WHITE PAPER

Using Antibody Arrays for Assessing the Integration of Multiple Signaling Pathways and off-Target Inhibitor Responses

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THE PROBLEM

Using time-consuming, traditional techniques such as Western blot to get a broad view of the phosphorylation status of many proteins in interrelated signal transduction pathways

THE SOLUTION

Proteome Profiler™ Antibody Arrays can rapidly uncover new relationships between signaling pathways, or reveal unexpected, off-target pharmacological responses that can confound results and waste time. There are no gels to run and no proteins to transfer. If you can collect data from an immunoblot, you have the equipment necessary to perform an array experiment today. For details please visit www.RnDSystems.com/ProteomeProfiler

INTRODUCTION

Kinases are enzymes that regulate protein activity by transferring phosphate groups to serine, threonine, or tyrosine residues in a wide range of substrates. They impact most cellular activities, and much effort is placed on studying how they affect such processes as cell proliferation, survival, differentiation, and motility. Tight regulation of kinase activity is crucial for maintaining cellular homeostasis, and aberrant kinase activity can directly contribute to cellular transformation. Consequently, kinases are often targets for drug development.

Common experiments associated with drug discovery include screening pharmacological candidates for efficacy or off-target activity. Also of interest to researchers is assessing the interrelationship of multiple signaling pathways. Traditional single analyte assays, such as Western blot, do not have the throughput necessary for these types of experiments.

THE PROBLEM

- Assessing phosphorylation changes in multiple interrelated pathways
- Performing pharmacological screens in a timely manner
- Identifying off-target pharmacological effects

THE SOLUTION

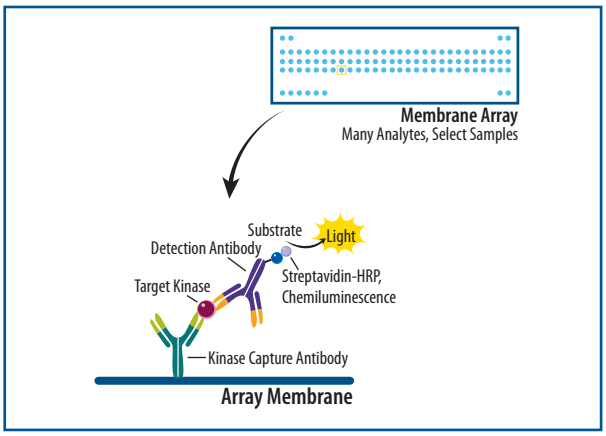
Proteome Profiler™ Phospho-Antibody Arrays are nitrocellulose membrane-based assays designed to measure the relative phosphorylation of many proteins simultaneously. They are the most referenced phospho-antibody arrays, and they have been used successfully by numerous laboratories. To highlight their utility, we screened a panel of PI 3-kinase and MEK inhibitors to assess their effects EGF-mediated signaling. Dysregulated EGF R and pathways such as PI 3-Kinase/Akt and Raf/MEK/ERK are often associated with tumor formation, and interplay between signaling cascades may contribute to pharmacological resistance. Our results showed variations in the efficacy of inhibitors. In addition, array data revealed cross-talk between pathways or off-target effects that may have been missed using single analyte assays.

Features of Proteome Profiler™ Phospho-Antibody Arrays

- Detect phosphorylation changes in many proteins simultaneously
- Reveal associations between interrelated pathways
- Easier to perform than a Western blot
- Requires no specialized equipment beyond what is used for Western blot data acquisition
- Only 3.5 hours of hands-on time

MATERIALS AND METHODS

Proteome Profiler Array Assay Principle



Arrays are composed of capture and control antibodies spotted in duplicate on nitrocellulose membranes. Cell lysates were diluted, mixed with a cocktail of biotinylated detection antibodies, and incubated overnight with the Proteome Profiler Phospho-Kinase Array (Catalog # ARY003B). Streptavidin-HRP and chemiluminescent detection reagents were applied to the membrane, and the signal produced at each capture spot corresponded to the amount of phosphorylated protein bound. The data was collected using x-ray film and analyzed using image analysis software.

