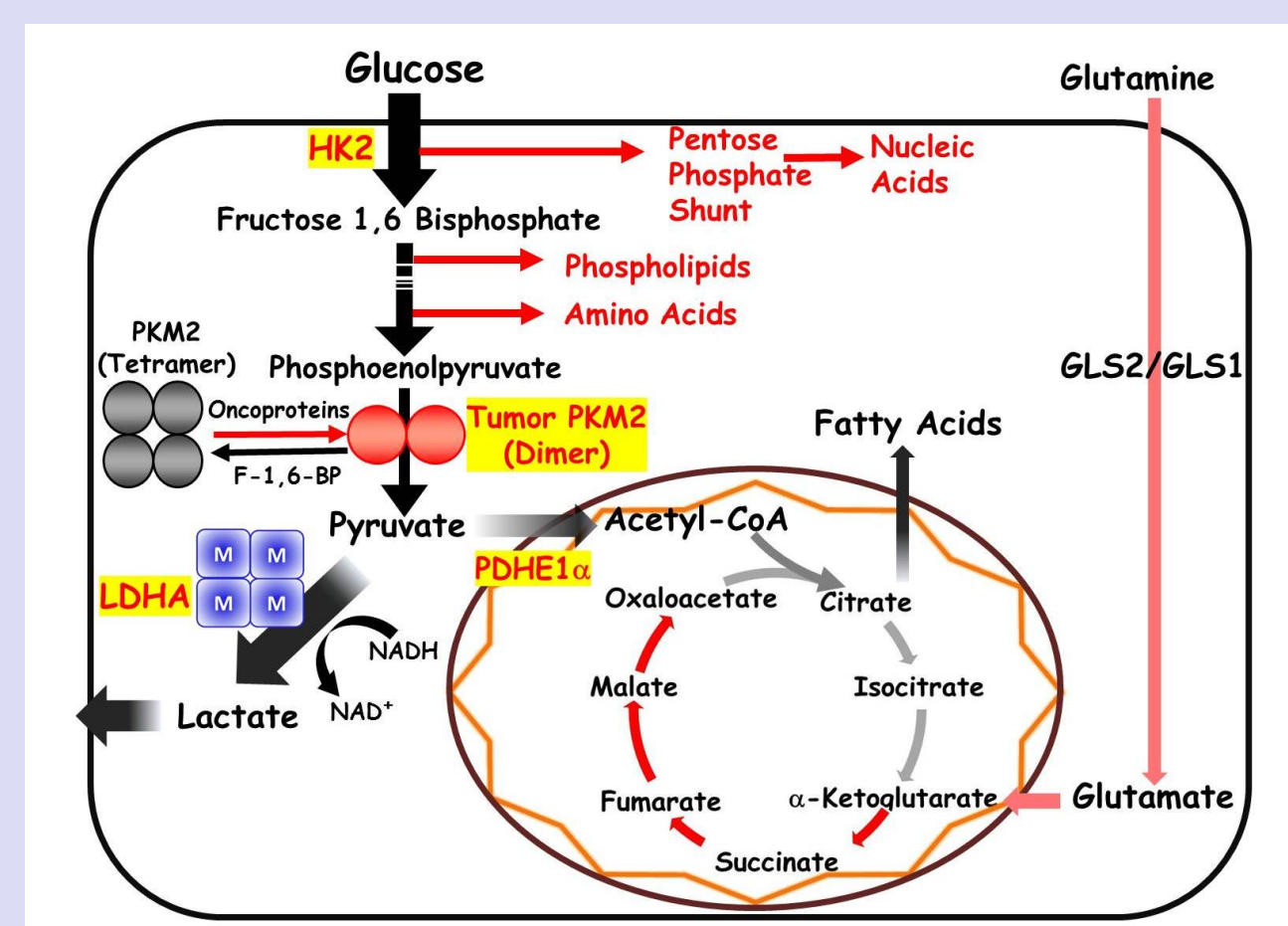




<sup>1</sup>SAIC-Frederick, Frederick National Laboratory for Cancer Research, Frederick, MD;  
<sup>2</sup>Biological Testing Branch, National Cancer Institute, Frederick, MD; and  
<sup>3</sup>Division of Cancer Treatment & Diagnosis, National Cancer Institute, Bethesda, MD

