Advancing Development of Anti-cancer Therapies with Unparalleled Measurement & Monitoring of Protein Kinase Pathways

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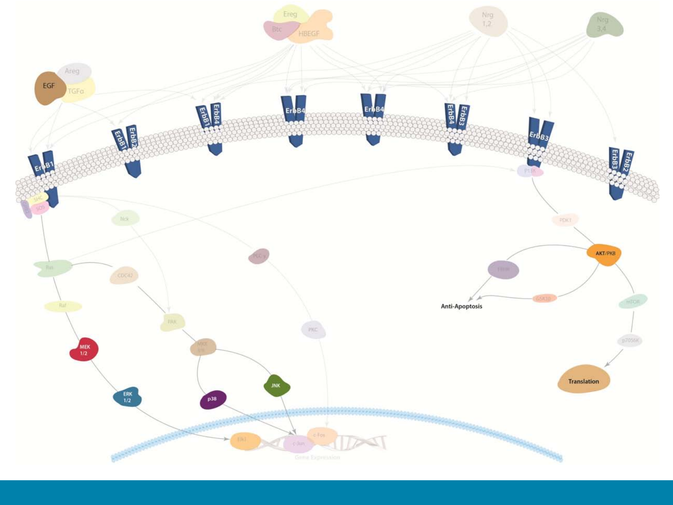
Abstract:
Aberrant activity of the MAP and PI3 kinases is implicated in many forms of cancer. The ability to gather quantifiable information on the activation state of these kinases from limited *in-vitro* and *in-vivo* tumor samples would accelerate drug development and ultimately, the treatment of cancer. Here non-optical, AMMP (acoustic membrane microparticle) technology is used to quantitate the activity state of multiple kinases including EGFR, MEK, ERK, AKT, p38 and JNK in a single assay plate. Lysates from multiple unstimulated tumor cell lines were compared with those from the same cell lines specifically stimulated with ligands to several well-known surface receptors for expressed changes in their phosphorylation states. The data show that the AMMP assay technology can be used to monitor the MAP Kinase pathway activation from EGFR stimulation through ERK phosphorylation following EGF stimulation. Additionally, the activation states of PI3 kinase, p38 and JNK were determined from the same lysate samples. Subsequently, assays were developed to monitor kinase dimerization (e.g., MEK-ERK) by rearranging the pairing of antibodies from the kinase activity assays described above. Using fewer than 2000 cells per assessment, multiple kinases were measured, in their native states, in a single assay using AMMP technology.

