# The PAH-CALUX®; a dedicated bioassay for the detection of carcinogenic PAHs

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## Background

A central mediator with respect to PAH-induced toxicity in vertebrate species is the aryl hydrocarbon receptor (AhR). Mechanistic studies in vertebrate models confirmed the role of this receptor in genotoxicity and carcinogenicity (12), cell cycle regulation (2; 9), and adverse developmental effects (4; 16). Therefore, reporter cell lines for the quantification of PAH-induced, AhR-mediated activity are obvious candidates for toxic potency estimation of PAH-mixtures (e.g.: 1; 8). A well known reporter assay for the quantification of AhR-mediated activity is the DR CALUX. In the current study an optimised CALUX is presented for the detection of PAHs. This cell line will be referred to as the PAH CALUX. Whereas the DR CALUX cell line contains a construct in which the reporter gene luciferase is promoted by a fragment from the mouse CYP1A1 promoter, the promoter in the PAH CALUX is composed of a quadruplicate repeat of DRE from the rat CYP1A1 gene (6;15). With this minimal promoter luciferase expression mediated by other promoter fragments than AhR can be avoided.

## Cell line comparison and exposure time

Since PAHs may be metabolised during the exposure, resulting in a decreased signal, exposure times were assessed for measuring PAHs on both the DR CALUX and the PAH CALUX. Already after 2 hours of exposure a good and steep sigmoidal dose responsive relation is observed in the PAH CALUX assay. No clear maximum could be observed in the DR CALUX cells at this early time point. Upon increasing the incubation period, the PAH-mediated dose-response curves of the DR CALUX cells were becoming less steep and EC50 values more difficult to determine. For the PAH CALUX cells, this decline occurred to some extent at 6 hours and clearly at 24 hours. In all cases the PAH CALUX cell line gave more reliable EC50 estimates.

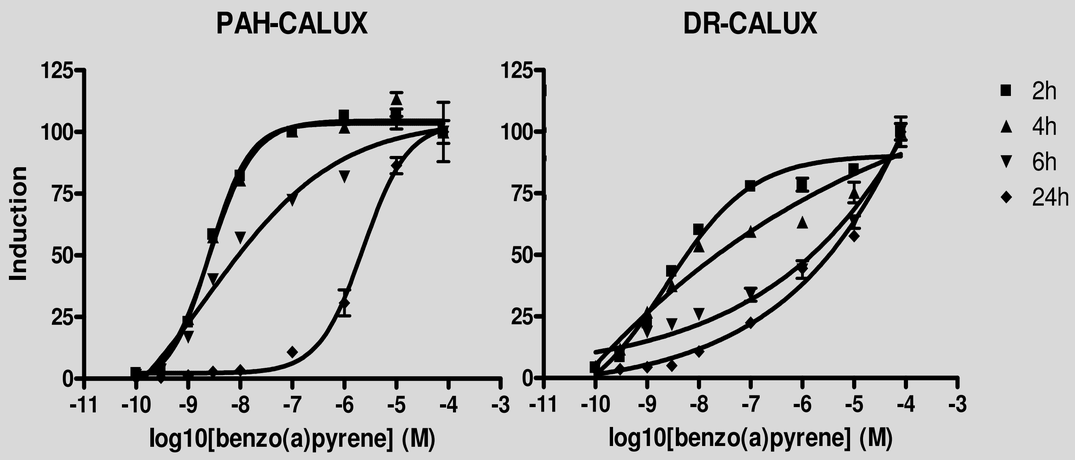


**Figure 1**. Dose response curves for benzo(a)pyrene using the PAH CALUX cell line and the DR CALUX cell line at different exposure times.

## PAH-sensitivity

For all analyses with the PAH CALUX, a benzo(a)pyrene concentration series was included in the experiment as reference. Potencies of individual PAHs are related to BaP with a relative potency value (REP), and the potency of samples is expressed in BaP equivalences (BEQ). Analysis of the EPA-PAHs on the PAH CALUX indicated that not all PAHs are detected by this cell line. There was a clear correspondence with the IARC classification of carcinogenic compounds (3): Whereas PAHs that are not, or very weak ligands for the AhR receptor have been classified as probably not carcinogenic or not classifiable as carcinogenic, the PAHs that are strong ligands for the AhR receptor have been classified as (probable / possible) carcinogens. Tested PAHs that appeared non- or weak ligands for the AhR receptor were designated a toxicity equivalence factor (TEF; relative to benzo(a)pyrene) below 0.01 according to Nisbet and Lagoy (13). At concentrations higher than 10µM some response towards fluoranthene and pyrene was observed. However, no complete dose-response curves could be established for these compounds.

