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The development of rapid immunoassays for the urinary analysis of 1-hydroxypyrene glucuronide facilitate both laboratory and on-site polycyclic aromatic hydrocarbon biomonitoring

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) are produced during the incomplete burning of organic materials. PAH sources include vehicle exhaust, tobacco smoke and waste incineration. Environmental and occupational exposures to PAHs are known to occur. Cancer is a significant endpoint of PAH exposure and several occupations associated with high PAH exposure have been classified by IARC as *carcinogenic to humans* (Group 1).

Pyrene is a common component of PAH mixtures and metabolism of pyrene leads to the excretion of 1-hydroxypyrene glucuronide (1-OHPyrG) in urine. Laboratory measurement of urinary 1-OHPyrG is employed in occupational and environmental biomonitoring programmes. The production of an anti-1-OHPyrG monoclonal antibody would allow the development of a PAH biomonitoring ELISA facilitating large scale laboratory screening and routine testing.

The development of a lateral flow immunoassay and the production of a field test (*point of use test*) would greatly increase the value of biomonitoring. A novel Lateral Flow has been developed which employs an anti-1-OHPyrG sheep monoclonal antibody (Mab) to capture the PAH metabolite. The captured metabolite is visualised through a second Mab raised against the Mab-1-OHPyrG immune complex. This sandwich assay provides a positive correlation between the assay signal and biomarker concentration.

A Smartphone camera allows signal measurement and a carefully considered 'app' provides result interpretation and data analysis. Results are provided in an exposed/not-exposed format. Performance of the lateral flow was confirmed through a comparative study and field trial. The development of a lateral flow test provides "real-time" analysis to occupational health professionals. On-site screening allows the immediate confirmation of safe working practice, provides immediate reassurance to those involved in potentially hazardous activities and greatly increases the efficacy of biomonitoring.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a ubiquitous group containing hundreds of chemically related, environmentally persistent, organic compounds. PAH's are composed of two or more fused benzene rings. PAHs do not contain a hetero atom or carry substituents. PAHs containing up to four rings are referred to as light PAHs and those that contain more than four rings are heavy PAHs (Lawal and Fantke, 2017).

PAHs have limited industrial use and their major source is the incomplete combustion of organic material such as coal, oil and wood (Kim et al., 2013). Occupational exposure may occur in workers involved in oil refining, mining, and iron and steel production. Breathing exhaust fumes may increase exposures for mechanics, street vendors and motor vehicle drivers. Environmental exposure and exposure due to lifestyle habits (tobacco smoke, consumption of grilled food) also occur (Louro et al., 2022). For most people PAH exposure happens on a regular basis.

PAHs exhibit various toxic effects. Different PAHs have been classified as carcinogenic, mutagenic and teratogenic. Most of the individual PAHs are classified by IARC as either *probably carcinogenic to humans* (Group 2A) or *possibly carcinogenic to humans* (Group 2B) (IARC, 2010). Some have been shown to be potent immuno-suppressants. Others may

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lead to CVD development, such as coronary artery disease, peripheral arterial disease, stroke, and myocardial infarction (Mallah et al., 2022). Inhalation is the major route of exposure although ingestion and dermal absorption can occur. There is a need to monitor both occupational and environmental exposure to PAHs.

PAHs tend to occur in complex mixtures containing many similar compounds. Pyrene is a common component of PAH mixtures and tends to be present in relatively high concentrations. In the body pyrene is metabolised to 1-hydroxypyrene (1-OHPyr) and excreted in the urine as 1-OHPyr Glucuronide (1-OHPyrG). Urinary 1-OHPyr correlates well with several genotoxic endpoints (Boogaard, 2012) and urinary analysis has been successfully employed in many biomonitoring studies.

The original method for the analysis of 1-OHPyr was developed by Jongeneelen (1997). His method involved the enzymatic hydrolysis of the urine sample with b-glucuronidase/arylsulfatase followed by a solid phase extraction and chromatography with fluorescence detection. Due to its sensitivity this method became widely employed in both occupational and environmental studies.

Urine samples collected in 1999 and 2000 as part of the USA National Health and Nutrition Examination Survey (NHANES) provide an example of environmental 1-OHPyr concentrations. More than 99 % of these samples contained 1-OHPyr and the overall geometric mean concentration for 1-OHPyr in the USA was 79.8 pg/ml, with a 95 % confidence interval (CI) of 69.0–92.2 pg/ml (Huang et al., 2004). Occupational exposures can be much higher than environmental exposures. For example, employees at a coke oven facility had a median 1-OH-Pyr concentration of 15,400 pg/ml urine (Ochoa-Martinez et al., 2016).

Traditional methods of analysis, such as HPLC with fluorescent detection after enzyme hydrolysis of the urine sample, provide analytical excellence but do not facilitate the development of easy-to-use test kits, the growth of routine laboratory screening programmes and the implementation of *point of use* testing. So, we began to consider an antibody-based approach which would overcome these limitations and increase the availability and value of testing.

Over 95 % or more of the 1-OHPyr is excreted in the urine as the 1-OHPyr-Glucuronide conjugate and our approach was to produce a sheep monoclonal antibody (sMab) that recognised this biomarker. We choose to produce antibodies in sheep rather than rodents because sheep produce ultra-high affinity antibodies to low molecular weight compounds. The affinities of sMabs can be several orders of magnitude higher than mouse Mabs enabling the development of assays with high sensitivity and improved specificity (Osborne et al., 1999).

1-OHPyrG is a small moleculer hapten, which means it only has the size and structure for one antibody to bind to. This limits assay design and leads to the development of competitive negative read immunoassays (meaning that a positive signal would give a negative result and a negative signal a positive result). This understandably is counter intuitive and difficult to quantify. To improve assay performance and provide the option for a positive read sandwich assay an antibody pair would be needed.

