Design of microarray experiments

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Experiments

Scientists deal mostly with experiments of the following form:

- A number of alternative conditions / treatments
- one of which is applied to each experimental unit
- an observation (or several observations) then being made on each unit.

The objective is:

- Separate out differences between the conditions / treatments from the uncontrolled variation that is assumed to be present.
- Take steps towards understanding the phenomena under investigation.

Statistical thinking

Uncertain knowledge + Knowledge of the extent of uncertainty in it = Useable knowledge

Measurement model

$m = \mu + e$

m – measurement with error, μ - true but unknown value

What is the mean of e?
What is the variance of e?
Is there dependence between e and μ?
What is the distribution of e (and μ)?

Decisions on the experimental design influence the measurement model.

Typically but not always: $e \sim N(0,\sigma^2)$

Gaussian / Normal measurement model

Main requirements for experiments

Once the *conditions / treatments*, *experimental units*, and the *nature of the observations* have been fixed, the main requirements are:

- Experimental units receiving different treatments should differ in no systematic way from one another – Assumptions that certain sources of variation are absent or negligible should, as far as practical, be avoided;
- Random errors of estimation should be suitably small, and this should be achieved with as few experimental units as possible;
- The conclusions of the experiment should have a wide range of validity;
- The experiment should be simple in design and analysis;
- A proper statistical analysis of the results should be possible without making artificial assumptions.

Taken from Cox DR (1958) *Planning of experiments*, Wiley & Sons, New York (page 13)

The most simple measurement model in microarray experiments

Situation: m arrays (Affimetrix) from *control* population
n arrays (Affimetrix) from population with
special condition /treatment

Observation of interest: Mean difference of log-transformed gene expression (ΔlogFC)

$$\Delta\log FC_{obs} = \Delta\log FC_{true} + e$$

$$e \sim N(0, \sigma^2 \cdot [1/n+1/m])$$

In an experiment with 5 arrays per population and the same variance for the expression of a gene of interest, the above formula implies that the variance of the ΔlogFC is only 40% (1/5+1/5 = 2/5 = 0.4) of the variability of a single measurement – ***taming of uncertainty***.

Separate out differences between the conditions / treatments from the uncontrolled variation that is assumed to be present.

Is $\Delta \log FC_{true} \neq 0$? – How to decide?

Special Decision rules: Statistical Tests

- When the probability model for the mechanism generating the observed data is known, hypotheses about the model can be tested.
- This involves the question: Could the presented data reasonable have come from the model if the hypothesis is correct?
- Usually a decision must be made on the basis of the available data, and some degree of uncertainty is tolerated about the correctness of that decision.
- These four components: data, model, hypothesis, and decision are basic to the statistical problem of hypothesis testing.

Quality of decision

	True state of gene	
Decision	Gene ***is*** diff. expr.	Gene ***is not*** diff. expr.
Gene ***is*** diff. expr.	**OK**	**false positive decision** happens with probability α
Gene ***is not*** diff. expr.	**false negative decision** happens with probability β	**OK**

Two sources of error: False positive rate α
False negative rate β

Power of a test: Ability to detect a difference if there is a true difference
Power – true positive rate or Power = 1 - β

