EDC WFD

EBMs Validation

ISPRA Istituto Superiore per la Protezione e la Ricerca Ambientale

Sistema Nazionale per la Protezione dell'Ambiente

22nd February 2023

EDC WFD

Experimental design

- Two methods implying the same sample preparation procedure combined to two different bioassays (i.e. ERα-CALUX and A-YES) were optimized and validated within the Project.
- A method validation experimental design was planned in accordance with CEN/TS 16800:2020 to improve the comparability of estrogen measurements.
- The methods performance characteristics were assessed by analyses on six matrices at three different concentrations level each (i.e. low, medium and high concentration) spiked with the targeted estrogens (i.e. b-E2, a-E2, a-EE2, E1, E3).

Samples scheme

Batch	Matrix	EDC (ng/L)	Performance Characteristic
0	Calibration verification	Calibration Solutions (8 solutions measured in triplicate) obtained by CRMs dilution	Calibration verification
		1st Verification Calibration Solution corresponding to approximately EC50.	
		2nd Verification Calibration Solution corresponding to approximately the highest calibration level .	
1	EVIAN+DOC 1 mg/L	EVIAN water as Procedural Blank	reagents check
		EVIAN + DOC 1 mg/L without the addition of hormones	"Background"
		*Milli-Q + 10 LOQ-V E2 eq	Matrix Effect
		*EVIAN +DOC 1 mg/L + 10 LOQ-V E2 eq +interferents	Interferents effect
		EVIAN +DOC 1 mg/L +10 LOQ-V E2 eq	Precision, Trueness, LOQ_v
		EVIAN +DOC 1 mg/L + 3LOQ-V E2 eq	Precision, Trueness
		EVIAN +DOC 1 mg/L + 10 LOQ-V E2 eq	Precision, Trueness, DOC effect
2	EVIAN+DOC 7 mg/L	EVIAN water as Procedural Blank	reagents check
		EVIAN + DOC 7 mg/L without the addition of hormones	"Background"
		*Milli-Q + 10 LOQ-V E2 eq	Matrix Effect
		*EVIAN +DOC 7 mg/L + 10 LOQ-V E2 eq +interferents	Interferents effect
		EVIAN +DOC 7 mg/L +LOQ-V E2 eq	Precision, Trueness, LOQV
		EVIAN +DOC 7 mg/L + 3 LOQ-V E2 eq	Precision, Trueness
		EVIAN +DOC 7 mg/L + 10 LOQ-V E2 eq	Precision, Trueness, DOC effect
		EVIAN + DOC 14 mg/L without the addition of hormones	"Background" in DOC effect
		EVIAN +DOC 14 mg/L + 10 LOQ-V E2 eq	DOC effect

Batch	Matrix	EDC (ng/L)	Performance Characteristic
3	EVIAN+DOC 7 mg/L + TSS 50 mg/L	EVIAN water as Procedural Blank	reagents check
		EVIAN + DOC 7 mg/L +TSS without the addition of hormones	"Background"
		*Milli-Q + 10 LOQ-V E2 eq	Matrix Effect
		*EVIAN +DOC 7 mg/L + TSS + 10 LOQ-V E2 eq +interferents	Interferents effect
		EVIAN +DOC 7 mg/L + TSS + LOQ-V E2 eq	Precision, Trueness, LOQ_v
		EVIAN +DOC 7 mg/L + TSS + 3LOQ-V E2 eq	Precision, Trueness
		EVIAN +DOC 7 mg/L +TSS + 10LOQ-V E2 eq	Precision, Trueness
4	1st Natural/syntetic sample spiked at three different level of concentration (partner choice)	EVIAN water as Procedural Blank	reagents check
		Natural sample without the addition of hormones	"Background"
		*Milli-Q + 10 LOQ-V E2 eq	Matrix Effect
		*Natural sample + 10 LOQ E2-V eq + interferents	Interferents effect
		EDC at LOQ E2-V eq	Precision, Trueness, LOQV
		EDC at 3 LOQ E2-V eq	Precision, Trueness
		EDC at 10 LOQ E2-V eq	Precision, Trueness
5	2nd Natural/syntetic sample spiked at three different level of concentration (partner choice)	EVIAN water as Procedural Blank	reagents check
		Natural sample without the addition of hormones	"Background"
		*Milli-Q + 10 LOQ E2 eq	Matrix Effect
		*Natural sample + 10 LOQ E2 eq + interferents	Interferents effect
		EDC at LOQ level each	Precision, Trueness, LOQ_v
		EDC at 3 LOQ level each	Precision, Trueness
		EDC at 10 LOQ level each	Precision, Trueness
6	3rd Natural/syntetic sample spiked at three different level of concentration (partner choice)	EVIAN water as Procedural Blank	reagents check
		Natural sample without the addition of hormones	"Background"
		*Milli-Q + 10 LOQ E2 eq	Matrix Effect
		*Natural sample + 10 LOQ E2 eq + interferents	Interferents effect
		EDC at LOQ level each	Precision, Trueness, LOQV
		EDC at 3 LOQ level each	Precision, Trueness
		EDC at 10 LOQ level each	Precision, Trueness

