Development of a Novel Positive Signal Control for p-AKT and p-ERK

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ABSTRACT

Over the past decade, the use of flow cytometry in clinical studies has grown substantially and has lead to new and innovative flow cytometric methods for biomarker evaluation. Unfortunately there are no commercially available controls to evaluate assay reproducibility and reliability for many biomarkers of interest, especially intracellular proteins. Measurement of phospho (p)-AKT/PKB and p-ERK protein expression has been of increasing interest due to their key roles in cell growth, survival, and proliferation. However, p-AKT/PKB and p-ERK proteins are not readily detected in PBMC samples from normal donors due to the lack of the phosphorylated form under these conditions. In order to validate a method for evaluating these biomarkers by flow cytometry, a control was developed to show a positive signal over background in normal human PBMC. ERK was stimulated with PMA and AKT was stimulated with IGF-1 to produce phosphorylated forms, which can be detected by monoclonal antibodies directed against specific phosphorylated epitopes. The stimulated samples were fixed and frozen and then evaluated at weeks 1 and 2, and months 2, 3, and 4 post stimulation to determine if the signal would be stable and reproducible within the specified storage conditions. Here we present data in which stability and reproducibility (precision) of p-AKT/PKB and p-ERK in a frozen control specimen was evaluated by flow cytometry. Stability testing results showed that subject specimens can be tested up to a 3 month timeframe and precision of less than 10% CV was consistently attained.

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INTRODUCTION

AKT, is considered to link extracellular survival signals (growth factors) with the apoptotic machinery (BAD). Akt is also a key mediator of the metabolic effects of insulin, and often considered an oncogene because it has increased activity in a number of tumors. Phospho-AKT (p-AKT) promotes cell survival by inhibiting apoptosis.

The ERK cascade functions in cellular proliferation, differentiation, and survival. The inappropriate activation of AKT is a common occurrence in human cancers.

The signals for p-AKT and p-ERK can be measured when phosphorylated through stimulation by a broad stimulator, targeted stimulator, by a disease state, or a test compound.

Measurement of p-AKT and p-ERK protein expression has been of increasing interest due to their key roles in cell growth, survival, and proliferation. There are no commercially available controls to evaluate assay reproducibility and reliability for many biomarkers, especially intracellular proteins. In order to validate a method for evaluating the p-AKT and p-ERK biomarkers by flow cytometry, a control was developed to show a positive signal over background in normal human PBMC.

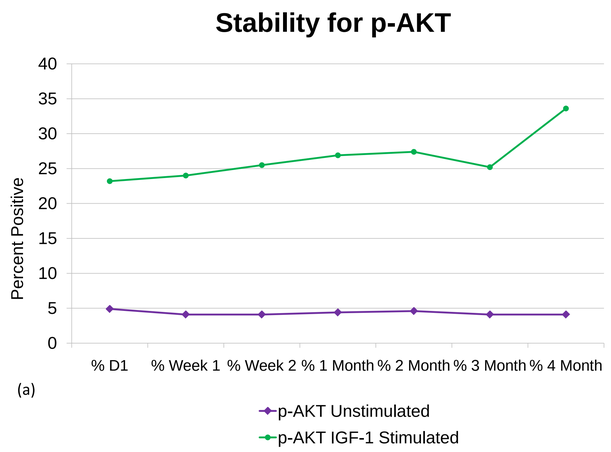
MATERIALS AND METHODS

Stimulation of Cells for p-AKT and p-ERK signal
Cells were stimulated for p-AKT (20 – 25 min) and p-ERK (15 – 30 min) signals. Cells were fixed, washed with Stain Buffer (FBS), and then frozen in 1 mL aliquots in Stain Buffer (FBS) with 10% DMSO. Samples were stored in liquid nitrogen until testing.