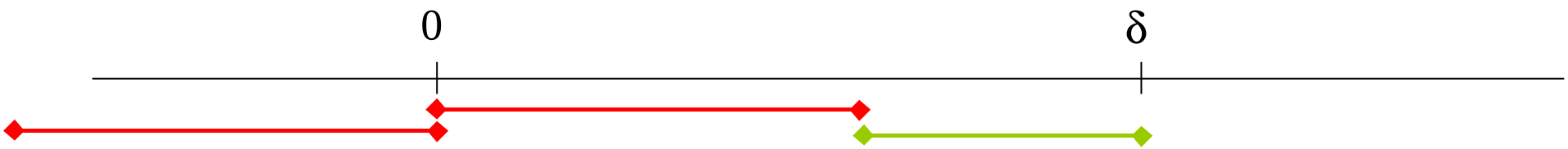
The Statistical test

- Question of interest (*Alterative*): Is the gene G differentially expressed between two cell populations?
- Answer the question via a ***proof by contradiction***: Show that there is no evidence to support the logical contrary of the *alternative*. The logical contrary of the *alternative* is called *null hypothesis*.
- *Null hypothesis*: The gene G is not differentially expressed between two cell populations of interest.
- A *test statistic* **T** is introduced which measures the fit of the observed data to the *null hypothesis.*
  The test statistics T implies a *prob. distribution* **P** to quantify its variability when the *null hypothesis* is true.
- It will be checked if the *test statistic* evaluated at the observed data t_{obs} behaves typically (not extreme) with respect to the *test distribution*.
  The *p-value* is the probability under the null hypothesis of an observation which is more extreme as the observation given by the data: $\mathbf{P}(T \geq t_{obs}) = p$.
- A criteria is needed to asses *extreme behaviour* of the test statistic via the *p – value* which is called the *level of the test:* **a .**
- The observed data does not fit to the null hypothesis if $p < \mathbf{a}$ or $|t_{obs}| > t^*$ where t^* is the $1-\alpha$ or $1-\alpha/2$ quantile of the prob. distribution P. t^* is also called the *critical value.*
  The conditions $p < \mathbf{a}$ and $t_{obs} > t^*$ are equivalent. If $p < \mathbf{a}$ or $t_{obs} > t^*$ the null hypothesis will be rejected.
- If $p \geq \mathbf{a}$ or $t_{obs} \geq t^*$ the null hypothesis can not be rejected – this does not mean that it is true
  Absence of evidence for a difference is no evidence for an absence of difference.

Controlling the power – sample size calculations

The test should produce a significant result (level α) with a power of $1-\beta$ if $\Delta\text{logFC}_{true} = \delta$

null hypothesis: $\Delta\text{logFC}_{true} = 0$ alternative: $\Delta\text{logFC}_{true} = \delta$



$z_{1-\alpha/2}\ \sigma_{n,m}$ $z_{1-\beta}\ \sigma_{n,m}$ $\sigma^2_{n,m} = \sigma^2 \cdot (1/n+1/m)$

The above requirement is fulfilled if: $\delta = (z_{1-\alpha/2} + z_{1-\beta}) \cdot \sigma_{n,m}$

or

$$\frac{n \cdot m}{n+m} = \frac{(z_{1-\alpha/2} + z_{1-\beta})^2 \cdot \sigma^2}{\delta^2}$$

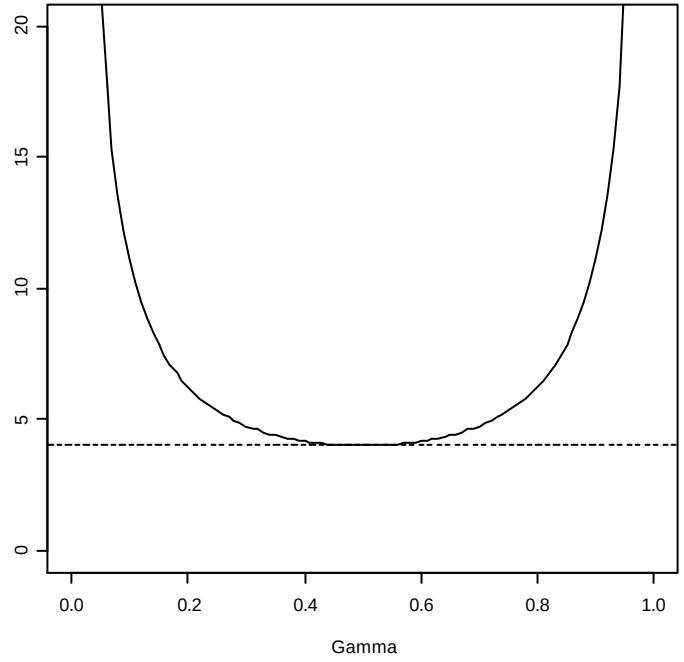
Controlling the power – sample size calculations

$$\frac{n \cdot m}{n+m} = \frac{(z_{1-\alpha/2} + z_{1-\beta})^2 \cdot \sigma^2}{\delta^2}$$

n = N·γ and m = N·(1-γ) with M – total size of experiment and $\gamma \in]0,1[$

$$N = \frac{1}{\gamma \cdot (1-\gamma)} \cdot \frac{(z_{1-\alpha/2} + z_{1-\beta})^2 \cdot \sigma^2}{\delta^2}$$

The size of the experiment is minimal if g = ½.



