Supporting Information for

Glyco-seek: Ultrasensitive Detection of Protein-Specific Glycosylation by Proximity Ligation PCR

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Materials

All chemicals and biological reagents were purchased from Sigma-Aldrich unless otherwise noted. All antibodies were purchased from R&D Biosystems (Minneapolis, MN). Anhydrous DMSO, SYBR Green Master Mix, Platinum Taq, Click-it O-GlcNAc labeling kit, and DTT were purchased from Life Technologies (Grand Island, NY). Uracil-DNA Excision mix and Ampligase were purchased from Epicentre Bio (Madison, WI). Sulfosuccinimidyl-4-(N-maleimidomethyl)cyclohexane-1 carboxylate (SMCC) and Zeba 7k MWCO Desalting Columns were purchased from Pierce Biotechnology (Rockford, IL). Amicon Ultra Centrifugal Filter columns were purchased from EMD Millipore (Billerica, MA). iQ 96-Well Optical PCR plates and MicroSeal Adhesive Seals were purchased from Bio-Rad (Hercules, CA). All oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA).

Name	5' Mod	Sequence	3' mod	Notes
Set 2- 5' Fragment	thiol	GGC CTC CTC CAA TTA AAG AAT CAC GAT GAG ACT GGA TGA A	none	
Set 2- 3' Fragment	phosphate	TCA CGG TAG CAT AAG GTG CAG TAC CCA AAT AAC GGT TCA C	thiol	
Set 2 Bridge	none	CUA CCG UGA UUC AUC CAG	none	U = Deoxy-ribouracil
Set 2 F	none	GGC CTC CTC CAA TTA AAG AA	none	
Set 2 R	none	GTG AAC CGT TAT TTG GGT AC	none	
Set 4- 5' Fragment	thiol	TCG TGG AAC TAT CTA GCG GTG TAC GTG AGT GGG CAT GTA GCA AGA GG	none	
Set 4- 3' Fragment	phosphate	GTC ATC ATT CGA ATC GTA CTG CAA TCG GGT ATT AGG CTA GTG ACT ACT GGT T	thiol	
Set 4 Bridge	none	GAA UGA UGA CCC UCU UGC UA	none	U = Deoxy-ribouracil
Set 4 F	none	CGT GGA ACT ATC TAG CGG TGT A	none	
Set 4 R	none	AAC CAG TAG TCA CTA GCC TA	none	

Table S1. DNA Sequences. For the 5' and 3' fragments, **Red** is the forward primer binding site, **Green** is the bridge binding site, and **Blue** is the reverse primer binding site.

General Glyco-seek Procedure

1 fmol of paired antibody-DNA conjugates was resuspended in 2 μL of incubation buffer C (2% BSA, 0.2% Triton X-100, 8 mM EDTA in PBS). 2 μL of analyte is added to the conjugates and then incubated at 37 °C for 40 min. 116 μL of ligation mix was added, and then incubated for 15 min at 30 °C. 25 μL of the ligation mix was added to 25 μL 2x PCR Master Mix (Qiagen) with 10 nM primers and then amplified by PCR (95 °C for 10 min, 60 °C for 30s, 95 °C for 15s, 13 cycles). The PCR reaction was then diluted 1:20 in ddH_2O. 8.5 μL of the diluted PCR samples were added to 10 μL 2x qPCR Master Mix (Life Technologies) with 1.5 μL primers (final concentration 690 nM). qPCR was performed with either a Bio-Rad CFX96 or a Bio-Rad iQ5 with standard thermocycling.

Ct, or the threshold cycle, is a measurement of signal intensity for qPCR experiments. In a qPCR experiment, PCR is performed in the presence of a fluorogenic intercalating dye. As more double-stranded DNA is produced by the PCR each cycle, the dye increases in fluorescence intensity. Once the fluorescence intensity of a reaction reaches a threshold value, the cycle number is recorded by the instrument as the Ct value. Samples containing a large amount of DNA will reach the fluorescence threshold at a much earlier cycle number than samples containing relatively small amounts. Therefore lower numbers correspond with high amounts of DNA.

In a Glyco-seek experiment, we perform two reactions. One contains the sample to be analyzed and the other a PBS blank. The ∆Ct is the difference between these two values. In this way, we control for random variation between samples, as non-specific changes in the signal intensity should occur at the same rate in both samples, leaving only the “true” intensity value.

General Cell Culture Procedure

Jurkat (human T-cell lymphoma) cells were purchased from ATCC and cultured in RPMI-1640 media supplemented with 10% fetal bovine serum, streptomycin (0.1 mg/mL) and penicillin (100 units/mL). Cells were grown in the presence of 5% CO_2 and maintained at densities between 1 x 10^5 and 2 x 10^6 cells/mL.