## Introduction

Cancer cells have an altered glucose metabolism that not only permits them to survive under hypoxic conditions, but also confers a distinct growth advantage by sustaining higher proliferation rates, invasiveness, and subsequent distant metastasis. This unique difference in glucose metabolism between healthy and cancerous cells opens up a selective mechanism for killing cancer cells that bypasses normal healthy cells. Consequently, several enzymes in glucose metabolism are attractive targets for the development of first-in-class therapeutic agents. We describe development of a multiplex panel comprising biomarkers involved in glucose metabolism to support drug development and to interrogate the glycolytic pathway in patients with cancer.



**Figure 1. Cancer cells typically exhibit the Warburg effect,<sup>1</sup> in which glycolysis and lactate production are elevated regardless of oxygen availability.** Glycolysis is a series of metabolic processes in which one mole of glucose is catabolized to two moles of pyruvate. As indicated above, cancer cells' reliance on glycolysis is presumably to fuel cell proliferation by diverting intermediates to the biosynthesis of building block nucleic and amino acids. Several intermediates can fuel the pentose phosphate pathway or lead to amino acid production. Accumulation of these intermediates is favored by the rate-limiting activity of Pyruvate Kinase M2 (PKM2), which is overexpressed in many cancers. The majority of pyruvate in cancer cells is converted into lactate, thereby generating NAD<sup>+</sup> from NADH. A small amount of pyruvate can be imported into the mitochondrial matrix to feed the TCA cycle. This step is controlled by PDHE1a, regulating pyruvate conversion into acetyl-CoA and further feeding the TCA cycle. The enzymes highlighted are part of the multiplex panel developed in this study, except for PDHE1a, for which an assay is available commercially on the Luminox platform.

## Materials and Methods

**Immunoassay development.** Antibodies specific to HK2, PKM2, and LDHA were coupled to Magplex Luminescence beads using Mix&Go™ Coupling Reagent (Anteo Diagnostics, Brisbane, Australia). Full-length recombinant HK2, LDHA, and PKM2 proteins were produced in both bacterial and HEK293 cells. Reporter antibodies specific to an epitope distinct from the capture antibodies were labeled with phycoerythrin using Prozyme Phycolink Kit (Hayward, CA) and used for sandwich immunoassay development. The panel is designed to measure protein concentrations in fractionated cell extracts from flash-frozen tumor biopsies.

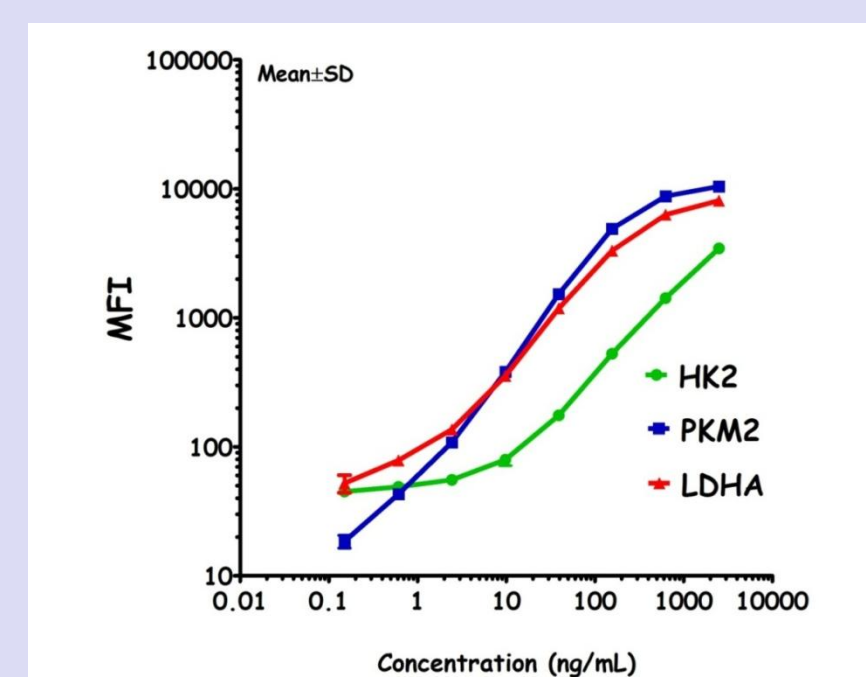
Specific inhibitors of PKM2 and LDHA were used in vitro to demonstrate fit-for-purpose utility of the multiplex panel.

