PAQMAN: Protein-nucleic acid affinity quantification by MAss spectrometry in nuclear extracts

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ABSTRACT

In recent years, various mass spectrometry-based approaches have been developed to determine global protein-DNA binding specificities using DNA affinity purifications from crude nuclear extracts. However, these assays are semi-quantitative and do not provide information about interaction affinities. We recently developed a technology that we call Protein-nucleic acid Affinity Quantification by MAss spectrometry in Nuclear extracts or PAQMAN, that can be used to determine apparent affinities between multiple nuclear proteins and a nucleic acid sequence of interest in one experiment. In PAQMAN, a series of affinity purifications with increasing bait concentrations and fixed amounts of crude nuclear extracts are combined with isobaric stable isotope labeling and quantitative mass spectrometry to generate Hill-like K_d curves for dozens of proteins in a single experiment. Here, we apply PAQMAN to determine apparent affinities for a genetic variant, rs36115365-C, which regulates TERT expression and is associated with an increased risk to develop various malignancies. Furthermore, we describe a detailed protocol for this method including important quality checks.

1. Introduction

The human genome contains a large number of non-coding elements such as promoters and enhancers, which play an important role in regulating gene expression in a dynamic and spatio-temporal controlled manner, for example during embryonic development but also during adult life. These regulatory elements contain motifs that are recognized and bound by proteins, including transcription factors. Transcription factors can subsequently recruit or inhibit the recruitment of other transcription factors or chromatin modifying enzymes, thereby regulating gene expression [1,2]. In recent years, it became apparent that mutations and single nucleotide polymorphisms (SNPs) can influence transcription factor binding in regulatory elements [3]. Therefore, deciphering the molecular mechanisms of transcription factor binding across the genome is not only essential from a fundamental point of view, but is also important to unravel molecular mechanisms of disease.

In recent years, many techniques have been developed to study the complex interplay between proteins and DNA. These techniques are mainly semi-quantitative and provide information about the specificity but not the affinity of an interaction [4]. Specificity refers to the relative strength of a protein-DNA interaction, whereas affinity refers to the absolute strength of this interaction. Combined information regarding specificity and affinity *in vitro* can help to predict if and when a specific interaction between a transcription factor and DNA occurs *in vivo* [1].

The binding affinity of a protein to its ligand, in this case DNA or other nucleic acids, can be described by using the dissociation constant (K_d^{app}). The K_d^{app} is a numeric representation of the strength with which a protein binds to a given DNA motif, usually expressed in M (moles/liter). One way to obtain the K_d is to determine the concentration of DNA that is required to bind half of the total amount of a certain protein, which is equal to the K_d [5]. Several methods have been developed to determine the binding affinity of proteins to DNA. However, most of these techniques are laborious due to the use of purified recombinant proteins, which can only be investigated on a case-by-case basis [6,7]. However, in recent years, several techniques have been developed that can be used to determine binding affinities on a larger scale [8,9]. In addition, mass spectrometry-based methods have been developed that can be used to obtain information concerning absolute affinities between proteins and small molecules [10,11]. Recently, we adapted these methods for protein-nucleic acid interactions [12]. We name this method Protein-nucleic acid Affinity Quantification by MAss spectrometry in Nuclear extracts or PAQMAN. PAQMAN can be used to determine dozens of apparent affinities between proteins and a particular DNA sequence of interest in the context of crude nuclear extracts. Here, we applied PAQMAN to study a major oncogenic driver event, which enables replicative immortality, the transcriptional reactivation of telomerase reverse transcriptase or TERT. In previous work, we identified a SNP in an enhancer of the TERT gene, which is associated with altered TERT expression and an increased risk to develop various

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malignancies. A PAQMAN experiment for this SNP revealed that ZNF148 interacts with the SNP with a high affinity. This protein was previously shown to regulate TERT expression in a SNP dependent manner, thus illustrating the usefulness of PAQMAN to identify transcription factors that are likely to interact with functional DNA elements in vivo. Furthermore, we provide a detailed protocol to perform a PAQMAN experiment, including important suggested quality checks.