Fitting: Graph-Pad Prism 9.0 (1)

Most Friendly Approach

↓

Ordinary Least Squares

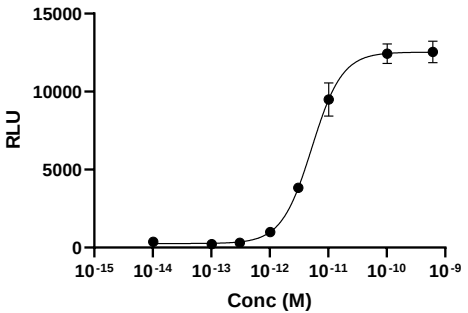
↓

Non Linear Regression

↓

4 Parameters Logistic Function

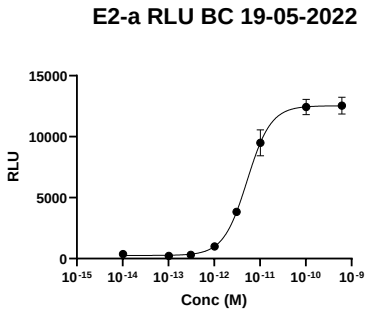
$$Y = Bottom + \frac{Top - Bottom}{1 + \left(\frac{EC_{50}}{X}\right)^{Hill\ Slope}}$$



Calibration checked with two independent standard solutions

Fitting: Graph-Pad Prism 9.0 (2)

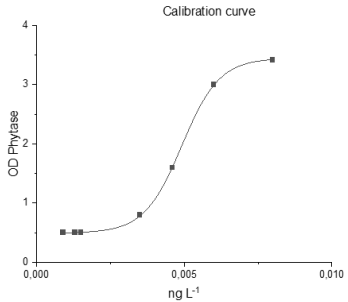
CALUX



ERα-CALUX calibration

- 8 levels measured in triplicate
- Calibration range in wells (ng/L): 0.0028 ng/L-170 ng/L with 0.1% of DMSO

A-YES



A-YES calibration levels in wells

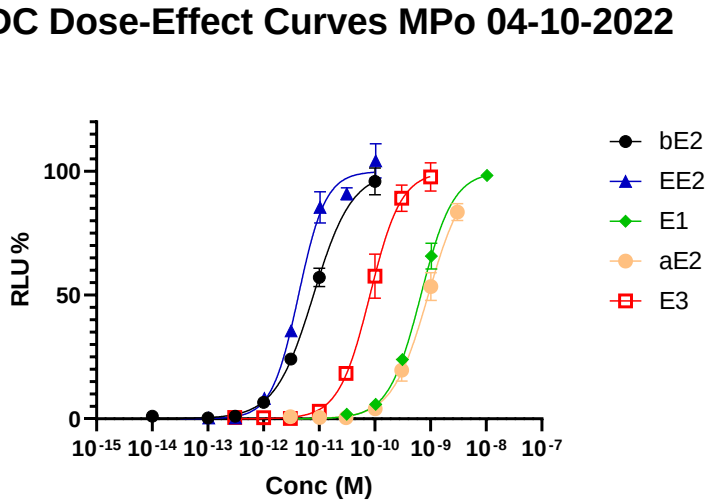
- 7 levels measured in triplicate
- Calibration range in wells (ng/L): 0.0009 ng/L-0.008 ng/L.

Batch 0 Calibration verification

CALUX Relative Potencies Assessment (1)

Relative potencies assessment was performed twice:

- Change of the operator involved
- Stability over the time.