This was achieved by raising a second Mab which binds the Mab-1-OHPyrG immune complex, allowing a novel non-competitive ELISA to be developed and a test kit for laboratory analysis manufactured. This test kit will enable the quick and cost-effective analysis of large sample numbers, facilitating the development of environmental population studies and routine occupational testing programmes.

The development of a novel Lateral Flow test will facilitate the production of a *point of use* test kit. The development of a novel *point of use* test and its integration with mobile technology will provide new opportunities and platforms for data storage and handling, result interpretation and presentation and service fulfilment.

The performance of the *point of use* test and Smartphone app was evaluated in a field trial. The field trial was undertaken in collaboration with a company with known PAH exposures. This company already performs biological monitoring and employs a traffic light system to interpret the results based upon the UK HSL Biological Monitoring Guidance Values (BMGV). The UK HSE BMGV of 4μmol 1-hydroxypyrene /mol creatinine is based on the 90th percentile of data from a survey of PAH exposure across a wide range of UK industries.

For the field trial we set a cut-off or an action point of 3umol/mol to help eliminate false negative samples. Samples containing less than 3umol/mol were classified as OK and samples containing more than 3umol/mol were classified as positive. When we employ the lateral flow immunoassay we do not correct for urinary concentration and based upon the ACGIH value of 0.5umol/mol = 1ug/l OH-pyr = 1.8ug/l OHPyrG we set a cut-off of 10ug/l OHPyrG in the app.

Incorporation of the field trial partners requirements and a survey of potential end-users has supported the development of a '*fit for purpose*' tool.

2. Materials and methods

2.1. Immunogen preparation

Sheep were immunised with a custom immunogen consisting of 1-OHPyrG conjugated to Keyhole Limpet Hemocyanin (KLH) produced by Fleet Bioprocessing. Immunisation protocols were carried out by previously described methods (Osborne et al., 1999) according to the UK Animal Scientific Procedures Act.

2.2. Monoclonal antibody production

Monoclonal antibodies were developed as described by Osborne et al. (1999). Candidate hybridomas were first selected for 1-OHPyrG specificity by screening against 1-OHPyrG conjugated to bovine serum albumin (BSA). Top candidates were then selected based on inhibition of their binding by free 1-OHPyrG and cross-reactivity with structurally similar compounds. Chosen hybridomas were subjected to sequential rounds of cloning to improve productivity and cell line stability. Final chosen sheep monoclonal antibodies were purified by protein A affinity chromatography.

2.3. Optimisation of urinary 1-OHPyrG ELISA

A novel ELISA has been developed which employs an anti-1-OHPyrG sheep monoclonal antibody (Mab 1) to capture the PAH metabolite. The captured metabolite is then visualised through a second Mab raised against the Mab-1-OHPyrG immune complex. The anti- Mab-1-OHPyrG immune complex antibody was produced by Bio-rad (Knappik et al., 2000; Prassler et al., 2011). The assay architecture of this non-competitive ELISA is described in Fig. 1.

2.4. ELISA optimisation and characterisation

Chequerboard and antibody binding studies allowed the optimisation of Mab 1 antibody concentration, Mab 1-biomarker complex

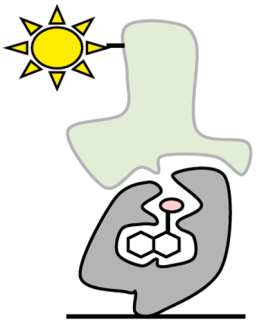

Fig. 1. 1-OHPyrG ELISA Architecture.

antibody concentration and assay diluent. The optimised assay is described below.

Briefly, a 96 well microtitre plates was coated with Mab 1 (2.5 μg/ml, phosphate buffered saline (PBS) pH 7.35) overnight at 4 °C. The wells were emptied and washed with a PBS, Tween20 (0.1 %) solution before blocking with a solution of PBS, Milk Powder (0.2 %) and sucrose (10 %) at 37 °C for ≥ 30 minutes. Wells were emptied and dried overnight at 4°C before use.

To complete an assay 100 μl/well of a 1-OHPyrG calibrator (range 0–1000 pg/ml), or urine sample was pipetted in duplicate, the plate covered and incubated for 1 hour at room temperature. Sample/Calibrator diluent was a solution of PBS, bovine serum albumin (BSA)(1 %) and Tween20 (0.5 %). After 1 hour the plate was washed 3 times with 300 μl/well of wash buffer and tapped dry. 100 μl of the HRP-conjugated anti Mab-1-OHPyrG immune complex antibody (100 ng/ml, PBS, gelatine (0.1 %) and Tween20 (0.5 %)) was added to each well. The plate was incubated for a further 1 hour at room temperature. The plate was emptied and washed 9 times with 300 μl/well wash buffer. 100 μl/well of TMB substrate was added and after incubating for 1 hour at room temperature the reaction was stopped by the addition of 100 μl/well of stop solution. The plate was read at 450 nm using a Thermo Scientific Multiskan FC plate reader.

Urinary matrix effects were eliminated by sample dilution and the use of an assay diluent designed with sufficient buffering capacity to maintain an assay pH of 7.4 and a constant salt concentration.

The optimised assay employs eight standards and allows up to 40 samples to be determined in duplicate.

2.5. The determination of assay characteristics

The optimised ELISA was characterised in terms of Assay Range, Limit of Detection (LOD), Limit of Quantification (LOQ) and Inter- and Intra-assay CV (%). To determine these assay characteristics the assay protocol, described above, was performed with 4 columns of 8 standard concentrations (0, 10, 21.5, 46.5, 100, 215, 465, 1000 pg/ml) and 32 urine samples in duplicate. The same assay was performed in triplicate on separate microtiter plates.

