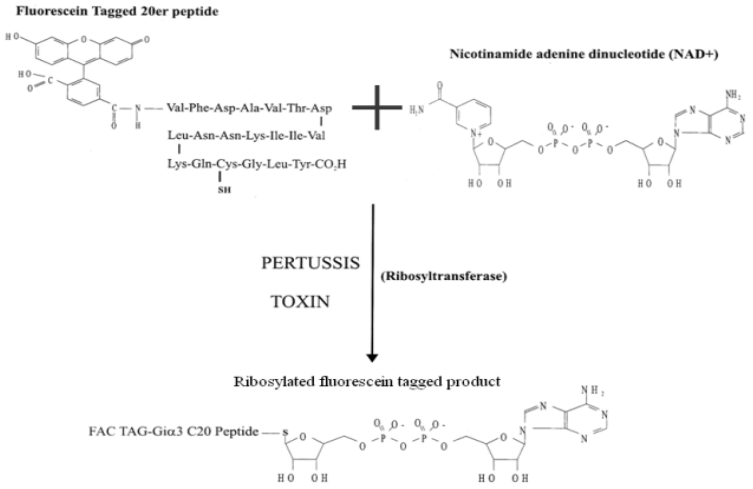
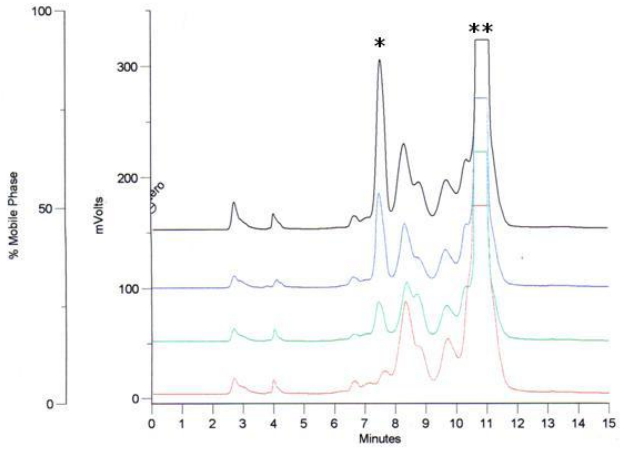
International collaborative study on evaluation of an *in vitro* assay system as an alternative to current histamine sensitization test for acellular pertussis vaccines