Molecules and Phosphorylation Sites Detected			
Akt (S473)	Fgr (Y412)	p38α (T180/Y182)	STAT2 (Y689)
Akt (T308)	Fyn (Y420)	p53 (S15)	STAT3 (S727)
AMPK α1 (T174)	GSK-3 α/β (S21/S9)	p53 (S46)	STAT3 (Y705)
β-catenin	Hck (Y411)	p53 (S392)	STAT5a (Y694)
Chk-2 (T68)	HSP27 (S78/S82)	p70 S6 Kinase (T421/S424)	STAT5a/b (Y694/Y699)
c-Jun (S63)	HSP60	PDGF Rβ (Y751)	STAT5b (Y699)
CREB (S133)	JNK pan (T183/Y185, T221/Y223)	PLC γ1 (Y783)	STAT6 (Y651)
EGF R (Y1068)	Lck (Y394)	PRAS40 (T246)	TOR (S2448)
eNOS (S1177)	Lyn (Y397)	Pyk2 (Y402)	WNK-1 (T60)
ERK1/2 (T202/Y204, T185/Y187)	MSK1/2 (S376/S360)	RSK1/2/3 (S380)	Yes (Y426)
FAK (Y397)	p27 (T198)	Src (Y419)	

Additional Kits and Reagents

Recombinant Human EGF was from R&D Systems (Catalog # 236-EG). Inhibitors were from Tocris, an R&D Systems Company: AS 605240 (Catalog # 3578), LY 294002 (Catalog # 1130), PI 103 (Catalog # 2930), PD 0325901 (Catalog # 4192), PD 98059 (Catalog # 1213), SL 327 (Catalog # 1969), U0126 (Catalog # 1144). ELISA development kits were from R&D Systems: Phospho-CREB (S133) DuoSet® IC ELISA (Catalog # DYC2510), Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) DuoSet IC ELISA (Catalog # DYC1018B). Antibodies were from R&D Systems: Anti-Human/Mouse/Rat Phospho-ERK1 (T202/Y204)/ERK2 (T185/Y187) Affinity-Purified Polyclonal Antibody (Catalog # AF1018), Anti-Human/Mouse/Rat Total ERK1/ERK2 Monoclonal Antibody (Catalog # MAB15761).

RESULTS

Measuring Efficacy and Off-Target Responses to PI 3-Kinase Inhibitors

Protein phosphorylation profiles of A549 human lung adenocarcinoma cells were obtained to assess their response to EGF and several PI 3-Kinase Inhibitors. The data revealed a range of signaling molecules that were phosphorylated in response to EGF treatment, some of which are highlighted in array images (Figure 1A). Histogram profiles were generated for select analytes to look more closely at the effects of inhibitors (Figure 1B). The phosphorylation of CREB appeared to be unaffected, while the phosphorylation of the PI 3-Kinase effector Akt was suppressed by all inhibitors. In this context, the commonly used inhibitor LY 294002 was least effective. We also found that EGF R phosphorylation increased in response to two of the inhibitors. This type of observation could prove important to data interpretation or when assessing mechanisms of resistance, and might be missed if single analyte assays were used.

Measuring Efficacy and Off-Target Responses to MEK Inhibitors

The T47D human ductal breast epithelial cell line was used to generate an EGF-induced phosphorylation profile and to assess the effects of several MEK inhibitors. Select analytes are highlighted in array images (Figure 2A). A histogram profile was generated showing the effects on ERK1/2, Akt, STAT3, and c-Jun phosphorylation (Figure 2B). The results indicated varied levels of efficacy and possible cross-talk between traditional pathways. For instance, PD 0325901 was the most potent inhibitor of ERK1/2 phosphorylation, while T47D cells displayed resistance to the MEK inhibitors PD98059, U0126, and SL372. In addition, we noted potential off-target inhibitor effects. These included an increase in phospho-Akt in the presence of 3 of the 4 inhibitors, and decreased STAT3 and c-Jun phosphorylation in the presence of PD 98089. Again the array revealed inhibitor-related changes in phosphorylation that could be biologically relevant, but may have been missed using single analyte assays.

Comparing Array Results to Western Blot and ELISA Data

The results obtained from array experiments can provide direction for the design of hypothesis-driven single analyte assays used to support or expand on array data. For instance, in TD47 cells the efficacy of MEK inhibitors on phospho-ERK1/2 was tested with similar results using Western blot (Figure 3A), or using phospho-ELISA or phospho-array (Figure 3B). Phospho-CREB, an ERK1/2 effector, also shows a similar phosphorylation pattern using phospho-array or phospho-ELISA (Figure 3C). In all instances, PD 0325901 was the most effective inhibitor.

