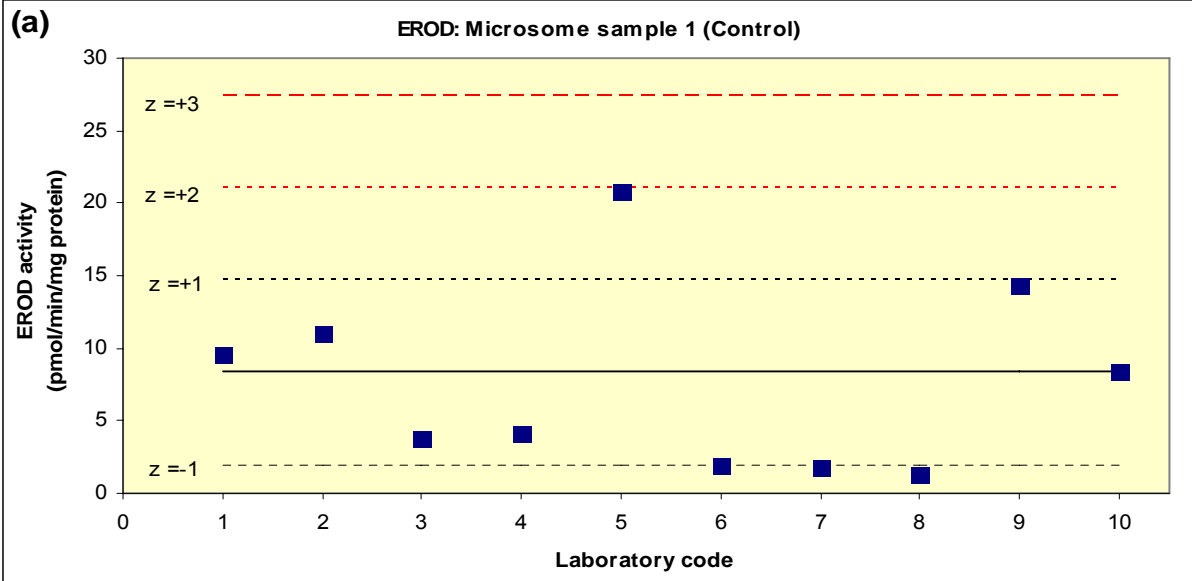
This inter-calibration exercise clearly showed that inter-laboratory differences exist even in samples that have been prepared identically. However, with the exception of one laboratory (laboratory 5), there was reasonable agreement in EROD activity between laboratories for the nine microsomal fractions with all Z scores within the satisfactory criteria of ± 2 .

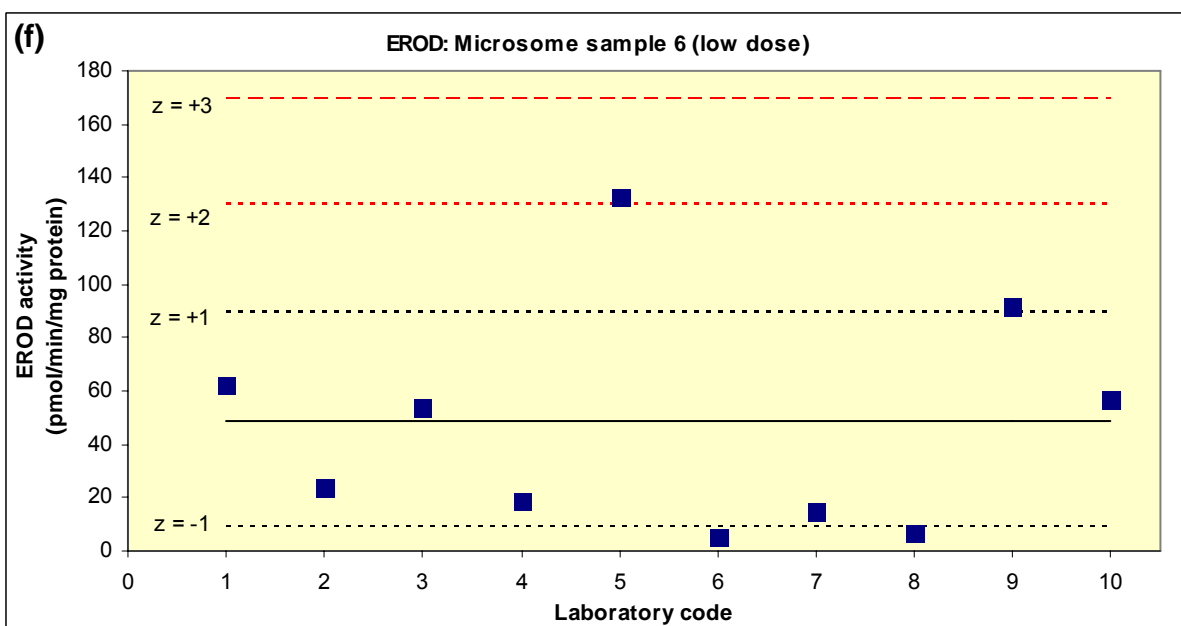
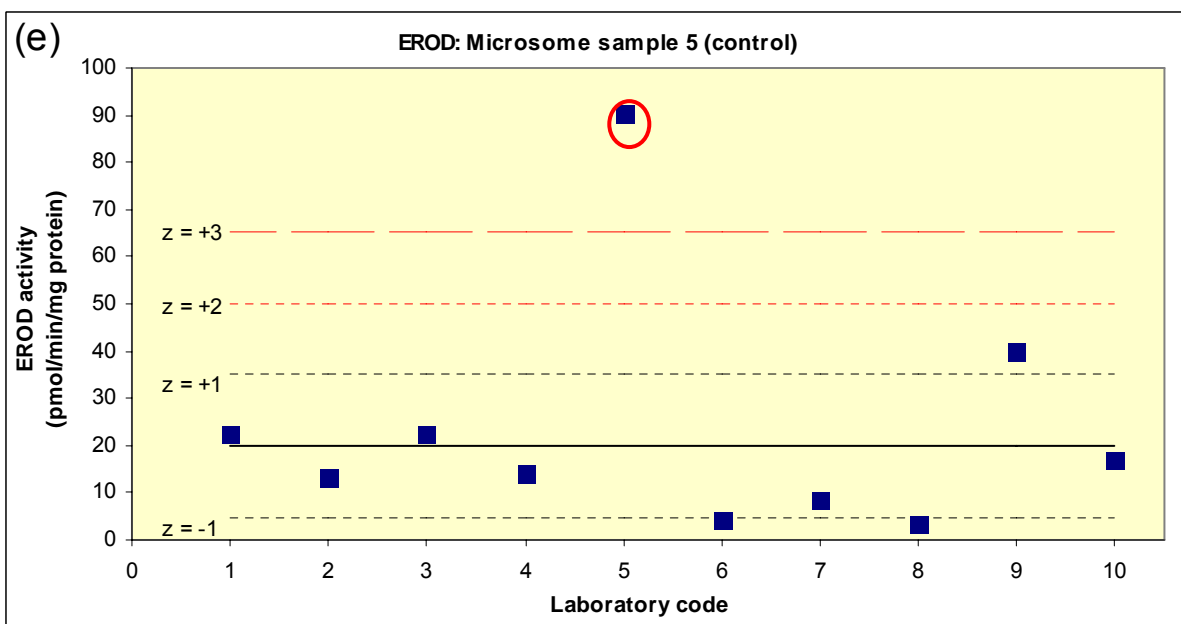
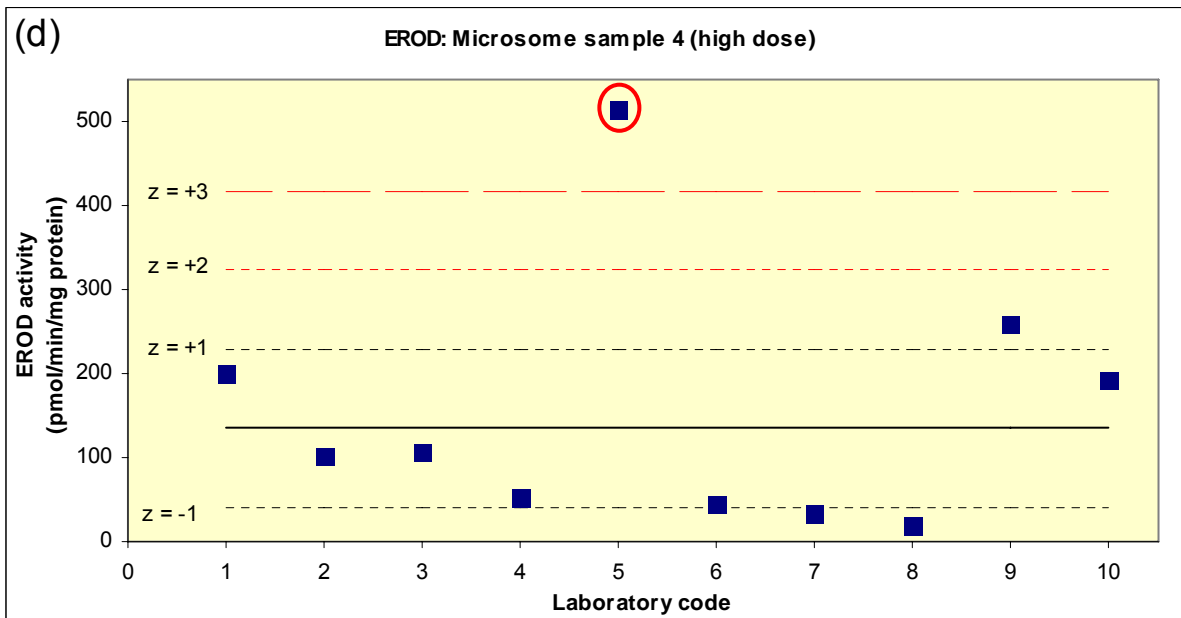
The reasons for the significantly higher EROD values reported by laboratory 5 were unclear. This laboratory provided data for both resorufin standards where even higher EROD values were reported when the resorufin standard provided by the co-ordinating laboratory was used. Therefore, a difference in the purity of the resorufin standard was not responsible for the higher activities reported. Calculation errors may be responsible and it is recommended that laboratory 5 should recheck their spreadsheets to see if calculation errors had occurred, which would explain the differences.

It was unfortunate that comparisons between the microsomal and S9 fractions could not be made. However, all labs successfully reported no or extremely low activity in the S100 fractions distributed as would be expected from these samples (data not presented).

The results should be used by participating laboratories to assess their internal protocols. No statements of performance will be issued for this inter-calibration.

Figure 1 (a-i): EROD activity reported by each laboratory from prepared liver microsome samples of the *S. maximus*. Solid line represents overall mean, dotted lines denote the appropriate Z score values. Circled data points denote excluded outliers based on Dixon's Q-test at 95% CL.