Ref. Biologicals 40 (2012) 456-465

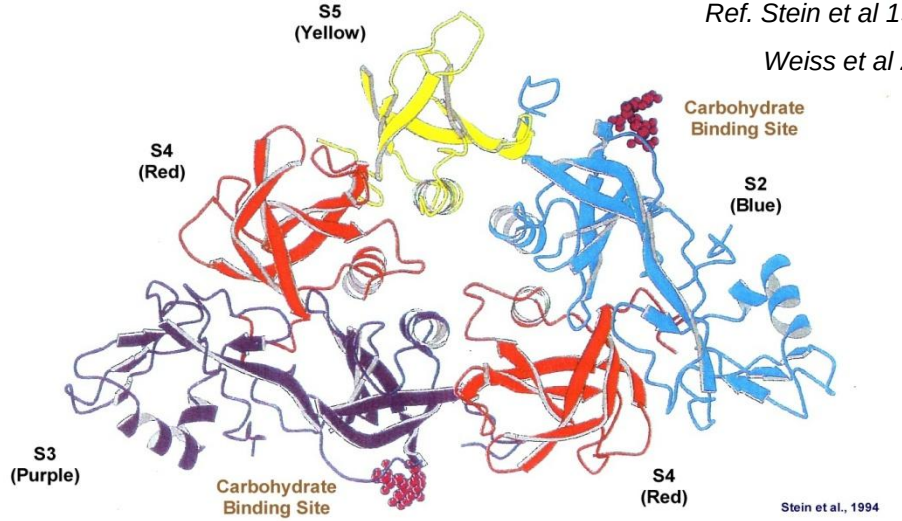
In vitro biochemical assays

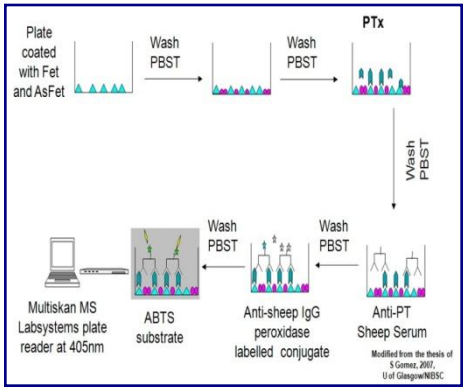


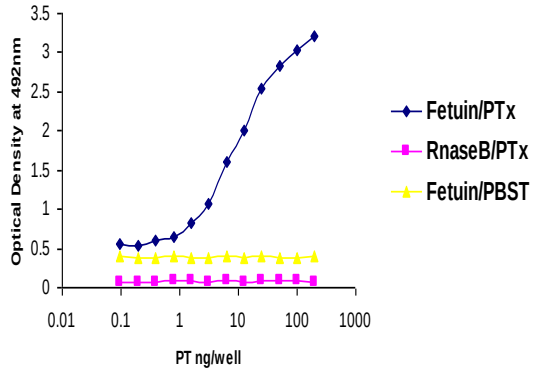


Ref. Stein et al 1994

Weiss et al 2010







Ref. Cyr T, Menzies AJ, Calver J, Whitehouse LW, Biologicals 2001, 29:81-95.

CT Yuen, C Canthaboo, JA Menzies et al.,Vaccine 2002, 21, 44-52.

Gomez SR, Yuen C-T, Asokanathan C et al., Vaccines 2007, 25, 3311–3318.

Ref. S R. Gomez, D K-L. Xing, M J. Corbel et al., Anal. Biochem 2006, 356, 244-253

CT Yuen, Horiuchi Y, Asokanathan C et al., Vaccine 2010, 28:3714-3718

Aims of the study

- To assess the methodology transferability of the enzyme coupled-HPLC (E-HPLC) and carbohydrate-binding assays
- To further assess the suitability of the *in vitro* assay system for three different types of ACV products
- To further evaluate the relationship between the *in vivo* activity by HIST (temperature reduction assay) and the enzymatic and binding activities in the *in vitro* assay system

Participants

Laboratories that are currently involved in quality control test of acellular pertussis containing vaccines by HIST and are willing to perform the in vitro assays were invited

16 *participants, from* ***9*** *countries: EU (5), North American (3*); Asia(3)*

including:

- National Control Laboratories (9*)
- Vaccine manufacturers (8#)

Each laboratories is identified by a randomly assigned code number from 1 to 16 in all analysis to maintain confidentiality

* *Two participants were from the same organisation, but different department*

\# *Laboratory 15 did not submit result due to staff shortage problem*

Sample information

Vaccine sample Code	Description
Samples 1, 6, 8	A purified acellular pertussis vaccine (three-component), detoxified with **glutaraldehyde and formaldehyde**, in combination with diphtheria and tetanus.
Samples 2, 4, 7	A purified acellular pertussis vaccine (five-component), detoxified with **glutaraldehyde**, in combination with diphtheria, tetanus, inactivated poliomyelitis and Haemophilus influenza type b conjugate vaccine
Samples 3, 5, 9	A co-purified acellular pertussis vaccine detoxified with **formaldehyde** in combination with diphtheria and tetanus.

Other materials included in the study

Critical reagents

- *Pertussis toxin reference preparation (NIBSC code 90/518) for HIST (temperature) assay*
- *Pertussis toxin antigen preparation for in vitro assays*
- *Anti-PT sheep serum preparation (NIBSC code 97/572)*
- *PT ADP-ribosyltransferase substrate working solution*

Documents

- Sample/document List
- Study Design
- Instruction for use (IFU) (5)
- Protocols:
  - i) E-HPLC assay
  - ii) Carbohydrate Binding assay
- Reporting sheets:
  - i) E-HPLC
  - ii)Binding Assay
  - iii)HIST Temperature

Study Design Outline-1

In vitro assays:

Primary Step: *In vitro assay system set up:*

- The detailed protocols for the E-HPLC assay and the carbohydrate binding assay provided
- Critical reagents provided, including pertussis toxin reference, substrate and detection antibody
- *Participants are encouraged :*
  - *First to use the pertussis toxin reference only and other critical reagents to set up their assay system*
  - *To get hands on experience of the assay system*
  - *To set up preliminary in-house validity criteria before starting analysis of the vaccine samples provided*

Study Design Outline-2

In vitro assays:

Second step:

Analysis of the 9 vaccine samples using the in vitro assay system established in their laboratory

For each vaccine sample

- A minimum of **three** independent assays of enzyme-HPLC assay
- A minimum of **three** independent assays of carbohydrate binding assay
- To use the pertussis toxin reference, substrate and detection antibody supplied by the study