**Cell lines.** Human cancer cell lines HepG2, HCT116, PC-3, A375, MCF7, Jurkat, and SW620 were obtained from American Type Culture Collection (ATCC) or the Division of Cancer Treatment & Diagnosis Repository, National Cancer Institute (NCI), Frederick, MD, and were maintained according to ATCC recommendations.

**Tumor extracts preparation.** Sample handling procedures were developed to generate cytosolic and mitochondrial (+nuclear) fractions of tissue lysates that maintain the non-covalent association of different proteins during the assays.

**Assay optimization and analytical performance.** Assay optimization and analytical validation are ongoing using tumor cell lysates, to address reproducibility, recovery, and sample stability.

## Results



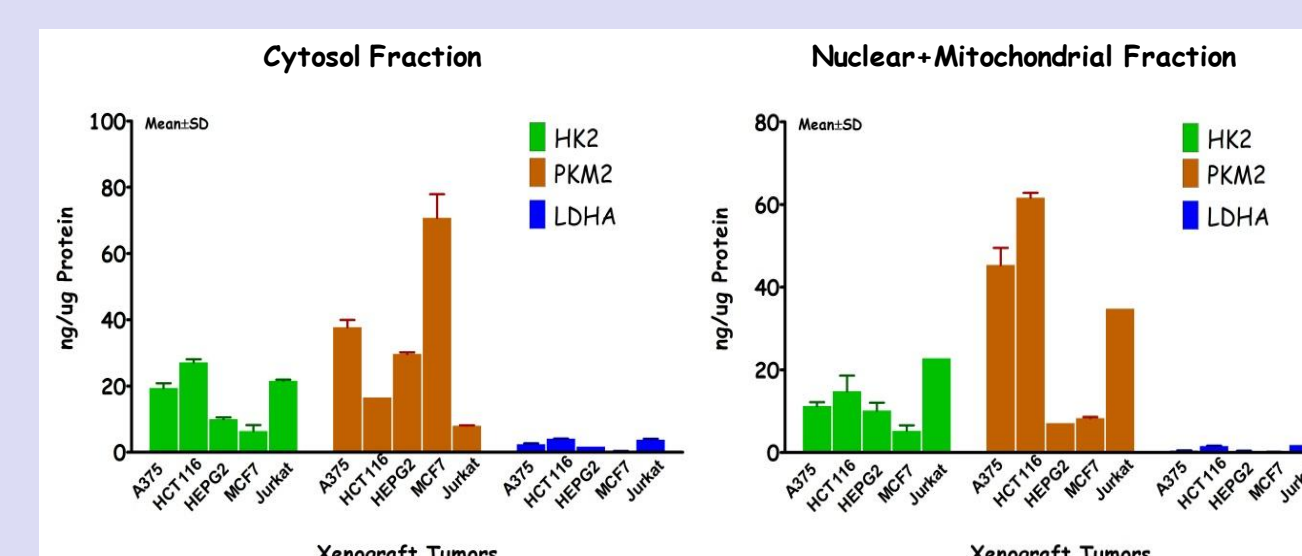
**Figure 2. Calibration curves for HK2, PKM2, and LDHA, using highly specific antibody pairs and full-length recombinant proteins as calibrators.**