2.6. Assay range

The assay range was determined from the linear portion of the standard curve.

2.7. Limit of Detection (LOD) and Limit of Quantification (LOQ) calculations

The LOD and LOQ of the assay was calculated from the mean and standard deviation of the 12 blank ODs from the standard concentrations across triplicate assay plates.

The following formula was used to calculate the LOD OD:

$$LOD = Mean_{blank} + 3.3 \times SD_{blank}$$

The following formula was used to calculate the LOQ OD:

$$LOQ = Mean_{blank} + 10 \times SD_{blank}$$

The ODs calculated from these formulas were read from the assay standard curve to give the LOD and LOQ concentrations described in Table 1.

Table 1
1-OHPyrG ELISA Assay Characteristics. (The ELISA was developed with sMab 1).

Limit of Detection (LOD)	10 pg/ml
Limit of Quantification (LOQ)	20 pg/ml
Assay Range	20–220 pg/ml
Inter-assay CV	6.10 %
Intra-assay CV	2.60 %

2.8. Intra-assay CV

The mean and standard deviation was calculated for all duplicate ODs (n = 48) from one of the assay plates. The CV was then calculated for each duplicate using the CV formula -

$$CV(\%) = \left(\frac{SD}{\bar{x}}\right) \times 100$$

The mean of all calculated CVs for each duplicate OD was then taken as the intra-assay CV.

2.9. Inter-assay CV

The inter-assay CV was calculated from two standard concentrations (21.5 and 215 pg/ml) across three assays. The mean and standard deviation of repeat (n = 12) ODs at each standard concentration was calculated and the above formula used for the calculation of CV:

The mean of the calculated CVs at both standard concentrations was then taken as the inter-assay CV.

2.10. Assay Specificity

Assay specificity was assessed in competitive binding studies with structurally similar compounds (Fig. 2). These included pyrene, 1-hydroxy-pyrene, 1-amino-pyrene, 1-napthalene-glucuronide and 3-hydroxybenzo-a-pyrene glucuronide. Potential cross-reactants were included in the ELISA at x100 excess concentration.

3. Sample analysis

3.1. Non-occupationally exposed laboratory workers

Urine samples (10–20 ml) were collected from 3 groups of laboratory workers into sterile plastic pots. Samples were immediately frozen and stored at −20 °C until analysis. After thawing and mixing well samples were determined by ELISA using the protocol described above. Based upon a questionnaire samples were classified as non-smokers, smokers and a group of laboratory workers who had ingested a lunch time BBQ.

3.2. ELISA comparative study with potentially exposed workers

A comparative study was completed with support from the UK Health and Safety Laboratory (UK HSL). Post-shift samples (8 h TWA) were collected (approximately 10–20mls in sterile plastic pots) from workers potentially exposed to PAHs. Before providing the sample workers were instructed to remove PPE and wash their hands. Samples were immediately split in to two aliquots and frozen at −20 °C.

Frozen samples were transported at ambient temperature (overnight in cool boxes with ice packs). One aliquot was later thawed, mixed well and determined by ELISA using the protocol described above. The second frozen aliquot was transported to the UK HSL laboratory and determined by an established HPLC-Fluorescence procedure (Unwin et al., 2006).

3.3. Optimisation of urinary 1-OHPyrG Lateral Flow

Sensitive and specific Mabs that recognise 1-OHPyrG (Mab 1) and Mab 1-biomarker complex were developed and characterised by ELISA. These reagents were then employed to develop a simple and rapid Lateral Flow immunoassay (LF). The LF assay architecture is shown in Fig. 3 and briefly described below.

Mab 1 is conjugated to gold nanoparticles and stored in the conjugate pad. A second antibody, with specificity to the Mab 1-Biomarker

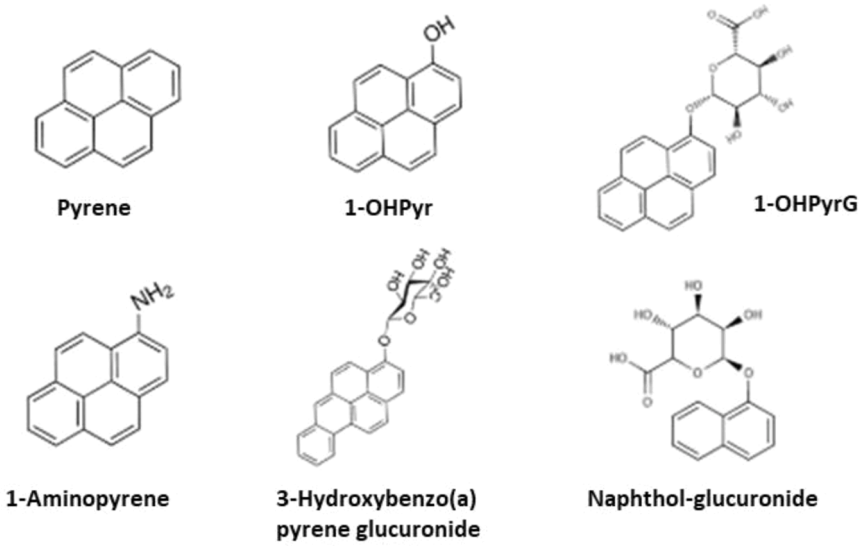

Fig. 2. 1-OHPyrG and Structurally Similar PAHs Employed in Cross-reactivity Studies.

