SUPPLEMENTAL MATERIAL

"*On silico*" peptide microarrays for high-resolution mapping of antibody epitopes and diverse protein-protein interactions.

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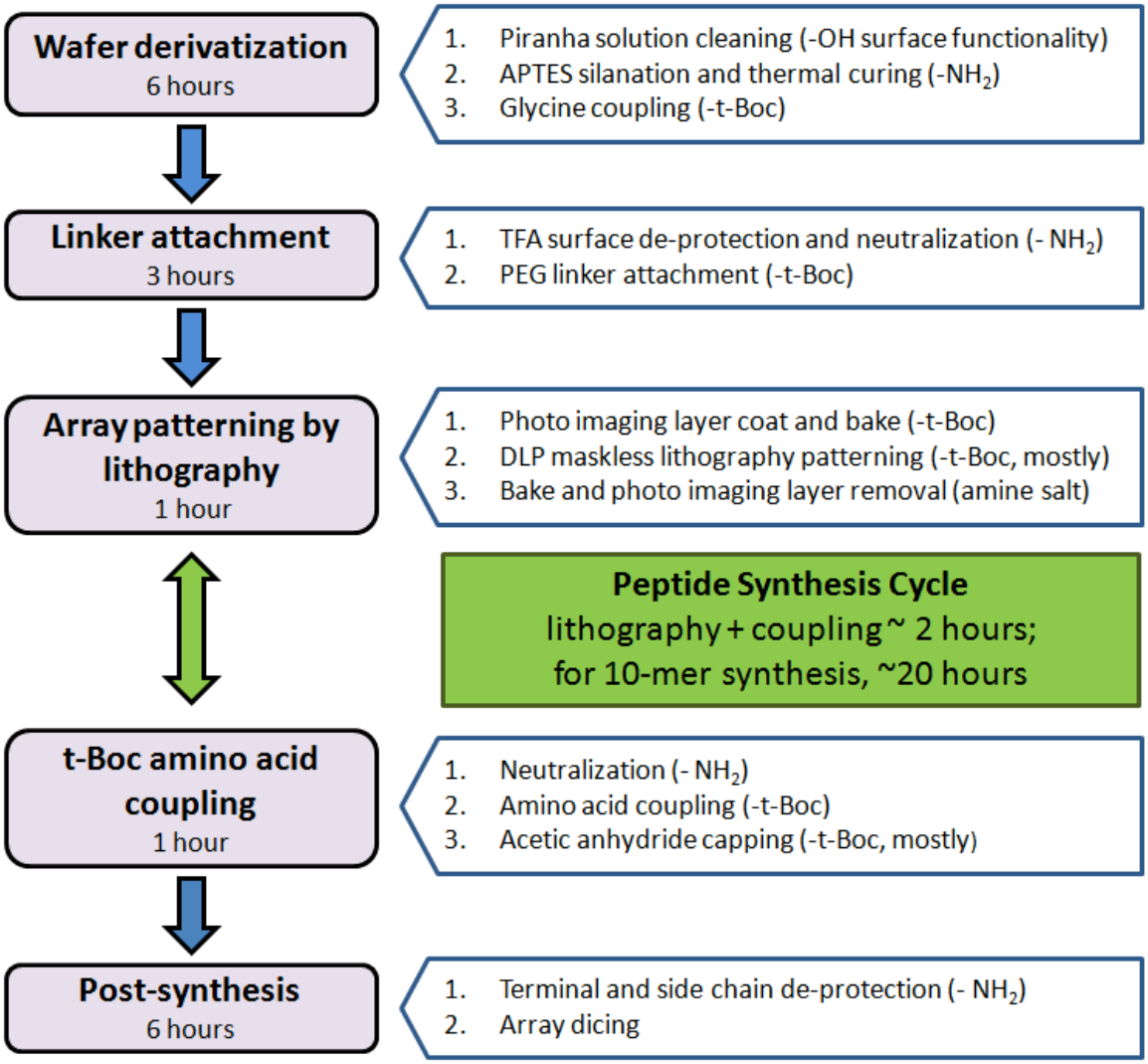
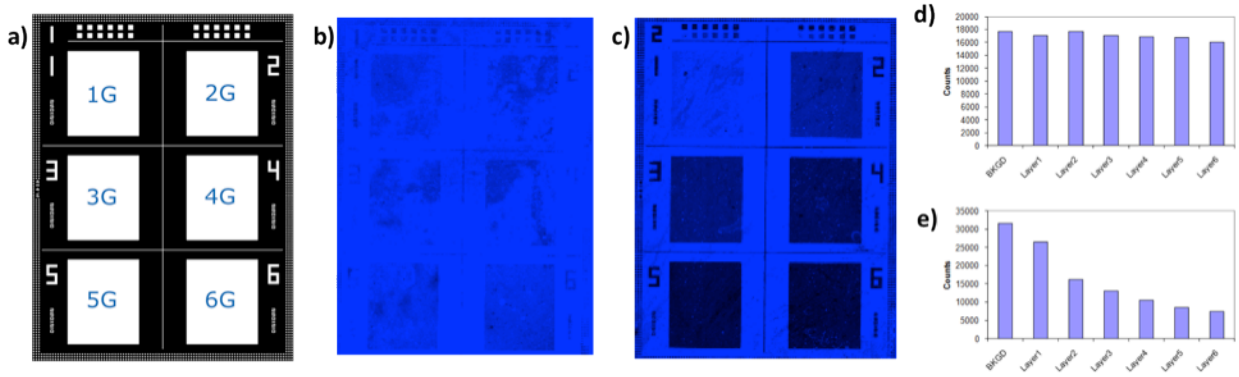


