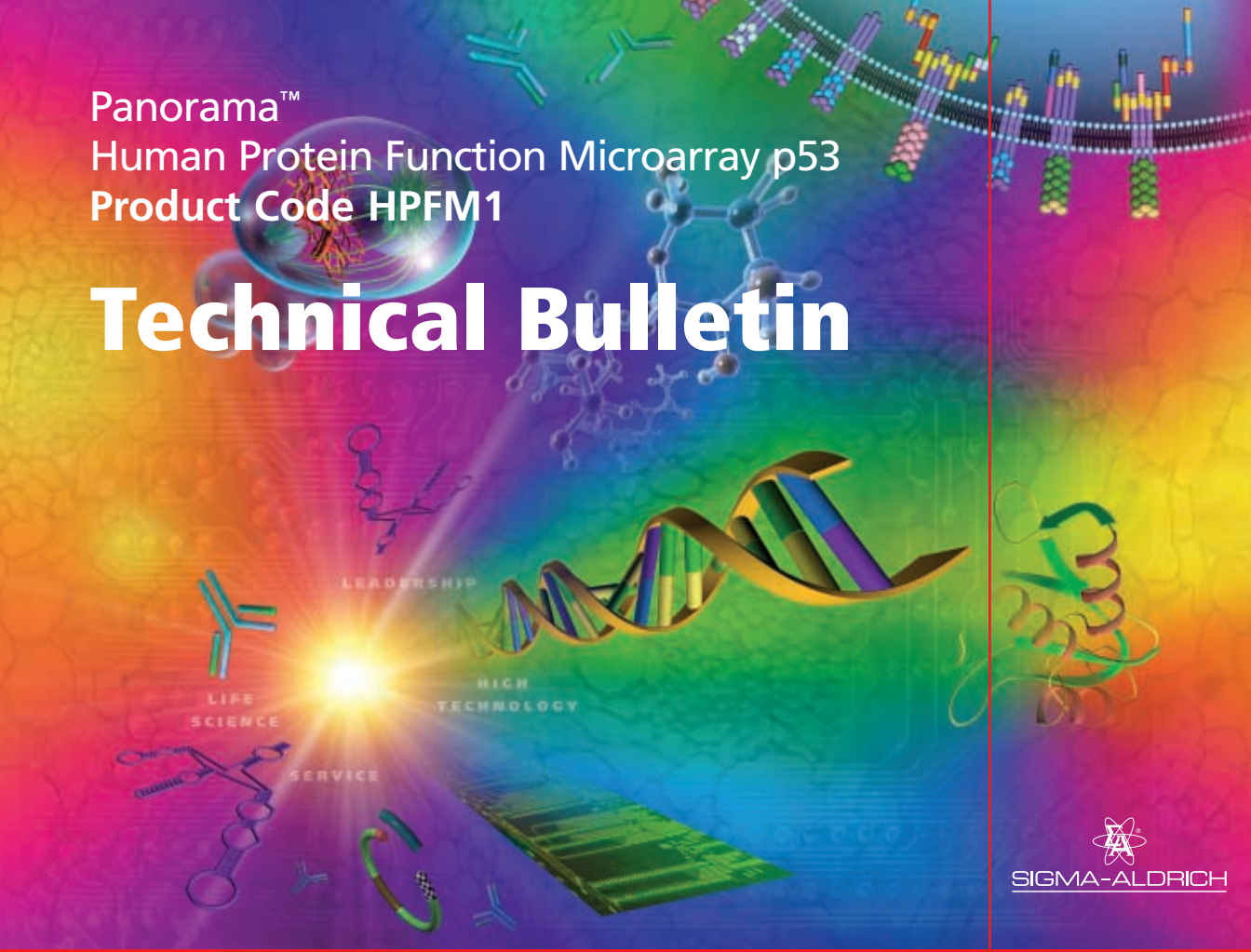
Panorama™
Human Protein Function Microarray p53
Product Code HPFM1

Technical Bulletin



SIGMA-ALDRICH

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INTRODUCTION

The human p53 protein is a transcription factor with an important role as a tumor suppressor. Loss of p53 function through mutation is responsible for more than 50% of human cancers. The functions of p53 are mediated via a range of different direct interactions including protein-DNA and protein-protein. The latter include direct binding (e.g. by MDM2), and post-translational modifications by enzymes including kinases and acetylases.

