**Supplemental Figure 1. Expression cloning with B7-1-Ig isolates PD-L1 cDNA clones**

In round one, 8 million cDNAs of divergent sizes (0.4-6 kb) from a CD28/CTLA4 -/- activated T cell library were transfected into COS cells. After three rounds of immunoselection with B7-1-mIgG2a, individual plasmids were digested with Xba, which cuts just 5' and 3' of the cDNA insert and the DNAs separated by gel electrophoresis. All plasmids with an intact vector band (3 KB) contained the same size cDNA insert band. Sequencing of six of those showed all 6 were PD-L1. The six differ in the exact start point of the 5' end and in the length of the poly(A) indicating they are independent isolates.

**Supplemental Figure 2. Surface plasmon resonance data**

**A)** Raw surface plasmon resonance data collected for immobilized PD-L1-Ig with B7-1-Ig as the analyte injected at concentrations between 0.3 and 21 μM. Pre-injection baseline has been subtracted, but data were not otherwise manipulated (i.e., not corrected for the reference channel or buffer blanks). Average of ~40 buffer blanks is shown (dashed line).

**B)** Equilibrium binding responses for CTLA-4-Ig to immobilized B7-1-Ig were measured using surface plasmon resonance. The calculated dissociation constant (Kd) is shown under the curve.

**Supplemental Figure 3. Confirmation of PD-L1:B7-1 interactions by Analytical ultracentrifuge**

Binding constants obtained via surface plasmon resonance can differ from solution-phase binding because one of the binding partners is necessarily affixed to a

planar surface Analysis of the gradient curves for B7-1 or PD-L1 alone ruled out any significant self-association.

We used a Beckman XL-A/XL-I instrument to perform sedimentation equilibrium experiments. PD-L1-hIgG1, B7-1-mIgG2a, CTLA-4-mIgG2a (3.6 μM) were loaded into a 6 sample rotor along with PBS blanks. The rotor was equilibrated to 20 °C and run at speeds of 4000, 5000, 6000, and 7000 rpm for 18 hours before the cartridges were scanned at two wavelengths (260 nm and 280 nm) in triplicate. Data were obtained at four rotational speeds (shown on graph) and at two wavelengths (only the 280nm wavelength data are shown). The data were analyzed using SEDPHAT from the Schuck lab at NIH (Schuck, 2003). Curve fits were replicated independently using Matlab.

**A)** Radial position (x axis) refers to the location of the analytical ultracentrifuge cell from the center of the rotor. B7-1:PD-L1 shows hetero-association with a dissociation constant of 1.58 μM.

**B)** Sedimentation equilibrium for B7-1 + CTLA-4 is shown as a positive control for the method. B7-1:CTLA-4 shows hetero-association with a dissociation constant of 0.34 μM, similar to published values (Collins et al., 2002).

**Supplemental Figure 4. Dynamic light scattering**

To further confirm the B7-1:PD-L1 interaction, we employed another solution-phase approach to measure macromolecular hetero-association, dynamic light scattering, which measures the Brownian motion and rotation of macromolecules in solution by measuring the Doppler shift of scattered laser light. The size of a rotating molecule is determined by fitting an autocorrelation function to the experimental data, resulting in a hypothetical sphere radius. Hetero- and homo-complexes are larger than monomeric, monodisperse proteins.

