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# Supporting Information



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# Direct Readout of Protein-Protein Interactions by Mass Spectrometry from Protein-DNA Microarrays

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## Supporting Experimental Section

**Protein Expression:** The coding sequence for Ras (H-Ras, aa 1-180) and the RBD (Ras-binding domain of cRaf-1, aa 51-134), respectively, were cloned into the pTXB1-vector (New England Biolabs) and expressed in *E.coli* BL21(DE3) as fusion proteins with an intein construct from *Mycobacterium Xenopis* and a chitin-binding domain. Protein expression was induced with IPTG. The cells were harvested by centrifugation and lysed in a M110S Microfluidizer (Microfluidics International Corporation, Newton). Cell debris and insoluble proteins were removed by centrifugation and the supernatant was loaded onto chitin-beads (New England Biolabs). Proteins were released as their C-terminal thioesters by treatment with 3% 2-mercaptoethanesulfonic acid (w/v, MESNA) in buffers consisting of 400 mM NaCl, 20 mM Tris/HCl, 5 mM $MgCl_2$, 500 μM GDP at pH 8 for Ras (buffer 1) and 150 mM NaCl, 50 mM Tris/HCl, pH 7.4 for the RBD (buffer 2) for 14 h. Electrospray mass spectrometry was performed on an LCQ ion trap system (Thermo Electron). C-terminally truncated Ras (aa1-166) was expressed and purified as described elsewhere [1]. The Ras-specific antibody was bought from Santa Cruz Biotechnology and fluorescently labeled with a Cy5-protein labeling kit (Invitrogen).

**Ligation:** 0.8 equivalents of cysteine-modified oligonucleotide **9** were added to C-terminal thioester proteins **4** and **5** (c = 1.5 mg/ml) in buffer 1 and 2, respectively containing 100 mM MESNA and the reaction mixtures were gently shaken for 16 h at 4°C. Non-ligated oligonucleotide was removed by gelfiltration on a Superdex 75 10/30 (Amersham Biosciences) using buffer 1 or 2 for Ras and RBD, respectively.

**MDCK cells:** Cells were grown until they formed a confluent layer, harvested and resuspended at $4x10^8$ cells/ml in TES buffer (20 ml Tris-HCl pH 7.4, 1 mM EDTA, 225 mM sucrose) supplemented with a mixture of protease inhibitors (CompleteTM, Roche). The cells were homogenized on ice in a Dounce homogenizer (30 strokes). The cell homogenate was centrifuged (600xg, 10 min, 4°C) to remove nuclei and cell debris. The supernatant was centrifuged (16000 xg , 20 min 4°C) and the resulting supernatant was referred to as

cytoplasm. The pellet from the 600xg centrifugation step was solubilized with buffer containing 10 mM Tris-HCl pH 7.4, 1mM EDTA and 0.5% Triton X-100. Insoluble material was removed by centrifugation (16000xg, 20 min, 4°C) and the supernatant was referred to as membrane fraction.

**Nucleotide exchange:** For Ras-DNA conjugates **10** the following protocol was applied: A 5 fold excess of EDTA over $Mg^{2+}$ was used to capture $Mg^{2+}$ present in the protein buffer. A 50x excess of GppNHp over Ras-DNA was added to achieve replacement of GDP bound to Ras-DNA against GppNHp at $4^{o}$C over night. EDTA and unbound nucleotides are removed by dialysis (24 h) using a 12-14 kDa cut-off membrane. Nucleotide exchange for unmodified Ras has been described previously [2]. The nucleotide content was checked by HPLC at 259 nm using a RP-C18-column (buffer A: 100 mM $KP_i$, 10 mM TBAB, pH 6.5; buffer B: 100 mM $KP_i$, 10 mM TBAB, pH 6.5, containing 50% Acetonitrile, isocratic 15% buffer B in A).