**Figure 3. Recombinant PKM2 and LDHA, used as calibrators, exist as higher molecular weight moieties in solution. Sandwich immunoassays capture these high-molecular-weight dimers and tetramers.** Oligomeric forms of recombinant PKM2 and LDHA were stabilized by crosslinking with 0.025% glutaraldehyde for 4 min at 4°C. Proteins were separated on SDS-PAGE (4–20%) and probed by Western blot with (A) anti-PKM2 and (B) anti-LDHA antibodies. Western blot analysis of non-crosslinked (lane 1) and crosslinked (lane 2) proteins indicates that higher molecular weight forms of both PKM2 and LDHA were present in solution.

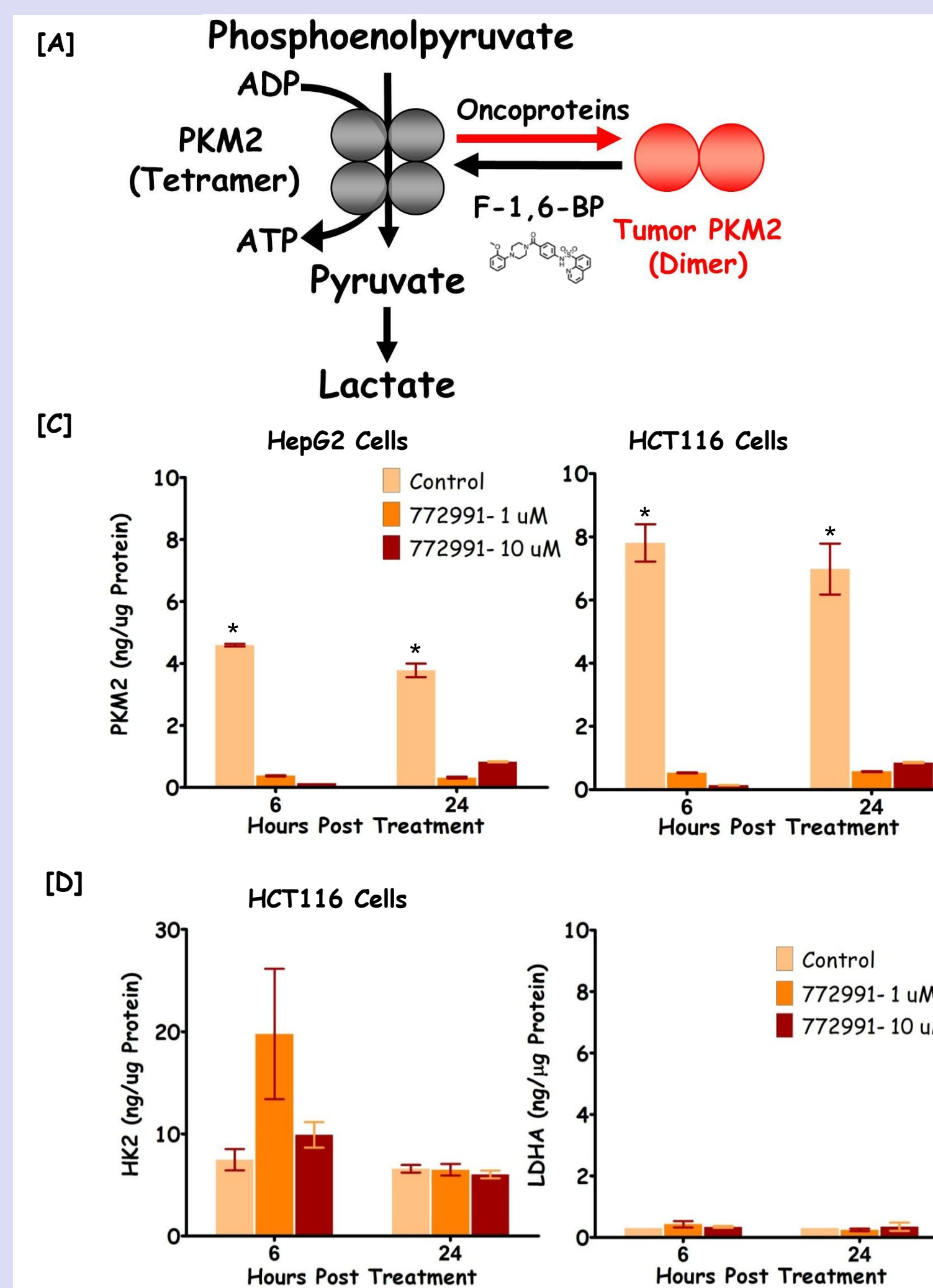
**Table 1. Reproducibility of multiplex panel: within assay variability.**

