Introduction to the AfCS Antibody Database:
Tabulation of Our Experience with Commercially Available Antibodies

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Abstract: To further the goals of the Alliance for Cellular Signaling (AfCS), the Antibody Laboratory has tested numerous commercially available antibodies for their utility by Western immunoblotting. We have tabulated results in database form and posted the information on the AfCS/Nature Signaling Gateway Web site (see link to database in sidebar). To date we have tested over 200 antibodies. Our goal is to provide the signaling community with an immediate resource on our experience with commercial antibodies and to supply updates as we progress. This communication is intended to instruct readers on how to navigate the database and to provide information on how we judge the utility of the antibodies that we test.

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Introduction

We routinely test the utility of antibodies by Western immunoblotting of whole cell lysates. The information in the current database, which will be updated periodically, is accumulated mostly from antibodies that are specific for phosphorylated epitopes (as part of our effort to quantify ligand-induced changes in phosphorylated proteins). A nonexclusive collaboration with Cell Signaling Technology has provided for many of the antibodies that we have tested thus far. We have combined some of the best phosphospecific antibodies for multiplex Western blotting, which have been employed in ligand screens conducted by the AfCS. Those results are available online in the DataCenter of the AfCS Nature Signaling Gateway.

A Word of Caution

Western immunoblotting for analysis of endogenous proteins in whole cell lysates is a highly stringent test, therefore, it may not always be an indicator of an antibody's utility in other settings. Furthermore, the conditions used in most of our tests are geared specifically for use in multiplex Western immunoblotting. For example, a phosphorylation site-specific antibody may not perform well in our assay but may function quite well if the protein target were first immunoprecipitated with a conventional, or pan, antibody (that is not directed to a site of modification). When we report favorable Western blot results, there is some likelihood that other investigators may adapt conditions to find the same antibody useful in their own work. On the other hand, if we report poor experience with an antibody that does not necessarily indicate that other investigators will find it without utility.

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Methods

The following procedures (indexed by AfCS protocol number) were used in the experiments described.

Method		AfCS Protocol ID
Preparation of primary cells	Cardiac myocytes	PP00000125, PP00000126
	B lymphocytes	PP00000001, PP00000017
Cell line culture	WEHI-231	PP00000118
Preparation of cell lysates	Cardiac myocytes	PP00000132
	B lymphocytes	PP00000010
	WEHI-231	PP00000117
	RAW 264.7	PP00000177
SDS PAGE		PP00000002, PP00000003
Western immunoblotting		PP00000007
Phosphatase treatment of blots		PP00000142

Navigating the Antibody Database

The database is organized into three levels

1. Index of Antibody Targets, Listed alphabetically
   - Click on target name to view next level, the list of antibodies tested
   - Alternatively, the search function at bottom of page may be utilized to find antibodies of interest by criteria other than the target.
2. List of Antibodies to Chosen Target
   - Click on antibody name to view table that summarizes our Western immunoblot results for that antibody.
   - Alternatively, click on "All antibodies tested" to see tables for all antibodies tested against the chosen target.
   - The search function at the bottom of page may be utilized to find antibodies by criteria other than the target.
3. Antibody Tables:
   - If "All antibodies tested" is chosen, the tables are shown divided into groups of phosphospecific and conventional antibodies
   - A separate table is shown for each antibody tested.
   - Each row in a table represents results from a separate experiment.

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Antibody Tables

Each row in an antibody table summarizes the conditions used and results obtained from a single experiment with the antibody. Each column is named, numbered, and linked to specific descriptions of the information displayed. The results determine how we number score and color-code the experiment in order to highlight conditions for which we obtained promising results (lower numerical score). In slides following the example tables, number scoring and color coding is defined separately for conventional and phosphospecific antibodies.