**Microarray preparation:** The oligonucleotides were spotted using a commercial microarrayer (Nanoplotter 1.2, Gesim, Dresden, Germany) on glass or silica slides covered with a dendritic intermediate layer as previously described. The spotted dropsize was 50 nL/spot. The 5'-amino modified oligonucleotides (fully complementary: 5'-TCCTGTGTGAAATTGTTATCCGCT-3' **1**, truncated complementary: 5'-CCTGTGTGAAATTG-3' **2** and not complementary: 5'-TGATAGGGTGCTTGCGAGT-3' **3**) were covalently coupled to the slides and a blocking step was introduced using 200 mM NaCl, 20 mM Tris/HCl, 5 mM $MgCl_2$, at pH 7.4 with 100 µg/ml BSA and 0.1% Tween20 (buffer 3) for 30 min at RT. Subarrays were spatially separated from each other by plastic frames that are glued to the slides to allow individual treatment of the subarrays. The subarrays are incubated with protein-DNA conjugates at a concentration of 100 nM for 45 min at RT and then washed with buffer 1 or 2, respectively. The protein-DNA conjugates were removed by a flow wash step with buffer 3 (BSA free).

**Microarray processing:** Application of the matrix solution (10 mg/ml sinapinic acid in 1:1 acetonitrile:$H_2O$ + 0.1% TFA) was done manually for every subarray. After evaporation of the solvents the microarray plate was fixed on the MALDI sample stage and transferred into the mass spectrometer. Measurements were usually started with subarray I at position A1 (Figure 2B). Calibration of the MALDI parameters was based on matrix signals.

**Fluorescence scans:** The fluorescence measurement was carried out by the use of microarray laser scanning system GenePix 4000B (Axon Instruments) with the aid of GenePix Pro 4.1 software (Axon Instruments). The scanner settings for the measurement were PMT 500 and 100% laser power.

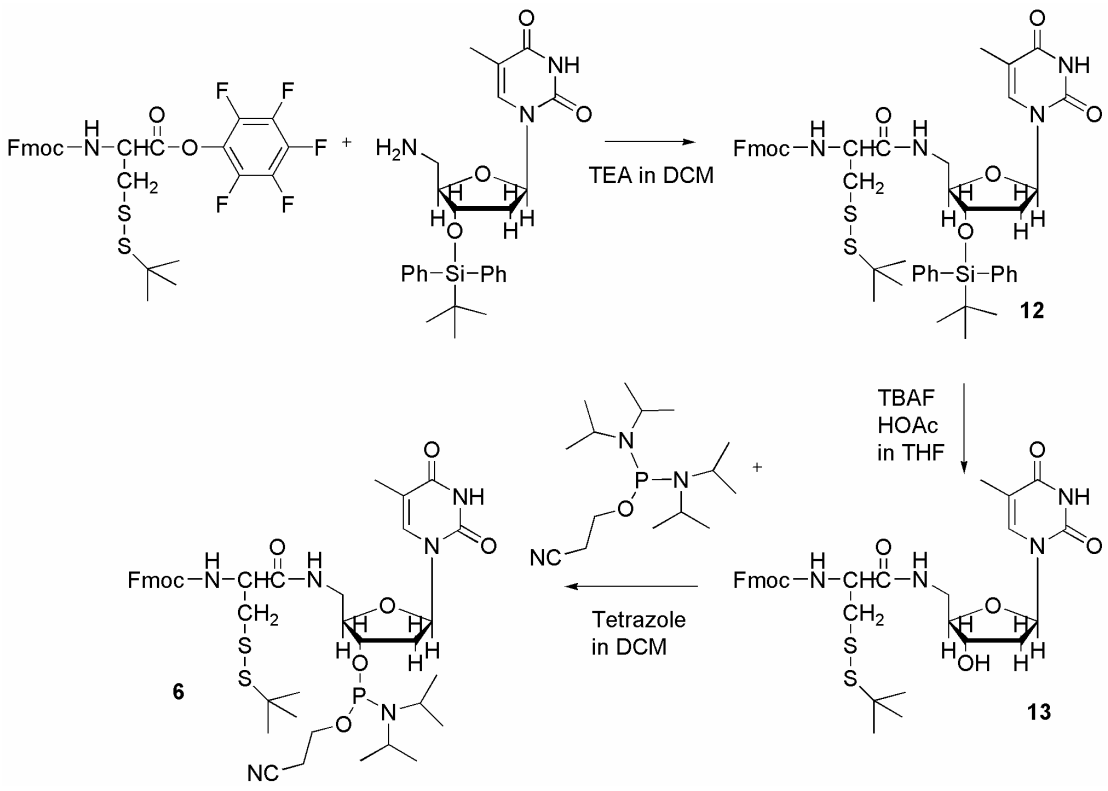
**MALDI mass spectrometry:** Spectra were collected with a home-built time-of-flight mass spectrometer in linear mode of operation and positive ion mode, 'ALADIM II', equipped with a nitrogen laser (wavelength 337 nm, 3 ns pulse duration, 250 µJ per laser pulse) [3;4]. Laser pulse energy was adjusted somewhat above the threshold for ion detection, to optimize analyte signal intensity. The laser focus diameter of the ALADIM II instrument was around 15 µm. Data analysis was performed with the software package Ulisses (vers. 8.2, copyright Bernhard Spengler, 1985-2005). Sinapinic acid (10 mg/ml in $H_2O$:acetonitrile 1:1, +0,1% TFA) was used as a matrix for MALDI analysis. Sub-microliter volumes of matrix solution were applied on the microarrays using micro-pipettes. The surface was then dried in air and analysed by mass spectrometry. Home-built sample stages were used to introduce the microarrays in object holder format into the mass spectrometer.