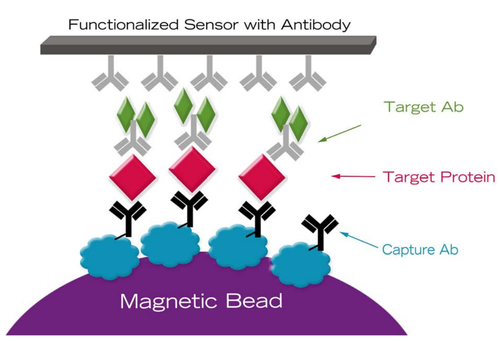


MAP Kinase Pathway

Materials

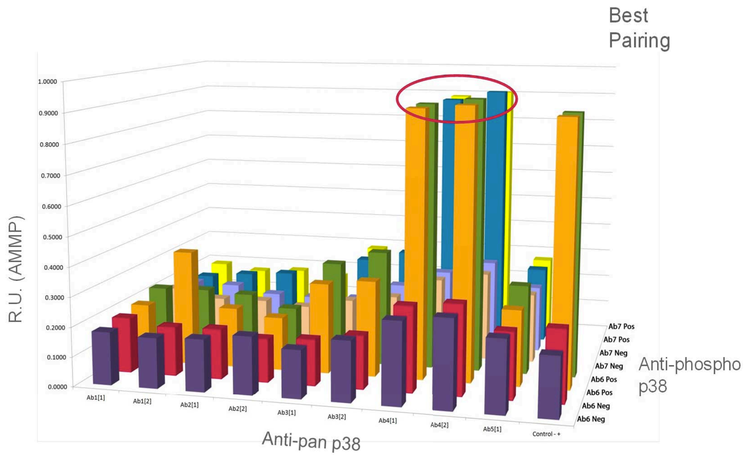
ViBE™ Protein Analysis Workstation

AMMP™ Assay Format



AMMP Assays work at the molecular level using sound versus optics to both sensitively and accurately detect and quantitate proteins.
AMMP measures the resonant frequency shift of a vibrating membrane to interactions at the sensor's surface.

Antibody Pairing Determination

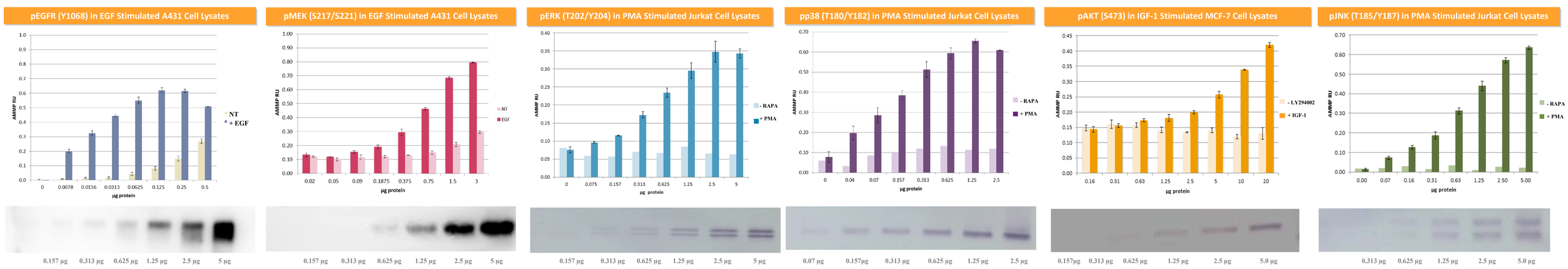


This chart illustrates how antibodies were chosen to perform each kinase analysis. A single concentration of analyte (positive cell lysate) was used in each well of the plate. Each column represents a single bead preparation conjugated with an antibody from a commercial source. For pair analysis each row utilized a different detector antibody. Subsequent to the AMMP analysis sandwich antibody pairs were chosen based on signal over noise. Assay compatible pairs (anti-phosphoprotein and anti-Total protein) were chosen that gave the most significant signal to noise ratios.

The ViBE Workstation is uniquely suited to allow efficient assay development of highly sensitive assays. Determining antibody pairs for AMMP immunoassays is a straightforward screening process on the ViBE Workstation as shown. Many antibodies and assay configurations can be probed within a microtiter plate to determine the best antibody pairs and bead/hapten configurations with which to move forward into assay development. This assay, in addition to determining the best antibody pairing and configurations, is done with the proteins in the native state. The data from AMMP assays also shows the non-specific binding and the Antibody-Analyte binding strength.

