

# ***Parallel techniques for the detection and identification of plant pathogens***

Neil Boonham



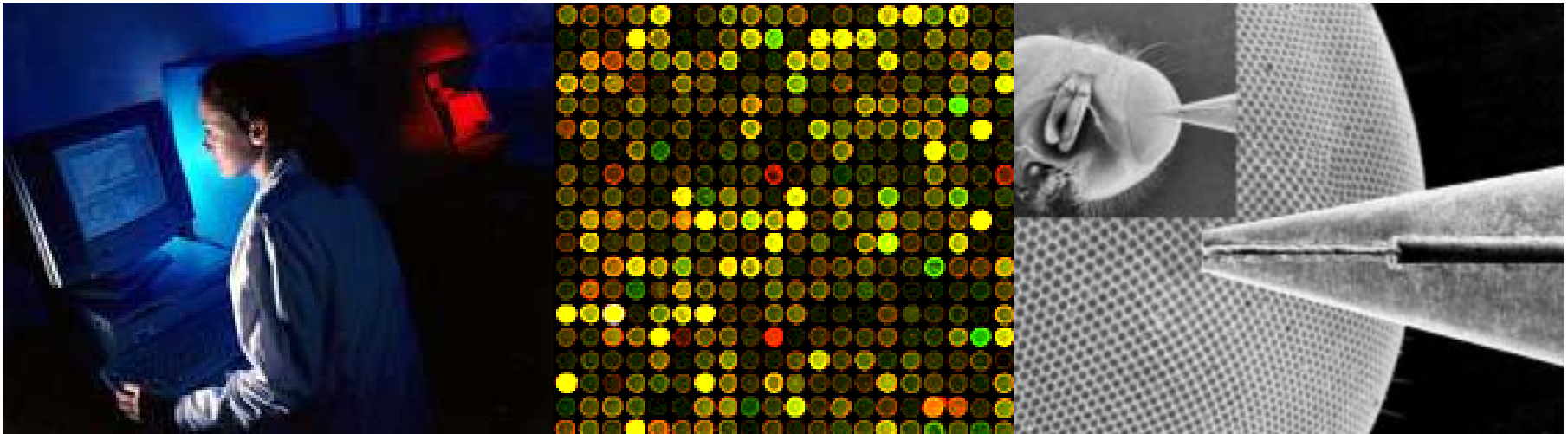
# *Introduction*

- ◆ Background – what is a parallel method ?
- ◆ Array techniques
- ◆ Labelling approaches
- ◆ Example of a potato pathogen array



# *Philosophy – two approaches*

- ◆ High throughput – surveys and monitoring
- ◆ Highly parallel – identification/unknowns



# *EU Plant Health Directive*

## *(2000/29/EC)*

- ◆ “requires that each member state needs to develop and maintain effective diagnostic provision for all the organisms listed”
- ◆ Estimated losses that might accrue in the absence of EU Plant Health controls in the potato crop would equate to some 464 M Euro p.a. for the whole community



# Objectives

- ◆ Methods can be
  - ◆ faster
  - ◆ cheaper
  - ◆ more accurate
  - ◆ more robust/reliable
  - ◆ widening scope
  - ◆ more generic



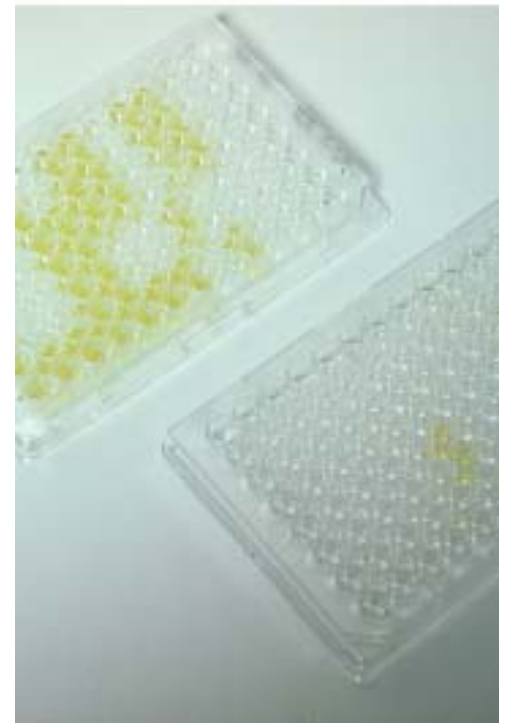
# *Examination of symptoms*

- ◆ Simplest method
- ◆ Inexpensive
- ◆ Potentially very rapid
- ◆ Extremely parallel
  - ◆ Fungal
  - ◆ Viral
  - ◆ Bacterial
  - ◆ Pest
  - ◆ Other problems
- ◆ Generic



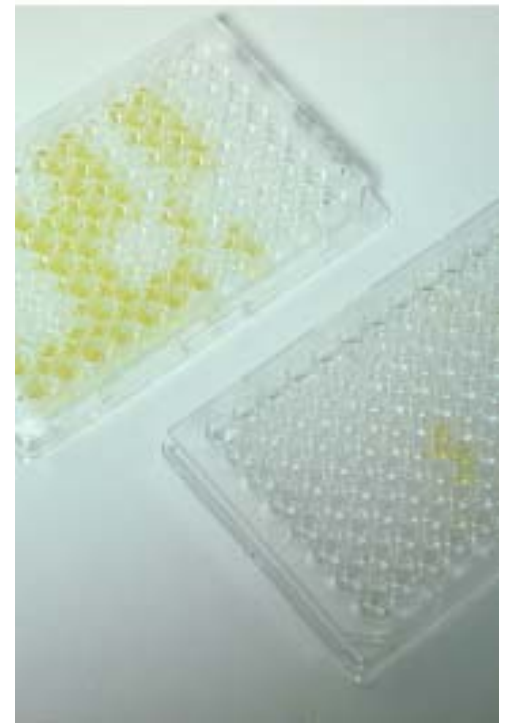
# *Immunological tests*

- ◆ Range of formats
  - ELISA
  - Gel diffusion
  - Lateral flow device
- ◆ Inexpensive per test
- ◆ Generic protocol
  - Different reagents
- ◆ Suited to large scale testing
- ◆ Quantitative



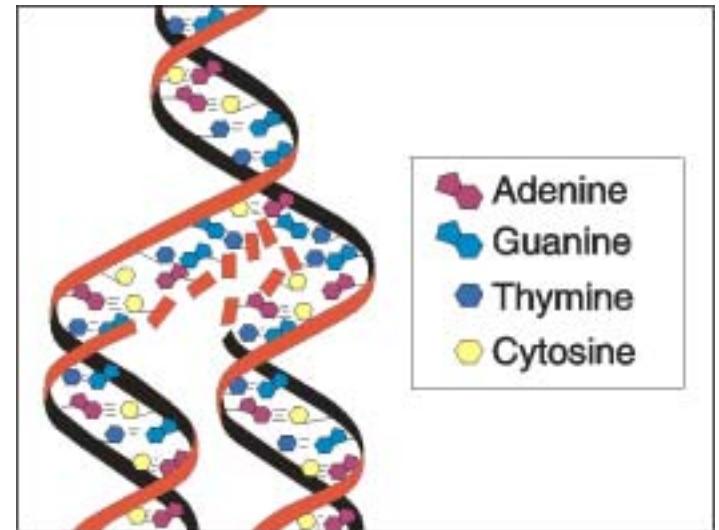
# *Problems with immunological tests*

- ◆ Detect proteins
  - not all pathogens have proteins
- ◆ Needs good quality antiserum
  - not available for all pathogens
- ◆ Lacks of sensitivity
- ◆ Not parallel



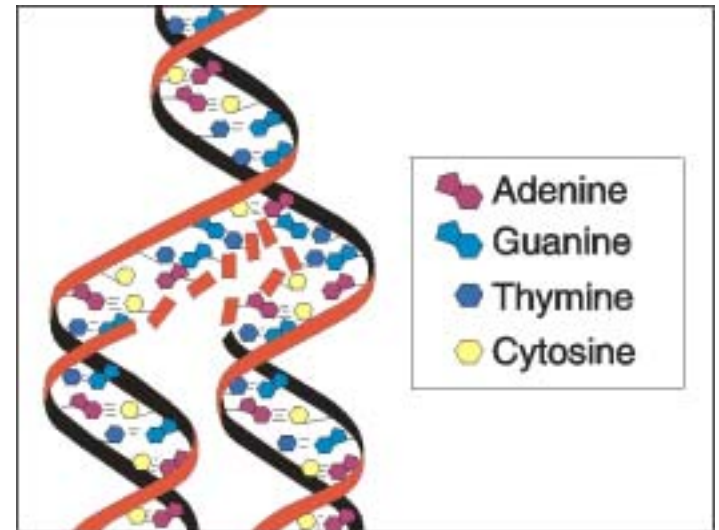
# *Molecular diagnostics*

- ◆ Based around the detection of nucleic acid
  - can be DNA or RNA
- ◆ Use of DNA methods are becoming more common in the diagnostics lab
- ◆ Generic nature
  - all organisms have DNA or RNA



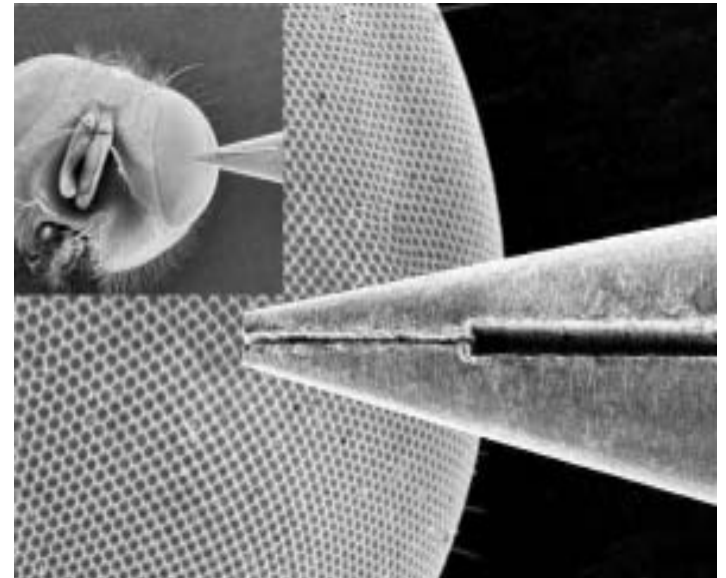
# *Molecular diagnostics*

- ◆ Techniques in common use
  - PCR (or variations of)
  - Real-time PCR
  - Hybridisation (dot blots etc.)
- ◆ Highly sensitive
- ◆ Highly specific
- ◆ Quantitative
- ◆ High throughput
  
- ◆ None are parallel



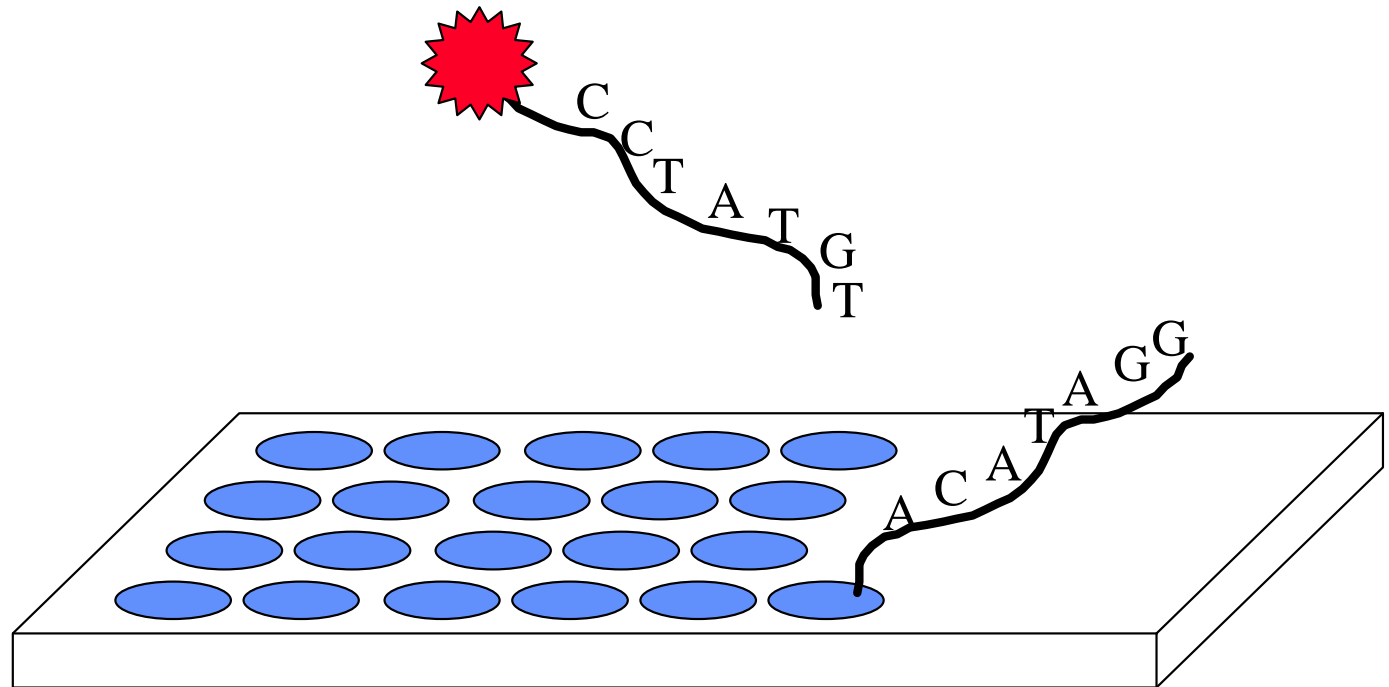
# *Micro-array technology*

- ◆ Post-genomics technology
- ◆ Highly parallel technique for expression profiling
- ◆ Printing DNA capture probes onto solid support
  - ◆ PCR products
  - ◆ Oligonucleotides



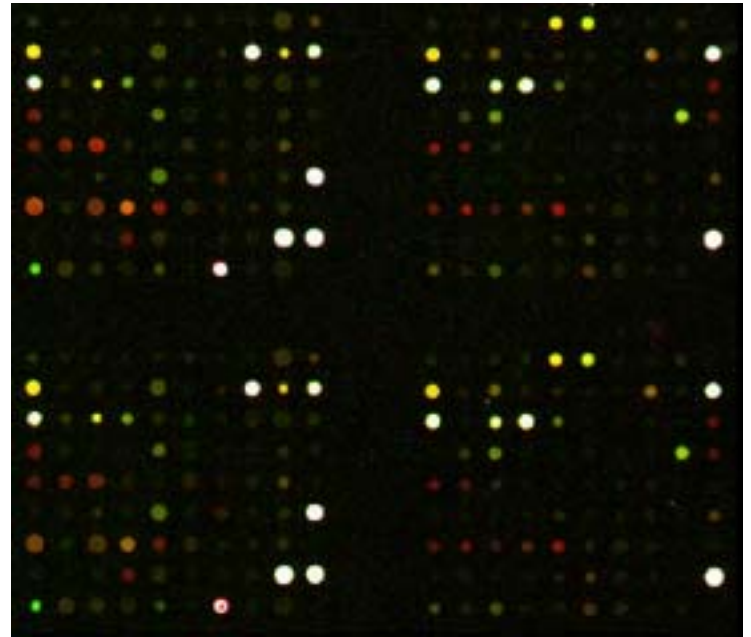
# Micro-array technology

- ◆ Fluorescent labelling of sample nucleic acid
- ◆ Hybridisation to the array



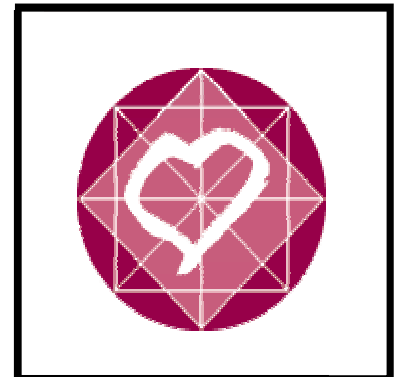
# *Micro-array technology*

- ◆ Scan array using a laser - identify target



# *EU FP5 funded project*

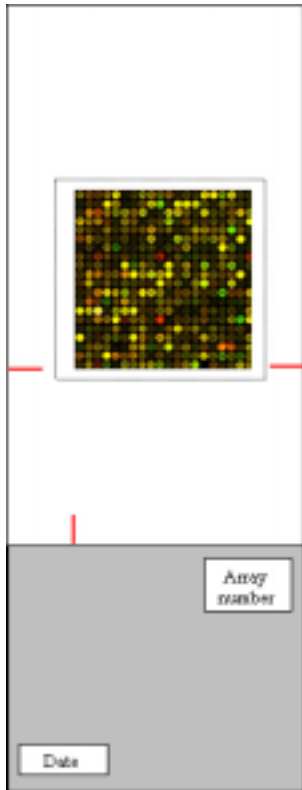
- ◆ Investigate the feasibility of detecting the EU quarantine potato pathogens using array techniques
  - ◆ 12 Viruses
  - ◆ 2 Bacteria
  - ◆ 1 Fungi
  - ◆ 6 Invertebrates
  - ◆ 1 viroid
  - ◆ 1 phytoplasma



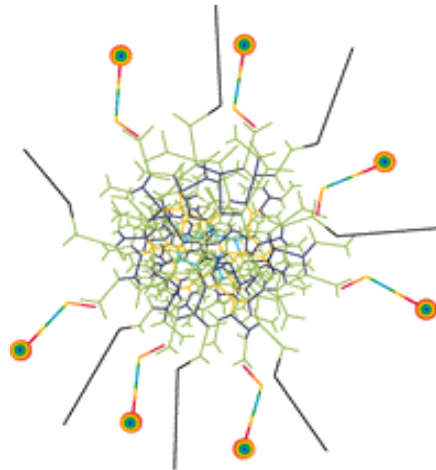
# Consortium – complementary skills



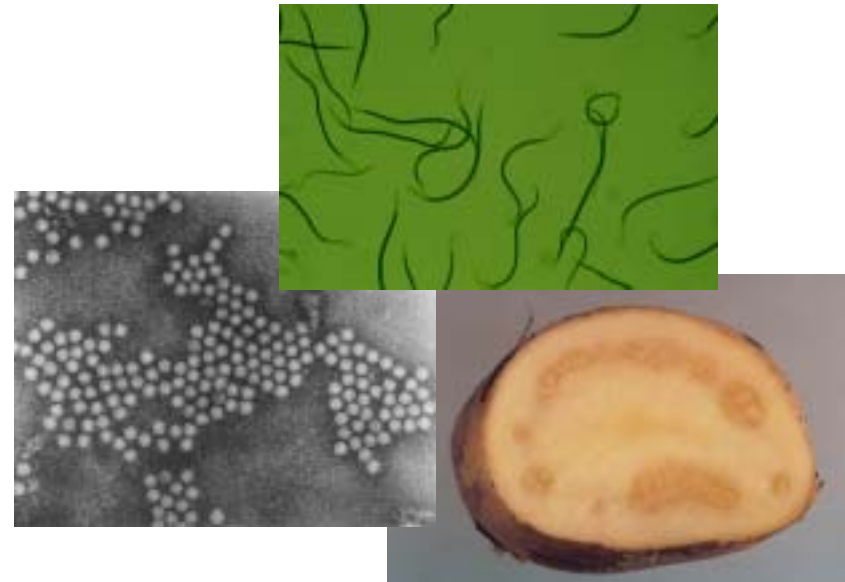
Arrays



Protocols



Pathogens/Pests



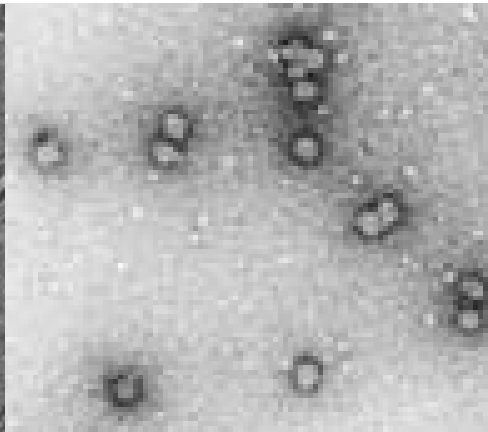
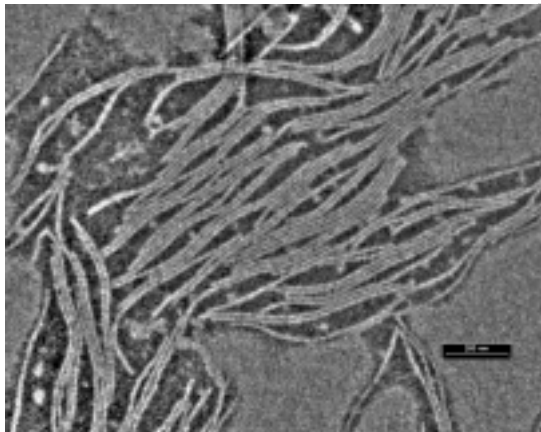
# *Nematodes under study*

- ◆ *Meloidogyne chitwoodi*
- ◆ *Meloidogyne fallax*
- ◆ *Globodera pallida*
- ◆ *Globodera rostochiensis*
- ◆ *Nacobus aberrans*
- ◆ *Ditylenchus destructor*