	E2	EE2				E1				aE2				E3			
ECx level (%)	ECx(M)	ECx (M)	REP	Mean	RSD % REP	ECx (M)	REP	Mean	RSD % REP	EC(x)	REP	Mean	RSD % REP	EC(x)	REP	Mean	RSD % REP
50	8.01E-12	4.26E-12	1.878			6.51E-10	0.0123			9.12E-10	0.00878			8.18E-11	0.09793		
40	5.76E-12	3.40E-12	1.697			5.09E-10	0.0113			6.73E-10	0.00857			6.29E-11	0.09161		
30	4.03E-12	2.65E-12	1.519	1.501	21	3.90E-10	0.0103	0.010	17	4.83E-10	0.00834	0.0083	5	4.73E-11	0.08519	0.084	14
20	2.60E-12	1.96E-12	1.327			2.82E-10	0.0092			3.23E-10	0.00806			3.34E-11	0.07795		
10	1.35E-12	1.25E-12	1.082			1.73E-10	0.0078			1.76E-10	0.00767			1.98E-11	0.06821		

Relative Potencies Assessment (2)

Compound	ISO 19040:3	Sonnesveld et al . 2005	ISPRA REP50 October 2022	ISPRA REP50 May 2022
bE2	**1**	**1**	**1**	**1**
E1	**0.02**	**0.016**	**0.012**	**0.014**
aE2	**0.1**	**0.011**	**0.0088**	**0.010**
E3	**0.017**	**0.13**	**0.098**	**0.084**
EE2	**1.3-1.5**	**1.88**	**1.9**	**1.4**

CV% ranged between 9-11%, with the exception of EE2 (21%)

Matrices and concentrations levels tested (1)

1	EVIAN water + 1 mg/L DOC
2	EVIAN water + 7 mg/L DOC
3	EVIAN water + 7 mg/L DOC + 50 mg/L TSS
4	Tap water BAM – Adlerhof (DOC 10 mg/L, pH 7.1)
5	Teltow canal water (DOC 12 mg/L, pH 6.8)
6	Non sparkling comercially available mineral water (Lidl, Saskia - Source „Leissling“ in Germany, DOC 3 mg/L, pH 6.5)

Matrices and concentrations levels tested (2)

Samples preparation and extractions carried out by BAM. The procedure followed was the one implemented by BAM within the Project (i.e. SPE disk + MiSPE purification)

CALUX concentrations levels	
Low Concentration	0.12 ng/L bE2 eq.
Medium Concentration	0.38 ng/L bE2 eq.
High Concentration	1.23 ng/L bE2 eq.
A-YES concentrations levels	
Low Concentration	0.14 ng/L bE2 eq.
Medium Concentration	0.40 ng/L bE2 eq.
High Concentration	1.40 ng/L bE2 eq.

ISPRA REP10 are used to determine the cumulative effect of the spiked concentrations

ISO 19040-2 REP50 are used to determine the cumulative effect of the spiked concentrations

Matrices and concentrations levels tested (3)

- Measured values were corrected for the absolute recoveries weighted mean.
- Absolute recoveries of estrogens are known from the MS method validation studies carried out by BAM.
- The weighted mean was calculated taken into account the relative potencies of each estrogen.

Absolute recoveries:
bE2= 79%
aE2=80%
EE2=81%
E1=73%
E3=63%

Corrected values are considered in the uncertainty evaluation (i.e. trueness assessment)

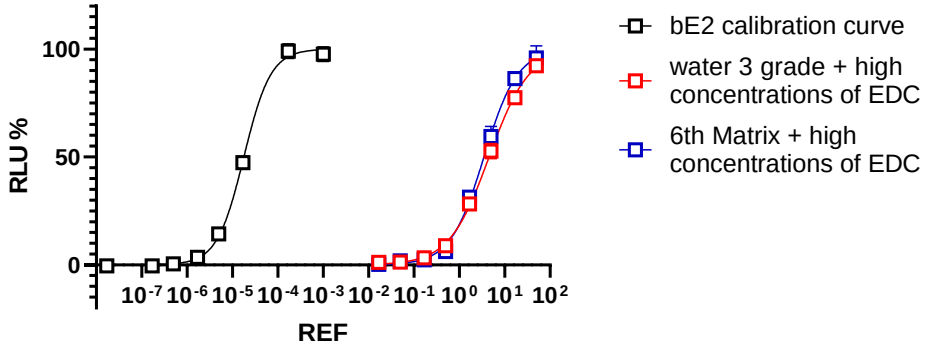
Selectivity: Matrix, interferents and DOC effects (1)