Supplementary Figure 1. Surface modification process for Intel array wafers. The fabrication of Intel Arrays entails wafer-scale cleaning, silanation, linker attachment, and a peptide synthesis cycle that involves a series of coupled photolithography and amino acid linking steps and post-processing. Selective UV light exposure results in the removal of the protective Di-*tert*-butyl dicarbonate (*t*-BOC) groups at specific array features allowing for incorporation of an amino acid of interest at the de-protected sites during the subsequent coupling step. This cycle of photolithography and amino acid coupling is repeated to achieve peptide arrays with desired amino acid sequences.

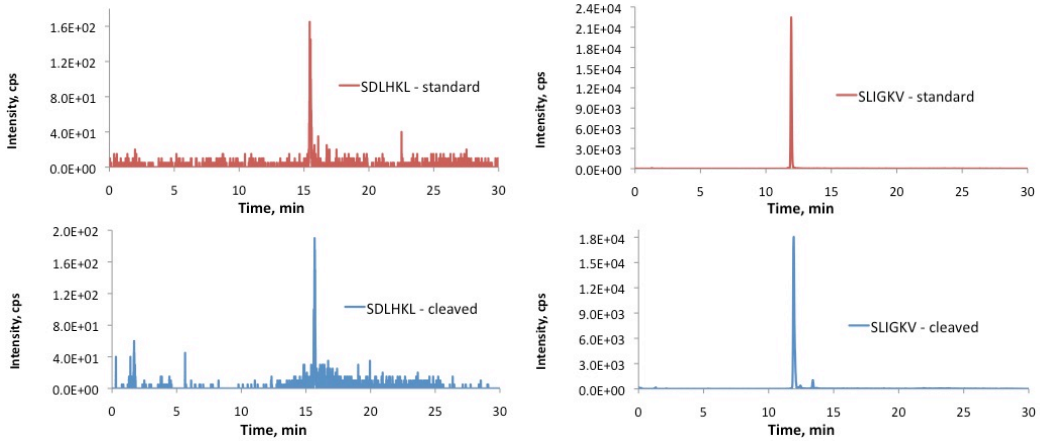


Supplementary Figure 2. Comparison of high and low synthesis yield of 1-mer to 6-mer glycine peptides. (**a**) mask design used with regions numbered "1" to "6" for different length peptides and regions in black used to pattern areas terminated with background linker, (**b**) fluorescence image of carboxy-fluorescein-labeled Gly1–Gly6 peptides and background linker on high-yield chip, (**c**) fluorescence image of carboxy-fluorescein-labeled Gly1–Gly6 peptides and background linker on low-yield chip (**d**) average MFI for each region of the high-yield chip corresponding to each synthesis step with accumulated yield of 90% across six steps, an average of >98% step-wise yield, and (**e**) average MFI for each region of the low-yield chip corresponding to each synthesis step with accumulated yield of 24%, a step-wise yield of 79%.

a

Peptide	Molecular Mass	Monoisotopic mass, $[M+H]^+$	Monoisotopic mass, $[M+H]^{2+}$
SDLHKL	711.4 g/mol	712.3993	356.7035
SLIGKV	615.4 g/mol	616.4033	308.7055

b **c**



Supplementary Figure 3. Confirmation of peptide sequences synthesized on surface. Comparison of LC-MS/MS-MRM TIC spectra from peptide control samples and peptides synthesized on surface prior to cleavage: (**a**) Table of expected masses for peptides synthesized on the Intel array platform. (**b**) +MRM TIC from m/z 357.0 → m/z 397.3 transition monitored from peptide SDLHKL standard above (15.44 min, ~160 cps) and cleaved sample below (15.67 min, ~190 cps) and (**c**) +MRM TIC from m/z 308.8 → m/z 173.0 transition monitored from peptide SLIGKV (11.94 min, ~2.2 x 10^4 cps) compared to solution containing cleaved peptide (11.93 min, ~1.8 x 10^4 cps).

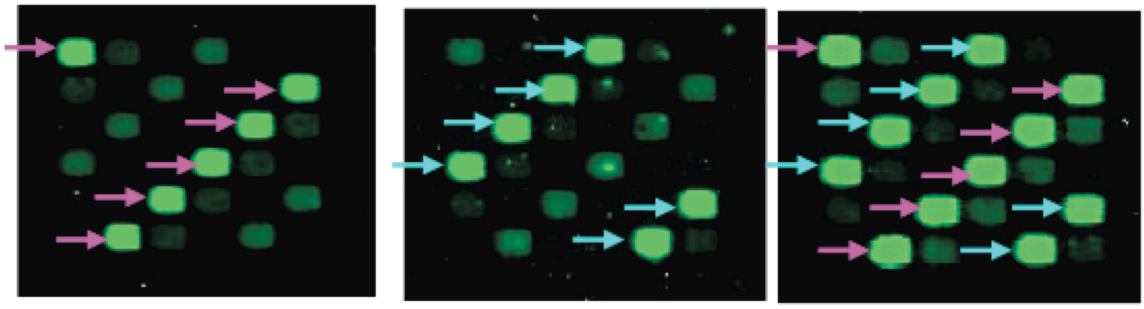
a

Peptide Number	Type	Peptide Sequence
1	Abl WT	AIYA APFK
2	Abl Mt 1	ARYA APDK
3	Abl Mt 2	AIGA APFK
4	Src WT	EIYG EFKK
5	Src Mt 1	EAYG EAKK
6	Src Mt 2	EIAG EFKK

