

# Using exogenous volatile organic compound (EVOC) probes to target tumour-associated aldo-keto reductase activity: a potential tool to detect lung cancer

Mariana Ferreira Leal<sup>1</sup>, Alexandra Martin<sup>2</sup>, Ben Taylor<sup>2</sup>, Rory Stallard<sup>2</sup>, Connor Clarke<sup>2</sup>, Christiaan F Labuschagne<sup>1</sup>, Rob Smith<sup>2</sup>, Matthew Hart<sup>3</sup>, Max Allsworth<sup>3</sup>, Billy Boyle<sup>3</sup>

<sup>1</sup>Owlstone Medical, Clinical and Translational Science, Cambridge, United Kingdom, <sup>2</sup>Owlstone Medical, Analytical Science, Cambridge, United Kingdom, <sup>3</sup>Owlstone Medical, Leadership team, Cambridge, United Kingdom. \*email: breathbiopsy@owlstone.co.uk

## Aims

Investigate aldo-keto reductase (AKR) activity in lung cancer cells as an initial step towards developing an EVOC® Probe-based breath test for cancer screening and early detection by:

- Evaluating the expression of AKRs associated with aldehyde metabolism in lung cancer and non-neoplastic cancer samples.
- Detecting AKR-associated alcohols and aldehydes from in vitro headspace using gas chromatography-mass spectrometry (GC-MS) workflow as used from clinical breath samples.
- Assessing the impact of AKR inhibition on VOC levels in headspace samples.

## 1. Background and Objectives

Cancer metabolism represents a promising and largely untapped focus for diagnostic testing. What's more, while the genomics of cancer can vary extensively, these changes converge onto key metabolic pathways bringing about survival benefits that enable cancer cells to survive in the harsh tumour microenvironment. The use of agents such as UDP-glucose in PET scans serves to illustrate how we can target metabolic pathways with exogenous compounds to provide high sensitivity cancer detection.

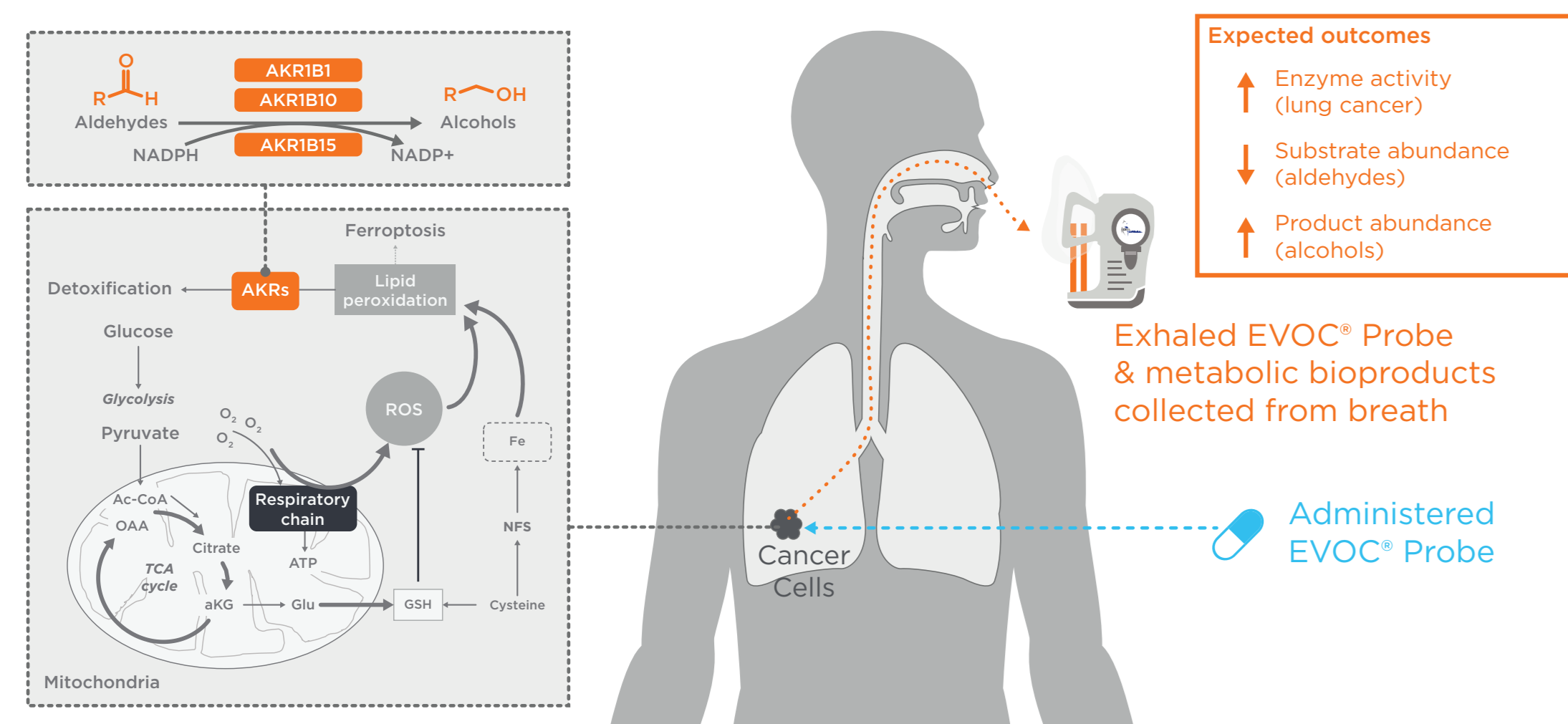
Our hypothesis is that a similar approach can be used in combination with non-invasive breath sampling to provide reliable early detection of cancer in a form that is well suited to the type of screening programme that could dramatically improve cancer survival.

