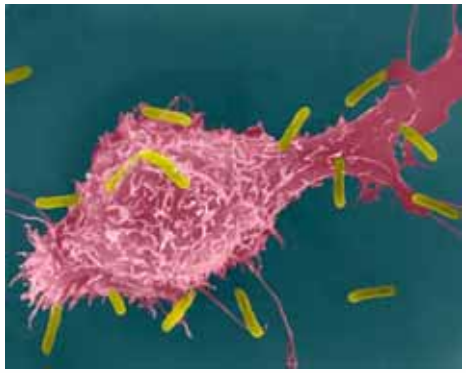


In vitro monitoring of vaccine (antigen) quality

Coenraad Hendriksen

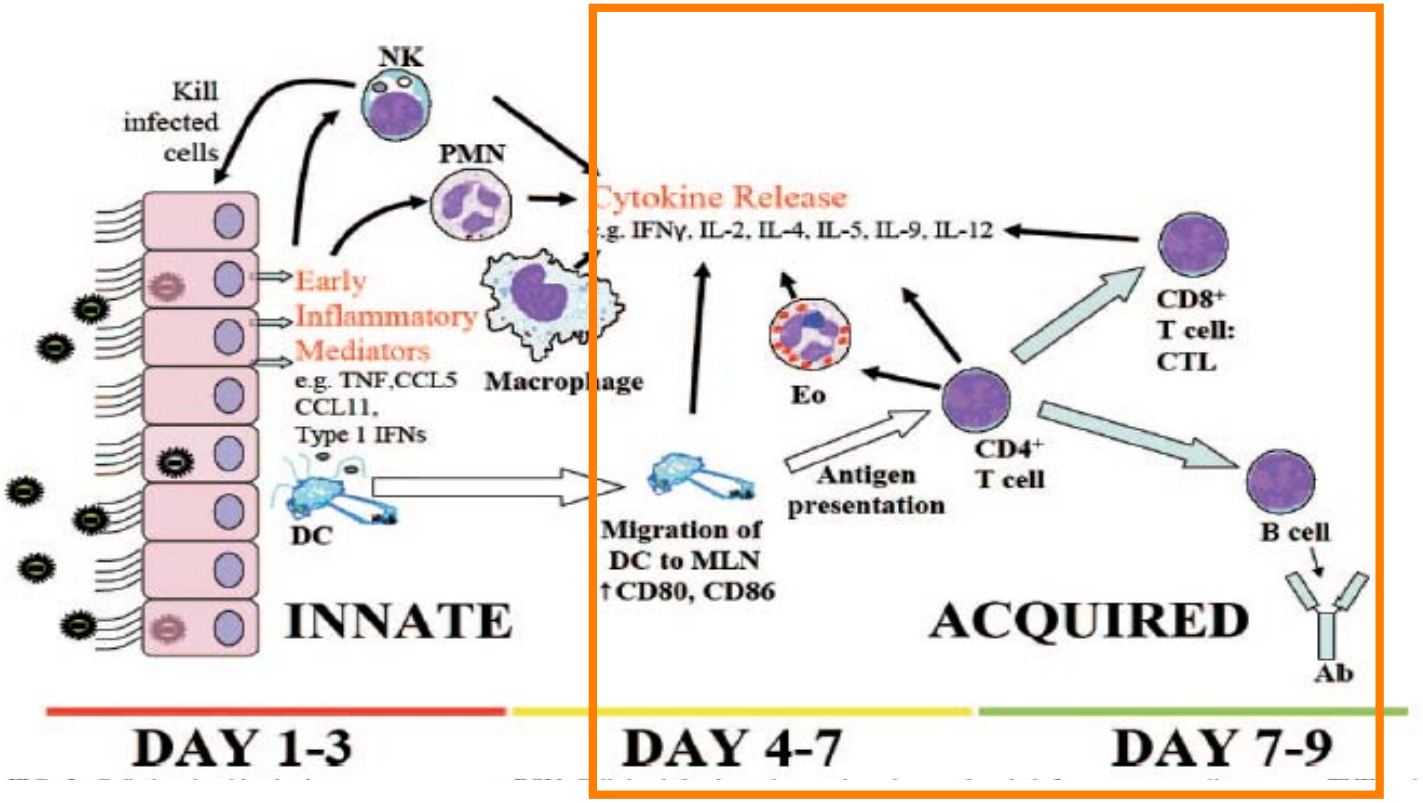
Netherlands Vaccine Institute (NVI)

&

Netherlands Centre Alternatives to Animal use (NCA), Utrecht University

Provisional title given to me: *In vitro* monitoring of antigen immunogenicity

Immunogenicity = the extent to which an antigen is capable of eliciting a specific type of immune response in the host animal

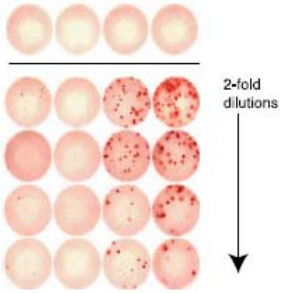


Openshaw *et al*, Clin Microb. Reviews 2005

In vitro monitoring of vaccine (antigen) quality

Evaluation of vaccine (antigen) quality (antigenicity and immunogenicity) by a set of analytical, immunochemical and functional *in vitro* tests

Antigenicity



Immunogenicity

Immunogenicity : the extent to which an antigen is capable of eliciting a specific type of immune response in the host organism

Antigenicity : the extent to which an antigen will react with the immune response elicited by the immunogen (affinity, avidity)

What will be discussed

- General information about vaccine production and quality control
- Three R's developments and results in vaccine quality control
- Limitations and obstacles in replacing animals
- A paradigm shift in vaccine quality control, 'the consistency approach': a new strategy for in vitro monitoring of vaccine (antigen) quality
- The non-animal tests in the consistency approach to monitor vaccine antigenicity and immunogenicity
- Conclusions

An expert is someone: * who knows almost everything of nearly nothing

* who knows nearly nothing of almost ...

Take home message

- Consider the Three Rs rather than focussing on one of the Rs.
- Be open for new ideas about research and testing strategies

What will be discussed

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Definition of a vaccine

- A preparation consisting of an antigen (immunogen) to which an effective immune response (humoral and/or cellular) must be induced after administration of the vaccine to the host.
- Liquid vaccines contain the antigen(s) and the excipient and generally also an adjuvant, preservatives and a stabiliser.
- A traditional relationship exists with laboratory animals

(ESI meeting, Spain, Sept.29-39, 2006)

Evolution of Vaccine Development

- Classical vaccines :

  Live attenuated : e.g. polio, measles, mumps, rubella

  Inactivated : e.g. pertussis, polio, diphtheria, tetanus, rabies

- Subunit & glyco-conjugaat vaccines

  Subunit : e.g. a-cellular pertussis, Hepatitis B

  Glyco-conjugaat : e.g. Haemophilus influenzae,

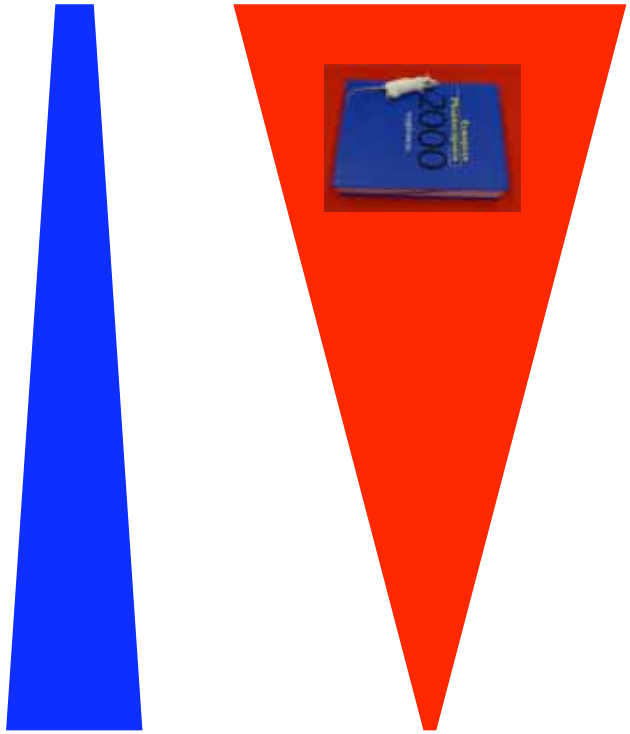
  Strep. pneumoniae

- 3rd Generation vaccines:

  Synthetic peptide : Foot and Mouth disease

  DNA Immunisation : Influenza

Extent of Animal Use



R&D + preclinical testing

Quality control

(ESI meeting, Spain, Sept.29-39, 2006)

Vaccine production

fermentation,
cultivation

purification)

Inactivation/
detoxification

(final) bulk

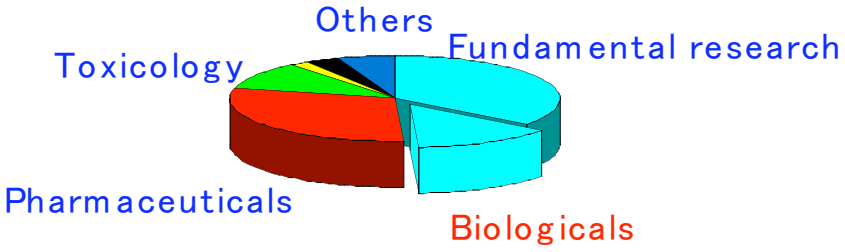
final lot

Seed strain
(starting material)

Characteristics of vaccine production & quality control

Characteristics:

- world-wide use (60% of production in 3rd world countries)
- used for both human and veterinary purposes
- undefined or poorly defined products
- batch-wise production
- batch-to-batch differences in quality
- mandatory batch-related quality control
- important aspects of q.c.: safety and potency
- Extensive use of animals and high % of animal pain and distress



eSI meeting, Spain, Sept.29-39, 2006)