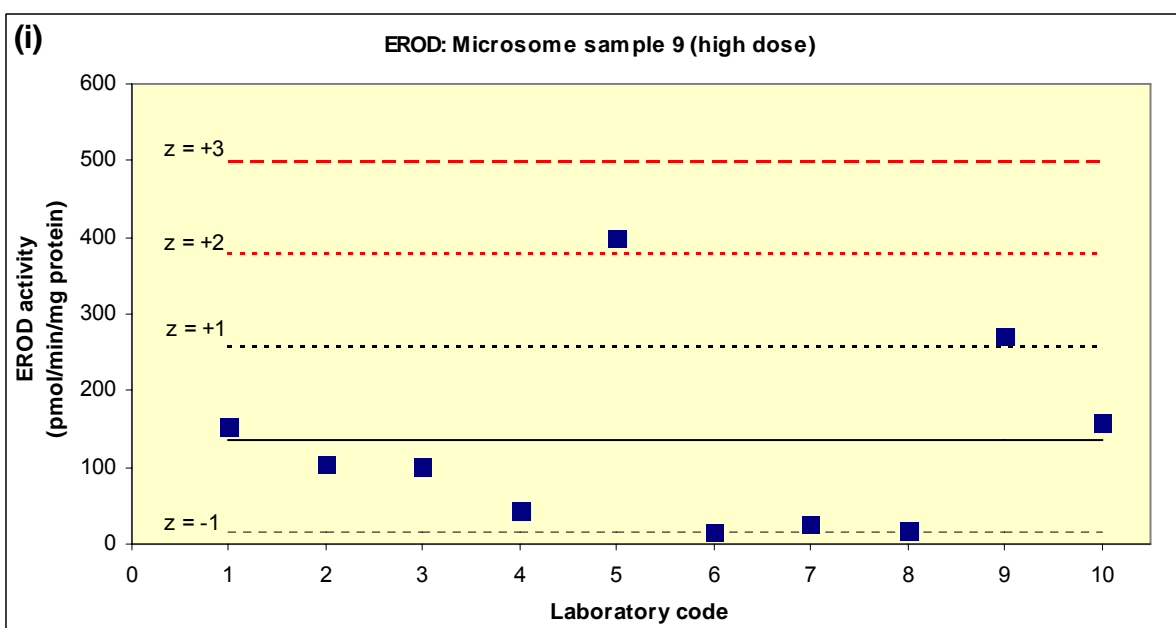
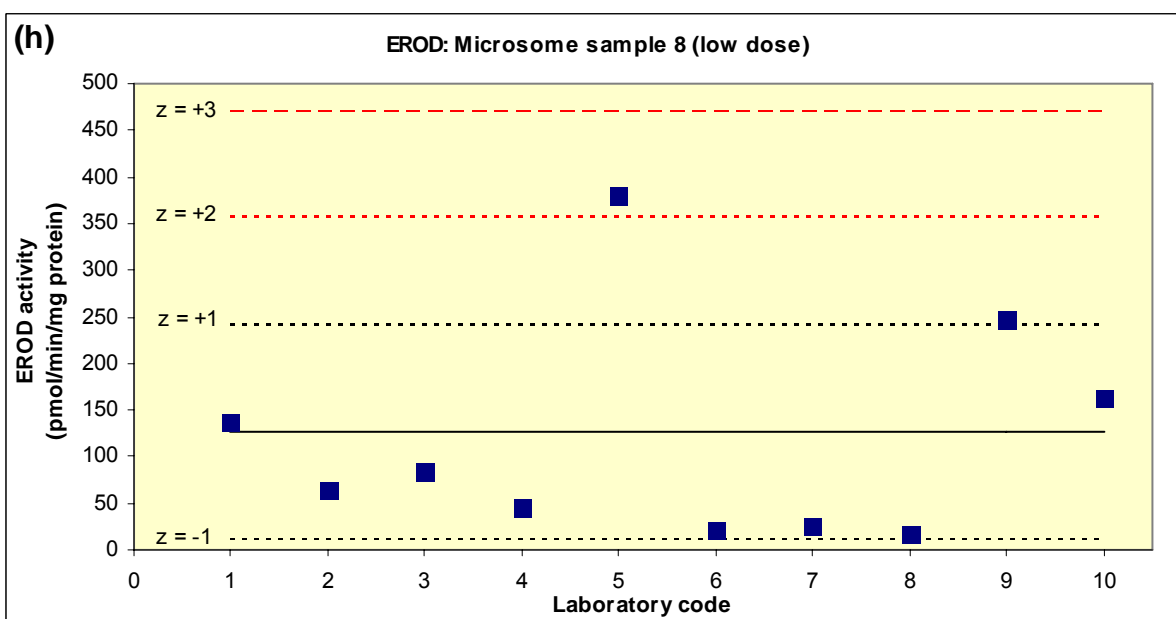
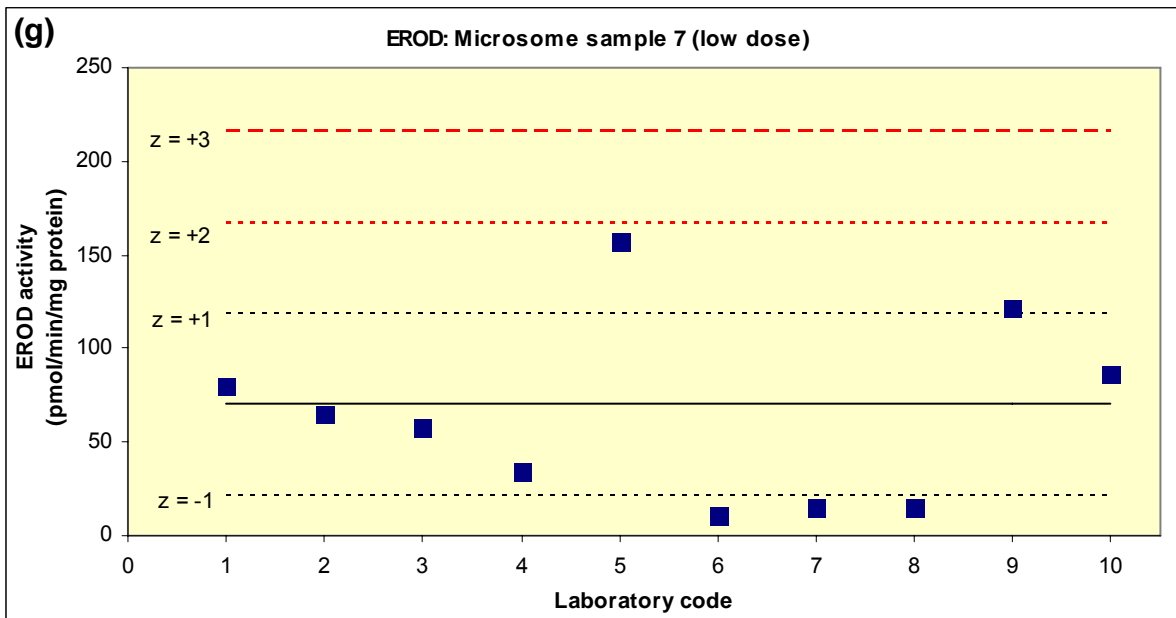