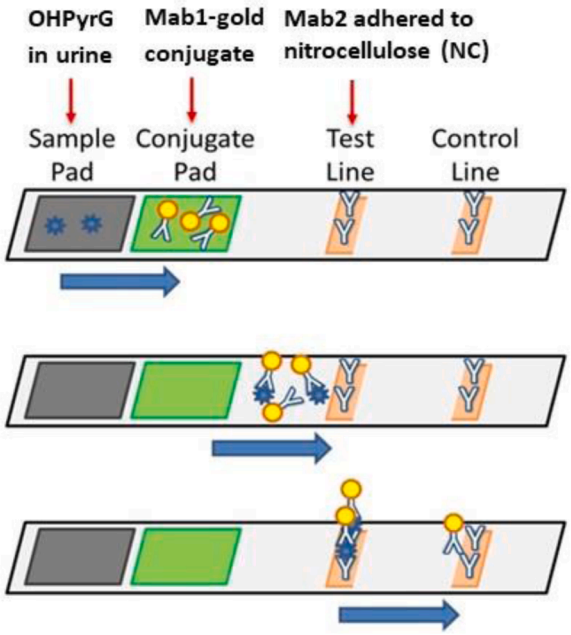

Fig. 3. Lateral Flow Assay Architecture. The gold conjugated sMab1 recognises free 1-OHPyrG in urine. The Mab1-OHPyrG complex is bound by immobilised Mab2. A red stripe proportional to the urinary concentration of OHPyrG develops at the test line.

complex is immobilised on the test line. Urine samples containing 1-OHPyrG are applied to the sample pad and move through the test by capillary action. As the sample moves through the test, the 1-OHPyrG in the sample complexes with the conjugated primary antibody and forms a 'sandwich' with the immobilised second antibody. This concentrates the gold nanoparticles on the test strip, forming a red line. The density of the red line is positively correlated to the amount of biomarker in the sample. A second control line is included to ensure the assay runs correctly, the control line consists of an immobilised rabbit anti-mouse antibody which binds to a gold conjugated mouse antibody in the conjugate mixture.

An optimised test kit has been developed which consists of a test cassette, single use 40 μl plastic pipette, a disposable dropper bottle prefilled with 1560 μl of assay buffer and a mobile phone plus lightbox for recording test results.

The assay is completed by transferring 40 μl of sample with a 40 μl disposable plastic pipette to a dropper bottle containing 1560 μl of assay buffer. The dropper bottle is mixed by gently inverting several times and three drops transferred to the well of the test cassette. The test is incubated at room temperature for 60 mins. After 60 mins the density of the red test and control lines are recorded using the Smartphone camera mounted in the lightbox.

3.4. Lateral flow validation

The repeatability of the LF was determined by inter-assay variation studies employing defined concentrations of 1-OHPyrG standards.

Assay specificity was assessed by competitive binding studies with common structurally similar compounds. Compounds included paracetamol, caffeine, aspirin, vitamin A and gentisic acid (antioxidant in food and pharmaceuticals). These compounds were included in the assay in excess concentrations.

3.5. Lateral flow biological monitoring field trial

A LF comparative study was completed with support from the UK Health and Safety Laboratory (UK HSL). On-site biomonitoring was supervised by an Occupational Hygienist. Post-shift samples (8 h TWA) were collected from workers potentially exposed to PAHs. Before providing the sample workers were instructed to remove PPE and wash their hands. Samples were split in to two aliquots. One aliquot was determined on-site by LF. The LF assay was performed in an office environment by an occupational health professional. The second aliquot was frozen, transferred to the UK HSL and determined by HPLC-Fluorescence (Unwin et al., 2006).

Aliquots determined by LF were recorded as either positive or negative based upon the UK HSE BMGV. Samples recording less than 10 pg/ml of 1-OHPyrG were recorded as negative (non-exposed), Samples containing more than 10 pg/ml of 1-OHPyrG were recorded as positive (exposed). The results were then compared using a 2×2 matrix and the LF Sensitivity and Specificity determined as follows:

	Gold Standard Positive	Gold Standard Negative
LF Positive	TP	FP
LF Negative	FN	TN

$$Sensitivity = \frac{TP}{TP + FN}$$

$$Specificity = \frac{TN}{TN + FP}$$

95 % confidence interval:

$$p \pm z \sqrt{\frac{p(1-p)}{n}}$$

where:
p = sensitivity or specificity value
z = z-value for confidence level (1.96 for 95 % CI)
n = sample size

4. Results

The conjugation of 1-OHPyrG to carrier protein (KLH) has enabled the production **a** sheep Mab (sMab 1) which binds to free 1-OHPyrG in urine. A second Mab which binds the Mab-1-OHPyrG immune complex was produced and a novel urinary non-competitive ELISA developed.

A positive relationship between biomarker concentration and signal generation was observed. As can be seen in Fig. 4 the assay was linear over the measuring range of 20 – 220 pg/ml.

The characteristics of the ELISA are summarised in Table 1 and described below.

4.1. Limit of Detection and Limit of Quantification calculations

The assay of 12 blank samples (mean OD = 0.077 SD = 0.005) enabled the calculation of the ELISA LOD (10 pg/ml) and LOQ (20 pg/ml).

4.2. Intra-assay and Inter-assay CV

The Intra-assay percentage coefficient of variation (CV) for sample duplicates was 2.6 %.

The Inter-assay CV (%) was calculated from two standard concentrations (21.5 and 215 pg/ml) measured across three assays. The Inter-assay CV (%) at 21.5 pg/ml was 7.5 % and the Inter-assay CV (%) at 215 pg/ml was 4.7 %. The mean Inter-assay CV (%) was 6.1 %.

Careful formulation of assay buffers has overcome the heterogeneous nature of urine. Competitive binding studies with structurally similar compounds (Fig. 2) did not cause significant antibody displacement (Fig. 5).