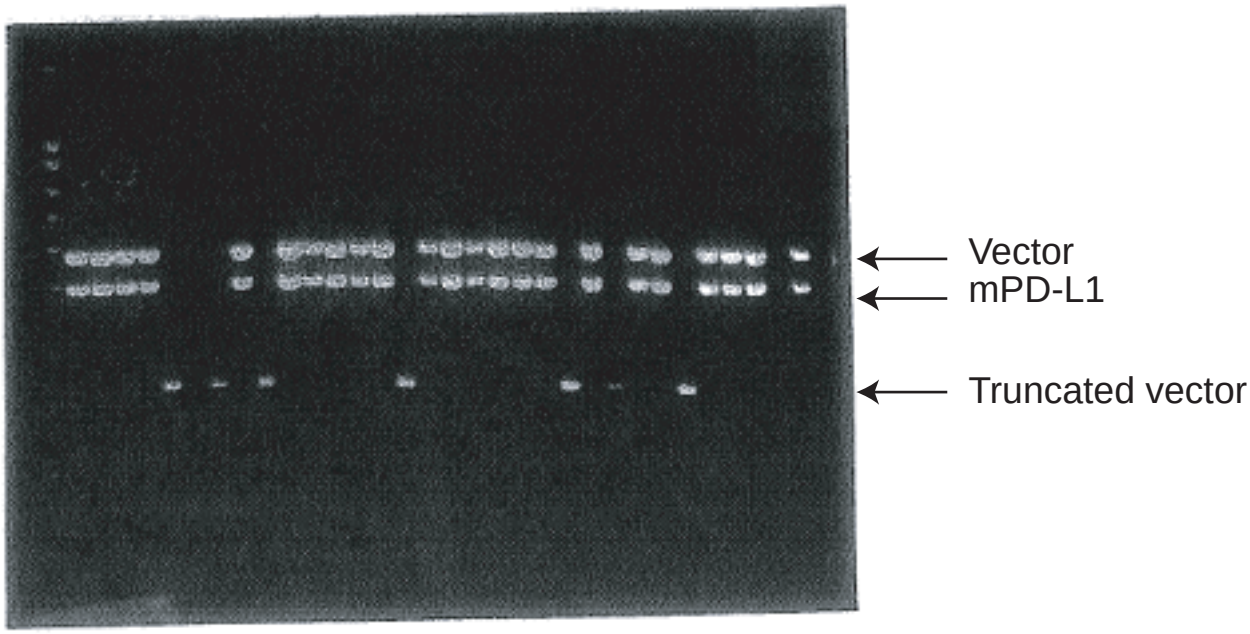
We used a Wyatt DynaPro dynamic light scattering device at 25 °C as per the manufacturer's directions. Solutions of B7-1-Ig, PD-L1-Ig, CTLA4-Ig, and control Ig at 4 μM concentration were centrifuged at 4 °C for 30 min at 18,000 x *g* to remove aggregates, and equilibrated to room temperature in a quartz cuvette individually and together. Samples (n > 20 in each case) were obtained every 10 seconds and a stable baseline was always observed. The DYNAMICS software (Wyatt) was used to fit the autocorrelation data and produce the mean hydrodynamic radius of the particles.

Mean hydrodynamic radius of particles for individual molecules and their complexes are shown. There was a statistically significant increase in the complex size for B7-1:PD-L1 ($p = 0.01$) and B7-1:CTLA-4 ($p < 0.001$) versus that of B7-1, PD-L1, or CTLA-4 alone. The PD-L1:mIgG2a mixture showed a radius that was the average of the two radii, indicating lack of association.

**Supplemental Figure 5. List of all cross-linked peptides matched between B7-1 and PD-L1**

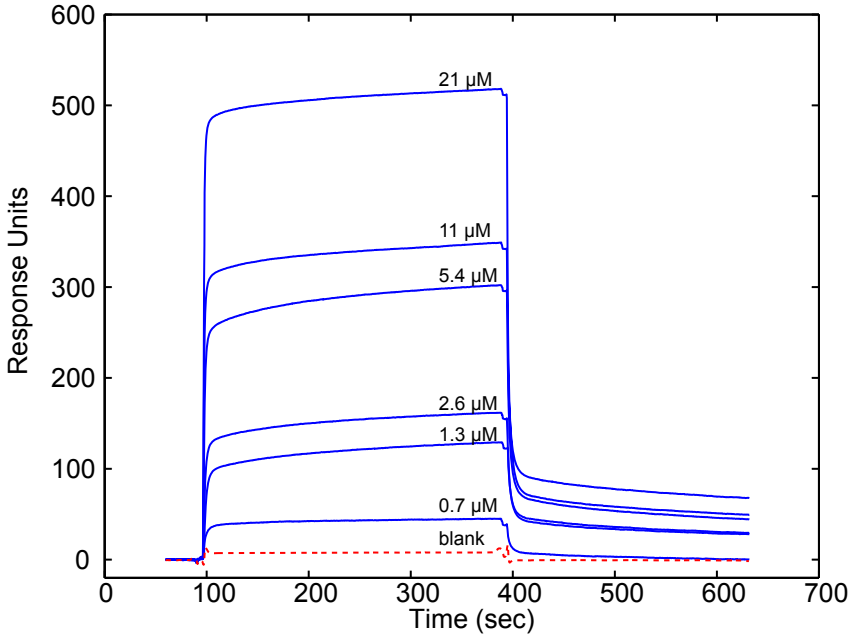
Mass spectrometry of fragments of chymotrypsin-digested 341 kDa band, whose uncharged molecular weights (MW) were identified as the MW sum from peptides of B7-1 and PD-L1 and the sulfo-SBED cross-linker are presented.