Many p53 mutations have been identified via genetic analyses, but little mechanistic data exists to help elucidate mechanisms of pathology. Where individual mutations have been characterized, comparison of data is often complicated by differences in experimental protocol. For example, the cell-based functional analysis of separated alleles in yeast (FASAY) method provides information on the transactivational functionality of variants, but without detailed mechanistic information. The electrophoretic mobility shift assay (EMSA) provides quantitative information on DNA binding, but is relatively low-throughput and therefore unsuitable for parallel analysis of many proteins. These issues are addressed effectively by the protein microarray format since many proteins can be assayed in the same experiment under identical conditions.

The Panorama™ p53 protein array contains human wild-type p53, 49 variants, and experimental controls. Each p53 variant contains a mutation or single nucleotide polymorphism (SNP) resulting in an amino acid change or a truncation and 45 of the variants are known germ-line mutations associated with cancer.[1-3] All of the proteins have been expressed in recombinant form in *Escherichia coli* and immobilized on the slide surface via an affinity tag. In many studies, the mutated proteins contained within the p53 protein array show different functional properties to the wild-type p53 and these differences observed *in vitro* may underlie the resulting cancer phenotype.

PROTEIN ARRAY KIT CONTENTS

The p53 protein array kit consists of:

Panorama Human p53 Microarrays Product Code P 2123	2 each
GADD45 DNA Probe-Cy3 Product Code G 3170	15 µl
Anti-p53-Cy5 Product Code P 2248	15 µl

Array Substrate

The proteins are arrayed onto a streptavidin-coated, low fluorescence glass slide (25 x 75.6 x 1 mm). The upper side and orientation is indicated by a label (Appendix A, page 13). The array has been pre-washed and blocked to reduce non-specific binding and immersed in buffer for long-term storage at –20 °C.

Array Content

The array contains two identical sub-arrays each containing 128 features. Each protein within the sub-arrays has been printed in duplicate. A map and key of the p53 array is shown in Appendix B, page 14.

Each sub-array contains:

- Wild-type human p53 protein
- 45 germ-line mutant p53 proteins
- 4 control p53 proteins
- 8 Cy3-labeled bovine serum albumin (BSA) marker spots for array alignment
- 2 negative control spots consisting of the immobilization tag alone
- 2 negative control spots printed with lysis buffer only
- 16 negative control spots printed with spotting buffer only

Spots are approximately 500 μm in diameter and are spaced at intervals of 1,100 μm.

Cy3-labeled GADD45 DNA

A DNA binding assay using Cy3-labeled GADD45 DNA is recommended as a control assay to test function of proteins on the p53 array. A Cy3-labeled GADD45 oligonucleotide representing the GADD45 DNA promoter element is provided. It is a double stranded Cy3-labeled DNA fragment (upper strand 5'-GTACAGAACATGTCTAAGCATGCTGGGGAC-3') that binds to wild-type p53. The DNA will also bind the p53 variants to different extents when used with the recommended DNA Assay Buffer (Appendix C). A typical profile of Cy3-GADD45 DNA binding to the full complement of p53 variants is shown on page 8, Section III.

Cy5-labeled p53 antibody

A Cy5-labeled monoclonal p53 antibody that binds all arrayed p53 variants is provided as a positive control. The p53-specific antibody can be used to probe the same control or experimental array after the assay for function. This will demonstrate that p53 protein is immobilized at each spot. The binding assay must be carried out using the Antibody Binding Assay Buffer (Appendix C). An example image of antibody binding is shown on page 8, Section IV.

Not included with the p53 protein array:

The following materials are not included in the protein array kit, but are required to perform the described DNA and antibody binding assays.

- DNA Assay Buffer (Appendix C)
- Antibody Binding Assay Buffer (Appendix C)
- Phosphate buffered saline (PBS), pH 7.4
- Non-fat dried milk powder (Product Code M 7409)
- Plastic containers for slide processing
- 70% ethanol
- Hybri-slip cover slips
  (Product Code Z37,027-4, 60 x 22 mm)
- Microarray scanner or fluorescence imager
- Microarray analysis software
- Forceps
- Powder-free gloves

STORAGE CONDITIONS

Proteins on the array are sensitive to heat and oxidation. To preserve protein activity, the arrays are shipped on dry ice in screw-capped Coplin tubes filled with 30 ml of storage buffer containing dithiothreitol and glycerol.