Study Design Outline-3

HIST - measuring reduction in body temperature after histamine challenge

- Laboratories who currently perform the HIST temperature reduction measurement assays
- The PTx activities expressed relative to the PTx reference (90/518)
  - *Quantitative values*
  - *Two independent HIST assays on the vaccine preparations*
  - *use their own procedure, reagents and calculation methods, and use assay runs that meet internal validity criteria.*

Results returned by participants

Laboratories		
E-HPLC	**Carbohydrate binding**	**HIST (temperature reduction assay)**
14	15	11

In which

All 3 assays	E-HPLC + binding	HIST	HIST + binding
9	5	1	1

Assays		
E-HPLC	**Carbohydrate binding**	**HIST (temperature reduction assay)**
42*	46*	28

* In addition, three laboratories also submitted data using their in-house in vitro assay protocols

Statistical methods

- Raw data were returned to NIBSC for analysis, to ensure as far as possible consistent calculation of results.

- ADP-ribosyltransferase activity (E-unit) and binding activity (B-unit) in vaccine samples were calculated from the standard curve, constructed from the results using PTx reference preparation (90/518). For all assays the data for each vaccine preparation were analysed separately against the reference preparation.

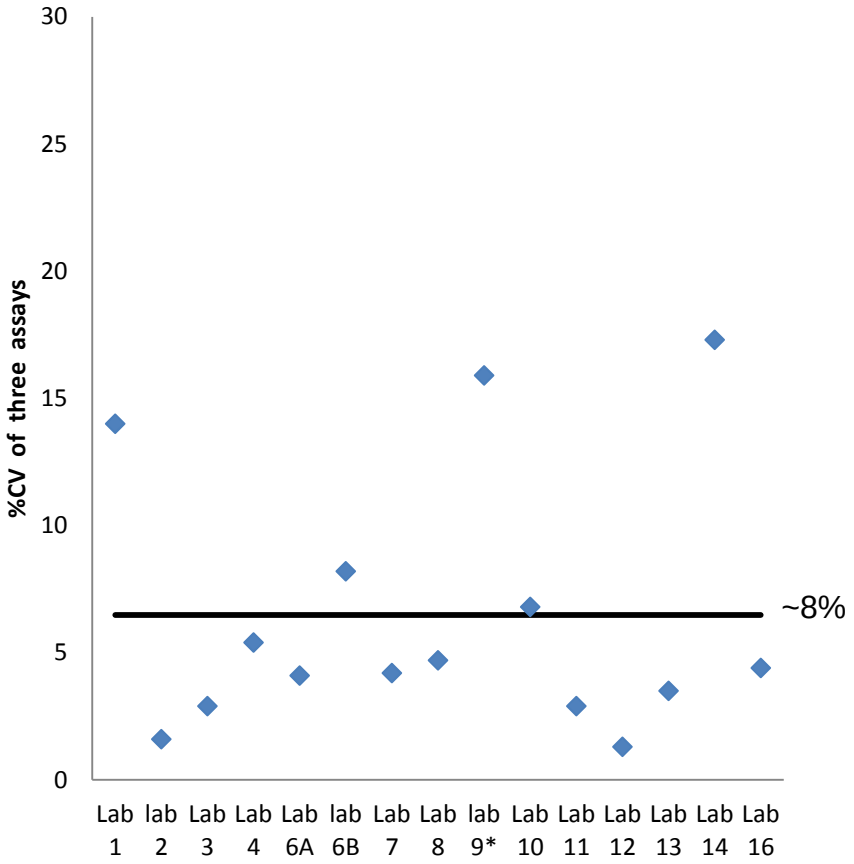
- Homogeneity of the results for each sample was examined. Data which showed out of homogeneity range were defined as outliers which were eliminated from the analysis.
  - *Ref. Sokal, R.R, Rohlf F. J. Biometry, 3rd ed. W. H. Freeman and Co., New York. 1995.*