Expanding on these results, concentration-response experiments were performed using PD 0325901. Measuring EGF-induced phosphorylation of ERK1/2 using Western blot (Figure 4A), or phospho-ELISA or phospho-Array all gave comparable results (Figure 4B).

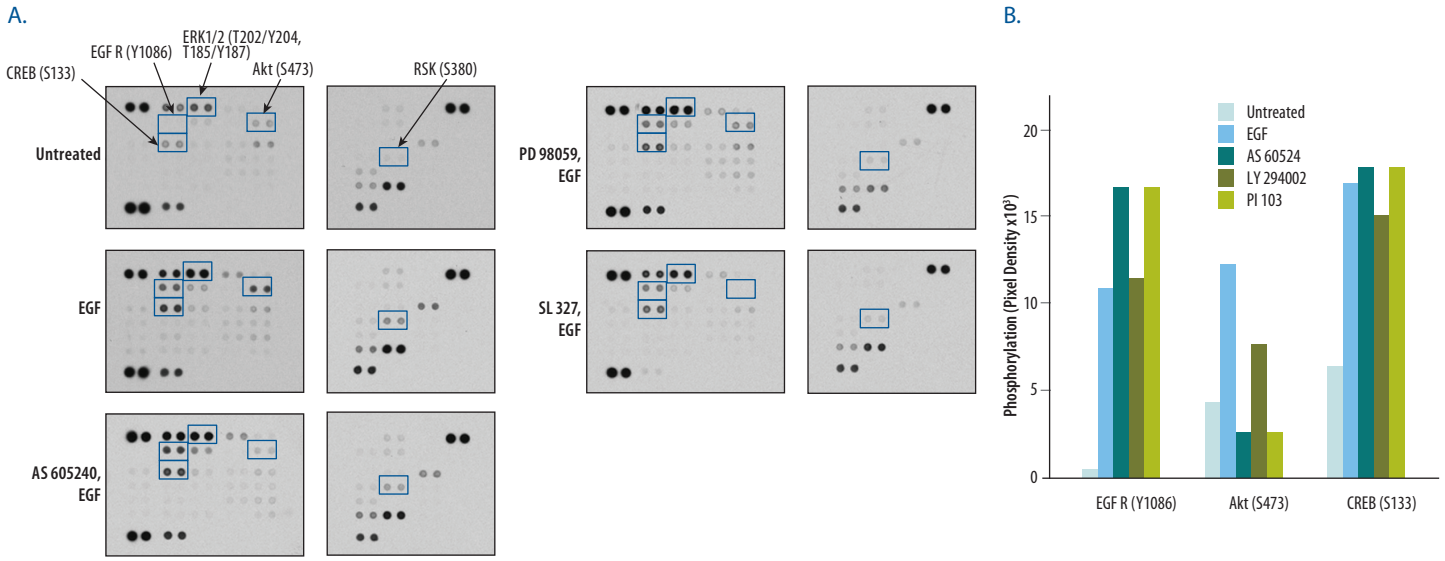


Figure 1. Induction and Inhibition of Kinase Phosphorylation in Lung Adenocarcinoma Cells. The A549 human lung adenocarcinoma cell line was untreated, treated with 100 ng/mL EGF alone for 15 minutes, or treated with EGF following a 3 hour pretreatment with 5 µM of the PI 3-Kinase inhibitors AS 605240, LY 294002, or PI-103. Images of the Proteome Profiler Human Phospho-Kinase membrane array **(A)** and corresponding histogram profiles of select analytes are shown **(B)**. CREB phosphorylation was unaffected, while LY 294002 was the least effective inhibitor of Akt phosphorylation. An increase in the phosphorylation of EGF R was observed in the presence the inhibitors AS 60524 or PI 103.

