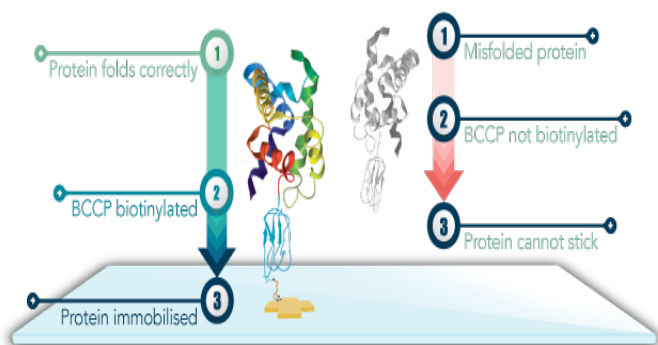


# Rapid Determination of the Effect of Genetic Mutations on Protein Function

## Technology background

Recombinant protein expression is a fundamental technique that underpins clinical diagnostics, drug discovery and screening, vaccine development and pure research for elucidating mechanisms of disease development and progression. However, high-throughput production of correctly folded and functional, full-length human proteins has a very high failure rate. Protein folding is a highly complex process requiring a combination of an aqueous environment, chaperones, post-translational modifications and the formation of multimeric structures held together by covalent bonds. Any deviation from the correct sequence of events can result in a misfolded protein. Loss of protein function is directly linked to misfolding. Use of misfolded proteins in downstream assays and interactions studies can result in identification of false positive biomarkers.

The Sengenics KREX™ technology utilises the biotin carboxyl carrier protein (BCCP) as a folding marker and solubility enhancer which results in high-throughput expression of full-length, correctly folded and functional proteins. BCCP-protein fusions are capable of being biotinylated either *in vivo* or *in vitro*, allowing the use of highly specific biotin-streptavidin interaction for surface capture. As biotinylated proteins bound to a streptavidin-coated surface show negligible dissociation, this interaction therefore provides a vastly superior means for tethering proteins to a planar surface and is ideal for applications such as protein microarrays, glass micro-titer plates, SPR and bead-based assays.



**Figure 1.** The BCCP folding marker acts as a marker for correctly folded proteins. Proteins will be immobilised on the array only when they are properly folded and biotinylated on the BCCP folding marker.

## Introduction

Changes in DNA comprise SNPs, Indels, domain shuffling or copy number variations which can affect diverse protein properties, such as stability, catalytic activity or the ability to interact with other molecules. Mutations play a major role in the onset and development of various diseases. However, it is difficult to correlate how mutation can affect protein structure and function as conducting mutagenesis experiments on physical proteins is expensive and time consuming.

### ADVANTAGES

- Access to variant proteins in recombinant form (either purified or over expressed within a cellular environment)
- Evidence that these variant proteins exhibit expected or known differential functional activities
- A more rapid, sensitive and parallel method for assaying protein variants compared to conventional methods.
- correctly folded BCCP-fusions.

### TECHNICAL PERFORMANCE

- Expression of correctly folded and functional proteins with a 98% success rate.
- Fully quantitative clinical-grade assay metrics. Dynamic range is linear up to five orders of magnitude.
- Excellent reproducibility and precision with a mean CV% below 4% between replica spots.
- Highly sensitive with a limit of detection of approximately 1:1,000,000 serum dilution and autoantibody titer of 190 pg/mL.
- Exceptional specificity and accuracy: non-specific binding eliminated as all proteins are immobilised as functional and correctly folded BCCP-fusions.

Furthermore, only very few methods are capable of assessing how multiple amino acid substitutions affect a protein's structure and stability. Conventional methods for correlating the effect of genetic mutations on protein function include:

1. Newman et al. performed a high throughput mutagenic analysis of yeast sumo structure and function by developing a versatile library of more than 250 mutant alleles of *S. cerevisiae* SUMO (Smt3) using biochemical gene synthesis technologies. The library was screened using traditional colony-forming assays and also high-throughput approach that take advantage of individual barcodes associated with each mutant allele. They identified 45 conditional smt3 alleles and generated a comprehensive structure-function based map of smt3 and residues that are critical for viability and responses to a variety of cellular stresses.
2. Restoration of protein function: Uggenti et al. demonstrated how they restored the function of four bestrophin-1 mutant proteins associated with retinopathy to that of wild-type (WT) protein using a clinically approved drug, 4PBA. They previously showed that nine bestrophin-1 mutant proteins have significantly reduced Cl<sup>-</sup> conductance compared with the WT protein and were able to rescue the expression, localization and function of the mutant proteins by treatment of cells with 4PBA. They concluded that 4PBA has the potential for long-term correction of protein folding and function for a variety of diseases.