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Example of Antibody Tables

p38 MAPK (T180/Y182) 28B10 **Cell Signaling Technology 9216**
Expected MW (kDa) = 43

1 MW (kDa)	2 Back-ground	3 Extra bands	4 Cell treatment	5 Conc.	6 Time (min)	7 Other treatment	8 Phospho-sensitivity detected?	9 Lot#	10 Ab Conc or dilution	11 Score	12 Cell type
40	+	22,50	LPS	10 µg/ml	30		yes	1	1000X	1	RAW264.7
42	none	none	LPS	10 µg/ml	9		yes	1	1000X	1	RAW264.7
42	+	80,112	PVD	25 µM	30		yes	2	1000X	1	RAW264.7
42	none	none	40L	65 nM	2.5,5,15,30		yes	1	1000X	1	B lymphocyte
42	+	none	CPG	0.05, 0.5, 5 µM	5		yes	1	1000X	1	B lymphocyte
40	none	none	CLA	100 nM	45		yes	2	1000X	1	Myocyte
41	+	none	CLA	100 nM	45		yes	3	1000X	1	Myocyte
43	++	none	40L	65 nM	5,15		yes	2	1000X	2	WEHI-231
38	none	23	AIG	1 µM	5,15		no	1	1000X	3	B lymphocyte
42	+	none	TGF	1, 10, 100 ng/m	5		no	1	1000X	3	B lymphocyte
42, weak	none	none	PEH	20 µM	5,15,60,240		no	2	1000X	3	Myocyte
42, weak	none	52	CLA	20 nM	45		no	3	1000X	3	WEHI-231
43	none	none	CLA	20 nM	45		no	2	1000X	3	WEHI-231
42, weak	none	none	I04	0.34 nM	9		no	1	1000X	4	RAW264.7
42	++	none	M3A	120 nM`	2.5,5,15,30		no	2	1000X	4	B lymphocyte

Scoring of Antibody Experiments

Experiments are scored so that the potential suitability of the test antibodies can be tracked. We score experiments numerically from 1 to 4 with lower values indicating better results. The criteria used for scoring phosphospecific antibodies differ from that of conventional antibodies because of the additional consideration of phosphosensitivity of the former. Conventional antibodies are scored on two specific criteria, assigned 1 (blue) or 2 (purple), while phosphospecific antibodies are scored on three criteria, assigned 1(red), 2 (green), or 3 (blue). In both cases, a score of "4" is assigned if the desired criteria are not met.

Sample blots and the specific criteria used to score and color code them are shown on the next two pages.

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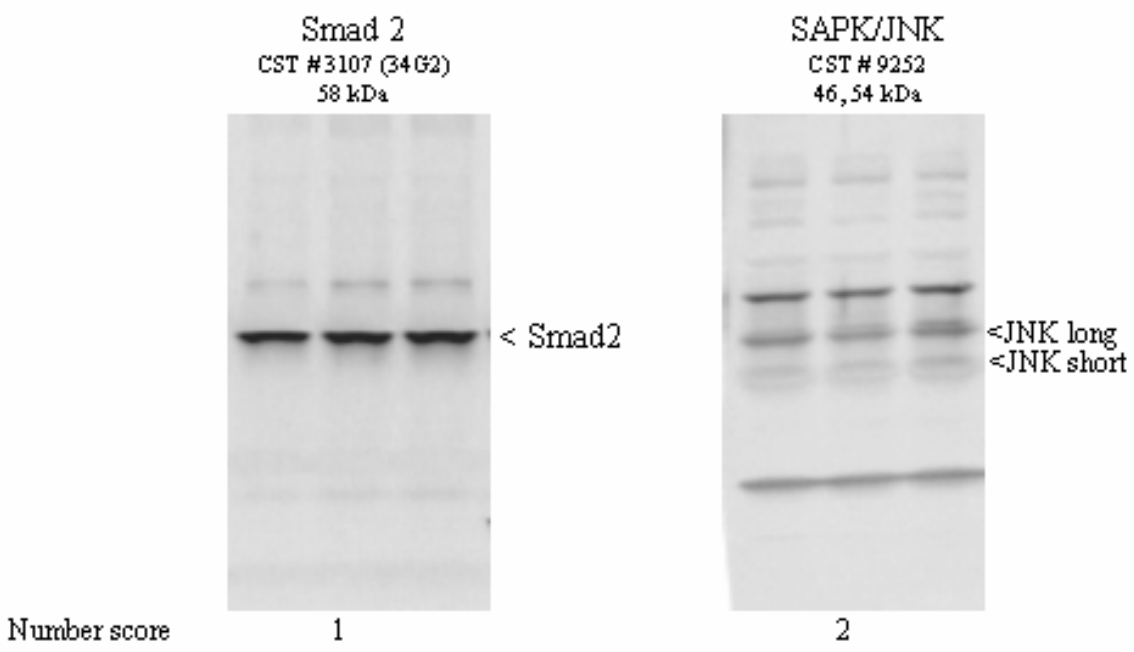
Number Scoring of Conventional Antibodies