Lung cancer is an area where there is significant unmet need to improve early detection through screening of at risk populations. Incidence and mortality in these cancers are high

and the majority of cases are diagnosed in the later stages. In addition, the location of lung cancers makes it particularly well suited for detection via breath sampling. As such, we have chosen to make this the initial focus of our work.

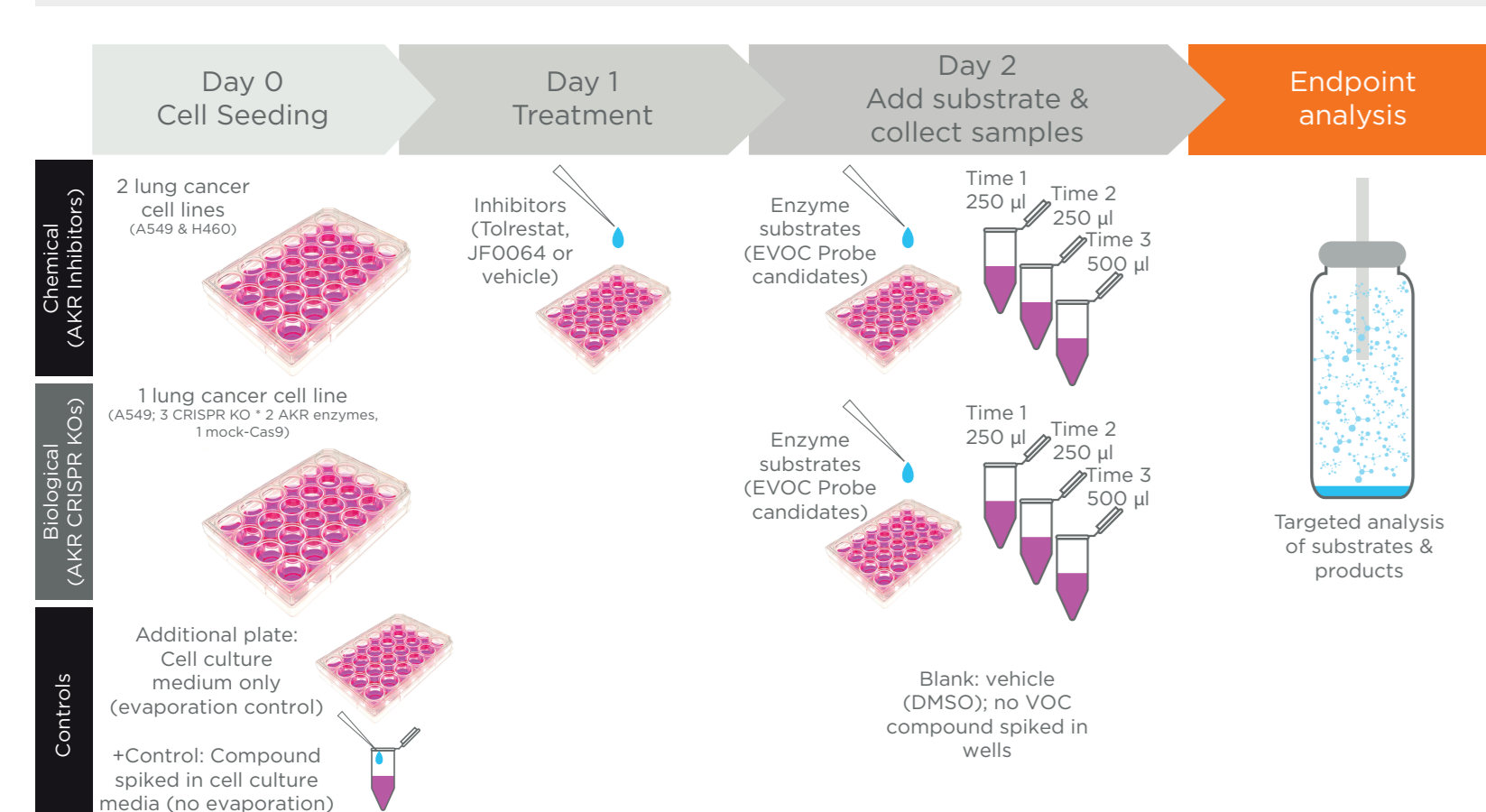
Rapid growth, poor blood flow and persistent genetic errors in cancer cells contribute to a high level of oxidative stress characterized by an increase in reactive oxygen species (ROS). In turn, ROS promote destructive processes such as lipid peroxidation which produce aldehydes. Human lung cancers increase expression of aldo-ketoreductase (AKRs) enzymes to help process these excess aldehydes and reduce them to alcohols.

