Diagnostic microbial microarrays

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Diagnostic microbial microarrays:

- Oligonucleotide microarrays
- Target evolutionary conserved genes (16/18S rRNA, *rpo*B, *pmo*A, *rbc*L, *nif*H)
- Enable the fast and parallel detection of hundreds to thousands of microorganisms from virtually any sample.
- Resolution: Subspecies or even strain level

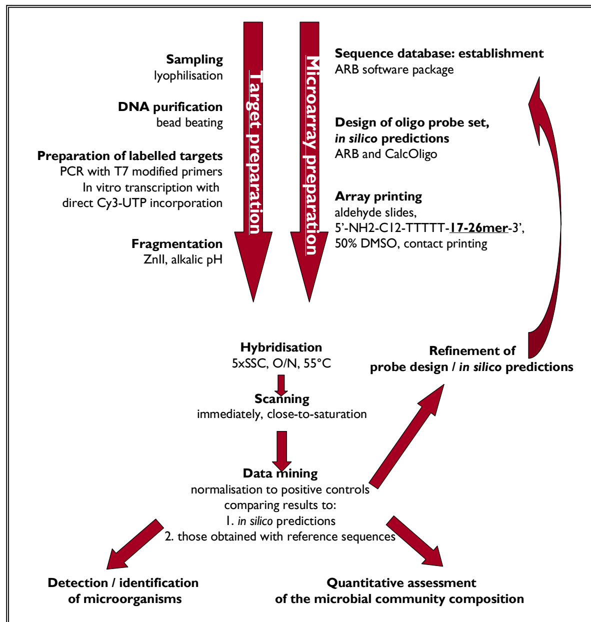
Application potential of diagnostic microarrays:

- Detection of human pathogens in clinical samples and wastewaters
- Detection of food-borne pathogens
- Detection of animal pathogens in veterinary samples
- Environmental analysis, ecotoxicology
- Environmental microbiology
- etc...

A complete set of techniques and procedures was developed for the design, production and application of diagnostic microbial microarrays

Reference: L.Bodrossy, Diagnostic oligonucleotide microarrays for microbiology In: E.Blalock (ed.): Microarrays and Bioinformatics for Beginners, Kluwer Academic Publishers, New York. In Press.

Outline of the experimental approach



Oligo set design

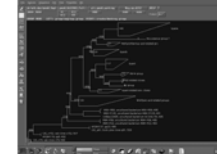
The design of oligo probe sets for diagnostic microarrays is a fairly laborious process. It is, however, well worth the investment of time and effort as all subsequent analyses are based on a set of assumptions regarding the specificity of each single probe. If these assumptions are false, all subsequent conclusions may be wrong and misleading. There is so far no single software package, which covers the entire process. We use the ARB phylogenetic software package, CalcOligo and Excel for this purpose.

Factors influencing the hyb. behaviour of oligo probes

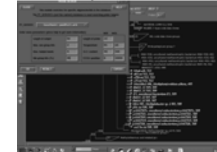
- Length, GC content and exact sequence of the probe - these together are considered when predicting T_m for the oligos by using the nearest neighbour method. Note: the predicted T_m applies for free oligos in solution.
- Position of GC and AT pairs. The middle of the probe is more important in stabilising hybridisation. A probe where the middle contains all Gs and Cs binds its target much stronger than another one with homogenous GC distribution (but with identical length and GC content).
- Secondary structure of the probe and of the corresponding target. When any of these two are of significant strength compared to the strength of hybridisation between the probe and the target, significant drop in hybridisation efficiency occurs.
- The exact nature of the overhanging nucleotides on the target. This comes from the nearest method model, but isn't normally accounted for as the overhangs of the target sequence are not considered.
- Number and type of mismatches. Some mismatches have little, others have very strong destabilising effect.
- Position of mismatches. Mismatches in the middle decrease hybridisation efficiency much stronger than mismatches in end positions.
- Factors arising from the immobilised nature of the probes. Steric effects can hinder the formation of hybrids between the target and the bound probe. This effect is much stronger for the bound end of the probe. Thus, effectively, the bound end of the probe plays a lesser role in the hybridisation than the free end.
- Hybridisation between DNA oligos and RNA fragments as in our case has got slightly different thermodynamics than DNA-DNA hybridisation.