2. Materials

2.1. Buffers for nuclear protein extraction

Buffer	Reagents (final concentration)
Buffer A	10 mM Hepes KOH pH 7.9 1.5 mM MgCl ₂ 10 mM KCl Additives: 0.15% NP-40* EDTA-free Complete Protease Inhibitor (Roche-04693132001)*
Buffer C	420 mM NaCl 20 mM Hepes KOH pH 7.9 20% v/v glycerol 2 mM MgCl ₂ 0.2 mM EDTA 0.1% NP-40* EDTA-free Complete Protease Inhibitor (Roche)* 0.5 mM dithiothreitol (DTT)*

2.2. Materials for nuclear protein extraction

Buffer	Reagents (final concentration)
Phosphate Buffered Saline (PBS) Dulbecco's Phosphate Buffered Saline (DPBS) without Calcium or Magnesium	Lonza (17-512F)

2.3. Buffers for PAQMAN

Buffer	Reagents (final concentration)
TE Buffer (pH 8.0)	10 mM Tris 1 mM EDTA
2X Oligo Annealing Buffer	100 mM NaCl 20 mM Tris, pH 8.0 2 mM EDTA
DNA Binding Buffer	1 M NaCl 10 mM Tris, pH 8.0 1 mM EDTA 0.05% NP-40*
Protein Incubation Buffer	150 mM NaCl 50 mM Tris, pH 8.0 EDTA-free Complete Protease Inhibitor (Roche)* 0.25% NP-40*
Washing Buffer	1 mM Tris(2-carboxyethyl)phosphine (TCEP)* 150 mM NaCl 100 mM TEAB
Elution Buffer	20% MS-Grade Methanol* 80 mM TEAB* 10 mM TCEP*

2.4. Materials for DNA pull-downs

Material	Manufacturer
DNA baits	Custom synthesized by Integrated DNA Technologies (IDT)
96-well filter plate, 1.2 µm pore 550 mM iodoacetamide	Millipore/Merck (MSBVS1210)

Trypsin (0.1 µg/ul)	Costar® (3599)
96 Well Cell Culture Plate	GE Healthcare (17-5113-01)
Streptavidin-sepharose high performance	Thermo Scientific™
TMT10plex™ Isobaric Label Reagent Set	

2.5. Buffers for StageTipping

Buffer	Reagents (final concentration)
Buffer A (mass spectrometry-grade)	0.1% formic acid
Buffer B (mass spectrometry-grade)	80% acetonitrile 0.1% formic acid

2.6. Materials for StageTipping

Material	Manufacturer
Solid phase extraction disk, Empore™, C18, 47 mm	3 M

*add freshly before use.

3. PAQMAN protocol

In PAQMAN, ten DNA affinity purifications are performed with a titration series of predefined oligonucleotide concentrations and a fixed amount of nuclear extract, followed by TMT labeling of bound proteins and identification as well as relative quantification of proteins by mass spectrometry (Fig. 1).

3.1. Nuclear protein extraction

UACC903 were cultured as described previously [13]. For one set of ten affinity purifications, 1 mg of nuclear extract is needed. The ratio of cells/nuclear extract may vary depending on the cell type that is used. For example, about 30x10⁶ HeLa cells are needed to obtain 1 mg of nuclear extract.

All steps are performed on ice and with precooled centrifuges.