Dataset	n	Control Level – 1		Control Level – 2	
		Mean ± SD	CV (%)	Mean ± SD	CV (%)
HK2	20	235 ± 42	18	139 ± 30	22
PKM2	20	1211 ± 252	21	671 ± 142	21
LDHA	20	17 ± 3	14	9 ± 1	17



**Figure 4. Levels of glycolytic panel proteins in xenograft tumor samples determined by multiplex panel.** Xenograft tumors were grown in nude mice until they reached a tumor volume of 200–300 mm<sup>3</sup>. Tumor quarters were then harvested and flash frozen. SOPs were developed to obtain fractionated cell extracts from tumor samples. Cytosol and nuclear+mitochondrial fractions were analyzed separately by the multiplex panel at a total protein concentration of 250 µg/mL. As indicated, LDHA was mainly observed in the cytosol fraction, whereas HK2 and PKM2 were observed in both the cytosol and nuclear+mitochondrial fractions (n=2 per xenograft).

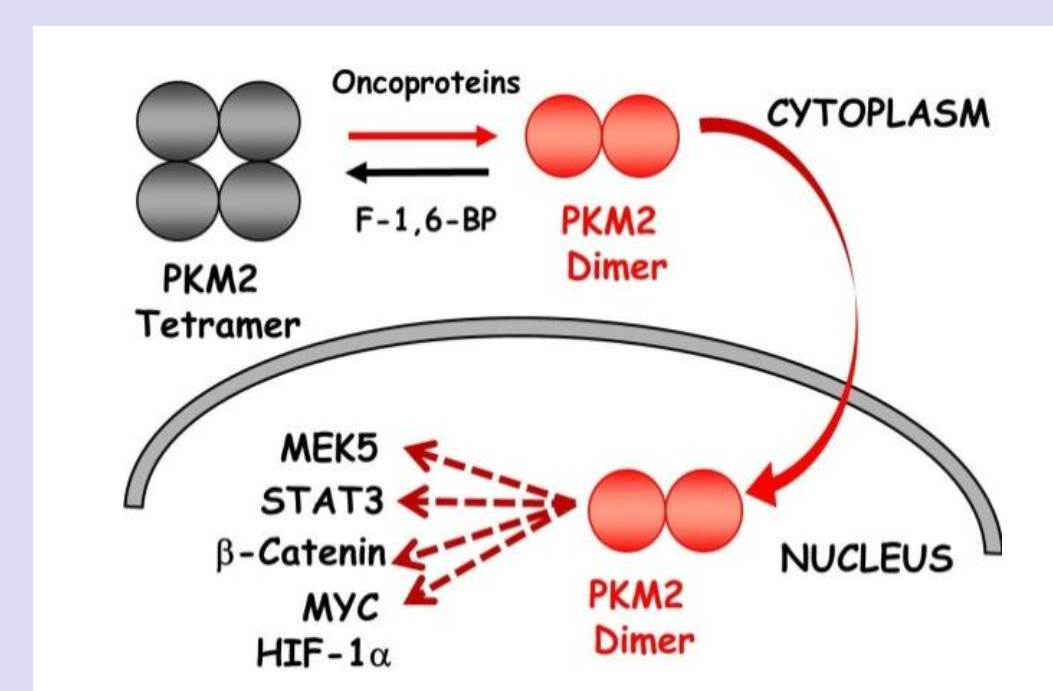
## Pharmacodynamic Assay for PKM2 Activators