We are seeking to investigate whether AKRs can be targeted using an exogenous volatile organic compound probe (EVOC® Probe), the metabolism of which can be monitored on breath using Breath Biopsy®.



**Figure 1: The EVOC Probe approach.** An administered EVOC Probe is introduced and monitored non-invasively on breath along with its metabolic bioproducts. Aldo-keto reductases (AKRs) are a potential EVOC Probe target that are upregulated in some cancers as an adaptation to oxidative stress and lipid peroxidation.

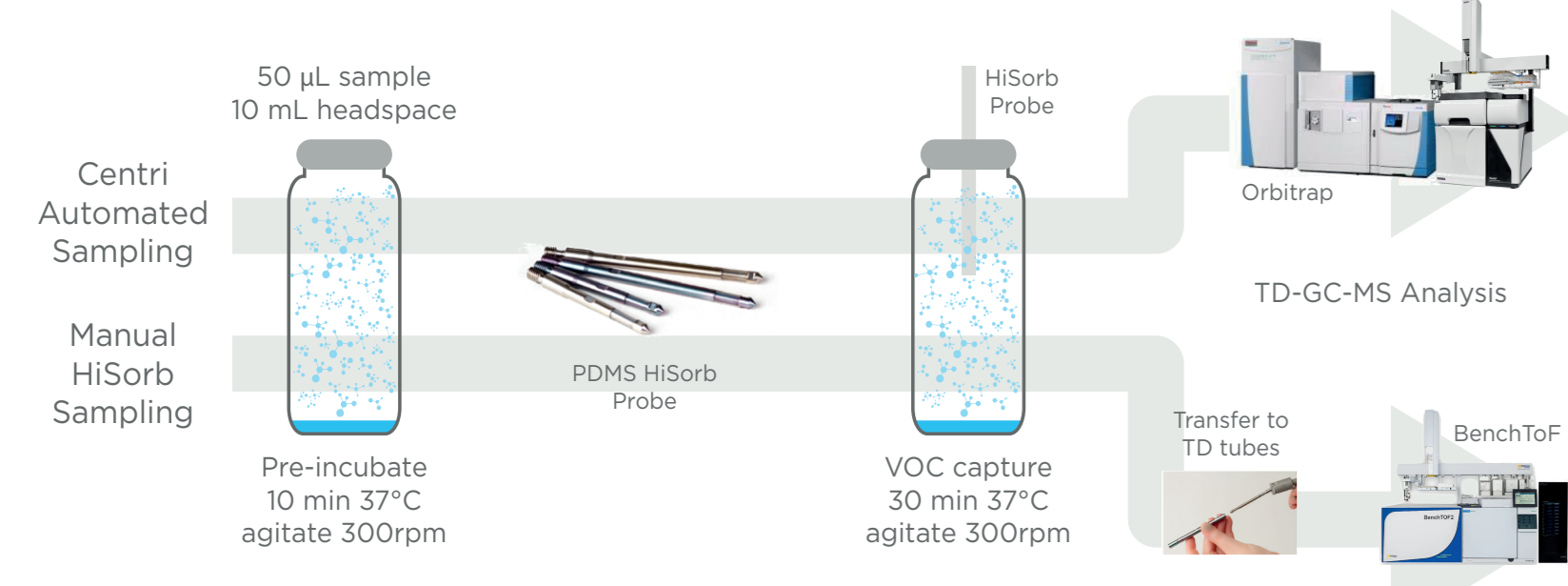
## 2. Method



**Figure 2: Method overview of biological and chemical in vitro methods used to investigate the effect of AKR activity modulation on released headspace VOCs.** For inhibition of the catalytic activity of AKR1B1/B10/B15, two lung cancer cell lines were treated for 24h with two AKR inhibitors (Tolrestat; IC50 and/or IC75 based on AKR activity assay; JF0064; 10µM). For permanently abrogated AKR1B10 or AKR1B15 expression, three clones of AKR1B10 knockout and three clones of AKR1B15 knockout in the background of A549 cells were developed using CRISPR-Cas9 system. A Mock-Cas9 was also used as control (wild-type cells). Cells were treated with 10µM of aldehydes (30 µM for Alkanal 1), and aliquots of cell culture media were collected at 3 timepoints for headspace analysis. Samples were stored at -80°C before analysis. An additional plate was run in parallel in each experiment for the control of evaporation and/or cross-contamination. Aldehydes were added to this plate following the plate layout of each experiment set. Positive controls: compound spiked in cell culture media in a closed vial. Blanks: vehicle DMSO.