- Harvest cells in 50 ml tubes and spin them down for 5 min at 400 g and 4 °C.
- Wash cells twice with PBS while combining all cells in one 15 ml falcon tube.
- Determine the pellet volume and add 5 volumes of cold Buffer A without additives. Resuspend the cells and incubate them for 10 min on ice. Afterwards, centrifuge the cells for 5 min at 400 g and 4 °C.
- The cell volume should have increased due to osmotic uptake of water. Add two cell pellet volumes of cold Buffer A plus additives (EDTA-free Complete Protease Inhibitors, 0.15% NP-40). Resuspend cells and transfer the suspension to a dounce homogenizer (type B pestle).
- Perform 40 strokes with the douncer while pausing every ten strokes to cool the sample on ice for 30 s. Afterwards, centrifuge the suspension for 15 min at 3200 g and 4 °C.
- The supernatant is the cytoplasmic extract which can be collected in a separate tube. For nuclear protein extraction, continue with the pellet which contains the crude nuclei.
- Wash the pellet once with 5 ml of DPBS, which is mass spectrometry-grade, without resuspending the pellet. Centrifuge for 5 min at 3200 g and 4 °C.
- Determine the pellet volume and add 2 volumes of Buffer C. Resuspend the pellet and transfer the suspension to Eppendorf tubes.
- Rotate the suspension for 60 min at 4 °C. The suspension will become viscous due to the lysis of the nuclei and the release of chromatin.

Fig. 1. Schematic overview of the PAQMAN method. 1. Nuclear extract is prepared from cultured cells (Section 3.1). 2 and 3. Custom synthesized 5'-biotinylated DNA oligonucleotides are titrated and immobilized using streptavidin beads on a filter plate (Sections 3.2.1–3.2.4). 4–7. Nuclear extracts are incubated with oligonucleotides, after which unbound proteins are washed away. Bound proteins are on-bead digested with trypsin and eluted from the filter plate (Sections 3.2.5 and 3.2.6). 8 and 9. Peptides are labeled using isobaric 10-plex TMT after which samples are combined and prepared for mass spectrometry using StageTipping (Sections 3.2.7 and 3.2.8). 10. Peptides are measured by mass spectrometry, TMT reporter ions are quantified in MS3 (Section 3.2.9). 11. K_d^{app} s are determined by calculating the DNA oligonucleotide concentration at which 50% of the protein is bound to DNA (Section 3.2.10).

- Centrifuge the nuclear suspension and the cytoplasmic extract, if kept, for 30 min at 20,000 g and 4 °C.
- Collect the supernatant which contains the nuclear proteins and snap freeze this extract in liquid nitrogen. Store the extract at –80 °C until further use.
- If the cytoplasmic extract is kept, add glycerol to a final concentration of 10% v/v and NaCl to a final concentration of 150 mM. Snap freeze in liquid nitrogen and store at –80 °C until further use.
- Determine the protein concentration of the extract, for example by using a Bradford assay.

3.2. PAQMAN assay

3.2.1. DNA oligonucleotide design and preparation

In principle, PAQMAN can be used to determine binding affinities for any (modified) nucleic acid sequence, including RNA or nucleosomal DNA [12]. Here, we use 5' biotin-labeled DNA oligonucleotides containing the SNP rs36115365-C (see Supplementary Table 1). The flanking sequence for a motif of interest can either be random or as here, taken from the genome. Bait length can be adjusted as desired; we have used baits varying in length between 20 and 50 base pairs. In principle, there is no limitation with regard to oligonucleotide bait length, but the longer the sequence the more proteins are pulled down, making it challenging to sequence and quantify all proteins by mass spectrometry.

3.2.2. Oligonucleotide annealing and dilution

- For one PAQMAN experiment performed in duplicate, 2.4 nmol of double-stranded oligonucleotides are needed.
- DNA oligonucleotides are custom synthesized (IDT) with the 5' end of the forward strand being biotinylated. Upon arrival, dilute oligonucleotides to 100 μ M following manufacturer instructions in TE buffer pH 8.0 and store at –20 °C until further use.
- Anneal oligonucleotides with a 1.5X molar excess of the reverse strand in 2X Annealing Buffer. To generate a 20 μ M double-stranded DNA oligonucleotide stock, combine 15 μ l of the 100 μ M forward oligonucleotide stock with 22.5 μ l of the 100 μ M reverse oligonucleotide stock in 37.5 μ l 2X Oligo Annealing Buffer. This is enough to do one titration series.
- Incubate the mixture at 95 °C for 10 min to denature oligonucleotides. Then allow the oligonucleotides to anneal by cooling them to room temperature slowly, preferably over night.
- To prepare a 3 μ M stock of the oligonucleotides, combine 60 μ l of 20 μ M annealed oligonucleotides with 340 μ l DNA Binding Buffer (DBB). This will be the highest concentration in the titration series.
- The dilution series can either be prepared in Eppendorf tubes or in a 96-well plate. Dilute 100 μ l of the 3 μ M oligo stock in 200 μ l of DBB for a three-fold dilution. Prepare a series of nine 3-fold dilutions by diluting 100 μ l of the previous oligonucleotide concentration with 200 μ l DBB, which makes ten samples in total (see scheme below).

