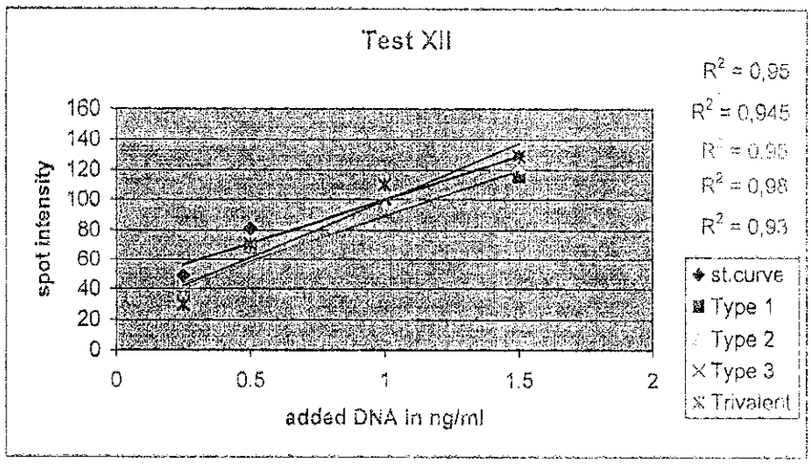
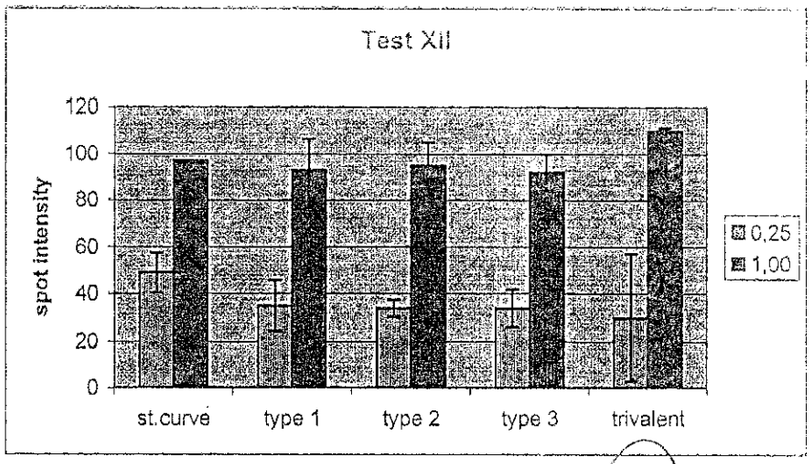
APPENDIX 3 GRAPHS TEST XII





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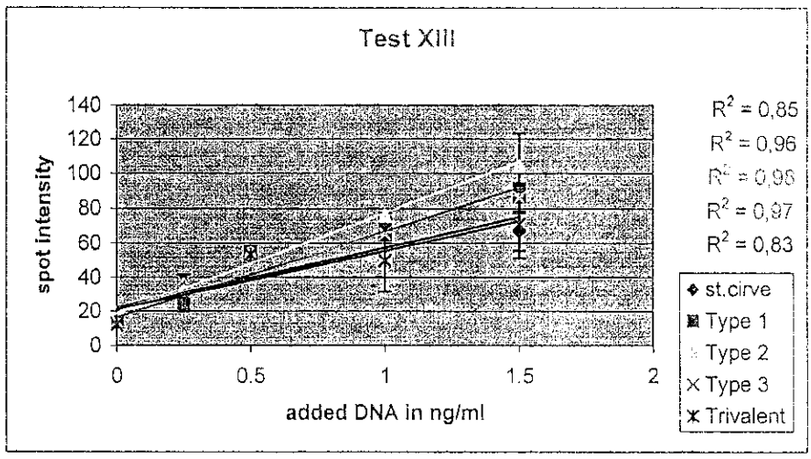
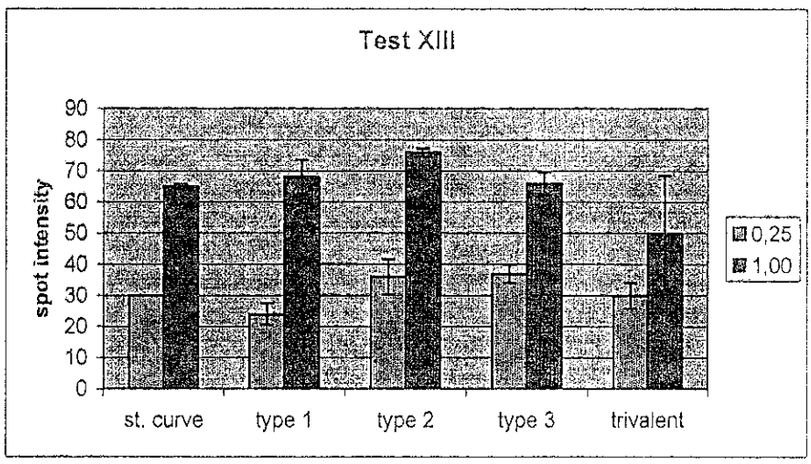
APPENDIX 4 GRAPHS TEST XIII