Flow Cytometry Staining
On day of testing, cells were thawed, washed and permeabilized. Tubes for isotype and antibody stain were created and incubated for 45 – 60 minutes at room temperature. After incubation, cells were washed and acquired/analyzed on the flow cytometer.

The following antibodies were used: **IgG1 AF488 and IgG1 PE, p-AKT AF488 and p-ERK PE.**

RESULTS



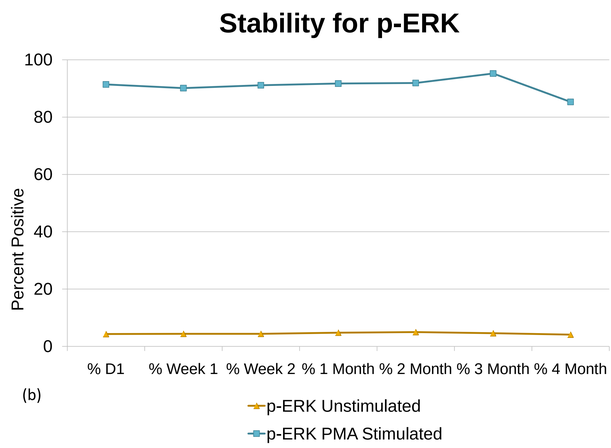
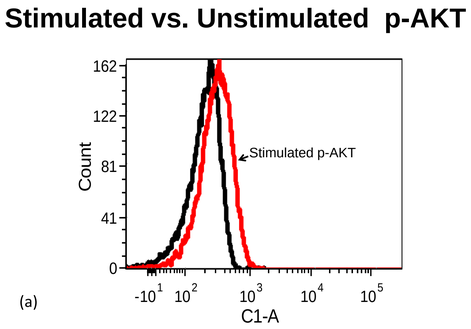


Figure 1. (a) Stability testing for p-AKT showed that the frozen specimens can be stored up to 3 months and compared across this testing range. (b) Stability testing for p-ERK showed that the frozen specimens can be stored up to 3 months and compared across this testing range.



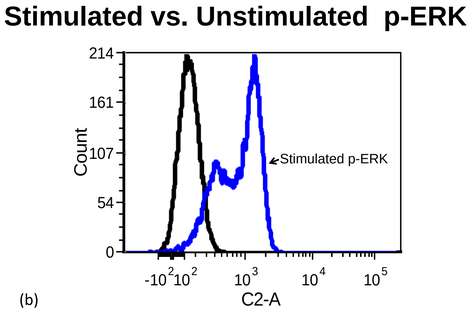


Figure 2. (a) Stimulated p-AKT compared to unstimulated background. (b) Stimulated p-ERK compared to unstimulated background.

RESULTS (cont.)

Inter-Assay Precision

Antibody	Condition	Analyst	Time point	Mean	SD	%CV	Pass/Fail
p-AKT	IGF-1	1,2	1	27.43	1.03	3.74	Pass
p-AKT	IGF-1	1,2	2	27.25	0.83	3.04	Pass
p-AKT	IGF-1	1,2	3	26.83	2.06	7.66	Pass
p-ERK	PMA	1,2	1	54.07	1.38	2.55	Pass
p-ERK	PMA	1,2	2	53.92	1.38	2.55	Pass
p-ERK	PMA	1,2	3	64.28	1.21	1.88	Pass

Table 1. Shows that the p-AKT and p-ERK assay is reproducible with a CV of <10% by multiple analysts and across three time points.

CONCLUSIONS

pAKT: The testing for p-AKT showed a positive signal over background with the degree of positive signal highly dependent on the batch of stimulator. The positive signal ranged from 8.6% - 23.2%.

pERK: The testing for p-ERK showed a positive signal over background ranging between 65.1% - 74.2% depending on the batch prepared.

This testing showed that fixing and freezing stimulated PBMC specimens can preserve a p-AKT and p-ERK signal. In addition, these frozen samples can serve as controls for use to verify the assay used in clinical trials.

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