The Sengenics KREX™ technology can be leveraged to develop a purpose-designed solid support platform (such as protein microarray) which could afford simultaneous screening of over thousands of mutant proteins for evaluating small molecule compounds for their potential in restoring mutant protein function, which could be valuable in designing therapeutics. This platform would enable a superior, low cost, high throughput method for studying the effect of genetic mutations on protein function.

## Case study: parallel characterisation of p53 mutants using protein microarray

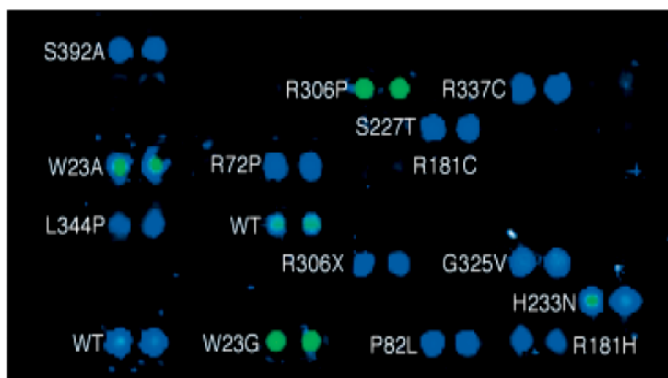
The main objective of this study is to compare the effects (e.g.: catalytic efficiency, affinity) of naturally occurring variants of p53 with SNPs in a high-throughput manner. The array was fabricated by cloning and expressing p53 variants via inverse PCR with a downstream His-tag and BCCP-tag followed by printing on the array surface. The p53 protein microarray was then used to evaluate DNA

binding affinity using a cy-3 labelled GADD45 promoter oligo sequence, protein interaction activity of MDM2 and phosphorylation of CKII.

Reduced/abolished DNA binding activity in p53 variants (Table 1 and Fig. 2) was observed with the exception of R181C/H, S227T and H233N/D variants which have mutations in solvent exposed positions and in regions distal from the protein-DNA interface. Variants R248Q/W, R273C/H and R280K exhibited low affinities which were consistent with loss of specific protein-DNA interactions and steric hindrance. Reduced MDM2 binding to p53 was also observed in W23A/G variants hence demonstrating a change in protein-protein interaction as a consequence of mutation.

**Table 1.** Partial summary data from microarray experiment.

Mutation	DNA binding		MDM2
	B <sub>max</sub> /% wild-type	K <sub>d</sub> /nM	
Wild-type	100 (90–110)	7 (5–10)	+
W23A	131 (119–144)	7 (5–10)	–
W23G	84 (74–94)	5 (3–9)	–
R181C	88 (81–95)	11 (8–13)	+
R181H	48 (40–57)	11 (6–21)	+
S227T	101 (94–110)	7 (5–9)	+
H233N	60 (52–68)	5 (3–8)	+
H233D	70 (58–84)	7 (3–14)	+
N235D	32 (25–40)	27 (15–49)	+
N235S	46 (36–56)	9 (4–20)	+
R248W	107 (95–120)	12 (8–17)	+
R248Q	85 (77–95)	17 (12–23)	+
R273C	70 (56–85)	20 (11–37)	+
R273H	59 (40–79)	54 (27–106)	+
P278L	ND		+
R280K	54 (40–70)	21 (9–46)	+