Vaccine Quality Control: background information

NVI nederlands vaccin instituut

Production of the classical (inactivated) vaccines

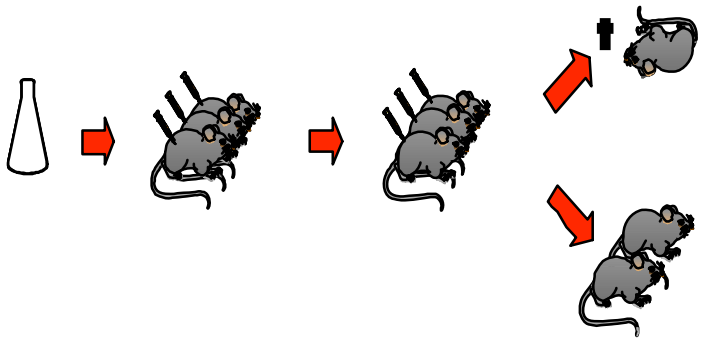
Virulent micro-organism / toxine

Detoxification / inactivation

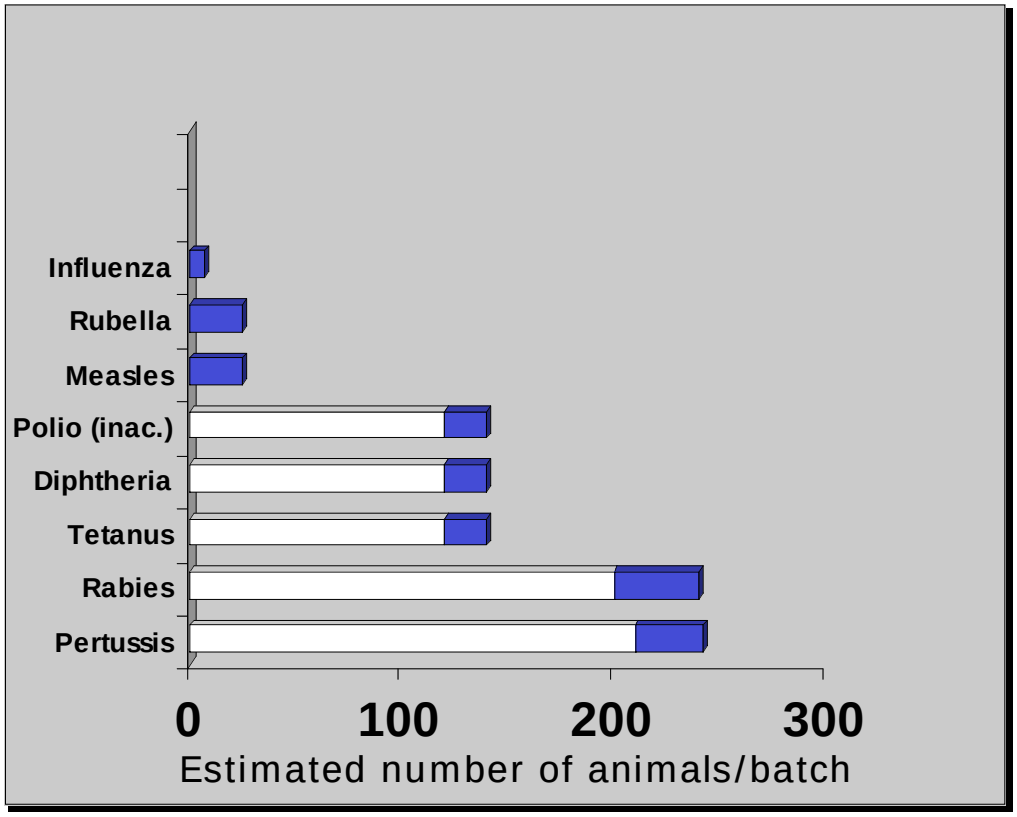
Attenuation

Inactivated vaccine / toxoid (Final lot)

- ✓ **Safety tests**
- ✓ **Potency tests**



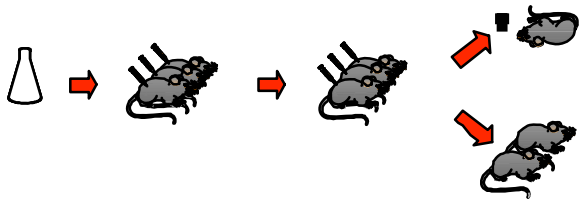
Estimated number of animals required for testing one vaccine batch

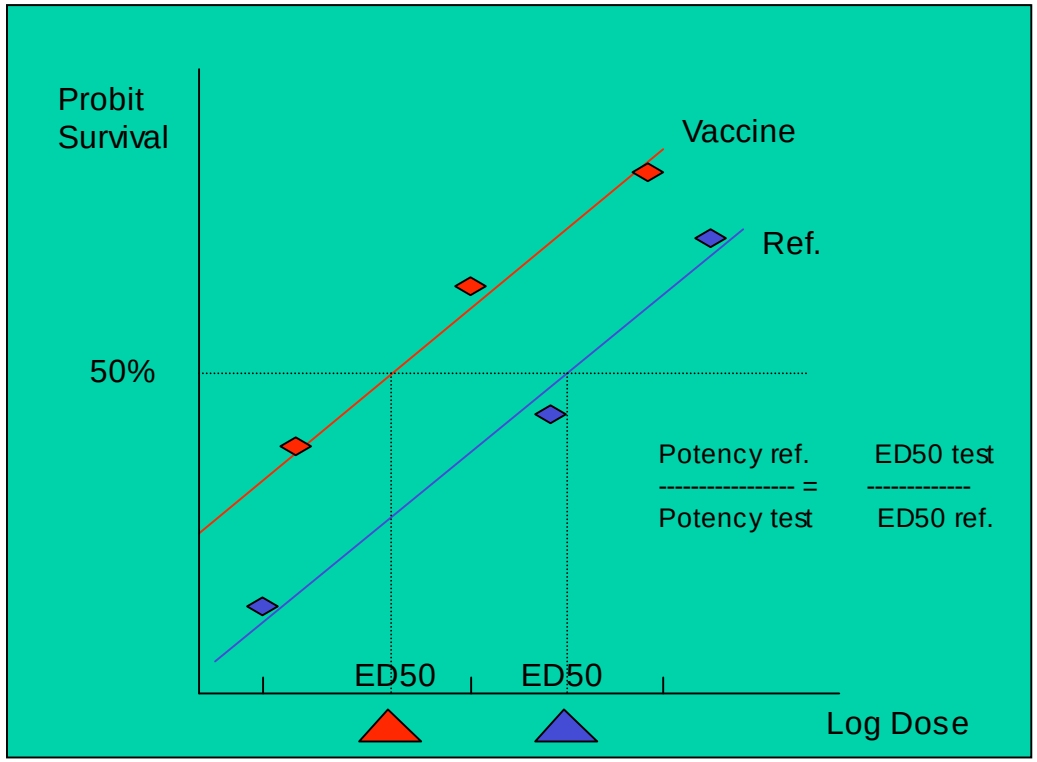


White = potency testing

Blue = safety testing

Principle of potency test based on challenge procedure





Characteristics challenge test

- high no.animals/test
- death specified as endpoint
- severe suffering
- approx.50% deaths/ severe clinical signs

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Vaccine Quality Control: Recent 3R's developments

3Rs in vaccine quality control

Replacement

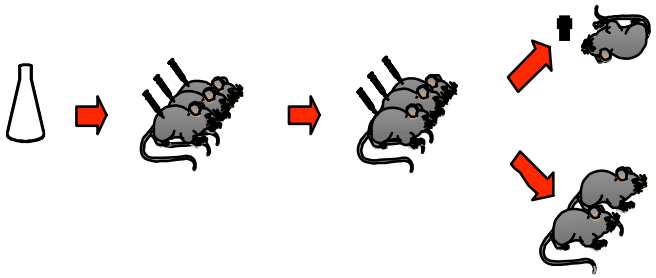
- in vitro antigenicity test
- cell culture tests (potency, safety)

Reduction

- *serological tests*
- test optimisation
- combination of tests
- single dose testing

Refinement

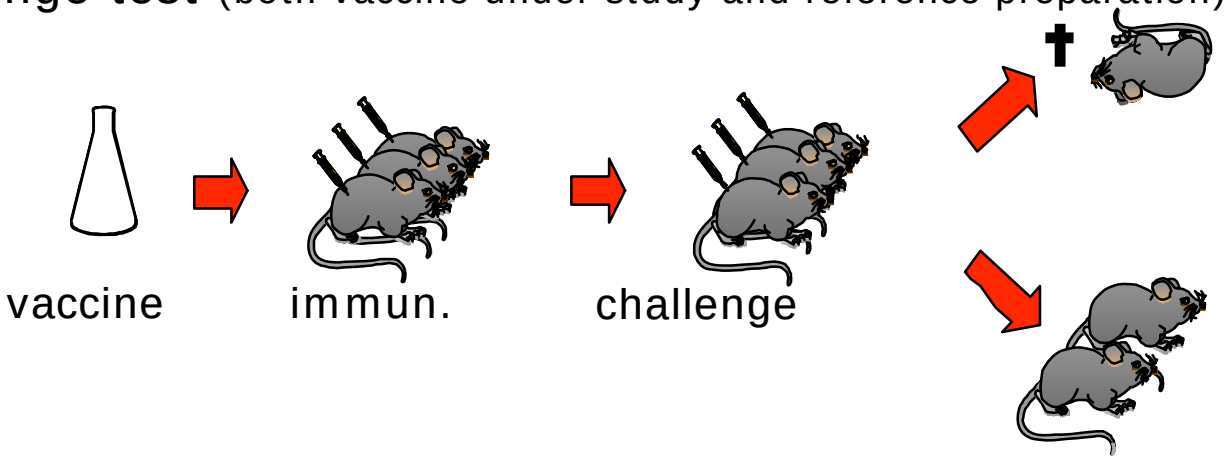
- *serological tests*
- *humane endpoints*