Action	Calculations
Matrix effect	The Matrix Effect (ME) was assessed via comparison between the samples and water grade 3 dose-response curves at EEQ iso concentrations (i.e. high concentration) and the obtained results.
Interferents effect	The interferents effect was assessed via comparison between the samples and samples + interferents dose response curves at iso concentrations of hormones (high concentration). Ramified NP and BPA at French P95 selected as interferents and concentration levels respectively and the obtained results.
DOC effect:	The DOC effect is assessed via comparison between the observed curves (i.e. in-house reference materials containing EVIAN + DOC 1 mg/L, EVIAN + DOC 7 mg/L and EVIAN + DOC 14 mg/L spiked at high EEQ concentration) and the obtained results.

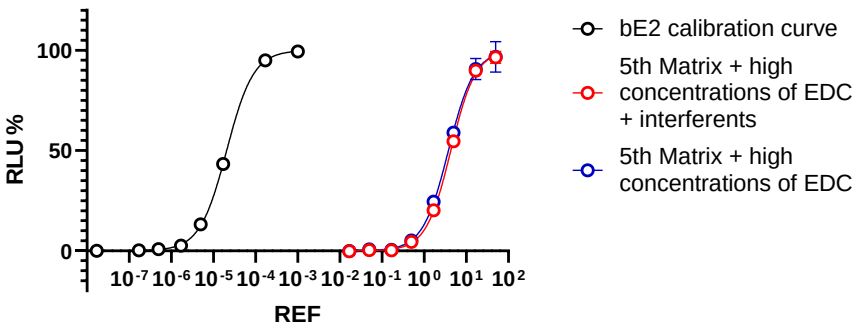
Selectivity: Matrix, interferents and DOC effects (2)

CALUX

Matrix effect: Dose-response curves for the 6th matrix

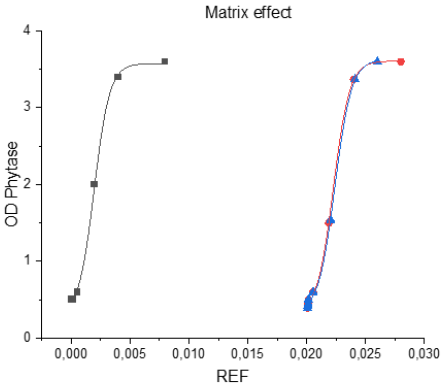


Interferents effect: comparison among normalized dose-response curves for the 5th matrix

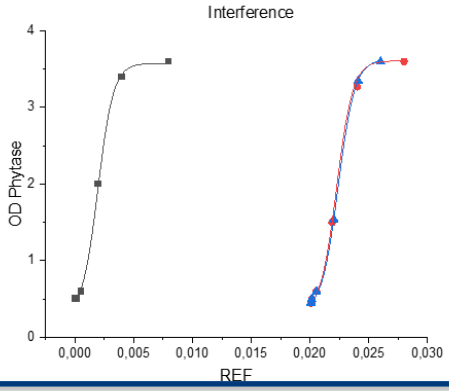


Selectivity: Matrix, interferents and DOC effects (3)

A-YES



Dose-response curves associated to the calibration curve, water 3 grade spiked sample and the 6th matrix sample

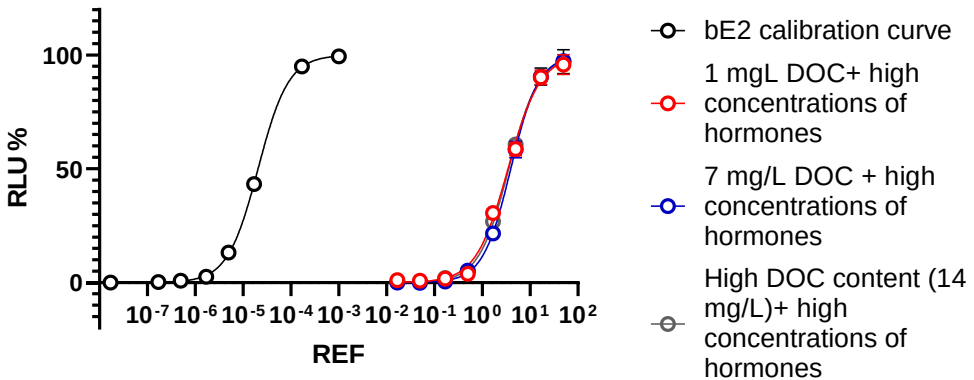


Dose-response curves associated with the calibration curve, water 3 grade spiked sample and the 5th matrix samples