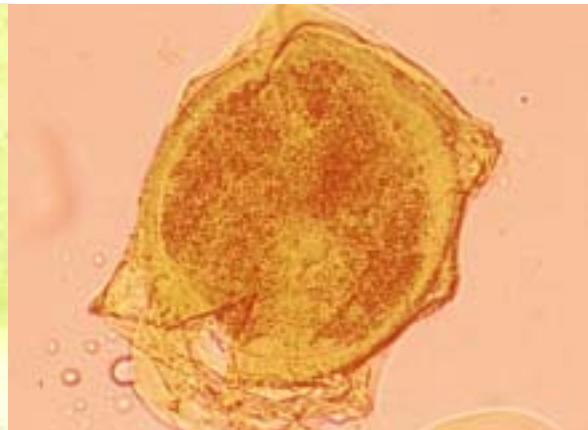
# *Virus isolates under study*

- ◆ Trichovirus (PVT)
- ◆ Nepoviruses (AVB and PBRSV)
- ◆ Potyviruses (PVA & PVY)
- ◆ Tymovirus (APLV)
- ◆ Alfamovirus (PYV)
- ◆ Carlavirus (PVS)
- ◆ Viroid (PSTVd)



# *Fungal pathogens under study*

- ◆ *Synchytrium endobioticum*
- ◆ Soil borne fungal pathogen
- ◆ Obligate parasite
- ◆ The thick walled winter sporangium can remain viable for up to 30 years



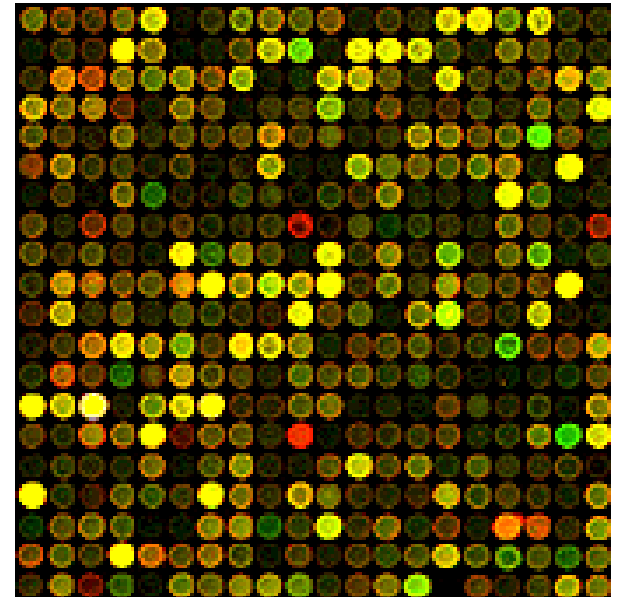
# ***Bacterial pathogens under study***

- ◆ ***Ralstonia solanacearum***
- ◆ ***Clavibacter michiganensis subsp. sepedonicus***



# *Techniques*

- ◆ Solid support
- ◆ Capture probes
- ◆ Labelling approaches
- ◆ Processing
- ◆ Analysis of results



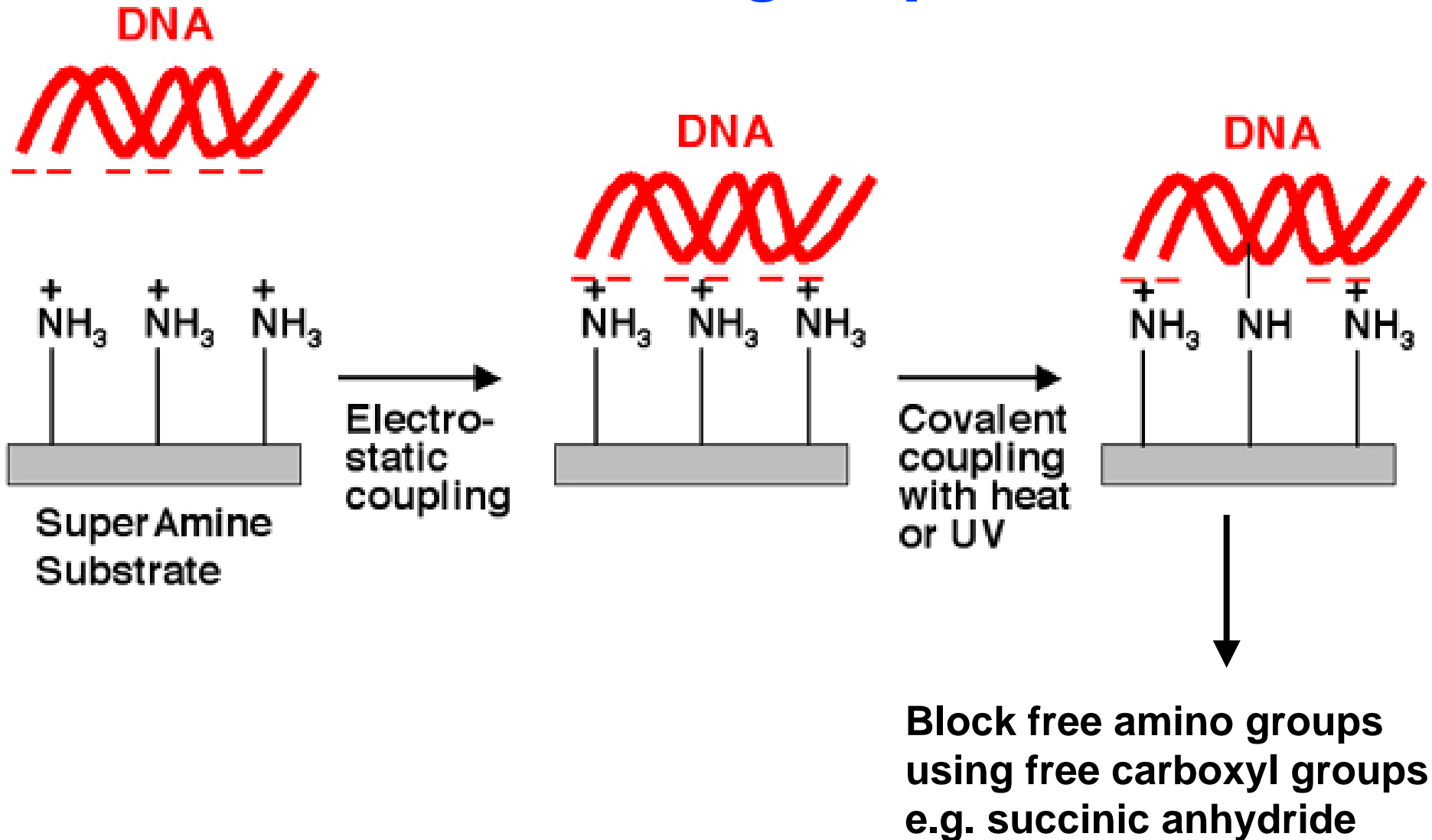
# ***Solid support***

- ◆ Surface used to carry out hybridisation
  - ◆ Nylon membrane
  - ◆ Porous metal oxide (e.g. Pamgene)
  - ◆ Glass slide (various coatings)

# *Slide types*

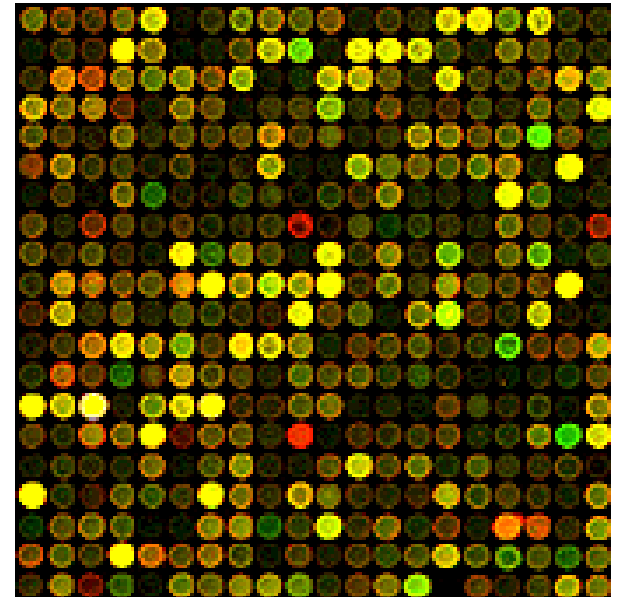
- ◆ GAP slides
  - Gamma amino propyl silane coating
- ◆ Poly-l-lysine slides
  - poly-l-lysine coating
- ◆ Epoxy coated slides
  - Free epoxide rings
- ◆ Aldehyde (silylated) slides
  - Free aldehyde groups

# Coupling chemistry – free amino groups

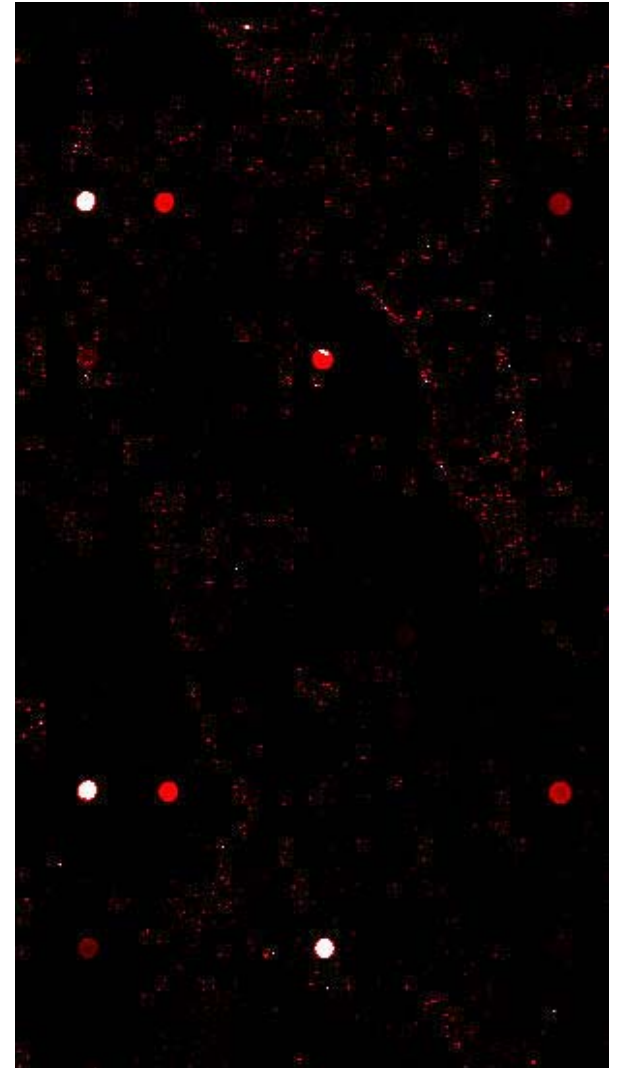
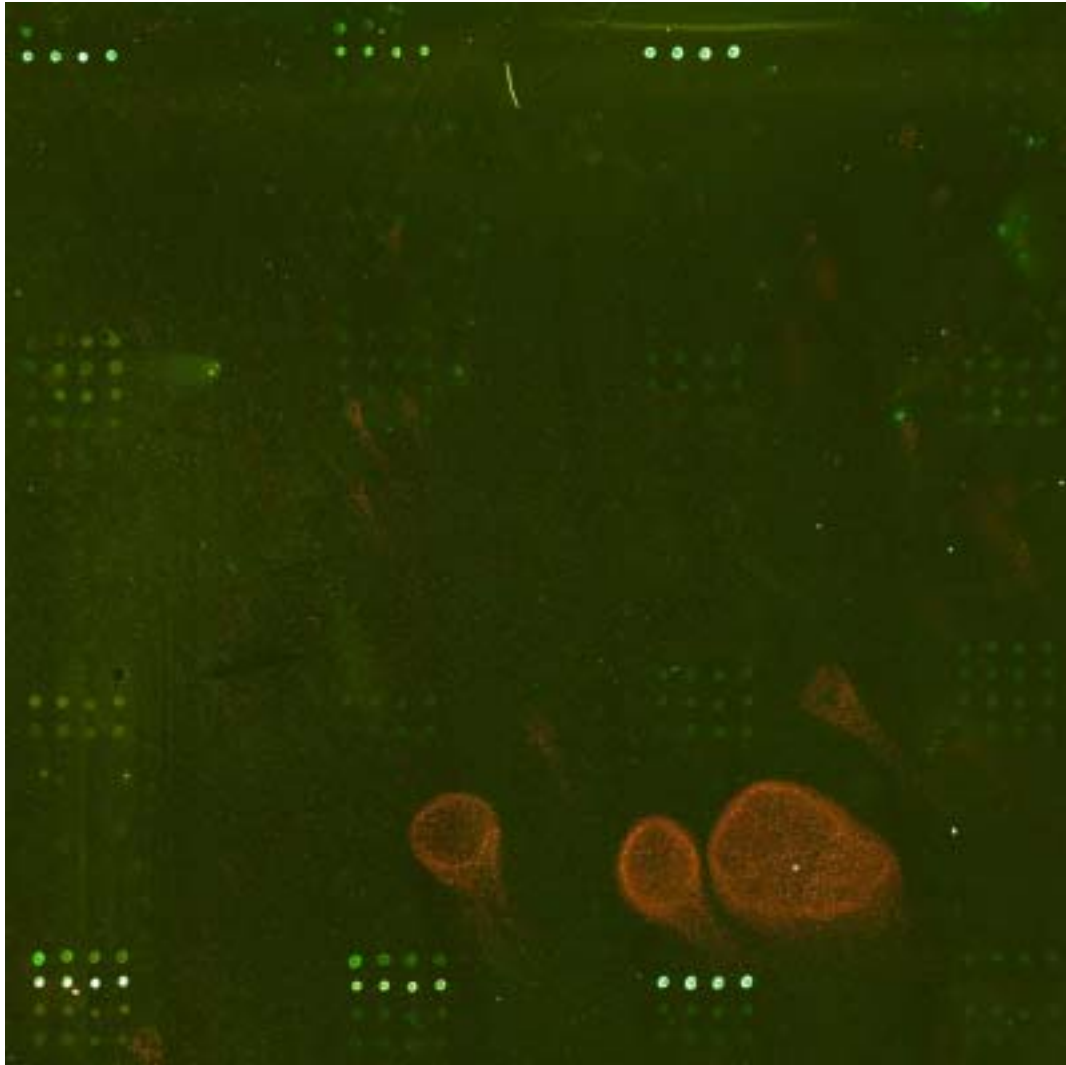


# *Capture probes*

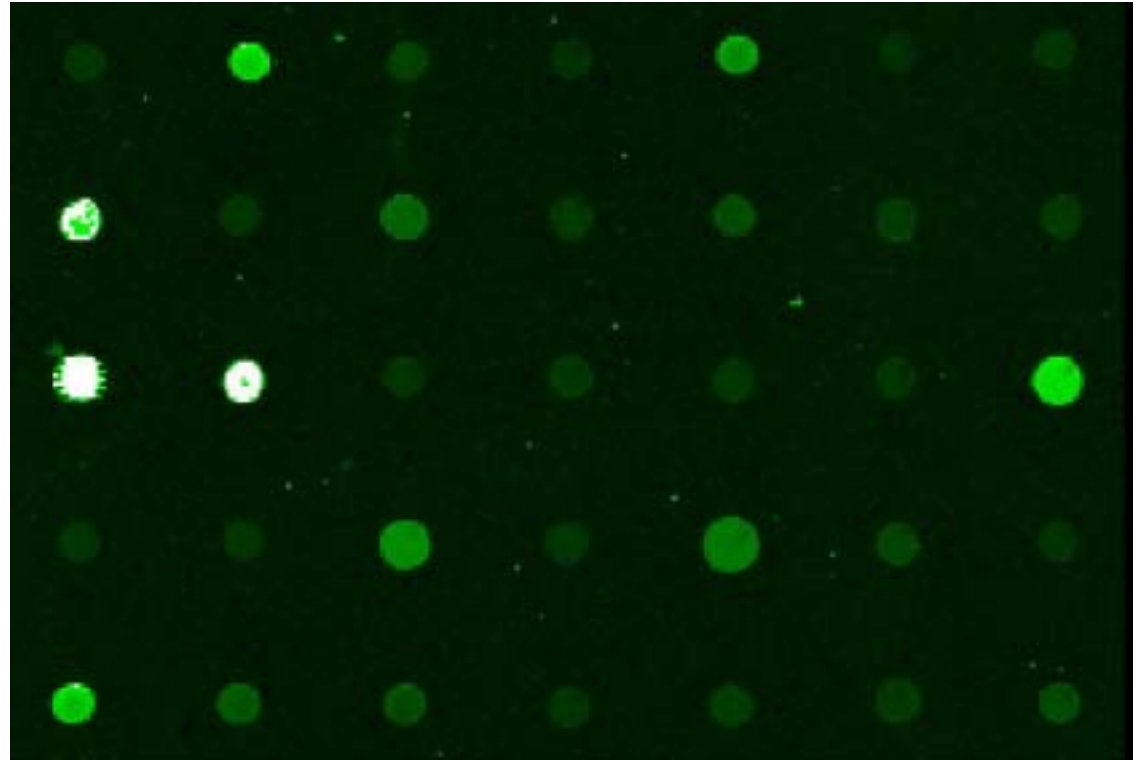
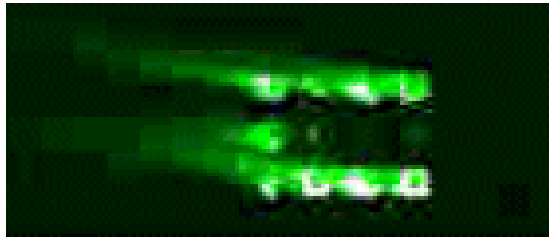
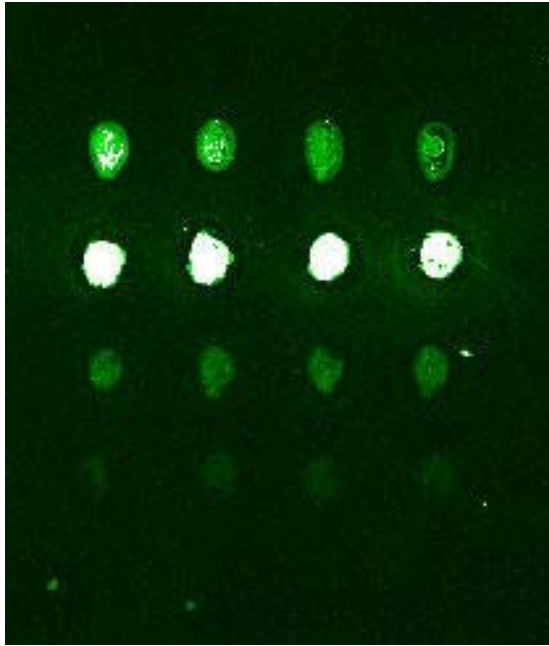
- ◆ PCR products
- ◆ Synthetic oligonucleotides
  
