p470 Kinase Expression Testing Report **12/12/2014**

Methods

Expression was in Rosetta2 cells grown in MagicMedia (Invitrogen, an autoinducing medium) with 100 μg/ml carbenicillin and 100 μg/ml spectinomycin as selection antibiotics. Single colonies of fresh transformants were picked into 900 μl medium in a 96-well block which was sealed with a gas-permeable seal. The block was incubated at 37°C for 4 h then 16°C for 40 h with shaking. Cells were pelleted by centrifugation and frozen at -80°C overnight. Cells were thawed and lysed by addition of 700 μl Solulyse (Genlantis) supplemented with 400 mM NaCl, 20 mM imidazole and protease inhibitors. Lysis proceeded for 1 h at room temperature on a rotating platform. Samples of the total cell lysate were saved for SDS-PAGE. Insoluble material was pelleted by centrifugation and samples of the soluble cell lysate were saved for SDS-PAGE.

For the nickel pulldown, 500 μl of the soluble lysate was added to 25 μl Ni-NTA resin (settled volume) in a 96-well filter plate. To this, 200 μl of Nickel Buffer A was added (25 mM HEPES pH 7.5, 5% glycerol, 400 mM NaCl, 20 mM imidazole, 1 mM BME) and the block was mixed by rotation at room temperature for 30 mins. The resin was then washed with a total of 2 ml Nickel Buffer A. To elute untagged target proteins, 10 μg TEV protease (untagged) in 80 μl Nickel Buffer A was added per well. This was incubated at room temperature with gentle shaking for 2 hours, after which the cleaved proteins were recovered. A subsequent wash with 40 μl Nickel Buffer A per well was performed which helps to release more target protein. To check for TEV protease resistant material remaining on the resin, an elution was then performed with Nickel Buffer B (as for Nickel Buffer A but at 400 mM imidazole). Samples from this elution were saved for running on SDS-PAGE.

Untagged protein eluted by TEV protease was run on a LabChip GX II Microfluidic system to analyze the major protein species present. Several samples of total cell lysate, soluble cell lysate and Nickel Buffer B elutions were run on SDS-PAGE to aid analysis.

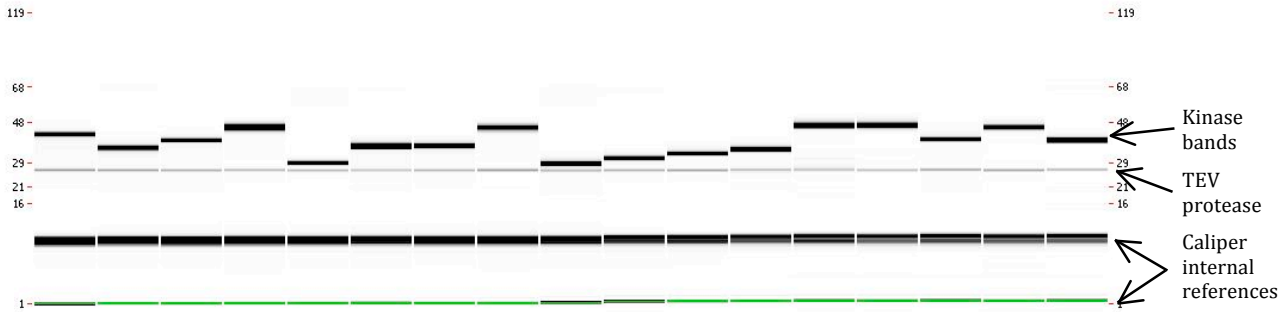
Results

The next few pages contain gel images and a table listing the top hits. The criteria for selection were a high concentration (>200 ng/μl) of the appropriate band, and no contaminating bands (other than the TEV protease itself, typically present at about 20 ng/μl). No phosphatase contamination was detected in any samples.