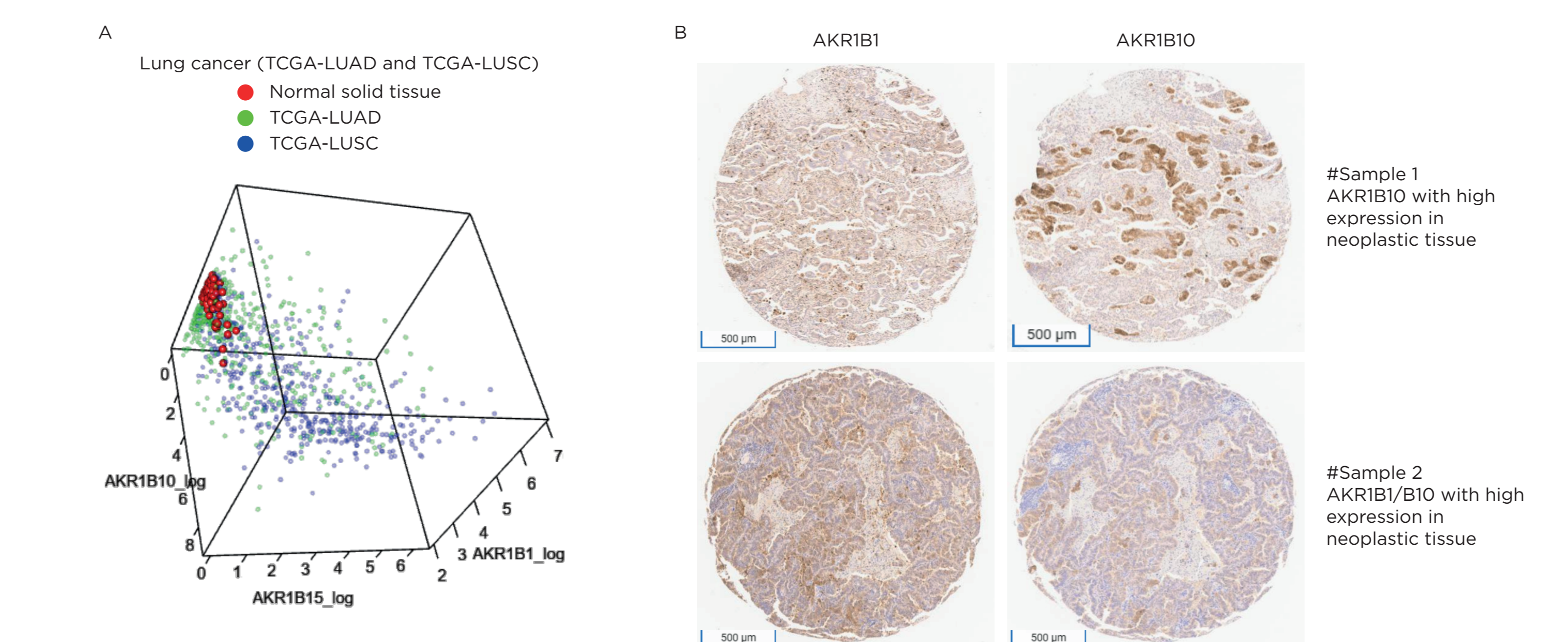
Inhibitor	IC50 (µM)		
	AKR1B15	AKR1B10	AKR1B1
Tolrestat	>100	0.006	0.01
JF0064	0.034 ± 0.005	1	0.3

**Table 1: Inhibitory effect of different compounds on enzymatic activity in non-cell based assay.** D,L-glyceraldehyde as a substrate. JF0064: 2,2',3,3',5,5',6,6'-octafluoro-4,4'-biphenyldiol. Adapted from Giménez-Dejoo et al. (2015; DOI:10.1371/journal.pone.0134506).

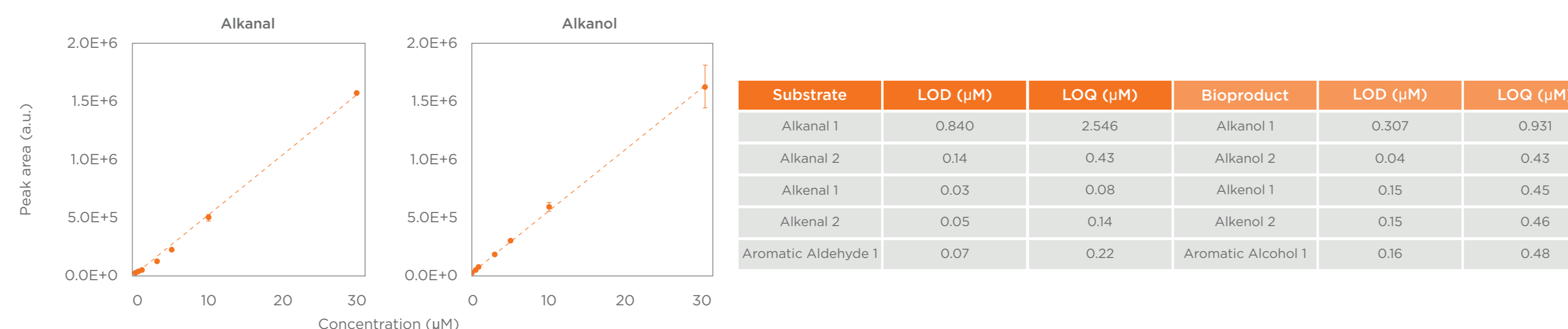


**Figure 3: Automated and manual headspace VOC sampling methods coupled with a mass spectrometry system.** 50 µl of aqueous samples containing the volatiles are used for headspace analysis. Quality control mix (Deuterated standards and a panel of aldehydes and alcohols) are injected onto sorbent tubes and run throughout the sequence. The mass spectrometry method is selected based on the higher sensitivity and specificity for each pair of aldehydes and alcohol products. TD-GC-MS: Thermal desorption-gas chromatography-mass spectrometry. TOF-MS: Time-of-flight mass spectrometry.