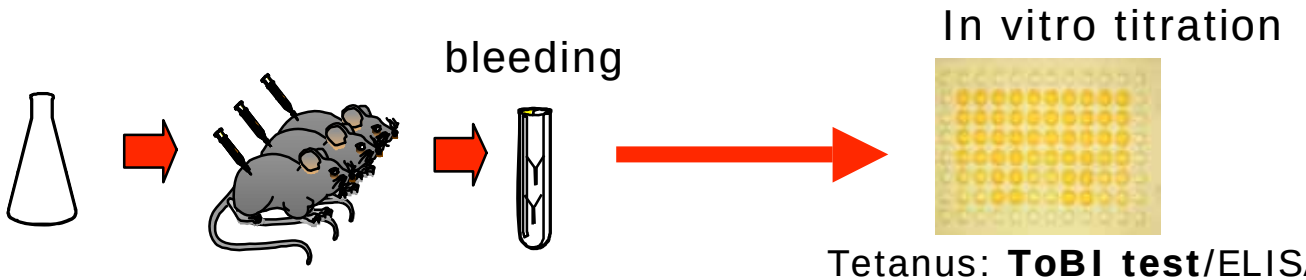
[e]SI meeting, Spain, Sept.29-39, 2006)

Recent developments: Reduction & Refinement

Challenge test (both vaccine under study and reference preparation)



Serological test (both vaccine under study and reference preparation)



Tetanus: **ToBI test**/ELISA

Diphtheria: **Vero cell**/ELISA/**ToBI**

Whole cell pertussis : **ELISA**

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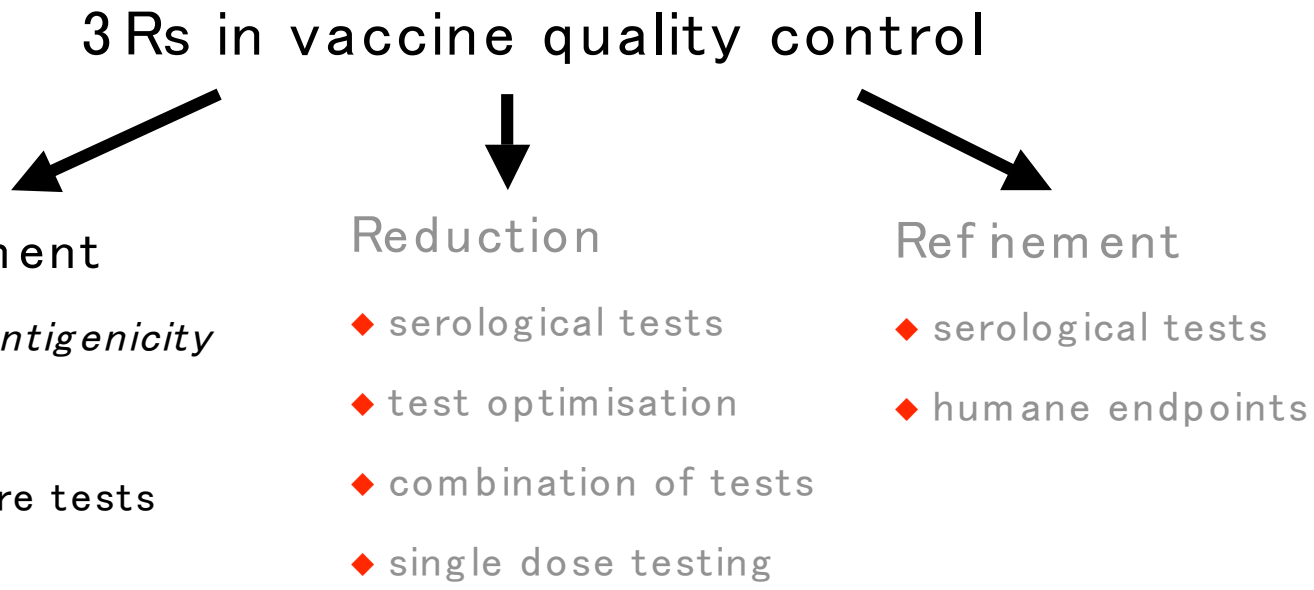
Vaccine Quality Control: Recent 3R's developments



Cell culture tests : in vitro titration of live particles (live attenuated va
safety tests (e.g. diphtheria vaccine)

In vitro antigenicity test : quantitation of amount of antigen with Mab
(examples: rabies vaccine, Hepatitis B vacc
other immunoassay.

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Disadvantages of the current Three R's development

- Huge investment in time and money
- Slow progress when replacing 1 by 1
- Scientific limitations to the development of alternatives
- Complex validation studies needed
- Are current animal studies always relevant (what are we measuring and why?)

Eur.Phar./ECVAM Collaborative Study to the Use of in-vitro Serological Test Systems for Potency Testing of Tetanus Toxoid Vaccines for Human Use

DESIGN STUDY

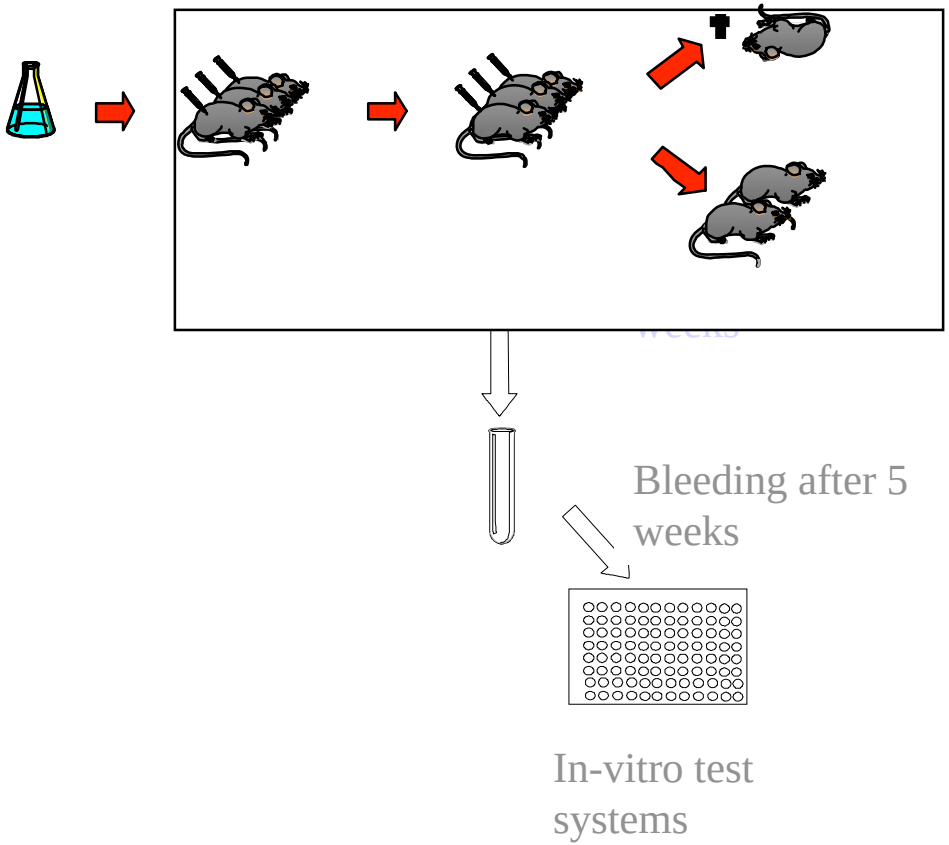
Management: 4 partners/2 bio-statisticians

Study was divided in 4 phases

Pre-validation	: 4 laboratories
Phase 1	: 3 laboratories
Phase 2	: 3 laboratories
Phase 2b	: 2 laboratories
Phase 3	: 26 laboratories

Time required : approx. 4 years

POTENCY TEST



Revision of monograph on tetanus vaccine of European Pharmacopoeia

2.7.8. Assay of tetanus vaccine (adsorbed) The potency of tetanus vaccine is determined by administration of the vaccine to animals (guinea-pigs or mice) followed either by challenge with tetanus toxin (method A or B) or by determination of the titre of antibodies against tetanus toxoid in the serum of the guinea-pigs (method C). In both cases the potency of the vaccine is calculated by comparison with a reference vaccine, calibrated in International Units. For methods A and B, in countries where the paralysis method is not obligatory the LD_{50} method may be used. For the LD_{50} method, the number of animals and the procedure are identical with those described for the paralysis method but the end-point is the death of the animal rather than paralysis. The International Unit is the activity contained in a stated amount of the International Standard for tetanus toxoid (adsorbed). The equivalence in International Units of the International Standard is stated by the World Health Organisation. Tetanus vaccine (adsorbed) BRP is calibrated in International Units with reference to the International Standard. The method chosen for assay of tetanus vaccine (adsorbed) depends on the intended purpose. Method A or B is used:

1. during development of a vaccine, to assay batches produced to validate the production;

2. wherever revalidation is needed following a significant change in the manufacturing process.

Method A or B may also be used for routine assay of batches of vaccine but in the interests of animal welfare, method C is used wherever possible.

Method C may be used, except as specified under 1 and 2 above, after verification of the suitability of the method for the product. For this purpose, a suitable number of batches (usually 3) are assayed by method C and method A or B. Where different vaccines (monovalent or combinations) are prepared from tetanus toxoid of the same origin, suitability demonstrated for the combination with the highest number of components can be assumed to be valid for combinations with fewer components and for monovalent vaccine. For combinations with a whole-cell pertussis component, a separate demonstration of equivalence must be made for the highest combination. The design of the assays described below uses multiple dilutions for the test and reference preparations. Based on the potency data obtained in multidilution assays, it may be possible to decrease the number of animals needed to obtain a statistically significant result by applying a simplified model using a single dilution for both test and reference preparations. Such a model enables the analyst to determine whether the potency of the test preparation is significantly higher than the minimum required but does not give information on the dose-response curves and their linearity, parallelism and significant slope. The simplified model may lead to a considerable reduction in the number of animals required and its use must be considered in accordance with the provisions of the European

Disadvantages of the current Three R's development

- Huge investment in time and money
- Slow progress when replacing 1 by 1
- Complex validation studies needed
- Scientific limitations to the development of alternatives
- Are current animal studies always relevant (what are we measuring and why?)