Selectivity: Matrix, interferents and DOC effects (4)

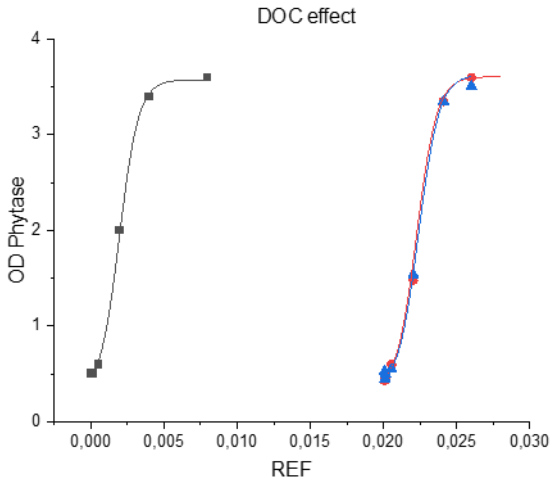
CALUX

DOC effect: comparison among normalized dose-response curves at increasing DOC content



Selectivity: Matrix, interferents and DOC effects (5)

A-YES



Dose-response curves associated to the calibration curve and matrices at increasing level of DOC spiked samples

Selectivity: Matrix, interferents and DOC effects (6)

- Similar behaviour in terms of EC50 and Hill's slope (Observed variability less than 20% and 15% for A-YES and CALUX respectively)
- Similar behaviour in terms of results (observed variability less than 20% and 25% for A-YES and CALUX respectively). *Reference values are obtained taking into account the targeted hormones*.
- A two tailed t-test was applied on the results, and the outcomes supported the hypothesis that the means were not significant different.

Precision assessment-CALUX

Precision was evaluated by following both NF T90-210 Standard and the ANOVA and applying the template implemented by ISPRA.

Matrix	Replicate 1 [M]	Replicate 2 [M]	Replicate 3 [M]	Square root of MSi within groups [M]	Intermediate precision standard deviation [M]	Mean of the means [M]	Within-laboratory reproducibility (%)
				$S_{i,rep}$	$S_{i,Between}$	$\bar{\bar{X}}_i$	$u_{i,rw}$
DOC 1 mg/L	3.189E-13	2.623E-13	2.743E-13	4.41091E-14	2.33804E-14	2.76971E-13	18.0
DOC 7 mg/L	2.576E-13	2.518E-13	2.369E-13				
DOC 7 mg/L+SPM	3.572E-13	2.352E-13	2.327E-13				
Natural 1*	2.228E-13	2.361E-13	2.587E-13				
Natural 2*	2.249E-13	3.462E-13	2.567E-13				
Natural 3*	3.084E-13	3.264E-13	3.783E-13				
DOC 1 mg/L	9.585E-13	1.016E-12	8.197E-13	9.6285E-14	7.76387E-14	7.80384E-13	15.8
DOC 7 mg/L	7.435E-13	8.607E-13	6.222E-13				
DOC 7 mg/L+SPM	7.264E-13	7.068E-13	9.628E-13				
Natural 1*	7.67E-13	6.878E-13					
Natural 2*	5.724E-13	7.024E-13	6.668E-13				
Natural 3*	8.026E-13	8.198E-13	8.316E-13				
DOC 1 mg/L	2.822E-12	2.415E-12	3.406E-12	5.18936E-13	4.32047E-13	2.8161E-12	24.0
DOC 7 mg/L	2.075E-12	2.286E-12	2.652E-12				
DOC 7 mg/L+SPM	2.332E-12	2.093E-12	2.756E-12				
Natural 1*	2.721E-12	2.234E-12	2.728E-12				
Natural 2*	2.957E-12	2.914E-12	3.029E-12				
Natural 3*	4.108E-12	2.583E-12	4.576E-12				

Precision assessment-A-YES

Precision was evaluated by following both NF T90-210 Standard and the ANOVA and applying the template implemented by ISPRA.