- ◆ Either just DNA
- ◆ Modified DNA
  - Amino labelled
  - Biotin labelled



# *PCR arrays vs. Oligo arrays*

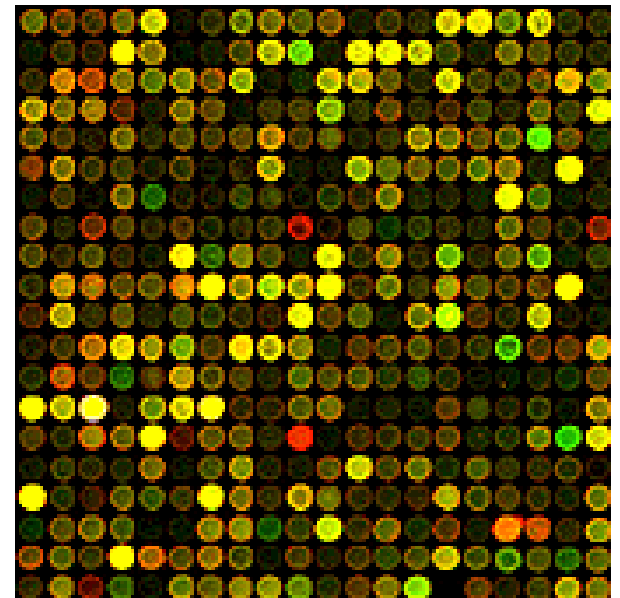


# *PCR arrays vs. Oligo arrays*

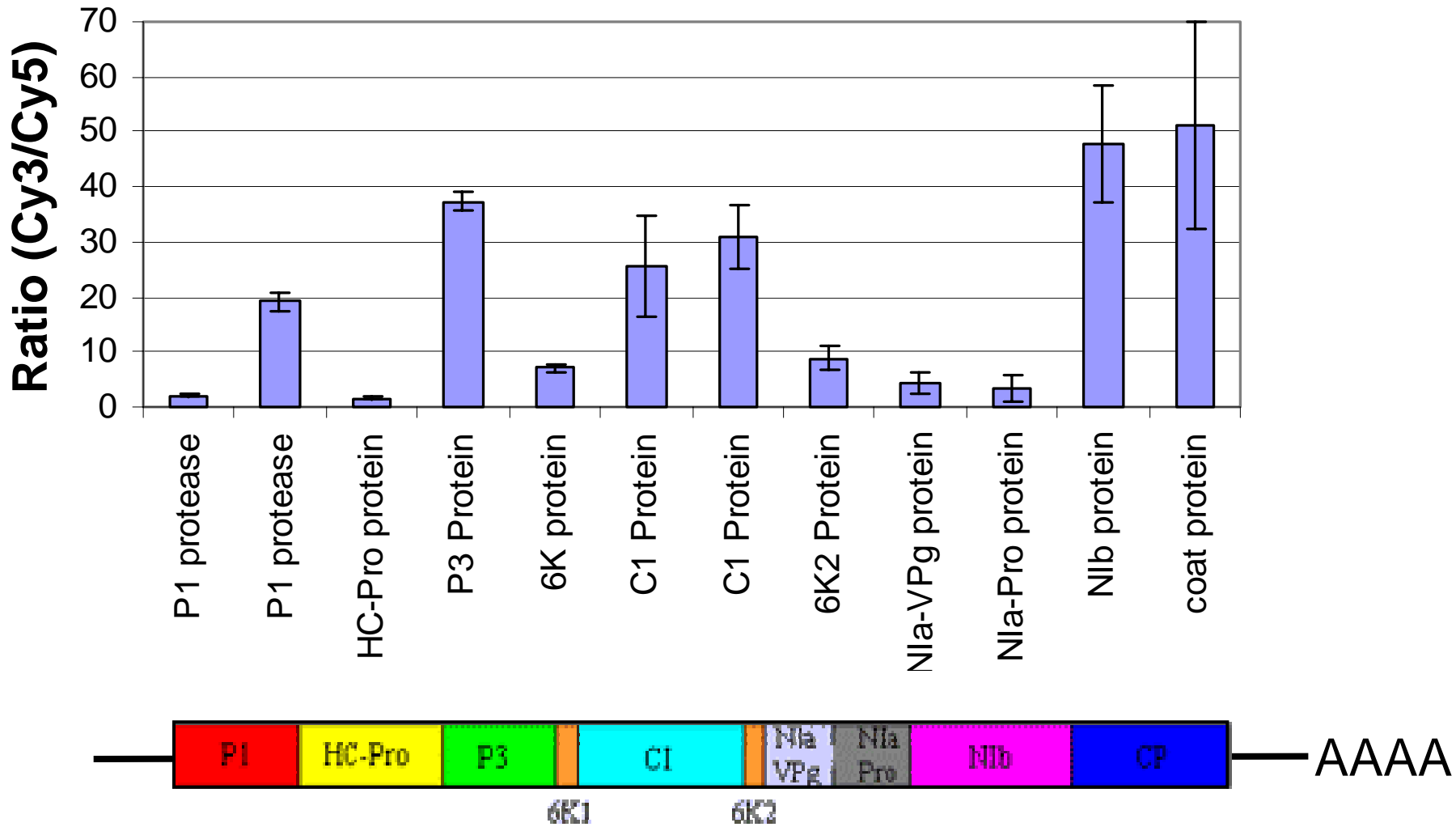


# *Capture probes: design*

- ◆ Synthetic oligonucleotides
- ◆ Similar melting temperatures
- ◆ No secondary structure
- ◆  $T_m$  70-90°C
- ◆ 50nt length
- ◆ 40-55% G/C content
- ◆ Specificity ?



# Oligo comparison



# *Labelling approaches*

- ◆ RNA or DNA
- ◆ Amplification ?
- ◆ Direct incorporation
- ◆ Post labelling / hybridisation

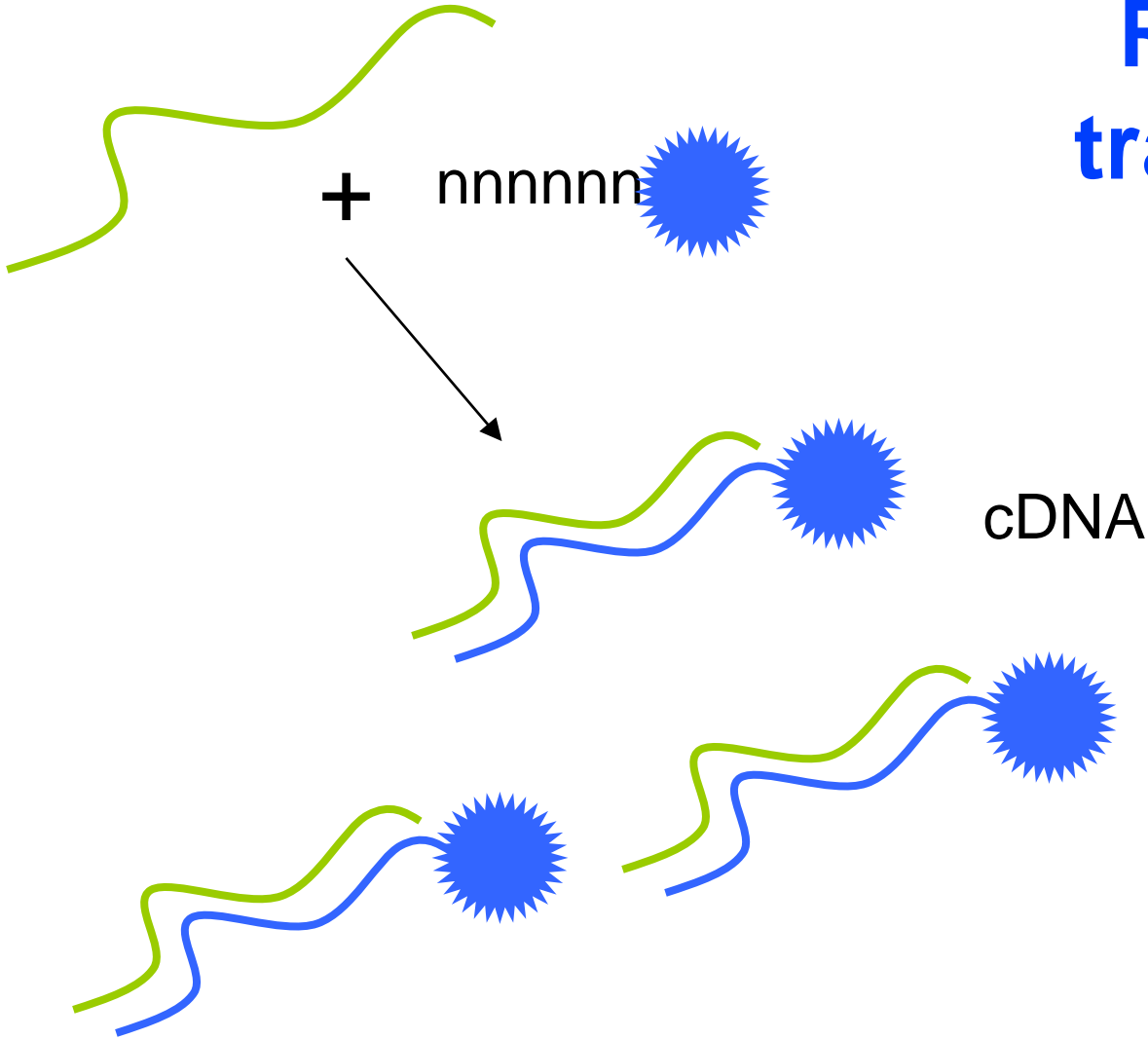
# *RNA Labelling approaches*

- ◆ Copy RNA into cDNA
- ◆ Incorporate label directly or in-directly into the cDNA strand
- ◆ Simplest approach
- ◆ Lower sensitivity

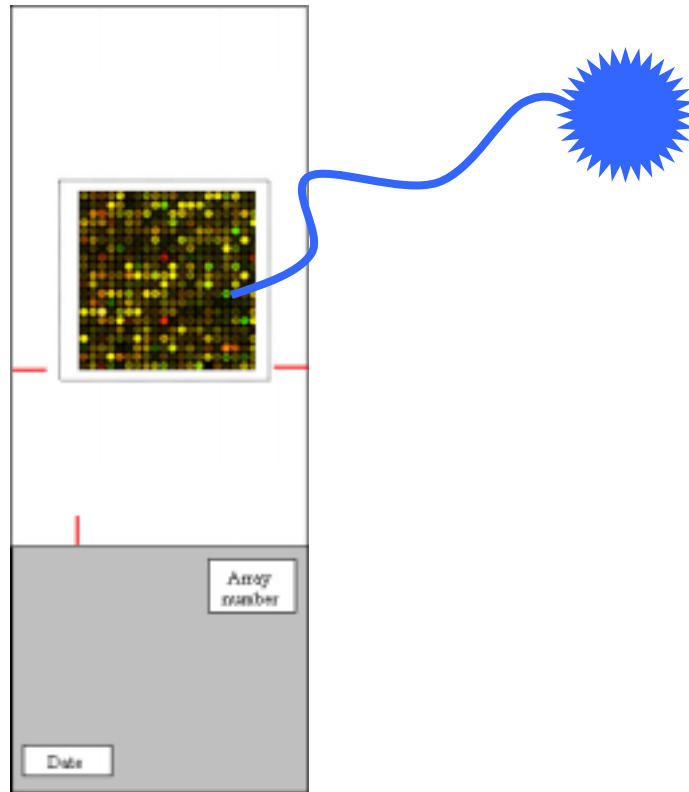
# *Direct labelling*

- ◆ Incorporation of labelled primers
  - ◆ Cy3 labelled random primers and oligo dT
    - Short protocol ✓
    - Poor incorporation ?

# Reverse transcribe RNA

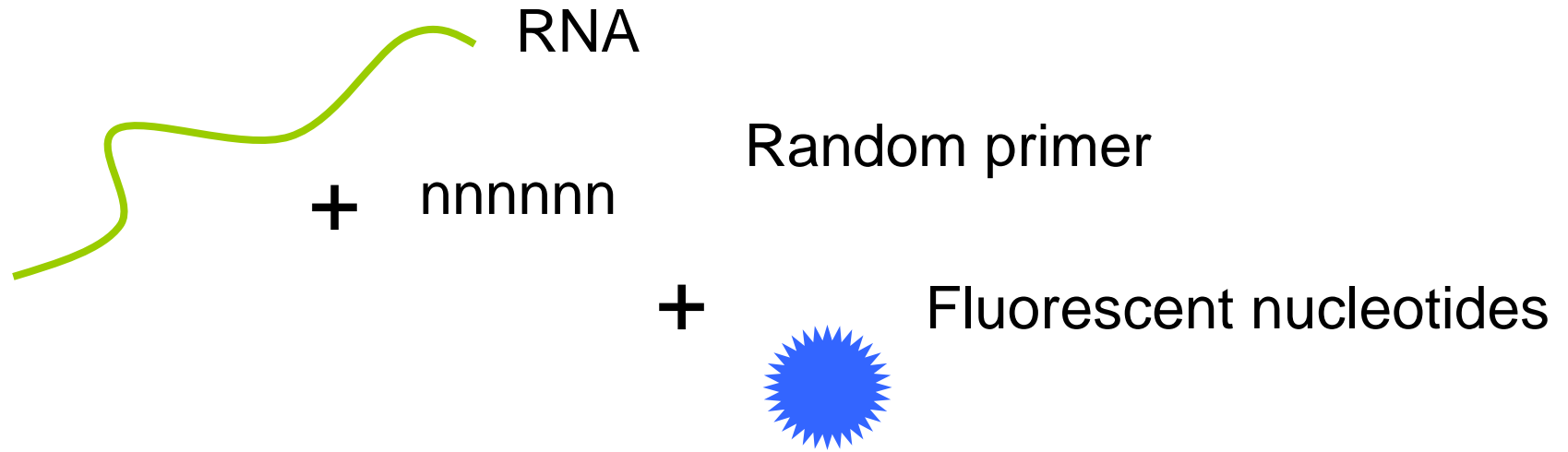


# Hybridise cDNA directly to array

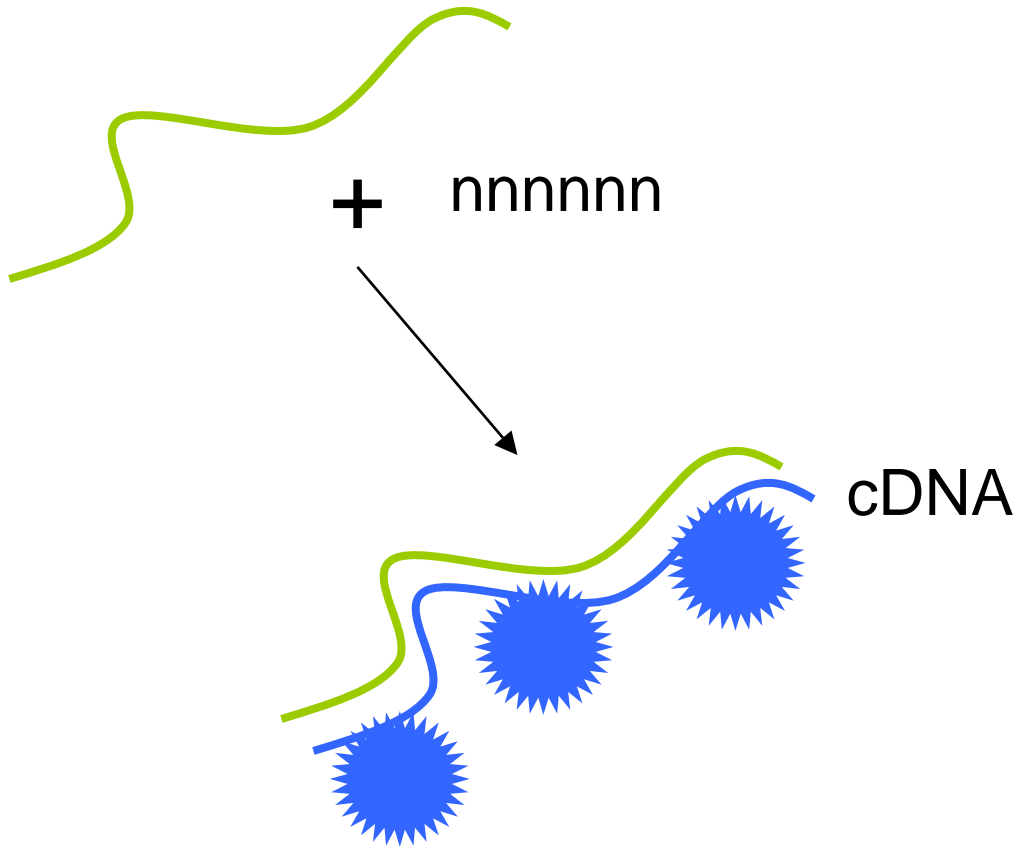


# *RNA - Direct labelling*

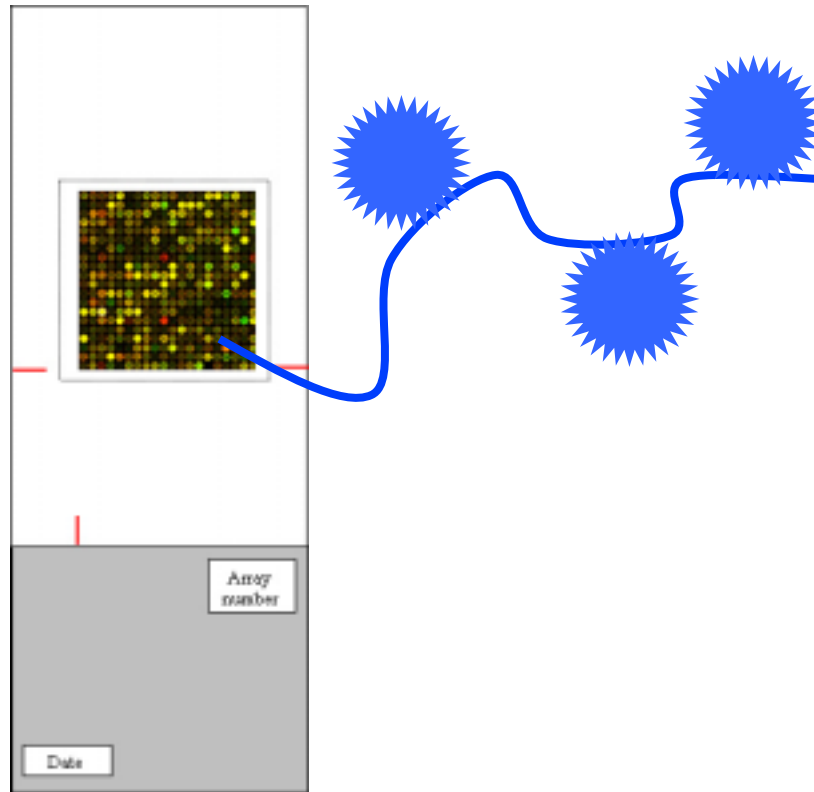
- ◆ Direct incorporation of Cy labelled dNTP's
  - ◆ Cy scribe kit or individual components
    - good incorporation of Cy3 ✓
    - simple protocol ✓
    - expensive Cy labelled nucleotides X
    - poor incorporation of Cy5 nucleotides X



# Reverse transcribe RNA



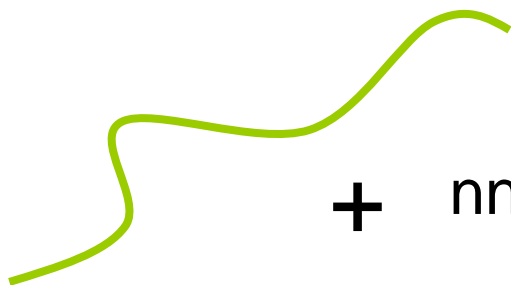
# Hybridise labelled cDNA to array



# *RNA - Amino allyl labelling*

- ◆ Direct incorporation of amino labelled dNTP's – post linking with reactive Cy dye
  - good incorporation of both Cy dyes ✓
  - less expensive ✓
  - slightly longer protocol X