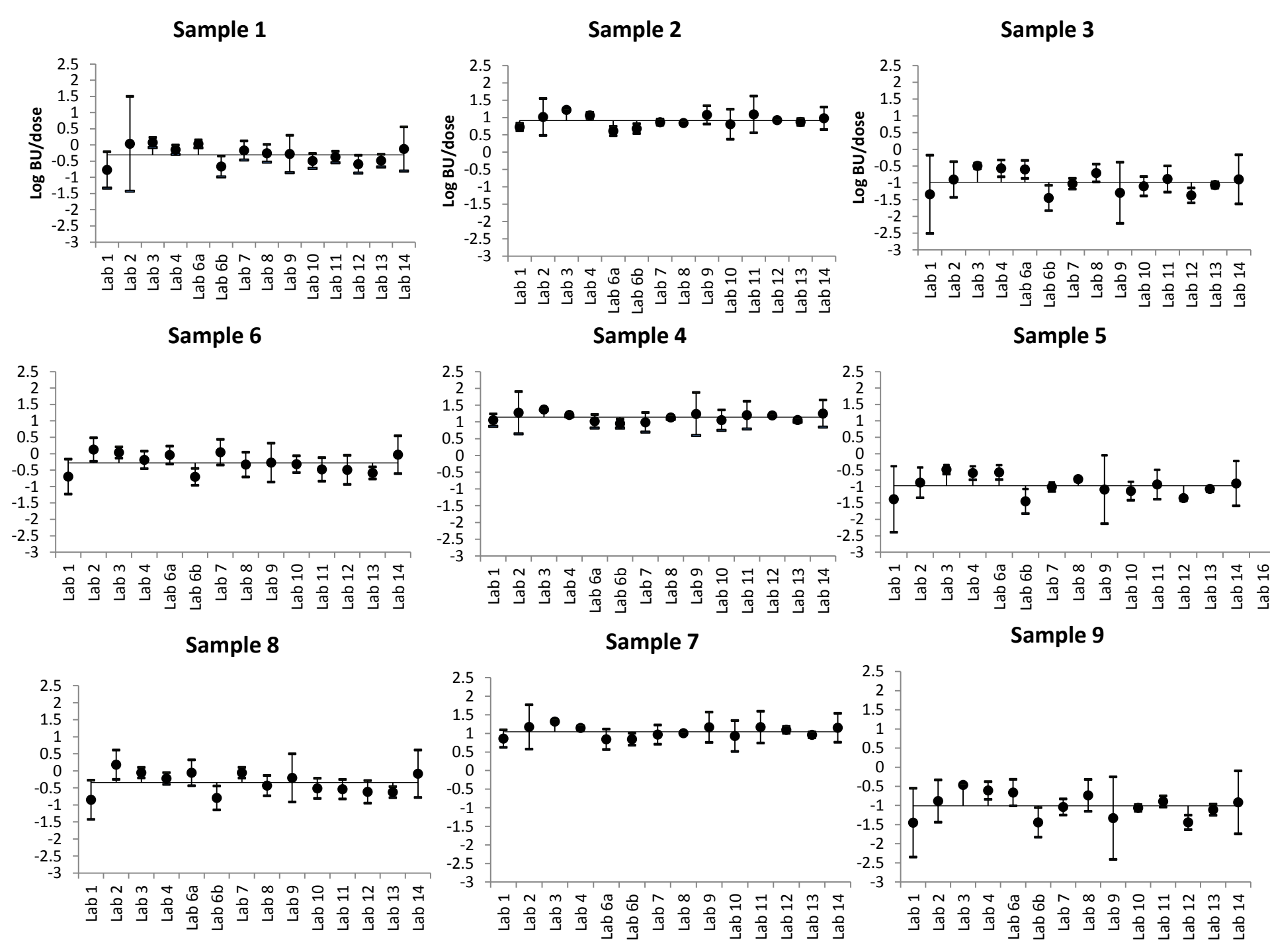
- The HIST activity in the test vaccine was calculated in comparison with that of the reference toxin groups. Relative potency estimates were calculated by parallel line analysis of log transformed response against log dose using the linear section of the dose-response curve and expressed as IU/dose.

Summary of slopes and regression for PTx standard in carbohydrate binding assays

Laboratory	Std curve slope		Std curve regression (r)	
	Log mean (n=3)	%CV	Mean	%CV
1	-0.66	14%	0.9965	0.28%
2	-0.54	1.6%	0.9985	0.07%
3	-0.55	2.9%	0.9995	0.03%
4	-0.42	5.4%	0.9995	0.03%
6A	-0.47	4.1%	0.9977	0.10%
6B	-0.61	8.2%	0.9995	0.01%
7	-0.51	4.2%	0.9963	0.07%
8	-0.53	4.7%	0.9986	0.01%
9*	-0.78	15.9%	0.9822	1.89%
10	-0.55	6.8%	0.9987	0.08%
11	-0.53	2.9%	0.9981	0.04%
12	-0.48	1.3%	0.9986	0.04%
13	-0.52	3.5%	0.9977	0.09%
14	-0.56	17.3%	0.9982	0.03%
16	-0.56	4.4%	0.9977	0.23%