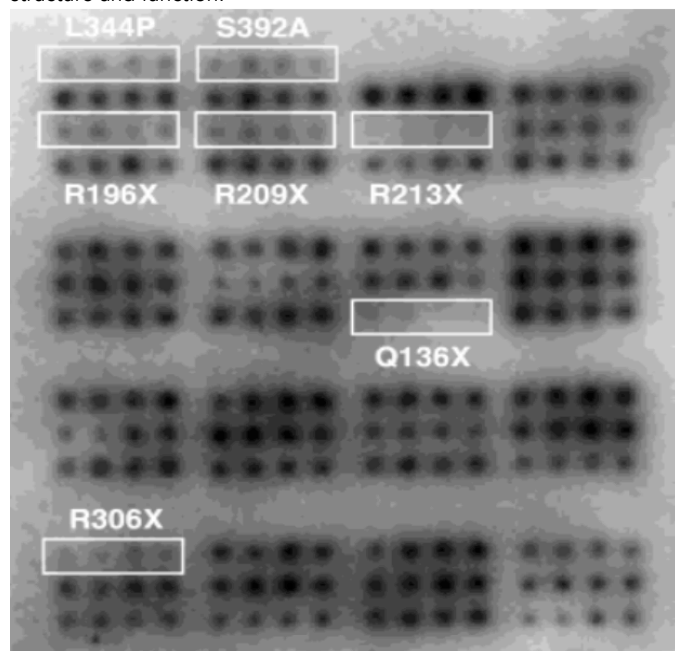


**Figure 2.** DNA binding of Cy3- labelled GADD45 on p53 custom protein microarray.

In order to determine functional variation of p53 variants, CKII phosphorylation of p53 at the C-terminal residue S392 was performed. The p53 variants were phosphorylated with CKII and the phosphorylation signals were detected using anti-phosphoserine 392 antibodies. Phosphorylation signals were detected in all proteins on the array with the exception of L344P, 392A (negative control) and variants with mutation X. Variants with mutation X had premature stop codons introduced at different regions and lack the S392 region required for CKII interaction, therefore no phosphorylation signals were detected in these variants.

In conclusion, the reduction of GADD45, MDM2 binding and phosphorylation demonstrated by p53 variants has provided insight

on how genetic variation leads to change or disruption of protein structure and function.



**Figure 3.** CKII phosphorylation assay on p53 protein microarray.

## References

1. Duarte, J. G. & Blackburn, J. M. (2017) Advances in the development of human protein microarrays, *Expert Review of Proteomics* 14:7, 627-641
2. Boutell, J.M., Darren, J.H., Godber, B.L., Kozłowski, R.Z. & Blackburn, J.B. (2004) Functional protein microarrays for parallel characterisation of p53 mutants. *Proteomics* 4, 1950-1958
3. Newman HA, Meluh PB, Lu J, Vidal J, Carson C, Lagesse E, et al. 2017. A high throughput mutagenic analysis of yeast sumo structure and function. *PLoS Genet* 13(2): e1006612
4. Uggenti C et al. 2016. Restoration of mutant bestrophin-1 expression, localisation and function in a polarised epithelial cell model. *Dis Model Mech.*; 9(11): 1317–1328

## Terms and conditions

Patented KREX functional proteomics technology which utilises the BCCP folding marker for the production of full-length, correctly folded and functional proteins. Protected by the following patents: EP1203238, JP4730804, GB2361698, US7816098, EP1470229, AU2003238441, US8999897, JP4377242, CA 2474457, EP1485411, CA2518927C, EP1456668, AU2002352355, JP4781628. Trademarked in the United Kingdom UK00003167383 under classes 05, 10 and 16. Except as otherwise agreed to by us in writing, the purchase of Products from us only conveys to you the non-transferable right for you to use the quantity of Products purchased in compliance with any applicable limited use statement or limited label license, as detailed in our catalogues, on our website, or on the label or other documentation accompanying the goods (all such statements or licenses being incorporated herein by reference as if set forth herein in their entirety). Unless otherwise authorized by us in writing, Products purchased from us may not be resold, modified for resale, or used to manufacture commercial products. All products and results from services are supplied / handed over by us to you on the condition that they may only be used by you alone (and no other third parties for and/or on your behalf) as instructed and directed in writing by Sengenics for your own internal, non-commercial and non-revenue and non-fee generating research purposes only. They are not in any circumstances to be used for drug or diagnostic purposes, nor are they intended for use in or on humans. By accepting delivery of our products or services, you are expressly agreeing to use our products or services for internal, non-commercial and non-revenue and non-fee generating research purposes only as specified in this paragraph. Products are not to be repackaged or resold and results from services are not to be used for any purpose apart from the research purposes specified in this paragraph. Any non-research use requires an ITAP license, the cost of such license is based upon the type of application of any Sengenics technologies, products or services for any purpose other than the internal, non-commercial and non-revenue and non-fee generating research purposes specified in this paragraph. You represent and warrant to us that the Products sold to you (i) will be used only for your own internal research, (ii) will only be used in compliance with any applicable limited use statement or limited label license or applicable law and (iii) will not be resold or otherwise transferred or conveyed to any third party. No license or immunity under any patent is either granted or implied by the sale of any of our Products except to the extent expressly granted in any respective label license or limited use statement (all such statements or licenses being incorporated herein by reference as if set forth herein in their entirety). You should evaluate whether your use of Products purchased from Sengenics requires permission or license from any third parties. Nothing in these Terms shall be deemed or construed (i) as a license or grant of any intellectual property, whether implied, by estoppel or otherwise except to the extent expressly granted under any applicable intended use statement, limited use statement or limited label license; (ii) to limit our rights to enforce our Intellectual Property, including, without limitation, as to use of any Product beyond that granted under any label license or statement applicable to the Products; (iii) as granting you any right to be supplied with goods or component thereof beyond those ordered by you and supplied by us in accordance with these Terms; or (iv) as a license or grant of any right to you to manufacture or to have manufactured the Products.

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