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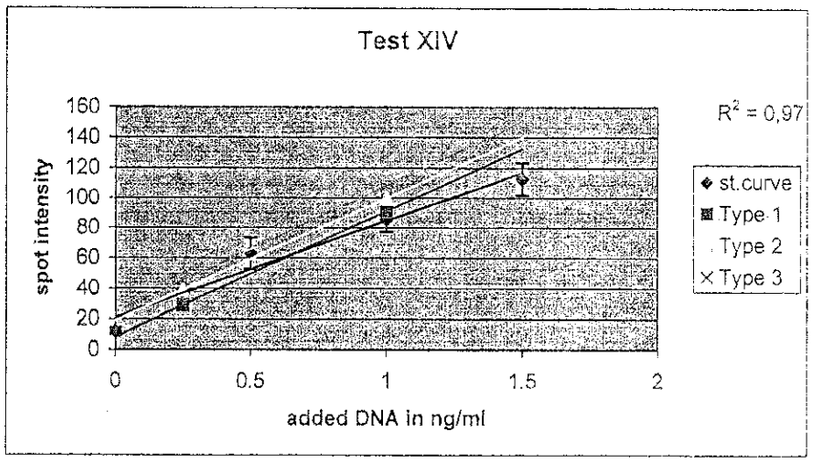
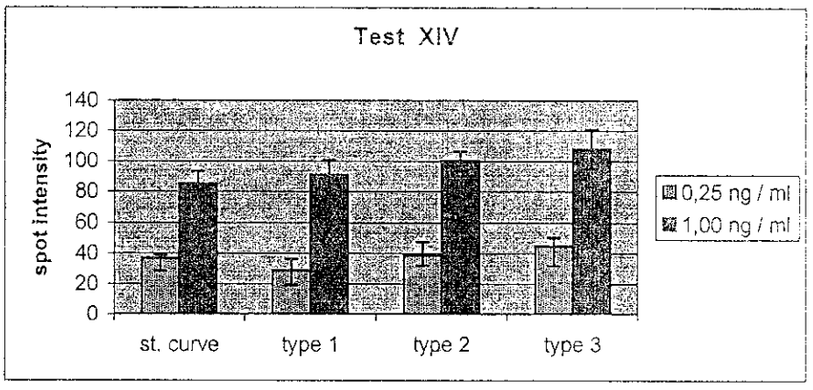
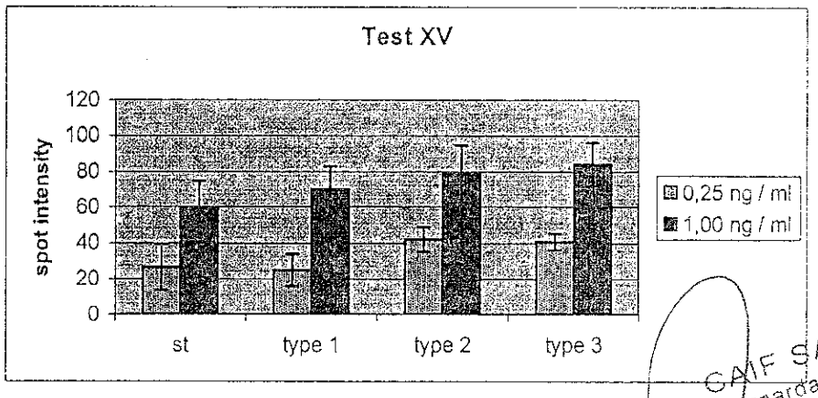
APPENDIX 5

