Assay Development on the NanoPro Platform: 4E-BP1 and 4E-BP2

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Abstract

We present the development of novel nanoimmunoassays for the translational repressor proteins 4E-BP1 and 4E-BP2 using NanoPro technology. Both the PI3 kinase/Akt pathway and FRAP/mTOR kinase pathway regulate 4E-BP1 activity, making 4E-BP1 a focal point for these two important signaling pathways. 4E-BP2 regulation is poorly understood, partially due to the lack of specific anti-phospho 4E-BP2 antibodies. Our assay, developed on the Cell Biosciences NanoPro platform, enables detailed differential investigation of 4E-BP1 and 4E-BP2 phosphorylation and signal transduction.

NanoPro Technology Overview

The CB1000 is an automated, capillary-based NanoPro immunoassay system. As in Western blot analysis, proteins from complex biological samples are separated, immobilized, and probed with antibodies. However, the CB1000 system uses an IEF separation to resolve the various phosphorylation states of signaling proteins. Separated proteins then are linked to the capillary wall through UV irradiation and probed with primary and secondary antibodies. The secondary antibody is HRP-labeled, which enables ultrasensitive chemiluminescence detection. Even low-abundance proteins can be rapidly measured in tiny samples.

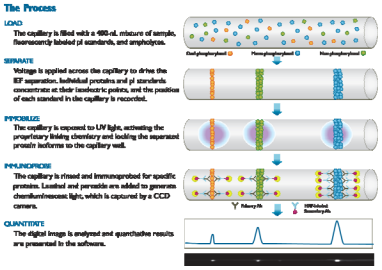
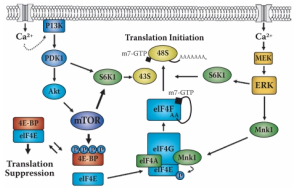


Figure 1: Assay principle of capillary based NanoPro platform

4E-BP1 and 4E-BP2

4E-BP1 and 2 are signaling molecules tightly regulated through several pathways, and act downstream of mTOR/Raptor. Upon activation, 4E-BP1 and 2 are phosphorylated and dissociate from eIF-4E, subsequently controlling protein translation. NanoPro technology combines IEF separation of different phosphorylation states with highly sensitive detection. Different phosphorylated states can be studied without specific anti-phospho antibodies for each phosphorylation site. 4E-BP1 and 2 are used to show steps in the NanoPro assay development process.



General Assay Conditions

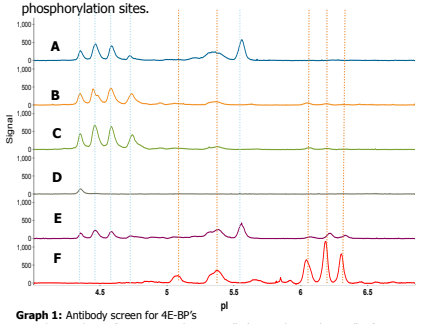
A detailed protocol can be found on the Cell Biosciences website.

Protein concentration in capillary	0.2 mg/mL
Gradient:	CB 5-8
UV-exposure (immobilization):	100s
Standard ladder:	3 (pI 4.9, 6.0, 6.4, 7.0, 7.3)
Separation conditions:	40 min, 15000 µW
Primary antibody incubation time	2h
Secondary antibody incubation time	1h

Table 1: Assay conditions used unless stated in legend

Antibody Screen

More than 50% of the antibodies screened against 4E-BP1 and 4E-BP2 showed positive results. Differential antibody specificities against different isoforms are apparent. In antibody selection, reproducibility of peak profiles, signal/noise and response to biological stimuli are the main criteria. The α-pSer65-4E-BP1 antibody (CS9451) recognizes only a single peak at the most acidic pI in the profile. One can infer that phosphorylation on Ser65 requires prior phosphorylation of all other active phosphorylation sites.