RNA



+

nnnnnn

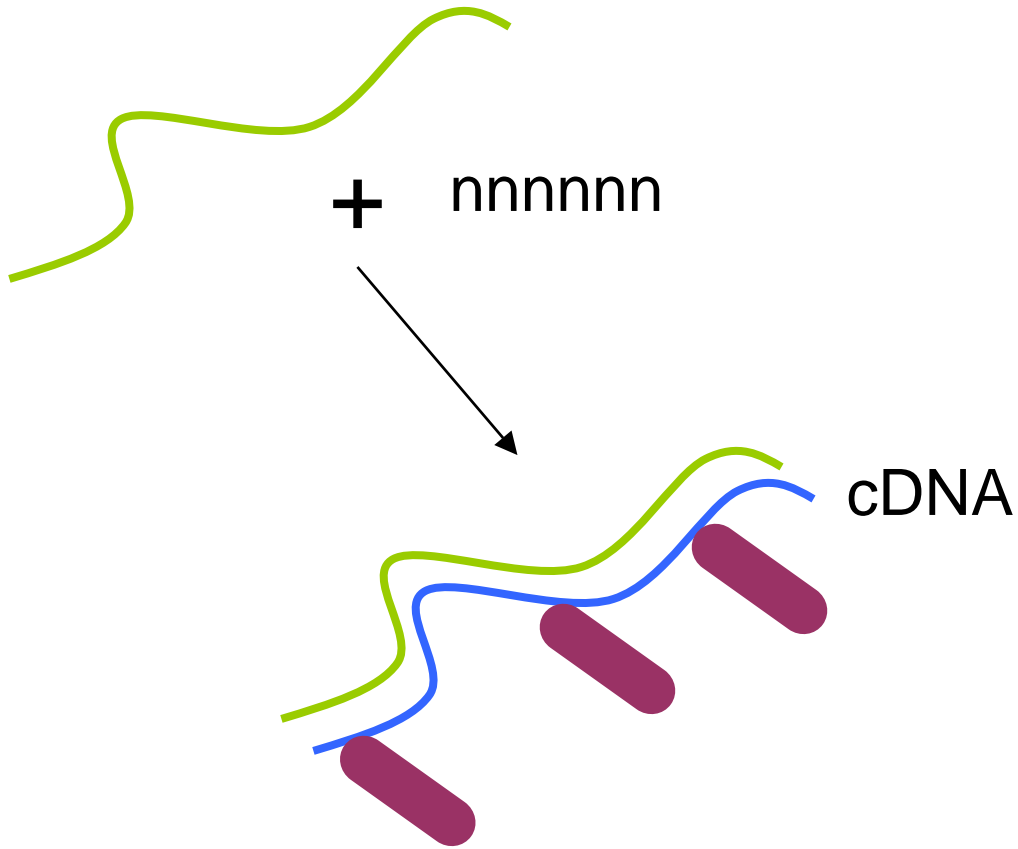
Random primer

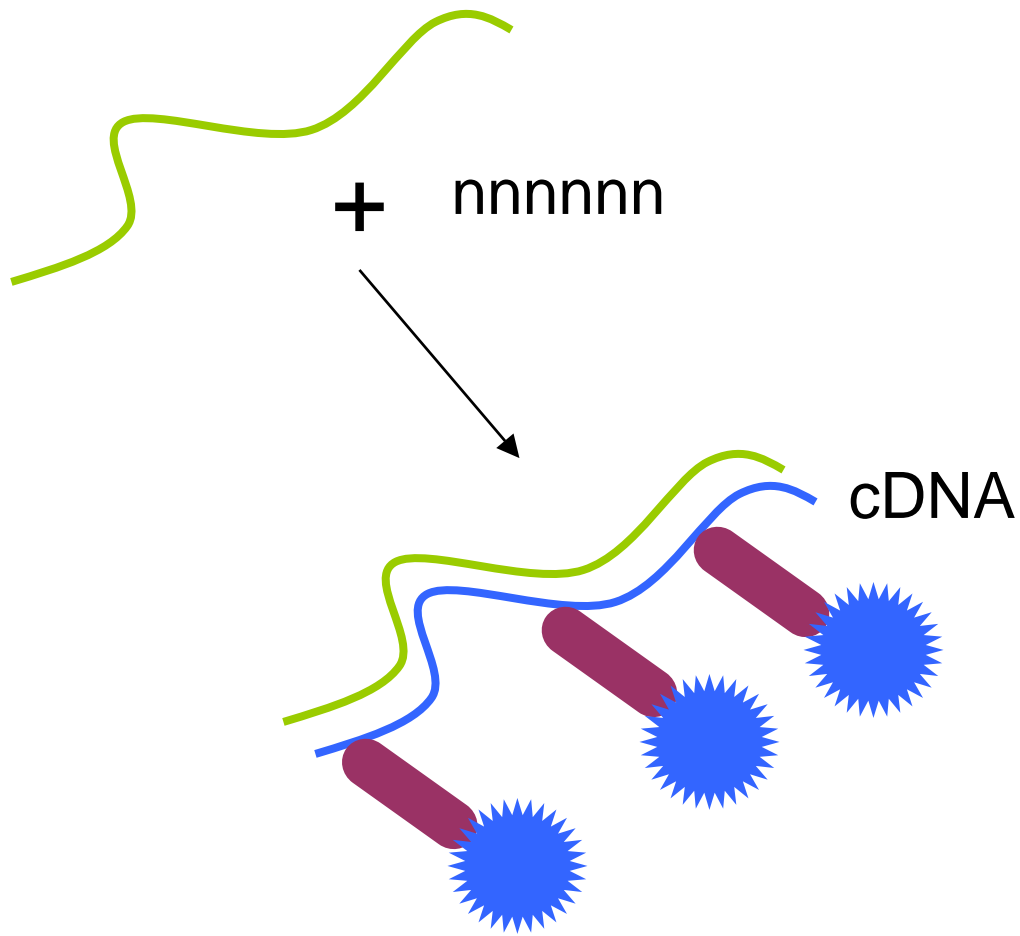
+

Amino allyl nucleotides



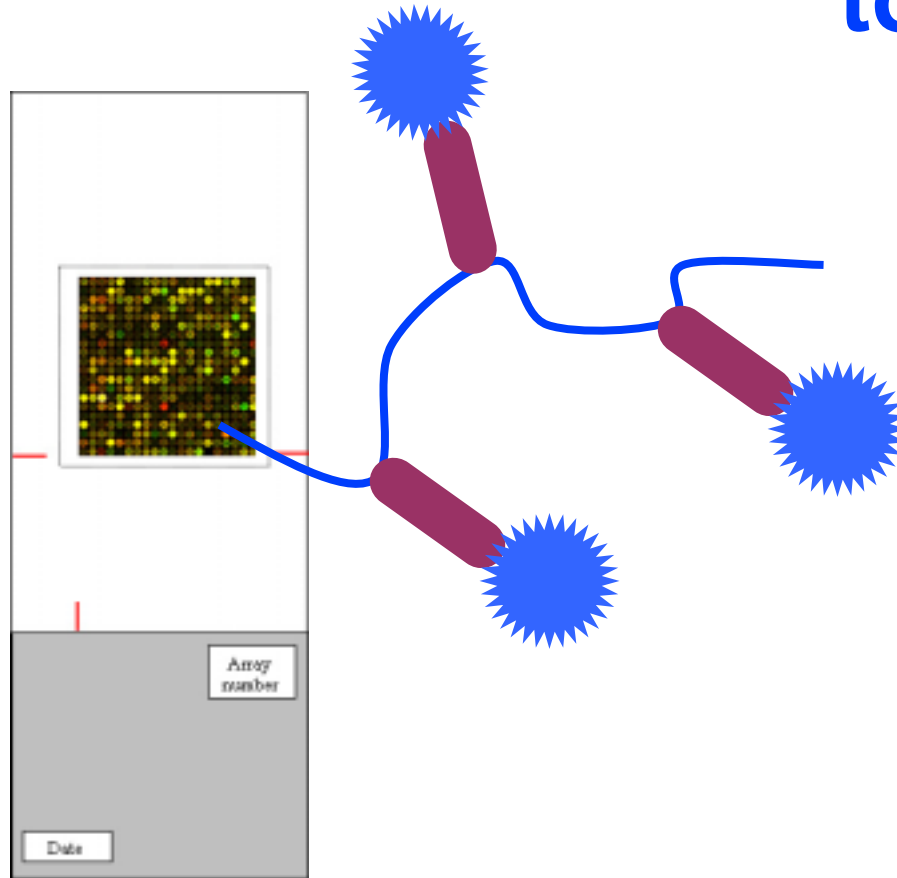
# Reverse transcribe RNA





# Chemical linkage of reactive Dye

# Hybridise labelled cDNA to array



# 3DNA - labelling

- ◆ Post incorporation of labelled dendrimer

- ◆ 3DNA Array 350RP kit

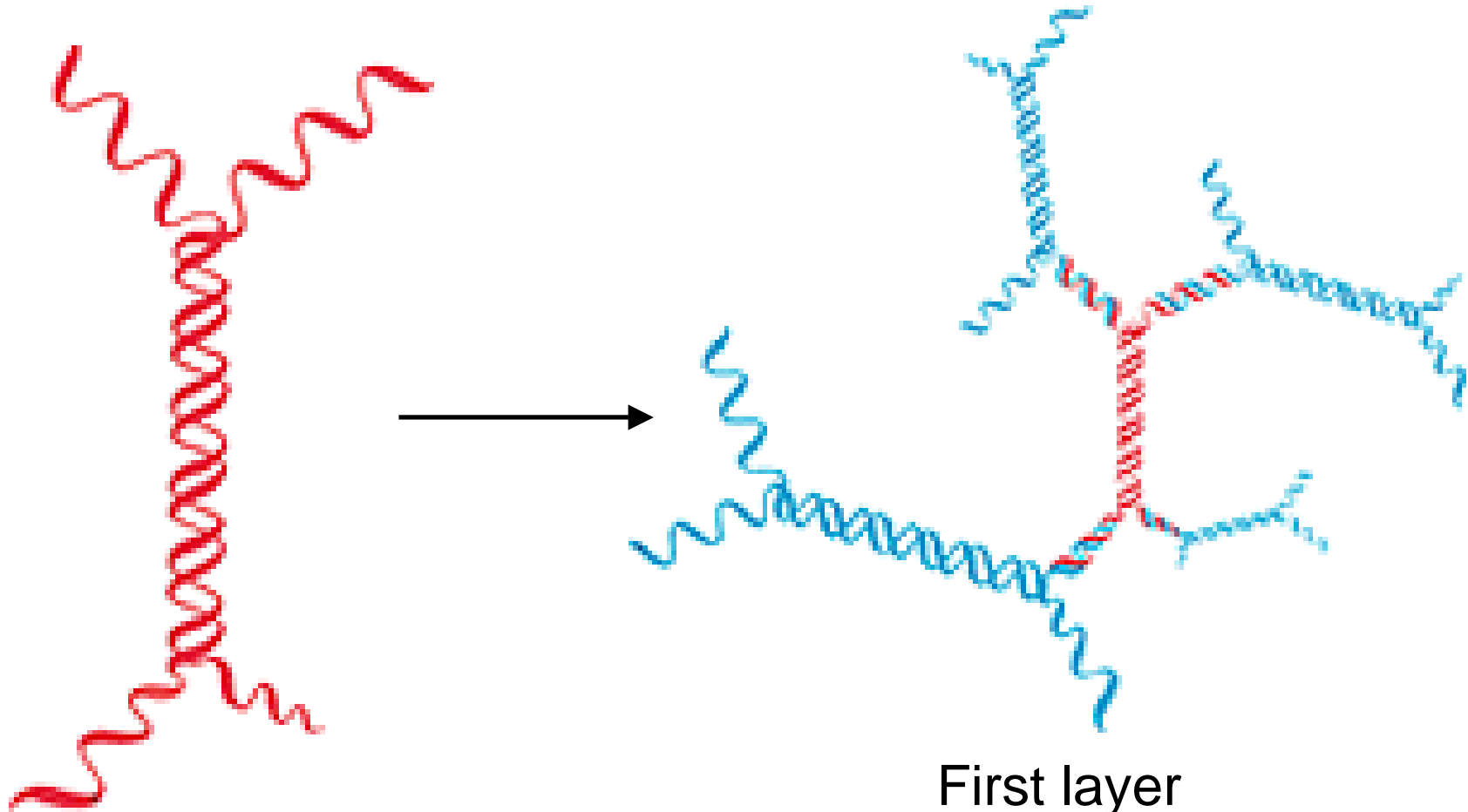
- good incorporation of Cy3 and Cy5 ✓

- long protocol X

- expensive kit X

- amplification of signal ?