Influence of mouse strain on assayed T potency

Mouse strain	Assayed potency (IU/ml)
NIH	223
CFW	185
CDF1	142
BALB/c	105

Hardegree et al. (1972)

Influence of animal species on assayed T poteny

Lab.no.	Assays in mice	Assays in g-ps
1	171 (152-193)	357 (289-442)
2	69 (58-82)	293 (198-429)
3	227 (181-285)	-
4	-	378 (278-497)
5	104 (75-145)	241 (180-321)

Lyng & Nyerges (1984)

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Vaccine quality control: a different way to look at it

Production of the old (inactivated) vaccines

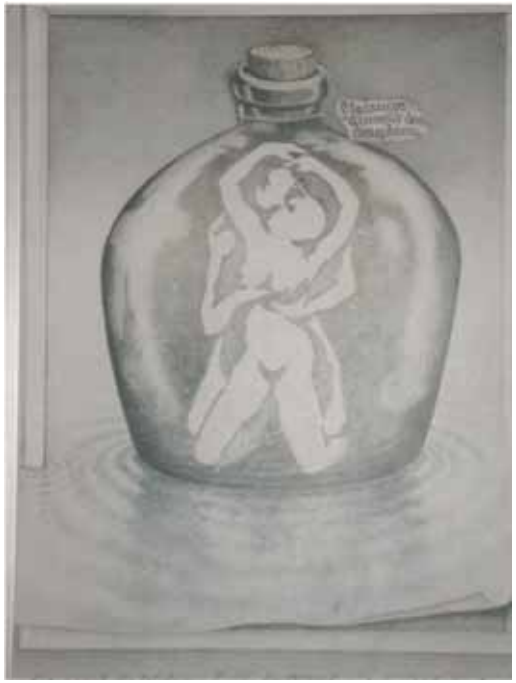
Virulent micro-organism / toxine

Detoxification / inactivation

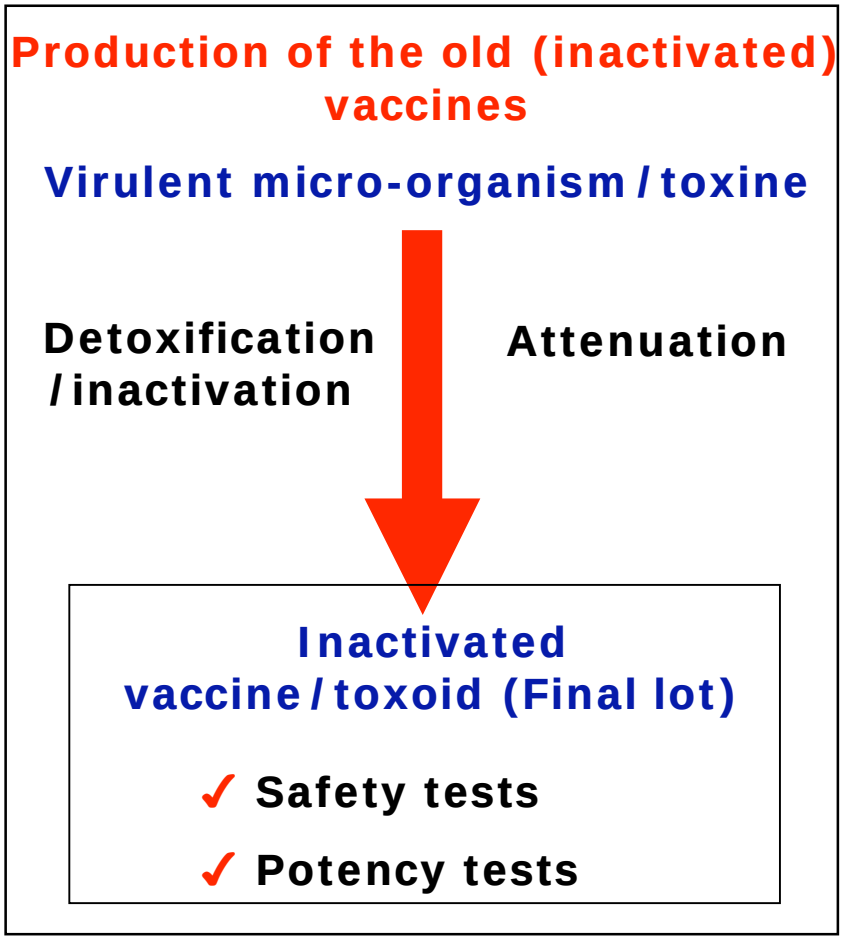
Attenuation

Inactivated vaccine / toxoid (Final lot)

- ✓ **Safety tests**
- ✓ **Potency tests**



Vaccine quality control: a different way to look at it



Two concepts are dominant in current vaccine quality control:

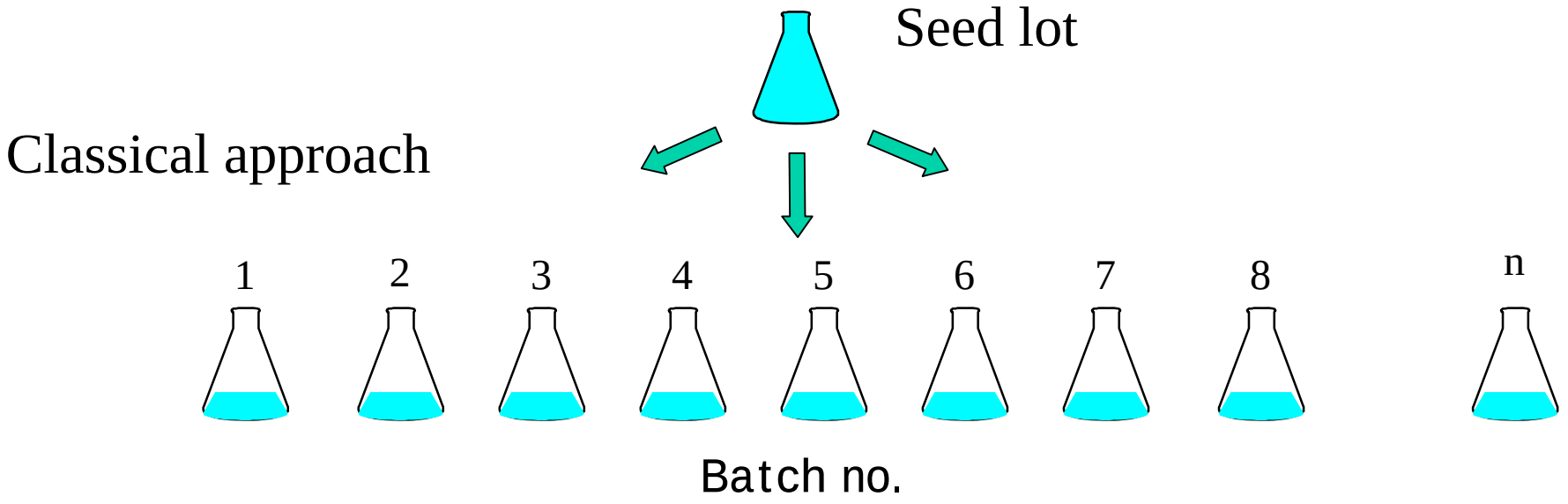
- A vaccine batch is a unique product
- Batch release should be based on testing of final lot.

Lot release based on:

- functional tests for potency
- functional tests for safety

(ESI meeting, Spain, Sept.29-39, 2006)

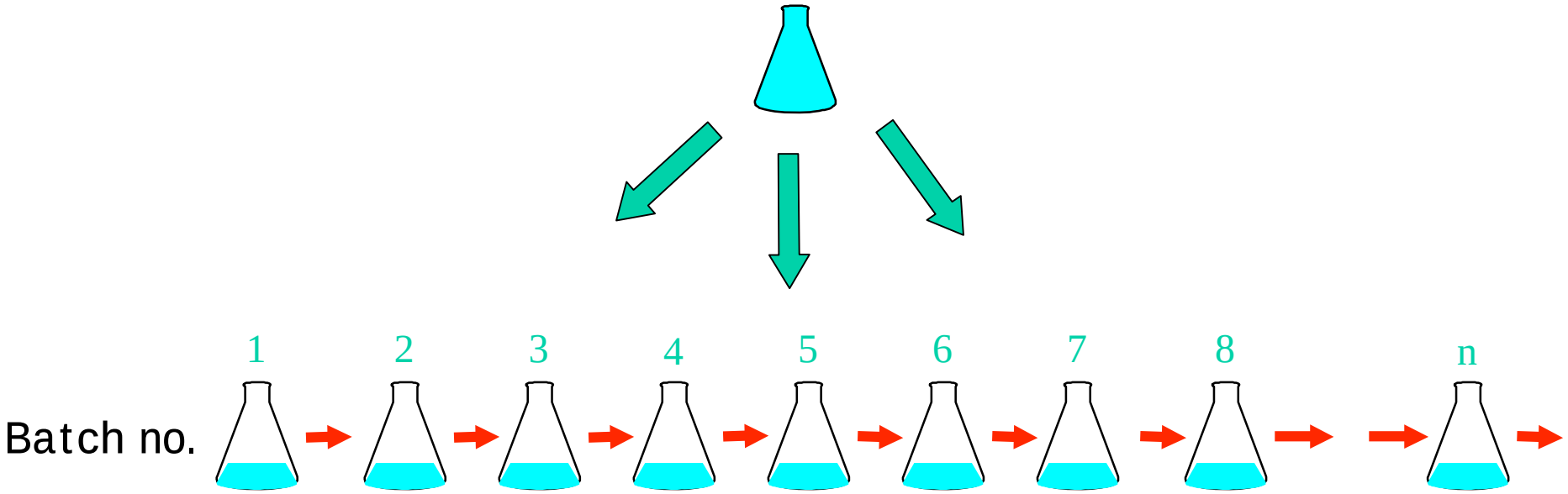
Consistency approach: the challenge of the uniqueness of a vaccine batch



However, at the manufacturer's level all batches are derived from the same bacterial/viral strain (seed-lot)

given to the q.c. of each final product (safety and potency).

Principle 'Consistency approach'



New strategy: quality control is seen as an instrument to monitor manufacturer's consistency in production.