5. Sample analysis

5.1. Non-occupationally exposed workers

Urinary samples collected from three non-occupationally exposed populations are shown in Fig. 6. Here we can see the biomarker concentration in 3 groups of people classified as non-smokers (n = 34, range = 37–499 pg/ml), smokers (n = 9, range = 233–1414 pg/ml) and a group of laboratory workers who ingested a lunch time BBQ (n = 20, range = 275–3135 pg/ml).

5.2. ELISA comparative study with potentially exposed workers

Results obtained in a comparative method study are shown in Fig. 7. During this study occupational samples were collected post-shift and determined by both ELISA and an established HPLC/fluorescence procedure (UK HSE Lab). Urines were determined over a wide range of biomarker concentrations, including some very high occupational samples (n = 20, range = 520–36,000 pg/ml). The correlation coefficient was greater than 0.9.

Having confirmed the characteristics of the antibody reagents in a non-competitive ELISA these were then employed in the development of a LF immunoassay. The LF is described in the Materials and Methods section. In the LF a positive correlation between assay signal and concentration can be visually observed (Fig. 8).

Determining the density of the gold which forms the red test line enabled the development of a semi-quantitative test. An assay developed in LF format has a measuring range of 1–10 pg/ml (Fig. 9).

The LF correlates well with established methods and has allowed the development of an occupational biomonitoring test kit. Like the ELISA the LF shows little or no-cross-reactivity with closely related pyrenes. The LF also demonstrates no cross-reactivity with the following potential con-founders - paracetamol, caffeine, aspirin, vitamin A and gentisic acid.

The performance of the LF has been evaluated in a field trial/comparative study. The field trial was undertaken in collaboration with a heavy industry company with known PAH exposures. A box plot

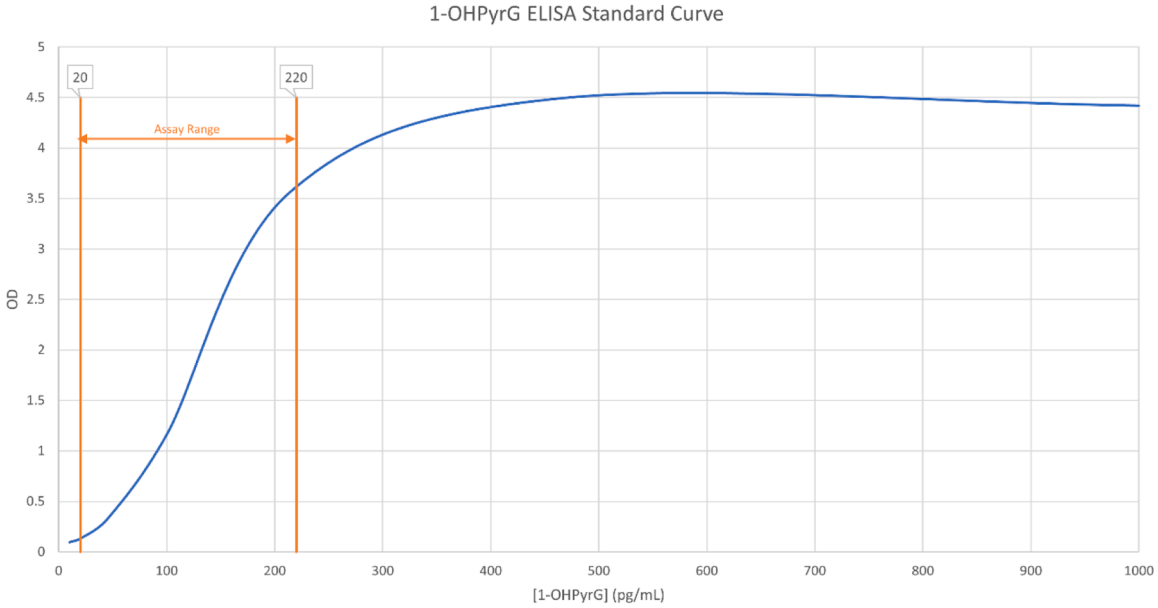

Fig. 4. 1-OHPyrG calibration plot developed with sMab 1. The assay was performed as described in the Materials and Methods.

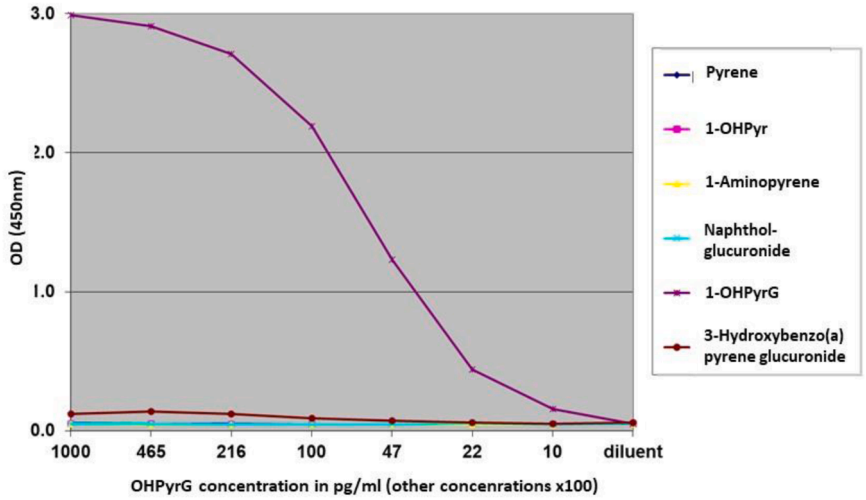

Fig. 5. 1-OHPyrG ELISA Sensitivity & Specificity in Binding Studies. 1-OHPyrG measuring range 10–220 pg/ml. Potential cross-reactants are shown in the legend to the right of the graph.