Cell Lysis Procedure

Cells were grown as above in media supplemented with 10 uM thiamet G or DMSO vehicle. After 3 d, 5e6 cells/sample were washed 3x in PBS and lysed by incubation with passive lysis buffer (Promega) with protease inhibitors (CalBioChem) and additional thiamet G to prevent proteolysis and O-GlcNAc degradation. The insoluble fraction was pelleted by centrifugation (14000 rcf, 15 minutes) at 4° C and the supernatant was collected. The protein concentration of the lysates was determined by DC Assay (BioRad).

Immunodepletion Procedure

Lysates were prepared as above. After quantification, 100-200 ug of lysate was resuspended to 1 mg/mL in PBS. 20 ug of antibody (anti-C-Rel or isotype control) was added to the lysate and incubated overnight at 4° C. 50 uL (25 uL settled volume, Pierce) of protein G resin was washed 3x in PBS and added to each sample and incubated for 1 h at RT. Resin was pelleted by centrifugation (2500 rcf, 3 min). The depleted supernatant was collected and analyzed.

Antibody-DNA Conjugate Synthesis

Antibody was resuspended to make a 1-2 mg/mL solution in Antibody Reaction Buffer (55 mM sodium phosphate, 150 mM sodium chloride, 20 mM EDTA, pH 7.2). 1 μL of a 4 mM solution of SMCC in anhydrous DMSO was added 10 μL of the protein solution and incubated at RT for 2 h. Thiolated DNA was purchased in 250 nmol scale from IDT and resuspended to 100 μM

solution in Antibody Reaction Buffer. 3 μL of the 100 μM stock was added to 50 μL of Antibody Reaction buffer. To this solution, 4 μL of a 100 mM solution of DTT was added to reduce the oxidized thiolated DNA. The solution was then incubated at 37 °C for 1 h. Four 7k MWCO gel microspin columns were equilibrated to Antibody Reaction buffer by adding 300 μL of buffer and centrifuging for 1 min at 1500 rcf and then repeating. The antibody solution and the reduced oligonucleotides were separately desalted by the equilibrated microspin columns. The solutions were then mixed and let react overnight at 4 °C. Conjugates were purified from unconjugated DNA by dilution and concentration with centrifugal filter. Conjugation efficiencies were analyzed by SDS-PAGE and silver staining as described previously. Antibody-DNA conjugates were stored at 4 °C for short-term usage or aliquoted for storage at -80 °C indefinitely.

Western Blot Analysis

Streptavidin-HRP was purchased from Pierce Biotechnologies (Rockford, IL).

Samples were resolved by gel electrophoresis in a 3-8% Tris-Acetate gradient gel (Bio-Rad) for 105 min at 100 V at 4 °C in Tricine running buffer (Bio-Rad). Mass-resolved proteins were then transferred to a nitrocellulose membrane by semi-dry transfer for 1 h at 15 V and then 1 h at 20 V. The membrane was then briefly rinsed in ddH$_2$O and then blocked in 5% bovine serum albumin (BSA, Sigma Aldrich) in PBST (Hyclone PBS with 0.1% Tween) for 1 h to overnight. Anti-biotin blots were then incubated with 1:100,000 Streptavidin-HRP in 5% BSA in PBST for 1 h, briefly rinsed in PBST and then thoroughly washed three times for 10 min in PBST for and then analyzed by standard chemiluminescence methods (Pierce).

To ensure equal loading, total protein stain was performed by rinsing the blots briefly in ddH$_2$O, and then incubated in 1:1000 India Ink dilution for 1-3 h.

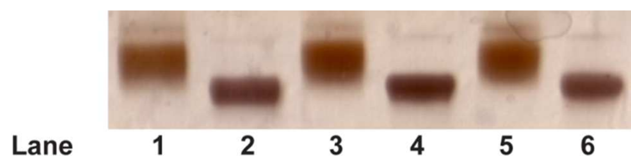

Figure S1. **Silver stain of antibody-DNA conjugates.** Lyophilized antibody was resuspended to 2 mg/mL in phosphate buffer and treated with SMCC crosslinker. After removing unreacted small molecules, the maleimide-activated antibody was incubated with reduced thiol-DNA and then purified using centrifugal filtration. Conjugated antibodies were resolved by PAGE and then reductively stained using silver nitrate. Lanes 1, 3, and 5 are the anti-biotin, anti-alpha-crystallin, anti-c-Rel DNA conjugated antibodies, respectively. Lanes 2, 4, and 6 are the appropriate unconjugated antibody for reference. A ~15 kDa mass shift confirmed the conjugation of DNA to the antibody.

