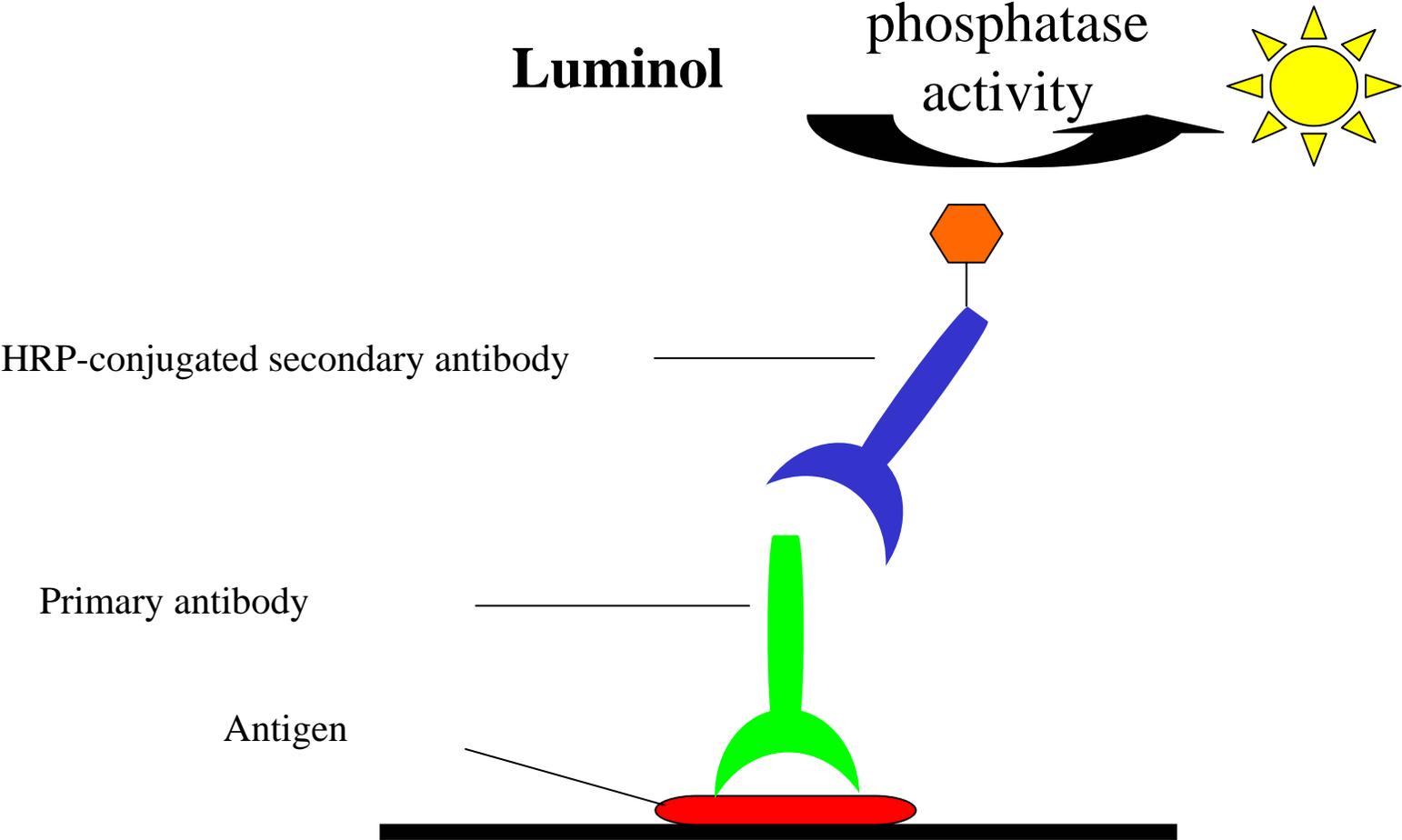


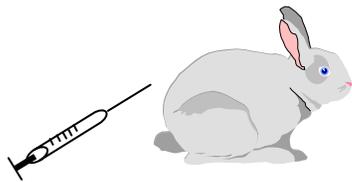
Immunodetection

Trinity College, December 2007

Principles of Immunodetection



Raising antibodies



rabbit anti- antigen Ig

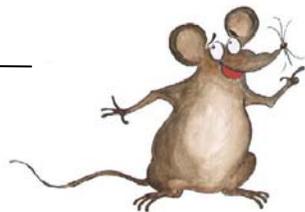
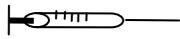
Antigen in *Freunds* adjuvant



donkey anti- rabbit Ig

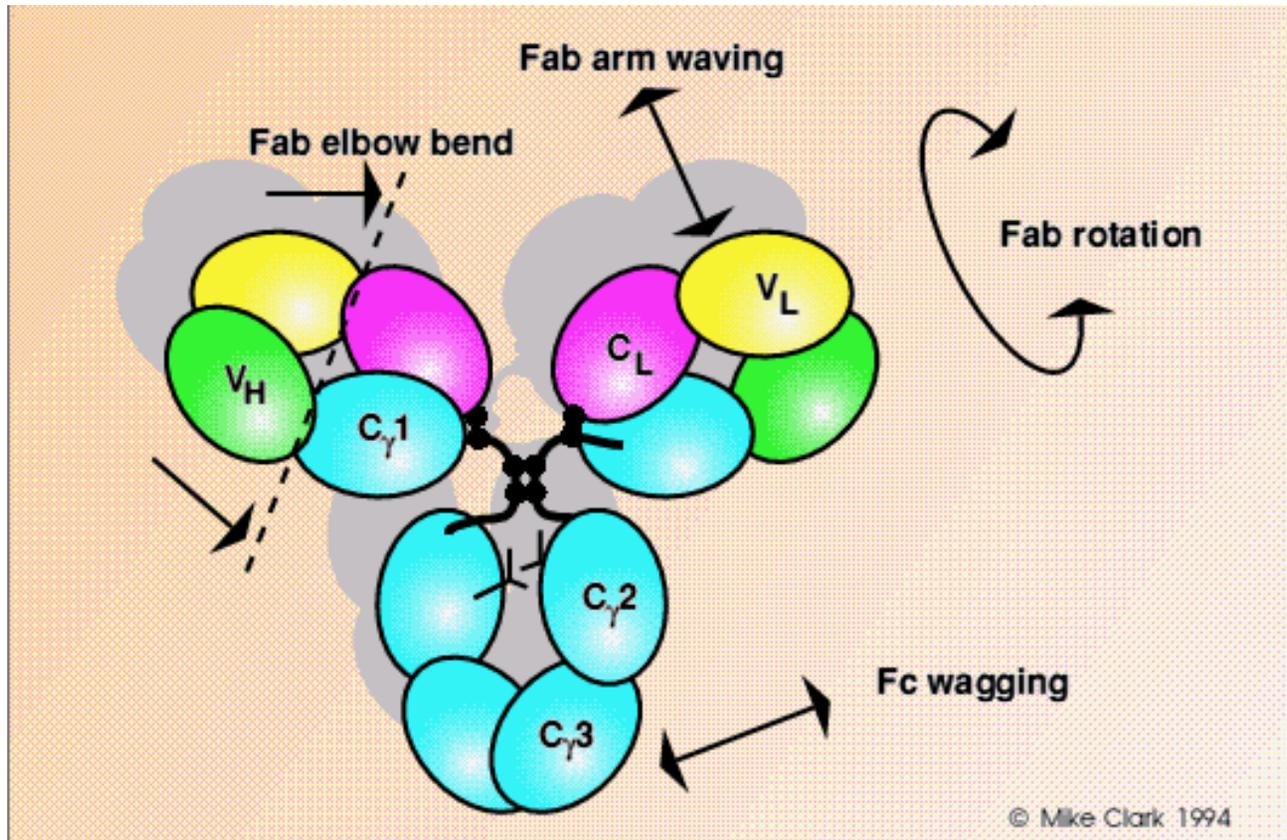
} Polyclonal antibodies

Antigen



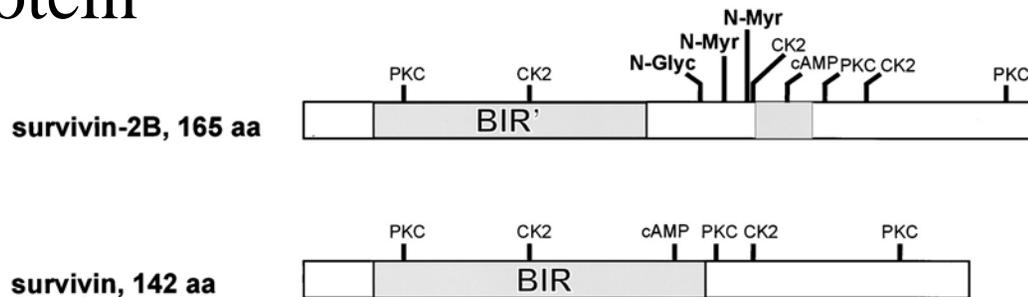
hybridoma secretes mAb monoclonal antibodies