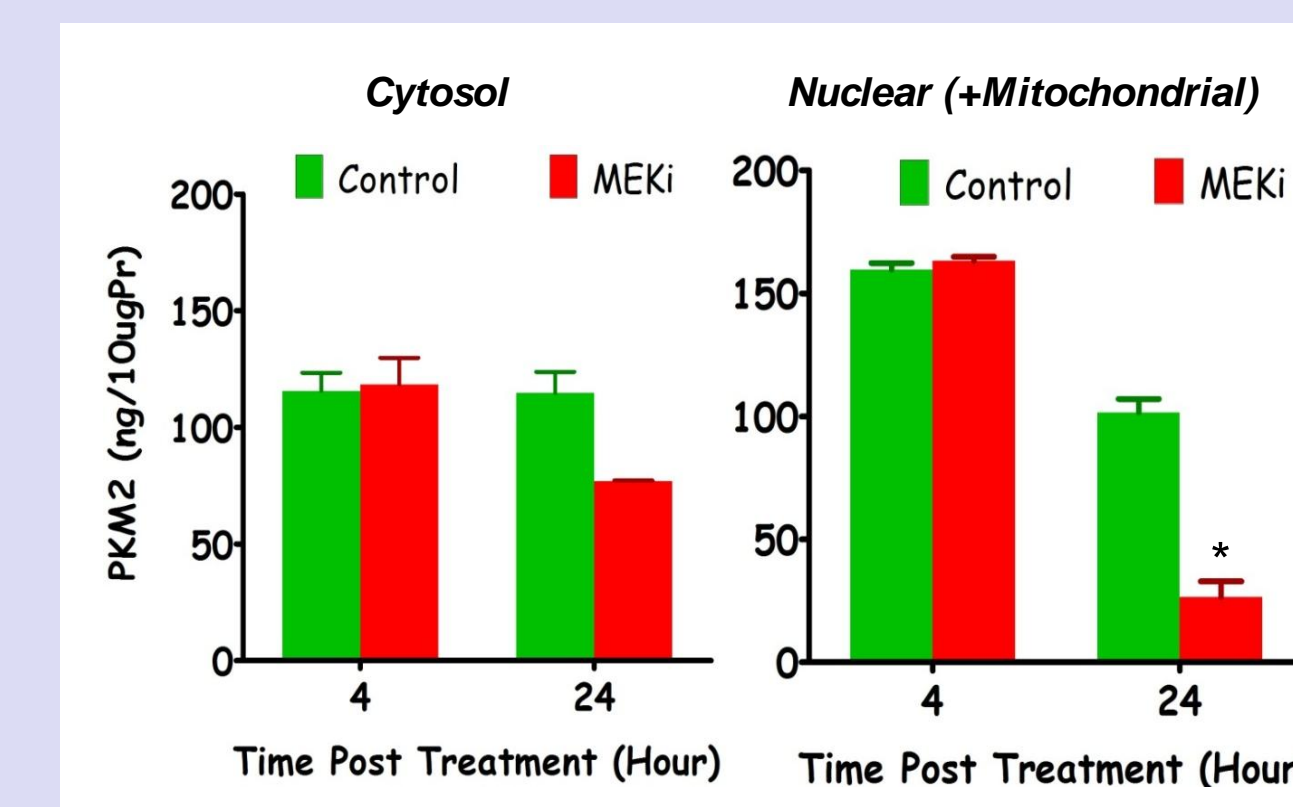
**Figure 5. In vitro pharmacodynamic monitoring of pyruvate kinase M2 (PKM2) by the multiplex panel.** (A) PKM2 is known to exist in two conformations: a tetramer, which is enzymatically active as a pyruvate kinase, and a dimer, which is inactive. In cancer cells, oncoproteins shift tetramer↔dimer equilibrium to inactive dimeric PKM2 by posttranslational modifications (phosphorylation, acetylation, oxidation of PKM2). Conversely, a new class of small molecule PKM2 activators bind to PKM2 subunit interface and enhance association of PKM2 subunits into stable tetramers. Thus, activating PKM2 to a tetramer form<sup>2</sup> that reverses the Warburg effect and impedes the ability of cancer cells to form tumors in mice.<sup>2</sup> (B) We utilized a small-molecule allosteric activator of PKM2, NSC 772991 (compound #9 from Ref. #3) to test in vitro modulation of PKM2. (C & D) HEPG2 (hepatocellular) and HCT116 (colon) human cancer cells were treated with 1 µM and 10 µM of NSC 772991 for 6 and 24 hrs, and the cytosolic fractions of the cell lysates were analyzed by the multiplex panel. (C) PKM2 levels in HepG2 and HCT116 cells treated with NSC772991 were significantly lower compared to vehicle treated controls. (D) HK2 and LDHA levels in HCT116 cell treated with NSC772991. \*p<0.05 for control vs. all drug-treated groups (n=2); F-1,6,BP = Fructose-1,6-bisphosphate