Supplemental Figure 1.

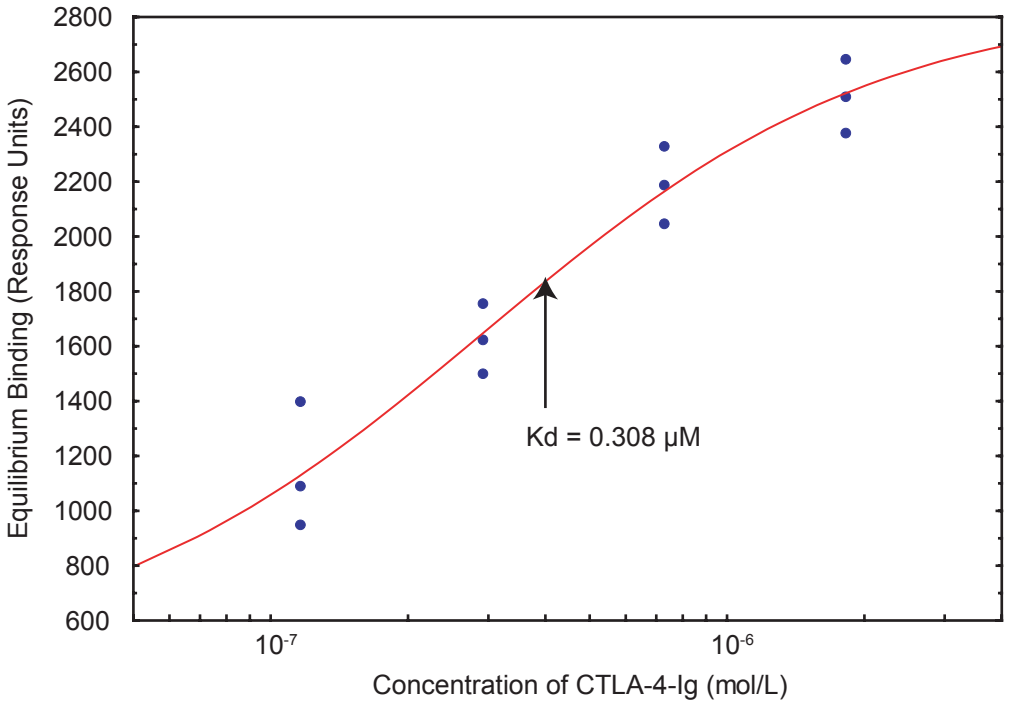


# Supplemental Figure 2.

A.