Structure of an immunoglobulin



Deciding which antibody

- Antibody raised against which portion of the protein

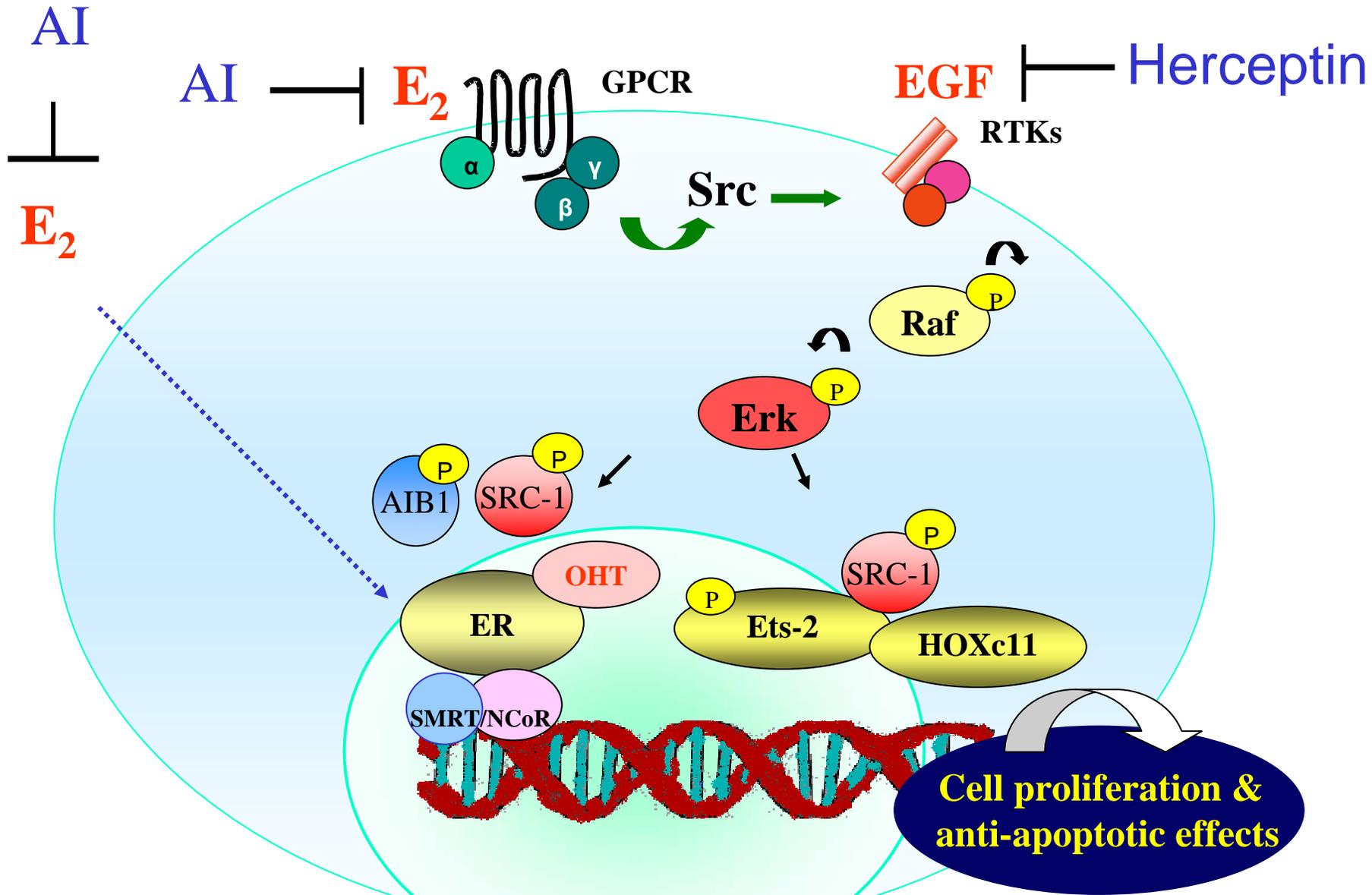


- Monoclonal or polyclonal
- Species cross reactivity
- Host animal

Application of Immunodetection

- Western Blotting
- Co-immunoprecipitation
- Immunohistochemistry
- ELISA
- Antibody Array/Protein Arrays
- Immunofluorescence/*in-situ* FRET
- EMSA super-shifts
- ChIP

Cell signalling in breast cancer

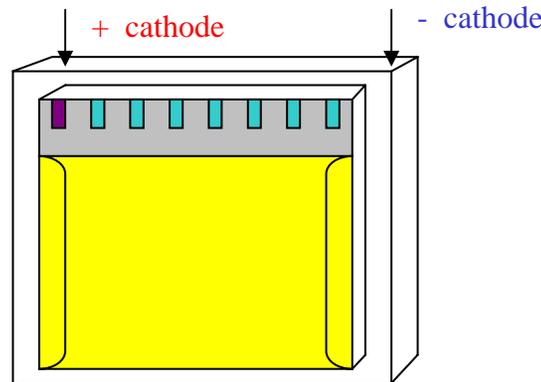


Western Blotting

- Protein extraction
 - Total protein – standard lysis buffer
 - Nuclear cytoplasmic extraction (NER-PER, extraction kit, Pierce)
 - To prevent degradation always centrifuge samples at 4⁰C and include a protease inhibitor eg Pefabloc (Roche diagnostics)
- Protein Quantification
 - Standard BCA assay (Pierce)

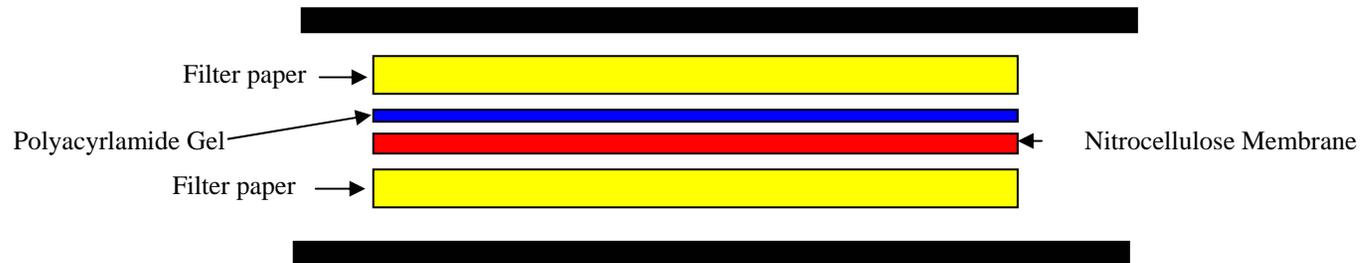
Electrophoresis

- SDS-PAGE carried out with discontinuous buffer system
 - Sample and stacking gel Tris-Cl (pH6.8)
 - Resolving gel Tris-Cl (pH8.8)
 - Buffer reservoirs Tris-glycine (pH8.3)



Transfer of Proteins to filter

Transfer of proteins from gel to filter can be
Semi-dry (ATTO)
Immersion (Bio-Rad)