Sample size calculation for a microarray experiment I

	Truth		
Test result	diff. expr. (H_1)	not diff. expr. (H_0)	
diff. expr.	D_1	D_0	D
not diff. expr.	U_1	U_0	U
Number of genes on array	G_1	G_0	G

$\alpha_0 = E[D_0]/G_0$ $\beta_1 = E[U_1]/G_1$ $FDR=E[D_0/D]$

E: expectation / mean number

family type I error probability: $\alpha_F = P[D_0>0]$

family type II error probability: $\beta_F = P[U_1>0]$

Sample size calculation for a microarray experiment II

Independent genes	Dependent Genes
$P[D_0=0] = (1-\alpha_0)^{G_0} = 1-\alpha_F$ $D_0 \sim \text{Binomial}(G_0, \alpha_0)$ $E[D_0] = G_0 \cdot \alpha_0$ Poissonapprox.: $E[D_0] \sim -\ln(1-\alpha_F)$ $P[U_1=0] = (1-\beta_1)^{G_1} = 1-\beta_F$ $E[U_1] = G_1 \cdot (1-\beta_1)$	Bonferroni: $\alpha_0 = \alpha_F / G_0$ No direct link between the probability for D_0 and α_F. $1-\beta_F \geq \max\{0, 1- G_1 \cdot \beta_1\}$ No direct link between the probability for U_1 and β_F.

Sample size calculation for a microarray experiment III

for an array with 33000 independent genes

What are useful α_0 and β_1?

$\alpha_F = 0.8$ $E[D_0] = - \ln(1-0.8) = 1.61 = \lambda$ $P(\text{exactly k false pos.}) = \exp(-\lambda) \cdot \lambda^k / (k!)$

false pos.	0	1	2	3	4	5
Prob.	0.200	0.322	0.259	0.139	0.056	0.018

P(at least six false positives) = 0.0062

32500 unexpressed genes: $\alpha_0 = 1.61/32500 = 0.0000495$

500 expressed genes, set $E[D_1] = 450$ $1-\beta_1 = 450/500 = 0.9$ $\beta_1 = 0.1$ $1-\beta_F = (1-\beta_1)^{G_1} < 10^{-23}$

E[FDR] = 0.0035 95% quantile of FDR: 0.0089 (calculated by simulation)

Sample size calculation for a microarray experiment IV

In order to complete the sample size calculation for a microarray experiment, information on σ^2 is needed.

The size of the experiment, N, needed to detect a $\Delta\text{logFC}_{\text{true}}$ of δ on a significance level α and with power $1-\beta$ is:

$$N = 4 \cdot \frac{(z_{1-\alpha/2} + z_{1-\beta})^2 \cdot \sigma^2}{\delta^2}$$

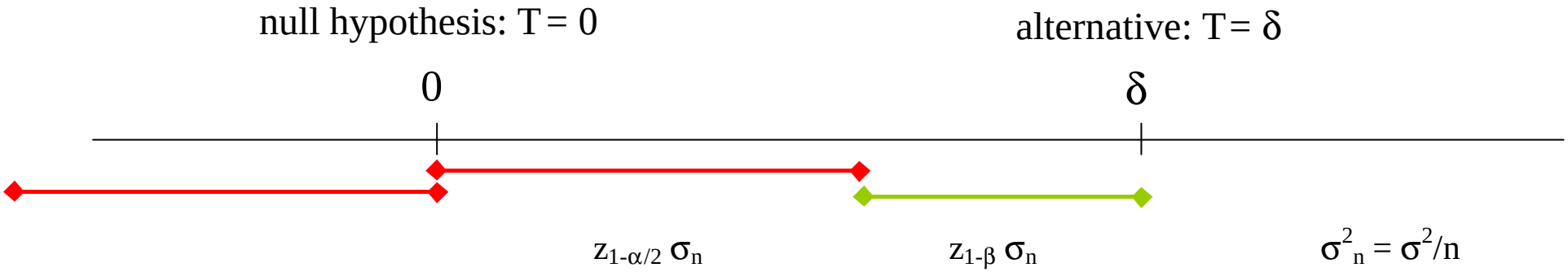
In a similar set of experiments σ^2 for a set of 20 VSN transformed arrays was between 1.55 and 1.85. One may choose the value $\sigma^2 = 2$.