b

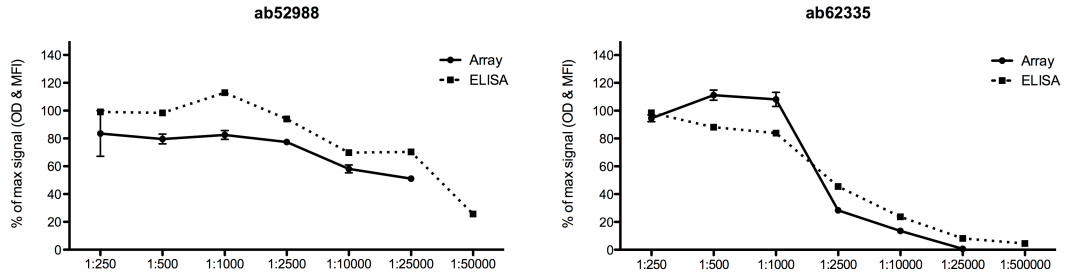
1	2	3	4	5	6
2	3	4	5	6	1
3	4	5	6	1	2
4	5	6	1	2	3
5	6	1	2	3	4
6	1	2	3	4	5

c d e

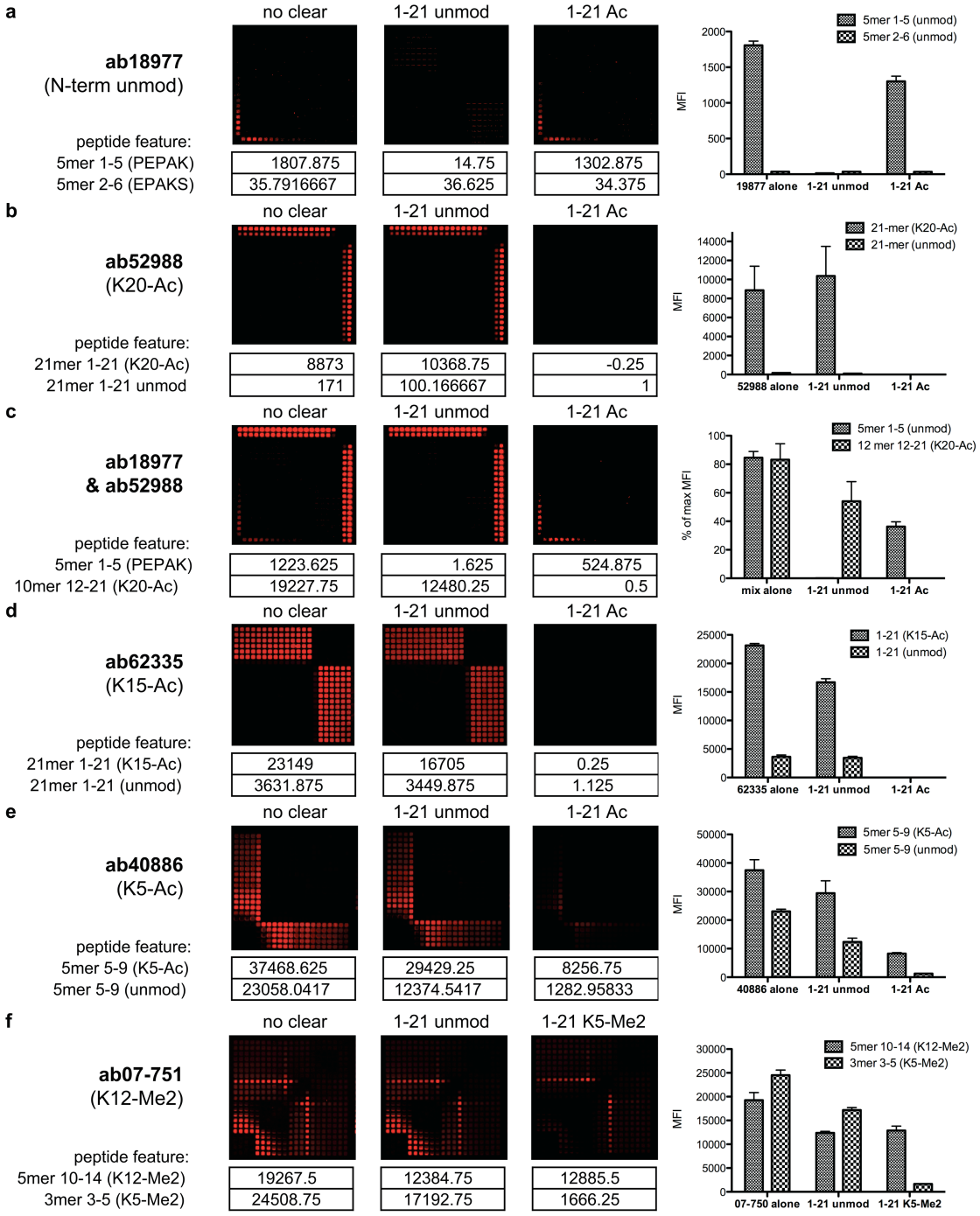


Supplementary Figure 4. Functional assessment of enzyme interaction with peptides on surface. (**a**) Table displaying Abl and Src kinase substrate peptide sequences. (**b**) Table showing peptide location on synthesized array. Fluorescent kinase assay results: (**c**) strong fluorescence for Abl WT peptide at position 1, as expected, with some interaction with Abl Mt peptide at location 2 and Src WT peptide at location 4; (**d**) strong fluorescence for Src WT peptide at position 4, as expected, with some interaction with Abl WT peptides at location 1 and Src Mt peptide at location 5, and (**e**) strong fluorescence for Src and Abl WT peptides in an assay containing both Abl and Src, as expected. Square sizes in the fluorescence images are approximately 50 µm x 50 µm with a 30 µm gap between squares.