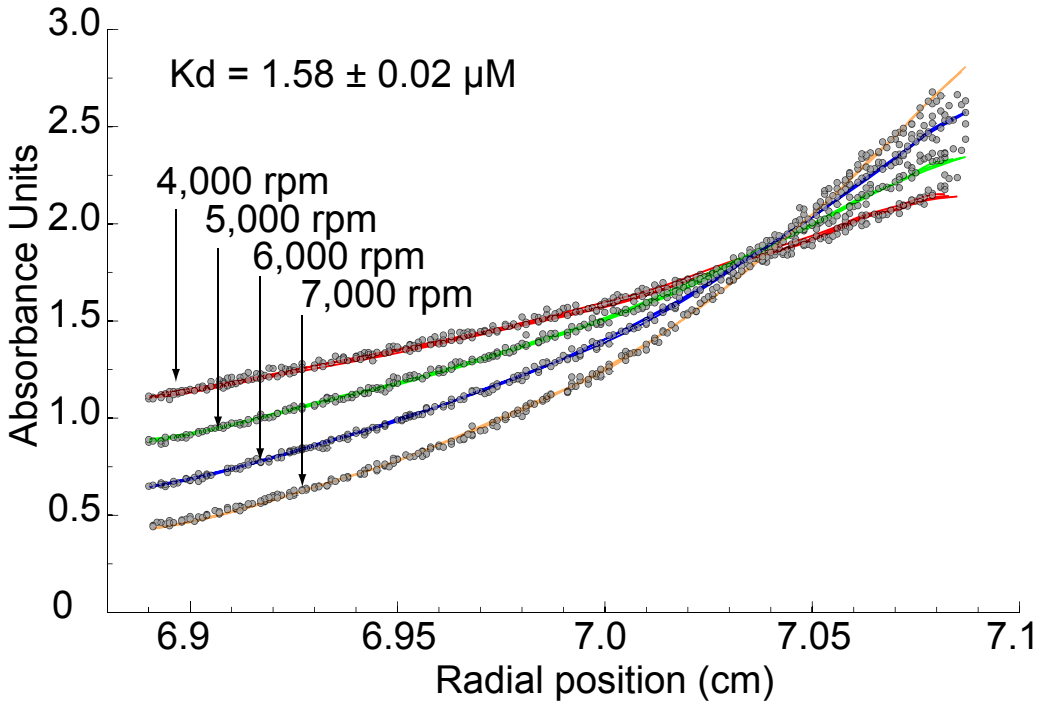



B.

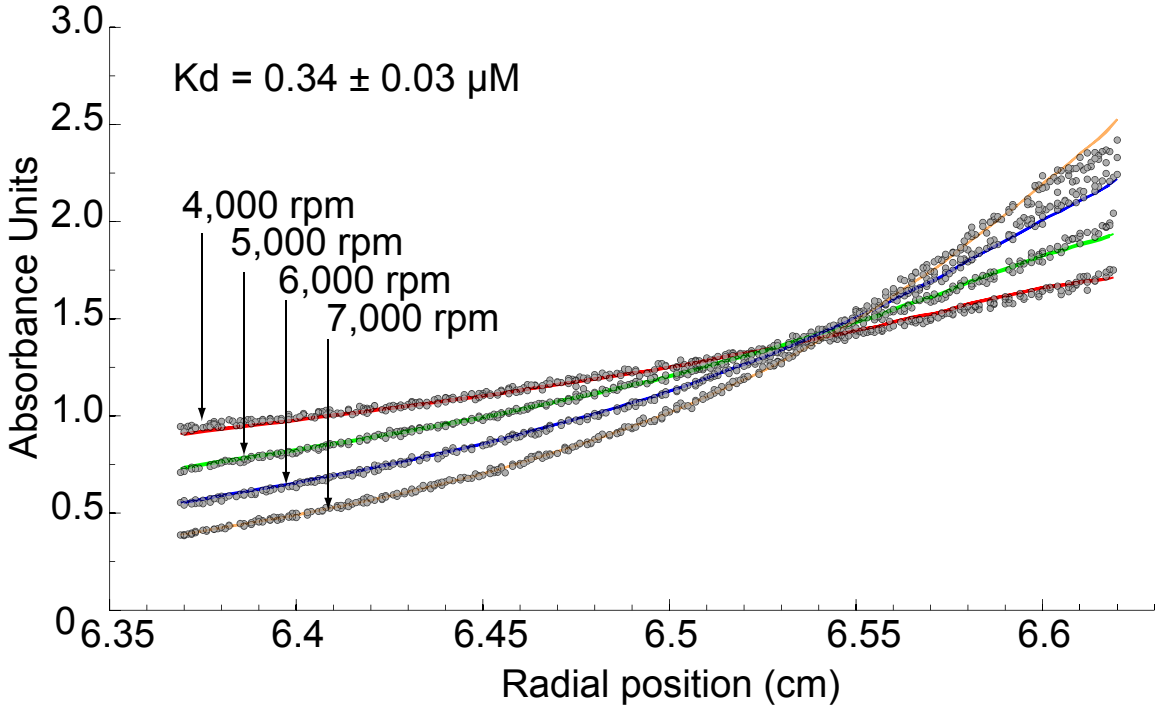


Supplemental Figure 3.