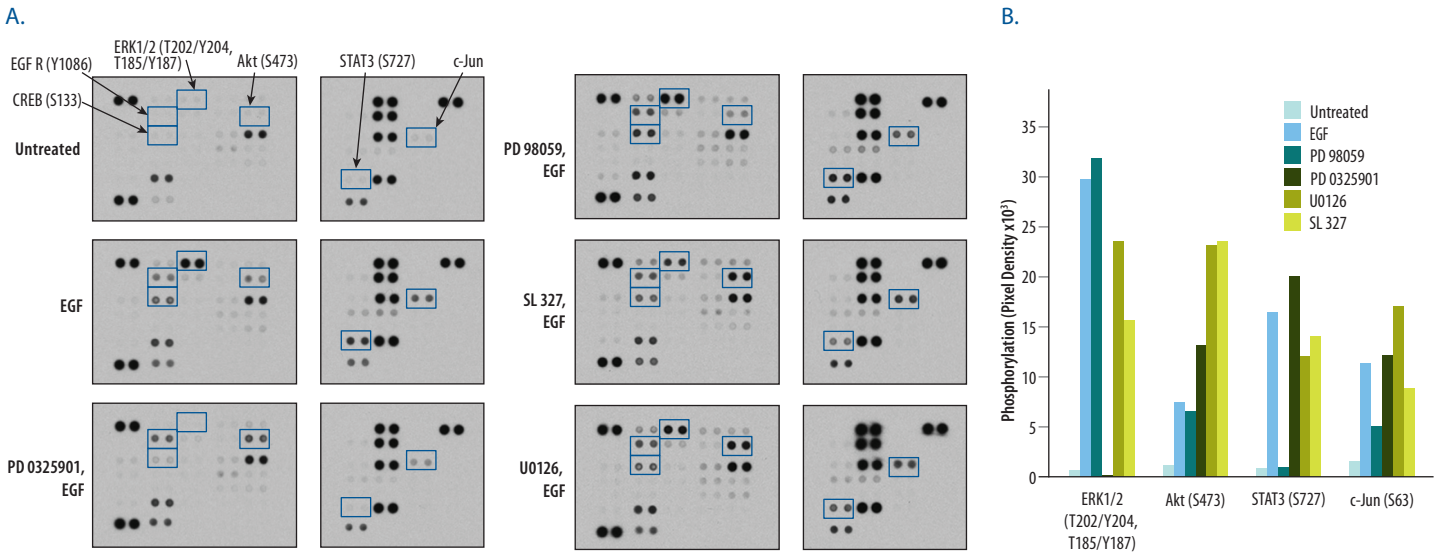


Figure 2. Induction and Inhibition of Kinase Phosphorylation in Breast Cancer Cells. The T47D human ductal breast epithelial cell line was untreated, treated with 100 ng/mL EGF for 15 minutes, or EGF following a 2 hour pretreatment with the MEK inhibitors 10 µM PD 0325901, 20 µM PD 98059, 10 µM SL 327, or 10 µM U0126. Images of Proteome Profiler Human Phospho-Kinase membrane arrays **(A)** and the corresponding histogram profiles are shown for select analytes **(B)**. PD 0325901 was the most effective suppressor of ERK1/2, while PD 98089 resulted in decreased phosphorylation of STAT3 and c-Jun. Increases in Akt phosphorylation were also observed in response to certain MEK inhibitors.

CONCLUSIONS

The Phospho-Kinase Array is an economical and time-saving alternative to traditional methods, such as Western blot, for pharmacological screens to assess efficacy and/or off-target effects. The arrays also provide a much better picture of the overall signaling response by revealing phosphorylation changes in multiple interrelated pathways. This information could prove important for data interpretation or when assessing compensation mechanisms associated with tumor resistance. Array assays are easy to perform. In addition, the data is collected from the array membrane using chemiluminescence in the same manner as an immunoblot and no additional specialized equipment is necessary. The arrays are sufficiently sensitive to measure changes in phosphorylation caused by both ligand and inhibitor treatment, and in follow-up experiments results are comparable to both Western blot and ELISA.