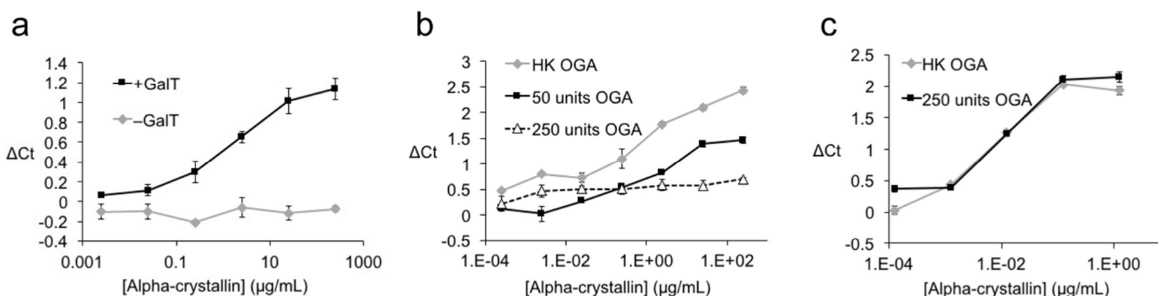

Figure S2. Glyco-seek can detect both abundance and glycosylation status of a model protein in buffer*.* (**a**) O-GlcNAc residues on alpha-crystallin were chemoenzymatically biotinylated and analyzed by a Biotin/Alpha-Crystallin proximity ligation assay. As a negative control, GalT was omitted. (**b**) Alpha-crystallin samples were treated with high or low amounts of recombinant O-GlcNAcase or the heat-killed enzyme as a negative control. (**c**) Samples treated with either active OGA or heat-killed OGA were analyzed for the levels of alpha-crystallin. As this assay does not respond to the protein's glycosylation status, no difference was observed.

The X-axis displays the concentration of the protein in the sample while the Y-axis is the Ct value in comparison to a blank. All data points are from technical triplicates and the error bars represent the standard deviation.

Figure S3. Western blot of chemoenzymatically biotinylated O-GlcNAcylated alpha-crystallin. To benchmark the performance of Glyco-seek against a comparable technology, alpha-crystallin was chemoenzymatically biotinylated using the commercially available Click-it O-GlcNAc detection kit. A 10 fold dilution series of the protein was then resolved by PAGE, transferred to a nitrocellulose membrane, and then imaged via standard chemiluminescence protocols following treatment with streptavidin-HRP.

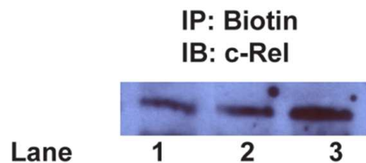

Figure S4. Inhibition of OGA increases O-GlcNAcylation of c-Rel. Jurkat cells were incubated with DMSO vehicle (Lane 1), thiamet-G only (Lane 2), or thiamet-G with glucose (Lane 3), washed. Detergent lysates were chemoenzymatically biotinylated as previous. Biotinylated proteins were enriched by incubation with neutravidin resin. The resin was rigorously washed and bound proteins were eluted, resolved by PAGE, and then transferred to a nitrocelluse membrane. The membrane was imaged via standard chemiluminescence protocols after treatment with anti-c-Rel primary and HRP-conjugated secondary antibody.

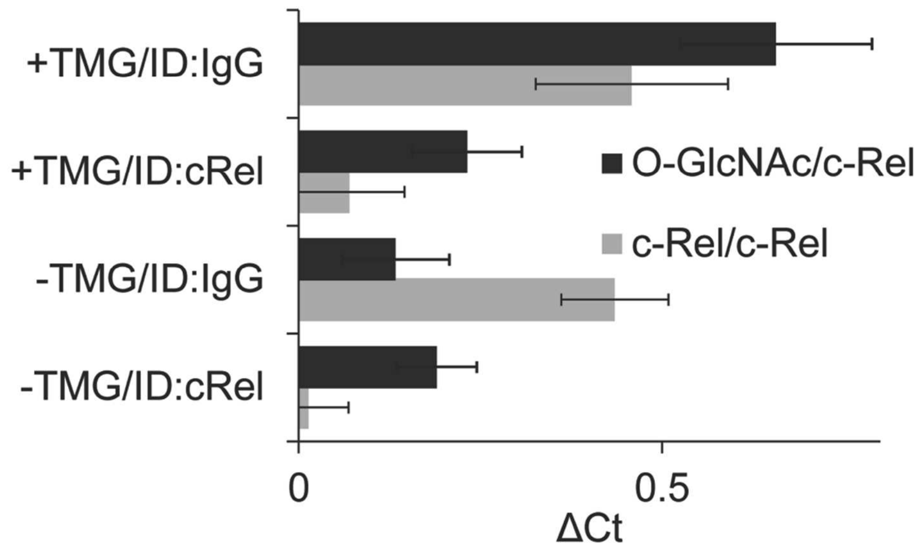

Figure S5. Immunodepletion diminishes signal in a c-Rel dependent manner. Jurkat cells were grown in the presence of the OGA inhibitor TMG or DMSO vehicle as a negative control. The lysates were then immunodepleted using an anti-c-Rel antibody or an IgG isotype control. All samples were then chemoenzymatically biotinylated and analyzed via Glyco-seek or a c-Rel PLA as shown.