a **b**

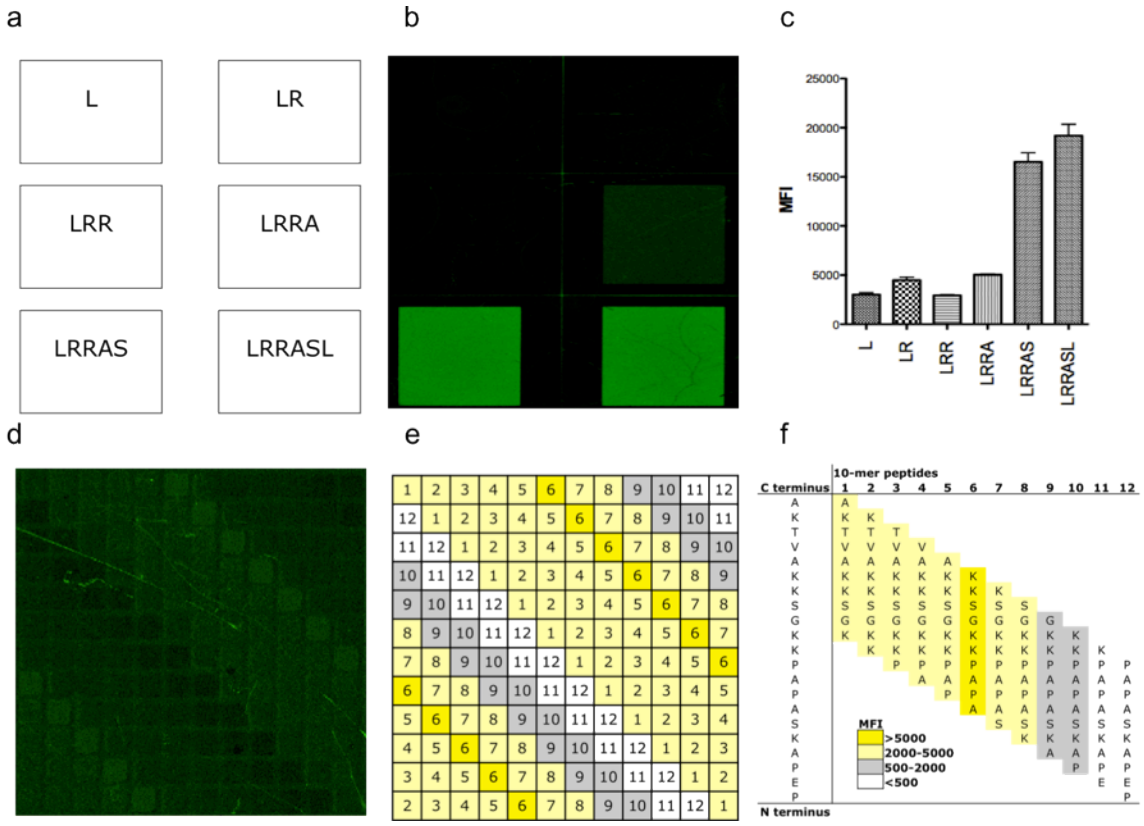


Supplementary Figure 5. Direct comparison of Intel array sensitivity with ELISA. Graphs displaying percent of maximum signal to full-length, 21-mer H2B peptide acetylated at lysine residues 5, 12, 15 and 20 (N_H3-PEPAK(Ac)SAPAPKK(Ac)GSK(Ac)KAVTK(Ac)A-COOH) as assessed by Intel array (MFI) or ELISA (OD) in a titration of (**a**) antibody 52988 (Abcam) or (**b**) antibody 62335 (Abcam). Each point represents the mean MFI or OD ± SEM of percent of highest signal feature (array) or well (ELISA) value in the 1:250 dilution condition. Error bars reflect mean ± SEM of 6 identical array features and triplicate ELISA wells, respectively.