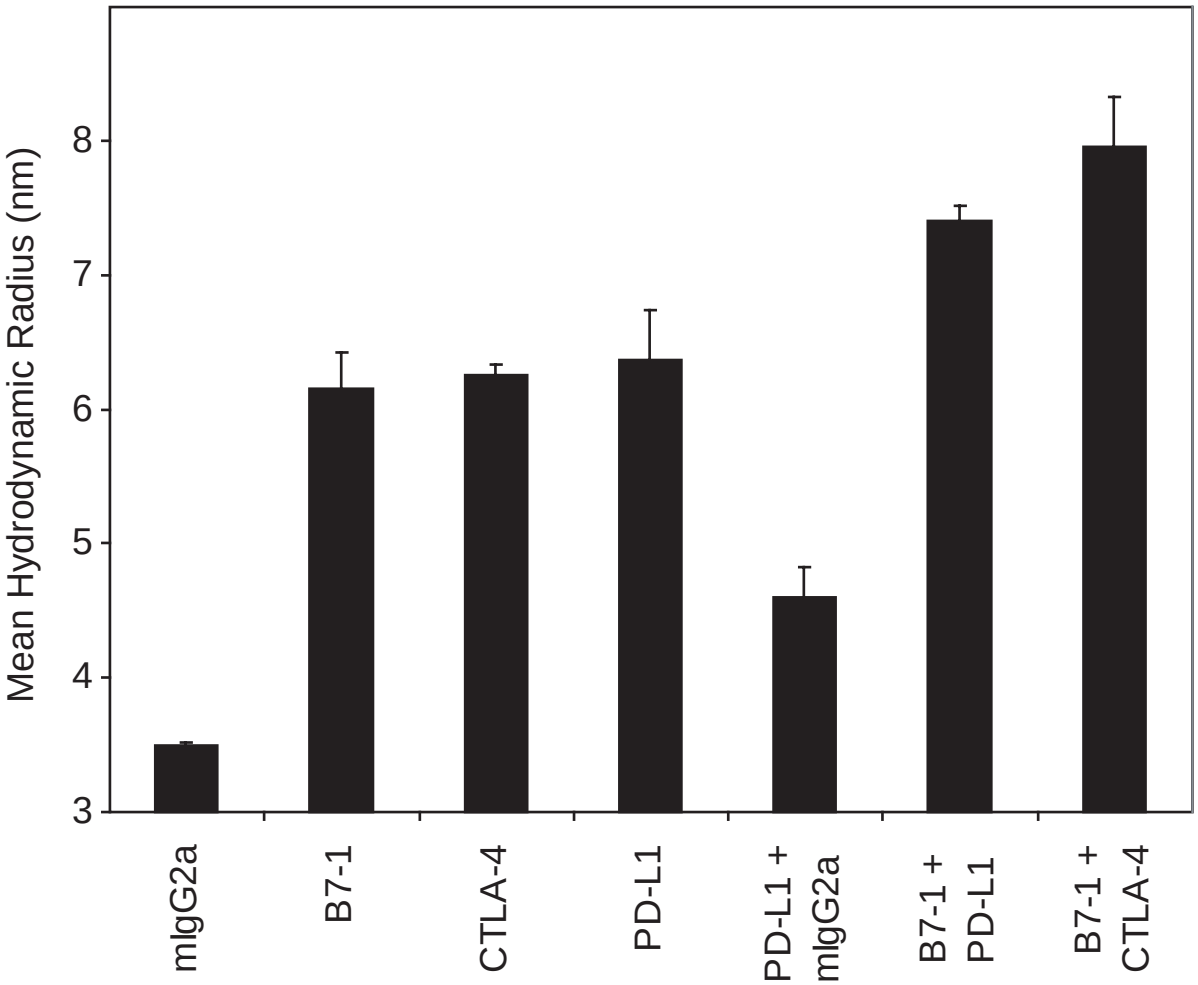
A.




B.