Consistency approach : the challenge of given emphasis to testing of the final lot

However, most laboratories:

- have improved their products
- have now well established production processess
- have implemented the principles of Good Manufacturing Processes (GMP) and Quality assurance (QA)
- do extensive in-process testing

Production of the old (inactivated) vaccines

Virulent micro-organism / toxine

↓

In-process (in vitro) testing

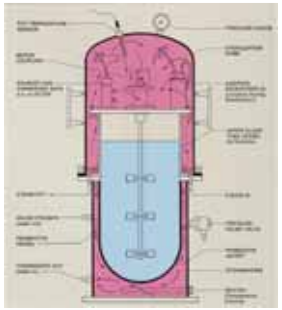
↓

Inactivated vaccine (Final lot = batch)

✓ **Safety tests**

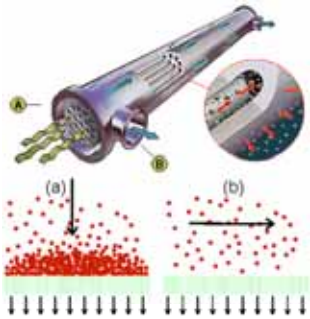
✓ **Potency tests**

In process testing for product and process monitoring



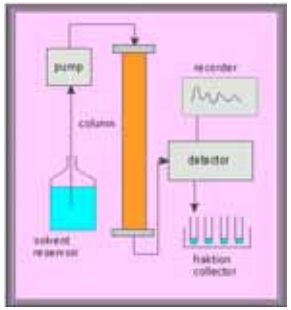
fermentation

temperature
stirrer speed
dO
substrate
LDH
free DNA
etc.



filtration

flow
back pressure
protein filtrate
protein retentate
etc.

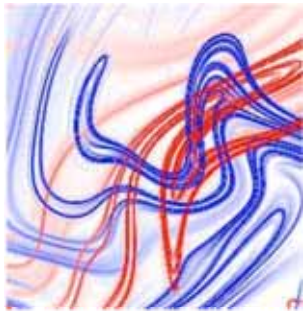


chromatography

plate number
protein
endotoxin
DNA
etc.

inactivation

temperature
formaldehyde
toxicity, titer
SDS-PAGE
etc.

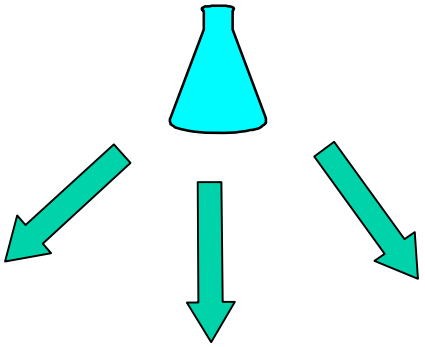


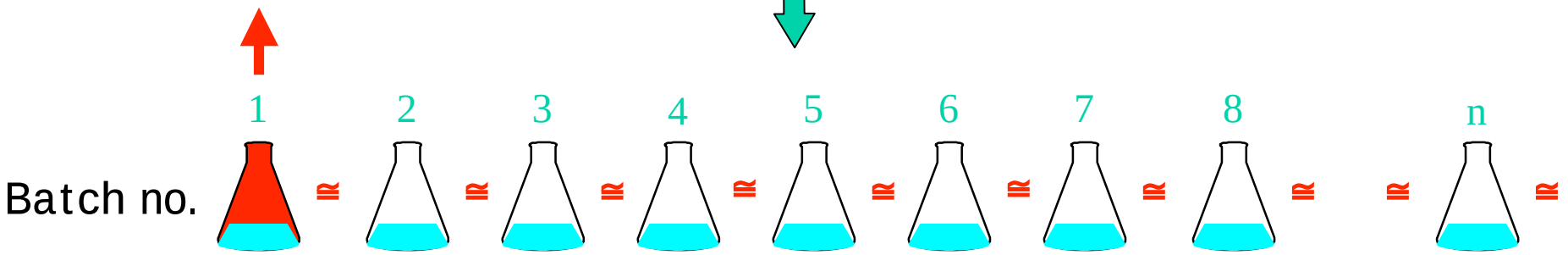
formulation

adsorption degree
homogeneity
residual water
transition temp.
etc.

Characterisation these vaccines (use of many in vitro techniques) is being used to support registration procedures to monitor improvements of production processes

Principle 'Consistency approach'



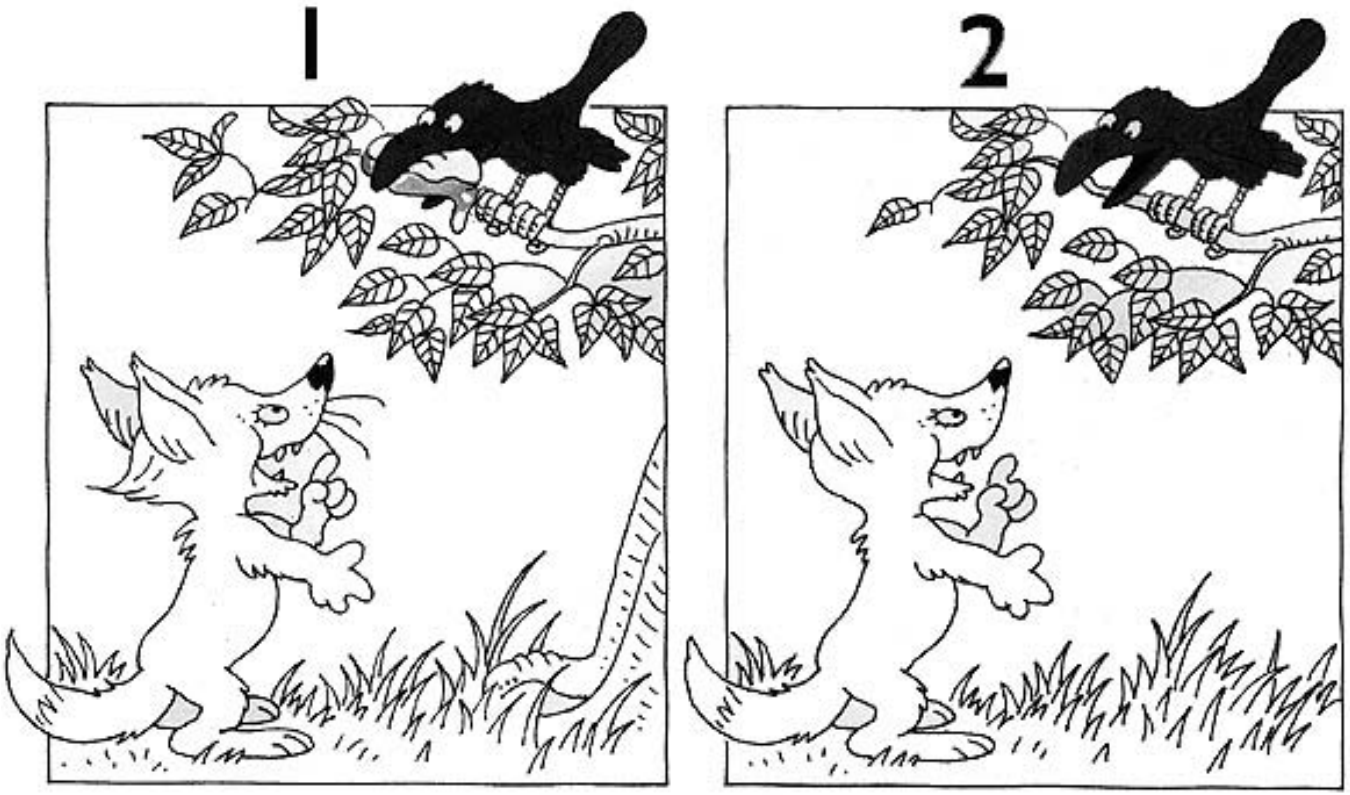


New strategy: quality control is seen as an instrument to monitor manufacturer's consistency in production.

The proposed testing strategy is to fully monitor the first final lots thoroughly, including in vivo tests and human clinical data (clinical lot) and to compare (fingerprint) subsequent batches of the same starting material (seed strain) with the final lot based on analytical and immunochemical and in vitro methods focussing on the critical steps in the production.

The consistency approach

Find the differences



Vaccine production: critical steps

fermentation,
cultivation

purification)

Inactivation/
detoxification

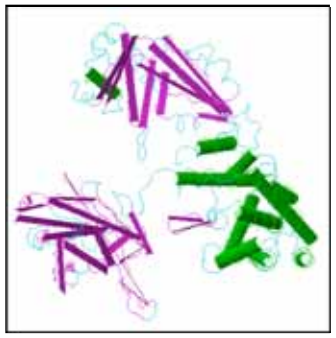
(final) bulk

final lot

Seed strain

eSI meeting, Spain, Sept.29-39, 2006)

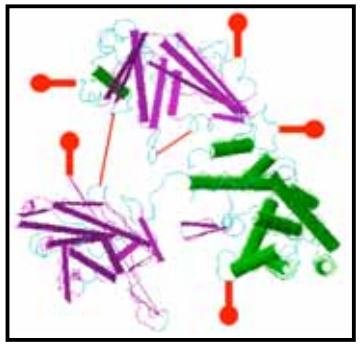
Effect of formaldehyde on characteristics of vaccine