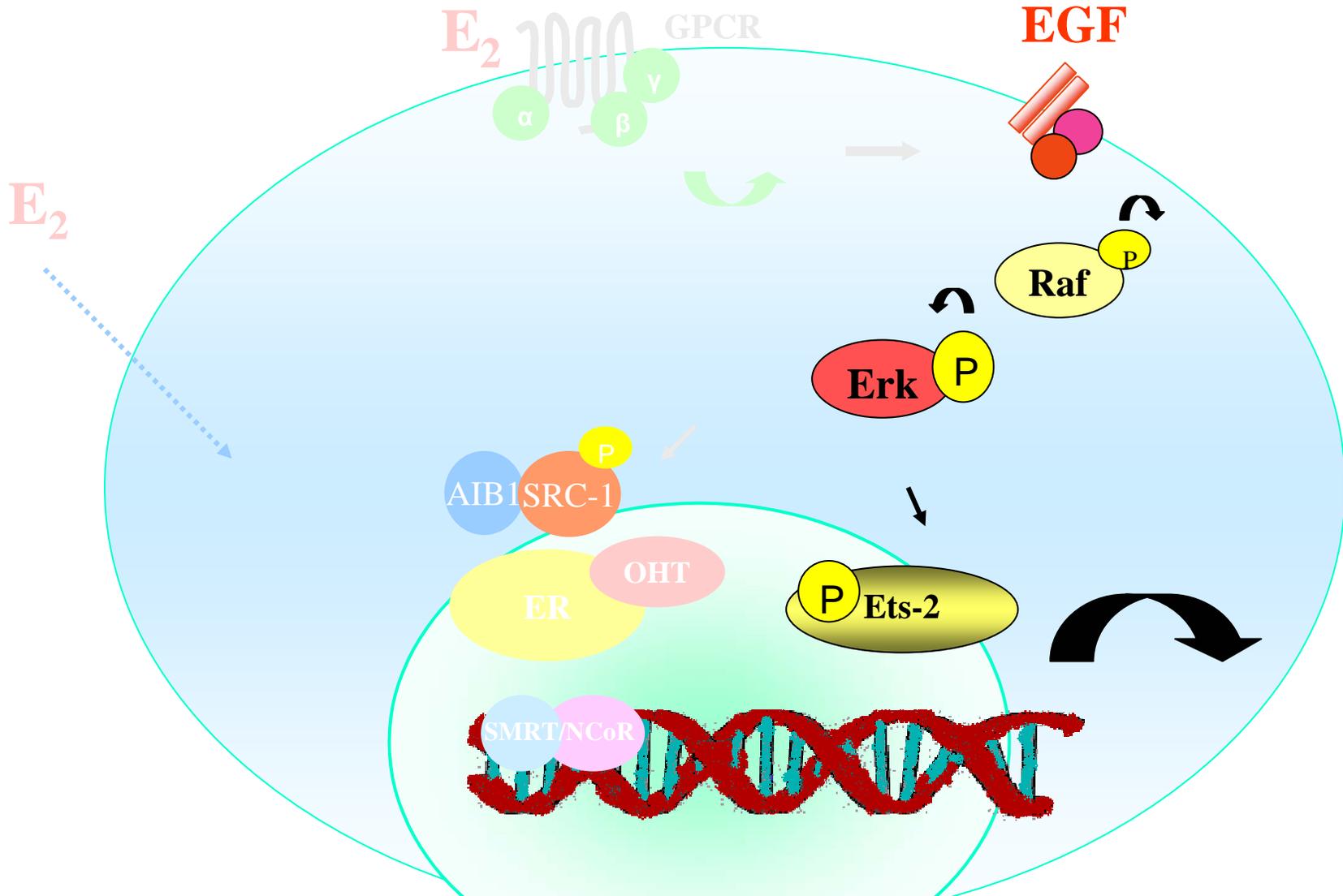
Types of Membranes

- Nitrocellulose (pore size $0.45 \mu\text{m}$) – standard membrane
- Nylon membranes – Binds protein with greater affinity, high background
- Polyvinylidene fluoride (PVDF) – high affinity binding, pre-wash with methanol
 - Immobilised protein can be visualised with ponceau S (not nylon membranes)

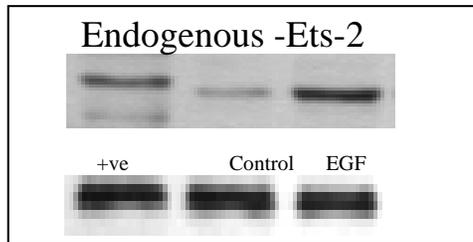
Immunoblotting Variables

- Blocking membranes – % Block and incubation time
- Primary antibody concentration and time: 1 hour @ room temperature; over-night @ 4⁰C
- Secondary antibody concentration
- Detection: Chromogenic or chemiluminescent

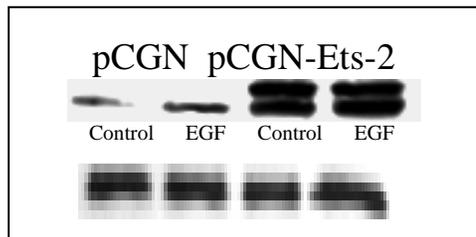
Ets-2 expression in breast cancer



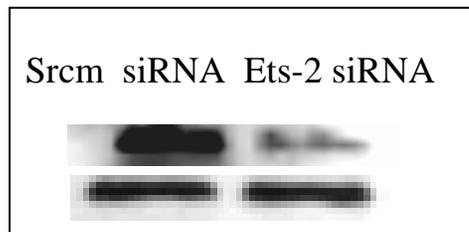
Ets-2 expression in breast cancer cells



Endogenous Ets-2



Over-expression Ets-2



Knockdown Ets-2

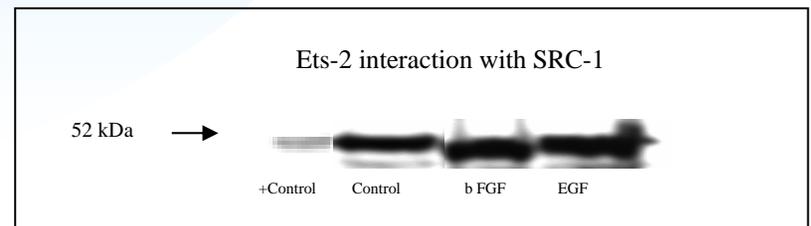
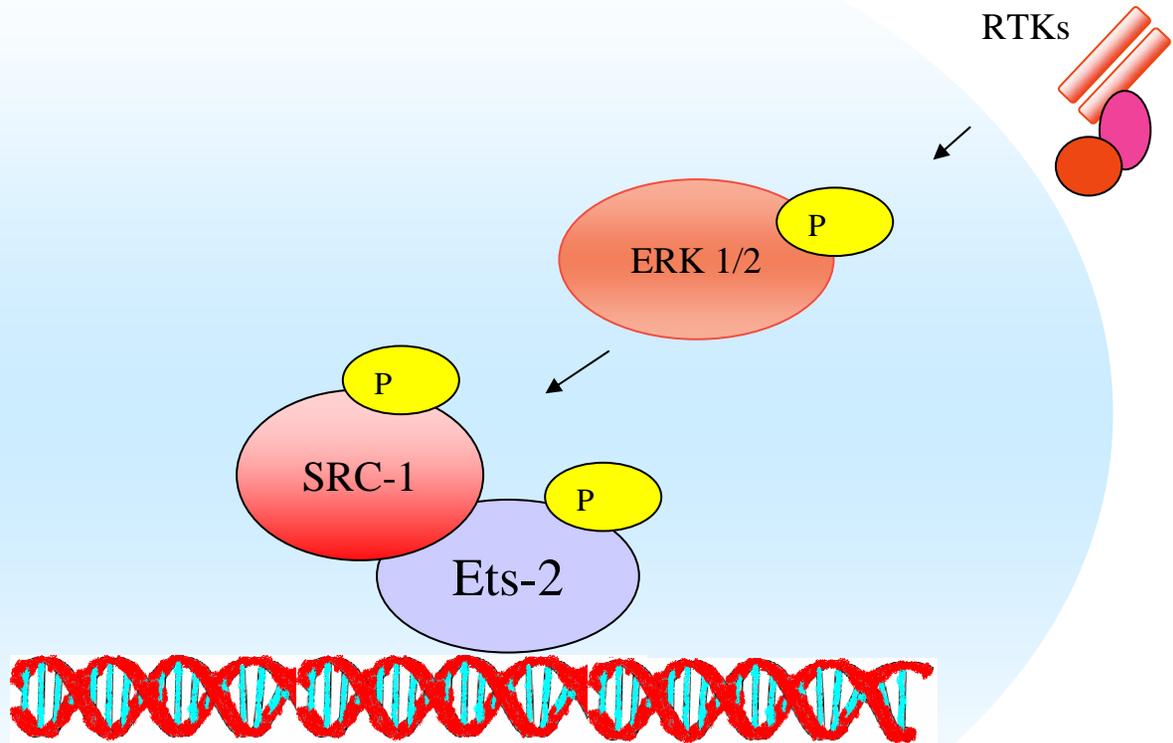
Immunoprecipitation

- Antibody directed against a particular antigen is added to cell lysate forming an antibody-antigen complex along with lysis buffer
- Protein A/G agarose complex is added to the lysate, binding the antigen-antibody complex and precipitating the complex out of solution.
- After centrifugation, non-specific protein will be removed in the supernatant.
- The complex is then washed to further remove any non-specific protein binding

Application of immunoprecipitation

- Co-immunoprecipitation is a process that allows the examination of a protein-protein interaction - precipitated out in the complex and detected by Western blotting
- Activity of immunoprecipitated proteins eg; acetylation assays, DNA footprinting