## 3. Results and Discussion

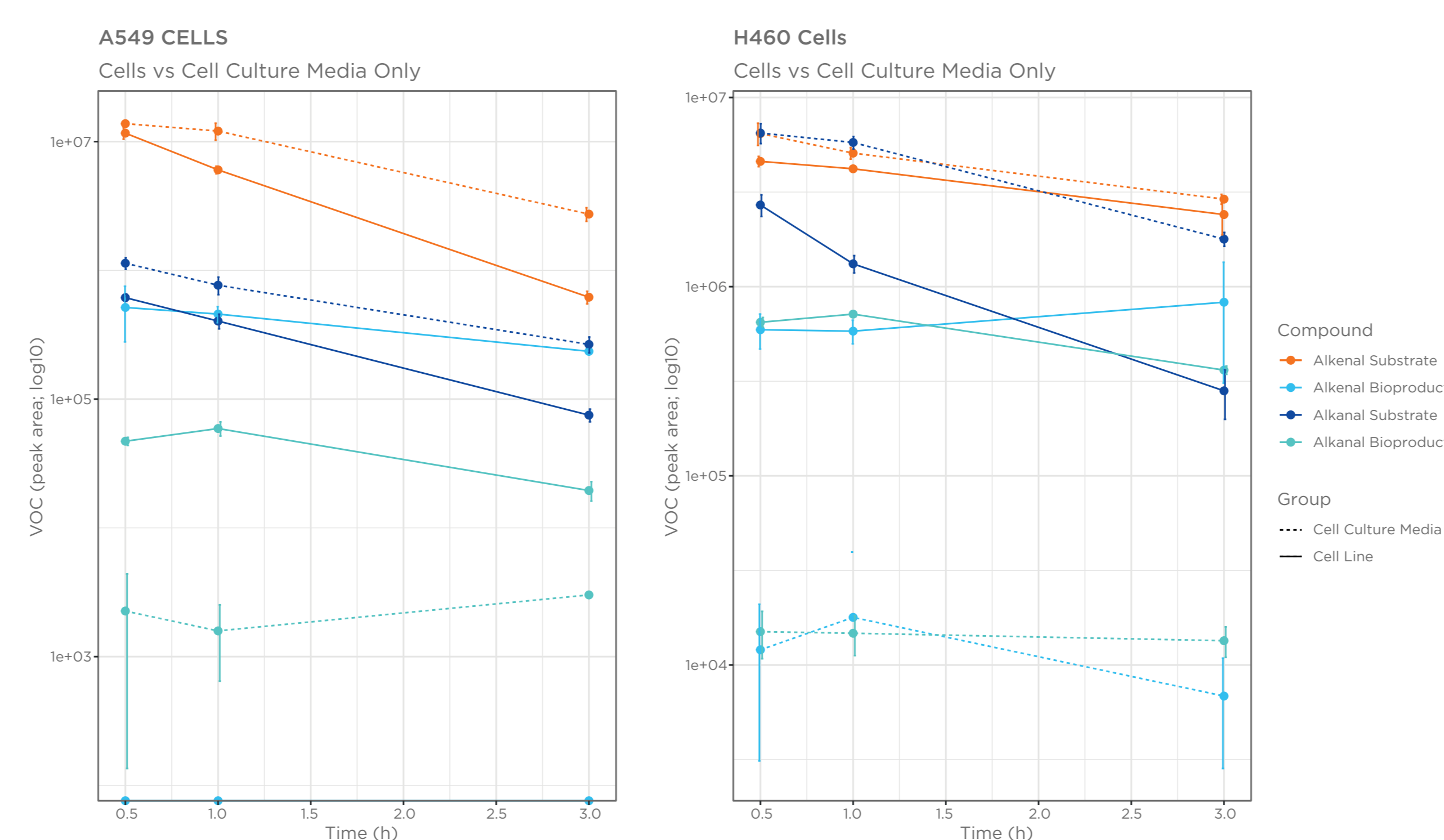


**Figure 4: Expression of AKRs in samples from lung cancer patients.** (A) *AKR1B1*, *AKR1B10* and *AKR1B15* expression in lung cancer samples and non-neoplastic samples (cancer free surgical margin) from patient with lung cancer from TCGA database; most of cancer samples discriminate from non-neoplastic samples based on these three AKR gene expression. (B) Representative immunostaining of a lung cancer specimens showing high expression of *AKR1B1* and/or *AKR1B10* in lung cancer cells than in the surrounding non-neoplastic lung cells. TCGA: The Cancer Genome Atlas (TCGA) (<https://www.cancer.gov/ccg/research/genome-sequencing/tcga>). TCGA-LUAD: Lung adenocarcinoma dataset. TCGA-LUSC: Lung Squamous Cell Carcinoma.