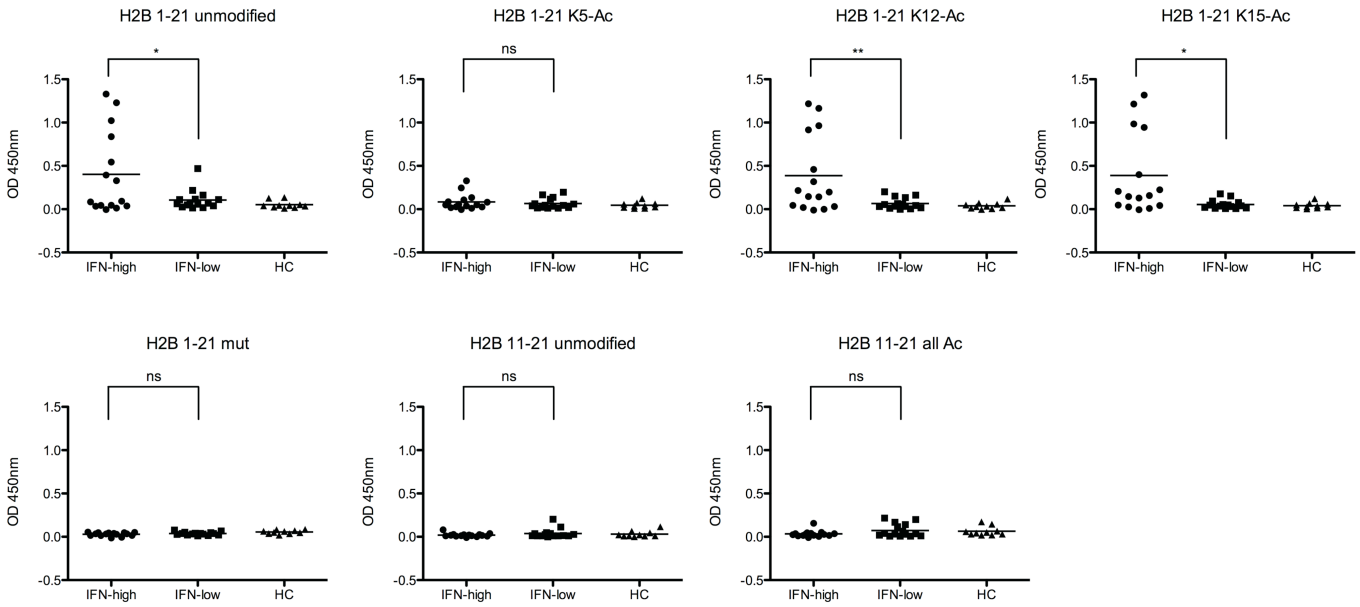


<u>Supplementary Figure 6. Pre-clearing of specific reactivity of commercial antibodies on the Intel array platform.</u> Array images showing reactive features and MFI displayed in table and histogram form for indicated Intel array peptides either not pre-cleared, or after five rounds of clearing with H2B peptide 1-21 unmodified, 1-21 Ac (H2B peptide acetylated at Lys5, Lys12, Lys15 and Lys20), or H2B Lys5-Me2 in the presence of (**a**) ab18977; (**b**) ab52988; (**c**) mixture of ab18977 and ab52988; (**d**) ab62335; (**e**) ab40886; (**f**) ab07-751. Each antibody was diluted 1:1000 before direct application to the Intel array or exposure to pre-clearing conditions. Values corresponding to MFI minus local background are provided in table form for each array shown. The peptides chosen for representation in table and histogram form for each array correspond to the brightest features observed in the non-pre-cleared condition, alongside

equivalent single amino-acid-shifted peptide (in the case of ab18977) or un-modified peptide (in the case of Abs 52988, 62335, 40886 and 07-751).



Supplementary Figure 7. Sequence-specific phosphorylation of peptides on Intel arrays. (**a**) Schematic depicting peptide sequence at each array location. Each block contains sequentially growing peptide that spans the kemptide PKA target sequence LRRASL. (**b**) Scanned image of a six-block Intel array incubated with bovine PKA and phosphorylation visualized with Pro-Q Diamond stain (Promega, Madison, WI). (**c**) Histogram displaying mean + SEM of MFI at each location in the six-block array. (**d**) Scanned image of an Intel array containing 10-mer overlapping peptides spanning the 21-mer H2B sequence incubated with bovine PKA and phosphorylation visualized with Pro-Q Diamond stain. (**e**) Schematic depicting the peptide identity of features in (d) corresponding to sequences displayed in (f). Color indicates MFI range corresponding to level of phosphorylation of peptide features (see key). (**f**) Peptide identity and sequence information for array image (d) and schematic (e) of 10-mer peptides spanning the 21-mer H2B sequence. Key indicates MFI range of array features.



Supplementary Figure 8. ELISA validation of IFN-high patient sera reactivity to unmodified and acetylated H2B peptides. Reactivity to the indicated peptides was assessed by ELISA as described in the materials and methods. Patient serum was diluted 1:250. * and ** indicate *P* values <0.05 and <0.01, respectively, in a two-tailed student's *t* test. Peptide sequences are as follows:
H2B 1-21 unmodified (N_H3-PEPAKSAPAPKKGSKKAVTKA-COOH);
H2B 1-21 K5-Ac (N_H3-PEPAK(Ac)SAPAPKKGSKKAVTKA-COOH);
H2B 1-21 K12-Ac (N_H3-PEPAKSAPAPKK(Ac)GSKKAVTKA-COOH);
H2B 1-21 K15-Ac (N_H3-PEPAKSAPAPKKGSK(Ac)KAVTKA-COOH);
H2B 1-21 mut (N_H3-PEPAASAPAPAAGSAAAVTAA-COOH);
H2B 11-21 unmodified (N_H3-KK GSKKAVTKA-COOH);
H2B 11-21 all Ac (N_H3-KK(Ac)GSK(Ac)KAVTK(Ac)A-COOH).