Sequence alignment

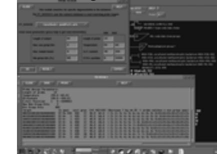
Tree construction



Probe design I

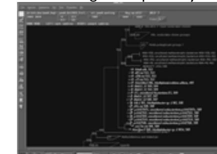


Probe design II



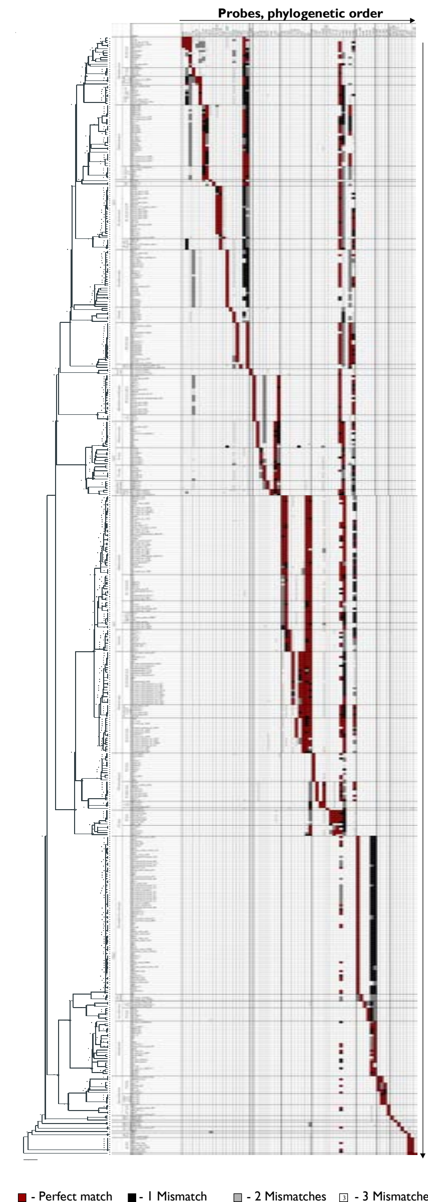
Probe design III

Probe design IV - specificity check



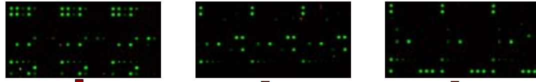
Oligo probe set for the methanotroph microarray:

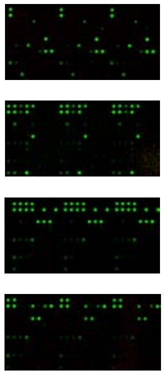
predicted hybridisation behaviour of the probe set



Oligo set : specificity testing

82% of the probes displayed hybridisation behaviour as predicted during *in silico* probe design; only 8% of them showed 1 or more unexpected positive or negative results when tested with selected reference strains.

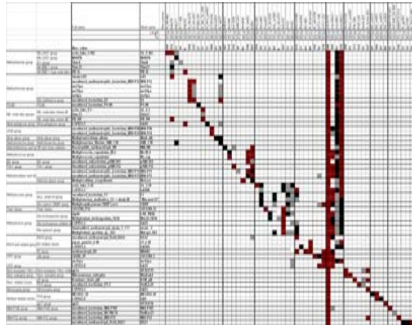




Oligo set for the methanotroph microarray:

Hybridisation behaviour of the probe set with reference strains

Probes, phylogenetic order

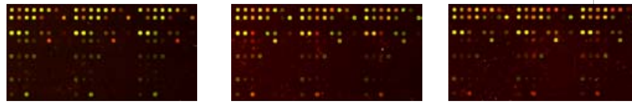


Strains, phylogenetic order

Hybridisation signals: ■ >50 arbitrary units ■ 20-50 arb.units □ 10-20 arb. units

Quantification of microbes with the microbial diagnostic chip

Artificial mixtures of methanotrophs were quantified with a high accuracy; average standard deviation from expected results was only 26%.