Diphtheria toxin

Formaldehyde

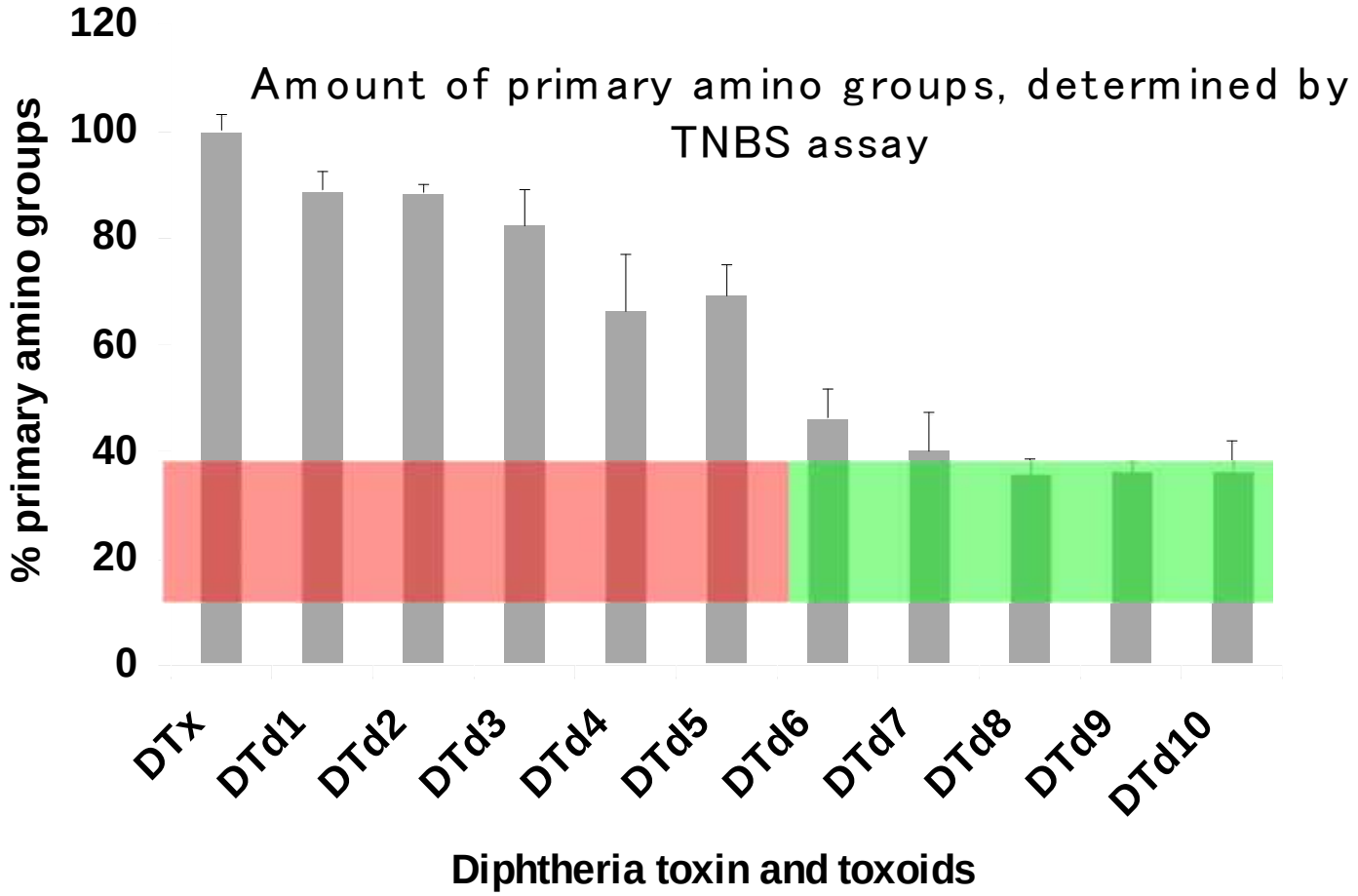
Glycine



Diphtheria toxoid

(eSI meeting, Spain, Sept.29-39, 2006)

Effect of detoxification on primary amino groups



Formaldehyde reacts with free amino groups in the protein, resulting in a labile Schiff base (imine)

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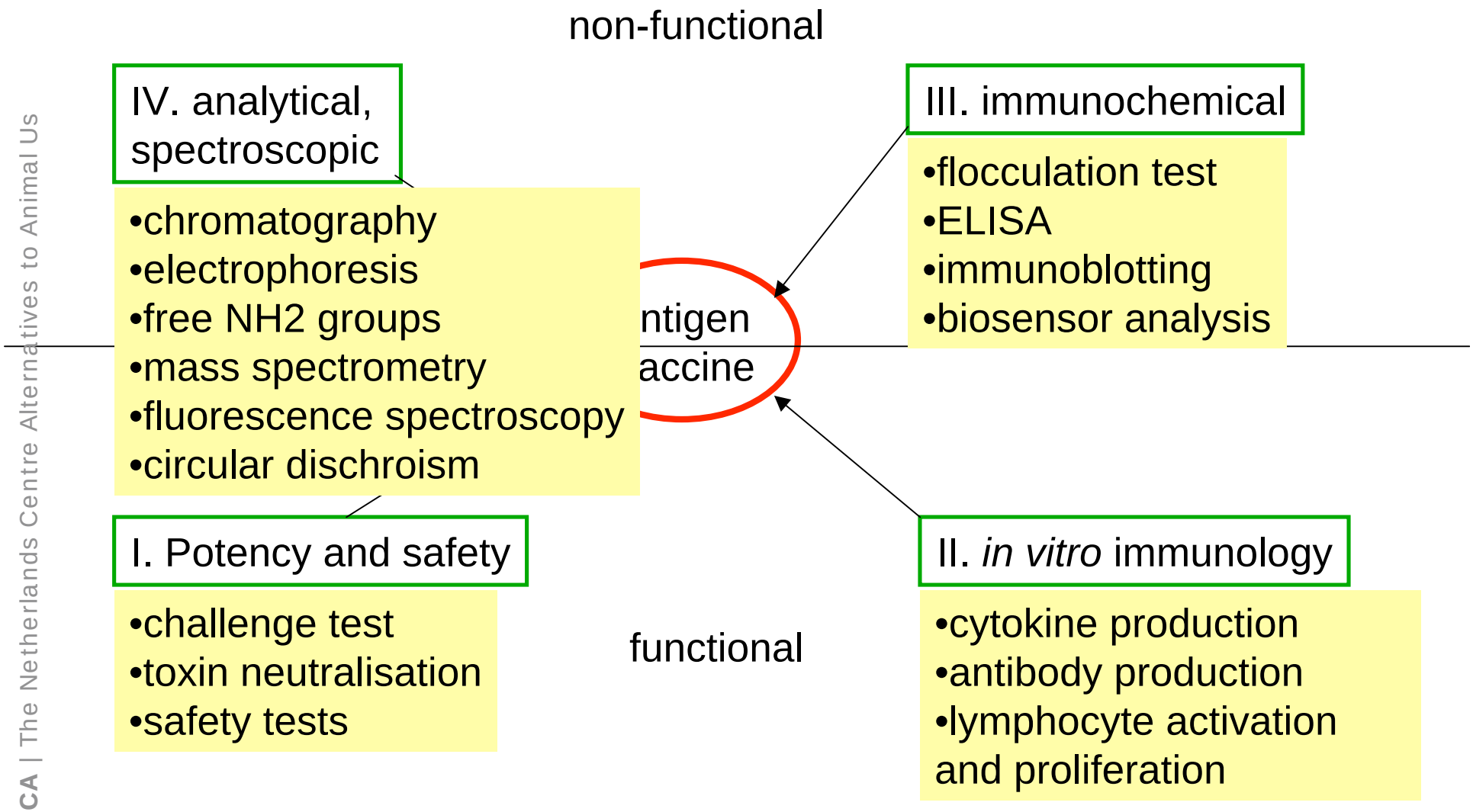
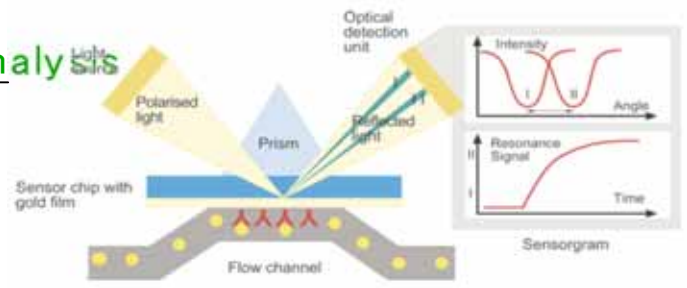
Monitoring of antigen and product quality: summary of potential test models

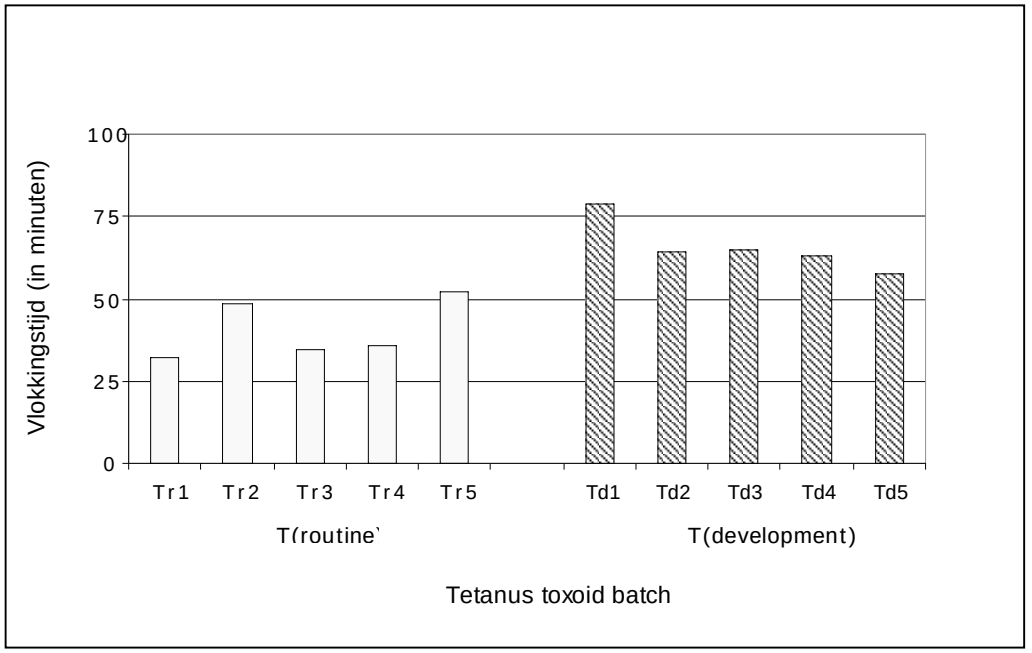


Immunochemical (antigenicity) tests

Test	Principle	Suitable for
Flocculation	visual immune complexes	antigen quantity, flocculation time: quality of antigen
ELISA	binding to coated solid phase	antigen quantity, epitope quality
Immunoblotting	size or charge based	antigen identification, antigen integrety
Biosensor kinetic	antibody-antigen interaction on custom sensor surface	antigen quantity, epitope quality, analysis