On receipt, the kit contents should be placed at –20 °C until use. The protein arrays may be frozen solid on arrival due to the dry ice used for shipping, but will thaw gently when placed at –20 °C.

GENERAL RECOMMENDATIONS PRIOR TO USING THE KIT

a. Remove foam insert before removing slides from tube.
b. Handle the protein arrays with care. Remove the arrays from their storage buffer by the labeled end using forceps. Do not touch the central portion of the slide surface.
c. Keep arrays in ice-cold buffer unless higher temperatures are required for assays.
d. Always keep the array label-side upwards. Wash the array for 5 minutes in ice-cold DNA Assay Buffer (5 ml minimum wash volume) with gentle shaking to remove components in the storage buffer.
e. Cover arrays completely in assay buffer/reagents to prevent them from drying out during the assay.
f. **Do not** use glass cover slips as they may sequester the sample. For low volume incubations where the sample is limited, use plastic cover slips (e.g. Hybri-slips, (60 x 22 mm). Pipette 50 µl of sample carefully onto the middle of the slide and lower the cover slip gently onto the surface using fine forceps.
g. If sample is not limited, perform incubations in small plastic containers with sufficient sample solution to immerse the arrays.
h. Protect Cy-dye-labeled DNA and antibody from light during the assay.

i. In order to keep p53 arrays under reducing conditions during use it is recommended that freshly prepared 1 mM DTT is included in all buffers and reagents.
j. Include 20% glycerol and 0.1% TRITON™ X-100 in assay buffers where it is compatible with the assay and detection methods.
k. p53 arrays have been pre-blocked with 5% milk powder 0.1% BSA, 20% glycerol, 0.1% Triton X-100 pH 7.6 (50 mM KCl, 25 mM HEPES). Additional blocking with 5% non-fat dried milk powder or other commonly used blocking agents may be necessary for specific applications.
l. If a high degree of background speckling is observed after processing, use de-speckling algorithms, which are present in most commercially available microarray analysis software. Please note that speckling affects the appearance only: it does not materially affect the data generated.

RECOMMENDED CONTROL ASSAYS

I. Protocol for Cy3-GADD45 DNA Binding Assay

Carry out all steps using freshly prepared, ice cold DNA Assay Buffer containing 1 mM DTT (Appendix C).

a. Remove the array from storage buffer and wash for 5 minutes in 25 ml of DNA Assay Buffer.
b. Dilute the Cy3-GADD45 DNA to a final concentration of 2.25 μM in DNA Assay Buffer (10 μl of reagent (Product Code G 3170) added to 168 μl of DNA Assay Buffer).
c. Remove the array from the DNA Assay Buffer and drain excess liquid by tapping gently the slide edge on lint-free tissue paper for several seconds.
d. Dry the back of the slide with lint-free tissue paper.
e. Immediately pipette 50 μl of 2.25 μM Cy3-GADD45 DNA carefully onto the middle of the slide, then lower a plastic cover slip gently onto the surface using fine forceps. Try to ensure that no air bubbles are trapped. **Do not** move the cover slip once in place as this can lead to displacement of the sample. If the recommended cover slips are used then any trapped air bubbles will generally disappear.
f. Place the array in an open tray in a refrigerator and incubate at 4 ºC for 30 minutes.
g. Perform all incubations using CyDyes™ away from direct light.
h. After incubation, lift the cover slip using a pair of fine forceps being careful not to slide the cover slip over the array surface. Place slides in a plastic container with 25 ml of ice cold DNA Assay Buffer. We recommend the use of watertight Pap jars which are available from Evergreen Scientific (www.evergreensci.com). Wash the arrays in ice cold DNA Assay Buffer three times with 25 ml for 5 minutes each time on an orbital shaker at ~80 rpm to remove unbound DNA. Change the buffer and invert the container several times at each wash step.

i. Pour off the wash buffer and add 25 ml of ice cold 70% ethanol to fix the DNA to the slide. Place the slides on an orbital shaker for 5 minutes on ice and then pour off the ethanol.
j. Dry the slides by centrifugation. Place each slide in a 50 ml disposable centrifuge tube **with the label at the bottom of the tube** then centrifuge at 4 °C for 2 minutes at 240 x *g*.
k. Carefully remove the slides from the centrifuge tubes with a pair of blunt-ended forceps touching only the end of the slide.
l. Scan the slides using a microarray scanner or imager with appropriate excitation and emission wavelengths for the fluorophore being used. The excitation and emission wavelengths for Cy3 are 550 and 570 nm, respectively.