**Oligonucleotide Synthesis:** Oligonucleotides containing a cysteine-modified thymidin at their 5'-end were synthesized on a Beckman Oligo 1000M. Assembly of oligonucleotides was carried out by standard 2-cyanoethyl phosphoramidite chemistry on long chain alkylamine controlled pore glass.

Synthesis of the 5'-cysteine-thymidine phosphoramidite **6**: To a slurry of 5'-amino-3'-O-[(1,1-dimethylethyl)diphenylsilyl]-2'-deoxy-thymidine (0.1 g) and $N_\alpha$-Fmoc-S-tert-butylthio-L-cysteine pentafluorophenyl ester (0.13 g) in 4 ml anhydrous dichloromethane (DCM) 32 µl triethylamine (TEA) were added and the resulting solution was stirred at room temperature over night. The reaction mixture was evaporated to dryness, dissolved in ethyl acetate and washed successively with ice-cold 5% citric acid solution, 5% sodium bicarbonate solution and brine. After washing the solution was dried over sodium sulfate and evaporated to produce a brown foam. The residue was purified by silica gel chromatography with a mixture of 70% cyclohexane and 30% ethyl acetate. Fractions were pooled and evaporated to give 170 mg of the desired product **12**. Yield: 90%, $^1$H-NMR ($CDCl_3$), δ 1.08 (s, 9H), 1.31 (s, 9H), 1.84 (s, 3H), 2.05 (d, 1H), 2.17-2.30 (m, 1H),.2.96-2.98 (m, 2H), 3.17-3.23 (m, 1H), 3.26-3.30 (m, 1H), 4.00-4.04 (m, 1H), 4.10-4.15 (dd, 1H), 4.20 (t, 1H), 4.21 (b, 1H), 4.34-4.44 (m, 2H), 5.70 (d, 1H), 6.98 (b, 1H), 7.29 (t, 2H), 7.36-7.44 (m, 8H), 7.58 (d, 2H), 7.62 (m, 4H), 7.74 (d, 2H); Mass spectrum: MALDI 895.4 g/mol (calc.: 893.2 g/mol).

**12** (0.1 g) was solubilized in 2 ml of THF and 2 equivalents of tetrabutylammonium fluoride (TBAF) and 2.5 equivalents of acetic acid were added to the solution. The reaction mixture was stirred over night at room temperature. The product was purified on a silica gel column using ethyl acetate**.** Appropriate fractions were pooled and evaporated to dryness to produce **13** (48 mg). Yield: 65%, Mass spectrum: MALDI 657.0 g/mol (calc.: 654.8 g/mol).