δ	log(1.5)	log(2)	log(3)	log(5)	log(10)
N ($\sigma^2 = 2$)	1388	476	190	88	44
N ($\sigma^2 = 1$)	694	238	96	44	22

Sample size with $\alpha = 0.0000495$, $\beta = 0.1$

Sample size formula for a one group test

The test should produce a significant result (level α) with a power of $1-\beta$ if $T = \delta$



The above requirement is fulfilled if: $\delta = (z_{1-\alpha/2} + z_{1-\beta}) \cdot \sigma_n$

or

$$n = \frac{(z_{1-\alpha/2} + z_{1-\beta})^2 \cdot \sigma^2}{\delta^2}$$

Measurement model for cDNA arrays

Gene expression under condition A – intensity of red colour,
Gene expression under condition B – intensity of green colour

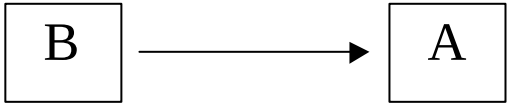
$$\text{Measurement: } m_{A/B} = \text{Log}_2\left(\frac{I_{red,A}}{I_{green,B}}\right) = \gamma_{A/B} + \delta + e$$

$\gamma_{A/B}$ – log-transformed true fold change of gene of condition A with respect to condition B
δ - dye effect, e – measurement error with E[e] = 0 and Var(e) = σ^2

Measurement $m_{A/B}$ is used to estimate unknown $\gamma_{A/B}$

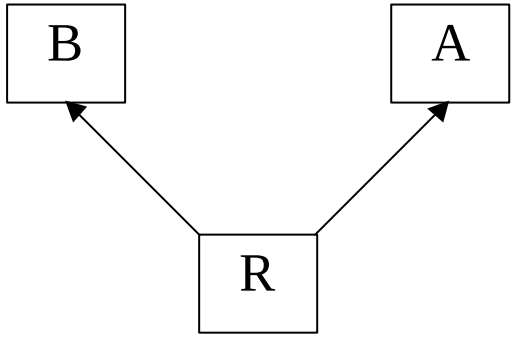
- *Vertices* mRNA samples
- *Edges* hybridization
- *Direction* Dye assignment

  Green ⟶ Red

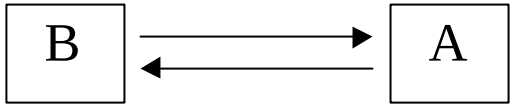


Estimation of *log fold change* $g_{A/B}$

Reference Design



Dye swap design



Estimate of $\gamma_{A/B}$

$$g^{R}_{A/B} = m_{A/R} - m_{B/R}$$

$$g^{DS}_{A/B} = (m_{A/B} - m_{B/A})/2$$

Variability of estimate

$$Var(g^{R}_{A/B}) = 2 \cdot \sigma^2$$

$$Var(g^{DS}_{A/B}) = 0.5 \cdot \sigma^2$$

Sample Size increases proportional to the variance of the measurement!

2x2 factorial experiments I

treatment / condition	Wild type	Mutation
before treatment	β	$\beta+\mu$
after treatment	$\beta+\tau$	$\beta+\tau+\mu+\psi$

β - baseline effect; τ - effect of treatment; μ - effect of mutation
ψ - differential effect on treatment between WT and MUT

treatment effect on gene expr. in WT cells: $\Delta^{WT} = (\beta + \tau) - \beta = \tau$
treatment effect on gene expr. in MUT cells: $\Delta^{MUT} = (\beta + \tau + \mu + \psi) - (\beta + \mu) = \tau + \psi$

differential treatment effect: $\Delta^{MUT} \neq \Delta^{WT}$ or $\psi \neq 0$

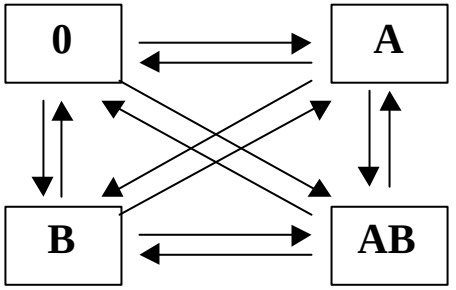
How many cDNA arrays are needed to show $\psi \neq 0$ with significance α and power $1-\beta$ if $|\psi| > \ln(5)$?

2x2 factorial experiments II

Study the **joint** effect of two conditions / treatment, A and B, on the gene expression of a cell population of interest.