II. Protocol for Antibody Binding Assay

After performing a functional assay, use the p53 monoclonal antibody to show that p53 protein is present in each p53 spot.

Carry out all steps at room temperature.

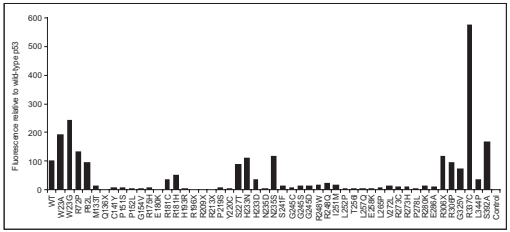
a. Prepare PBS-TWEEN® Washing Buffer (Appendix C).
b. Prepare Antibody Binding Assay Buffer (Appendix C).
c. Dilute the Cy5-anti-p53 antibody 1:1000 in Antibody Binding Assay Buffer. Prepare enough for 5 ml per slide.
d. Place slides in small plastic containers with 25 ml PBS-TWEEN Washing Buffer and wash for 5 minutes (25 ml per container on a shaker).
e. Remove the PBS-TWEEN Washing Buffer and add 5 ml of diluted Cy5-anti-p53 antibody per container.
f. Incubate for 30 minutes on an orbital shaker.
g. Remove the antibody solution and wash slides 3 x 5 minutes in PBS-TWEEN Washing Buffer, changing the buffer after each wash step.
h. Dry the slides by centrifugation. Using forceps place each slide in a 50 ml disposable centrifuge tube **with the label at the bottom of the tube** then centrifuge at 4 °C for 2 minutes at 240 x *g*.
i. Carefully remove the slides from the centrifuge tubes with a pair of blunt ended forceps touching only the end of the slide.
j. Scan the slides using a microarray scanner or imager with appropriate excitation and emission wavelengths for the fluorophore being used. The excitation and emission wavelengths of Cy5 are 649 and 670 nm, respectively.

III. Data Analysis for Cy3-GADD45 DNA Binding Assay

Analyze the images using a standard microarray analysis software package.

Figure 1. An example of a typical array image for the Cy3-GADD45 DNA binding assay is shown below (see Appendix B for array layout).

Figure 2. An example of a typical profile for the Cy3-GADD45 DNA binding assay is shown. The data was obtained by normalizing the binding exhibited by mutant and control proteins to the wild-type p53 binding.



IV. Data Analysis for Cy5-anti-p53 Binding Assay

Figure 3. An example of a typical array image for the Cy5-anti-p53 binding assay is shown below (see Appendix B for array layout). Please note that BSA marker spots and buffers do not appear in this analysis as these are not recognized by the antibody.

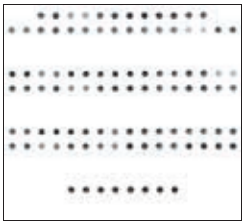
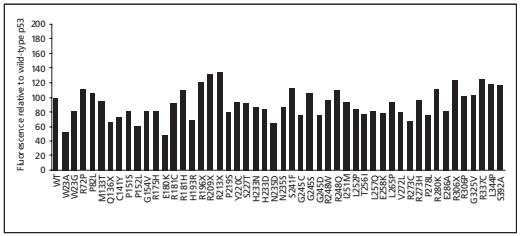


Figure 4. An example of a typical profile for the Cy5-anti-p53 binding assay is shown below. The data was obtained by normalizing the binding exhibited by mutant and control proteins to the wild-type p53 binding.



GUIDELINES FOR DIFFERENT ASSAY TYPES

I. DNA and Antibody Binding Assays

It is recommended that oligonucleotides are 5'-labeled with fluorophores during synthesis.

Antibody labeling may be performed using Cy5 mono-reactive dye for antibody labeling (Amersham Biosciences).

The DNA and Antibody Assay Buffers recommended in this booklet may not be suitable for other user experiments. Optimize all buffers for the binding assays in question.

The concentration of DNA recommended in the control assay has been optimized for demonstration purposes only. DNA binding to p53 is complicated by the fact that there is more than one DNA binding site. Those wishing to conduct further DNA binding experiments will need to optimize the probe concentration range according to their own experimental objectives.

II. Protein: Protein Interactions

The optimal conditions for studying protein: protein interactions on the array will vary according to the protein being studied. For example, the optimal concentration of protein probe will depend on the affinity of the interaction with arrayed proteins.