GRAPHS TEST XIV and XV







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Report RAP-21726
Version: 1

Validation of D-antigen ELISA (QC-QCV)

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1 Introduction

1.1 Background

The D-antigen concentration of poliovaccines is determined by an ELISA according to current EP guidelines as part of the release of polio-containing vaccines. The test is currently performed with an automated pipetting instrument and the results are calculated by means of linear regression with a calibration curve consisting of three consecutive dilutions of the standard, international reference PU91-01.
For the new method the dilutions will be performed manually and the calculations performed according to the 4 - parameter fit calculation model using seven dilutions of PU91-01.
Due to the extra dilutions of PU91-01 and the use of the 4-parameter fit calculation model, a more accurate calibration curve is obtained. This results in a more reliable D-antigen concentration of poliovaccines when compared to the current method. This report describes the validation of the new method.

1.2 Objective

The objective of this study is to record the validation of the manually performed ELISA using the 4 parameter fit calculation method. This manual test will replace the current automated test for the routine testing of D-antigeen concentration in polio vaccines. The raw data from the validation study will be kept by the department NVI-QCV.

2 Summary

Table 1: Summary

Parameter	Criteria	Result
Specificity: Cross reaction	No cross reaction between the different polio types.	No cross reaction was observed between the different polio types.
Specificity: Vaccine matrix effect	The vaccine matrix must have no interfering effect on the D-antigen ELISA test.	No interfering effect observed.
Specficity: Individual vaccine components	The individual vaccine components must have no interfering effect on the D-antigen ELISA test.	No interfering effect observed.
Linearity	The correlation coefficient (R^2) must be at least 0.990. The residuals (extinctions of the reference calculated back to the fitted curve) cannot deviate more than 10% with a maximum spread of 20%.	The R^2 is ≥ 0.990. The residuals are ≤ 10% with a maximum spread of 18%
Accuracy	The accuracy for IPV 785B, DaKTP, Monovalent IPV and DTP vaccines was determined. The accuracy must be within 10% of the expected value.	The recovery was within 10% of the expected value. The maximum deviation was 4.7%
Precision: repeatability	Three independent test were performed by one analist on one day. The maximum coefficient of deviation (CoV) is 10% per product.	The repeatability of the test was good. The maximum CoV was 7.8%
Precision: Intermediate Precision	Six tests were performed by two analists on different days, three tests per analist. The CoV is 10% per product.	The intermediate precision of the test was good. The maximum CoV was 9.2%.

On the basis of the results obtained from the validation study it is concluded that the D-antigen test conforms to the criteria and may be adopted for use as the D-antigen test for routine samples.

3 General information

3.1 Definitions & abbreviations

CoV	Coefficient of Variation
DU/ml	D-antigen units per millilitre
DaKTP	Diphtheria, a-cellular pertussis, Tetanus, Polio
DTP	Diphtheria, Tetanus, Polio
ELISA	Enzyme Linked Immuno Sorbent Assay
IPV	Inactivated Polio Vaccine
MoAb	Monoclonal antibody
PBS	Phosphate buffered saline
SD	Standard deviation
EP	European Pharmacopoeia
Old method	current test - performed with an automated pipetting instrument and calculated using linear regression
New method	test being validated – performed manually and calculated using the 4-parameter fit model

3.2 References

- European Pharmacopoeia :0214
- ANA-10102: Determination of the D-antigen content of inactivated polio vaccine by means of an ELISA. (Original dutch title: bepaling van het D-antigeen gehalte van geinactiveerd poliovaccin m.b.v. Enzyme Linked Immuno Sorbent Assay (ELISA))

3.3 Deviations from the European Pharmacopoeia (EP)

The EP states that vaccines containing aluminium phosphate should be desorbed before the D-antigen concentration determination. The NVI procedure deviates from this as the vaccine is not treated before testing. This validation study demonstrates that the untreated vaccines do not have an interfering effect on the test and therefore prior-treatment of the test samples is not necessary (See section 6.3).