## Nuclear Functions of PKM2

PKM2 also functions as a transcriptional co-activator by translocating to the nucleus to regulate the transcription of downstream genes<sup>4</sup> including β-catenin, MYC, CCND1, HIF-1α, and MEK5. We interrogated this mechanism by measuring PKM2 levels in cells treated with an MEK inhibitor, GSK1120212. Nuclear PKM2 has been reported to exist only as dimer, whereas, cytoplasmic PKM2 exists in dimer↔tetramer equilibrium.



**Figure 6. Nuclear functions of PKM2 are critical for the Warburg effect.**



**Figure 7. ERK1/2-dependent nuclear translocation of PKM2, which promotes the Warburg effect, is blocked by treatment with an MEK inhibitor.** SW620 human colon cancer cells were treated with 100 nM NSC 758246 (GSK1120212) or vehicle for 4 and 24 hrs. After cell lysis, PKM2 protein levels were assayed in fractionated cell extracts by the multiplex panel. PKM2 protein concentrations shown as mean±SD, \*p<0.05 vs. control by student t-test, n=2.

## Summary & Conclusions

➤ We describe the development of a multiplex panel to measure the protein levels of HK2, PKM2, and LDHA in frozen tumor biopsies.

➤ The sandwich immunoassay developed for PKM2 appears to be specific for PKM2 dimer (tumor-specific isoform).

➤ Sample handling SOPs were developed to obtain cytosolic and mitochondrial (+nuclear) fractions from tumor tissue; < 50 µg total protein was sufficient for biomarker analysis.

➤ PKM2 activator treatment of human cancer cells in vitro resulted in decreased levels of PKM2, as determined by the multiplex panel, which was consistent with published observations of decreased pools of dimeric PKM2.<sup>2</sup> These data suggest that the PKM2 assay measures the dimeric form of PKM2 and is ideally suited for pharmacodynamic monitoring of PKM2 activators. However, further evidence will be necessary to confirm the exact oligomeric form of PKM2 measured by the multiplex immunoassay.

➤ The in vitro modulation of markers presented in this study provide proof-of-concept for using this panel to support ongoing therapeutic strategies aimed at targeting the energy provision pathway in patients with cancer.

➤ The panel can also be used to survey metabolic phenotypes of different tumors to identify the types and stages of cancer that are most susceptible to inhibition of glycolysis.

## References

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- Richard A. Ward et al. Design and synthesis of novel lactate dehydrogenase A inhibitors by fragment-based lead generation. *J Med Chem* 55, 3285–3306, 2012.

All animals used in this research project were cared for and used humanely according to the following policies: the U.S. Public Health Service Policy on Humane Care and Use of Animals (2000); the Guide for the Care and Use of Laboratory Animals (1996); and the U.S. Government Principles for Utilization and Care of Vertebrate Animals Used in Testing, Research, and Training (1985). All Frederick National Laboratory animal facilities and the animal program are accredited by the Association for Assessment and Accreditation of Laboratory Animal Care International.

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