To demonstrate specific binding of a protein to the p53 array, the protein may be labeled directly with a fluorescent dye or detected indirectly using a labeled antibody to the probe protein. It is essential that experimental conditions are optimized for each protein. However, the following protocol provides a guideline for using a fluorescent dye to label the protein probe. **Please note that the following protocol is intended as a guideline only.**

a. Proteins can be labeled using commercially available fluorescent dyes e.g. CyDye™ and following the manufacturer's recommended protocols. In brief, the protein is dissolved in a buffer which is compatible with amine labeling reactions and with protein stability. Optimize the conditions to obtain the correct protein labeling ratio (1-2 molecules of dye per molecule of protein). Perform the incubation on ice to reduce protein degradation. Following labeling, separate the labeled protein from free dye using gel permeation chromatography using commercially available pre-packed columns (e.g. PD10 columns, Amersham Biosciences).
b. Check the concentration of the protein using spectroscopy.
c. Store labeled proteins frozen if compatible with protein stability. Aliquot labeled protein and thaw only once.

d. Dilute the labeled protein probe to the required concentration in a buffer which is compatible with both the probe and the protein array targets. The DNA assay buffer, pH 7.6 (Appendix C) is a suitable starting point for buffer development. Include 20% glycerol, 0.1% TRITON™ X-100, 0.1% BSA and 1 mM DTT in new buffers to aid protein stability. Optimize the probe concentration for each assay and protein.
e. Wash protein arrays immediately prior to assay. Remove the storage buffer by washing in an excess of assay buffer (25 ml) and shaking on an orbital shaker at ~80 rpm for 5 minutes to remove the storage buffer.
f. After washing, blot the slides along one edge onto lint-free tissue paper to remove excess liquid.
g. Dry the back of the slide with lint-free tissue paper.
h. Lay the protein arrays flat on a plastic tray and pipette diluted labeled probe onto the center of the slide (50 µl of probe is sufficient when using cover slips). Lower a cover slip gently onto the array surface using fine forceps (see General Recommendations). Ensure that no air bubbles are trapped. **Do not** move the cover slip once in place as this can lead to displacement of the sample. If the recommended cover slips are used then any trapped air bubbles will generally disappear. Where the availability of labeled probe is not limiting, assays can be performed in larger volumes (5-25 ml) using incubating dishes or tubes.
i. Perform the assay in a cold room or refrigerator. Carry out wash steps on ice. Orbital shaking at ~80 rpm is recommended for large volume assays to improve mixing, but is not helpful for assays carried out under cover slips.
j. Perform all incubations using fluorescent dyes away from direct light.
k. Incubate arrays for 30 minutes to several hours depending on the assay, and optimize this step if necessary.
l. After incubation, remove cover slips gently with forceps being careful not to slide the cover slip over the array surface. For large volume assays, remove the arrays from the assay solution with forceps.
m. Place the arrays in a plastic container (Pap jar) with 25 ml of ice cold assay buffer. Invert the container several times to mix the contents and then place the tube in a container of ice and shake for 5 minutes on an orbital shaker at ~80 rpm. Pour off the buffer and repeat the washing step twice with fresh ice cold buffer. Wash the slides briefly with ice cold water to remove buffer salts if this is compatible with the assay.
n. After the final wash, remove the arrays from the buffer and place them in 50 ml disposable centrifuge tubes **with the label positioned at the bottom of the tube.** Centrifuge at 4 °C for 2 minutes at 240 x *g*.
o. Carefully remove the slides from the centrifuge tubes with a pair of blunt ended forceps touching only the end of the slide.

p. Scan the slides using a microarray scanner or imager with the appropriate excitation and emission wavelengths for the fluorophore being used.

III. Post-translational Modification

p53 proteins may be modified enzymatically on the array and detected using labeled antibodies specific for a particular modification. It may also be possible to perform functional assays following protein modification.

TROUBLESHOOTING GUIDE

I. Signal is very low over the entire array after the DNA binding assay

Check that incubation times have been followed correctly and that wash steps have been carried out with ice cold assay buffer.

Always carry out the final wash in 70% ethanol. Failure to do this can lead to incomplete drying of the array allowing DNA to dissociate away from the p53 spot.

Check the position of the cover slip during incubation with DNA to avoid leakage of solution from the array surface.