Flocculation time of vaccins that differ in quality



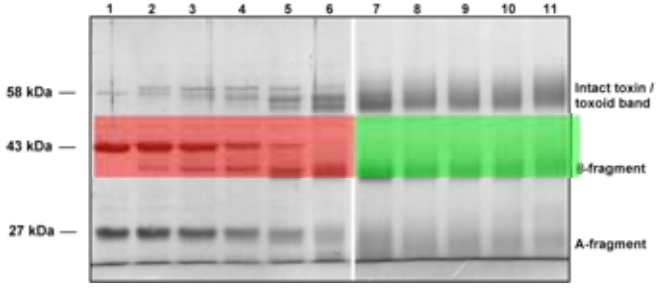
Flocculation time of 5 experimental batches of tetanus toxoid (Td) an 5 routine batches of tetanus toxoid (Tr)

N.B. Potency of routine batches of tetanus toxoid was higher than potency of experimental batches

eSI meeting, Spain, Sept.29-39, 2006)

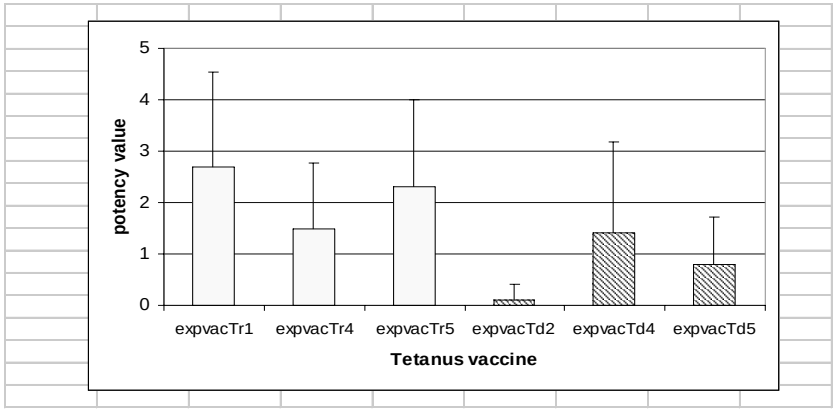
Analytical and spectroscopic methods (antigenicity)

Test	Principle	Suitable for
Chromato-graphy	Hydrophobicity based separation	purity, protein modifications, stability
Peptide mapping	enzymatic or chemical degradation	protein modifications, stability
Circular dichroism	differential absorption of left-and right handed circulary light	secundary and tertiary structure of proteins
Fluorescence	intrinsic fluorescence of proteins after excitation	protein conformation, protein modifications

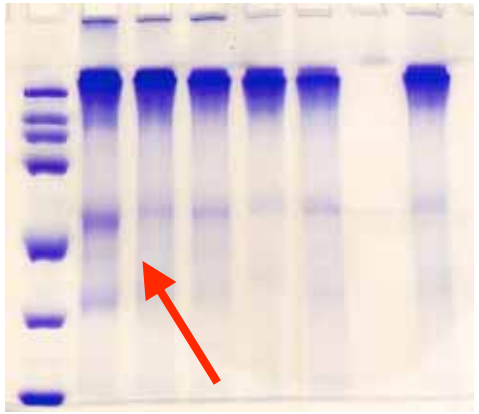


Effect of increasing formoldehyde concentrations

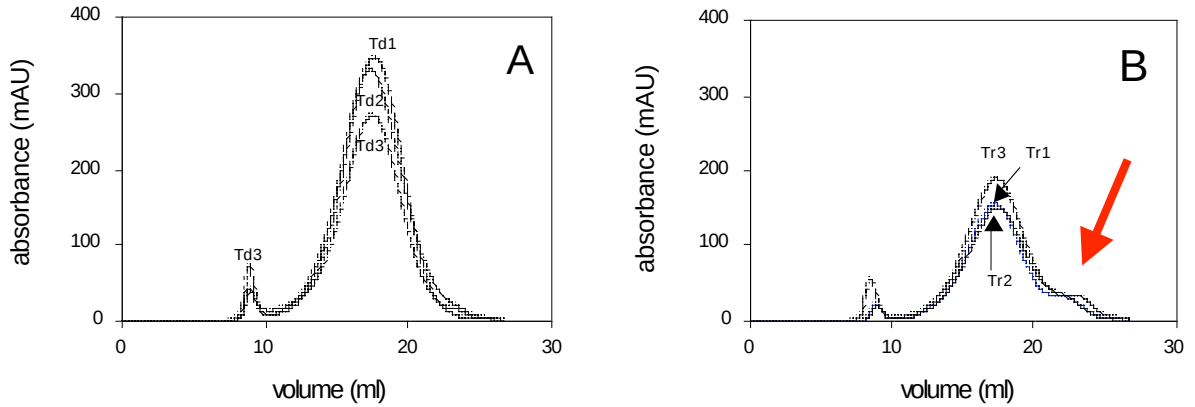
Results from routine (Tr) and experimental (Td) Tetanus toxoids



Potency



SDS-Page



Gelpermeation chromatography

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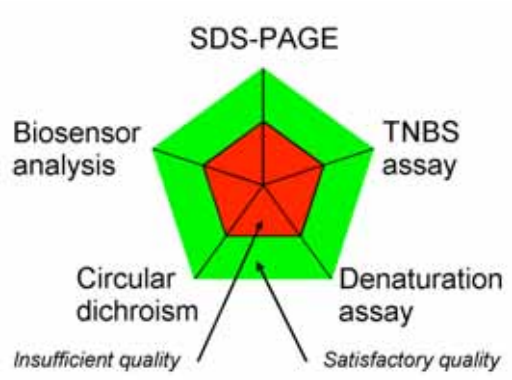
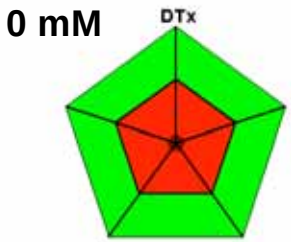
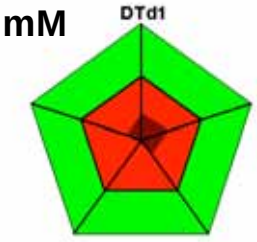
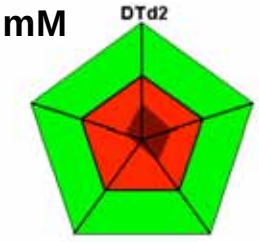
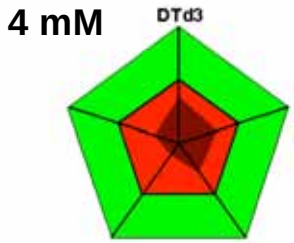
Radarplots of summarised data

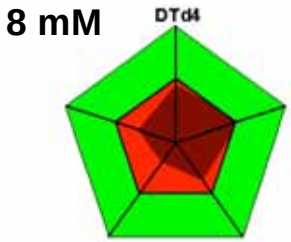


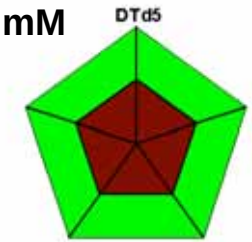


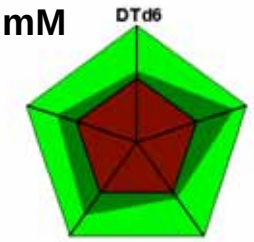


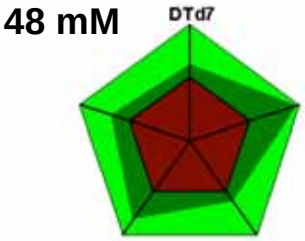


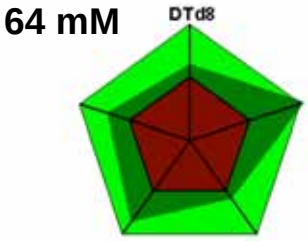


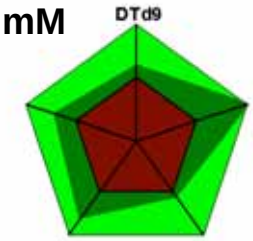












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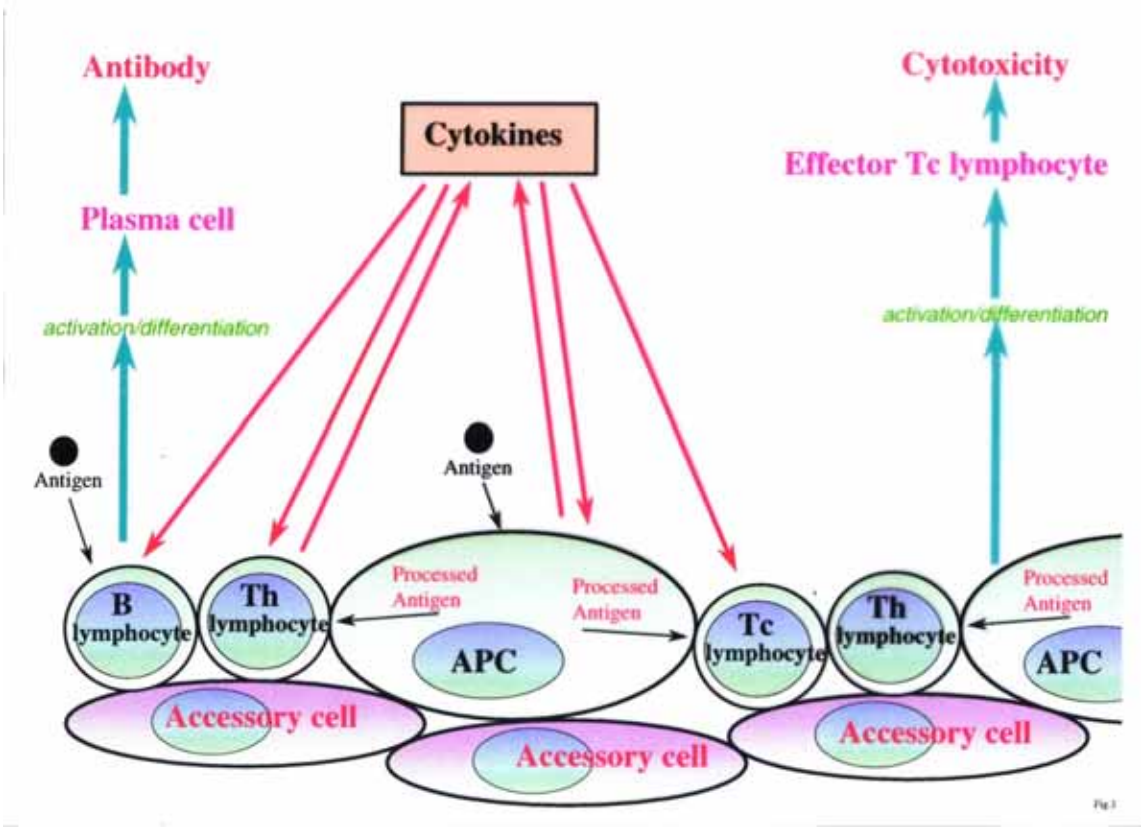
Functional in vitro immungenicity tests