4 Method

4.1 Principal of the test

The D-antigen content determination is performed following the immunochemical method (ELISA) described in the EP :0214.

The assay consists of a 5-step procedure:

1. The wells of a micro test system are coated with type-specific bovine-anti-polio antiserum.
2. Type-specific polio antigen binds to the coated wells.
3. Type-specific monoclonal mouse-anti-polio antibodies bind to the bound antigen.
4. Peroxidase labelled sheep-anti-mouse conjugates bind to the mouse monoclonal antibodies.
5. Tetramethylbenzidine (TMB) is a substrate for peroxidase and gives a colour reaction.

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4.2 Materials

The following reference materials are used for the test:

- Reference vaccine Polio trivalent PU91-01 with Poliomyelitis virus type 1 (430 DU/ml), Poliomyelitis virus type 2 (95 DU/ml) and Poliomyelitis virus type 3 (285 DU/ml).
- Internal standard Polio plain 785 B.

The following other materials are used for the test:

- Bovine anti polio type 1, 2 and 3 for the coating of the plates
- Monoclonal polio antibody (MoAb): Anti-poliomyelitis virus type 1, type 2 or type 3
- Conjugate: Sheep-anti-mouse Ig, Horseradish peroxidase labelled (reaction with MoAb anti-polio type 1, 2 and 3)
- Buffers and diluents:

  PBS 0.01 M, pH 7.2 (dilution bovine-anti-polio antisera for coating)
  PBS 0.01 M, pH 7.2 + 1% BSA (block buffer)
  PBS + 0.005% Tween 80 (antigen dilution buffer)
  PBS + 0.005% Tween 80 + 1% BSA (dilution monoclonal antibodies and conjugates)
  PBS 0.01M, pH7.2 + 0.05% Tween 80 (washing buffer)

- Tetramethylbenzidine solution (TMB),
- H_2SO_4 (0.2 M)
- For the bovine-anti-polio antiserum, monoclonal and the conjugate the actual practical dilution is determined by a dose-response curve each time a new batch is used.

4.3 ELISA procedure:

The samples are tested in parallel with a reference preparation to determine a calibration line.

1. Coating and blocking of the plates

All wells are coated with type specific bovine-anti-polio antiserum and incubated overnight at room temperature. The plates are washed with washing buffer. Hereafter, the plates are blocked with block buffer and incubated for a minimum of 30 minutes at 37°C and washed again.

2. Preparation of internal standard, reference vaccine and samples:

The reference is diluted 1/25 and the internal standard is diluted 1/2. The samples are diluted to a concentration equivalent to the reference. All samples are diluted with antigen dilution buffer. All samples are tested in duplicate.

3. Antigen incubation

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Reference vaccine, internal standard and samples are diluted with a 1/2 serial dilution in the ELISA plate with one line filled with antigen dilution buffer as a negative control. The plate is incubated for 2 hours at 37°C and subsequently incubated overnight at 4°C.

4. Monoclonal incubation

The plates are washed with washing buffer and then incubated with type-specific monoclonal mouse-anti-polio antibodies for 2 hours at 37°C.

5. Conjugate incubation

The plates are washed with washing buffer and then incubated with sheep-anti-mouse Ig, Horseradish peroxidase labelled, for 1 hour at 37°C.

6. Colouring of the plates

The plates are washed with washing buffer and substrate solution (TMB) is added to the wells. After 10 minutes of incubation H_2SO_4 is added to stop the reaction. The extinctions are measured at 450 nm.