There are four possible condition / treatment combinations:

AB: treatment applied to MUT cells
A: treatment applied to WT cells
B: no treatment applied to MUT cells
0: no treatment applied to WT cells



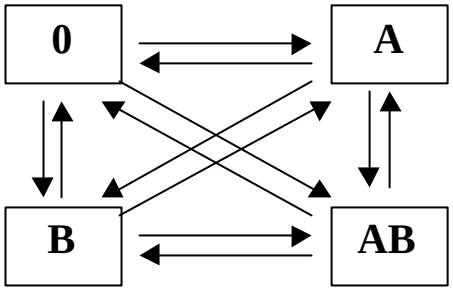
Design with 12 slides

2x2 factorial experiments III

Array	Measurement
$m_{A/0}$	$\gamma_{A/0} + \delta + e = \tau + \delta + e$
$m_{0/A}$	$-\gamma_{A/0} + \delta + e = -\tau + \delta + e$
$m_{B/0}$	$\gamma_{B/0} + \delta + e = \mu + \delta + e$
$m_{0/B}$	$-\gamma_{B/0} + \delta + e = -\mu + \delta + e$
$m_{AB/0}$	$\gamma_{AB/0} + \delta + e = \mu + \tau + \psi + \delta + e$
$m_{0/AB}$	$-\gamma_{AB/0} + \delta + e = -(\mu + \tau + \psi) + \delta + e$
$m_{AB/A}$	$\gamma_{AB/A} + \delta + e = \mu + \psi + \delta + e$
$m_{A/AB}$	$-\gamma_{AB/A} + \delta + e = -(\mu + \psi) + \delta + e$
$m_{AB/B}$	$\gamma_{AB/B} + \delta + e = \mu + \psi + \delta + e$
$m_{B/AB}$	$-\gamma_{AB/B} + \delta + e = -(\mu + \psi) + \delta + e$
$m_{A/B}$	$\gamma_{A/B} + \delta + e = \tau - \mu + \delta + e$
$m_{B/A}$	$-\gamma_{A/B} + \delta + e = -(\tau - \mu) + \delta + e$

- Each measurement has variance σ^2
- Parameter β is confounded with the dye effect

Regression analysis



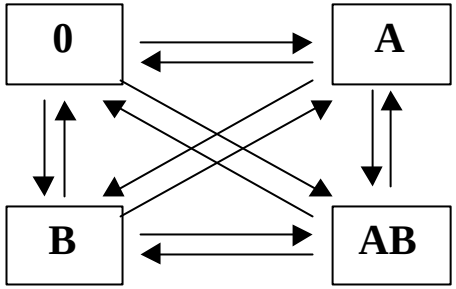
$$E\begin{pmatrix} M_{A/0} \\ M_{0/A} \\ M_{B/0} \\ M_{0/B} \\ M_{AB/0} \\ M_{0/AB} \\ M_{AB/A} \\ M_{A/AB} \\ M_{AB/B} \\ M_{B/AB} \\ M_{B/A} \\ M_{A/B} \end{pmatrix} = \begin{pmatrix} 1 & 1 & 0 & 0 \\ 1 & -1 & 0 & 0 \\ 1 & 0 & 1 & 0 \\ 1 & 0 & -1 & 0 \\ 1 & 1 & 1 & 1 \\ 1 & -1 & -1 & -1 \\ 1 & 0 & 1 & 1 \\ 1 & 0 & -1 & -1 \\ 1 & 1 & 0 & 1 \\ 1 & -1 & 0 & -1 \\ 1 & -1 & 1 & 0 \\ 1 & 1 & -1 & 0 \end{pmatrix} \bullet \begin{pmatrix} \delta \\ \tau \\ \mu \\ \psi \end{pmatrix}$$

- For parameter $\theta = (\delta, \tau, \mu, \psi)$ define the design matrix X such that $E(M) = X\theta$.
- For each gene, compute least square estimate $\theta^* = (X'X)^{-1}X'M$ (BLUE)
- Obtain measures of precision of estimated effects.
- Use all possibilities of the theory of linear models.

Design problem:

- Each measurement M is made with variability σ^2. How precise can we estimate the components or contrasts of θ?
  Answer: Look at $(X'X)^{-1}$

2 x 2 factorial designs IV



➢ total.2.by.2.design.mat

	delta	alpha	beta	psi
A/0	1	1	0	0
0/A	1	-1	0	0
B/0	1	0	1	0
0/B	1	0	-1	0
AB/0	1	1	1	1
0/AB	1	-1	-1	-1
AB/A	1	0	1	1
A/AB	1	0	-1	-1
AB/B	1	1	0	1
B/AB	1	-1	0	-1
B/A	1	-1	1	0
A/B	1	1	-1	0

```
> precision.2.by.2.rfc(x.mat)
$inv.mat

          tau     mu   psi
  tau   0.250  0.125 -0.25
   mu   0.125  0.250 -0.25
  psi  -0.250 -0.250  0.50

$effects
  tau    mu   psi   tau-mu
 0.25  0.25  0.50  0.25
```

$$\mathrm{Var}(A\text{-}B) = \mathrm{Var}(A) + \mathrm{Var}(B) - 2\cdot\mathrm{Cov}(A,B)$$