Matrix	Replicate 1 [ng/L]	Replicate 2 [ng/L]	Replicate 3 [ng/L]	Square root of MSi within groups [ng/L] $S_{i,rep}$	Intermediate precision standard deviation [ng/L] $S_{i,Between}$	Mean of the means [ng/L] $\overline{\overline{X}}_i$	Within-laboratory reproducibility (%) $u_{i,rw}$
DOC 1 mg/L	0.1650727	0.1413123	0.1575694	0.067636416	0.014876319	0.159680732	43.4
DOC 7 mg/L	0.1400617	0.1675738	0.1400617				
DOC 7 mg/L+SPM	0.1363101	0.1388112	0.1400617				
Natural 1*	0.15882	0.1377302	0.1238046				
Natural 2*	0.1087979	0.1513167	0.1367296				
Natural 3*	0.1399596	0.4301895	0.1600705				
DOC 1 mg/L	0.4552006	0.392673	0.4253556	0.026572505	0	0.407669747	6.5
DOC 7 mg/L	0.4202824	0.4122777	0.4256716				
DOC 7 mg/L+SPM	0.4014269	0.3739148	0.4439456				
Natural 1*	0.3739148	0.4134187					
Natural 2*	0.3776664	0.4264379	0.4226862				
Natural 3*	0.3739148	0.4139324	0.3776664				
DOC 1 mg/L	1.4215559	1.3723515	1.3661549	0.042867672	0.01326444	1.39773268	3.2
DOC 7 mg/L	1.3952597	1.3884838	1.3794961				
DOC 7 mg/L+SPM	1.3862193	1.3756061	1.4106215				
Natural 1*	1.4009297	1.373105	1.3618501				
Natural 2*	1.3773895	1.4037911	1.3830771				
Natural 3*	1.5644393	1.4061784	1.3926792				

Precision assessment-ANOVA

Anova: Single Factor

SUMMARY

Groups	*Count*	*Sum*	*Average*	*Variance*
Row 1	3	1E-12	3.59E-13	1.41164E-27
Row 2	3	9E-13	3.13E-13	1.80401E-28
Row 3	3	1E-12	3.46E-13	8.02794E-27
Row 4	3	9E-13	3.01E-13	5.22386E-28
Row 5	3	1E-12	3.47E-13	6.27272E-27
Row 6	3	1E-12	4.25E-13	2.09044E-27

ANOVA

Source of Variation	*SS*	*df*	*MS*	*F*	*P-value*	*F crit*
Between Groups	2.84197E-26	5	5.68E-27	1.842887822	0.17879371	3.105875239
Within Groups	3.7011E-26	12	3.08E-27			
Total	6.54307E-26	17				

F < F crit in all the three levels tested.

Bias evaluation-CALUX

Matrix	Mean [M]	EEQ Spiked Reference value [M]	Standard uncertainty associated to the EDC addition (%)	Matrix j Mean recovery (%)	Deviation from the complete recovery (%)	Mean square of the deviations at the ith level (%)^2	Mean of the relative standard uncertainties associated to EDC addition (%)	Relative uncertainty at the ith concentration associated to the bias (%)
	$X_{c+\Delta C,ij}$	ΔC	$u_{i,conc\ rel}$	$R_{i,j}$	$b_{ij,rel}$	$b^2_{i\ rms,\ rel}$	$\overline{u_{i\ conc,rel}}$	$u_{i,b}$
DOC 1 mg/L	3.590E-13	4.54193E-13	3.5	79	-21.0	601	3.5	24.8
DOC 7 mg/L	3.133E-13	4.49907E-13	3.5	70	-30.4			
DOC 7 mg/L+SPM	3.463E-13	4.50445E-13	3.5	77	-23.1			
Natural 1*	3.012E-13	4.51117E-13	3.5	67	-33.2			
Natural 2*	3.474E-13	4.53366E-13	3.5	77	-23.4			
Natural 3*	4.252E-13	4.59824E-13	3.5	92	-7.5			
DOC 1 mg/L	1.173E-12	1.37482E-12	3.5	85	-14.7	869	3.5	29.7
DOC 7 mg/L	9.344E-13	1.37765E-12	3.5	68	-32.2			
DOC 7 mg/L+SPM	1.006E-12	1.35568E-12	3.5	74	-25.8			
Natural 1*	9.158E-13	1.36122E-12	3.5	67	-32.7			
Natural 2*	8.149E-13	1.35962E-12	3.5	60	-40.1			
Natural 3*	1.030E-12	1.37046E-12	3.5	75	-24.9			
DOC 1 mg/L	3.628E-12	4.55488E-12	3.5	80	-20.4	663	3.5	26.0
DOC 7 mg/L	2.943E-12	4.55836E-12	3.5	65	-35.4			
DOC 7 mg/L+SPM	3.014E-12	4.54085E-12	3.5	66	-33.6			
Natural 1*	3.225E-12	4.54381E-12	3.5	71	-29.0			
Natural 2*	3.736E-12	4.55947E-12	3.5	82	-18.1			
Natural 3*	4.729E-12	4.59572E-12	3.5	103	2.9			

The expected EEQ values were obtained by summing each target analyte spiked concentration corrected for its relative potency estimated at 10% of the effect.