| PAH | MW | Relative potency (PAH CALUX) | IARC classification * | TEF |
|---|---|---|---|---|
| naphtalene | 128 | <0.001 | - | 0.001 |
| acenaphtylene | 152 | <0.001 | - | 0.001 |
| acenaphptene | 154 | <0.001 | 3 | 0.001 |
| fluorene | 166 | <0.001 | 3 | 0.001 |
| phenanthrene | 178 | <0.001 | 3 | 0.001 |
| anthracene | 178 | <0.001 | 3 | 0.01 |
| fluoranthene | 202 | <0.001 | 3 | 0.001 |
| pyrene | 202 | <0.001 | 3 | 0.001 |
| chrysene | 228 | 0.84 | 2B | 0.01 |
| benz(a)anthracene | 228 | 0.42 | 2B | 0.1 |
| benzo(b)fluoranthene | 252 | 13.9 | 2B | 0.1 |
| benzo(k)fluoranthene | 252 | 3.7 | 2B | 0.1 |
| benzo(a)pyrene | 252 | 1 | 1 | 1 |
| indeno(c,d)pyrene | 276 | 1.3 | 2B | 0.1 |
| benzo(ghi)perylene | 276 | <0.001 | 3 | 0.01 |
| dibenzo(a,h)anthracene | 278 | 1.7 | 2A | 5 |

*) IARC classification (1=carcinogenic to human; 2A=probably carcinogenic to humans; 2B=possibly carcinogenic to humans; 3=not classifiable as carcinogenic to humans)

## PAH measurement in mixtures

Since PAHs generally occur in mixtures, good additivity of the contribution of the individual PAHs to the signal that is produced by the assay is important. As is illustrated in Fig. 2, indeed good additivity can be observed for the PAHs that were identified as strong ligands for the Ah-receptor. In coexposures in which one of the strong ligands was used in combination with a weaker or non- ligand (anthracene, pyrene or fluoranthene) a diminishing effect on the total signal could be observed once the concentration of the weaker ligand was over $10^5$ times higher than that of the stronger ligand (results not shown). Since these fold differences are not likely to occur in most samples, this is not considered a problem.

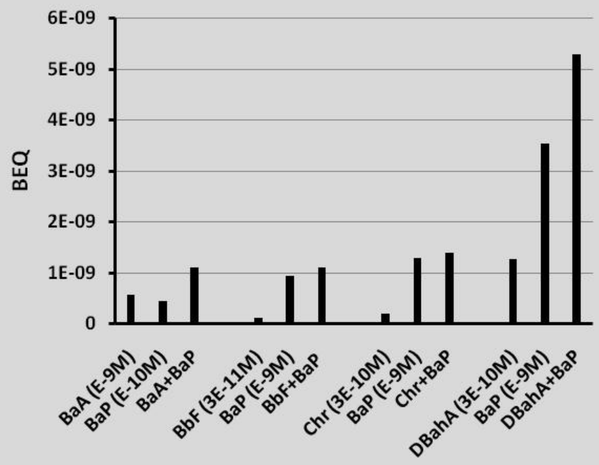


**Figure 2**. Assessment of the additive behaviour of the PAH CALUX by the measurement of two individual PAHs and a combination of identical concentrations of these two. BaA: benzo(a)anthracene; BaP: benzo(a)pyrene; BbF: benzo(b)fluoranthene; Chr: chrysene; DBahA:dibenzo(a,h)anthracene

Additional measurements were performed on eight synthetic mixtures that were composed out of the sixteen EPA-PAHs in equivalent ratios as reported in scientific studies on several soil sites. Moreover BaP-equivalents were determined for extracts from three reference samples. A comparison between the measured BEQ and the theoretical BEQ values, based on the chemical data and the relative potencies, is presented in the table below. The measured values for the synthetic mixtures were between 8 to 59% lower than the calculated values, which is a good performance considering the complexity of the mixtures. The measured values for the extracts from the reference samples were 232 to 416% higher than the theoretical values. This difference is probably introduced by the presence of AhR-ligands other than the limited set of EPA-PAHs that were reported for these samples.

| Mixture (ref) | Measured $BEQ_{PAH}$* | Calculated $BEQ_{PAH}$ | Deviation (%) |
|---|---|---|---|
| **A Industrial site, Holmsund, Sweden (11)** | 0,008 | 0.011 | -27 |
| **B Roadside, Agra, India (10)** | 0,015 | 0.028 | -47 |
| **C Industrial site, France (14)** | 0,011 | 0.012 | -8 |
| **D Industrial site, Germany (14)** | 0,008 | 0.018 | -55 |
| **E Agricultural soil, The Netherlands (5)** | 0,011 | 0.024 | -55 |
| **F Urban soil, United Kingdom (7)** | 0,011 | 0.025 | -56 |
| **G Industrial site, Portugal (14)** | 0,006 | 0.010 | -42 |
| **H Industrial site, Lulea, Sweden (11)** | 0,009 | 0.022 | -59 |
| **LGC6188 (LGC standards)** | 0.155 | 0.067 | 231 |
| **LGC6182 (LGC standards)** | 0.187 | 0.045 | 416 |
| **BCR524 (IRMM)** | 3.056 | 0.934 | 327 |

*) Median value in M $BEQ_{PAH}$.

## Conclusions

The PAH CALUX shows a good dose-reponsiveness towards (probably) carcinogenic PAHs. This trait makes the PAH CALUX a promising bioassay for the *in vitro* detection of the carcinogenic potency of PAH-mixtures.

**References**

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