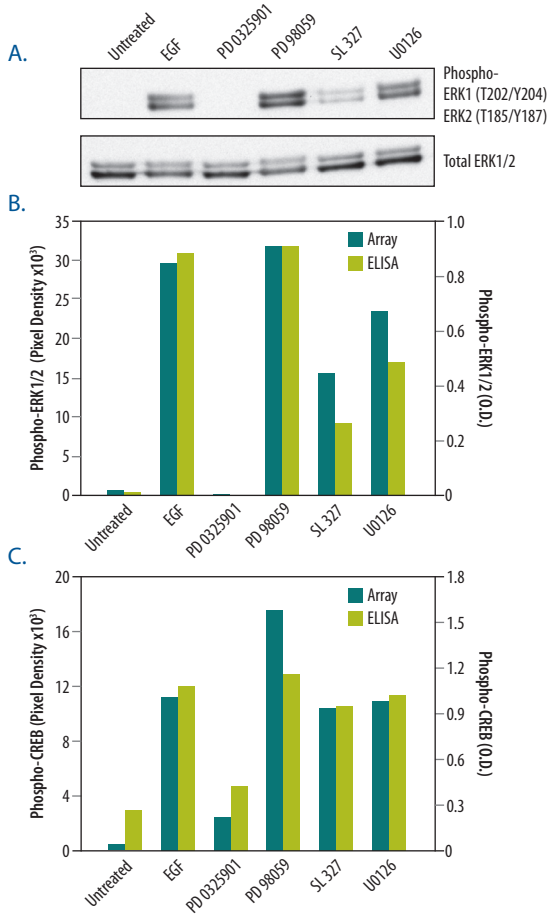


Figure 3. Comparing Array Data to Western Blot or Single Analyte ELISA in a MEK Inhibitor Screen. The T47D human ductal breast epithelial cell line was untreated, treated with 100 ng/mL EGF for 15 minutes or treated with the MEK inhibitors 10 µM PD 0325901, 20 µM PD 98059, 10 µM SL 327, or 10 µM U0126 for 2 hours, followed by treatment with EGF. Phosphorylation of ERK1/2 was measured using Western blot **(A)**. Similar results are shown in histogram profiles comparing the array data to ELISA for Phospho-ERK1/2 **(B)** or Phospho-CREB **(C)**.

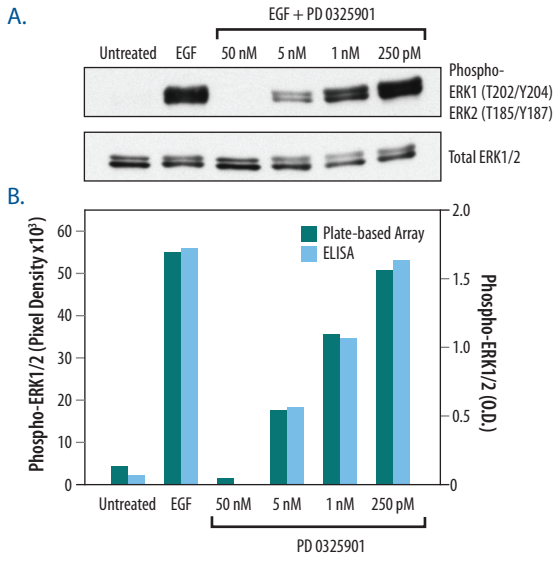


Figure 4. MEK Inhibitor PD 0325901 Concentration-Response Measurements. The T47D human ductal breast epithelial cell line was treated with different concentrations of PD 0325901 for 2 hours, followed by treatment with100 ng/mL EGF for 5 minutes. **A.** ERK1/2 phosphorylation was measured using Western blot. **B.** Histogram profiles obtained from the array were comparable to those obtained by Phospho- ELISA and Western blot.

PEER-REVIEWED REFERENCES: R&D SYSTEMS PROTEOME PROFILER™ PHOSPHO-ANTIBODY ARRAYS

Proteome Profiler Arrays are easier to perform than a Western blot and provide much more information. Find out why Proteome Profiler is the most referenced phospho-array in the literature. For details and a listing of all available arrays, please visit www.RnDSystems.com/ProteomeProfiler

1. **Proteome Profiler Human Phospho-RTK Array**
(Catalog # ARY001B)

Duncan J.S. *et al.* (2012) Dynamic reprogramming of the kinome in response to targeted MEK inhibition in triple-negative breast cancer. Cell **149**:307.
Sample: Human triple-negative breast cancer tissue homogenate

Kotani, N. *et al.* (2012) Fibroblast growth factor receptor 3 (FGFR3) associated with the CD20 antigen regulates the rituximab-induced proliferation inhibition in B-cell lymphoma cells. J. Biol. Chem. **286**:37109.
Sample: BJAB and Raji Burkitt's lymphoma cell lysates

Joshi, A. *et al.* (2012) Evaluation of tyrosine kinase inhibitor combinations for glioblastoma therapy. PLoS One **7**:e44372.
Sample: Human glioblastoma multiforme stem-like cell lysates

Sasaki, T. *et al.* (2011) A novel ALK secondary mutation and EGFR signaling cause resistance to ALK kinase inhibitors.
Cancer Res. **71**:6051.