Receptor Tyrosine Kinase Stimulation of Signaling Cytosolic Kinases

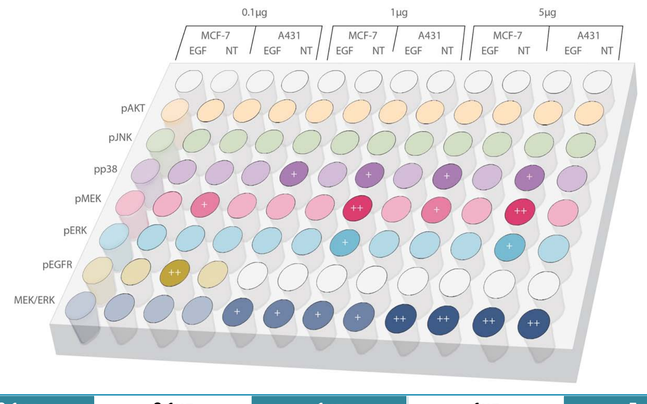
Six assays were developed to detect activation of the EGF tyrosine kinase receptor and 5 cytosolic kinases MEK, ERK, JNK, p38 and AKT. Assays were specific for the phosphorylation of EGF receptor at Y1068 , ERK 1/2 at T202/Y204, MEK at S217/S221, JNK at T185/Y187, p38 at T180/Y182, and AKT at S473. Each assay was verified for robustness through multiple repeats across multiple days and multiple cell preparations. Representative graphs are presented here for each assay developed on the AMMP platform; each cell lysate dilution was run in triplicate over 8 dilutions. For comparison, western blots were run for each analyte at the same lysate dilutions as measured in the AMMP assay. The results reveal increased sensitivity in the AMMP assay over that seen in the western blot. For the EGF receptor assay, as few as several hundred cells were used for the analysis, while maintaining significant signal to noise ratios at the cell numbers tested.



NT = Not treated

Pathway Analysis of EGF Stimulation of A431 and MCF-7 Cell Lines

On a single plate, lysates of A431 and MCF-7 cells were analyzed for the activation states of 7 kinases as illustrated. Using 0.1µg, 1µg or 5µg of cell lysate in each well and using different bead/ detector antibody pairs it was shown that EGF stimulation causes the phosphorylation the EGF receptor, ERK, MEK and p38 in A431 cells and ERK, MEK, and p38 in MCF-7 cells.



		0.1ug MCF-7		0.1ug A431		1ug MCF-7		1ug A431		5ug MCF-7		5ug A431	
		EGF 1	NT 2	EGF 3	NT 4	EGF 5	NT 6	EGF 7	NT 8	EGF 9	NT 10	EGF 11	NT 12
	A												
pAKT	B	0.1584	0.1107	0.1410	0.1765	0.1521	0.1470	0.1999	0.1696	0.1661	0.1396	0.1990	0.1568
pJNK	C	0.0241	0.0181	0.0249	0.0242	0.0425	0.0226	0.0268	0.0326	0.0715	0.0320	0.0439	0.0167
pp38	D	0.0923	0.1138	0.1074	0.0875	0.1542	0.0911	0.2125	0.1457	0.2123	0.1272	0.3329	0.2024
pMEK	E	0.20902	0.18867	0.27426	0.20876	0.21597	0.18735	0.54522	0.21344	0.3342	0.19468	0.65989	0.31738
pERK	F	0.0598	0.0726	0.1058	0.0754	0.1276	0.0503	0.2081	0.0808	0.1398	0.0992	0.3287	0.0638
pEGFR	G	0.0112	0.0000	0.5943	0.0486	ND	ND	ND	ND	ND	ND	ND	ND
MEK/ERK	H	0.150	0.109	0.184	0.168	0.252	0.218	0.430	0.398	0.643	0.626	0.589	0.560

ND=Not Done

Conclusions

- The AMMP assays detect a range of phosphoproteins with improved sensitivity over western blot and with much simpler workflow. Flexible development format allows quick screens for compatible antibody pairs in a simple, user friendly format. The assay format has no wash steps.
- The AMMP assay measures multiple phosphoproteins comprising a portion of the MAPK pathway on a single plate allowing the analysis of groups of analytes at the same time under the same conditions. Monitoring changes in expression of analytes due to stimulation by mitogens or specific agents such as receptor ligands provides significant advantages to drug discovery and development.
- AMMP assays were able to detect the MEK-ERK heterodimer which highlights the ability of the technology to detect weak and transient low affinity interactions.
- The assays presented here for detection of kinase or receptor analytes are semi-quantitative for both single and dimeric forms of the molecules. With the availability of recombinant kinases, full quantitative analysis can be achieved.
- Correlation between protein concentration and cell numbers allows an assessment of sensitivity based on cell number (<1000/assessment). This is advantageous when assaying tumor biopsy samples where the cell numbers collected are typically low.

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