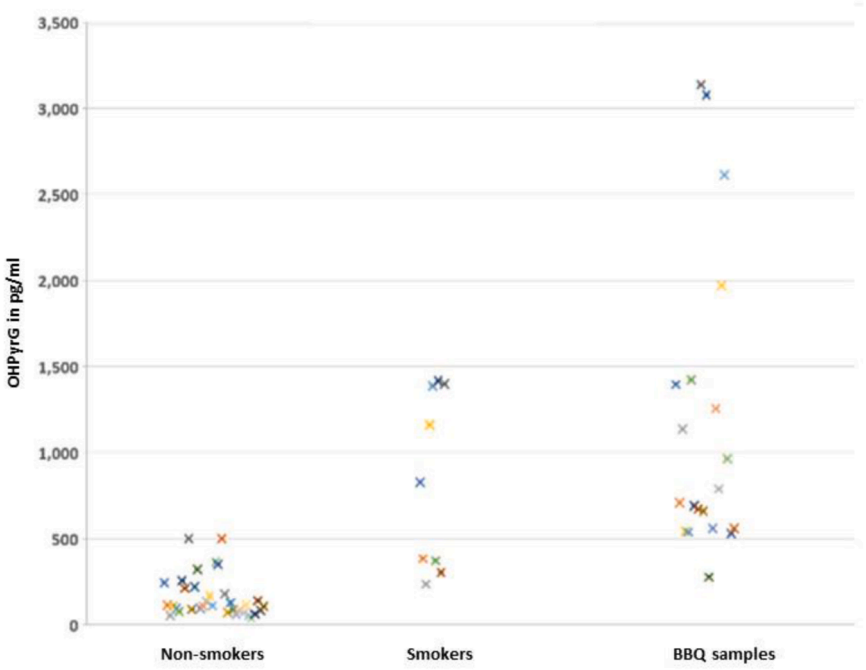

Fig. 6. PAH Biomonitoring Database. Urinary 1-OHPyrG concentrations determined by ELISA for non-smokers, smokers and a group of workers after ingesting a BBQ.

showing the distribution of negative and positive samples as determined by LF testing against a gold standard HPLC-fluorescence method is shown in Fig. 10. The figure also shows the pre-determined cut-off of 10 μg/l.

As can be seen from the 2×2 Contingency Table (Fig. 11) 82 % of the samples were correctly identified, there was 11 false positives (confirmed as negative by the HSL) and only a single false negative. Looking more carefully at the false positives, two contained high concentrations (9.5 and 9.9ug/l) when determined by HPLC-Fluorescence and 10/11 contained high creatinine concentrations (range 10.5–31.2 mmol/l).

Lateral Flow Sensitivity and Specificity is calculated below:

Sensitivity: 95.65 % (95 % CI: 0.8731–1.0000)

Specificity: 75.00 % (95 % CI: 0.6221–0.8779)

6. Discussion

A sensitive and specific sheep monoclonal antibody has been produced to 1-OHPyrG (sMab 1). A second sensitive and specific monoclonal antibody which recognises the Mab1-Biomarker complex has also been developed. These antibodies have enabled the production of two polycyclic aromatic hydrocarbon biomonitoring test kits. An ELISA designed for laboratory use and a LF test, which enables, for the first time, *point of use* testing.

In the ELISA the anti-OHPyrG monoclonal antibody is immobilised to the surface of the microtitre plate well. Calibrators and urine samples containing 1-OHPyrG are incubated with the immobilised sMab 1. The second antibody, conjugated with enzyme allows the immobilised antibody-HOP-G complex to be visualised. Addition of substrate initiates an enzyme reaction and a colour change. The colour change is directly

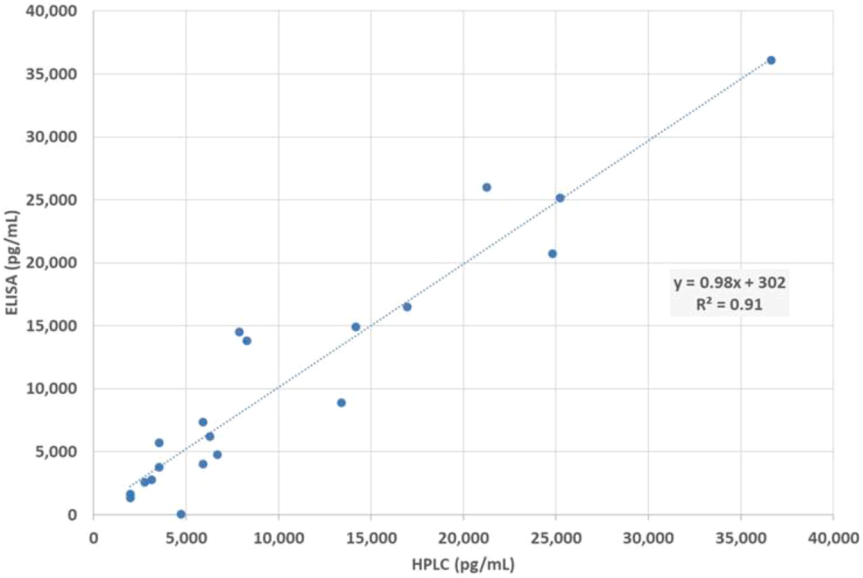

Fig. 7. ELISA Correlation with HPLC/Fluorescence in a Comparative Study. (n = 20, range 520–360 pg/ml, Corr Coeff >0.9).