Do not leave arrays in buffer or 70% ethanol wash. Dry immediately by centrifugation after the final wash.

Re-scan the array at a higher laser power or PMT voltage.

II. Signal is very low over the entire array after other assay types

The fluorescent labeling of the molecule used as probe may not have been efficient. Check the degree of labeling of the probe (1-2 molecules of dye per protein) and repeat the labeling or use a higher amount of probe.

The assay conditions used may be sub-optimal for any **new** interaction or reaction under study. Optimize the conditions as appropriate including blocking conditions, assay buffer, assay incubation time and temperature.

III. Signal for some spots is very low

Ensure that no air bubbles are trapped under the cover slip during the assay.

The presence of p53 in the spot may be confirmed using the Cy5-anti-p53 antibody provided in this kit.

IV. Signal between the two sub arrays is different

Ensure that the assay solution evenly covers the entire array surface.

V. Streaking (high signal) is observed on the array after scanning

This can occur when slides are dried by centrifugation by placing the slides in the centrifuge tube so that the array label is at the top. Always dry the slides with the label at the bottom of the tube.

VI. Spots on the array appear scratched

Ensure that cover slips are not dragged across the array surface.

VII. Binding is observed on the negative control spots

Further blocking before and/or during the assay may be required to prevent non-specific interactions for some assays. Blocking is assay and sample dependent. Try using commonly used blocking agents, e.g. 5% non-fat milk or increased concentrations of BSA.

VIII. The background is speckled

Use clean powder free gloves when handling the arrays.

Ensure assay containers are clean and free of fluorescent contaminants.

Ensure that buffers are prepared freshly before use.

Apply de-speckling algorithms present in most commercially available microarray analysis software packages (note: this does not materially affect the data generated).

IX. The background signal is high over the entire surface

Further blocking before and/or during the assay may be required to prevent non-specific interactions. Blocking is assay and sample dependent. Try using commonly used blocking agents e.g. 5% non-fat milk or increased concentrations of BSA.

X. There are localized patches of high background on the slides

Ensure that arrays are completely covered with buffer during wash steps.

Ensure that arrays are not allowed to dry out during assay or processing.

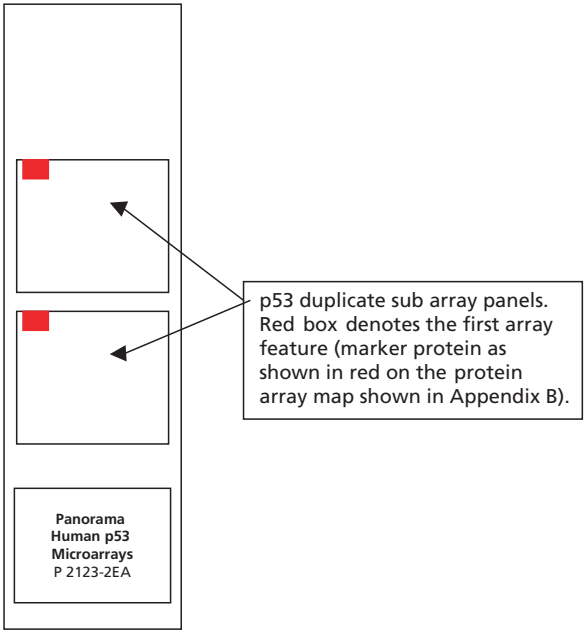
QUALITY CONTROL

The p53 variants included on the protein function array are produced from fully sequence verified clones.

A variety of QC tests are performed to check the performance of the p53 protein function arrays. Each slide is inspected visually for imperfections that are known to affect experimental results. Each batch undergoes tests that measure the performance of the variants on the array including the functional Cy3-GADD45 DNA binding assay and the Cy5-anti-p53 antibody binding assay.