Well	1	2	3	4	5	6	7	8	9	10
Final oligonucleotide concentration (nM)	0.15	0.46	1.37	4.1	12.3	37	111.1	333.3	1000	3000
DBB (μl)	200	200	200	200	200	200	200	200	200	–
Volume of oligonucleotide dilution (μl)	100 of well	100 of well	100 of well	100 of well	100 of well	100 of well	100 of well	100 of well	100 of well	300 of 3 μM stock
	2	3	4	5	6	7	8	9	10	

3.2.3. Preparation of filter plate

For each experiment, ten affinity purifications are performed consisting of nine data points (dilutions) plus the highest oligo concentration. Every step is performed at 4 °C. Perform each experiment at least in duplicate.

- Place the filter plate on a vacuum-system, for example on the MultiScreen Vacuum Manifold from MERCK. Covering unused wells with PCR tape can improve vacuum.
- Add 50 μl of 70% ethanol to each well and vacuum.
- Wash each well twice with 200 μl DBB and vacuum. Vacuum after each wash.

3.2.4. DNA oligonucleotide immobilization on streptavidin coated beads

- For each affinity purification, use 20 μl of Streptavidin-sepharose bead slurry (10 μl beads with a total binding capacity of about 3 nmol) diluted with DBB to a final volume of 200 μl.
- Add 200 μl of the bead mix (10 μl of beads diluted in 200 μl DBB) to each well. Vacuum.
- Take the filterplate off the vacuum and clean the bottom side with a tissue. Make sure that the bottom side is dry to prevent loss of sample.
- Add 150 μl of diluted oligonucleotide mix to the corresponding wells. Use the general scheme described in [Section 3.2.2](#).
- Incubate for at least 1 h at 4 °C at 90 rpm on a benchtop mixer, for example on an Orbit P2 Digital Shaker (S2020-P2-B). Alternatively, oligonucleotide immobilization can also be done overnight, which may improve oligonucleotide binding to beads.
- Oligonucleotide depletion after immobilization can be tested by DNA gel electrophoresis ([Fig. 2A](#)). To this end, after incubation of the oligonucleotides with the beads in the plate, spin down the plate and collect the flow-through. Then, load a fraction of the flow-through and the same amount of input oligonucleotide, for example leftover material from the dilution series, on an agarose gel. In this way, depletion of oligonucleotides from the solution on the beads can be manually inspected. Here, we used a 2% agarose gel and DNA was stained using ethidium bromide. Be aware that a small fraction of excess reverse oligonucleotide will always be visible after oligonucleotide depletion.

3.2.5. Affinity purification

- After DNA oligonucleotide immobilization, wash beads once with 200 μl DBB and twice with 200 μl Protein Incubation Buffer (PIB). Vacuum after each wash.
- Take the filter plate off the vacuum and clean the bottom side. Make sure that the bottom side is dry to prevent loss of sample.
- Prepare the nuclear protein solution by adding 100 μg of nuclear extract to a final volume of 150 μl PIB per pulldown. Make a mastermix for eleven (ten samples plus one extra) affinity purifications to ensure that the exact same amount of proteins is used per pulldown for one experiment. This is important for accurate K_d^{app} calculation.
- Apply 150 μl nuclear protein solution to beads.
- Incubate for 2 h at 4 °C shaking at 90 rpm on a benchtop mixer.
- After incubation, remove unbound proteins by vacuum and wash six times with 200 μl of Washing Buffer. Quick washes (< 2 s) are important to minimize disturbance of the binding equilibrium.
- After the last vacuum, dry the bottom side of the filterplate and place it on a collection plate (96 Well Cell Culture Plate).