Sample size for differential treatment effect (DTE) in a 2 x 2 factorial designs I

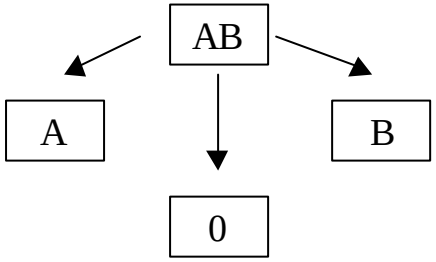
- Array has 20.000 genes: 19500 without DTE, 500 with DTE
- $\alpha_F = 0.9$, using Bonferroni adjustment: $\alpha = 0.9/20.000 = 0.0000462$
- Mean number of correct positives is set to 450: $1-\beta = 0.9$
- $\sigma^2 = 0.7$, taken from similar experiments
- A total dye swap design (12 arrays) estimates ψ with precision $\sigma^2/2 = 0.35$

$$N = [4.074 + 1.282]^2 \cdot 0.35 / \ln(5)^2 = 3.876$$

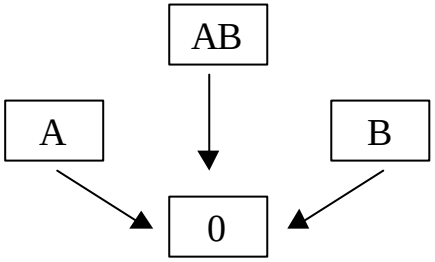
- The experiment would need in total 4 x 12 = 48 arrays
- Is there a chance to get the same result cheaper?

2 x 2 factorial designs V

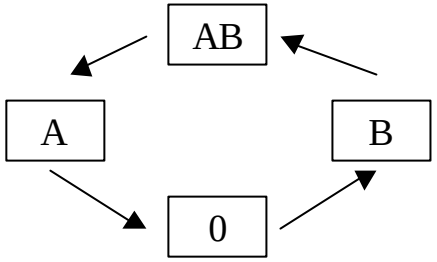
Design I
Common ref.



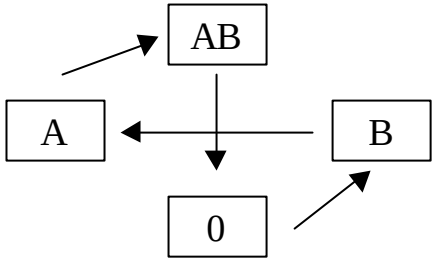
Design II
Common ref.



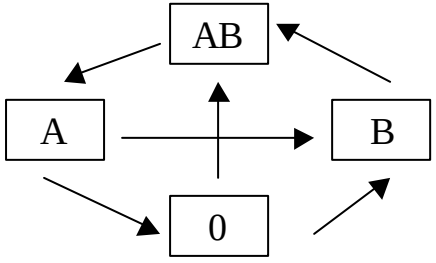
Design III
Connected



Design IV
Connected



Design V
All-pairs



Scaled variances of estimated effects

	D.I	D.II	D.III	D.IV	D.V	D.tot
tau	2	1	0.75	1.00	0.5	0.25
mu	2	1	0.75	0.75	0.5	0.25
psi	**3**	**3**	**1.00**	**2.00**	**1.0**	**0.50**
# chips	**3**	**3**	**4**	**4**	**6**	**12**

Sample size for differential treatment effect (DTE) in a 2 x 2 factorial designs II

Is there a chance to get the same result cheaper?

- Using total dye swap design, the experiment would need in total 4 x 12 = 48 arrays
- Using Design III, the effect of interest is estimated with doubled variance (4 $\rightarrow$ 8) but by using a design which need only 4 arrays (12 $\rightarrow$ 4).
- This reduces the number of arrays needed from 48 to 32.

Experimental Design - Conclusions

- Designs for *time course* experiments
- In addition to experimental constraints, design decisions should be guided by knowledge of which effects are of greater interest to the investigator.
- The unrealistic planning based on independent genes may be put into a more realistic framework by using simulation studies – speak to your bio – statistician/informatician
- How to collect and present *experience* from performed microarray experiments on which to base assumptions for planing (σ^2)?
- Further reading:

Kerr MK, Churchill GA (2001) *Experimental design for gene expression microarrays,* Biostatistics, 2:183-201

Lee MLT, Whitmore GA (2002), Power and sample size for DNA microarray studies, Stat. in Med., 21:3543-3570