APPENDICES

Appendix A: Protein Array Orientation



Appendix B: p53 Protein Array Map and Key

Layout of spot features for each sub array

M	M	WT	WT	W23A	W23A	W23G	W23G	R72P	R72P	P82L	P82L	M133T	M133T	M	M
Q136X	Q136X	C141Y	C141Y	P151S	P151S	P152L	P152L	G154V	G154V	R175H	R175H	E180K	E180K	R181C	R181C
R181H	R181H	H193R	H193R	R196X	R196X	R209X	R209X	R213X	R213X	P219S	P219S	Y220C	Y220C	S227T	S227T
H233N	H233N	H233D	H233D	N235D	N235D	N235S	N235S	S241F	S241F	G245C	G245C	G245S	G245S	G245D	G245D
R248W	R248W	R248Q	R248Q	I251M	I251M	L252P	L252P	T256I	T256I	L257Q	L257Q	E258K	E258K	L265P	L265P
V272L	V272L	R273C	R273C	R273H	R273H	P278L	P278L	R280K	R280K	E286A	E286A	R306X	R306X	R306P	R306P
SB	SB	Control	Control	G325V	G325V	R337C	R337C	L344P	L344P	S392A	S392A	LB	LB	SB	SB
M	M	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB	SB	M	M

Key	
M	Cy3 BSA marker
WT	Wild-type p53
SB	Spotting buffer only
LB	Lysis buffer only
Control	Immobilization tag only

For each p53 variant, the first letter and the number denote the amino acid identity and its position in the primary sequence of wild-type p53. The final letter denotes the amino acid replacement in each variant. X denotes a truncation in the sequence at this position.

Description of p53 Variants

The following sections describe the different p53 variants featured on the array, and their behavior in the DNA binding assay supplied with this kit. Databases relating to p53 mutations can be found in references 2 and 3.

Mutations Within the p53 Core DNA Binding Domain

Many of the mutations in the p53 core DNA binding domain result in reduction of DNA binding activity.

Examples include:

- Mutation of residues that directly interact with DNA (R^{248} and R^{273})
- Mutation of residues that do not directly interact with DNA, but affect DNA binding by conformational effects (R^{175} and G^{245})

Not all mutations in the core domain result in the same reduction of DNA binding activity.

The array also includes a number of naturally occurring mutations that result in truncations at the C-terminus. Four of these occur within the core DNA binding domain (Q136X, R196X, R209X, R213X) resulting in loss of DNA binding activity.

Mutations Outside the p53 Core DNA Binding Domain

A number of variants have been included with mutations in other regions of the p53 protein.

Examples include:

- Mutation of residues involved in p53 tetramerization (R337C and L344P)
- Mutation of a residue that results in truncation of p53 so that the core DNA binding domain is present, but the tetramerization and C-terminal domain are lacking (R306X)

Control p53 Variants

The control variants mutations include:

- A widely distributed polymorphism (R72P).

Three other p53 variants which are not naturally occurring or known to be cancer-associated involving:

- Mutation of residues that directly interact with MDM2 (W23A and W23G)
- Mutation of a residue that can be modified by phosphorylation (S392A)

Appendix C: Recommended Buffers

Assay buffers may be unsuitable for assays other than those for which they were optimized.

DNA Assay Buffer

Prepare freshly before use.

25 mM HEPES, pH 7.6 with 200 mM KCl
1 mg/ml BSA
20% (v/v) glycerol
0.1% (v/v) TRITON X-100

Before use, add fresh dithiothreitol to DNA Assay Buffer at a final concentration of 1 mM.

PBS-TWEEN Washing Buffer

Phosphate buffered saline (PBS), pH 7.4 with
0.1% (v/v) TWEEN 20

Antibody Binding Assay Buffer

5% milk powder
Phosphate buffered saline (PBS), pH 7.4
0.1% (v/v) TWEEN 20

REFERENCES

1. Boutell, J.M. *et al*., Functional protein microarrays for parallel characterization of p53 mutants. *Proteomics* **4**(7), 1950-1958 (2004).
2. Olivier, M. *et al*., The IARC TP53 Database: new online mutation analysis and recommendations to users. *Hum Mutat.* **19**(6), 607-614 (2002).
3. Sedlacek, Z. *et al*., A database of germline p53 mutations in cancer-prone families. *Nucl. Acids. Res.* **26**, 214-215 (1998).

TRADEMARKS

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2. TWEEN™ is a registered trademark of Uniqema, a business unit of ICI, Americas, Inc.
3. CyDye™ is a trademark of Amersham Biosciences.

RELEVANT PATENTS

1. WO 01/57198. Methods Of Generating Protein Expression Arrays And The Use Thereof In Rapid Screening
2. WO 02/27327. Rapid Profiling Of The Interactions Between A Chemical Entity And Proteins In A Given Proteome
3. WO 03/048768. Protein Arrays For Allelic Variants And Uses Thereof
4. WO 03/064656. Protein Tag Comprising A Biotinylation Domain And Method For Increasing Solubility And Determining Folding State

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