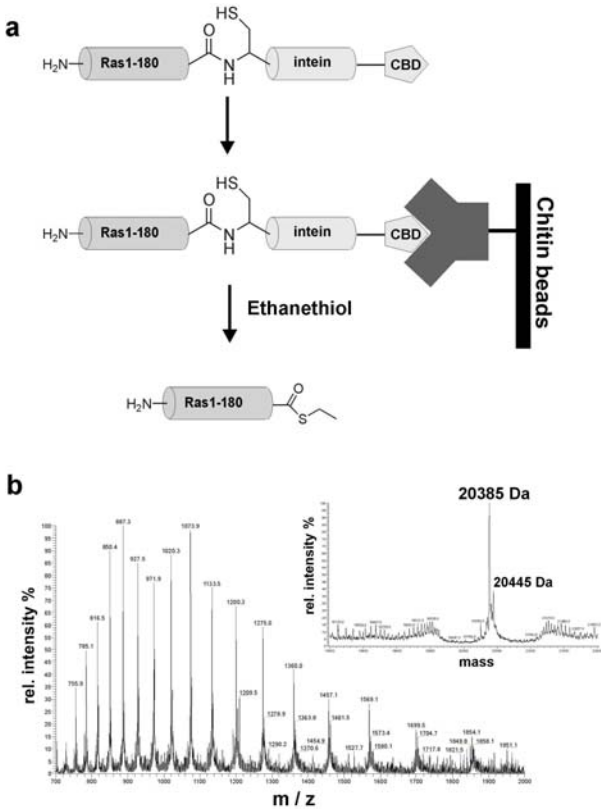
To a solution of **13** (48 mg) in 2.5 ml of anhydrous dichloromethane containing 1.5 equivalents of tetrazole and 5 eq. of 2-cyanoethoxy-N,N,N,N-tetraisopropyl phosphoramidite were added and the mixture was stirred over night at room temperature. DCM was removed by evaporation and the product was dissolved in ethyl acetate washed with 5% sodium bicarbonate solution and brine, dried over anhydrous sodium sulfate. Purification was performed on a silica gel column, eluted by a mixture of ethyl acetate:cyclohexane 85:15. Fractions were pooled and evaporated to dryness to produce **6**. Yield 35%; $^{1}$H-NMR ($CDCl_3$), δ 1.09 (m, 12H), 1.31 (s, 9H), 1.84 (s, 3H), 2.02-2.06 (m, 1H), 2.18-2.23 (m, 1H), 2.98 (d, 2H), 3.19-3.28 (m, 2H), 3.74-3.77 (m, 1H), 4.00-4.05 (m, 1H), 4.19 (t, 1H), 4.18-4.21 (b, 1H), 4.33-4.45 (m, 3H), 5.77 (d, 1H), 6.08 (b, 1H), 6.86 (t, 1H), 7.00 (b, 1H), 7.27 (d, 2 H), 7.38 (m, 2H), 7.58 (d, 2H), 7.63 (d, 2H), 7.74 (d, 2H), 9.18 (b, 1H); Mass spectrum: m/z 856 g/mol (calc.: 855 g/mol)