3.2.6. Reduction and alkylation of disulfide bonds followed by trypsin digestion

Note: The following steps are performed at room temperature.

- Add 50 μl of Elution Buffer to each well.
- Incubate for 30 min at room temperature shaking in a humidified incubator. If no humidified incubator is available, use a plastic box with a wet paper tissue.
- Add 5 μl of 550 mM stock of iodoacetamide (IAA) to a final concentration of 50 mM. Incubate for 10 min at room temperature in the dark and shaking in a humidified incubator.
- Carefully deposit 2.5 μl of 0.1 μg/μl stock of trypsin on top of the filter in the bottom of each well without damaging the filter (0.25 μg total). Incubate for 1 h at room temperature shaking in a humidified incubator.
- Elute the sample into a collection plate by centrifuging at 500 g for 5 min.
- Wash beads by adding 50 μl Elution Buffer to each filter plate well.
- Spin into the same collection plate at 500 g for 5 min.
- Incubate overnight at room temperature shaking in a humidified incubator.

Fig. 2. PAQMAN quality checks. A. Agarose gel electrophoresis of rs36115365-C oligonucleotide titration, before and after binding to streptavidin-sepharose beads, to visualize depletion of the oligonucleotides by bead immobilization. B. TMT labeling efficiency of peptides. For replicate 1, 12,454 out of 12,641 (98.5%) peptides were labeled, whereas for replicate 2, 12,778 out of 12,426 (97.1%) peptides were labeled.

3.2.7. In-solution TMT labeling

To quantify the 10 affinity purifications in one LC-MS run, we make use of 10-plex TMT labeling, but other MS2-based isobaric labeling strategies such as iTRAQ [14] or diLeu labeling [15] can also be used. Label-free approaches or MS-1 based labeling strategies such as SILAC are, however, less suitable because of increased mass spectrometry measurement time and increased run-to-run variability.

- Prepare samples for TMT labeling by reducing the sample volume to ~10 μ l using SpeedVac centrifugation at room temperature.
- Spin down each vial containing 0.8 mg TMT reagent and suspend in 101 μ l of MS-grade anhydrous acetonitrile.
- Add 10 μ l (0.08 mg) of TMT reagent to the relevant sample. Apply the TMT label as described in the following scheme:

Well	1	2	3	4	5	6	7	8	9	10
Final oligonucleotide concentration (nM)	0.15	0.46	1.37	4.1	12.3	37	111.1	333.3	1000	3000
TMT label	126	127C	127N	128C	128N	129C	129N	130C	130N	131

Note: The amount of TMT label might need to be adjusted depending on the length of oligonucleotides because more proteins are pulled down when using longer oligonucleotides. Label efficiency can be determined using ProteomeDiscoverer (Thermo Scientific) as described in Section 3.2.10 (Fig. 2B).

- Incubate for 1 h at room temperature shaking in the dark.
- Add 10 μ l 1 M Tris pH 8.0 to quench the reaction and incubate for 30 min shaking at room temperature.
- Combine all 10 related reactions.

3.2.8. Peptide purification and desalting

To prepare samples for mass spectrometry analysis, samples are concentrated and desalted using StageTipping [16].

- Add 200 μ l 10% TFA to prepare samples for StageTipping. Check that the pH is below 2.
- Prepare the StageTips by picking up two layers of C18 material using a blunt-end syringe and push these into a non-autoclaved 250- μ l pipette tip using a rod. Spin adaptors for the StageTips can be built by removing the lid and bottom of a 0.5-ml Eppendorf tube. Collection tubes can be prepared by cutting of the lid of a 2-ml Eppendorf tube. Place the spin adaptor holding the StageTips into the collection tube.
- Add 50 μ l 100% methanol to the StageTip. Centrifuge for 4 min at 1.5 g.
- Add 50 μ l Buffer B to the StageTip. Centrifuge for 4 min at 1.5 g.
- Add 50 μ l Buffer A to the StageTip. Centrifuge for 4 min at 1.5 g. Repeat once.
- Load the sample on the StageTip. Centrifuge for 10 min at 1.5 g. Continue the centrifugation if this is necessary to load the whole sample.
- Add 50 μ l Buffer A to the StageTip. Centrifuge for 4 min at 1.5 g.
- StageTips can be stored at 4 °C until measurement on a mass spectrometer.