Wafer derivatization. Blank six-inch silicon wafers were used for Intel array fabrication (p-type, boron-doped, <100> orientation, 1 ohm cm^{-1}, 675 ± 25 μm, polished front side, etched back side from Silicon Valley Microelectronics, Santa Clara, CA). Wafers were thermally oxidized according to standard procedures[1] to form a 2500nm-thick silicon oxide layer, and custom-designed chrome alignment marks were used to provide accurate alignment from layer to layer. Wafers were cleaned by immersion in piranha solution, consisting of a 1:1 v/v mixture of concentrated sulfuric acid (Sigma-Aldrich, St. Louis, MO) and hydrogen peroxide (Fisher Scientific, Pittsburg, PA), for 1 h at room temperature (RT). Following cleaning, wafers were thoroughly rinsed with distilled water (diH_20) and ethyl alcohol (EtOH, Sigma) and dried in a flowing nitrogen gas. For silanation, the wafers were immersed in a 0.5% v/v mixture of 3-aminopropyltriethoxysilane (Sigma) in 95% EtOH for 30 min at RT. Upon completion of the reaction, the wafers were rinsed with 95% EtOH, dried, and then cured at 100 °C for 1 hour under ultrapure and ultradry N_2 (Airgas, Sacramento, CA) to crosslink silane groups to each other and to the silicon surface. Prior to storage, a single layer of amino acid (typically glycine) was covalently bound to exposed amine groups on the wafer surface as described in the Peptide Synthesis Chemistry section below. The derivatized wafer was subsequently stored in a clean, dry container at RT prior to subsequent processing.

Linker attachment. In order to construct custom peptides on the wafer surface, polyethylene glycol (PEG)-based linker molecules were added to the wafer surface. Briefly, the wafer was treated with trifluoroacetic acid (TFA, Sigma) for 15 min at RT to remove the protective *t*-BOC group on glycine. The wafer was washed with 2-propanol (IPA, Sigma) and *N,N*-dimethylformamide (DMF, Sigma) and then neutralized in a solution of 5% *N,N*-diisopropylethylamine (DIEA,

Sigma) in DMF for 5 min before it was rinsed with EtOH and dried. A linker layer was deposited on the wafer by adding a solution of *t*-BOC-PEG or O-(N-Boc-2-aminoethyl)-O'-(N-diglycolyl-2-aminoethyl)hexaethyleneglycol (Novabiochem, San Diego, CA), 1-Hydroxybenzotriazole (CPC Scientific, Sunnyvale, CA), and *N,N'*-diisopropylcarbodiimide (CPC Scientific) in 1-methyl-2-pyrrolidinone (Sigma) at RT for 2 h with gentle shaking. The wafer was then dried and the surface was treated with 25% acetic anhydride (Ac_2O, Sigma) in DMF to cap unreacted terminal amine sites.

Array patterning by photolithography. For wafer-scale array patterning, a photosensitive imaging solution containing a photoacid generator and a sensitizer in propylene glycol methyl ether acetate (PGMEA, Sigma) was applied using a spin coater (Brewer Science, Rolla, MO). Wafers were baked on a hot plate at 80 °C to dry the photosensitive layer and then cooled to RT before being transferred to a computer controlled X-Y stage (Newport Corporation, Irvine, CA) capable of wafer movement with single-micron accuracy. In a class 10,000 cleanroom, array features were patterned by exposing selected areas to UV light through a virtual mask with a digital exposure tool modified from a Texas Instrument DLP device (Texas Instruments, Dallas, TX), which consisted of a 768x1024 array of 10x10 μm-wide microscopic mirrors with 0.75 μm pitch. The wafer was aligned from layer to layer using custom chrome alignment marks. After UV light exposure, the wafer was baked to ensure the photo-generated acid completely reacted with the *t*-BOC protective group, de-protecting the array features. Following this step, the photosensitive imaging layer was stripped off with PGMEA. The wafers were subsequently cleaned with IPA and transferred to a solution of 5% DIEA in DMF for neutralization, at which point the patterned

features consisted of terminal amine salt. The wafers were then rinsed in DMF and IPA and cycled through the next amino acid coupling.

Peptide synthesis chemistry. For each amino acid coupling step, *t*-BOC amino acids were coupled to arrayed patterns of exposed surface amine groups. Briefly, a solution containing equimolar amounts of the *t*-BOC amino acid of interest, hydroxybenzotriazole (HOBt, CPC Scientific), and *N*,*N*'-diisopropylcarbodiimide (DIC, CPC Scientific) were mixed and dissolved in *N*-Methyl-2-pyrrolidone (NMP, Sigma) with a final concentration of 0.1 M each. Silicon wafers with derivatized terminal amine groups from the previous step were incubated in coupling solution for 30 min at RT. After a brief rinse in DMF, wafers were transferred into capping solution (5% Ac_2O in DMF) for 15 min to cap most exposed amine groups with *t*-BOC. Wafers were then washed extensively in DMF and IPA before being dried in preparation for the next photolithography step.

Chip de-protection. All incubation steps were carried out at RT with 250 rpm orbital shaking. N-terminal *t*-BOC protection groups were removed by soaking chips in PGMEA for 3 min prior to centrifuging (300xg) for 5 min in microscope slide container tubes. Chips were then incubated in TFA for 10 min before transfer to a solution of 95.6% TFA, 3.8% hydrogen bromide (Sigma), 0.6% thioanisole (Sigma), and 0.46% (w/v) pentamethylbenzene (Sigma). After incubation for 1 h, chips were washed for 3 min each as follows: 2X TFA, 2X IPA and 1X DMF. Chips were then neutralized for 5 min in DIEA/DMF. Chips were rinsed once in DMF and then twice in IPA prior to use in subsequent experiments.