Bias evaluation-A-YES

Matrix	Mean [ng/L]	EEQ Spiked Reference value [ng/L]	Standard uncertainty associated to the EDC addition (%)	Matrix j Mean recovery (%)	Deviation from the complete recovery (%)	Mean square of the deviations at the i^{th} level (%)^2	Mean of the relative standard uncertainties associated to EDC addition (%)	Relative uncertainty at the i^{th} concentration associated to the bias (%)
	$X_{c+\Delta C,ij}$	ΔC	$u_{i,conc\ rel}$	$R_{i,j}$	$b_{ij,rel}$	$b^2_{i\ rms,\ rel}$	$\overline{u_{i\ conc,rel}}$	$u_{i,b}$
DOC 1 mg/L	1.547E-01	0.130900687	3.5	118	18.1	1277	3.5	35.9
DOC 7 mg/L	1.492E-01	0.129665409	3.5	115	15.1			
DOC 7 mg/L+SPM	1.384E-01	0.129820511	3.5	107	6.6			
Natural 1*	1.401E-01	0.130014389	3.5	108	7.8			
Natural 2*	1.323E-01	0.130662494	3.5	101	1.2			
Natural 3*	2.434E-01	0.13252372	3.5	184	83.7			
DOC 1 mg/L	4.244E-01	0.396230635	3.5	107	7.1	20	3.5	5.7
DOC 7 mg/L	4.194E-01	0.397044921	3.5	106	5.6			
DOC 7 mg/L+SPM	4.064E-01	0.39071343	3.5	104	4.0			
Natural 1*	3.937E-01	0.392311536	3.5	100	0.3			
Natural 2*	4.089E-01	0.391848999	3.5	104	4.4			
Natural 3*	3.885E-01	0.3949732	3.5	98	-1.6			
DOC 1 mg/L	1.387E+00	1.3127404	3.5	106	5.6	43	3.5	7.4
DOC 7 mg/L	1.388E+00	1.313743025	3.5	106	5.6			
DOC 7 mg/L+SPM	1.391E+00	1.308696665	3.5	106	6.3			
Natural 1*	1.379E+00	1.309549727	3.5	105	5.3			
Natural 2*	1.388E+00	1.314064308	3.5	106	5.6			
Natural 3*	1.454E+00	1.324511547	3.5	110	9.8			

The expected EEQ values were obtained by summing each target analyte spiked concentration corrected for the ISO 19040-2 stated relative potencies.

Uncertainty evaluation according to ISO 11352

CALUX

		Precision Component	Bias Component	Total Combined Std Uncertainty	
Compound	Concentration Level (ng/L)	%	%	%	**Rounded Relative Expanded Uncertainty (%)**
EEQ bio	0.12-0.38	18.0	24.8	30.6	61
	0.38-1.2	15.8	29.7	33.6	67
	>1.2	24.0	26.0	35.4	71

A-YES

		Precision Component	Bias Component	Total Combined Std Uncertainty	
Compound	Concentration Level (ng/L)	%	%	%	**Rounded Relative Expanded Uncertainty (%)**
EEQ bio	0.14-0.40	43.4	35.9	56.3	110
	0.40-1.4	6.6	5.7	8.7	17
	>1.4	3.2	7.4	8.1	16

Recommendations

- The analysis as sample of a bE2 dose-response curve independently prepared from the calibration curve is helpful in the assessment of the calibration curve validity over batches.
- The preparation of more than a reference plate decreases the risk of discharging all the other samples plates in case the reference curve does not fulfil the acceptance criteria.
- Sensitivity to different compounds is not always stable over the time, it is recommended that the laboratory should determine relative potencies and periodically check them.

Conclusions

- The applied Validation Experimental Design has proven to be fit for purpose for EBMs validation and their validation resulted more aligned to MS Methods validation.
- Matrix, interferents and DOC do not impact on the samples analyses in terms of results and dose-response curves when the implemented procedure is applied as preparation procedure of the samples.
- At concentrations close to the EQS, CALUX bioassay showed better results in terms of precision and bias component, whereas A-YES provided the lowest uncertainties when higher concentrations were considered.