3.2.9. Mass spectrometry

- Elute the sample from the StageTip using 30 μ l of Buffer B. Reduce the sample volume to 5 μ l by SpeedVac centrifugation at room temperature. Afterwards, add 7.5 μ l Buffer A to a total volume of 12.5 μ l. The sample is now ready to be measured by liquid chromatography-mass spectrometry (LC-MS).

Samples can be measured on various modern high-resolution mass spectrometers. TMT quantification can be performed at either MS2 or MS3 level, also depending on the instrument that is being used. Although MS2 quantification generally results in higher identification rates, MS3 quantification is more accurate [17]. Therefore, we recommend MS3-based quantification because accurate reporter ion quantification is important for K_d^{app} calculations. Below we describe settings and parameters for TMT quantification at the MS3 level on an Orbitrap-Fusion mass spectrometer from Thermo Scientific, connected online to an Easy-nLC 1000 system (also Thermo Scientific). 5 μ l sample is loaded onto a 30 cm column with an inner diameter of 75 μ m (New Objective; heated at 40 °C) packed in-house with 3 μ m Reprosil-Pur C18-AQ beads (Dr Maisch). Tryptic peptides are separated using a gradient from 7 to 15% Buffer B over 5 min, from 15% to 35% Buffer B

over 214 min, from 35% to 50% Buffer over 5 min, and eventually from 50% to 95% Buffer B in 1 min followed by 5 additional minutes isocratic flow at 95% Buffer B (flowrate 200 nl/minute). Tryptic peptides eluting from the LC column are directly sprayed into a Thermo Fusion Tribrid mass spectrometer with the built-in Thermo synchronous precursor selection (SPS)-MS3 method. Full MS scans are recorded in the Orbitrap at 120,000 resolution at a scan range of 380–1500 m/z . Target values for full MS are set to 2.0e5 AGC target and a maximum injection time of 50 ms. Precursors with a charge between 2 and 7, an intensity threshold of 5.0e3, and dynamic exclusion for 60 s are selected for MS2 analysis. MS2 is performed in top speed data dependent acquisition mode, with precursor priority given based on highest intensity and after isolation in the quadrupole using an isolation window of 0.7 m/z units. We use CID activation at a collision energy of 35% for fragmentation. Target values for MS2 are set to 1.0e4 AGC target with a maximum injection time of 50 ms. MS2 precursors in the mass range of 400–1200 m/z are selected for MS3 analysis using the Thermo TMT reagent isobaric tag loss exclusion property and MS2 precursor ions 18 m/z units low and 5 m/z units high are excluded. The mass spectrometer is operated for MS3 selection in top data-dependent acquisition mode. MS3 ions are selected with synchronous precursor selection activated for ten precursors. The isolation width of MS and MS2 is set to 2 m/z . The fragmented ions are detected in the Orbitrap with a resolution of 60,000 and a maximum injection time of 120 ms.

3.2.10. Data analysis and K_d^{app} calculation

After mass spectrometry, raw MS spectra can be analyzed using MaxQuant or Proteome Discoverer (Thermo Scientific) to identify and quantify proteins. Here, we provide a workflow for analysis using Proteome Discoverer version 2.2.