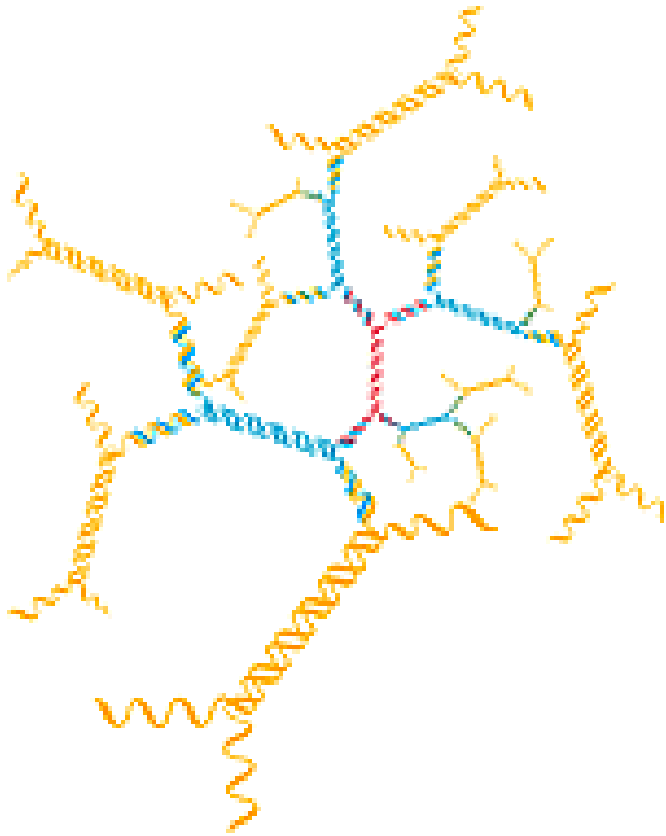
## *3DNA - labelling*



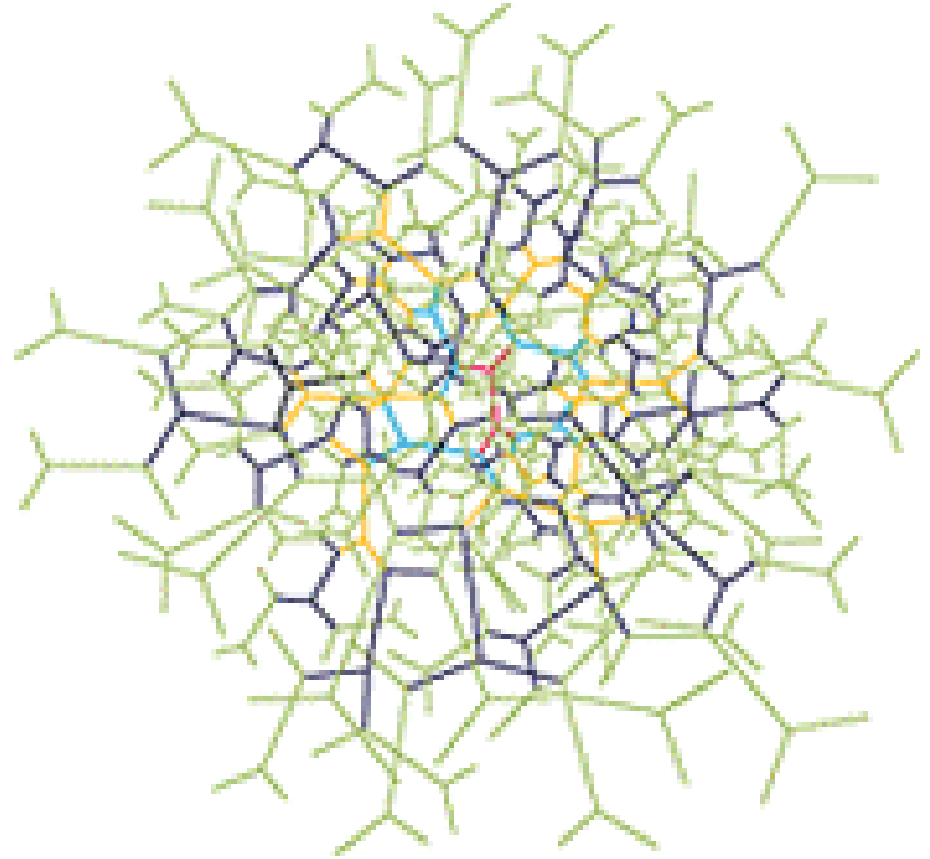
Monomer

First layer  
gives 12 free  
ends – this is  
chemically cross  
linked

# 3DNA - labelling

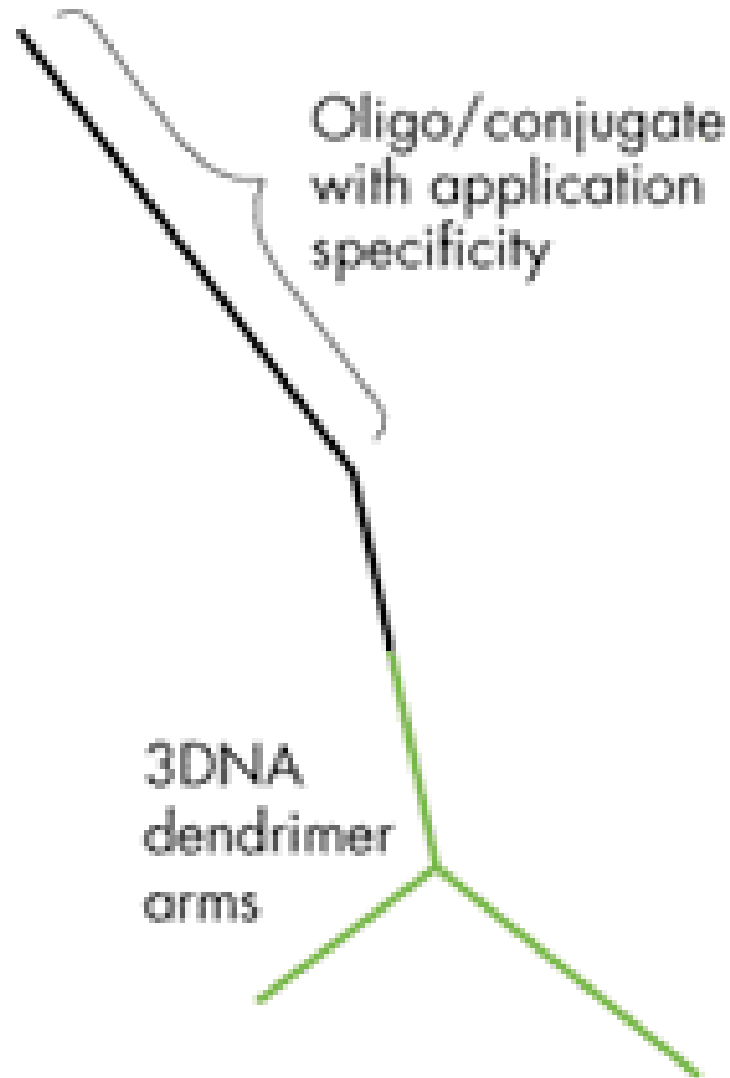


Second layer gives 36 free ends – this is chemically cross linked

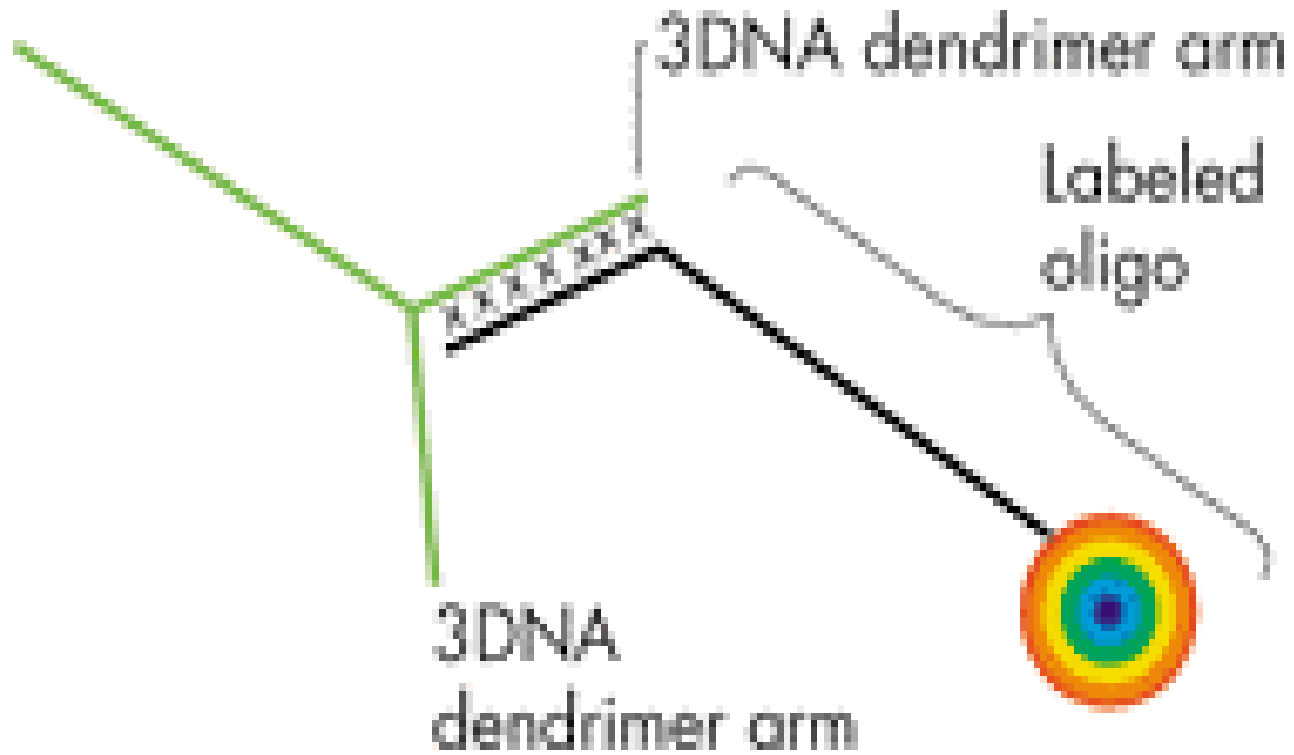


Layers 3 & 4 gives a dendrimer with 324 free ends

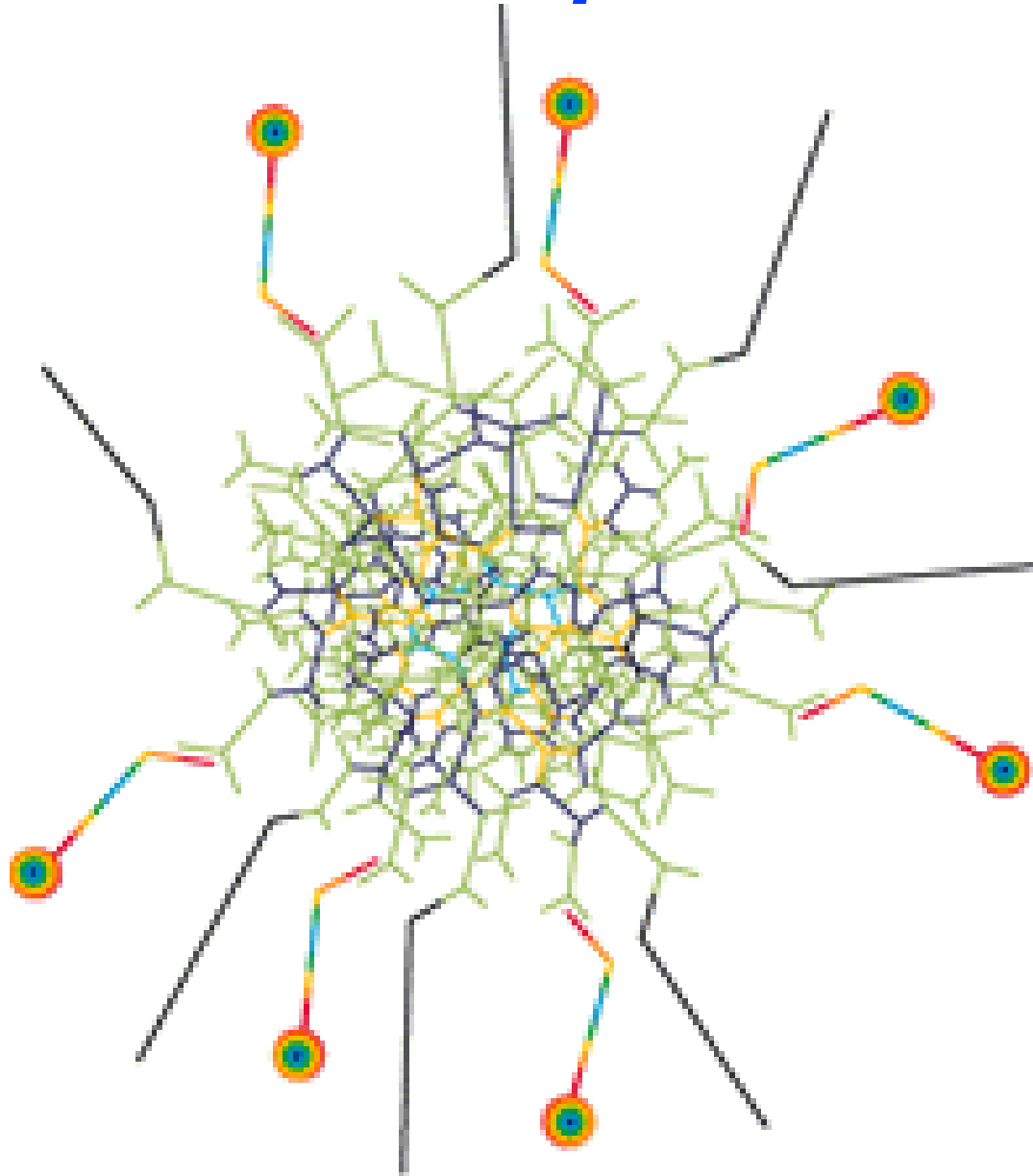
# *Add specificity*

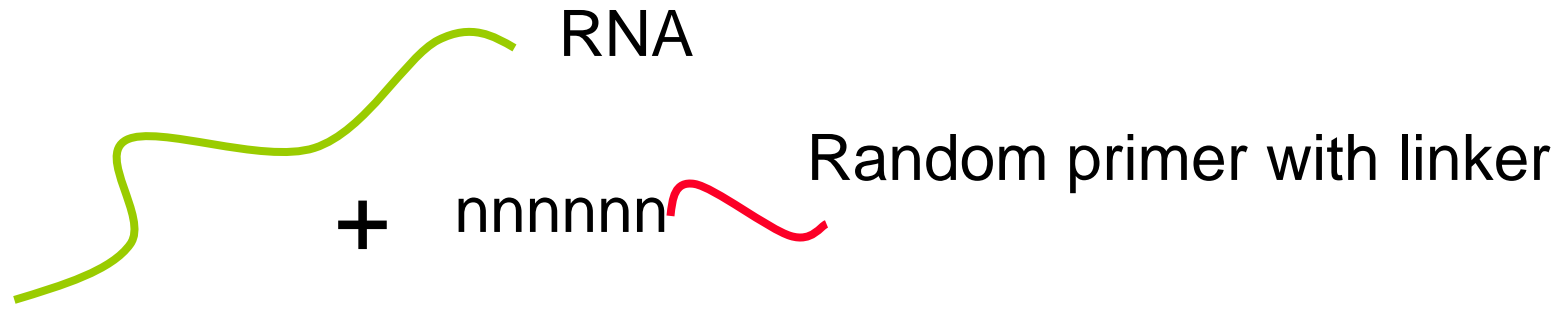


# *Add label*

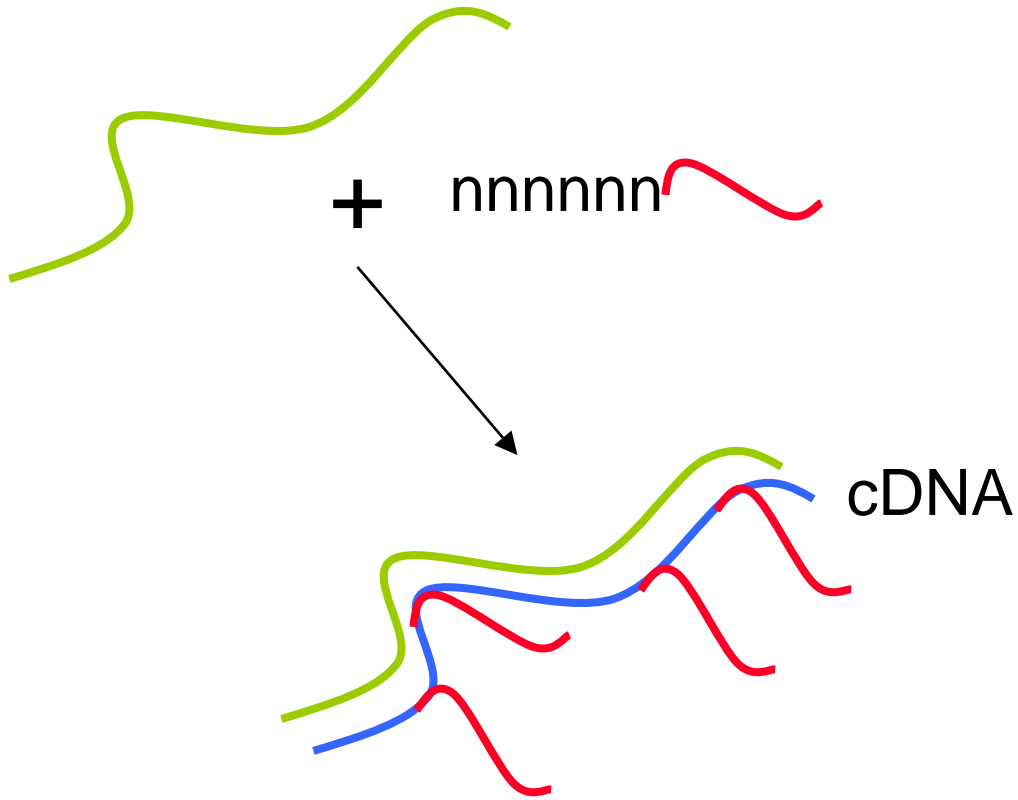


**>300x Amplification**

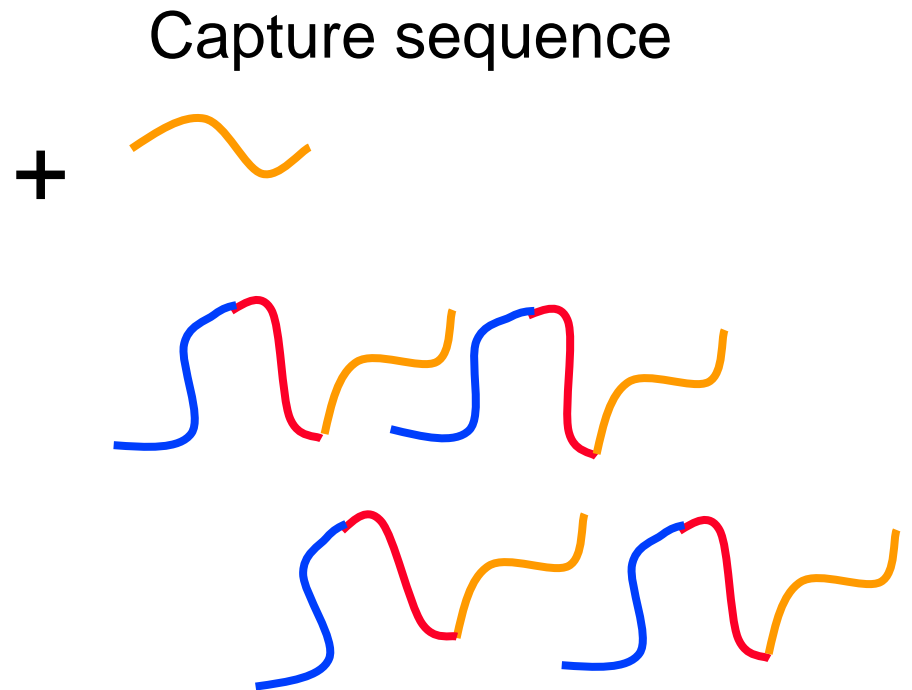
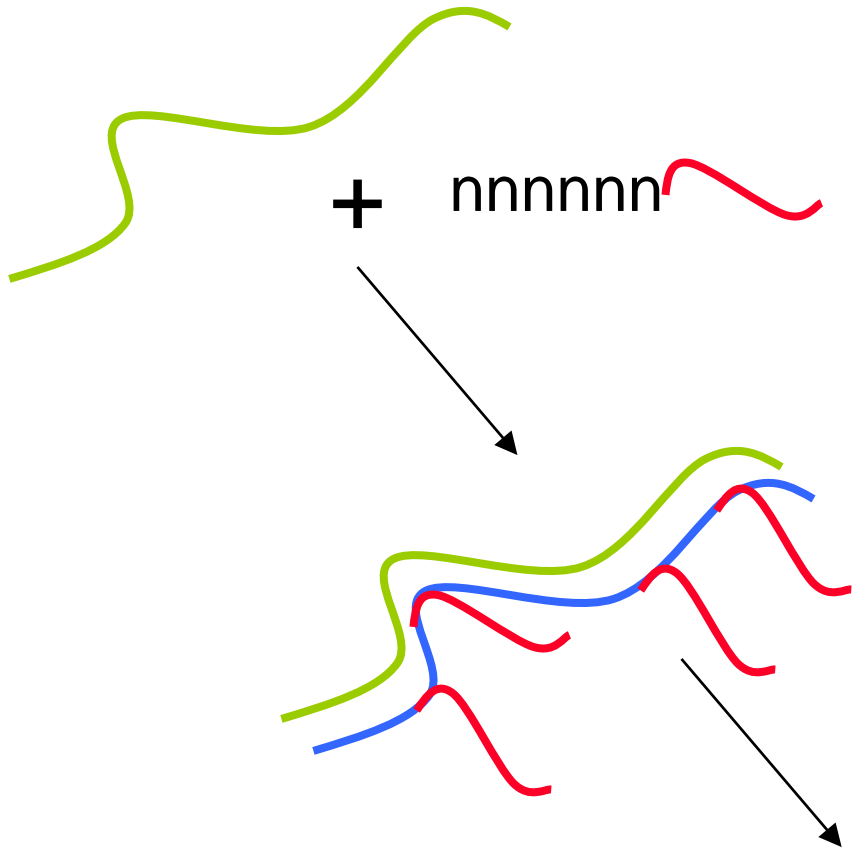