Protein-protein interactions



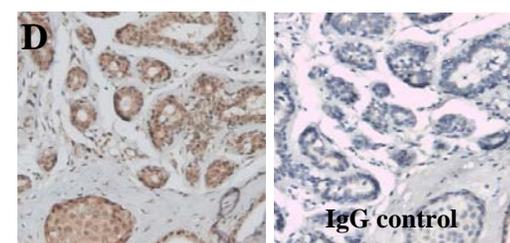
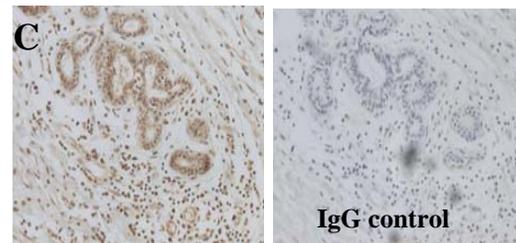
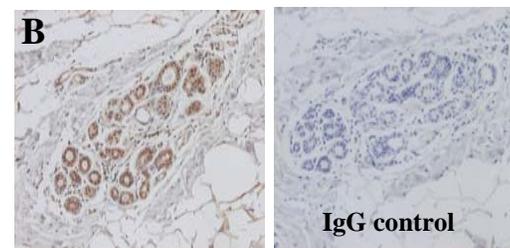
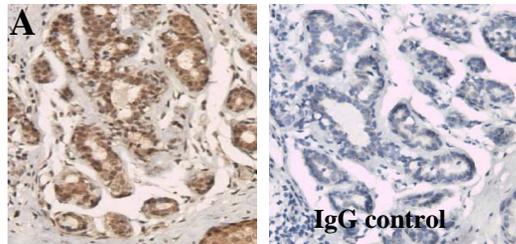
Immunohistochemistry

- Localisation of antigen within
 - Cellular and intracellular localisation
- Qualitative expression in patient populations; correlations with clinicopathological data

Immunohistochemical localisation

Ets-2

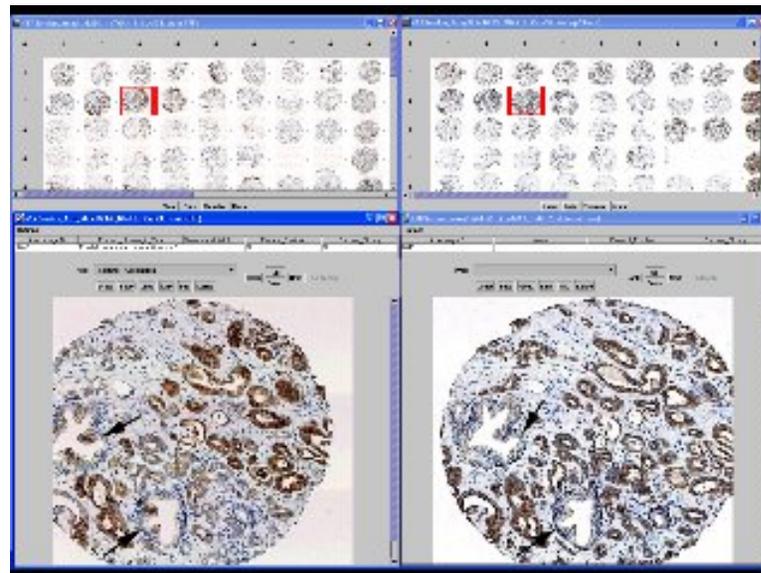
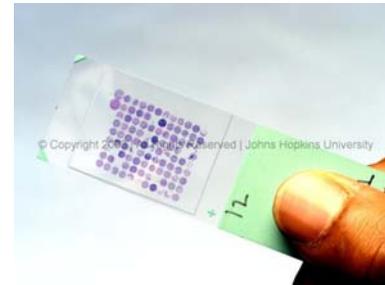
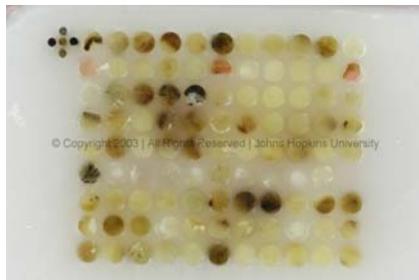
SRC-1



Immunohistochemistry variables

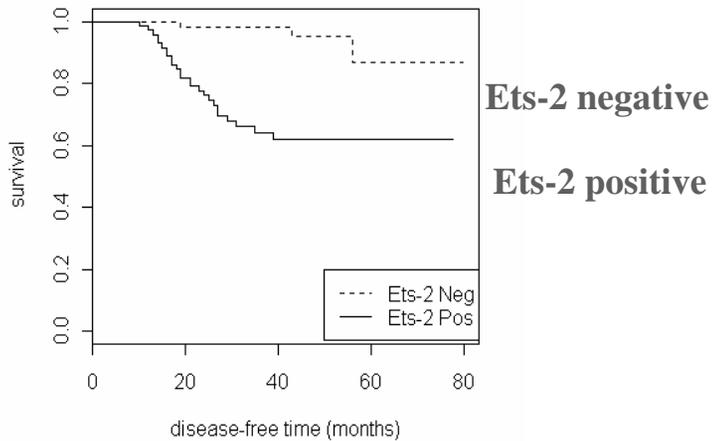
- Cryosections ($\sim 7\mu\text{m}$) from frozen sections or paraffin embedded sections ($\sim 5\mu\text{m}$)
- Blocking: endogenous peroxidase H_2O_2 ; non-specific binding with sera from animal in which 2⁰ antibody raised in
- Concentration of primary antibody (1-5 μg) and incubation time
- 2⁰ antibody, peroxidase conjugated/biotinylated – detection with DAB (**brown**)
- Counter-stain with haematoxylin (**blue**)