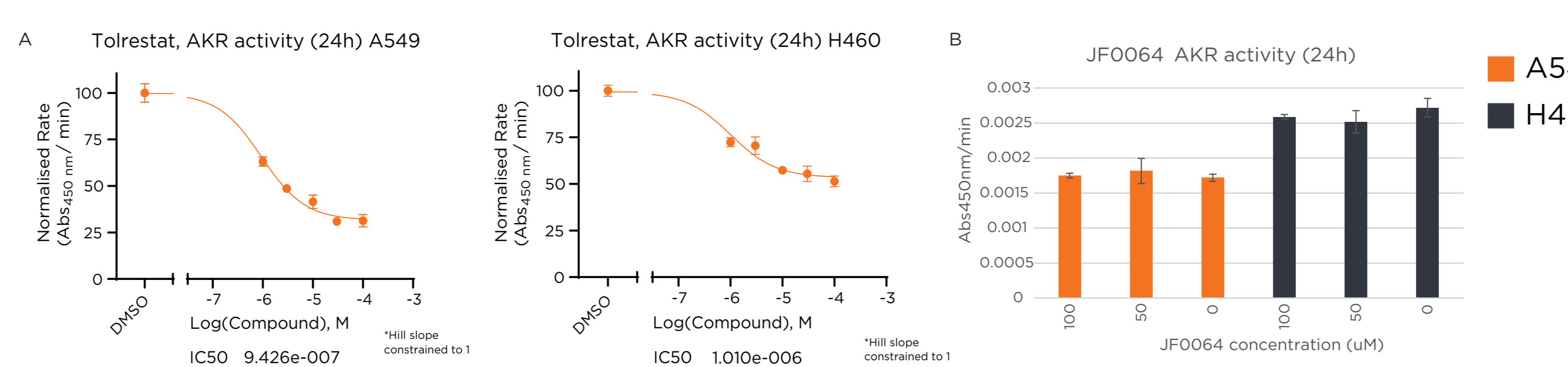


**Figure 5: Example of calibration curves for AKR alkanal substrate and the alkanol bioproduct.** High sensitivity and broad linearity can be observed.

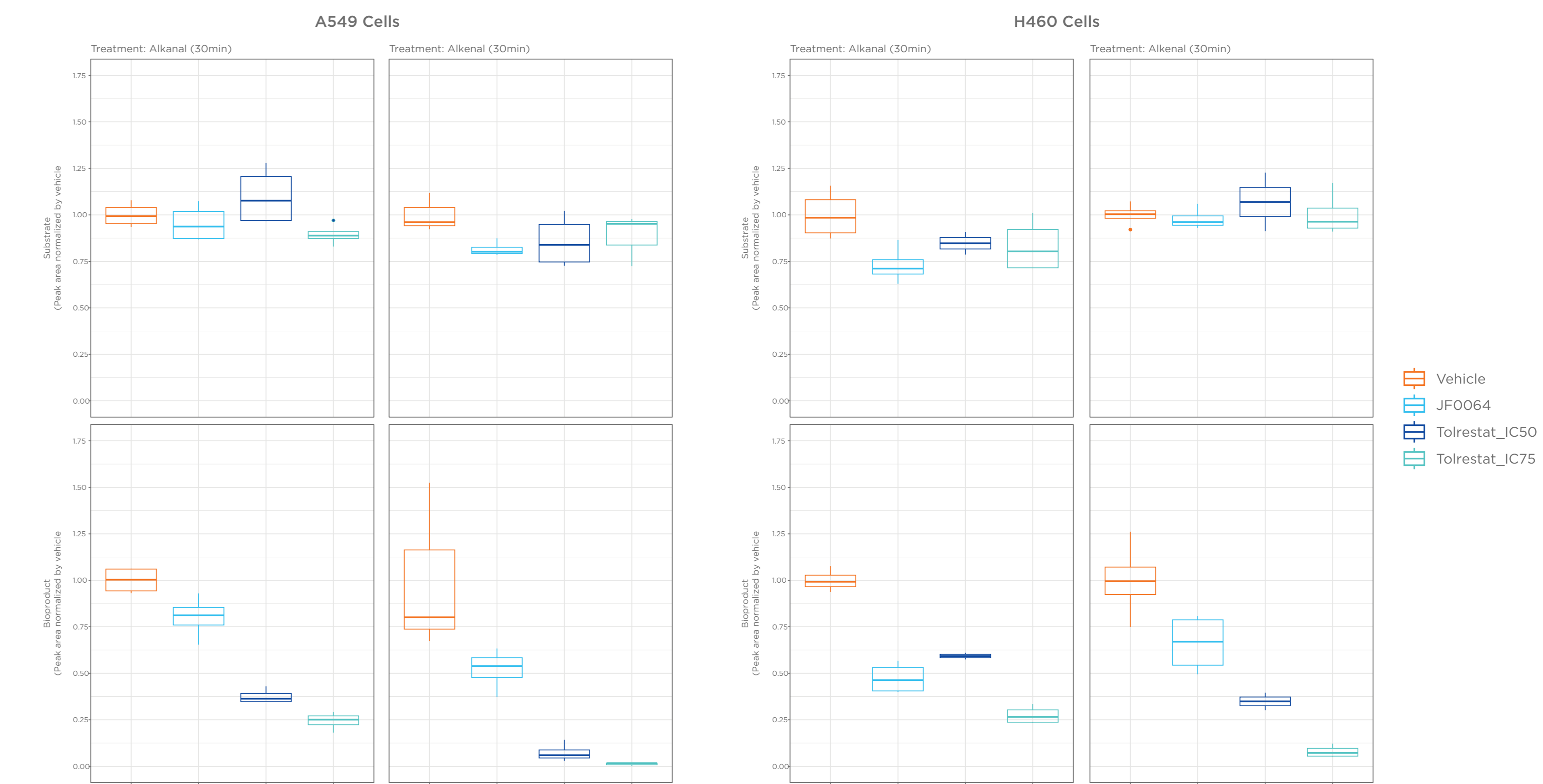
**Table 2: Sensitivity of analytical method to detect aldehyde substrates and alcohol bioproducts using high-capacity PDMS sorbent probes.**