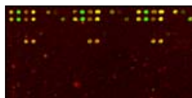


	Artificial Mixture #1		Artificial Mixture #2		Artificial Mixture #3	
	Ratios (%)	Measured ratios (%)	Ratios (%)	Measured ratios (%)	Ratios (%)	Measured ratios (%)
Clone SL-5.102	60	68	1	2.1	10	20
Methylocaldum szegediense OR2	30	21	3	4.7	1	2.3
Clone SL-5.70	10	6.1	60	36	3	3.6
Clone rbp46	3	4.0	30	43	60	51
Clone JY-6.48	1	1.8	10	12	30	22

Real life experiment, example

Analysis of the methanotroph community of Movile cave, a closed methane- and sulphur-dependent ecosystem.

The aim of this experiment was to analyse the community structure at the genus level



Community structure:

Methylobacter	5%
Methylomonas	40%
Methylococcus or Methylocaldum	5%
Methylocystis	50%

Conclusions

We can design, produce and apply diagnostic microbial microarrays for the rapid and parallel detection of microorganisms from virtually any sample.

Our arrays make it also possible to obtain quantitative data thus analysing microbial community structures.

Wet laboratory techniques

Target preparation

I. PCR amplification of DNA sequence for *in vitro* transcription

Design PCR primers to amplify the gene of interest. The primer of the strand to be labelled has to contain the T7 promoter site as well: 5'-TAATACGACTCACTATAG – actual primer-3'. In our case, this is always the reverse primer. Consequently, oligonucleotide probes are of the same sequence as the positive strand of the gene.

For each target start 3 PCR reactions of 50 µl volume each. Each 50µl reaction contains: 5µl 10x PCR buffer, 4µl dNTP mixture (2.5mM for each dNTP), 1.5µl 50 mM $MgCl_2$, 1-1µl of both primers (15pmol/µl ≈ 100ng/µl), 1U Taq polymerase (Gibco Life Sciences/Invitrogen). Leaving space for template DNA (10 ng for environmental DNA, 1 ng for genomic DNA (gDNA) or 0.1 ng for plasmid DNA), add ultrapure water to 50µl.

95°C, 5 min. Pause @95°C. Add template DNA ("hot start" to minimise mispriming). 32 cycles of 1 min @ 95°C; 1 min at the annealing temperature; 1 min @72°C for every 1000 bp to be amplified. A final 10 mins @72°C to allow the completion of all ongoing or unfinished amplification reactions.

Pool parallel PCR products (3x50µl) and purify with a commercial PCR purification kit according to manufacturer's instructions (we use the HighPure PCR purification kit from Macherey-Nagel). Dissolve or elute purified DNA in ultrapure water keeping in mind that the final concentration has to be adjusted to 50 ng/µl.

Measure the concentration of purified DNA by spectrophotometry (Concentration of dsDNA = (A_{260} x Dilution rate)/0.02 [ng/µl]). For example: Dilute 1µl DNA in 49µl water, measure absorbance at 260nm in a 50µl cuvette with 10mm light path. An A_{260} of 0.1 refers to (0.1x50)/0.02=250 ng/µl dsDNA concentration. Adjust concentration to 50ng/µl with ultrapure water. Store at-20°C.

II. In vitro transcription

Work under RNAse-free conditions.