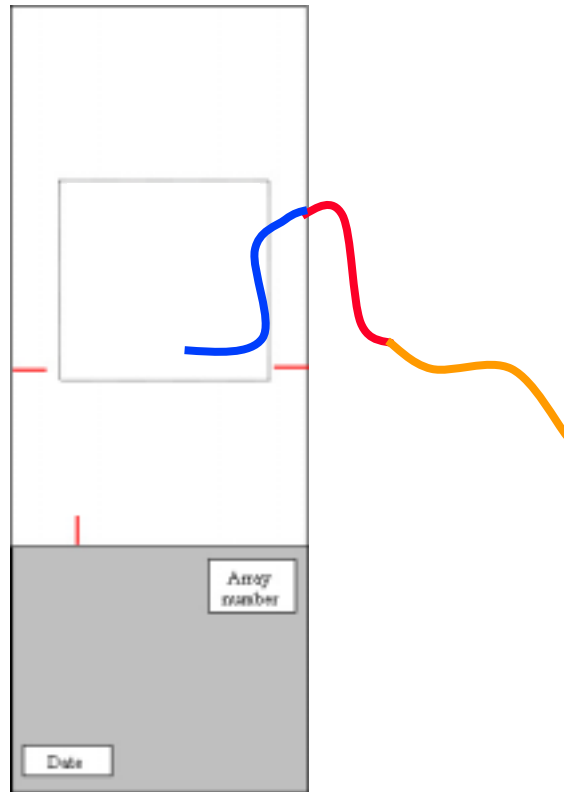
# Reverse transcribe RNA



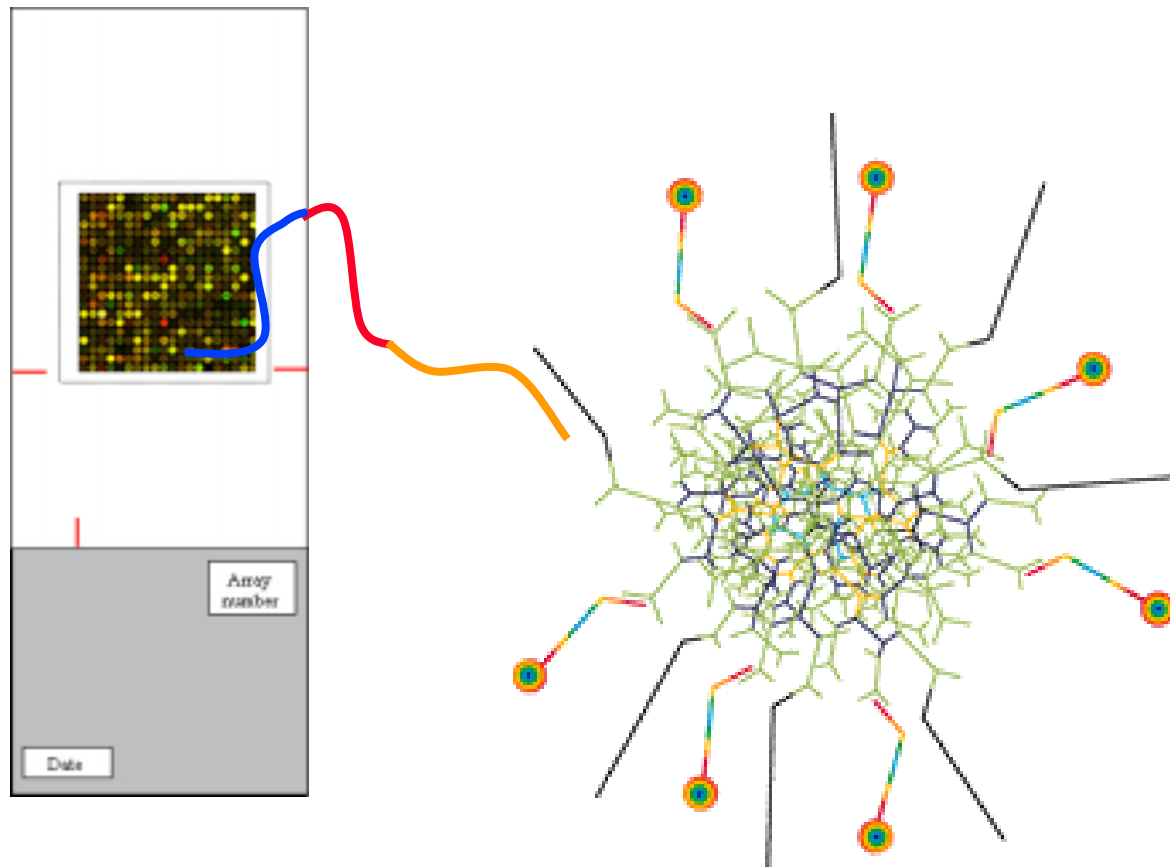
# Degrade RNA & ligate capture sequence



# Hybridise cDNA to array

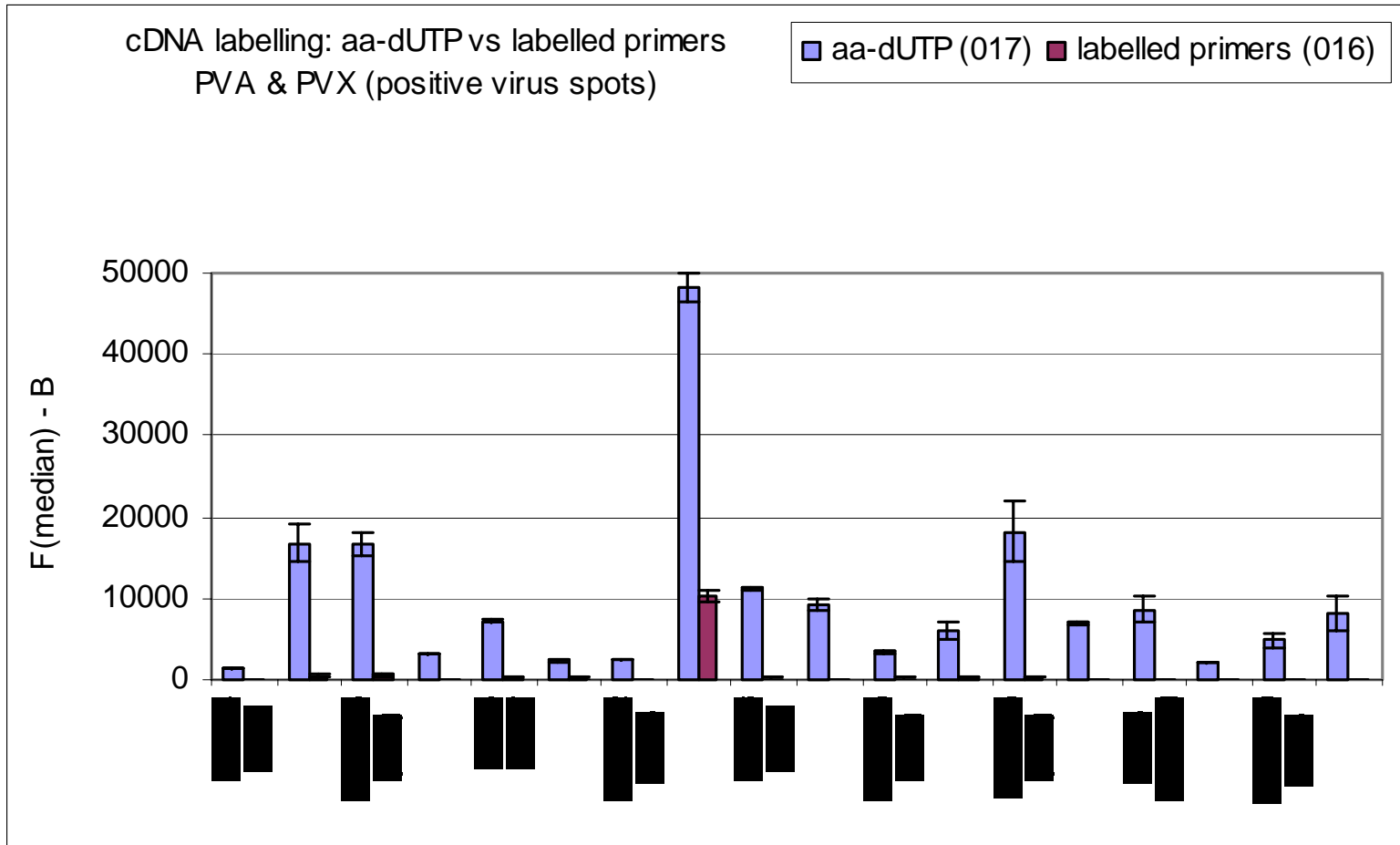


# Hybridise dendrimer to array



# Labelling - cDNA

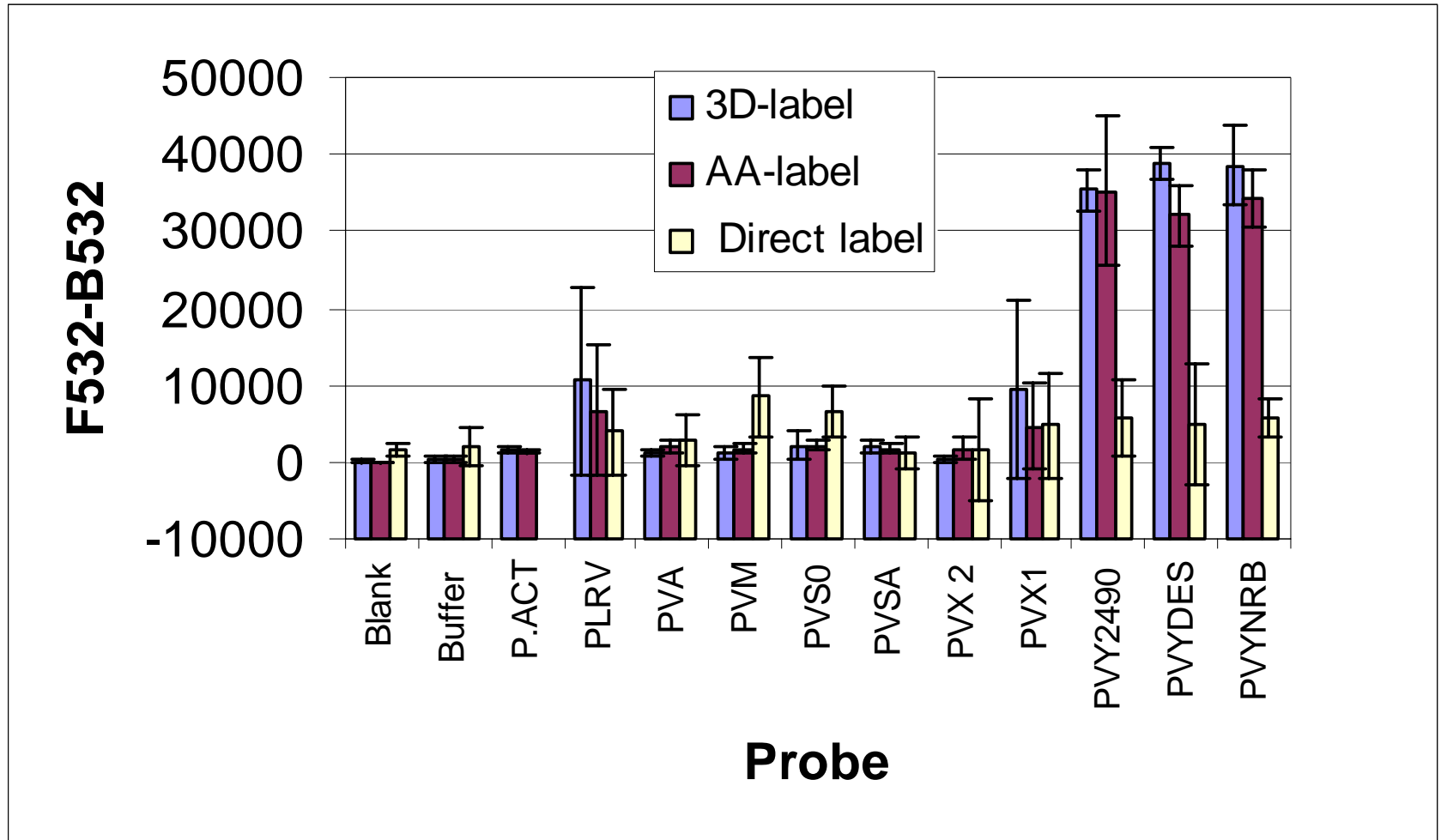
## ◆ AA dNTP's vs. Cy labelled primers





# Labelling - cDNA

- ◆ AA dNTP's vs. 3 DNA label vs. Cy dNTP's



# ***DNA Labelling approaches***

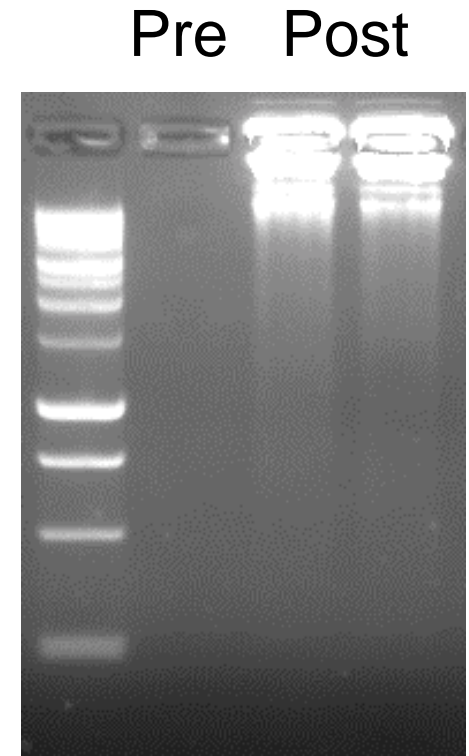
- ◆ Copy DNA e.g. Klenow & random primers
- ◆ Whole genome amplification e.g.  $\phi$ 29 polymerase & random primers
- ◆ PCR
  - Specific primers (1 set per pathogen)
  - Universal primers (e.g. ITS primers)
- ◆ Padlock probes

# ***Whole genome amplification***

- ◆ Based on multiple displacement amplification
- ◆ Exonuclease resistant random primers
- ◆  $\phi$  29 DNA polymerase – highly processive
- ◆ Isothermal

# *Whole genome amplification*

- ◆ Real time PCR shows amplification of at least 200x on a range of targets
- ◆ Low bias across genome
- ◆ Not getting increased signal to noise



# *Universal PCR*

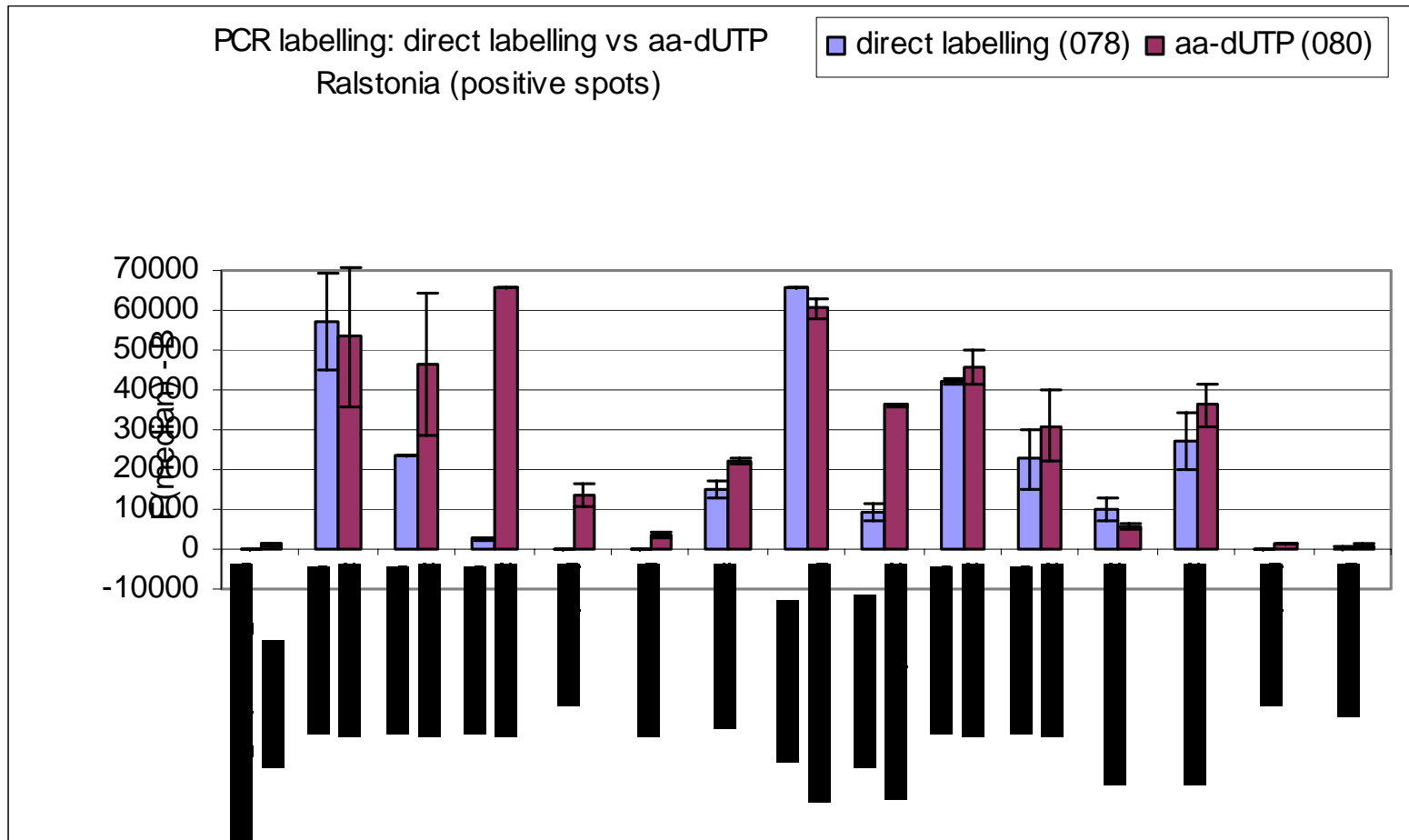
- ◆ PCR - Universal primers

- 16S primers for bacteria

- ITS primers for invertebrates and fungi

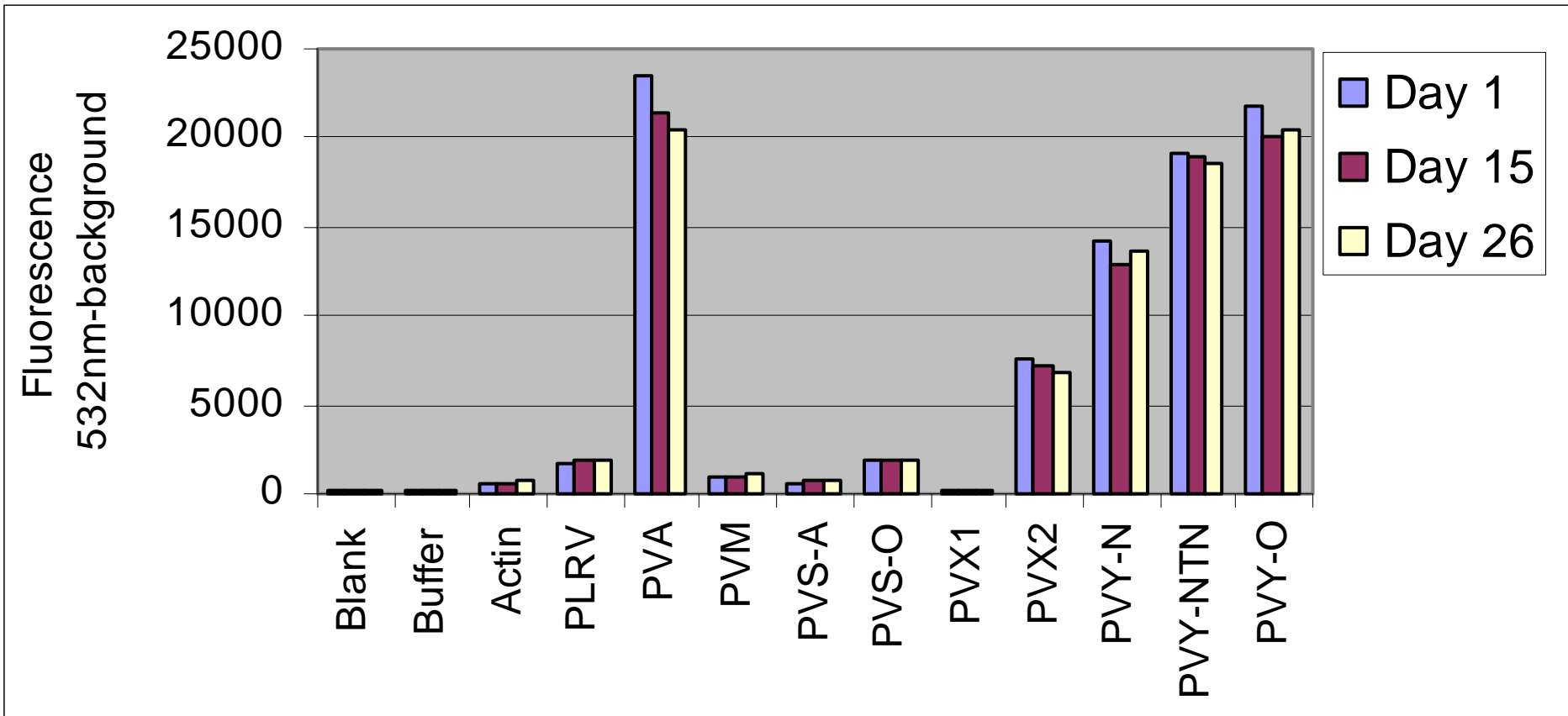
# Labelling - PCR

## ◆ AA dNTP's vs. Cy labelled dNTP's



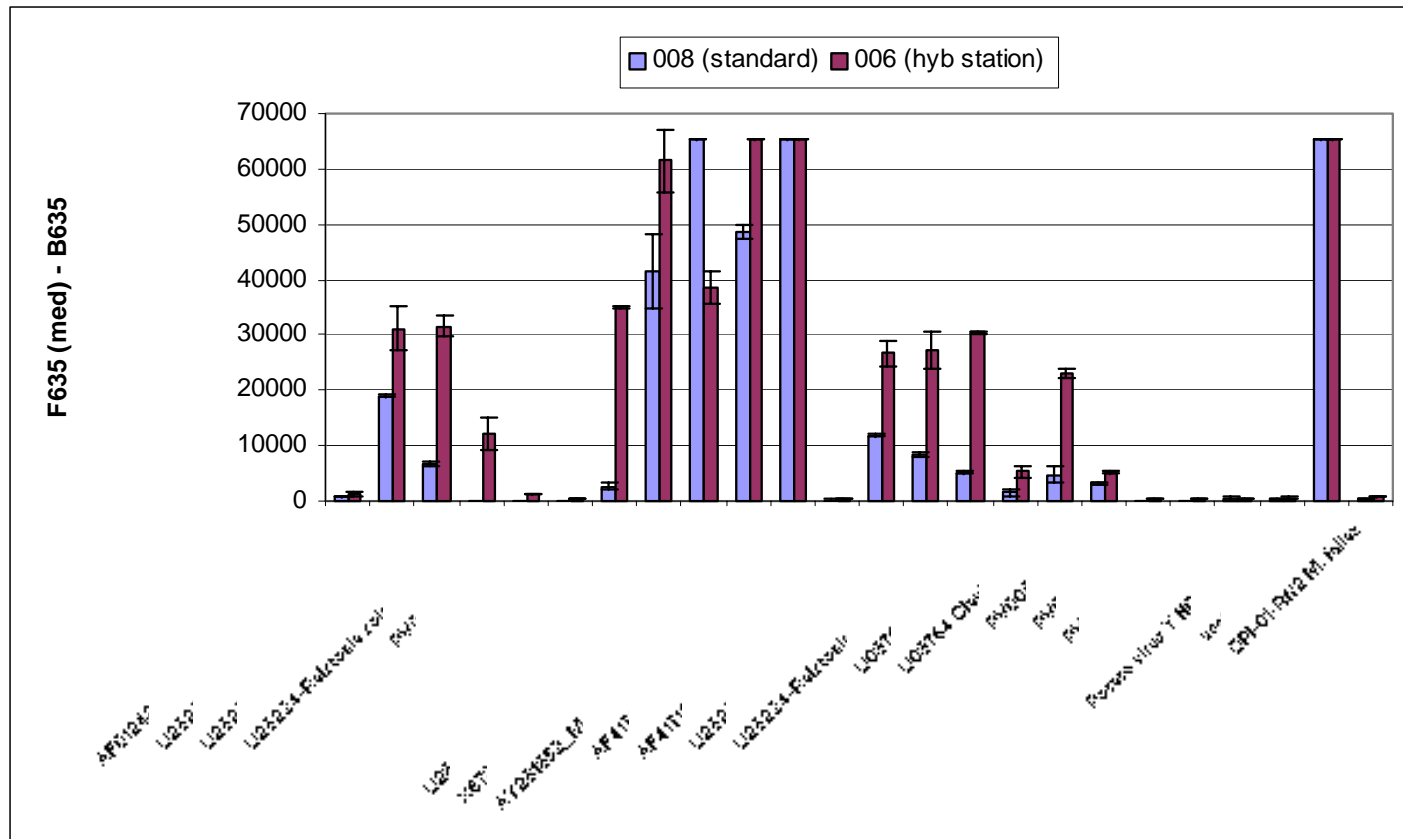
# Fluorescent decay

## ◆ Timing of slide scanning



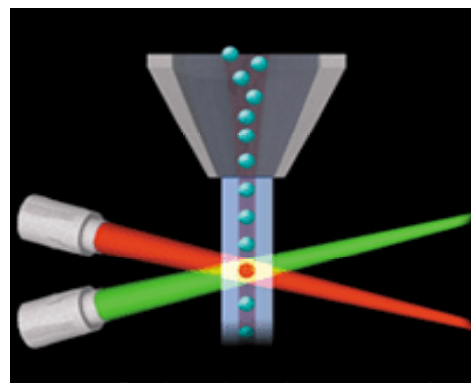
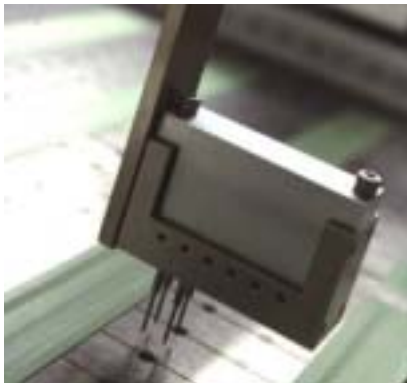
# Processing

- ◆ Hybridisation station
  - faster processing (2 hours)



# *Direction ?*

- ◆ Labelling methods direct / indirect ?
- ◆ PCR or oligo arrays ?
- ◆ Printed or synthesised ?
- ◆ Hybridisation / wash stations ?
- ◆ Glass slides / 'affy' chips / bead arrays ?
- ◆ Home made / manufactured ?
- ◆ Pre-amplification or direct labelling ?



# *Labelling approach*

- ◆ PCR labelling 16S and ITS regions for bacterial, fungal and nematode targets
  - ◆ Incorporating labelled dNTP's
- ◆ Reverse transcription labelling for viruses
  - ◆ Incorporating AA'dNTP's

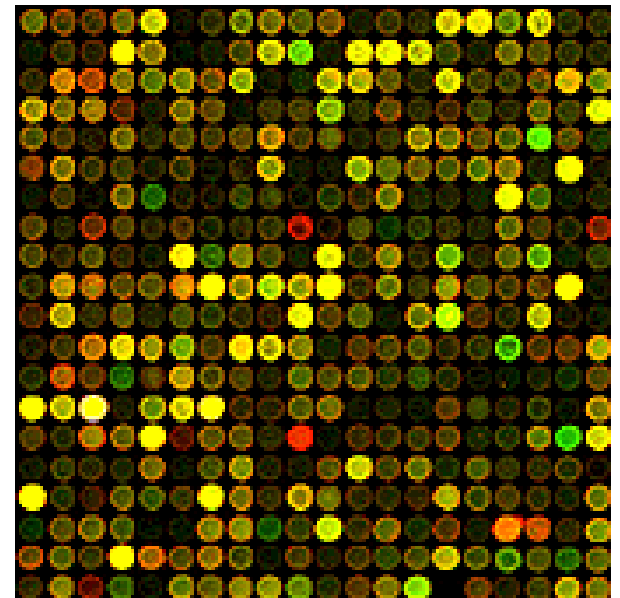
# *Define the assay format*

- ◆ Reproducible
  - ◆ Manufactured oligo arrays
- ◆ Little dedicated equipment
  - ◆ Disposable plasticware
- ◆ Low cost as possible
  - ◆ Glass slide format
- ◆ Simple labelling method
  - ◆ Suitable for all labs



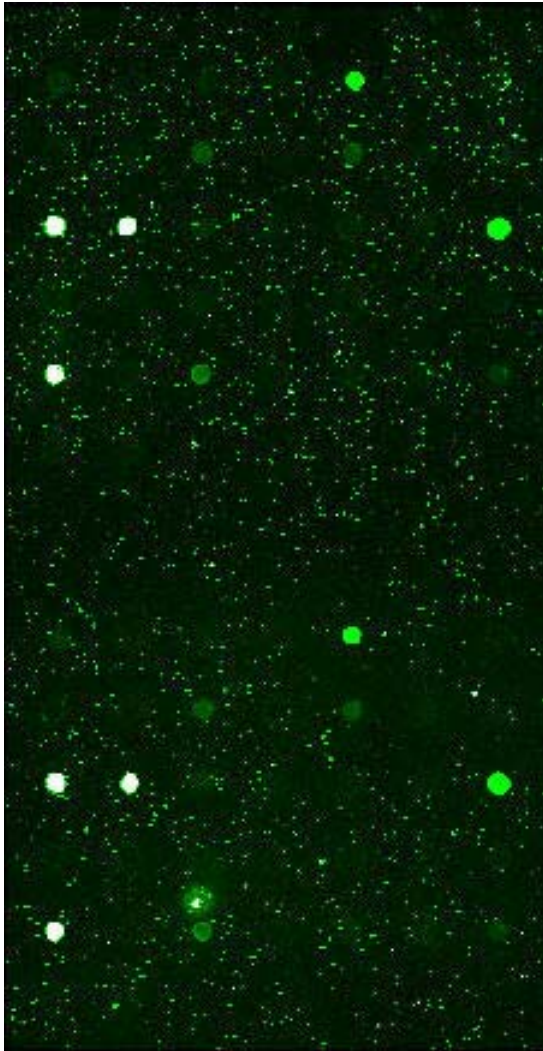
# *Interpretation of results*

- ◆ Direct observation of slide ?
- ◆ Exporting data
  - Fluorescent data
  - Background – local / global
  - Ratio data