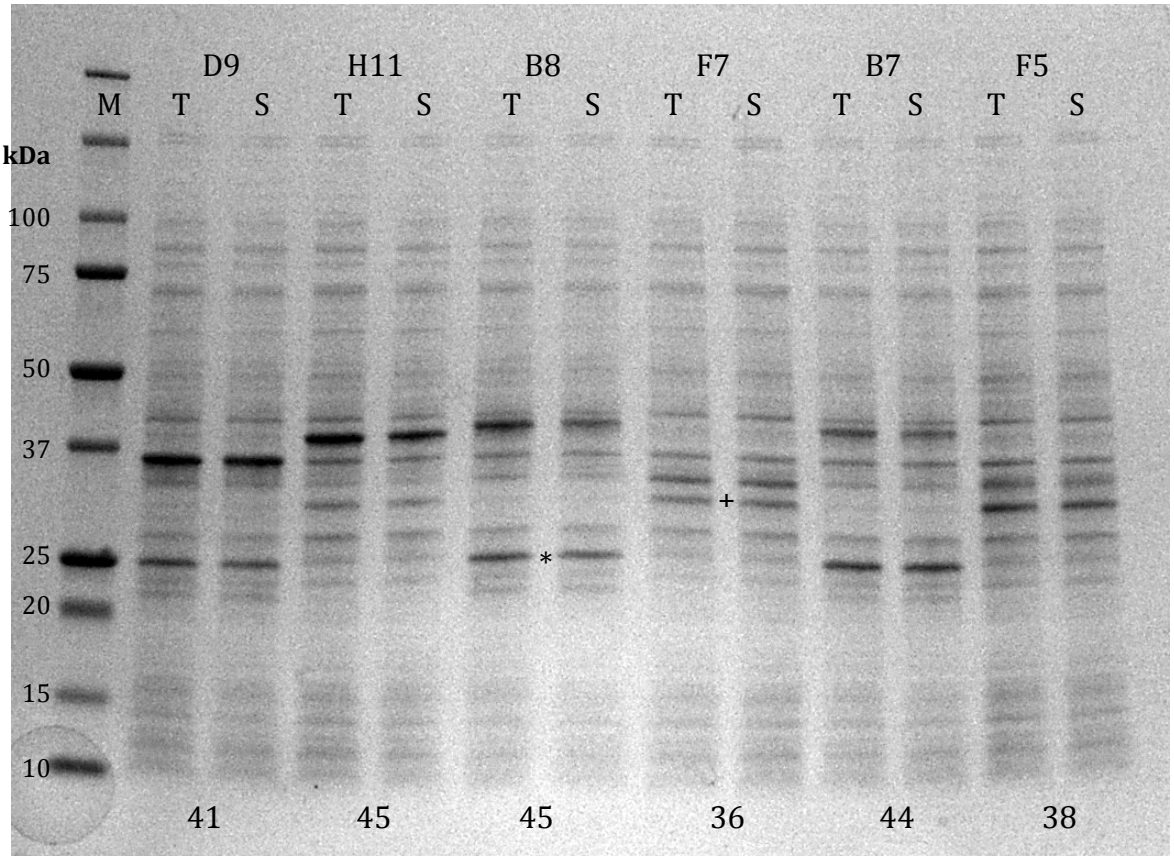
Best Hits (> 200 ng/ul in TEV elute)

Well	target ID	phosphatase	Calc. kDa (no tag)	Caliper kDa	Caliper Conc. (ng/ul)
H5	MK14_HUMAN_D0	Lambda	41.6	46.4	530
C12	VRK3_HUMAN_D0	Lambda	37.4	45.8	506
D3	GAK_HUMAN_D0	Lambda	37.9	36.4	485
E5	CSK_HUMAN_D0	YopH	30.0	29.0	469
H6	VRK1_HUMAN_D0	Lambda	41.8	46.7	467
D9	KC1G3_HUMAN_D0	Lambda	38.6	36.9	422
H11	FES_HUMAN_D0	YopH	42.8	39.8	330
D1	PMYT1_HUMAN_D0	Lambda	32.0	29.6	285
H8	MK03_HUMAN_D0	Lambda	43.4	46.0	273
G5	STK3_HUMAN_D0	Lambda	34.1	35.4	257
C6	DYR1A_HUMAN_D0	Lambda	42.0	39.5	256
C5	KC1G1_HUMAN_D0	Lambda	36.1	36.2	256
D10	MK11_HUMAN_D0	Lambda	39.8	45.7	238
H7	MK13_HUMAN_D0	Lambda	40.8	40.0	238
F7	EPHB1_HUMAN_D0	YopH	33.7	31.5	217
B8	MK08_HUMAN_D0	Lambda	42.2	42.3	214
G1	CDK16_HUMAN_D0	Lambda	36.5	33.5	202