This is achieved by observing the following basic rules:

- Always wear clean gloves when working with RNA
- Use filter tips from separate boxes used for RNA work only
- Use plasticware autoclaved at 121°C for 60 minutes
- Use RNAse free solutions
- Most solutions (and also glassware) can be made RNAse free by DEPC (diethylpyrocarbonate)-treatment (add 0.01% DEPC, shake, incubate at 37°C overnight, autoclave at 121°C for 60 minutes). Tris and other amine-containing buffers cannot be treated with DEPC; best is to buy them RNAse free from major suppliers. Warning: DEPC is toxic; Autoclaving is needed to inactivate it!!!
- Use chemicals dedicated to RNA work; try to measure them straight into RNAse free glassware without using spatulas. If spatulas have to be used, sterilise them by ethanol flaming.

Into an RNAse-free Eppendorf tube add:

- 8 µl 50 ng/µl purified PCR product;
- 4 µl 5x T7 RNA polymerase buffer;
- 2 µl 100mM DTT;
- 0.5 µl 40 U/µl RNasin (Promega);
- 1 µl each of 10mM ATP, CTP, GTP;
- 0.5 µl 10mM UTP;
- 1 µl 40U/µl T7 RNA polymerase (Gibco BRL);
- 1 µl 5mM Cy3 or Cy5-UTP

Incubate @37°C, for 4 hours.

Purify labelled RNA immediately.

III. RNA purification

We use the Quiagen RNe
asy kit .

This step removes unincorporated nucleotides, DNA template, T7 polymerase and salts.

Work under RNAse free conditions.

Add 80µl DEPC treated water to the IVT mix.

- Add 350µl RLT solution (provided with the kit); mix thoroughly.
- Add 250µl EtOH; mix thoroughly.
- Sample (700µl) into an RNeasy mini column. 15 secs @>10,000 rpm.
- Transfer column into a new 2ml collection tube. Add 500µl RPE solution (provided with the kit), 15 secs @>10,000 rpm.
- Add 500µl RPE, 2 minutes @>10.000 rpm.
- Transfer column into a 1.5 ml collection tube. Add 50µl RNAse free water. 1 minute @>10,000 rpm. Transfer the eluate into a new 1.5 ml Eppendorf tube.

IV. Zn2+ fragmentation of RNA

This procedure fragments RNA into pieces with an average length of 50 nt.

Work under RNAse free conditions.

To 50 µl purified RNA (in a 1.5 ml Eppendorf tube, from the previous step) add:

- 1.43 µl,1M Tris.Cl pH 7.4
- 5.71 µl,100mM $ZnSO_4$

Mix; incubate at 60°C, 30 minutes.

Note: Use dry block and do not mix during the incubation because the condensation on the lid of the tube is also included in the optimisation of the protocol (it causes gradual concentration of the reaction, thus influencing efficiency of fragmentation).

Add 1.43 µl 500mM EDTA pH 8.0 to stop the reaction (by chelating Zn^{2+}).

Put on ice for 1 min; add 1 µl 40 U/µl RNasin.

Fragmented, labelled RNA target can now be stored at -20°C for several months.

Hybridisation

To ensure good control over hybridisation temperature, a custom tailored aluminium block is used with holes of 11mm in depth for 14 slides. The block is used as an insert for a Belly Dancer (Stovall Life Sciences). 1-2 mm spacing between the block and the wall of the water bath chamber allows for the addition of about 20 ml water, which is required for reliable heat transfer to the aluminium block.

Method:

- Preheat hybridisation block to 55°C. Allow for at least 30 minutes for the temperature to stabilise.
- Preheat an Eppendorf incubator (dry block) to 66°C.
- Apply 200 µl HybriWell (Grace BioLabs) chambers onto the slides containing the arrays. Preheat assembled slides on top of the hybridisation block. The BellyDancer should be set to maximum bending.
- Per hybridisation add into a 1.5 ml Eppendorf tube:
  - 124 µl DEPC-water
  - 2 µl 10% SDS
  - 4 µl 50x Denhardt's reagent (Sigma)
  - 60 µl 20x SSC
  - 10 µl target RNA
- Incubate at 65°C for 1 - 15 mins.
- Apply preheated hybridisation mixtures onto assembled slides via the port in the lower positions (to minimise risk of air bubbles being trapped within the chamber). Seal chambers with seal spots.
- Incubate overnight in Belly Dancer (30-40 rpm circulation at maximum bending).
- Take slides one by one, remove sticky chamber and put them immediately into
- 2xSSC, 0.1% SDS at RT.
- Wash slides by shaking at RT for:
  - 5 min in 2xSSC, 0.1% SDS;
  - 2x5 min in 0.2x SSC;
  - 5 min in 0.1x SSC
- Dry slides one by one using an airgun with a cottonwool filter inside (to keep oil microdroplets away from the slide surface). Apply a modest stream to the area containing the array first to blow the drops down on the slide, rather than drying them onto it.
- Scan slides the same day.

Scanning

Prescan the whole slide without averaging. Start with a photomultiplier voltage likely to be near but still below saturation. When prescan is started, finish to the end of the spotted area as each round of scanning causes photobleaching of the spots. Partial scanning of the images may thus introduce variation. Find the optimal photomultiplier voltage that yields the highest signals possible without saturation.

Scan the spotted area at 3 lines to average, 10µm resolution. Save as multi-layer Tiff image (required to enable later analysis of the scanned image).

Array printing

50% DMSO is used as printing buffer for the following advantages

- It doesn't dry during long spotting rounds (a routine spotting of 100 oligos onto 100 slides takes about 6 hours) unlike aqueous solutions, such as 3xSSC or phosphate buffers.
- It provides uniform spots on the slides applied. Standard deviation in signal intensities between replicate spots is 10-15% as opposed to 20-30% for 3xSSC (Table C).

384 well plates are used because of the smaller evaporation rate and smaller volume required.

1 pin is applied to avoid variations inherent in spotting with multiple pins.

50% humidity and 22°C provides optimal conditions in our hands to yield uniform, homogenous spots from 50% DMSO.

Reduction of the free aldehydes makes prehybridisation with BSA or other aminated compounds (which then serve to block free aldehyde groups) unnecessary.

Method:

- Prepare a 384 well flat bottom plate with 30 µl 50 µM oligonucleotide solutions in 50% DMSO.
- Spot samples with an OmniGrid spotter (1 TeleChem SMP3 pin) at 50% relative humidity, 22°C. This pin takes 250 nl sample per run and deposits 0.6 nl of it per spot.
- Incubate spotted slides overnight at room temperature at <30% relative humidity. The formation of the Schiff base between the aldehyde and amino groups yields water. Low humidity levels help this reaction.
- Rinse slides twice in 0.2% (w/v) SDS for 2 min at room temperature (20-25°C), with vigorous agitation to remove the unbound DNA.
- Rinse slides twice in dH2O for 2 min at room temperature, with vigorous agitation.
- Transfer slides into dH2O at 95-100°C for 2 min to denature the DNA.
- Allow slides to cool at room temperature (~5 min).
- Treat slides in a freshly (right before use) prepared sodium borohydride solution for 5 min at room temperature to reduce free aldehydes. Sodium borohydride solution: Dissolve 0.5 g NaBH4 in 150 ml phosphate buffered saline (PBS; 8g NaCl, 0.2g KCl, 1.44g Na2HPO4, 0.24g KH2PO4, in 1000 ml H2O, pH 7.4, autoclaved), then add 44 ml of 100% ethanol to reduce bubbling.
- Rinse slides three times in 0.2% (w/v) SDS for 1 min each at room temperature.
- Rinse slides once in dH2O for 1 min at room temperature.
- Dry slides, one by one, using an airgun fitted with a cottonwool filter inside (to keep oil microdroplets away from the slide surface). Apply a modest stream of air first to the area containing the array, blowing the drops down on the slide, rather than drying them onto it. Dried slides can be stored at room temperature in the dark for several months.

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