4.4 Calculation

Calculation of the D-antigen concentration is performed using the 4-parameter fit method. The D-antigen concentrations of the samples are calculated in DU/ml and are based on the results of the reference calibration curve. The reference produces a sigmoid curve with a linear region when the reference concentration is plotted against the extinctions obtained. This linear region is used to calculate the D-antigen concentration (DU/ml) of the samples. The extinctions of the samples that fall within this region are converted to D-antigen concentrations using the KC junior calculation programme.

4.5 Validity criteria

The D-antigen concentration for each type of polio virus is calculated on the basis of the results of a valid assay.

The assay is valid if the following criteria are met:
1. The correlation coefficient of the calibration line of the reference vaccine and the internal standard is ≥ 0.990.
2. The coefficient of variation of the final result for the internal standard is ≤ 20%.
3. The internal standard complies with the criteria of the trend monitoring. These include:
   - The value falls within the ± 3SD area
   - The value must not fall outside the ± 2SD area for two consecutive tests
   - The range between the value and the previous value is smaller than 4SD
   - The value must not fall outside ± 1SD area for four consecutive tests
   - The value must not fall on one side of the average for ten consecutive tests
4. The average deviation of the residuals (extinctions of the standard calculated back to the fitted curve) must be between -10% and 10 %, with a maximum spread of 20%.

The test sample is valid if the following criteria are also met:
1. The correlation coefficient for each sample is ≥ 0.990.
2. The coefficient of variation of the average final result for the samples is ≤ 20%.

4.6 Test samples and reference

The reference and test samples used in this study have been previously routinely tested for D-antigen.

Table 2: Reference and test samples used in this study

Sample	Name	Value obtained with old method (DU/ml) (polio type 1 – type 2 – type 3)
Reference	PU91-01	430 – 95 - 285
Internal standard	785B	38 – 8 - 28

Sample		Name	Nominal value (DU/ml) (polio type 1 – type 2 – type 3)
IPV (final lot)		IPV 801 (IPVmkc)	40 – 8 - 32
		IPV 799 (IPVvero)	80 – 16 - 64
		IPV 795(IPVvero)	80 – 16 - 64
IPV (trivalent bulk)		IPV 05-129	400 – 80 - 320
DTP		DTP 130	40 – 4 – 7.5
		DTP 134	
		DTP 135	
		DTP 131	
DaKTP		DaKTP 001	80 – 16 - 64
		DaKTP 005	
		DaKTP 003	
		DaKTP 006	
Monovalent IPV	Polio Type 1	PV06-127-6.1	none (criteria for release: 1250 tot 3140)
		PV 06-128-6.1	
		PV 06-129-6.1	
	Polio Type 2	PV06-213-6.1	none (criteria for release: 430 tot 1460)
		PV 06-214-6.1	
	Polio Type 3	PV06- 313-6.1	none (criteria for release: 520 tot 2220)
		PV06-314-6.1	
		PV06-316-6.1	

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5 Specificity A: Cross-reaction

5.1 Criteria

No cross-reaction occurs between the different polio types.

5.2 Results

The specificity of the reagents used for the D-antigen test is studied here. The type specific coat and monoclonals are tested with three different types of antigens on coated plates of the three different types to determine if crossreactions occur between the types. The tables below report the extinctions obtained. Only the first three lines of the reaction curve are represented as these are the most relevant. The complete curves are represented in Appendix 1. A reaction is determined to be positive when the extinction obtained is two times the average background.