Tissue Microarray

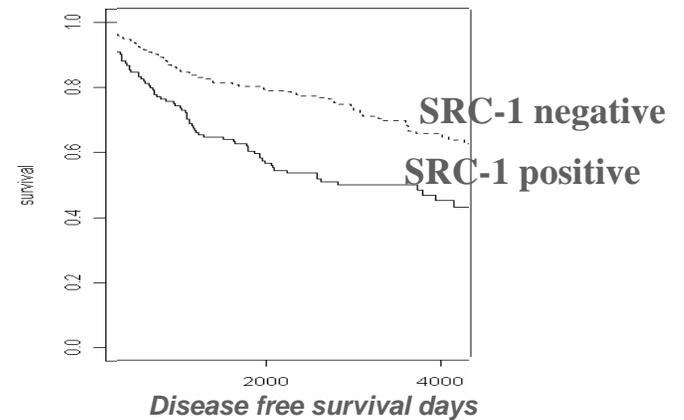


Expression of proteins in breast cancer patients

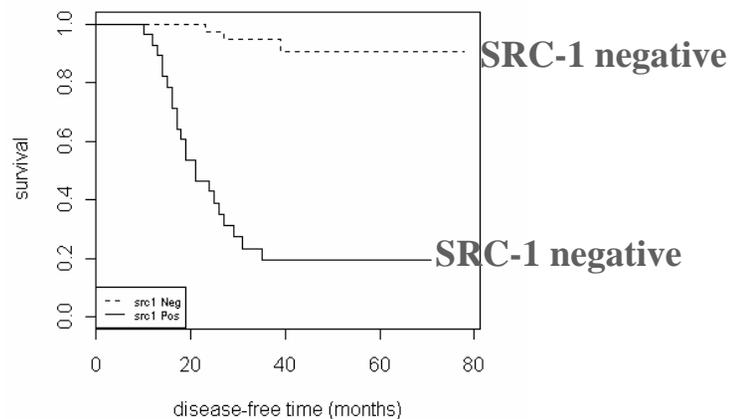
Ets-2



SRC-1



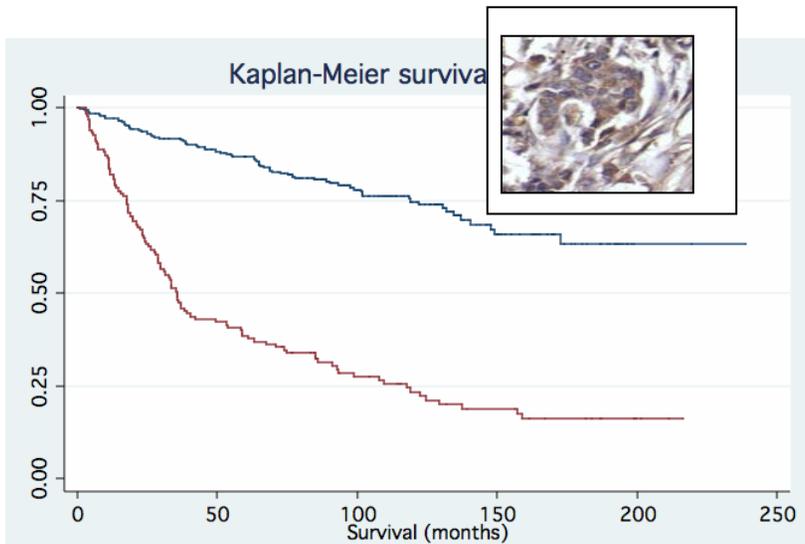
Ets2 positive



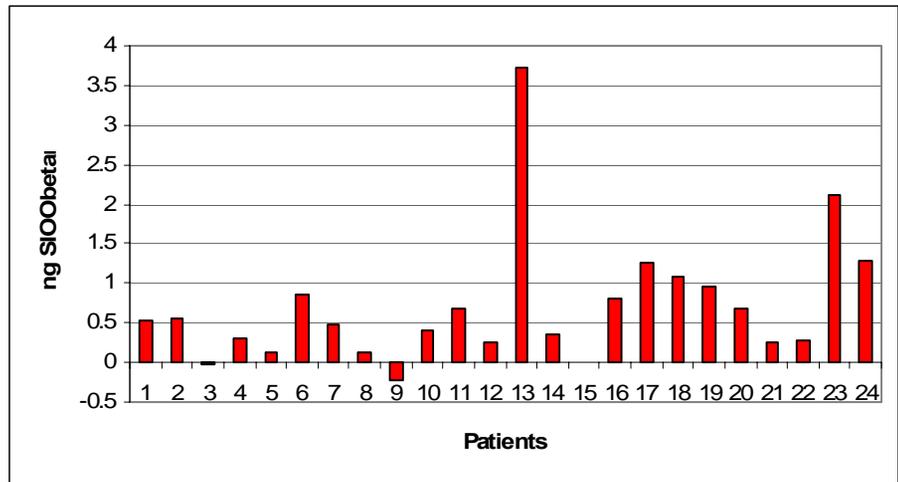
ELISA

- Enzyme-Linked ImmunoSorbent (ELISA) – detecting and quantitating substances such as peptides, proteins, antibodies and hormones
- Usually performed on a 96-well plate (Corning's/Nunc)
- Plate coated with capture protein (2-10 μ g/ml) in buffer Carbonate-Bicarbonate
- Incubated overnight at 4⁰C
- Non-specific sites blocked (1% BSA or Super block)
- Standards/samples added (50-100 μ l/well)
- Antibody directed against antigen of interest conjugated to enzyme HRP/AP
- Add substrate and detect

Detection of secreted protein in breast cancer patients



Immunohistochemistry on TMA



Elisa detection in patient sera

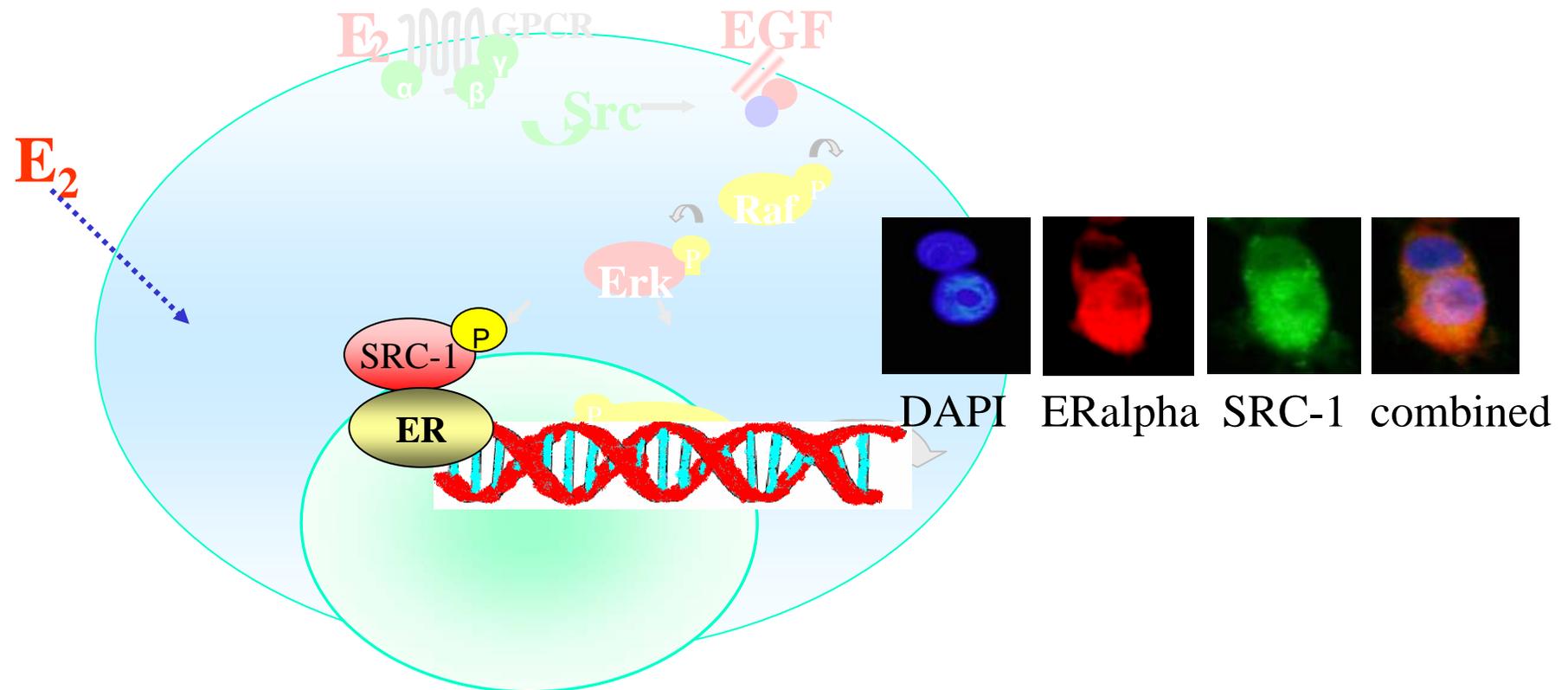
Immunofluorescence

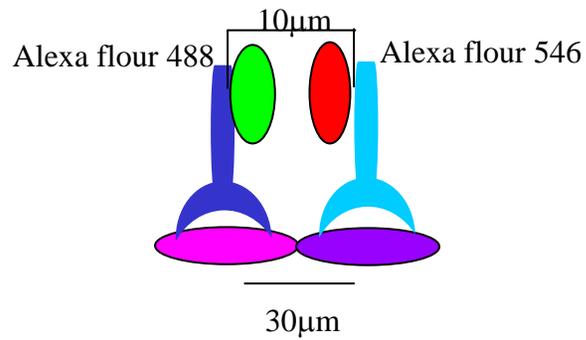
- Co-localisation of two different antigens within tissue
- Intracellular localisation of antigens

Immunofluorescence variables

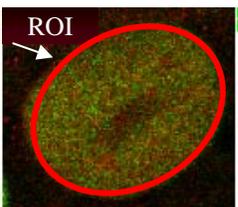
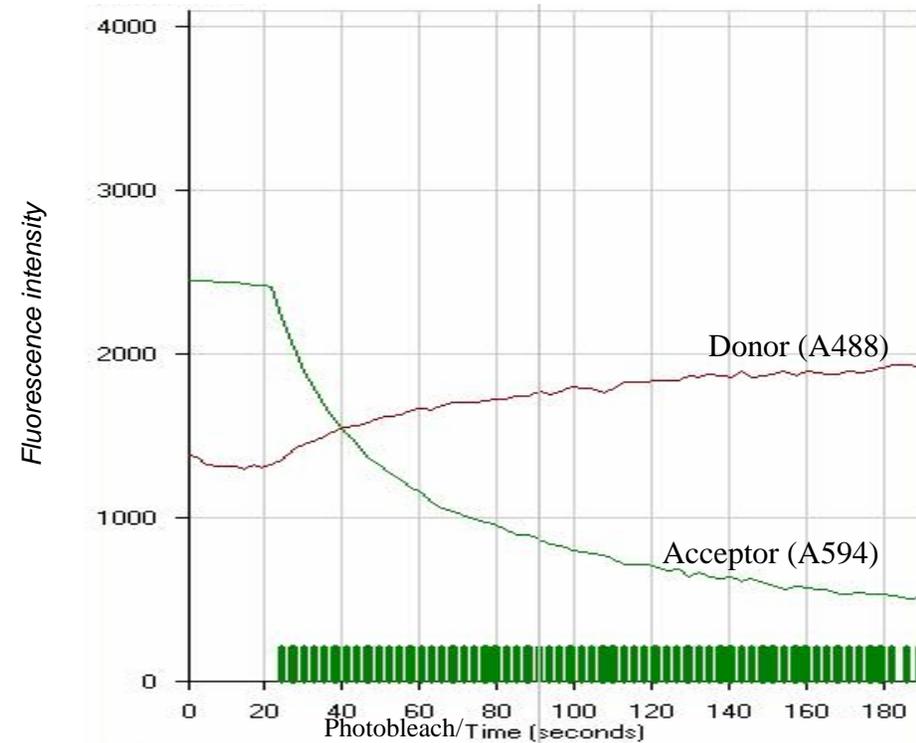
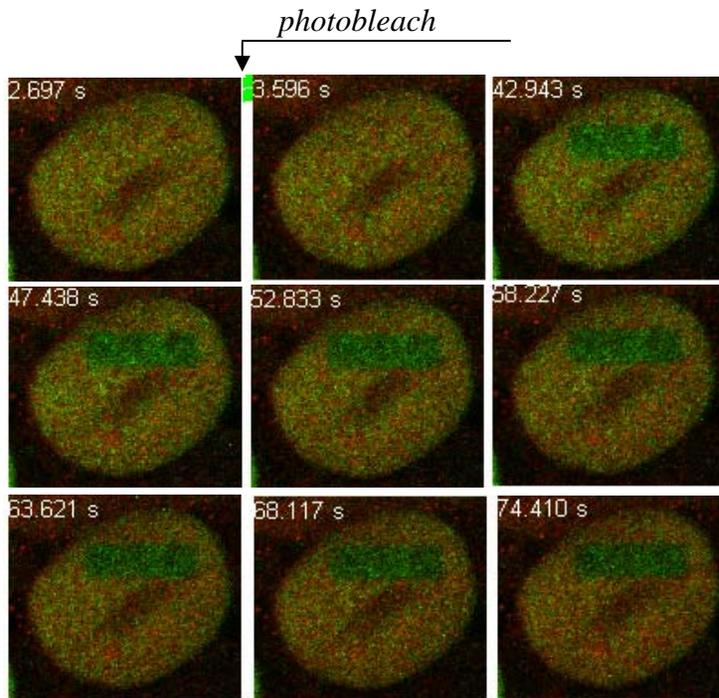
- Optimise staining first with immunohistochemistry
- Primary antibodies 10X concentration
- Different secondary antibodies with different fluoroscene label
- Sections mounted in DAKO mounting media, viewed under UV
- Sections can be mounted with DAPI, stains nuclei blue