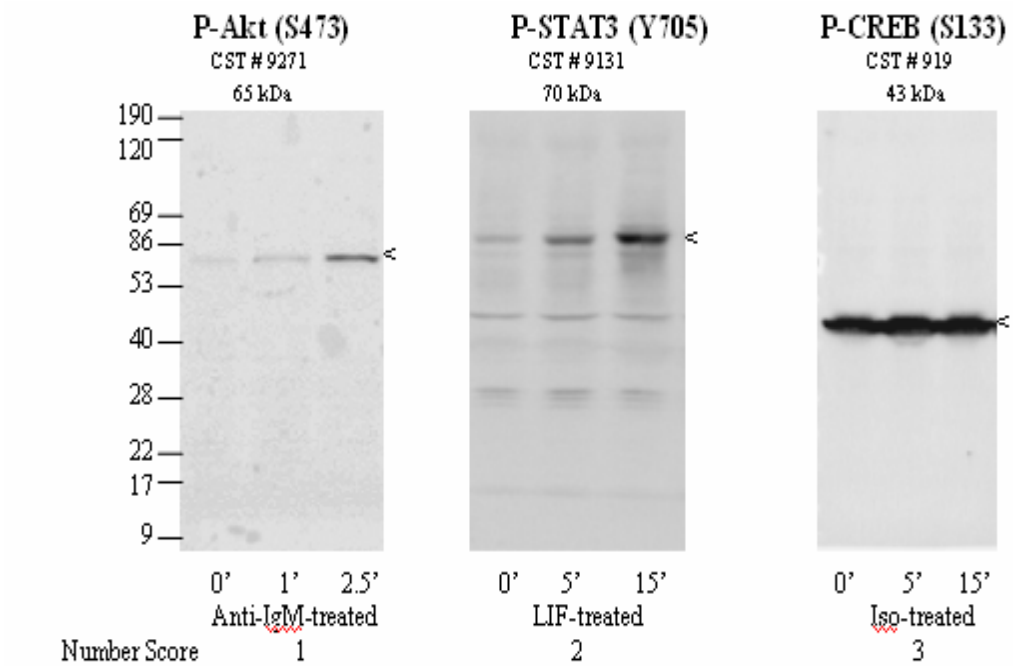
Two sample blots from triplicate samples are shown above. Definitions for scoring and color-coding of conventional (not modification site directed) antibodies are given below.

1 = Band(s) of appropriate molecular weight is (are) most prominent.

2 = Band(s) of appropriate molecular weight is (are) evident (but not most prominent), and we have reason to believe it is (they are) correct.

Number Scoring of Phosphospecific Antibodies

Example blots are shown below with definitions of number scores.



1 (red) = Unambiguous band(s), of expected molecular weight, very little or no background. Phosphosensitivity is evident. These antibodies are of quality necessary for multiplex Western blotting.

2 = (green) Band of expected size is observed but it is weak or accompanied by additional bands. Phosphosensitivity is evident.

3 = (blue) Prominent band(s) of expected size(s), but we do not see evidence of phosphosensitivity in the experiment.

Evidence of Antibody Phosphosensitivity

Many of the antibodies that we test are directed to a specific site of phosphorylation. We test these antibodies with lysates from cells exposed to an appropriate ligand or a phosphatase inhibitor (i.e., calyculin A and/or pervanadate), which is anticipated to increase phosphorylation of the protein. The best antibodies allow us to see a difference in intensity of the correctly sized band when comparing cells in the basal and stimulated states; this is considered evidence of phosphosensitivity and is designated as such in the antibody report. With some antibodies, we observe a prominent band of the correct size but do not observe the anticipated changes in response to ligand or phosphatase inhibitor (assigned a score of 3). Reasons for not observing phosphosensitivity may include the following:

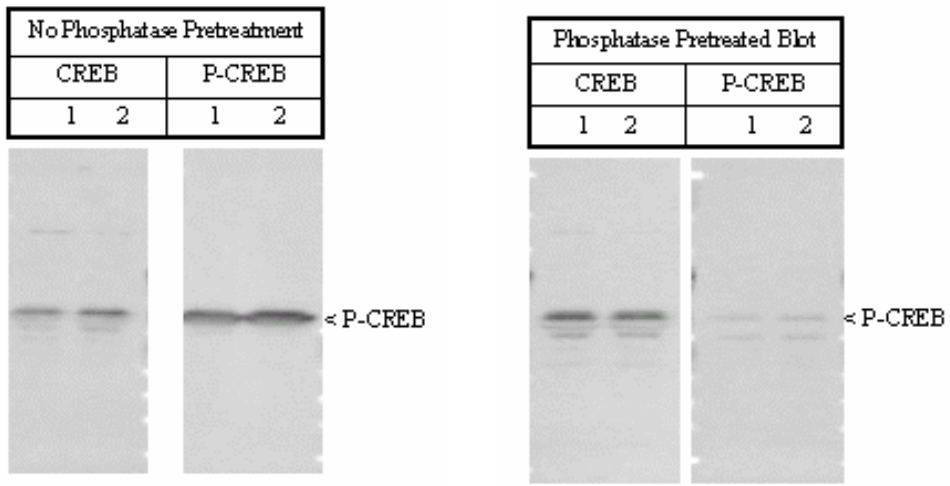
- The protein might be fully phosphorylated in unstimulated cells
- We are not using the correct timing or concentration of ligand or phosphatase inhibitor
- The antibody is not phosphosensitive

Phosphatase Pretreatment of Blots

To determine whether a protein is phosphorylated and an antibody is phosphosensitive, we can treat the blot with alkaline phosphatase before processing it with antibodies. This type of experiment is coded "A" in an antibody report under "Other Treatments". An example experiment with a phospho-CREB antibody is shown on the next page.

Phosphatase Pretreatment of Blots

Establishes Phosphosensitivity of Phospho-Ser 133 CREB Antibody



Duplicate extracts of untreated cardiac myocytes
Antibodies: Cell Signaling Technology 9191 and 9192

We routinely obtain a strong signal for phospho-CREB (P-CREB) in cardiac myocytes, but the signal does not change upon exposure of the cells to tested ligands. The blots above demonstrate that the P-CREB antibody is indeed phosphosensitive because when a blot is pretreated with alkaline phosphatase, the signal is lost. The blot with the conventional CREB antibody shows that the CREB protein was not lost during the pretreatment of the blot with phosphatase. The data suggest that CREB is substantially phosphorylated in our cultures of cardiac myocytes, without ligand treatment.