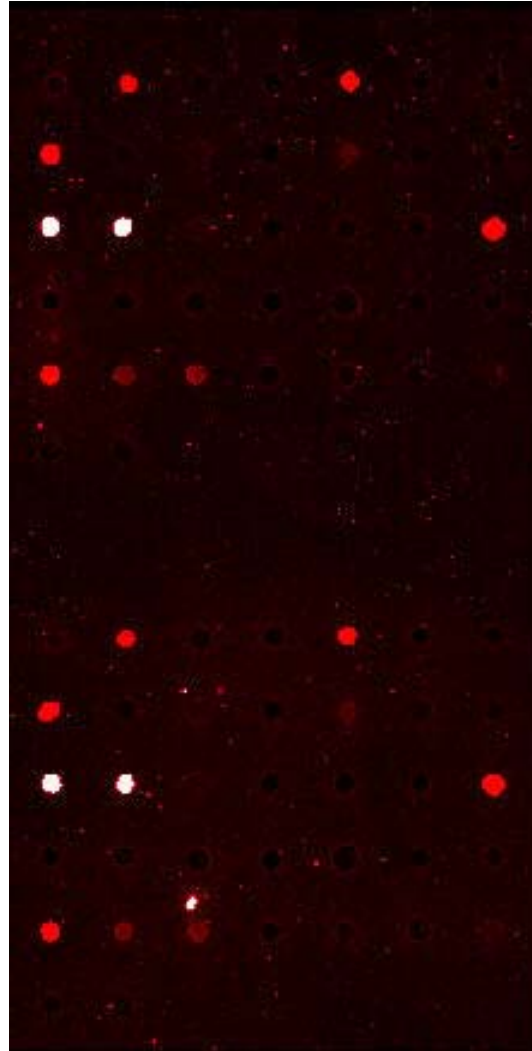


# *Interpretation - Two colour detection*

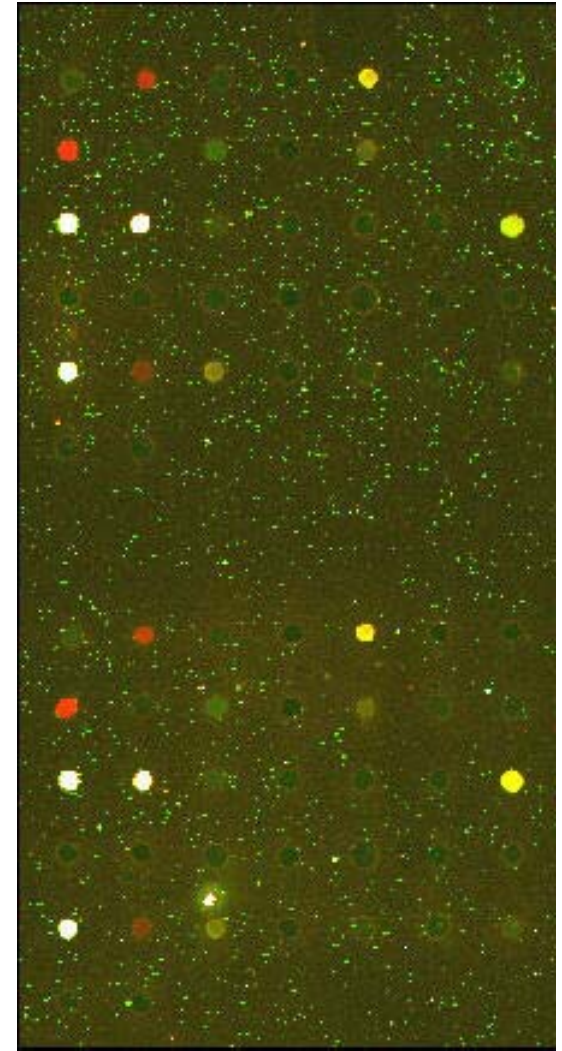
Healthy cDNA Cy3



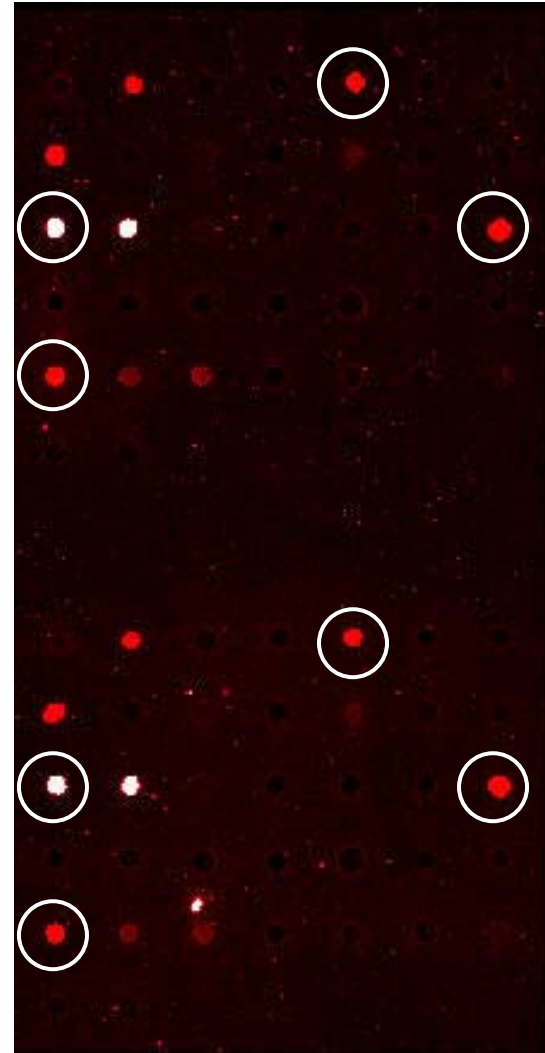
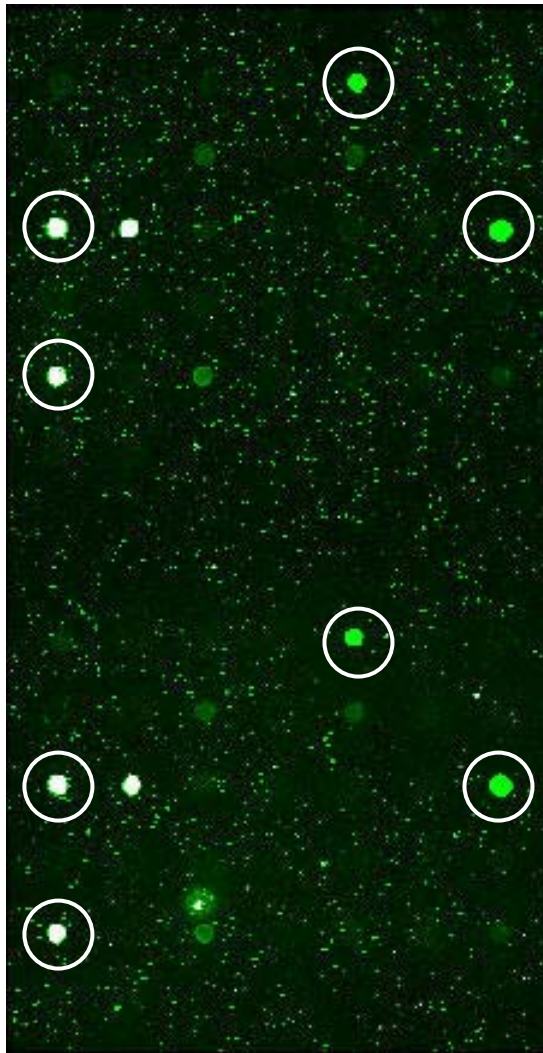
Test plant cDNA Cy5



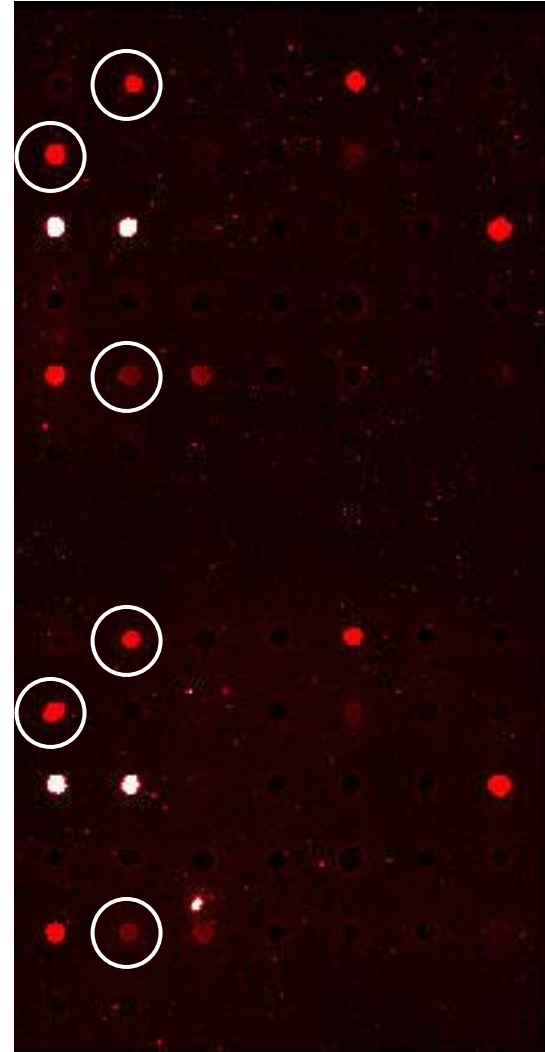
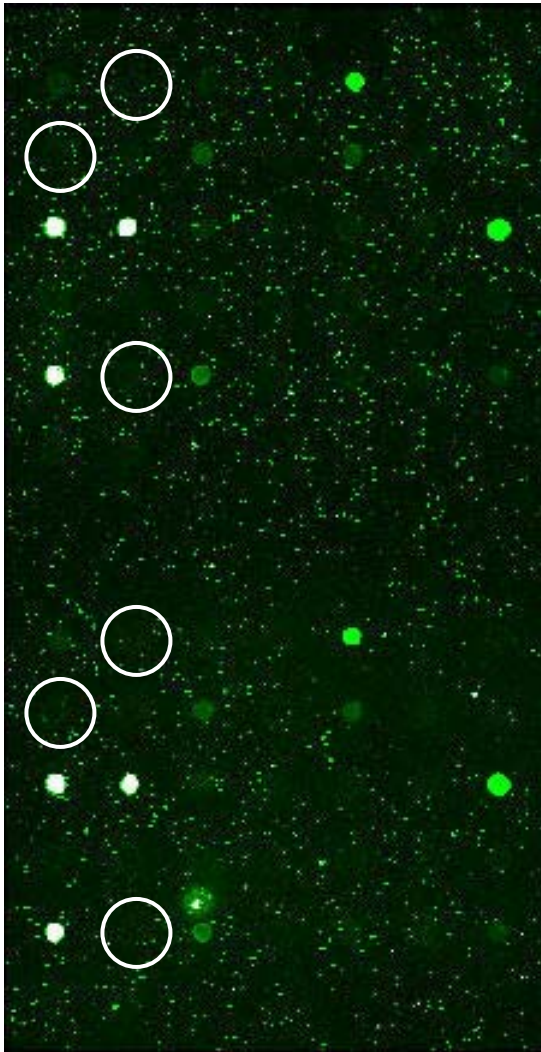
Ratio



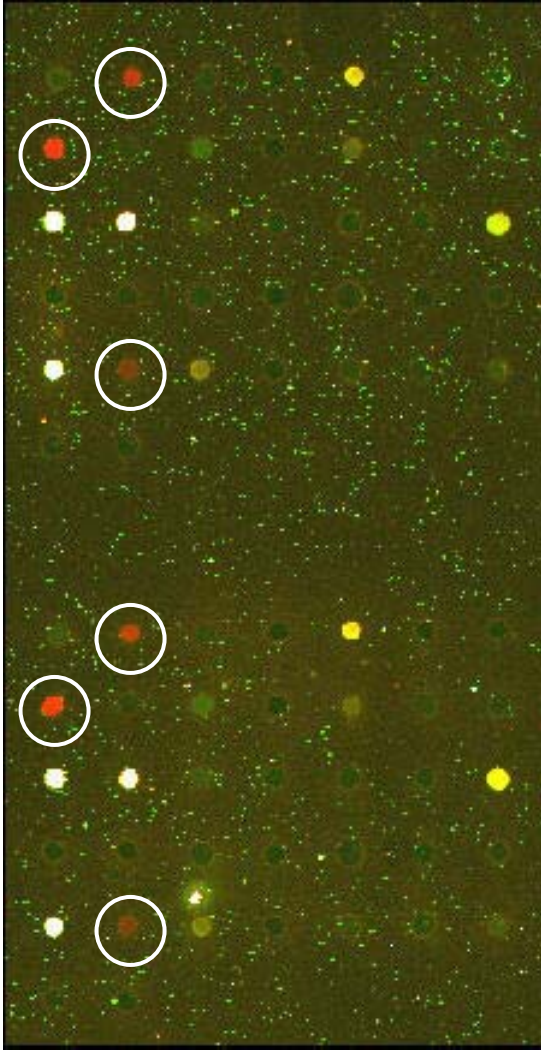
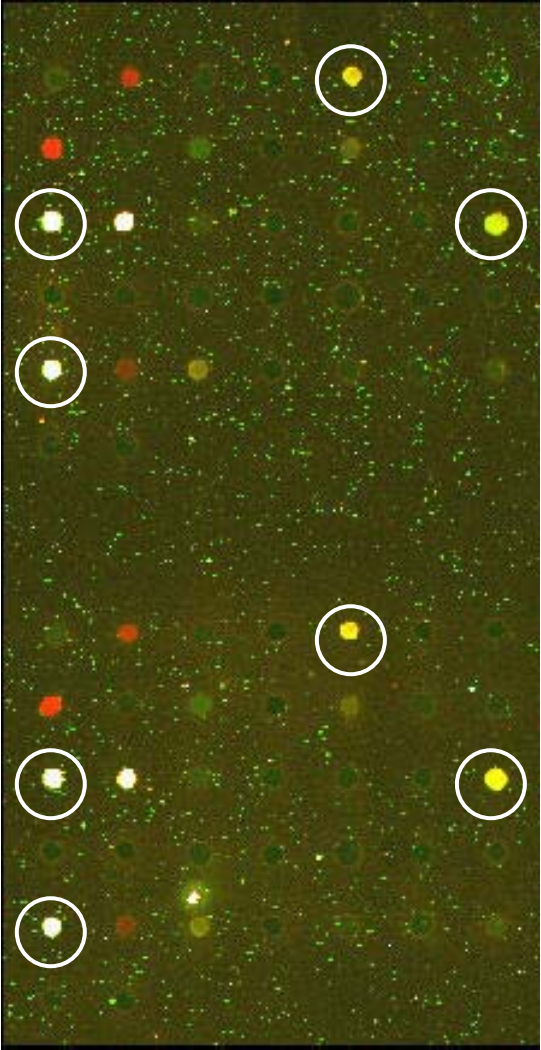
Control spots appear on both



Test spots appear on Cy5 not Cy3

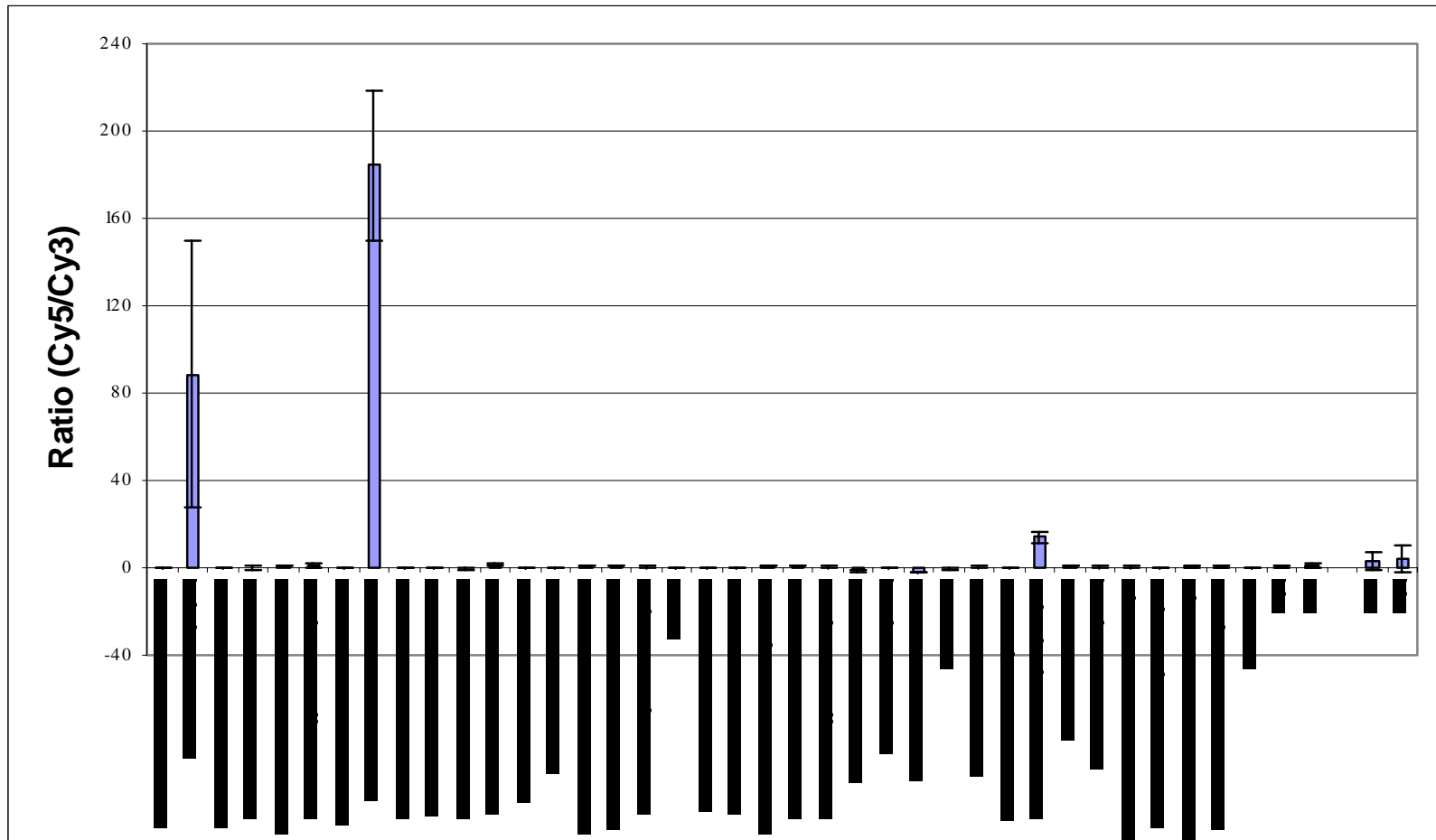


Control spots appear yellow Diagnostic spots appear red

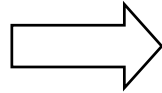




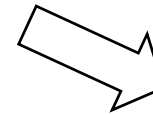
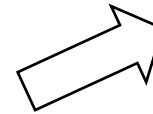
# Ratio data



**1** Sample



**2** Total Nucleic Acid Extraction



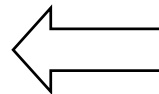
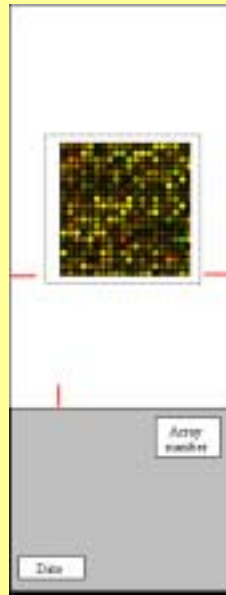
**3** RNA labelling



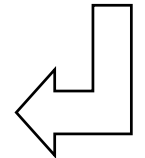
PCR labelling

Total time = 1.2 days  
(hands on  $\approx$  2 hours)