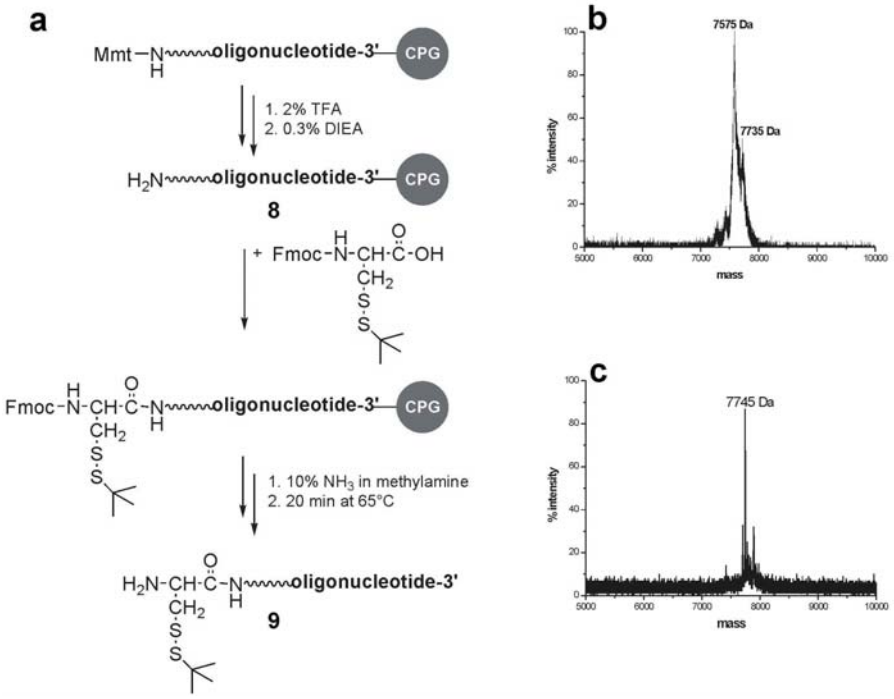

**Supporting Scheme 1:** Synthesis of 5'-cysteine modified thymidine phosphoramidite **6**.

Oligonucleotides with an Mmt-protected 5'-amino group (1 µmol scale) were ordered from Metabion (Martinsried, 5'-MTT-N-$C_6$-linker-AGCGGATAACAATTTCACACAGGA-3') and MTT was removed with 3% trichloroacetic acid in DCM. 5 eq. of Fmoc-Cys(StBu)-OH were coupled to the free amino group in the presence of HBTU (5 eq.) and DIEA (1 eq.) in 75% dimethylformamide and 25% acetonitrile for 2 h at RT. Cleavage from the solid support

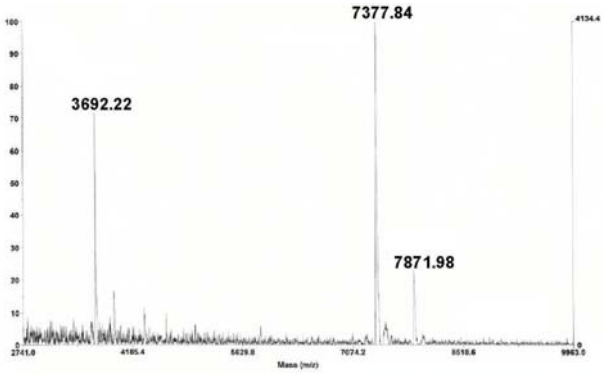
was achieved by treatment with 10% $NH_3$ in methylamine for 20 min at RT and additional 15 min at 65°C (see supplementary figure 1). The oligonucleotide **9** was initially purified over a PD10 column and a mixture of cysteine-modified oligonucleotide and unmodified oligonucleotide was used for ligation reactions with Ras and RBD thioesters.