Caliper Gel Image



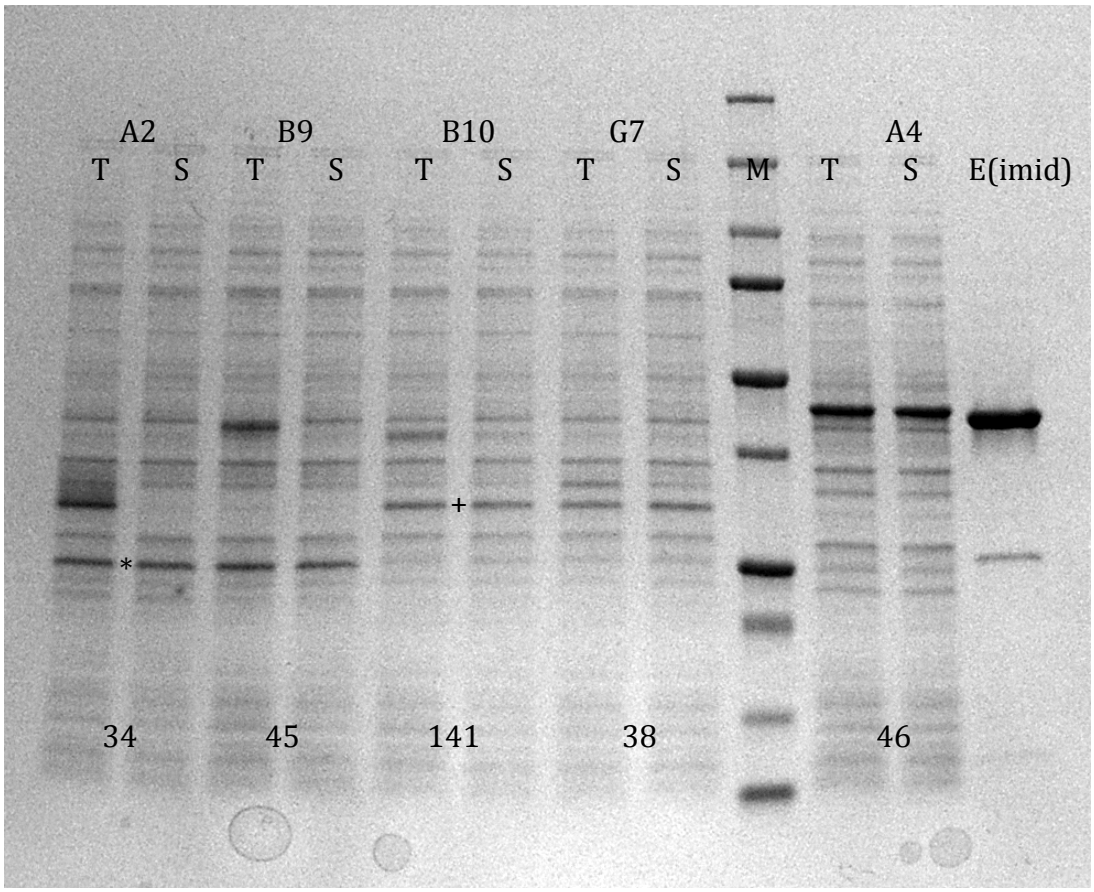
T and S for Good (D9, H11), Medium (B8, F7) and Low (B7, F5) pulldowns.
D9, B8, B7 are lambdaP. H11, F7 and F5 are YopH164.



Expected MWts (tagged) shown along bottom.

LambdaP at 25 (*), YopH164 at 33 (+).

T and S for negative pulldowns: A2, B9, B10, G7, then A4 ctrl and imid elute of A4. A2 and B9 are lambdaP. B10 and G7 are YopH164.