*Four assays were performed

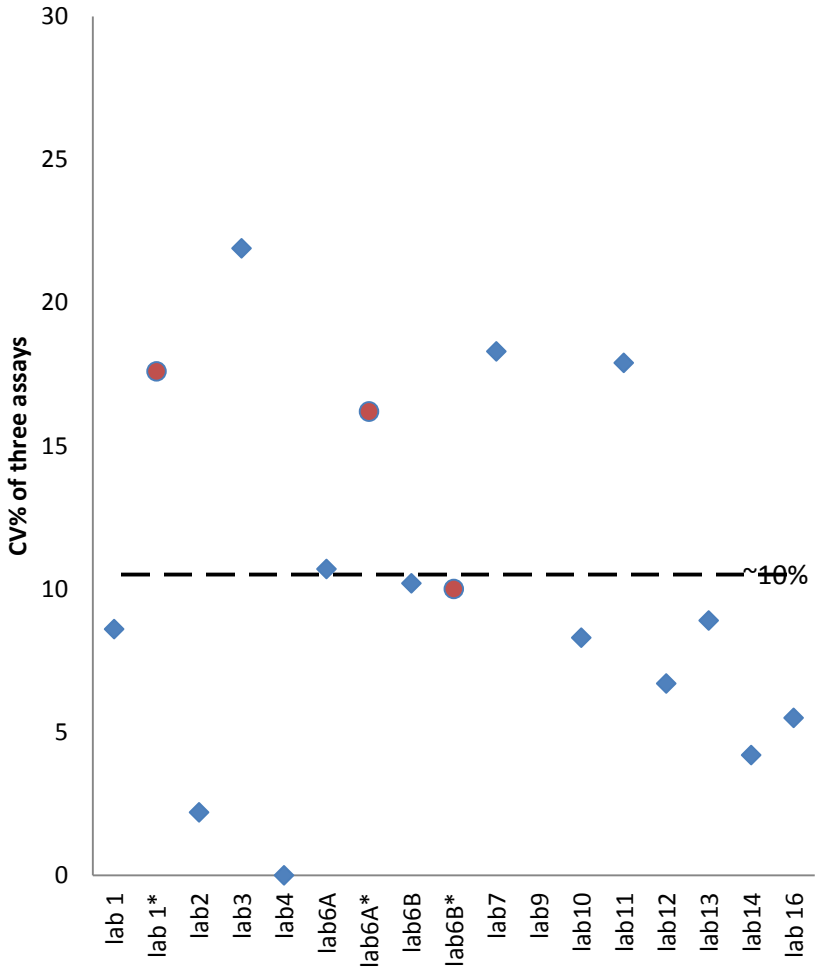


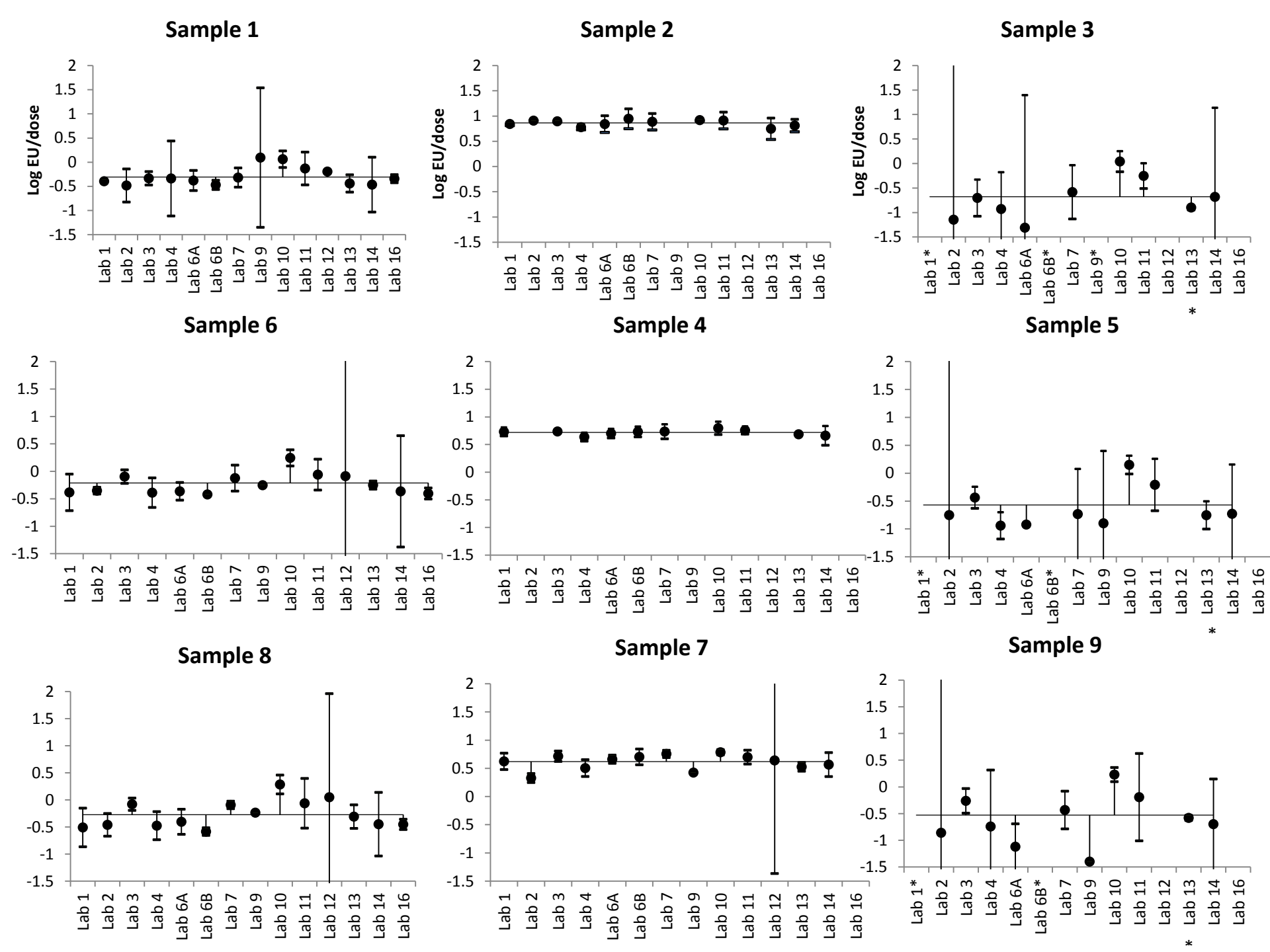

Sample 1
Log BU/dose
2.5
2
1.5
1
0.5
0
-0.5
-1
-1.5
-2
-2.5
-3
Lab 1
Lab 2
Lab 3
Lab 4
Lab 6a
Lab 6b
Lab 7
Lab 8
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Sample 2
Log BU/dose
Sample 3
Log BU/dose
Sample 6
Sample 4
Sample 5
Lab 16
Sample 8
Sample 7
Sample 9

Summary of slopes and regression for PTx standard in E-HPLC assays

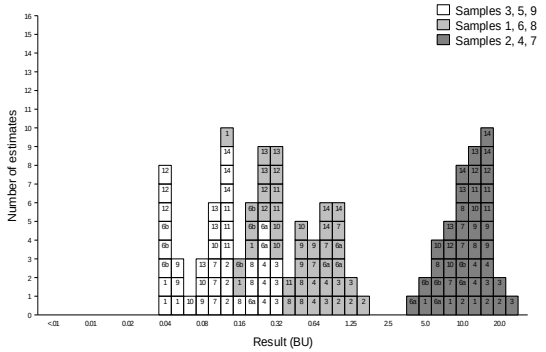
Laboratory	Std curve slope		Std curve regression (r^2)	
	Log Mean	%CV	Mean	%CV
1	-0.21	8.6%	0.999	0.06%
1*	0.09	17.6%	0.994	0.15%
2	4.87	2.2%	0.976	0.27%
3	3.07	21.9%	0.998	0.20%
4	6.30	0	0.998	0.06%
6A	5.95	10.7%	0.996	0.67%
6A*	4.61	16.2%	0.998	0.15%
6B	8.13	10.2%	0.985	1.67%
6B*	6.53	10.0%	0.995	0.41%
7	3.81	18.3%	0.982	1.50%
9#	4.48		0.998	0.21%
10	5.96	8.3%	0.969	2.84%
11	4.68	17.9%	0.983	0.62%
12#	6.87	6.7%	0.976	2.03%
13	1.90	8.9%	0.991	0.27%
14	3.53	4.2%	0.996	0.42%
16	-0.16	5.5%	0.986	1.23%