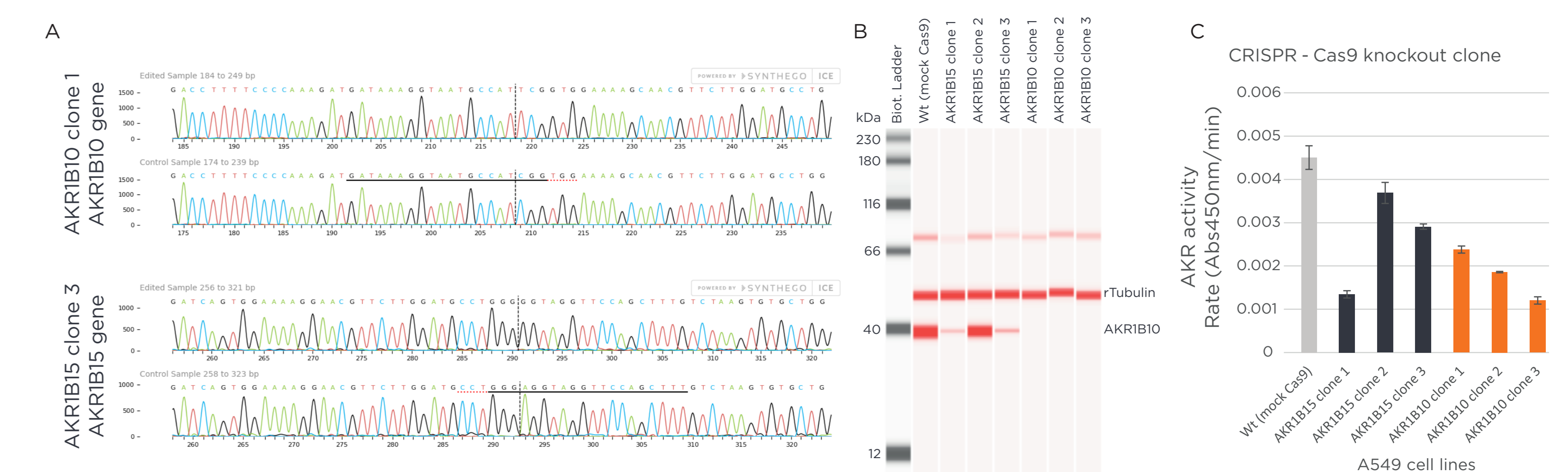
**Figure 6: Monitoring AKR activity in lung cancer cells using aldehydes as EVOC probes.** Aldehydes were added to cell culture media with and without lung cancer cells (evaporation controls) in 24-well plates. Headspace from A549 and H460 samples showed lower levels of aldehydes and higher levels of alcohol products than those observed in evaporation controls confirming that AKRs are active in the studied lung cancer cells and can be monitored using the headspace system. VOC: volatile organic compounds (aldehydes and alcohols).