Expected MWts (tagged) along bottom

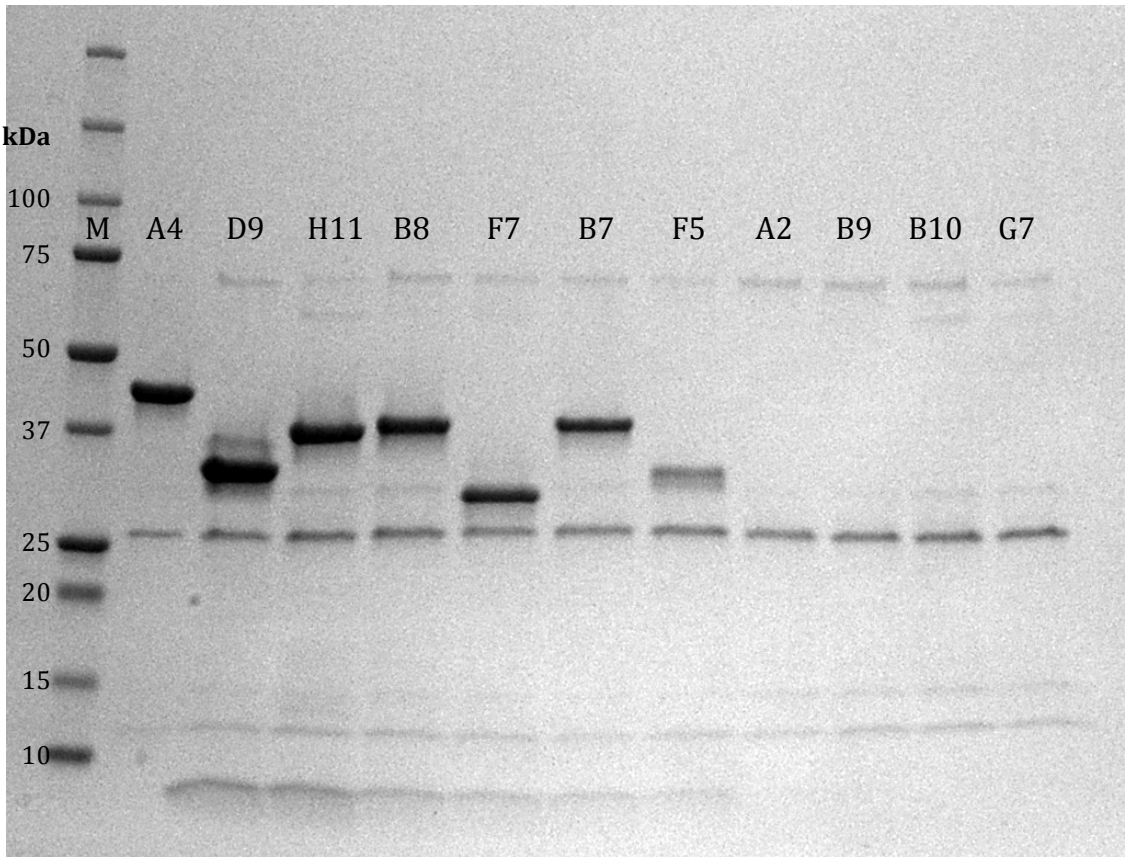
LambdaP at 25 (*), YopH164 at 33 (+).

Looks like no expression at all for B10.

A2, B9 and G7 are insoluble.

A4 is control (MBP).

Imidazole Elutions after TEV elutions (loads 4 ul for A4, 10 ul all others).



The band at 26 kDa is TEV protease. The usual host cell contaminants are seen at about 70 kDa and 12 kDa. The lowest band on the gel (<10 kDa) is the His10 tag.

For the well expressed kinases (D9, H11, B8, B7), there is a lot of tagged protein left behind on the resin. This is frequently seen in TEV elutions although it is not clear whether this is a steric issue or a time/quantity of protease issue. On scaling up to 2L purifications and off-column tag removal, this incomplete TEV reaction is rarely seen. However, keep in mind that yield estimates based solely on the TEV eluted band intensity will be underestimates.

In rare cases, a protein may bind to the nickel resin but be an extremely poor substrate for TEV. To confirm that this was not the case for some of the undetected proteins from the TEV elution step, the imidazole elutions were run here (A2, B9, B10, G7). Indeed, there is no tagged kinase in these instances.

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