Co-localisation of ERalpha and SRC-1



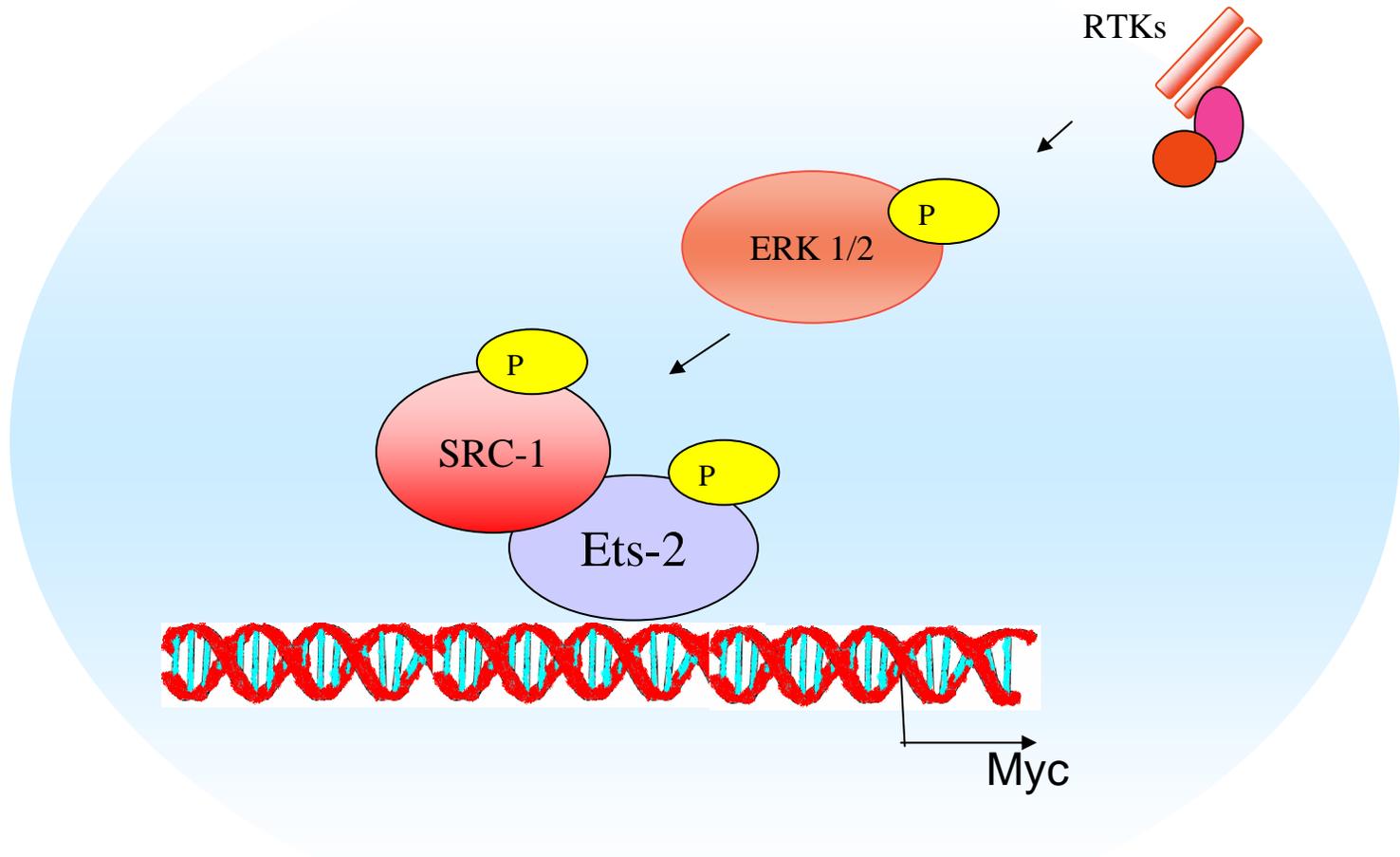


In-situ FRET

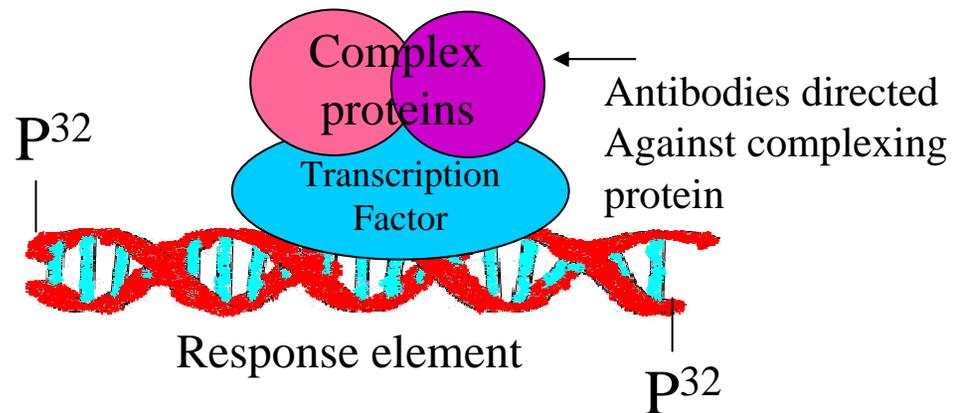
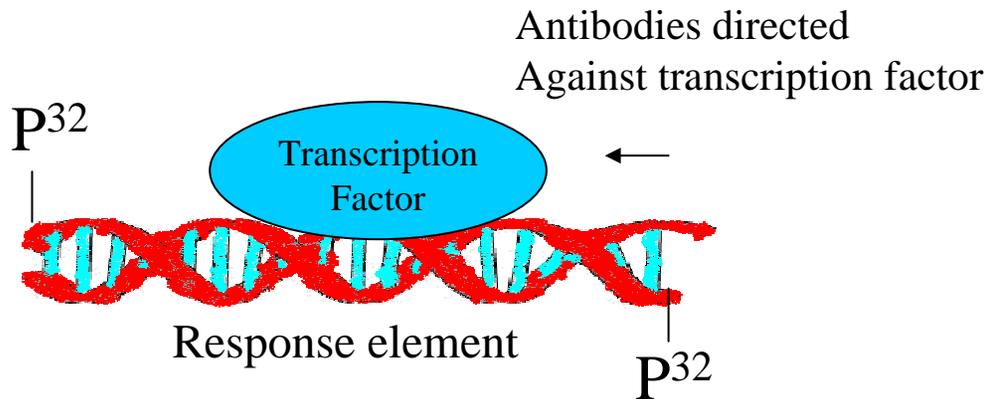
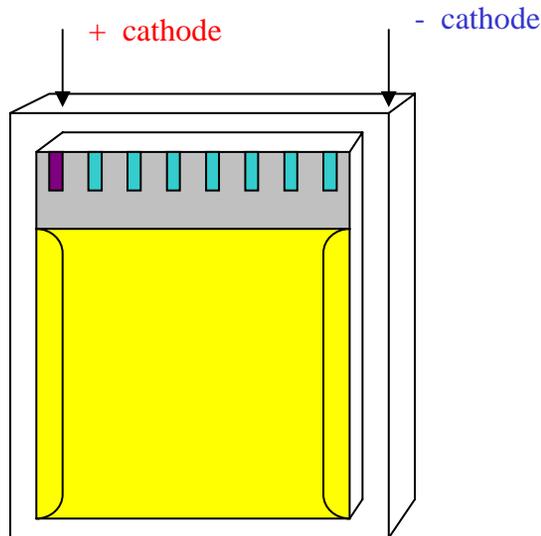
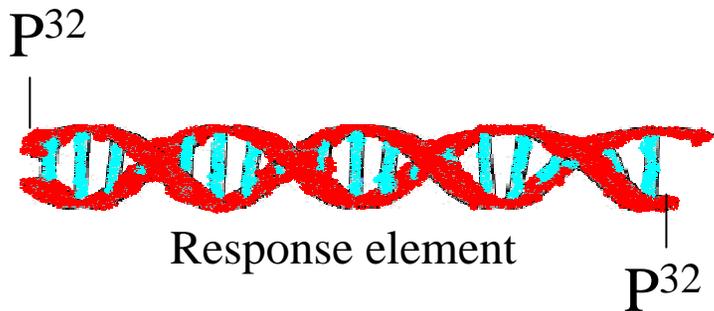