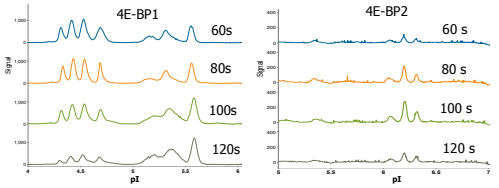


Graph 1: Antibody screen for 4E-BP's
Bicine/CHAPS lysate from untreated MCF-7 cells (0.1 mg/mL in the capillary) was separated according to conditions in Table 1, and probed with the indicated antibodies (each diluted 1:50 in Antibody Dilution Buffer). **A:** α-4E-BP1 (Cell Signaling 9644) is a pan antibody recognizing phospho and non-phospho peaks. **B:** α-phospho 4E-BP1 (pThr36/37, Cell Signaling 9459) is a rabbit polyclonal. **C:** α-phospho 4E-BP1/2/3 (pThr45, Epitomics 2334-1), a rabbit monoclonal antibody, is raised against close, but not identical, Threonine phosphorylation sites to antibody in B. Both antibodies recognize a very comparable profile. **D:** α-phospho-4E-BP1 (Ser65, Cell Signaling 9451) recognizes only the phospho peak with the lowest pI (highest phosphorylation grade, discussed above). **E:** α-4E-BP1 (Millipore 07-1416) is described as 4E-BP1 specific but clearly cross-reacts with 4E-BP2 when compared to F. **F:** α-4E-BP2 (Cell Signaling 2845).

Titrations

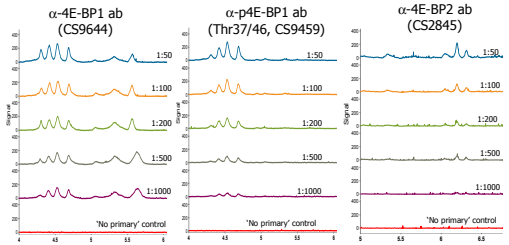
As with any immunoassay, certain assay parameters need to be optimized. Here, we show optimization of three parameters we found most important.

A: Immobilization Time
Following separation, proteins are immobilized to the coated capillary wall by UV light. Extended UV exposure might damage proteins, while too short exposure might leave immobilization incomplete. Hence, optimal exposure time must be determined for each protein. A 100 sec exposure is a robust condition for 4E-BP1 and 4E-BP2. In addition, both proteins can be run in the same cycle.



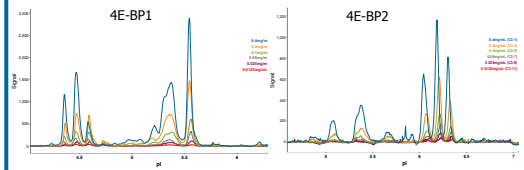
Graph 2: UV titration
MCF-7 cells treated for 15 min with 100 ng/mL IGF-1 were lysed and diluted to a final concentration of 0.1 mg/mL in the capillary. Proteins in capillaries were exposed to UV light for times indicated in graphs. Proteins in capillaries were probed with α-4E-BP1 (CS9644, 1:100) or α-4E-BP2 (CS2845, 1:50). All other assay conditions were as described in Table 1.

B: Antibody Conditions
Conditions for primary and secondary antibody incubation times and concentrations must be optimized to reach binding saturation. In Graph 3, we show titrations of different anti-4E-BP1 and 2 antibodies. Expected differences in affinity between antibodies are illustrated. For example, CS9644 can be used with confidence at much greater dilution than CS2845. Experiments of this type allow the user to identify the most stable conditions for their assay.



Graph 3: Primary antibody titration
MCF-7 cells treated for 15 min with 100ng/mL IGF-1 were lysed and diluted to final concentration of 0.1 mg/mL in the capillary. Proteins in capillaries were probed with α-4E-BP1 (CS9644), α-phospho 4E-BP1 (CS95459) or α-4E-BP2 (CS2845) antibodies at dilutions stated. All other conditions as described in Table 1.

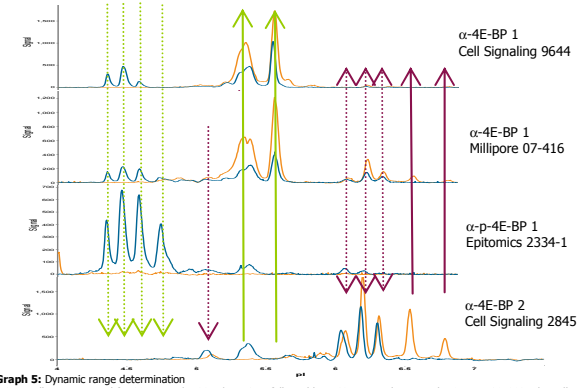
C: Lysate Concentration
Knowledge of dynamic range is crucial to relevant data analysis. It defines the signal range in which the signal changes linearly with protein concentration in the capillary. Above this range, potential biological changes might be missed as the signal does not change with change in protein concentration. Below this range, the signal might be too small.