- For data analysis in Proteome Discoverer version 2.2 select the processing workflow “PWF_Fusion_TMT_Quan_SPS_MS3_SequestHT_Percolator” and the consensus workflow “CWF_Comprehensive_EnhancedAnnotation_Reporter_Quan” with standard settings.
- Set the quantification method to ‘TMT 10plex’ with the TMT 131 label as control channel.
- Perform the “Spectrum Files RC” and “Sequest HT” searches against a uniprot curated human proteome.
- Add the 6-plex TMT reagent mass (229.163 Da) as dynamic modification on histidine, serine, and threonine. Make sure that the 6-plex TMT reagent mass is set as static modification on lysines and on the peptide N-terminus.
- Add a file containing contaminants in the section “ProteinMarker”.

Fig. 3. Data analysis pipeline. A. Grouped abundances obtained from ProteomeDiscoverer 2.2 for proteins identified in both replicates. B. Grouped abundance for ZNF148. C. Fraction bound calculated using grouped abundances. The highest titration point was used as references because binding is saturated at this point. D. Fraction bound is scaled between 0 and 1. E. A Hill-like curve is fitted to determine apparent binding affinities by calculating K_d^{app} , which is equivalent to the DNA concentration where half of the maximal protein fraction is bound (indicated in red). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

This file can be obtained from the MaxQuant software suite at www.MaxQuant.org.

- When analysis is finished, export the results by selecting 'Proteins' and create a tab limited text file with R friendly headers.
- TMT labeling efficiency can be tested by analyzing the raw data as described above with the difference that 6-plex TMT reagent mass is

added as dynamic modification on lysines and the peptide N-terminus. Labeling efficiency is calculated by comparing the total amount of identified peptides with peptides modified by TMT.

The binding curves and apparent binding affinities can be calculated as described below. To calculate the apparent binding affinities, ratios

are used. The highest concentration of bait used is 3 μM , labeled with the TMT reporter ion 131.

- For further downstream analysis, only ‘MasterProteins’ are used that are not marked as contaminant (based on the predefined list used in Proteome Discoverer).
- Most proteins are background binders or bind with a low affinity and therefore show no binding curve (Fig. 3A, Supplementary Table 2). However, some proteins bind with high affinity and show a Hill-like curve in the used concentration range (Fig. 3B).
- To obtain the fraction bound for identified proteins, ratios of protein binding are calculated relative to the highest titration point (3 μM , TMT 131) (Fig. 3C). Theoretically, the binding at this concentration is saturated for proteins that bind to the oligonucleotide bait with an apparent affinity up to 500 nM. Here, we used the ratios based on the normalized abundances that were calculated by Proteome Discoverer.
- Normalize protein ratios by min-max scaling between 0 and 1 (Fig. 3D).
- To generate protein binding curves, fit a Hill-like curve: $\Theta = \frac{1}{\left(\frac{K_d^{\text{app}}}{[L]}\right)^n + 1}$, where Θ is the fraction of bound protein, $[L]$ is the bait concentration, K_d^{app} is the apparent dissociation constant, and n is the Hill coefficient.
- Fit the parameters K_d^{app} and n using non-linear least squares with the mean Θ of each duplicate, with initial parameter estimates of $K_d^{\text{app}} = 100$ and $n = 1$. Plot the binding curves as the mean of replicates plus and minus error bars representing the standard error of the mean. Here, we calculated the standard errors using bootstrapping in the python package seaborn (Fig. 3E).
- The fit of binding curves should be tested to avoid artificial curve-

fitting of proteins that do not show binding kinetics. Here, we suggest the following quality checks described below: for analysis, only proteins for which all nine ratios are determined in all replicates are included. After curve fitting, obtained data is filtered by goodness of fit of the Hill-like curve, where an r^2 value of 0.95 for a linear regression between the fit binding model and the measured data is required. Furthermore, a predicted fraction bound of < 0.25 for the lowest titration point and more than 0.75 for the highest titration point is required. Care should be taken to not over fit a curve such that the data points appear to reach saturation when this effectively is not the case when looking at the raw data points