Area [$\mu\text{m} \times \mu\text{m}$]	Relative Area [%]	Mean Intensity 488	Mean Intensity 594	Standard Deviation 488	Standard Deviation 594	Colocalization Coefficient 488	Colocalization Coefficient 594	Weighted Coloc. Coefficient 488	Weighted Coloc. Coefficient 594	Overlap Coefficient	Correlation R	Correlation R x R
981.00	24.8	686	250	326	54							
21.26	0.5	341	475	37	75							
826.15	20.9	1920	1300	782	825	0.457	0.975	0.814	0.995	0.8	0.12	0.01

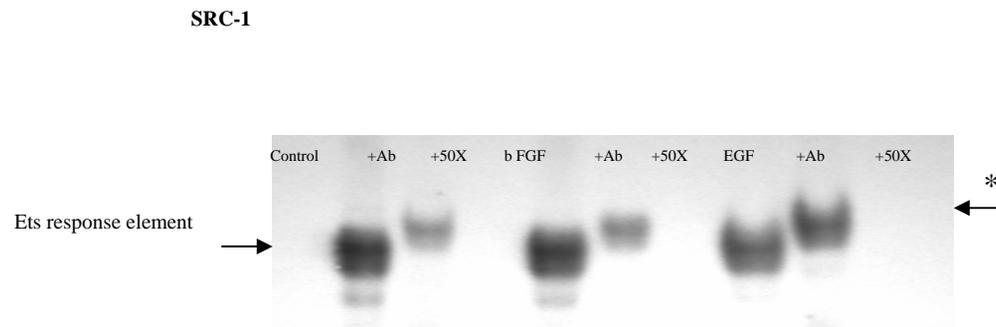
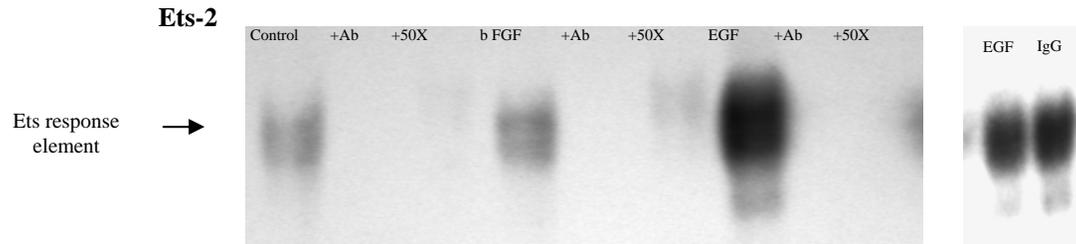
Recruitment of proteins to DNA



EMSA Super-shift



Ets-2 and SRC-1 recruitment to ets response element in breast cancer cells



Chromatin immunoprecipitation

- Allow detection of proteins at the DNA
 - Detection of transcription factors
 - Detection of co-regulatory proteins
 - Assessment of histone acetylation and deacetylation

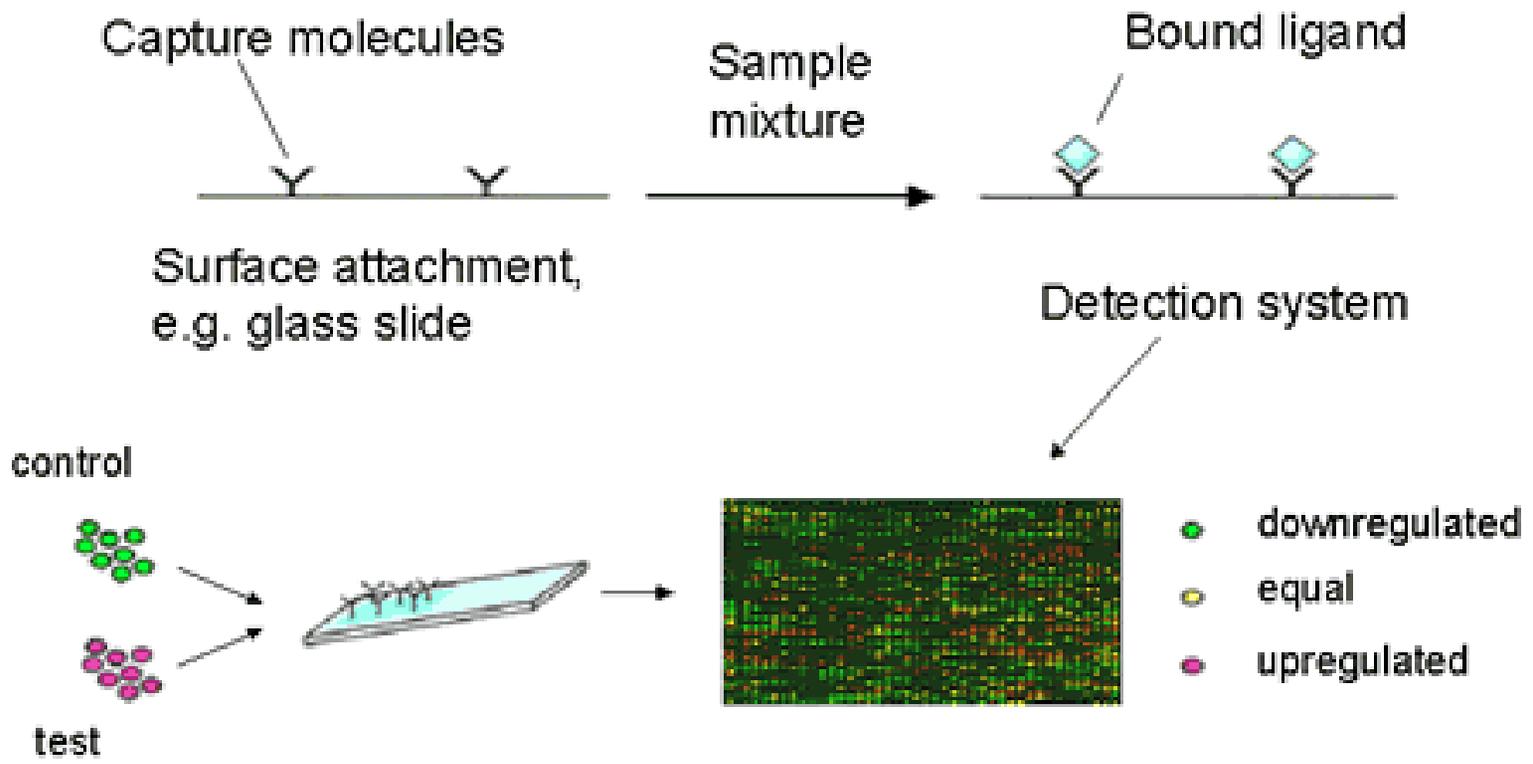
Antibody Array

- Efficient and sensitive high throughput protein analysis
- Antibody arrays are solid-phase ligand binding array systems using immobilised antibodies on surfaces (eg: glass)
- Direct or indirect sample labelling