Sample: Human non-small cell lung carcinoma cell lysates

2. **Proteome Profiler Human Phospho-MAPK Array**
(Catalog # ARY002B)

Lin, F. *et al.* (2012) RUNX3-mediated transcriptional inhibition of Akt suppresses tumorigenesis of human gastric cancer cells. Oncogene **31**:4302.
Sample: Human gastric carcinoma cell lysates

Oliveras-Ferraros, C. *et al.* (2012) Cross-suppression of EGFR ligands amphiregulin and epiregulin and de-repression of FGFR3 signalling contribute to cetuximab resistance in wild-type KRAS tumour cells. Br. J. Cancer **106**:1406.
Sample: A431 epithelial carcinoma cell lysates

Kotani, N. *et al.* (2012) Fibroblast growth factor receptor 3 (FGFR3) associated with the CD20 antigen regulates the rituximab-induced proliferation inhibition in B-cell lymphoma cells. J. Biol. Chem. **286**:37109.
Sample: BJAB and Raji Burkitt's lymphoma cell lysates

Schulze, D. *et al.* (2011) Anti-tumor effects of fibroblast growth factor-binding protein (FGF-BP) knockdown in colon carcinoma. Mol. Cancer **10**:144.
Sample: Human colon cancer cell lysates

Fryer, R.S. *et al.* (2011) Mechanisms underlying gemcitabine resistance in pancreatic cancer and sensitization by the iMiD lenalidomide. Anticancer Res. **31**:3747.
Sample: Human pancreatic cancer cell lysates

Wei, Q. *et al.* (2011) Sulfiredoxin–Peroxiredoxin IV axis promotes human lung cancer progression through modulation of specific phosphokinase signaling. Proc. Natl. Acad. Sci. USA **108**:7004.
Sample: A549 human lung carcinoma cell lysates

3. **Proteome Profiler Human Phospho-Kinase Array**
(Catalog # ARY003B)

Zhuang, G. *et al.* (2012) Tumour-secreted miR-9 promotes endothelial cell migration and angiogenesis by activating the JAK-STAT pathway. EMBO J. **31**:3513.
Sample: Human umbilical vein endothelial cell lysates

Oliveras-Ferraros, C. *et al.* (2012) Cross-suppression of EGFR ligands amphiregulin and epiregulin and de-repression of FGFR3 signalling contribute to cetuximab resistance in wild-type KRAS tumour cells. Br. J. Cancer **106**:1406.
Sample: A431 epithelial carcinoma cell lysate

Kotani, N. *et al.* (2012) Fibroblast growth factor receptor 3 (FGFR3) associated with the CD20 antigen regulates the rituximab-induced proliferation inhibition in B-cell lymphoma cells. J. Biol. Chem. **286**:37109.
Sample: BJAB and Raji Burkitt's lymphoma cell lysates

Joshi, A. *et al.* (2012) Evaluation of tyrosine kinase inhibitor combinations for glioblastoma therapy. PLoS One **7**:e44372.
Sample: Human glioblastoma multiforme stem-like cell lysates

Chandarlapaty, S. *et al.* (2011) AKT inhibition relieves feedback suppression of receptor tyrosine kinase expression and activity. Cancer Cell **19**:58.
Sample: Human breast, lung, and prostate cancer cell lysates

4. **Proteome Profiler Human Phospho-Immunoreceptor Array**
(Catalog # ARY004)

Wei, Q. *et al.* (2011) Sulfiredoxin–Peroxiredoxin IV axis promotes human lung cancer progression through modulation of specific phosphokinase signaling. Proc. Natl. Acad. Sci. USA **108**:7004.
Sample: A549 human lung carcinoma cell lysates

Kotani, N. *et al.* (2012) Fibroblast growth factor receptor 3 (FGFR3) associated with the CD20 antigen regulates the rituximab-induced proliferation inhibition in B-cell lymphoma cells. J. Biol. Chem. **286**:37109.
Sample: BJAB and Raji Burkitt's lymphoma cell lysates

5. **Proteome Profiler Human Apoptosis Array**
(Catalog # ARY009)

Schulze, D. *et al.* (2011) Anti-tumor effects of fibroblast growth factor-binding protein (FGF-BP) knockdown in colon carcinoma. Mol. Cancer **10**:144.
Sample: Human colon cancer cell lysates

For more information on Proteome Profiler Arrays, including our selection of arrays for secreted molecules, please visit **www.RnDSystems.com/ProteomeProfiler**