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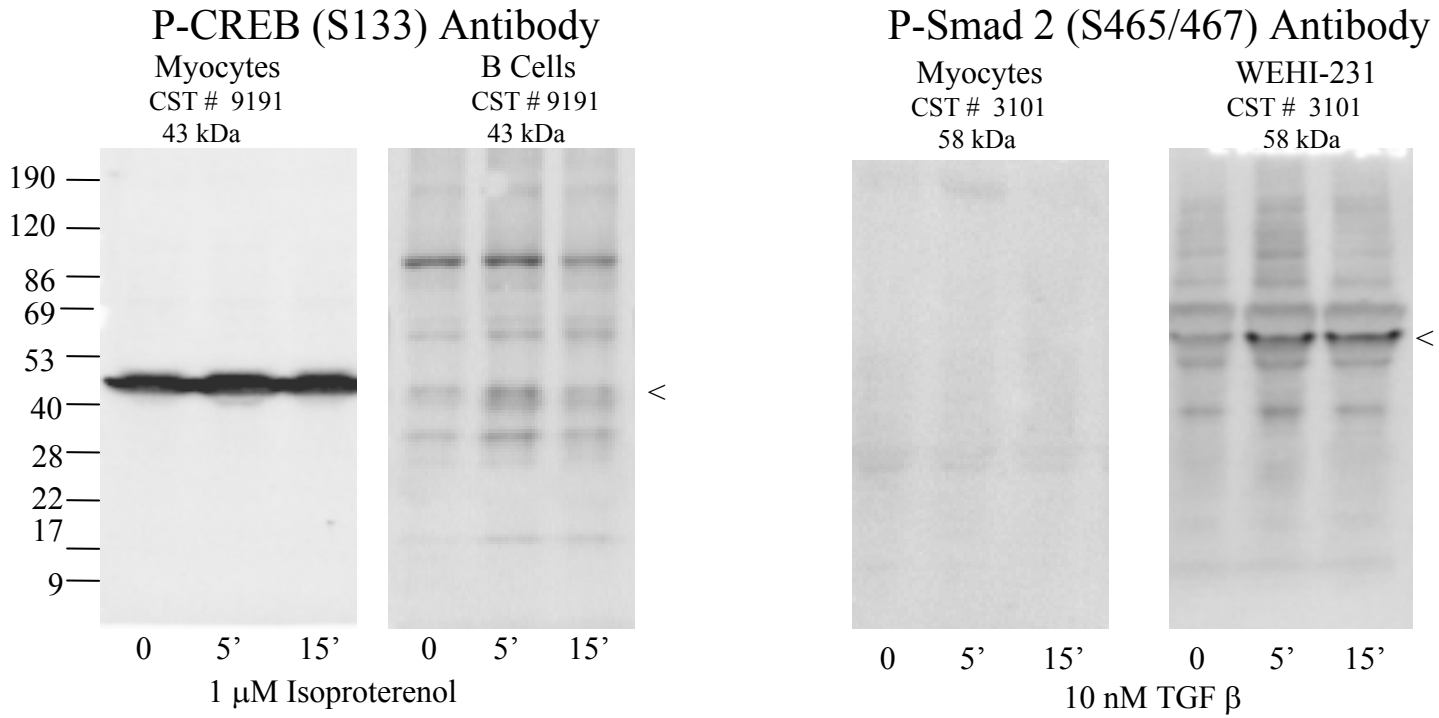
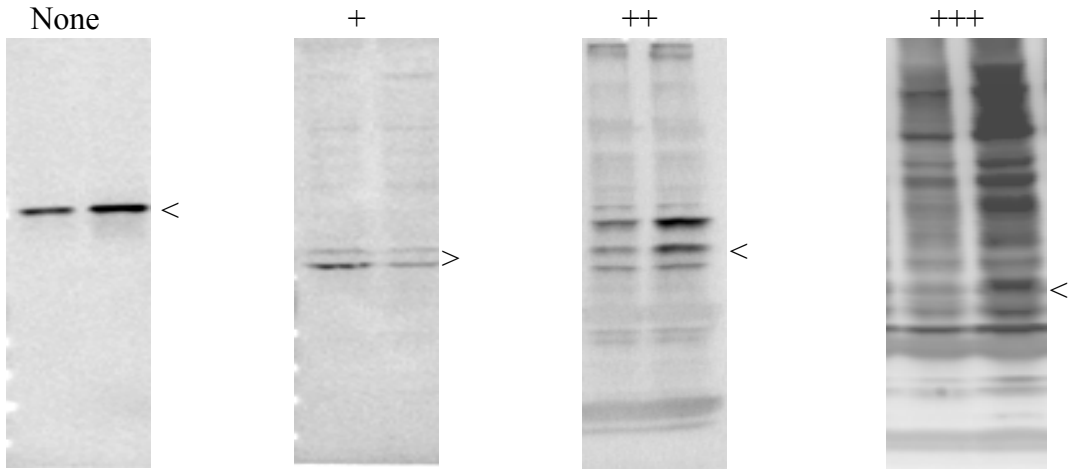
Cell Type Can Make a Difference



There are many reasons that an antibody may not "work" well. In this example, P-CREB gave clean results for myocyte lysates, but many extra bands were detected in B-cell lysates (left pair of blots). In the second example, we detected ligand responsive phospho-Smad 2 (P-Smad 2) in WEHI-231 cells but not in myocytes (right pair of blots). These results highlight the fact that an antibody that works well for one cell type may not do so for another.

Scoring Background



Extraneous bands or other background noise is often evident in images of Western immunoblots. Background can arise from a number of sources, including insufficient blocking of the blot before addition of antibodies, low stringency conditions for processing the blot, incomplete specificity of antibodies, or too high of a concentration (or too long of an incubation) of primary and/or secondary antibodies. We have scored the background in our experiments with none, +, ++, or +++, in order of increasing background that we observed. Although it is difficult to definitively and uniformly assign scores to background, here we show example blots for each score assigned. The arrowheads point to the expected band sizes. We are unable to assess background for some more broadly reactive antibodies (such as phosphotyrosine or kinase substrates), in which case we list “unknown” in the background column.

Conclusion

Please copy and save the link below to access the latest version of the database at your convenience.

AfCS Antibody Database

http://www.signaling-gateway.org/data/antibody/cgi-bin/targets.cgi

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