Supplemental Figure 4



Supplemental Figure 5.

| KEY | | | |
|---|---|---|---|
| | 2-way hit | **bold font** | counted |
| | 2-way hit, one protein was repeated twice | | |
| | 3-way hit with one outcome | | |
| | 3 way hit with multiple outcomes | | |
| | 3-way hit with no self-links | | |

| MW Peak | Protein | Site | Mods | Protein | Site | Mods | Protein | Site | Mods | Peptide | Peptide | Peptide |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **4019.1354** | B71 | 2 | pyroGlu | B71 | 2 | | PDL1 | 1 | AcetN | (W)QKHDKVVL(S) | (L)KVWPEY(K) | (-)RIFAGIIF(T) |
| **4035.1156** | B71 | 2 | pyroGlu | B71 | 2 | | PDL1 | 1 | | (W)QKHDKVVL(S) | (Y)KNRTLY(D) | (F)TITAPKDLY(V) |
| **4203.0682** | B71 | 2 | | B71 | 2 | | PDL1 | 3 | | (Y)WQKHDKVVL(S) | (L)SVIAGKL(K) | (Y)GGADYKRITL(K) |
| **4203.0682** | B71 | 2 | | B71 | 2 | | PDL1 | 1 | Acet | (W)QKHDKVVL(S) | (L)SVIAGKLKVWPEY(K) | (-)RIF(A) |
| 4019.1354 | B71 | 3 | | B71 | 2 | | PDL1 | 2 | | (L)ALVKLSIKADF(S) | (L)SVIAGKL(K) | (L)KPQHSNF(R) |
| **3905.1397** | PDL1 | 2 | | B71 | 2 | | | | | (L)QITDVKLQDAGVY(C) | (Y)WQKHDKVVLSVIAGKL(K) | |
| 4019.1354 | PDL1 | 2 | | B71 | 2 | | PDL1 | 3 | | (L)KGNAALQITDVKL(Q) | (L)SVIAGKL(K) | (L)KVNAPY(R) |
| **3890.1166** | PDL1 | 3 | | B71 | 2 | | | | | (Y)GGADYKRITLKVNAPY(R) | (L)SVIAGKLKVWPEY(K) | |
| **3890.1166** | PDL1 | 3 | | B71 | 2 | | PDL1 | 2 | | (Y)KRITLKVNAPY(R) | (Y)KNRTL(Y) | (L)LKGNAAL(Q) |
| **3905.1397** | B71 | 1 | | B71 | 3 | | PDL1 | 2 | | (-)DVDEQLSKSVKDKVL(L) | (L)VKL(S) | (L)KGNAAL(Q) |
| **4203.0682** | B71 | 1 | | B71 | 3 | | PDL1 | 2 | | (L)SKSVKDKVL(L) | (L)VKL(S) | (F)VAGEEDLKPQHSNF(R) |
| **3957.1259** | B71 | 3 | | B71 | 3 | | PDL1 | 2 | | (Y)EVKHLALVKL(S) | (L)SIKADF(S) | (L)KPQHSNF(R) |
| **3957.1259** | B71 | 3 | | B71 | 3 | | PDL1 | 2 | | (L)VKLSIKADF(S) | (Y)EVKHLAL(V) | (L)KPQHSNF(R) |
| **3957.1374** | PDL1 | 2 | | B71 | 3 | | PDL1 | 3 | | (L)KGNAALQITDVKL(Q) | (Y)EVKHL(A) | (L)KVNAPY(R) |
| 4019.1354 | PDL1 | 3 | | B71 | 3 | | B71 | 2 | | (Y)KRITLKVNAPY(R) | (Y)EVKHL(A) | (L)KVWPEY(K) |
| 4203.0682 | PDL1 | 3 | | B71 | 3 | | B71 | 2 | | (Y)KRITLKVNAPY(R) | (Y)EVKHLAL(V) | (L)KVWPEY(K) |
| **4019.1354** | B71 | 2 | | B71 | 5 | | PDL1 | 3 | | (W)QKHDKVVLSVIAGKL(K) | (L)IKY(G) | (L)KVNAPY(R) |
| **4035.1156** | PDL1 | 1 | | B71 | 5 | | | | | (-)MRIFAGIIFTACCHL(L) | (F)NTTRNHTIKCLIKY(G) | |