* In-house protocol
# data from 1 or 2 assay

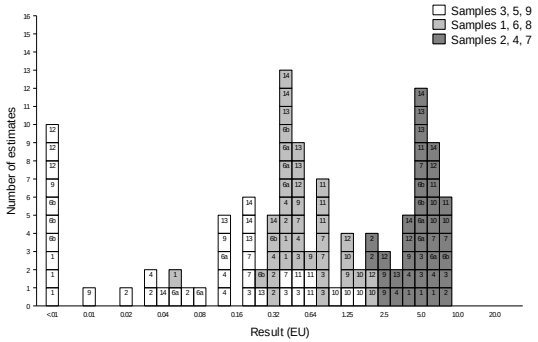



Sample 1
Log EU/dose
2
1.5
1
0.5
0
-0.5
-1
-1.5
Lab 1
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B
Lab 7
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
Sample 2
Log EU/dose
Lab 1
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B
Lab 7
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
Sample 3
Log EU/dose
Lab 1*
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B*
Lab 7
Lab 9*
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
*
Sample 6
Lab 1
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B
Lab 7
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
Sample 4
Lab 1
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B
Lab 7
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
Sample 5
Lab 1*
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B*
Lab 7
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
*
Sample 8
Lab 1
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B
Lab 7
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
Sample 7
Lab 1
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B
Lab 7
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
Sample 9
Lab 1*
Lab 2
Lab 3
Lab 4
Lab 6A
Lab 6B*
Lab 7
Lab 9
Lab 10
Lab 11
Lab 12
Lab 13
Lab 14
Lab 16
*

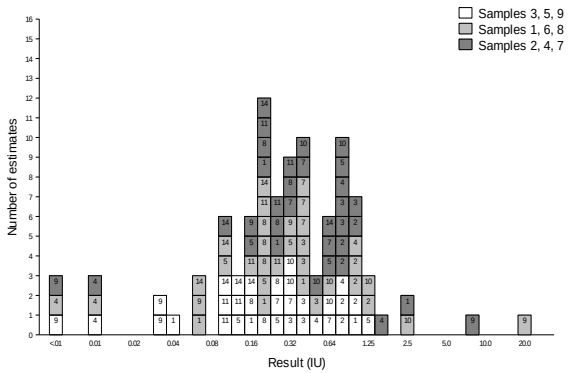
Carbohydrate binding assay



E-HPLC assay



HIST



Geometric mean (GM) and 95% Confidence Limits (CL) of carbohydrate binding activity (BU), enzymatic activity (EU) and HIST activity (IU) for the three types of acellular pertussis based vaccine products included in the study