Antibody-binding assays. De-protected chips were equilibrated by washing 3X in phosphate-buffered saline (PBS, Bio-Rad, Hercules, CA) + 0.1% Tween-20 (Sigma) (PBST), once in PBS and then once in diH_20. Commercial antibodies or

patient serum were diluted in peptide binding buffer (PBB): [50mM Tris (Mallinckrodt Baker, Phillipsburg, NJ) pH 7.5, 150mM NaCl (Mallinckrodt AR, Phillipsburg NJ), 0.05% NP-40 (Sigma) plus 2.5% fetal calf serum (FCS, Omega Scientific, Tarzana, CA)] and incubated on chips placed in 12-well tissue culture plates (Fisher Scientific, Pittsburg, PA) overnight at 4 °C on a rocking platform. After three washes in PBST, secondary goat anti-mouse IgG, goat anti-rabbit IgG, or goat anti-human IgG Cy5 conjugated antibodies (Jackson Immunoresearch, West Grove, PA) were diluted to 0.375 μg ml^{-1} in PBST plus 20% FCS and incubated on the chips for 45 min at RT on a rocking platform. Chips were washed 3 x 5 min in PBST and then briefly rinsed in PBS and the diH_20. Chips were spin-dried in microscope slide racks at 300 xg for 5 min at RT. Dry chips were immediately scanned using an Axon digital scanning system and analyzed using Genepix Pro 6.1 software (Molecular Devices, Sunnyvale, CA).

On-chip kinase assay. Phosphorylation experiments were performed by blocking de-protected chips in tris-buffered saline (TBS, Mallinckrodt Baker) with 0.05% tween-20 (TBST) plus 3% bovine serum albumin (BSA, Sigma) for 1 h at RT on a rocking platform. Blocked chips were rinsed thoroughly with diH_20 and dried with N_2. Chips were subsequently incubated for 1 h at 30 °C in a kinase reaction mixture containing 40 mM Tris, 20 mM $MgCl_2$, 0.1 mg ml^{-1} BSA, 200 μM ATP and 0.5U μl bovine protein kinase A (PKA), Abl or Src (Promega, Madison, WI) in 70 μl total reaction volume using adherent plastic microscope slide covers (Electron Microscopy Sciences, Hatfield, PA). Kinase reactions were terminated by transferring chips into a solution of 50 mM EDTA (Sigma) with 0.05% Tween-20 for 10 min at RT on a rocking platform. Chips were washed three times in TBST and once in diH_20 before being dried with N_2. Dried chips were stained using Pro-Q® Diamond Phosphoprotein/Phosphopeptide Microarray Stain Kit

(Invitrogen, Carlsbad, CA) according to manufacturer's protocol. Phosphorylation was visualized as fluorescent signal intensity at 532 nm using an Axon GenePix 4000B microarray scanner (Molecular Devices).

On-chip methylation assay. De-protected chips were incubated in a 12-well plate (Fisher) overnight on a rocking platform at 30 °C in a reaction mixture containing 60 µg of SETD7 or GST purified proteins, and 0.1 mM *S*-adenosyl-methionine (AdoMet, Sigma) in a methylation buffer containing 50 mM Tris-HCl (pH 8.0), 10% glycerol, 20 mM KCl, 5 mM $MgCl_2$, and 1 mM phenylmethanesulfonylfluoride (Roche Pharmaceuticals, San Francisco, CA) in 500 µl total reaction volume. Chips were washed three times with PBST followed by three washes with PBST plus 20% FCS. Chips were then incubated with rabbit polyclonal pan-methyl antibody (ab23366, Abcam, Cambridge, MA) for 1 h at RT followed by 1 h incubation with Alexa Fluor 647 chicken anti-rabbit IgG (Invitrogen) diluted in PBST with 20% FCS. The chips were washed six times with PBST followed by 1 wash with diH_20 and were dried at 300 xg for 5 min at RT. A GenePix 4000B scanner (Molecular Devices) was used to scan the arrays, and data were analyzed using Genepix Pro 6.1 software (Molecular Devices).

Pre-clearing and peptide competition assay. For pre-clearing experiments, SA-sepharose beads (GE Healthcare, Piscataway, NJ) were incubated with 0.2 mg ml^{-1} of biotinylated histone peptide H2B 1-21 unmodified; H2B 1-21 acetylated K5,12,15,20 or H2B 1-21 K5Me2 (W.M. Keck Foundation, Yale University) for 30 min at RT, washed twice in PBS + 10% FCS and then incubated with commercial antibodies diluted 1:1000 in PBB + 2.5% FCS for 20 min at RT. Samples were then centrifuged at 850 xg for 1 min to pellet the peptide-sepharose beads for removal. Pre-clearing of each sample was repeated 5X prior to incubation on the surface of peptide arrays using conditions identical

to those described above. Peptide competition assays were performed using the conditions described above for the antibody-binding assay except that 0.225 mg ml^{-1} of soluble peptide (H2B 1-21 K5-Ac) (W.M. Keck Foundation) was added to the arrays during the primary incubation step.