| **4035.1156** | PDL1 | 2 | | B71 | 5 | | PDL1 | 1 | | (L)LKGNAALQITDVKL(Q) | (L)IKY(G) | (F)TITAPKDL(Y) |
| **3957.1259** | B71 | 2 | | PDL1 | 1 | | B71 | 2 | | (L)KVWPEYKNRTL(Y) | (-)MRIF(A) | (L)SVIAGKL(K) |
| **3957.1259** | B71 | 2 | | PDL1 | 1 | | B71 | 2 | | (L)SVIAGKLKVWPEY(K) | (-)MRIF(A) | (Y)KNRTL(Y) |
| 4035.1156 | B71 | 2 | | PDL1 | 1 | AcetN | PDL1 | 3 | | (L)KVWPEYKNRTLY(D) | (-)RIF(A) | (L)KVNAPY(R) |
| **4035.1156** | B71 | 2 | | PDL1 | 1 | | PDL1 | 1 | | (W)QKHDKVVL(S) | (-)MRIF(A) | (L)RAFTITAPKDL(Y) |
| **4203.0682** | B71 | 2 | | PDL1 | 1 | Acet | B71 | 2 | | (W)QKHDKVVLSVIAGKL(K) | (-)RIF(A) | (L)KVWPEY(K) |
| **4203.0682** | B71 | 2 | | PDL1 | 1 | Acet | B71 | 2 | | (L)SVIAGKLKVWPEY(K) | (-)RIF(A) | (W)QKHDKVVL(S) |
| **4203.0682** | B71 | 2 | | PDL1 | 1 | Acet | B71 | 2 | | (W)QKHDKVVL(S) | (-)RIF(A) | (L)SVIAGKLKVWPEY(K) |
| **3890.1166** | B71 | 3 | | PDL1 | 1 | | | | | (Y)SCVVQKKERGTYEVKHLAL(V) | (-)MRIFAGIIF(T) | |
| **3888.1302** | B71 | 2 | | PDL1 | 2 | pyroGlu | | | | (Y)WQKHDKVVLSVIAGKL(K) | (L)QITDVKLQDAGVY(C) | |
| **3888.1302** | B71 | 2 | | PDL1 | 2 | | | | | (W)QKHDKVVLSVIAGKLKVWPEY(K) | (L)QITDVKL(Q) | |
| **4203.0682** | B71 | 2 | | PDL1 | 2 | | | | | (L)SVIAGKLKVWPEY(K) | (L)KPQHSNFRGRASLPKDQL(L) | |
| **1850.8514** | B71 | 3 | | PDL1 | 2 | | | | | (L)VKL(S) | (L)KPQHSNF(R) | |
| **3905.1397** | B71 | 3 | | PDL1 | 2 | | PDL1 | 2 | pyroGlu | (Y)EVKHLALVKL(S) | (L)LKGNAAL(Q) | (L)QITDVKL(Q) |
| 3957.1374 | B71 | 3 | | PDL1 | 2 | | PDL1 | 3 | | (L)VKLSIKADF(S) | (L)KGNAAL(Q) | (Y)GGADYKRITL(K) |
| 4019.1354 | B71 | 3 | | PDL1 | 2 | | PDL1 | 1 | | (L)ALVKLSIKADF(S) | (L)LKGNAAL(Q) | (F)TITAPKDL(Y) |
| **4035.1156** | B71 | 3 | | PDL1 | 2 | | B71 | 2 | | (Y)SCVVQKKERGTY(E) | (L)KGNAAL(Q) | (Y)KNRTLY(D) |
| **3890.1166** | B71 | 4 | | PDL1 | 2 | | | | | (F)ASGGFPKPRF(S) | (L)KPQHSNFRGRASLPKDQLL(K) | |
| 3888.1302 | B71 | 2 | | PDL1 | 3 | | PDL1 | 1 | | (W)QKHDKVVL(S) | (Y)KRITL(K) | (F)TITAPKDLY(V) |
| **4035.1156** | B71 | 2 | | PDL1 | 3 | | | | | (W)QKHDKVVLSVIAGKL(K) | (Y)GGADYKRITLKVNAPY(R) | |
| 4203.0682 | B71 | 2 | | PDL1 | 3 | | B71 | 4 | | (W)QKHDKVVL(S) | (Y)KRITL(K) | (F)ASGGFPKPRFSW(L) |
| 4203.0682 | B71 | 2 | pyroGlu | PDL1 | 3 | | PDL1 | 2 | | (W)QKHDKVVL(S) | (Y)KRITL(K) | (F)RGRASLPKDQLL(K) |
| **3905.1397** | B71 | 3 | | PDL1 | 3 | | B71 | 2 | | (Y)EVKHLALVKL(S) | (L)KVNAPY(R) | (Y)KNRTLY(D) |