Antibody Array

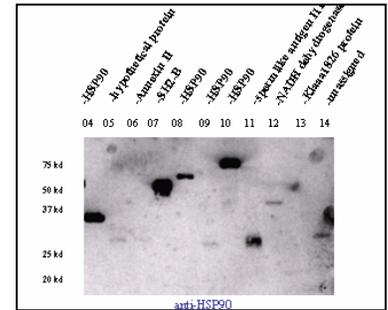
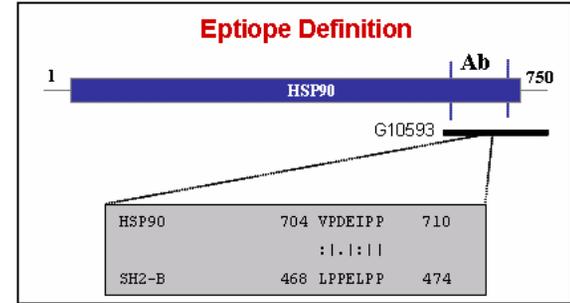
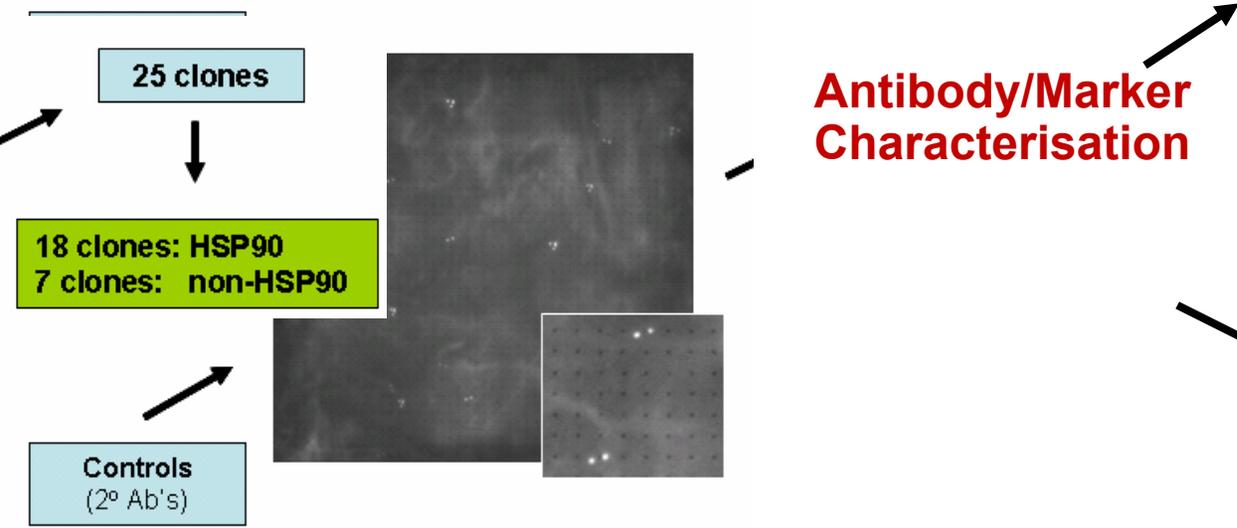
- Proteins from control and test samples are labelled with Cy3 or Cy5 respectively
 - Non conjugated free dye removed, spin columns
 - Labelled protein samples (test+control) are mixed and incubated on microarray
 - Slides are read for differential analysis- microarray scanning devices – ratio of 2 dyes ‘traffic light’ (red yellow green)

Antibody microarray principle

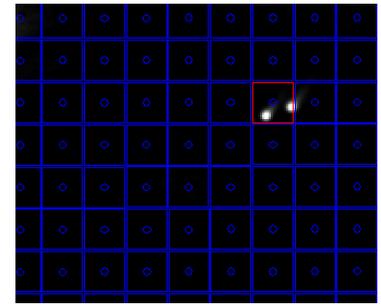
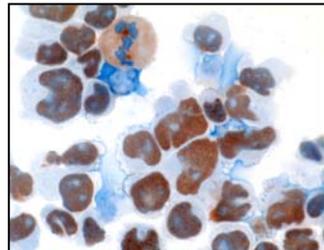


e.g. to compare protein expression, mix Cy3/Cy5 labelled samples

Protein Arrays

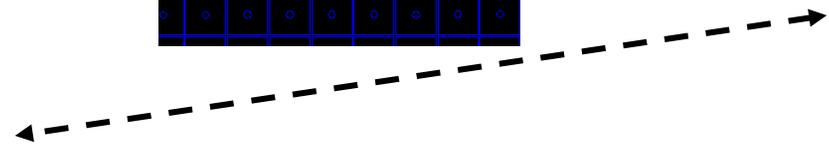


Marker 166
Nuclear fraction from Jurkat and JVM-2



Nuclear Mitotic Protein (1 Clone)

Promyelocytic Leukemia
t(11;17)(q13;q21)
NUMA-RARA



Identification of Disease-associated Autoantigens

Circulating Autoantibody (IgG) Response in Cancer (Lymphoma, Colon Cancer, Breast Cancer) Neurodegenerative Disease (Glaucoma)

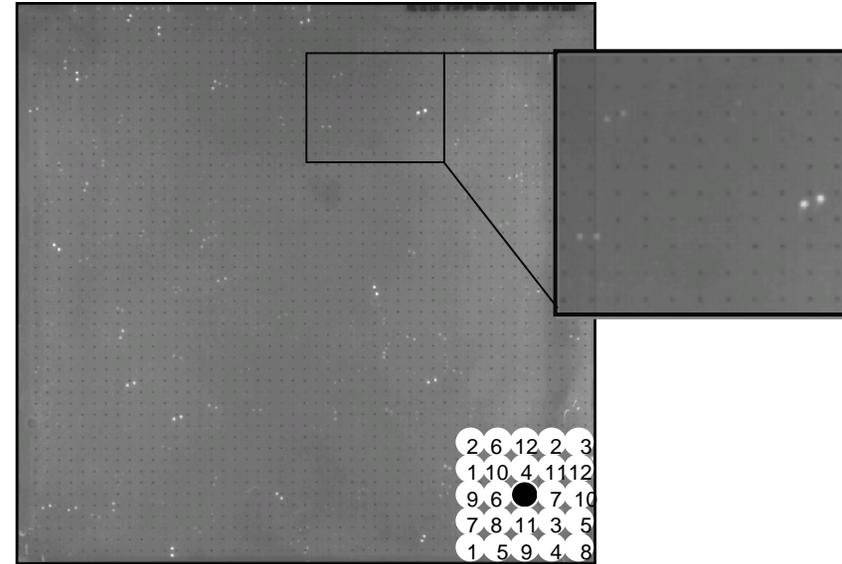
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Biomarkers (e.g. simple ELISA test)

Potential therapeutic targets

Understanding Molecular Mechanisms in Disease

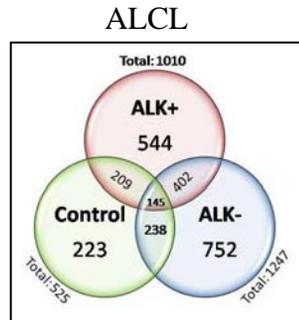
1. Screening (Serum, Plasma, CNS, etc.)



2. Reporting (Automated)

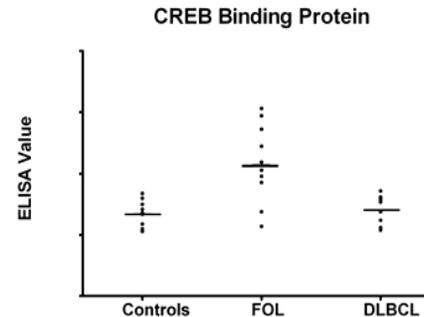
B-cell Lymphoma

Patients (%)	Controls (%)	Protein ID
72	0	Cyclin-dependent kinase inhibitor
76	20	PIP5K1 protein
60	0	6-phosphogluconolactonase
64	20	Exostosin family member
64	20	Nuclear mitotic apparatus protein
56	0	Nucleoside diphosphate-linked moiety
48	0	cAMP-regulated phosphoprotein
48	0	60S ribosomal protein LX
48	0	V-maf musculoaponeurotic fibrosarcoma oncogene
48	20	Nucleolar TGF-beta target protein
48	20	RAB11-like protein
44	0	Fas-associating protein
44	0	MAP7D1-like protein
44	0	Novel protein
44	20	Histone deacetylase
44	0	Huntingtin-interacting protein 1-related protein
44	20	Putative uncharacterized protein
40	0	Cyclin-dependent kinase associated protein
40	0	Islet cell autoantigen

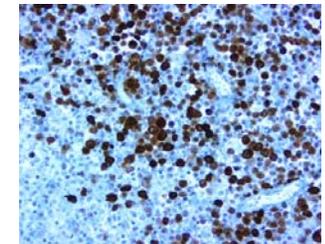


3. Validation

Reverse ELISA



IHC



ALK (ALCL)

Conclusions

To detect antigen of interest in your system choose carefully:

- Technique for detection
- Primary antibody
- Secondary antibody/conjugate
- Blocking
- Detection systems