THANKS FOR YOUR ATTENTION!

22nd February 2023

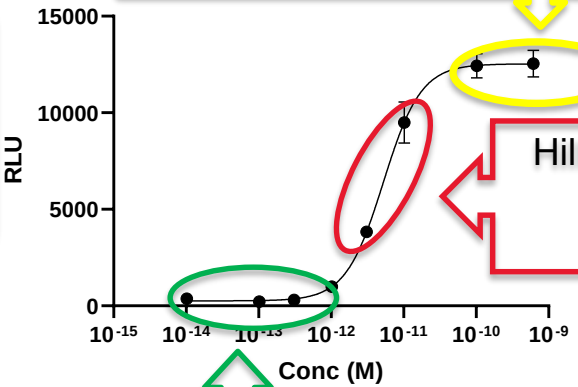
EDC WFD

Fitting: Graph-Pad Prism 9.0 (4)

$$Y = Bottom + \frac{Top - Bottom}{1 + \left(\frac{EC_{50}}{X}\right)^{Hill\ Slope}}$$

Top verified by QC Liv 7 Relative Induction (%)

Cytotoxity at 8th level? Only seven levels are taken into account in the final fitting



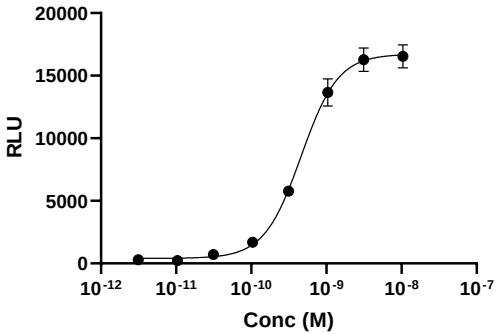
Hill's slope verified by QC Liv 4 interpolation

Bottom verified by X-Control Charts

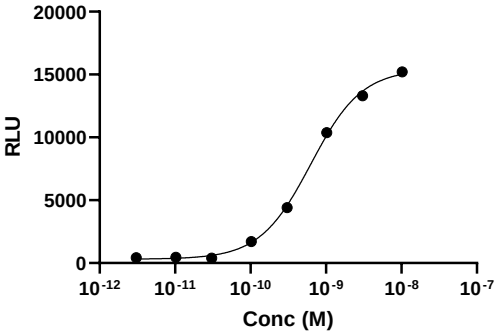
EDC WFD

Relative Potencies Assessment (2)

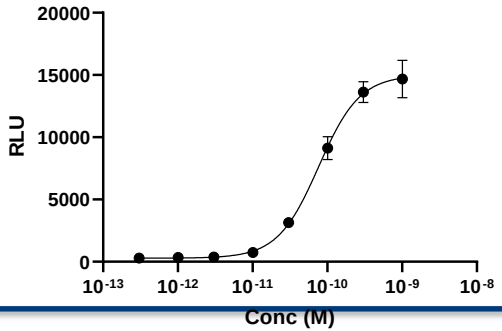
E1 RLU BC-GM 31-05-2022



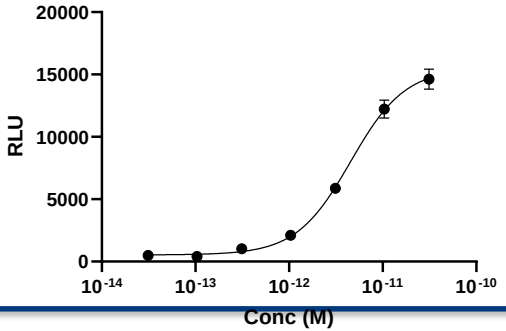
aE2 RLU BC-GM 31-05-2022



E3 RLU BC-GM 31-05-2022



EE2 RLU BC-GM 31-05-2022



EDC WFD

Calibration

Action	Calculations
Calibration: Measure the solvent in use, negative controls and at least 7 calibration levels in triplicate	The calibration function was established by calculating appropriate regression statistics.
Calibration verification:	Calculate the determination coefficient (R^2), the EC50 and the Z-factor (if requested). The following criteria should be verified: Erα-CALUX: R^2>0.98 2.10^{-12}M <EC50< 2.10^{-11}M Z factor > 0.6 A-YES: R^2>0.98 0.3 ng/L <EC50< 35 ng/L