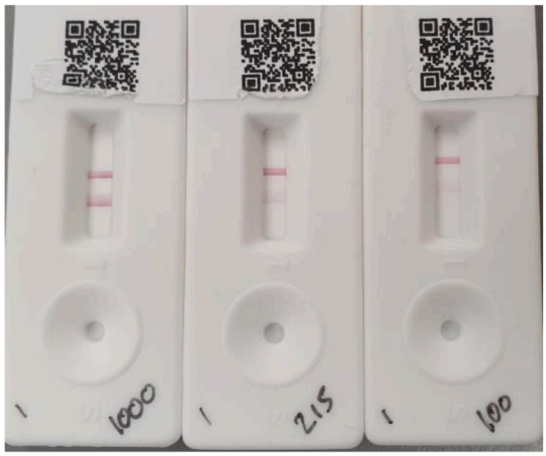

Fig. 8. Results from urinary 1-OHPyrG Lateral Flow Immunoassays. LF cassettes developed at three 1-OHPyrG concentrations (100–1000 pg/ml).

proportional to the amount of biomarker present.

Using one antibody coated microtitre plate and eight calibrators the ELISA allows up to 40 samples to be determined in duplicate in less than 4 hours. The assay has a measuring range of 10–220 pg/ml and is suitable for determining both occupational and environmental exposures to PAHs. Several assays may be performed in parallel enabling the rapid throughput of large batches such as those encountered in screening programmes.

The intra- and inter-assay coefficients of variation (2.6 % and 6.1 %) confirm the repeatability and robustness of the ELISA. While the cross-reactivity data confirm the specificity of the test.

Under the assay conditions employed the assay was able to differentiate between smokers and non-smokers. The determination of three non-occupationally exposed populations – non-smokers, smokers and a group of laboratory workers who had ingested a lunch time BBQ are shown in Fig. 7. As can be seen these samples measure on the linear portion of ELISA calibration plot allowing the accurate determination of low environmental PAH exposures.

According to NHANES the geometric mean concentration for 1-OHPyr in the USA was 79.8 pg/ml, with a 95 % confidence interval (CI) of 69.0–92.2 pg/ml (Huang et al., 2004). In general adult smokers in the USA have urinary 1-OHPyr levels three times higher than those of non-smokers. The measured concentrations for both non-smokers (37–499 pg/ml) and smokers (233–1414 pg/ml) are in-line with literature values (Huang et al., 2004).

According to both NHANES and the German Environmental Survey the 95thpercentile in the general population is around 500 pg/ml. Due to this range of values and the sensitivity of the test, samples often need diluting to bring them within the ELISA measuring range. It is recommended that non-occupationally exposed non-smokers (expected 1-OHPyrG <500 pg/ml) are diluted 1:3 in assay diluent, non-occupationally exposed smokers (expected 1-OHPyrG 500–1500 pg/ml) are diluted 1:10 in assay diluent and occupationally exposed workers (expected 1-OHPyrG >1500 pg/ml) are diluted 1:30 in assay diluent.

In a comparison of quantitative results generated by ELISA and HPLC-Fluorescence the two methods correlate (R=0.91). Confirming the performance of the ELISA test kit. The construction of a database for potentially exposed workers confirms the usefulness of the PAH biomonitoring assay in occupational studies. The sensitivity of the ELISA and the ability to screen large numbers of samples quickly and cost-effectively highlights its usefulness in longitudinal and cross-sectional studies.

Excitingly, the ELISA reagents have enabled the development of a LF test kit. This *point of use* (*point of care*) test will provide health and safety workers with "real time" results. "*On the job*" testing will provide the immediate confirmation of good working practice and immediate reassurance to workers involved in potentially hazardous tasks. This will significantly increase the value or efficacy of occupational biomonitoring.

The LF has a measuring range of 1–10ug/l (Fig. 9) and has enabled the development of a qualitative test kit. A control line is included in the assay and a positive stripe confirms the test procedure has been correctly followed and the test is working as expected. To support this *point of use* test we are developing a Smartphone App.

The Smartphone camera allows the intensity of the lateral flow endpoint to be recorded. To stop interference from outside light and to help position the camera and the cassette we have produced a plastic lightbox. A Smartphone is mounted at one end of the black plastic box and at the opposite end a small aperture allows the LF cassette to be slid into place. Once in place the camera scans the cassette detecting and measuring the colour density of the control line, the sample line and accessing the QR code for test details such as batch number and

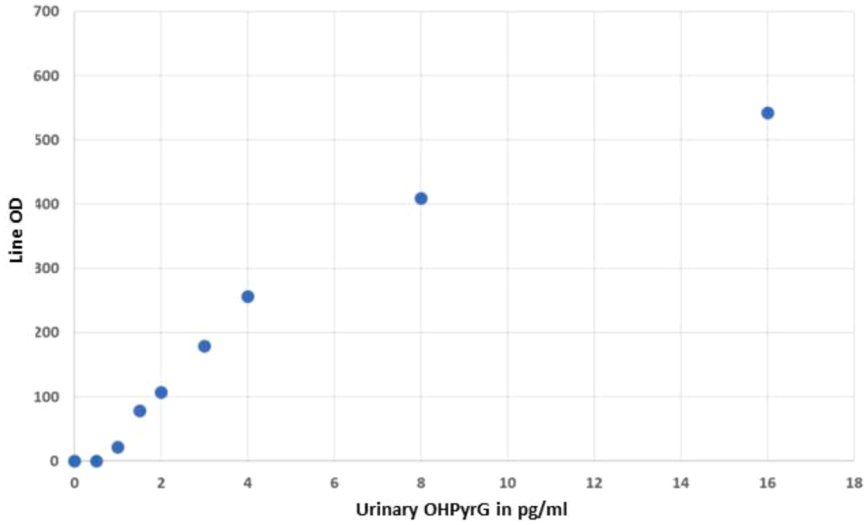

Fig. 9. 1-OHPyrG LF Calibration Plot. The LF immunoassay has a measuring range of 1–10 pg/ml.