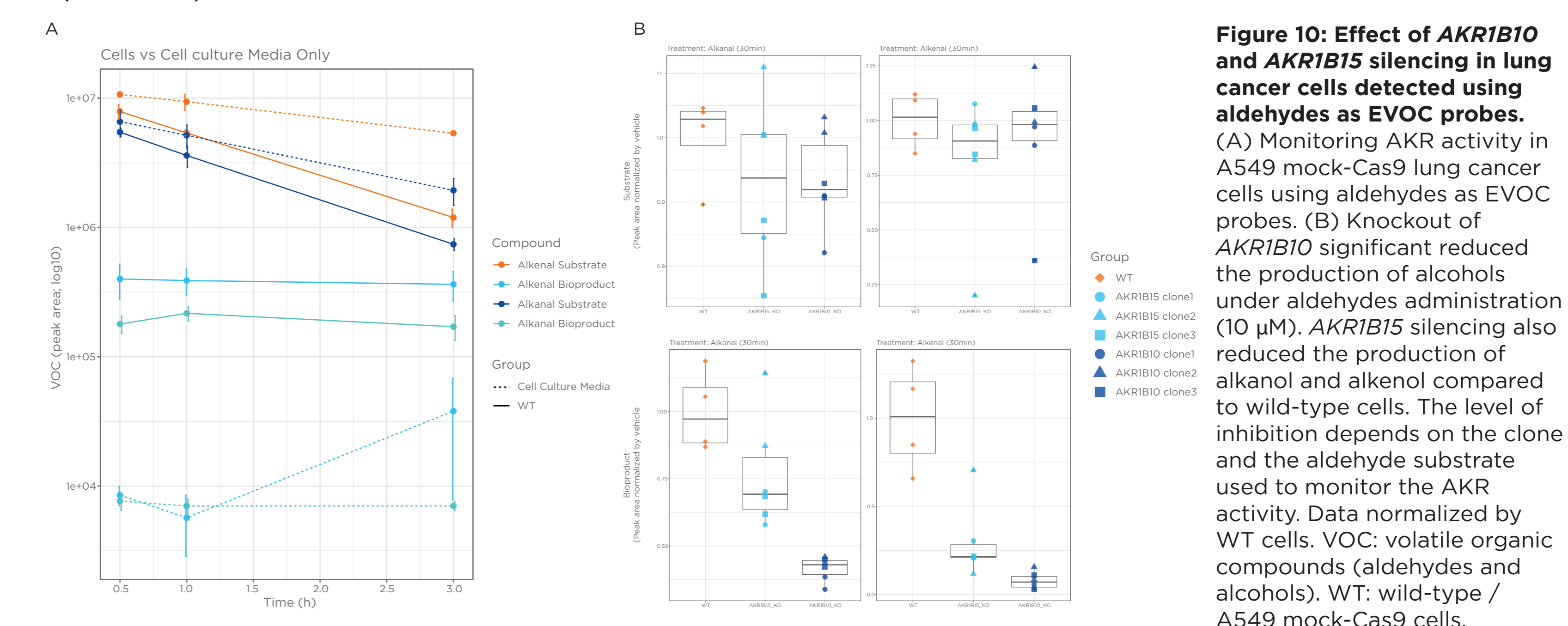
**Figure 7: AKR inhibitor effects in lung cancer cells.** A commercial colorimetric AKR activity assay validated to *AKR1B10*, *AKR1C1* and *AKR1C3* activity analysis was used to evaluate the effect of Tolrestat and JF0064 in two lung cancer cell lines. A) Dose-dependent effect of Tolrestat in AKR activity in A549 and H460 after 24h of treatment. B) JF0064 does not affect overall AKR activity after 24h of treatment in A549 and H460 cell lines.



**Figure 8: Effect of AKR inhibitors in lung cancer cells detected using aldehydes as EVOC probes.** No significant difference is observed in the analysis of substrate abundance; however, a dose-dependent response was observed between Tolrestat and the inhibition of alkanol and alkenol production in A549 and H460 cells treated with alkanal or alkenol 10 µM respectively (30 min). JF0064 also inhibited AKR activity. Different level of inhibitions are observed with this compound treatment depending on the substrate and the cell line used. Data normalized by vehicle.



**Figure 9: A549 *AKR1B10* and *AKR1B15* knockout cells.** (A) Representative images of Sanger sequencing used to confirm the presence of indels in the *AKR1B10/15* knockout; (B) Western blot analysis showing that two *AKR1B15* knockouts (without *AKR1B10* gene edition) show downregulation of *AKR1B10* protein expression. (C) Using a commercial colorimetric AKR activity assay (validated with *AKR1B10*, *AKR1C1* and *AKR1C3*), a reduction in AKR activity of over 50% was detected in *AKR1B10* knockouts and variable levels were observed in *AKR1B15* knockouts. The clone without reduced expression of *AKR1B10* (clone 2) showing AKR activity more similar to wild-type (mock-Cas9) cells.



**Figure 10: Effect of *AKR1B10* and *AKR1B15* silencing in lung cancer cells detected using aldehydes as EVOC probes.** (A) Monitoring AKR activity in A549 mock-Cas9 lung cancer cells using aldehydes as EVOC probes. (B) Knockout of *AKR1B10* significantly reduced the production of alcohols under aldehydes administration (10 µM). *AKR1B15* silencing also reduced the production of alkanol and alkenol compared to wild-type cells. The level of inhibition depends on the clone and the aldehyde substrate used to monitor the AKR activity. Data normalized by WT cells. VOC: volatile organic compounds (aldehydes and alcohols). WT: wild-type / A549 mock-Cas9 cells.

## 4. Conclusions

- AKR enzymes are potential targets for EVOC probes to detect lung cancer.
- We have demonstrated the capacity to detect VOCs from in vitro headspace. Using in vitro study of human lung cancer cells, we have demonstrated the potential to monitor the metabolic conversion of administered EVOC Probe aldehydes into alcohols by AKRs, and have used AKR inhibition/silencing to show that this relationship is specific and sensitive to manipulation.
- Pre-clinical samples were analysed using the same GC-MS workflow used for the detection of VOCs in clinical breath samples (Breath Biopsy workflow). We are now seeking to investigate the same relationships through in vivo and ex vivo sampling to further establish the potential to use EVOC Probes targeting AKR metabolism as a tool for early detection of lung cancer.