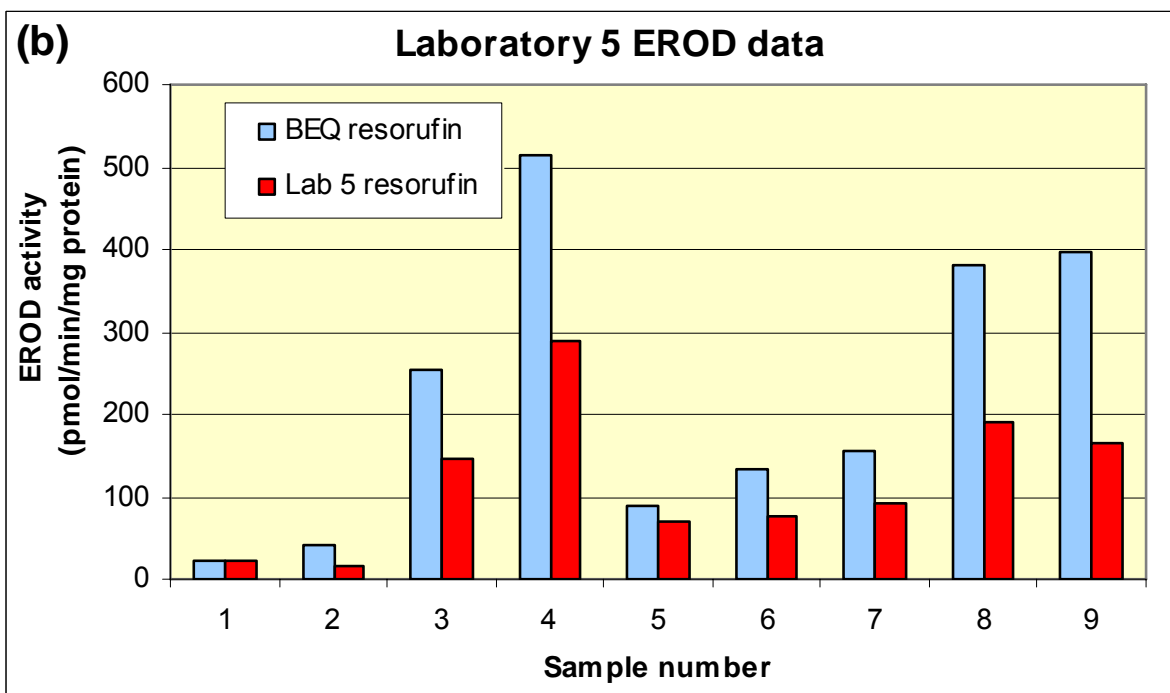
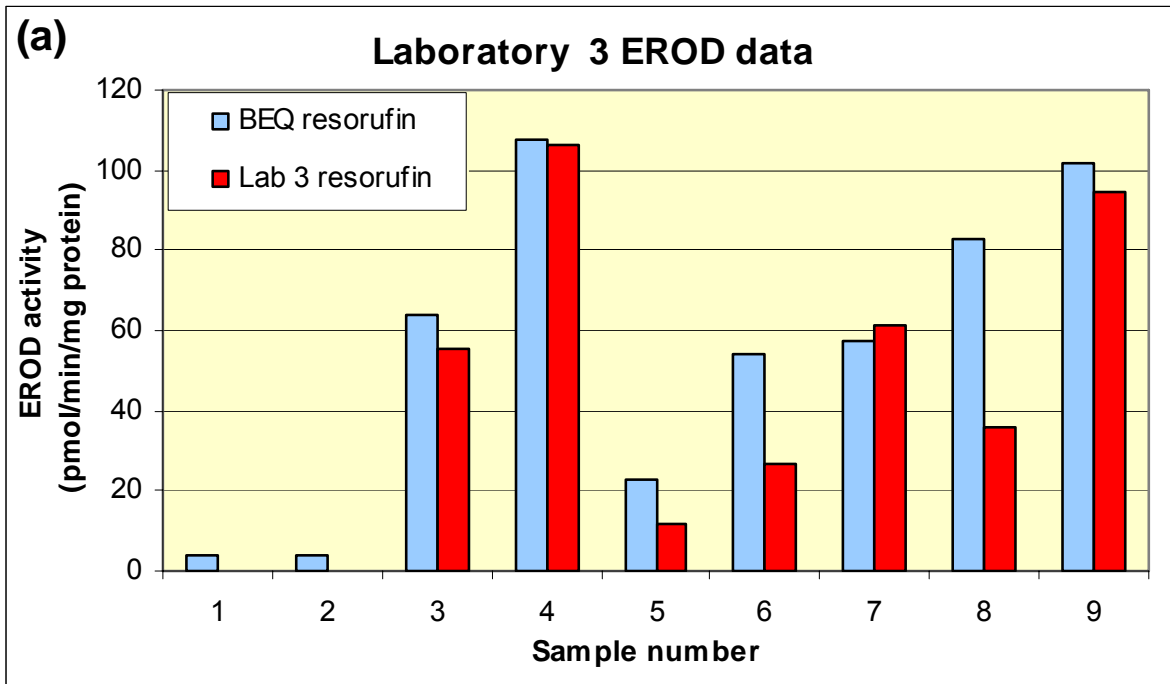


Figure 2 (a-c). Comparison of EROD activity measured using two resorufin standards. Data provided by three laboratories.



(c)

Laboratory 6 EROD data