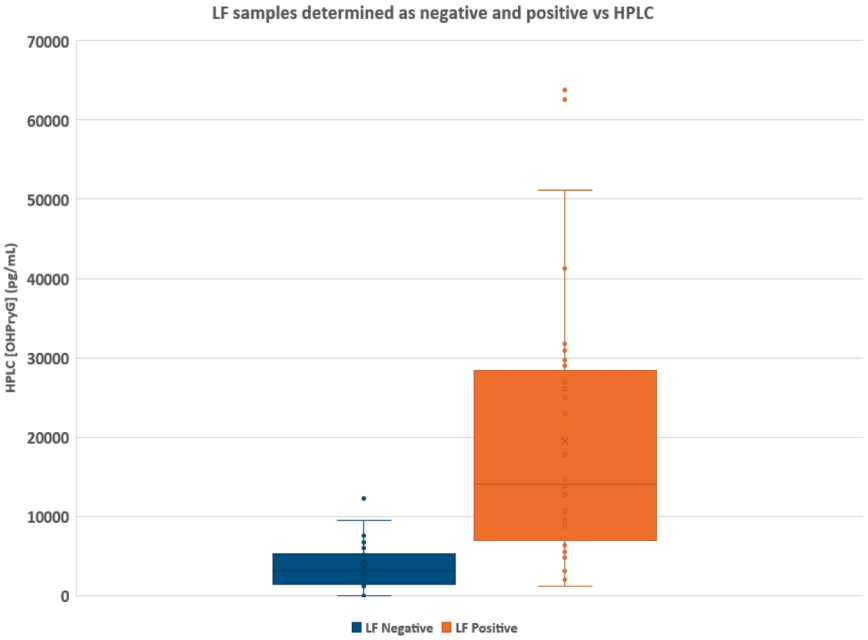

Fig. 10. A box plot showing the distribution of negative and positive samples as determined by Lateral Flow testing against a gold standard HPLC-fluorescence method.

	HPLC-Fluorescence Positive	HPLC-Fluorescence Negative
Lateral Flow Positive	22 (33% of samples)	11 (16% of samples)
Lateral Flow Negative	1 (1% of samples)	33 (49% of samples)

Fig. 11. 2×2 contingency table comparing lateral flow and HPLC-Fluorescence results. This table shows true positive, true negative, false positive and false negative results achieved by the lateral flow.

performance characteristics.

The development of *point of use* tests for biomonitoring and integration with mobile technology provides new methods and platforms for data storage and handling, result interpretation and presentation and service fulfilment. To help develop a "fit for purpose" tool we undertook a survey of potential end-users and have incorporated their answers into the app programme. These include a plot of individual results over time and a record of job description, age, gender and con-founding factors such as smoking and drinking.

The performance of the *point of use* test kit has been evaluated in a field trial. The field trial was undertaken in collaboration with a company with known PAH exposures. This company already performs biological monitoring and employs a traffic light system to interpret the results based upon the UK HSE BMGV 4μmol 1-hydroxypyrene /mol creatinine.

For the field trial/comparative study an action point of 3umol/mol or 10ug/l OHPyrG was set. The *point of use* test was completed by an occupational hygienist in an office environment demonstrating the assay can be successfully completed outside of a laboratory environment.

Samples determined by LF were also determined for 1-OHPyr and Creatinine by the UK HSL. In this field trial/comparative study 67 samples were collected and determined by both LF and HPLC-Fluorescence. Health professionals found the *point of use* test quick and easy to complete and the smartphone camera and app simple to use. As can be seen in Fig. 10, the LF correctly identified 82 % of the samples. Of the correctly identified samples 33/55 were above the cut-off and 22/55 below the cut-off of 10ug/l. There were 11 samples identified as positive by the lateral flow which were determined as negative by the HSL (false positives).

Looking more carefully at the false positives, two contained high concentrations (9.5 and 9.9 pg/ml) when determined by HPLC and 10/11 urines demonstrated high creatinine concentrations (range 10.5–31.2 mmol/l). These elevated values were generally 3–4x the normal creatinine concentration (5–13 mmol/l). There was only 1 false negative highlighting the reliability of the test.

Urinary analysis by LF takes approximately 30 mins providing results in real-time. This enables the immediate confirmation of safe-working practice and provides immediate reassurance to workers involved in potentially hazardous tasks. Positive samples can be re-tested. Positive samples may also be sent for confirmatory analysis. Sending selected samples for laboratory analysis will greatly reduce the costs associated with packaging, transport and laboratory determination and enable a greater number of samples to be determined at more regular intervals. This should greatly increase the benefit and efficacy of biomonitoring.

In conclusion, this study shows that the production of carefully characterised antibody reagents and the development of validated immunoassays contribute to the production of both laboratory and point of use biomonitoring test kits. These simple to employ test kits overcome the limitations of time and cost and support the development of routine testing, population studies and on-site screening services.

CRediT authorship contribution statement

Robert Porter: Writing – review & editing, Validation, Resources, Methodology, Formal analysis, Conceptualization. **Claire Baker:** Methodology. **Harry Carter:** Writing – review & editing, Project administration, Methodology, Investigation, Formal analysis. **Lathan Ball:** Writing – review & editing, Writing – original draft, Investigation, Formal analysis, Conceptualization.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Lathan Ball reports financial support was provided by Bioventix plc. Lathan Ball reports a relationship with Bioventix plc that includes: consulting or advisory and travel reimbursement. If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

Data availability

Data will be made available on request.

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