Table 3.1: Type 1 coat

	Moab: type1 Antigen: type 1		Moab: type 2 Antigen: type 2		Moab: type 3 Antigen: type 3		Moab: type 2 Antigen: type 1		Moab: type 3 Antigen: type 1		Moab: type1 PU91-01	
	1.9	2.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	2.0	1.6
	1.6	1.6	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	1.5	1.6
	1.0	1.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	1.1	1.2
Background:	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table 3.2: Type 2 coat

	Moab: type1 Antigen: type 1		Moab: type 2 Antigen: type 2		Moab: type 3 Antigen: type 3		Moab: type 1 Antigen: type 2		Moab: type 3 Antigen: type 2		Moab: type 2 PU91-01	
	0.3	0.2	1.2	1.2	0.2	0.1	0.1	0.1	0.1	0.1	1.3	1.3
	0.2	0.2	0.9	0.9	0.2	0.2	0.1	0.1	0.1	0.1	1.0	1.0
	0.2	0.2	0.7	0.6	0.1	0.2	0.1	0.1	0.1	0.1	0.6	0.7
Background:	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Table 3.3: Type 3 coat

	Moab: type1 Antigen: type 1		Moab: type 2 Antigen: type 2		Moab: type 3 Antigen: type 3		Moab: type 1 Antigen: type 3		Moab: type 2 Antigen: type 3		Moab: type 3 PU91-01	
	0.1	0.1	0.1	0.1	2.1	1.9	0.1	0.1	0.1	0.1	2.1	1.9
	0.1	0.1	0.1	0.1	1.9	1.8	0.1	0.1	0.1	0.1	1.9	1.9
	0.1	0.1	0.1	0.1	1.5	1.6	0.1	0.1	0.1	0.1	1.5	1.3
Background:	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

: positive signal (yellow and underlined)
: negative signal (white)
: light positive signal (orange)

The results show that the homologous antigen-antibody combinations for each type give an expected positive result. This positive result is also shown by the trivalent reference (PU91-01). Both are illustrated in yellow and underlined. A slight positive signal is shown by the combination antigen / monoclonal type 2 and coating antibody type 1. This is illustrated in orange. The negative signals in the other heterologous antigen-monoclonal combinations indicates that the monoclonals are type specific. These are illustrated in white.

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5.3 Discussion

The results show positive reactions for the homologous antigen-antibody combinations and negative for the heterologous antigen-antibody combinations. This proves the specificity of the coating and monoclonal antibody.

However, there is a slight positive reaction between coat 1 and antigen/monoclonal 2. This is indicative of a cross reaction. This can be explained by the fact that the coating antibody is polyclonal. A test was carried out to determine the effect of the cross reaction. Monovalent 1 and 2 were mixed in different ratios and subsequently tested for type 1 and 2 concentration and the % recovery determined. The recovery from both types was approximately 100% for the homologous antigen-antibody combinations. It was concluded that there is no competition between the two types and that the cross reaction for type 1 coat and type 2 antigen has no influence on the measured concentration of polio D-antigen type 1 and type 2. The little extra background caused by the aspecific reaction between type 1 coat and type 2 antigen does not lead to an overestimation in type 1 for NVI products.

The cross reaction occurs only in trivalent IPV samples on the type 1 coated plate. The cross reaction also occurs in the reference PU91-01. The extra background that occurs by the test samples also occurs by the reference. This compensates for the extra background in the test samples and thus does not lead to a false higher result for type 1.

5.4 Conclusion

This test shows that no cross reaction occurs between the different types with the exception of Coat type 1 and monoclonal type 2. These exhibit a slight positive reaction but is shown to have no effect on the determination of the D-antigen content of the vaccine.

6 Specificity B: Matrix effect (with polio component)

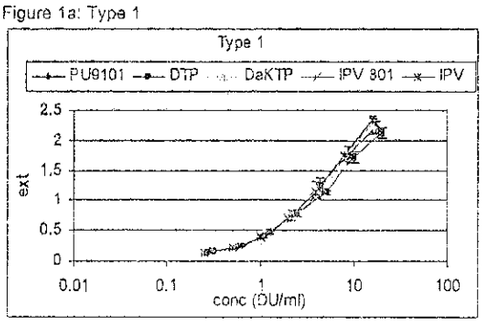
6.1 Criteria

The vaccine matrix must have no interfering effect on the D-antigen test.

6.2 Results

Any possible interfering effect that the vaccine components may have on the test is studied in this test. IPV (in two concentrations), DTP and DaKTP are tested in triplicate and compared to the reference PU91-01. The test samples are diluted to the same concentration as the reference.