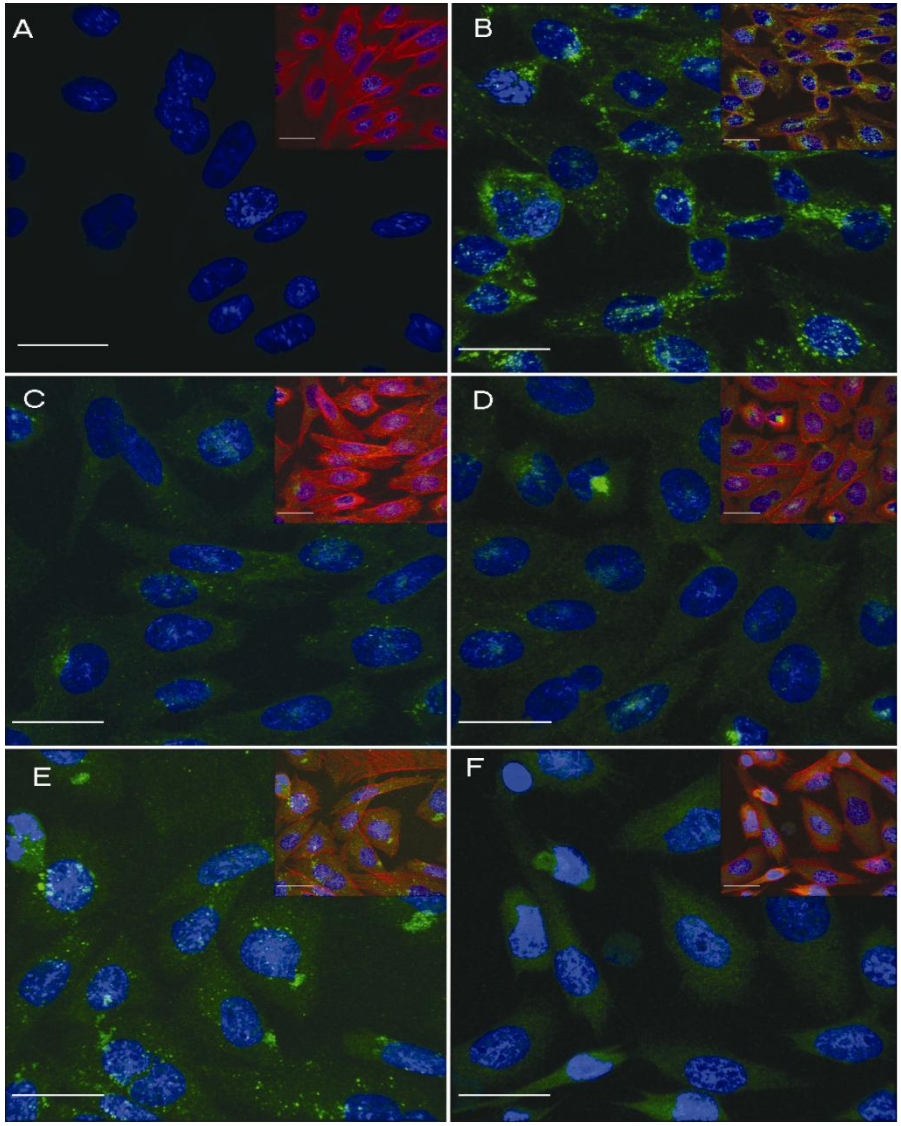
Sample Number		Activities		
		BU/dose	EU/dose	IU/dose
Sample 1, 6, 8	GM	0.49	0.27	0.24
	95% CL	(0.41-0.59)	(0.39-0.75)	(0.11-0.54)
Sample 2, 4, 7	GM	10.80	2.77	0.37
	95% CL	(5.57-20.93)	(2.39-12.83)	(0.13-1.09)
Sample 3, 5, 9	GM	0.10	0.12	0.20
	95% CL	(0.09-0.11)	(0.15-0.34)	(0.05-0.75)

Discussion

- The methodology of the E-HPLC and carbohydrate-binding assays are transferable between laboratories worldwide
- This system is suitable for the three types of ACV products included in the study.
- Although direct correlation between the *in vitro* assay system and the *in vivo* HIST for each individual vaccine lot cannot be established due to the small range of the activities/large variation in the HIST results, geometric mean estimates of the *in vitro* and *in vivo* activities gave the same rank order of the three vaccine types which is encouraging.
- The *in vitro* systems provide reproducible product specific profiles which proved the principal of this approach as a potential alternative to the HIST.
- Due to limitation in capacity, investigation of the adsorption/desorption effect on the assay system was not addressed in the present study. These factors may be product specific and have to be further evaluated.
- PT activities in the vaccine samples included in the study represented the range in most products in current market, they are relatively low and within a very narrow range. Further study using spiked sample may provide further information on assay sensitivity.
- In the present study only 3 batches of each product were examined because of restricted capacity for assay evaluation. Therefore further product specific validation study involving more batches under each laboratory setting would be needed, e.g. precise assay condition, desorption, detection limits, precision, validity criteria, specification etc.

Discussion

This in vitro assay system could not address directly the mechanism of translocation/internalisation of PTx



- Comparison of PTx and different PTds in translocation to CHO cells shown by the fluorescence intensity of target protein in confocal images.
- CHO cells were treated with PTx or PTd for 12 h.
- PTds with different detoxification procedures showed great reduction in their translocation/internalization activities compared to the native toxin.
- All PTds at higher concentrations could translocate into the target cells with differences in fluorescence intensity under the same conditions, indicating different detoxification methods could result in PTds with different activities.
- The differences in translocation capacity on CHO cells between PTx and PTds or between PTds are consistent with their carbohydrate binding activities which added further scientific evidence to support the developed in vitro assay system as a potential alternative to HIST as safety test for ACV quality control.

PT
Nucleus
F-actin of cytoskeleton

Scale bar =20µm

Ref. Human Vaccines & Immunotherapeutics, Volume 9 Issue 2, in press

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NIBSC Team

- CT Yuen
- Cathy Asokanathan
- Sheena R Gomez
- Peter Rigsby
- Roland Fleck
- Dorothy Xing

NIID, Japan

- Yoshi Horiuchi
- Masaki Ochiai

NIFDC, China

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