Graph 4: Dynamic range determination
Untreated MCF-7 cells were lysed in Bicine/CHAPS buffer. Lysate was diluted in the same buffer to the final concentration in the capillary depicted in graph. The immobilized protein was probed with α-4E-BP1 antibody (CS9644, 1:100) and α-4E-BP2 antibody (CS2845, 1:50). All other assay conditions as described in Table 1.

Peak Identification

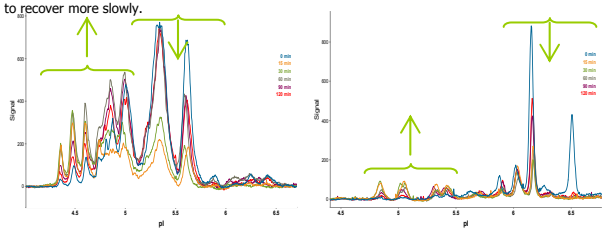
Differential specificities of pan and phospho antibodies along with an expected biological response, are valuable in understanding NanoPro profiles. MCF-7 cells were EGF stimulated and treated with or without the AKT inhibitor LY294002. For 4E-BP1, the phospho antibody (Epitomics 2334-1) only recognized the peaks from pI 4 to 5.5 and not the 5.7 peak, indicating the latter is a non-phospho, or at least not phosphorylated at Thr36/37. The same rationale is applied to the α-4E-BP2 profile. The peaks above pI 6.5 are designated as non-Thr36/37 phospho 4E-BP2, while the small acidic peaks of the 4E-BP2 profile also recognized by the phospho antibodies, correspond to phospho isoforms of 4E-BP2. In addition, the phospho peaks for 4E-BP2 are reduced with inhibitor treatment as the non-phospho peaks increase. All 4E-BP1 and 2 peaks are recognized by the Millipore α-4E-BP1 antibody, indicating this antibody is actually not as specific as described. However, it represents a good control for this system.



Graph 5: Dynamic range determination
MCF-7 cells were treated for 30 min with 100ng/mL IGF-I followed by a 60 min incubation with 50 µM LY294002. The cells were lysed in Bicine/CHAPS buffer. 0.2 mg/mL protein in the capillary was separated using the conditions in Table 1. Different treatments were probed with the antibodies as indicated. All were diluted 1:50 in antibody diluent, 1:100 for CS9644.

Biological Response

To prove that an observed change in profile is truly reflective of a change in biology, treat the cell system in a time course manner. In these experiments, MCF10A cells are treated with EGF between 0 and 120 min. The 4E-BP1 non-phospho peaks identified in Graph 5 diminish at 15 min, plateau out to 30 min and recover between 60 to 120 min. At the same time, the most highly phosphorylated (lowest pI) peaks increase out to 30 min and decrease again close to their initial size by 120 min. 4E-BP2 shows a similar bell shaped behavior, with the exception of the non-phospho peaks which seem to recover more slowly.



Graph 6: Time course of EGF treatment in MCF10A for 4E-BP1 and 4E-BP2
MCF10A cells were serum starved overnight, treated with 600 ng/mL EGF at the indicated times and lysed with Bicine/CHAPS buffer. After separation at 0.2mg/mL protein, the immobilized protein was probed with pan 4E-BP1 (CS9644, 1:100) or 4E-BP2 (CS2845, 1:50) antibodies.

Discussion

- NanoPro assays for 4E-BP 1 and 4E-BP2 were successfully developed.
- Optimization of immobilization time, antibody incubation, lysate concentration and other parameters ensures the stability of the results.
- The expected biological response for EGF-activation and inhibition of IGF-1 activation could be assessed in 2 different cell models.
- Unlike any other system, the response of different phospho isoforms could be assessed from a minute sample using pan α-4E-BP1 and α-4E-BP2 antibodies.
- The high sensitivity and low sample volume make this technology highly suitable for small sample applications.