ELISA. Nunc-Immuno Maxisorp 96-well plates (Thermo Scientific, Rochester NY) were coated overnight with H2B (1-21) unmodified or H2B (1-21) acetylated K5,12,15,20 at 1 μg ml^{-1} diluted in carbonate buffer, pH 9.5. The plates were washed three times in PBST and then blocked with PBST + 3% FCS for 30 min at RT. Plates were washed three times in PBST and commercial antibodies or patient serum was diluted in PBST plus 3% FCS, loaded in triplicate on the plates in a volume of 50 μl, and incubated for 2 h at RT. After washing the plates five times with PBST, peroxidase–conjugated goat anti-human IgG (H+L) for human serum samples, or peroxidase-conjugated donkey anti-rabbit IgG (H+L) secondary antibodies (Jackson ImmunoResearch) for rabbit primary antibodies were diluted to 0.16 μg ml^{-1} in PBST + 3% FCS, loaded into wells and incubated for 1 h at RT. Plates were washed five times in PBST and then TMB One-Step Substrate (Dako, Carpinteria, CA) was added for 5-15 min prior to terminating the reaction with 2 M sulfuric acid (Sigma). Colorimetric readings were obtained using a SpectraMAX 190 plate reader at λ = 450 (Molecular Devices, Sunnyvale , CA).

Sources of human samples and storage. Serum samples from SLE patients were collected as part of the Autoimmune Biomarkers Collaborative Network (ABCoN) and were provided by one of the authors (E.C.B.) and maintained at –80 °C. Interferon-responsive gene signatures in blood cells were characterized as described[2,3].

Significance Analysis of Microarrays (SAM). The SAM algorithm was applied to numerical MFI values. All peptide reactivity that significantly correlated with IFN-high (vs. IFN-low) or SLE (vs. healthy control) classes was determined by 10,000 or more permutations of repeated measurements to have a false discovery rate of 0 (*q*-value = 0). Binding reactivity heatmaps were generated using Multiexperiment Viewer (MEV TM4 Microarray Software Suite version 10.2, Dana-Farber Cancer Institute, Boston, MA) using k-nearest neighbor replacement and average linkage Euclidean distance hierarchical clustering.

On-chip analysis of peptide yield. The use of fluorescence labeling to measure the synthetic yield for peptides synthesized on substrates has been previously reported by several groups[4-6].

Off-chip analysis of peptide yield and purity. Using an analysis procedure modified from that used in short DNA sequence synthesis analysis[7], we treated the silanated wafer surface with a cleavable Rink linker (Novabiochem). A fluorescein-labeled lysine (Sigma) was attached to the surface and then 6 cycles of aspartic acid (D) coupling reactions were performed. Then the sample was cleaved from the surface and subjected to high-performance liquid chromatography (HPLC) analysis with fluorescence detection (Agilent 1100, Santa Clara, CA) against an internal fluorescein standard (5-Fam-labeled lysine, AnaSpec, custom sample, lot 39871, Fremont, CA).

Off-chip analysis of peptide purity (LC-MS/MS): Liquid chromatography followed by mass spectrometry (LC-MS) is an analytical technique widely used to analyze the identity and purity of synthetic compounds such as peptides and oligomers[8]. Using multiple reaction monitoring (MRM), a standard technique for quantitative LC-MS/MS, multiple mass-to-charge ratio (m/z) fragments are chosen for fragmentation and only these parent-to-fragment ion transitions are

measured and reported in order to facilitate quantitative measurement in a complex matrix[9]. Applying LC-MS/MS (Agilent 1100 autosampler, Agilent 1100 LC pump, and Applied Biosystems 4000 Q TRAP® (Foster City, CA)) to the analysis of peptide purity, two peptides (SDLHKL and SLIGKV) were synthesized on chip and then cleaved from the surface prior to injection into the LC-MS/MS system for MRM analysis.

REFERENCES

1. Jaeger, R.C. Thermal Oxidation of Silicon. in *Introduction to Microelectronic Fabrication*, Vol. 5 (ed. Pierret, R.F.a.N., Gerold W) (Prentice Hall, Upper Saddle River, 2001).
2. Bauer, J.W.*, et al.* Elevated serum levels of interferon-regulated chemokines are biomarkers for active human systemic lupus erythematosus. *PLoS Med* **3**, e491 (2006).
3. Baechler, E.C.*, et al.* Interferon-inducible gene expression signature in peripheral blood cells of patients with severe lupus. *Proc Natl Acad Sci U S A* **100**, 2610-2615 (2003).
4. Gao, X., Zhou, X. & Gulari, E. Light directed massively parallel on-chip synthesis of peptide arrays with t-Boc chemistry. *Proteomics* **3**, 2135-2141 (2003).
5. Shin, D.S.*, et al.* Automated maskless photolithography system for peptide microarray synthesis on a chip. *J Comb Chem* **12**, 463-471 (2010).
6. Li, S.*, et al.* Photolithographic synthesis of peptoids. *J Am Chem Soc* **126**, 4088-4089 (2004).
7. Barone, A.D.*, et al.* Photolithographic synthesis of high-density oligonucleotide probe arrays. *Nucleosides Nucleotides Nucleic Acids* **20**, 525-531 (2001).
8. Lee, M.S. & Kerns, E.H. LC/MS applications in drug development. *Mass Spectrom Rev* **18**, 187-279 (1999).
9. Aebersold, R. & Mann, M. Mass spectrometry-based proteomics. *Nature* **422**, 198-207 (2003).