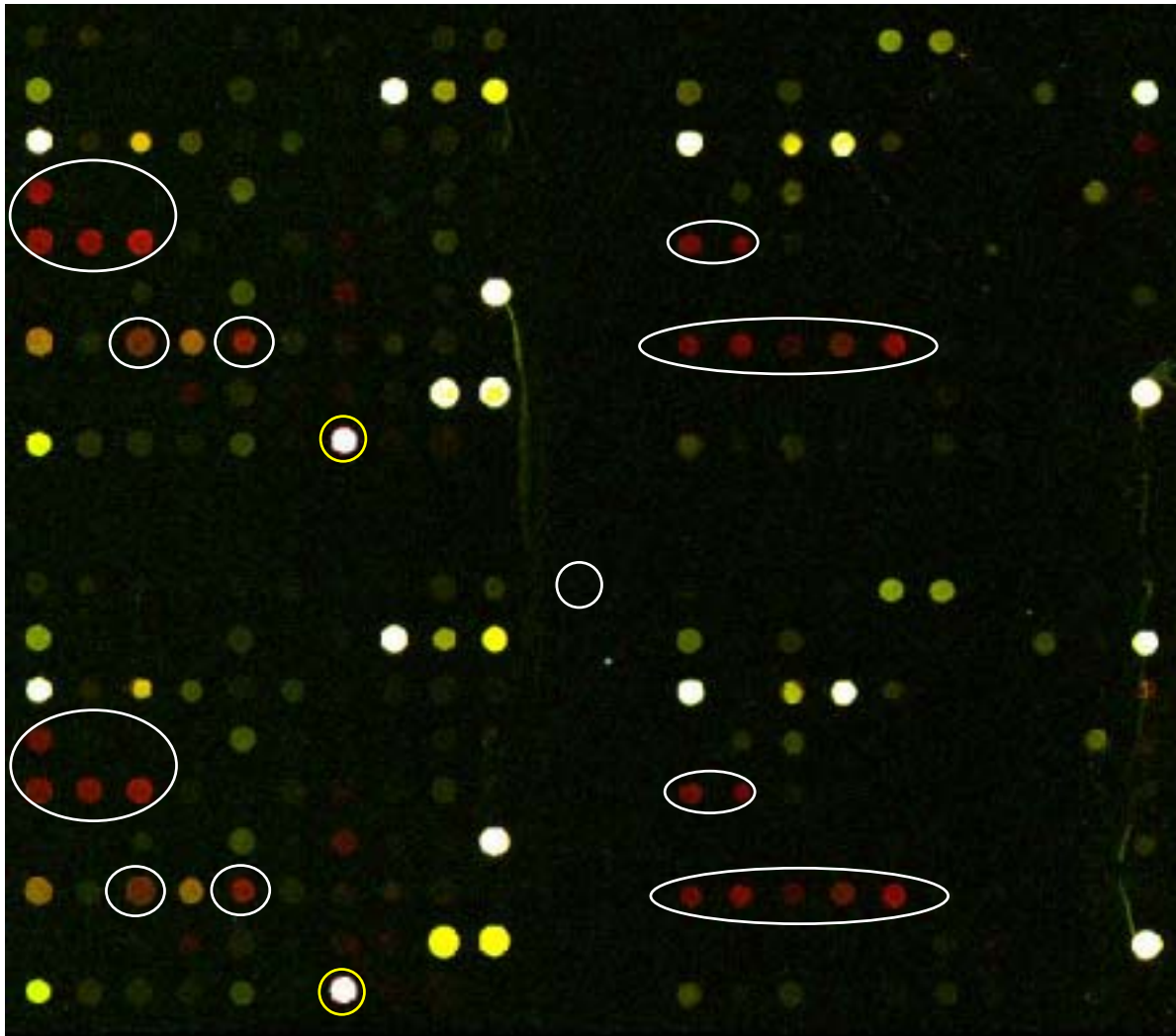
**5** Scan Arrays



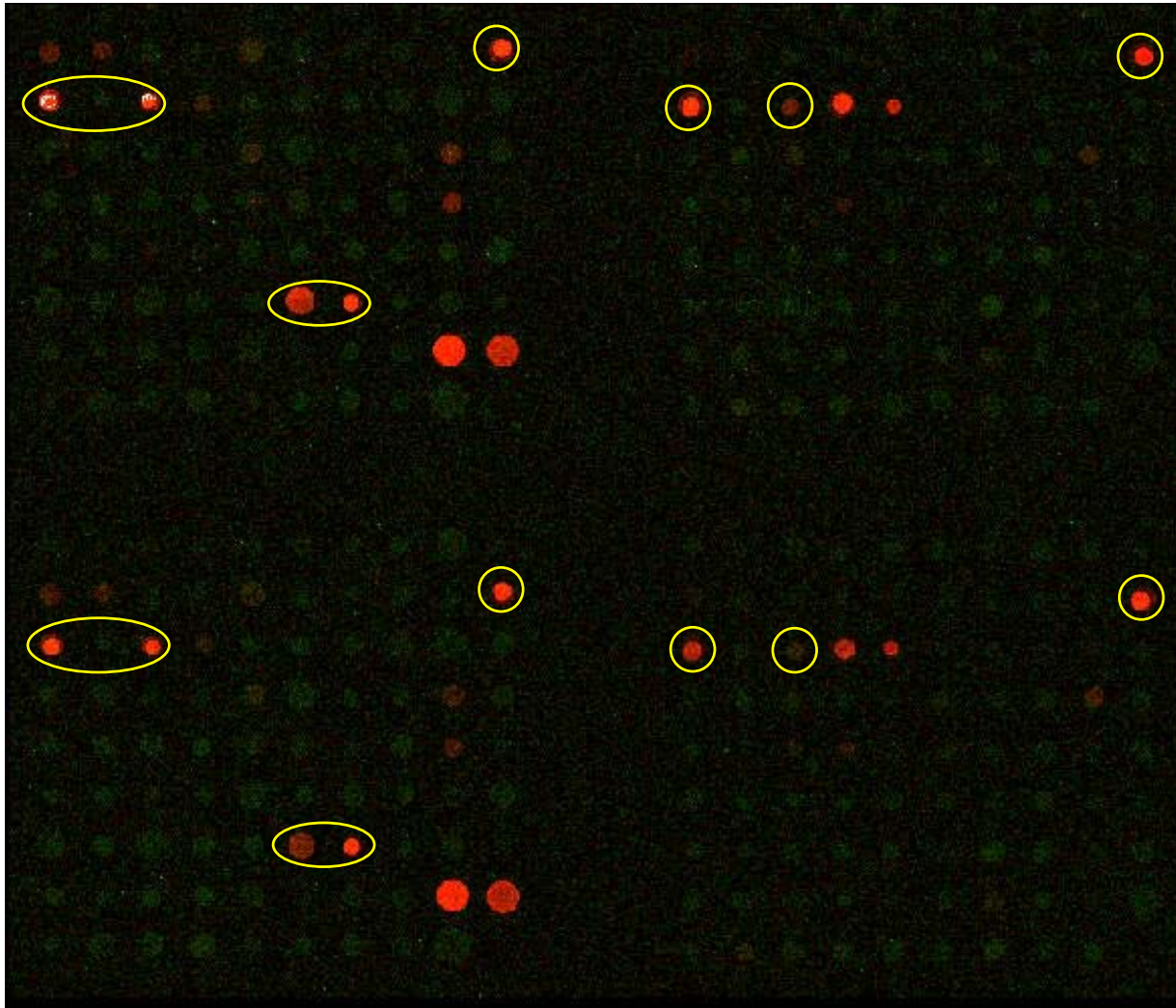
**4** Hybridisation



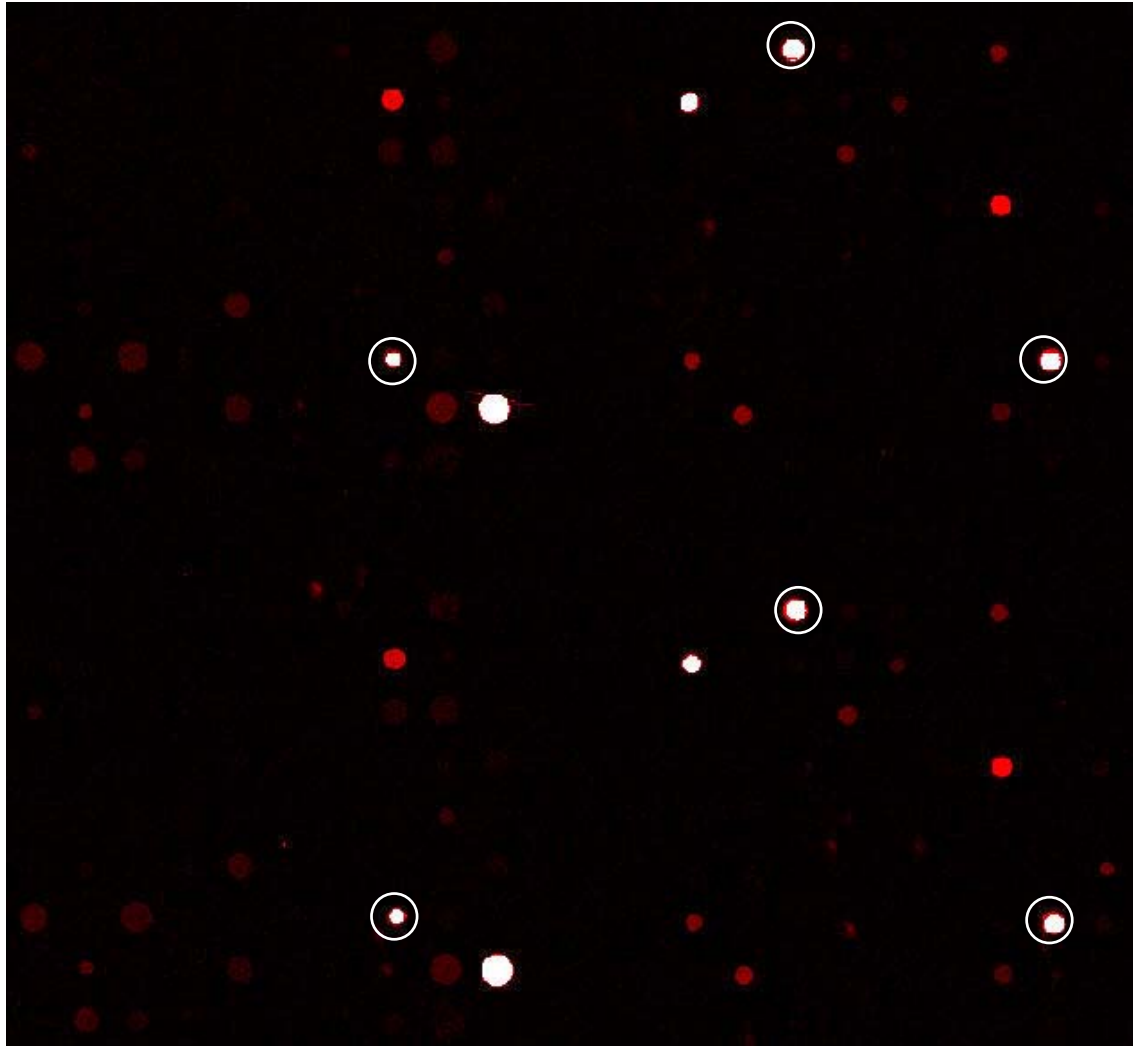
# *Results : PVY and PVX*



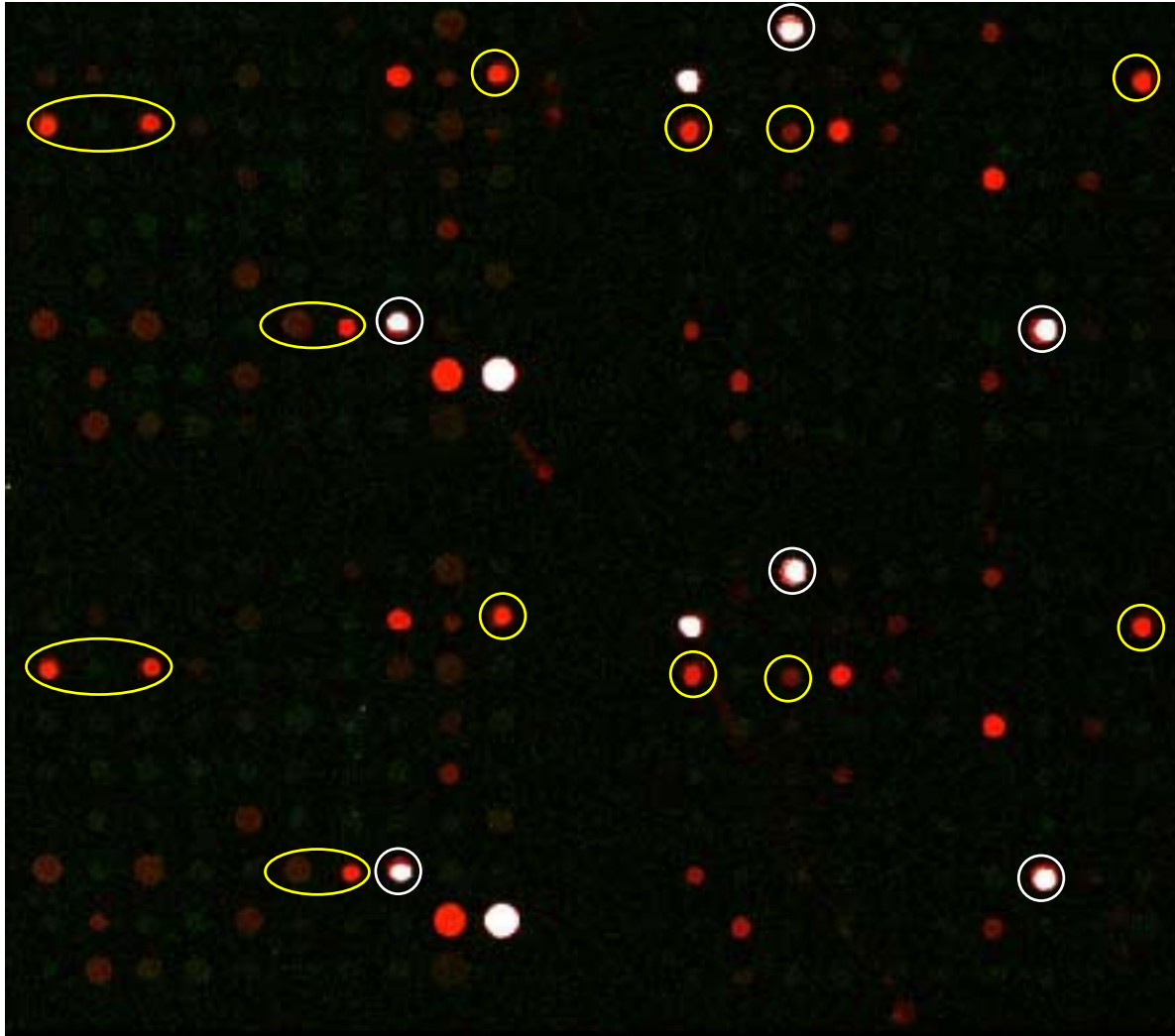
# *Results : Ralstonia*



# *Results : Melodogyne*

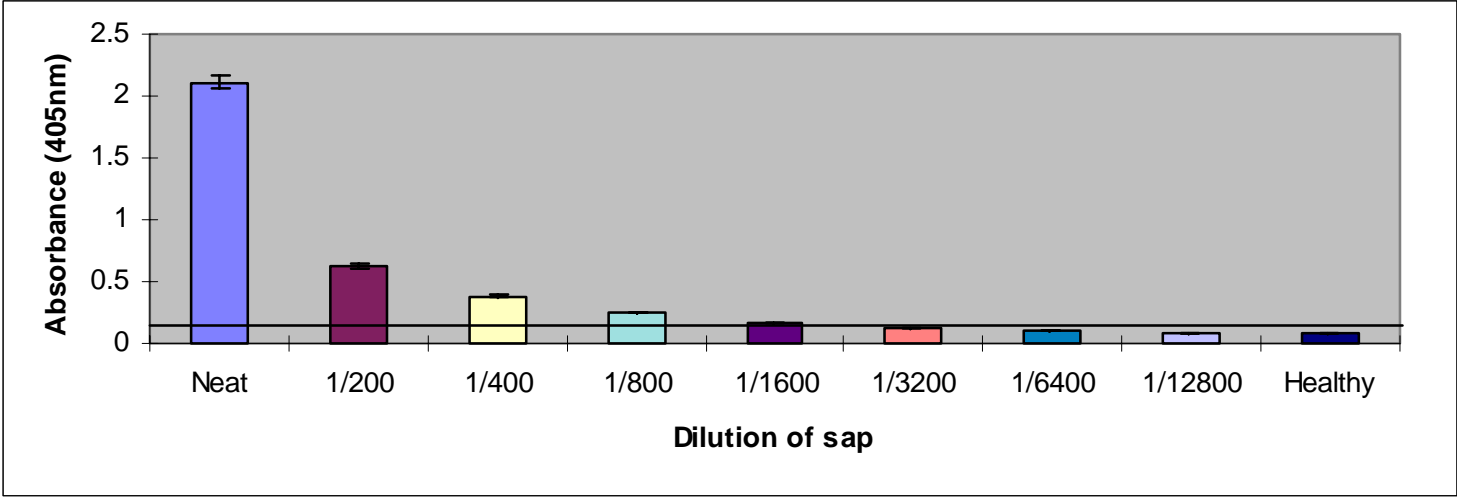


# *Results : multiplex nematode/bacteria*

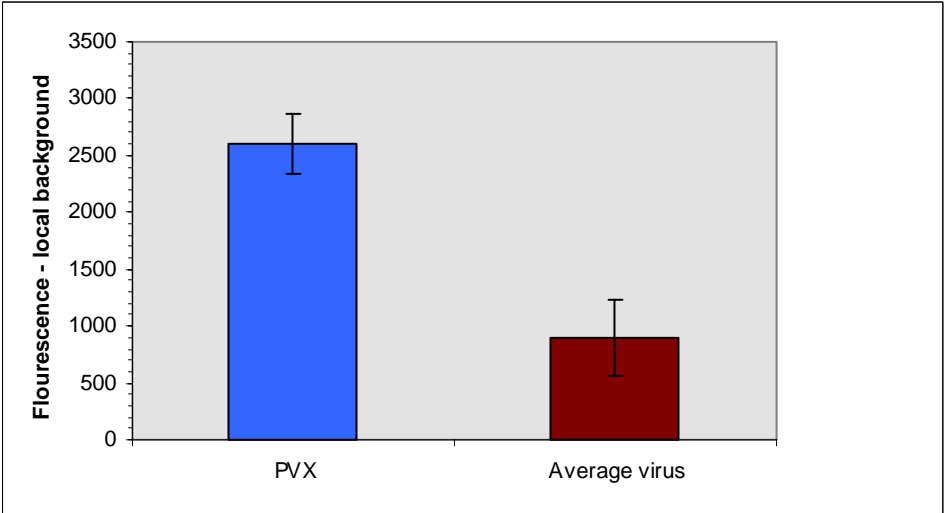


# Sensitivity

PVX  
ELISA

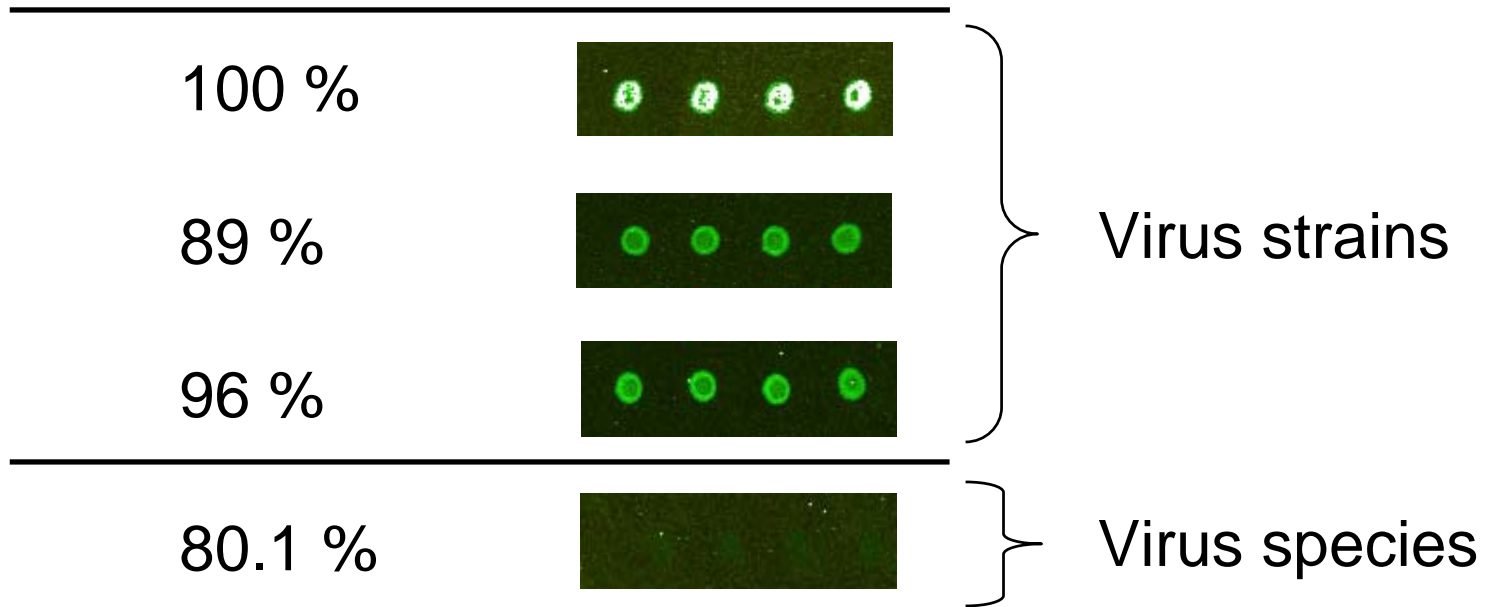


PVX  
Array (1/1600)



# Specificity

% Sequence  
Identity



# Conclusions

- ◆ Method works for multiple organisms
- ◆ Sensitivity is similar to ELISA
- ◆ Specificity to genus level using universal PCR



# *Future work*

- ◆ Need for generic amplification methods to increase specificity for all organisms  
e.g. Padlock probes
- ◆ Need for real applications  
e.g. Spherical virus array  
e.g. Petunia virus array



# Acknowledgements

Jenny Tomlinson

Kathy Walsh

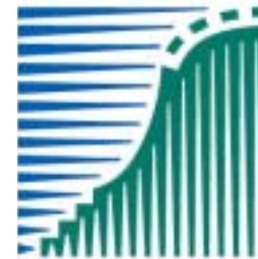
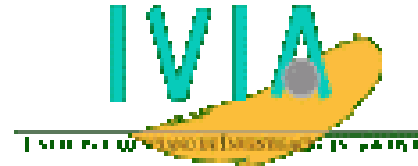
Ian Barker



# Acknowledgements

## ◆ EU consortium

- ◆ Ian Graham (CNAP)
- ◆ Kathryn Madagan (CNAP)
- ◆ Stephan Winter (DSMZ)
- ◆ Ismail Abdullahi (DSMZ)
- ◆ Philippe Castagnone (INRA)
- ◆ Maria Lopez (IVIA)
- ◆ Pablo Llop Perez (IVIA)
- ◆ Kevin Daish (MWG)
- ◆ Tony Gordon (MWG)
- ◆ Linda Kox (PD)



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