Requires:

- APC,
- T lymphocytes,
- B lymphocytes,
- Accessory cells (e.g. endothelial cells) not necessary

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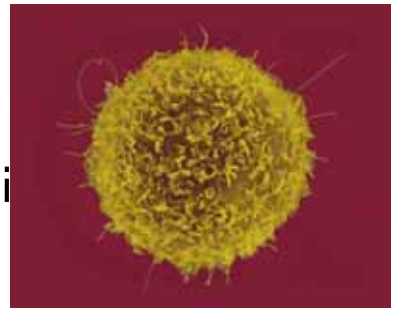
Functional in vitro tests studying essential parts of immunogenicity

- Semi-functional tests: epitope mapping of vaccine antigen with monoclonal antibodies for identifying and monitoring of B cell epitopes
- Critical aspects of the immune respons
  - ✓ Innate respons: antigen uptake and processing
  - ✓ Cytokine responses
  - ✓ Acquired immune respons (in vitro antibody production, , T cell proliferati T cell activation markers, etc.)



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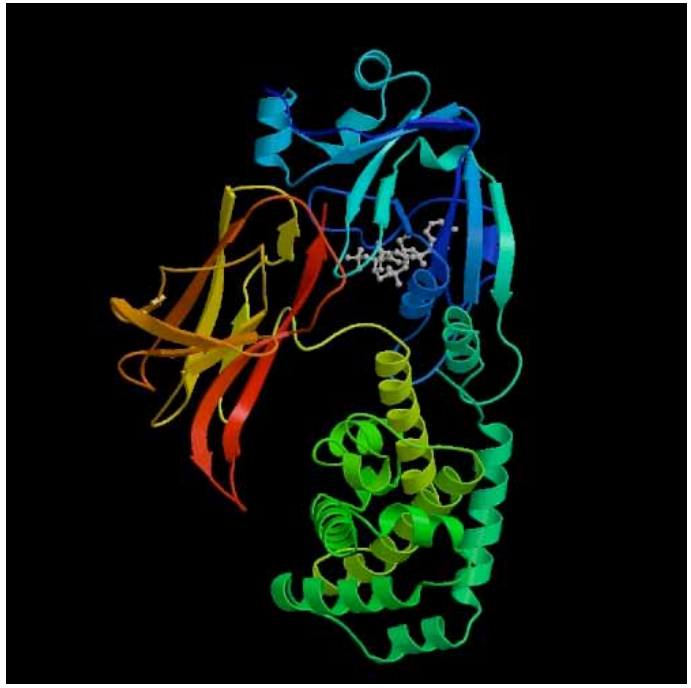
Epitope mapping and Mabs

Evaluation of 20 monoclonal antibodies binding to different epitopes of the diphtheria toxin/toxoid (total and fragment A and B), of which some:

Code	Dx	Dd	N	Cat
Dim 1		X		1
Dim 2	X	X		3
Dim 3		X		5
Dim 4	X		X	2
Dim 5		X		8
Dim 6		X		5
Dim 7	X	X		4
Dim 8	X		X	7



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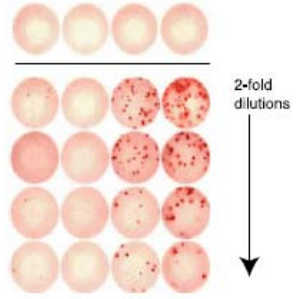

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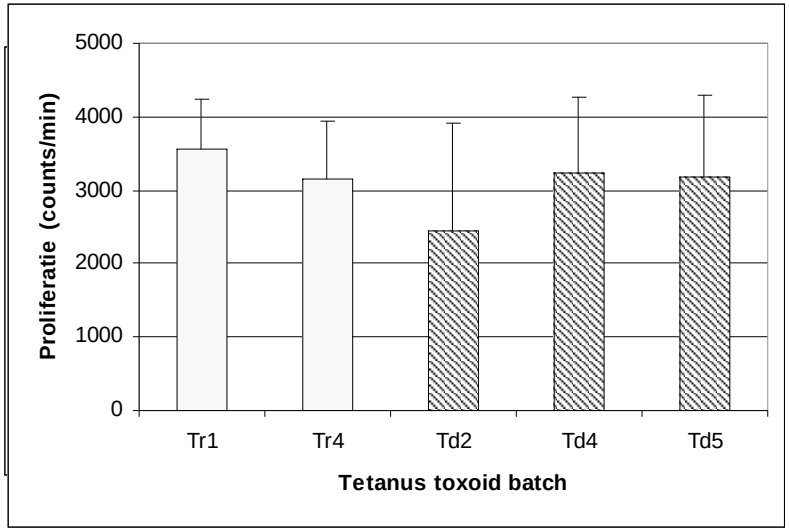
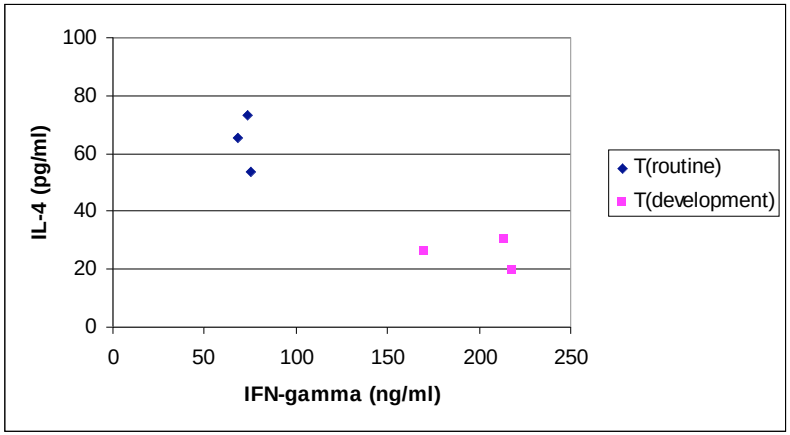
Use of cytokine responses for characterising vaccine quality

Relative estimates of potency and cytokine responses (IFN-gamma and IL-4) for three batches of routinely produced tetanus toxoid (Tr) and for three batc of tetanus toxoid produced during a production development process (Td)



Estimates of potency



IFN-gamma and IL-4 production in cultures of murine spleen cells

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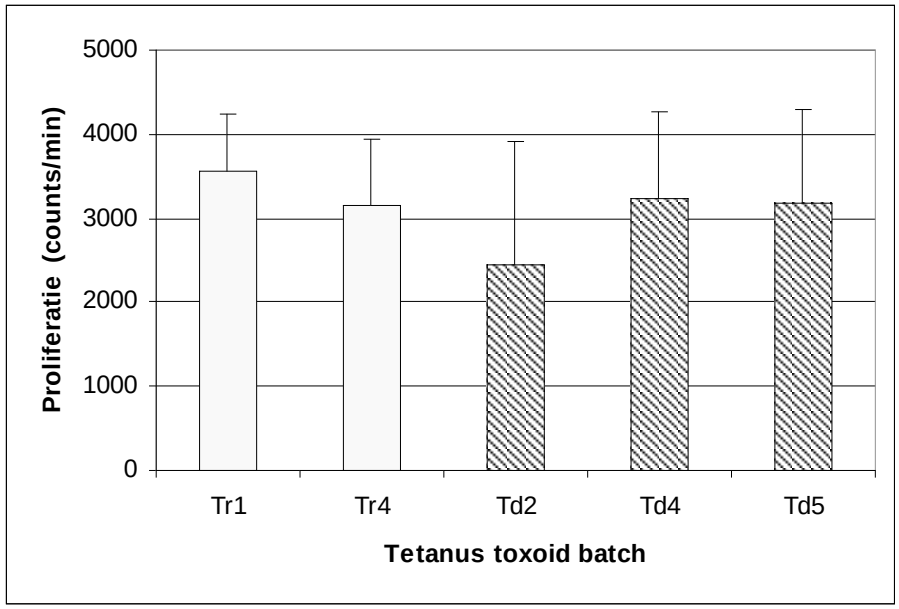
Proliferation assays with mouse spleen cells



Proliferation of mouse spleen PBMCs of non-immunised animals, after culture for 3 days with routine produced tetanus toxoid (Tr) and Tetanus toxoid from process development (Td). Incorporation of 3H thymidine

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Conclusions

- Traditionally, mandatory required vaccine quality control is an area of extensive animal use
- With regards to Three Rs a lot has been achieved, but replacing individual tests by in vitro alternatives might be a too difficult goal
- An better approach might be the shift in paradigm of vaccine q.c.: from final lot testing to the consistency approach
- Consistency testing means demonstrating that the new vaccine batch produced is similar to the clinical lot that has been shown to be safe and effective
- Immunochemical, analytical and in vitro functional are now becoming available that could demonstrate consistency

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Problems to overcome

- Antigen concentrations are normally small
- Antigens are adsorbed onto an adjuvant
- Some preservatives used are not chemically inert and might react with antigen
- Interaction with other vaccine antigens
- Other way of thinking

Studies underlying the development and implementation of Three R's methods in vaccine quality control in our institute have been performed by many:

Johan van der Gun
Gideon Kersten
Marlies Leenaars
Bernard Metz
Bjorn Steen

NVI nederlands vaccin instituut

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