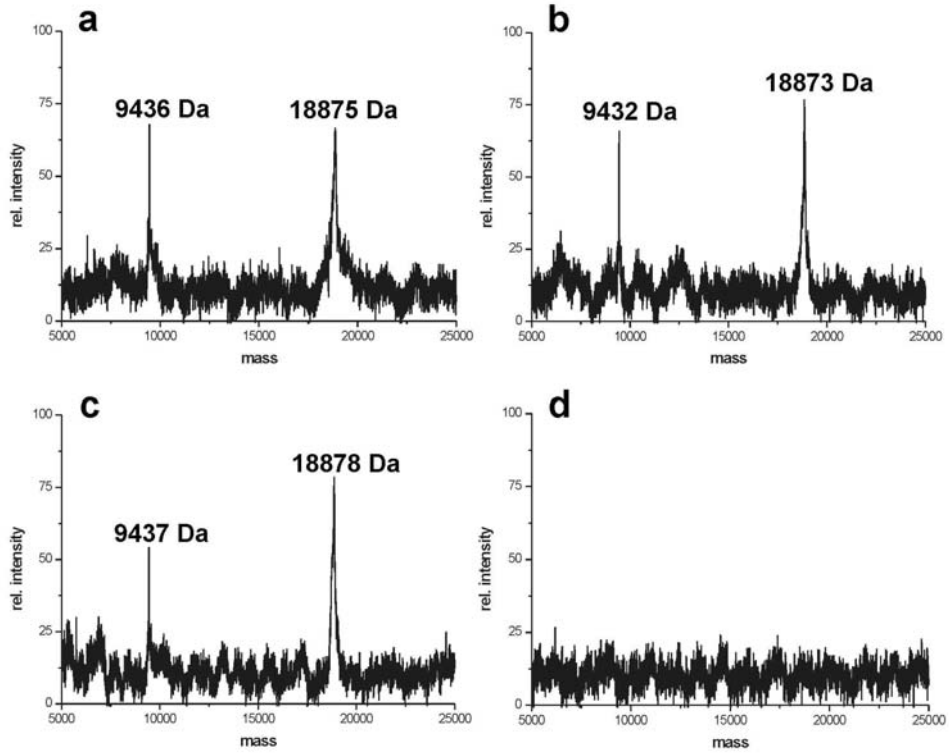

**Supporting Figure 1:** Generation of protein thioesters. a) Affinity purification of Ras-intein fusion construct and subsequent ethanethiol (MESNA was used preferably because it is odorless) induced generation of the Ras-thioester. b) ESI-MS analysis of the Ras-thioester. The observed molecular weight of 20,385 Da is in very good agreement with the calculated mass of 20,387 Da. The second largest peak at 20,445 Da corresponds to the Ras-thioester forming a mixed disulfide with excess ethanethiol during the cleavage reaction.



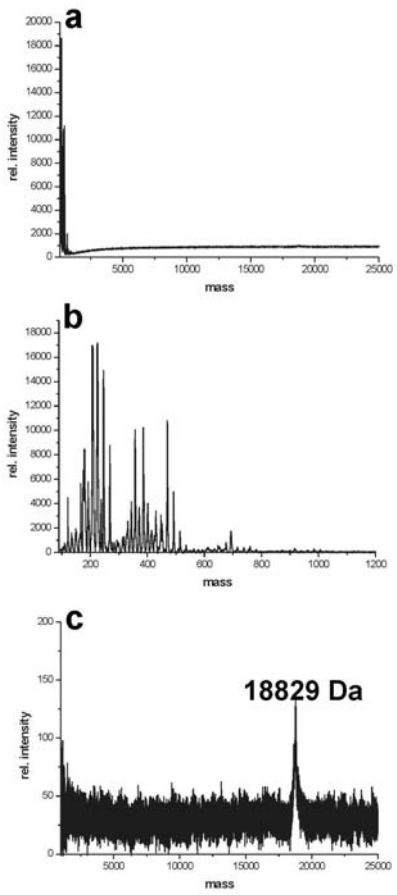

**Supporting Figure 2:** Synthesis of cysteine-modified oligonucleotide **9**. a) Synthesis scheme of 5'-cysteine modified oligonucleotide **9**; b) MALDI mass spectrum of crude **9** (expected MW of 5'-amino oligonucleotide: 7558 Da, expected MW of **9**: 7749 Da); c) MALDI mass spectrum of HPLC-purified **9**.




**Supporting Figure 3:** MALDI-MS of crude oligonucleotide **7** after cleavage from solid support. Calculated MW of unmodified oligonucleotide: 7380 g/mol, observed MW: 7377.8 g/mol (m/2 3692.2) and calculated MW of oligonucleotide **7**: 7877 g/mol, observed MW: 7872 g/mol.




**Supporting Figure 4:** Detection limit for MALDI-based readout of the analyte Ras:GppNHp immobilized via interaction with the surface-attached capture agent RBD-DNA.
a) Incubation with a 300 nM (a), 30 nM (b), 3 nM (c) and 0.3 nM (d) solution of Ras:GppNHp in buffer 1. The detection limit is below 3 nM for activated Ras. At a concentration of 0.3 nM no Ras signal could be detected.




**Supporting Figure 5**: Typical MALDI spectrum obtained for a subarray with surface-attached RBD-DNA as the capture agent and incubated with the analyte Ras:GppNHp. a) Complete spectrum ranging from 100 to 30000 Da; b) Magnification of the 100-1200 Da mass range with signals corresponding to matrix peaks; c) Mass range from 5000 to 25000 Da showing only a single peak for the Ras protein.

Reference List

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[2.] J. John, R. Sohmen, J. Feuerstein, R. Linke, A. Wittinghofer, R. S. Goody, *Biochemistry* **1990,** *29*. 6058-6065.

[3.] P. Chaurand, F. Luetzenkirchen, B. Spengler, *Journal of the American Society for Mass Spectrometry* **1999,** *10*. 91-103.

[4.] B. Spengler, *Journal of Mass Spectrometry* **1997,** *32*. 1019-1036.