4. Results

To determine apparent binding affinities for proteins binding to the C-variant of rs36115365, we performed PAQMAN in UACC903 nuclear extract, which is a melanoma cell line. We identified 40 proteins binding with a $K_d^{\text{app}} < 500$ nM (Fig. 4A, Supplementary Table 3) of which ZNF148 and VEZF1 were identified previously as specific binders [13]. ZNF148 binds with a higher affinity than VEZF1 (Fig. 4B, C). Other proteins binding with a high affinity were previously identified as non-sequence specific. For ZNF281, previously identified as a specific interactor of the C-variant, K_d^{app} could not be calculated, probably because the binding affinity is too low. Another specific binder, ZNF740, was only quantified in one replicate; however, no binding kinetics were observed in this replicate. Follow-up experiments performed previously revealed that ZNF148 regulates TERT expression and telomere length in a SNP-specific manner. This illustrates that PAQMAN experiments are informative and can predict which sequence specific transcription factors identified to interact with a certain motif

Fig. 4. ZNF148 binds to rs rs36115365-C with high affinity. A. Grouped abundances obtained from ProteomeDiscoverer 2.2 of proteins for which we can calculate a K_d^{app} , which is plotted in red. B. Binding curve of ZNF148. C. Binding curve for VEZF1. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

in vitro, regulate gene expression associated with that motif in vivo.

5. Conclusion and outlook

Here, we present a detailed protocol for PAQMAN, which is a mass spectrometry-based method that can be used to determine the apparent binding affinities for dozens of proteins to a DNA sequence of interest in a single experiment. Of note, the protocol we present is not limited to protein-DNA interactions but can also be applied to measure affinities between proteins and DNA secondary structures, nucleosomes or RNA [12]. Furthermore, cells treated with a certain stimulus can be used to investigate how this stimulus potentially affects binding affinities.

To illustrate the usefulness of PAQMAN, we profiled a SNP, rs36115365-C, located in an enhancer associated with the *TERT* gene, which is associated with an increased risk to develop multiple types of cancer. Previous work resulted in the identification of ZNF148 as the transcription factor that, through binding rs36115365-C, drives increased *TERT* expression. In that study, multiple proteins were identified that bind specifically to the SNP and elaborate follow up experiments were necessary to study these proteins to eventually determine the main regulator, ZNF148 [13]. Here, we determined apparent binding affinities between nuclear proteins and the rs36115365-C SNP and show that amongst all specific rs36115365-C interactors, ZNF148 has the highest apparent binding affinity.

In its current format, PAQMAN is limited to determine apparent binding affinities in the range of up to 500 nM with, in theory, no lower limit. It should be noted that at low bait concentrations, binding competition between multiple nuclear proteins, which display binding affinity for this bait, might occur. Apparent binding constants determined by PAQMAN may thus deviate from measurements done with purified recombinant proteins. However, the apparent binding constants determined by PAQMAN are in principle more informative compared to experiments with single purified recombinant proteins, as these are reflective of the competitive binding environment in vivo. The affinity range of PAQMAN could theoretically be increased to identify lower binding affinities by increasing the concentration of bait DNA. However, in this case the duration of washing steps needs to be decreased to determine low affinities accurately. Low (> 500 nM) affinity interactions are typically characterized by a fast off-rate, which means that the product (DNA-protein complex) will dissociate with a half-time of < 1 s [5]. However, washes after protein extract incubation in PAQMAN normally take about 2 s, thus resulting in disruption of the binding equilibrium for low affinity interactors.

Taken together, information about protein-DNA binding specificity and affinity can now be obtained by mass spectrometry-based proteomic approaches. In the future, we aim to establish a method, which integrates non-isobaric stable isotope labeling and isobaric stable isotope labeling [18] to obtain DNA-protein binding specificity and affinity information in a single LC-MS run.

CRedit authorship contribution statement

Cathrin Gräwe: Conceptualization, Formal analysis, Investigation,

Data curation, Project administration, Writing - original draft. **Matthew M. Makowski:** Conceptualization, Supervision. **Michiel Vermeulen:** Conceptualization, Funding acquisition, Resources, Supervision, Writing - review & editing.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ymeth.2019.12.003>.

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