Figure 1a: Type 1



	PU91-01	DTP	DaKTP	IPV 801	IPV
Conc. (DU/ml)	430	40	80	40	80
Dilution	25	2	5	2.5	5
Start conc.	17.2	20	16	16	16
R^2 value	0.997	0.998	0.996	0.999	1.000

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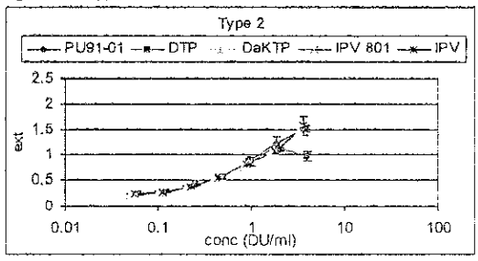
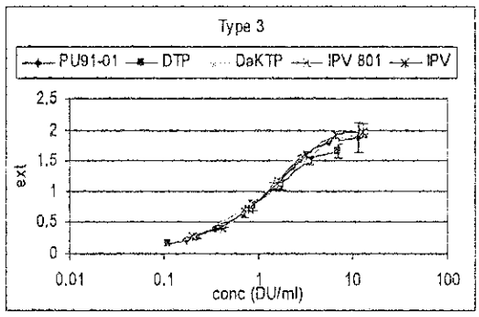
Figure 1b: Type 2



	PU91-01	DTP	DaKTP	IPV 801	IPV
Conc. (DU/ml)	95	4	16	9	18
Dilution	25	1	5	2.5	5
Start conc.	3.8	4	3.2	3.6	3.6
R^2 value	0.995	0.995	0.999	1.000	0.997

Figure 1c: Type 3



	PU91-01	DTP	DaKTP	IPV 801	IPV
Conc. (DU/ml)	285	7	65	33	65
Dilution	25	1	5	2.5	5
Start conc.	11.4	7	13	13.2	13
R^2 value	0.997	0.998	0.998	1.000	0.998

Figure 1: The antigen curves of the different vaccines and PU91-01 are shown above. The concentrations of the vaccines are represented on the horizontal axis and the extinctions on the vertical axis. The errorbars in the graphs represent the standard deviation within the triplicate.

The graphs show that all vaccines tested show the same response curve as the reference. This indicates that the vaccine components have no effect on the test. DTP type 2 however, shows a plateau at the highest concentration in figure 1b. The R^2 is above 0.990 and is within the criteria.

6.3 Discussion

The graphs (figure 1a - 1c) show that all curves for the three polio types increase at the same rate. The R^2 value for the curves is above 0.990. All test samples increase with the reference without having a effect on the test. DTP type 2 however, exhibits a plateau by the highest concentration. The graphs show that this effect is only to be seen by the higher concentration and not in the linear range and thus has no effect on determining the concentration of the test sample.
The EP states that test samples should be desorbed before the D-antigen concentration determination. The NVI procedure deviates from this as the vaccine is not treated before testing. The results in figure 1a – 1c illustrate that aluminium phosphate does not influence the test. PU91-01, IPV 801 and IPV are products without aluminium phosphate and DTP and DaKTP contain aluminium phosphate. The curve of all five products overlap with each other for all the three types. This demonstrates that aluminium phosphate does not have an interfering effect on the test and that desorpstion is not necessary.

6.4 Conclusion

This test shows that the vaccine matrix has no interfering effect on the test.

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7 Specificity C: Matrix effect (without polio component)

7.1 Criteria

The vaccine components have no interfering effect on the D-antigen test.

7.2 Results

This test determines if there is a matrix effect from the individual vaccine components. The individual components of the vaccine, namely, diphtheria bulk, tetanus bulk and aluminium phosphate, and the DaKT bulk were spiked with PU91-01 and diluted in the ELISA plate so that the component concentration remained the same and PU91-01 was diluted. Aluminium phosphate without PU91-01 and antigen-dilution buffer spiked with PU91-01 were also tested as controls. The following graphs report the results.

Figure 2a: Type 1

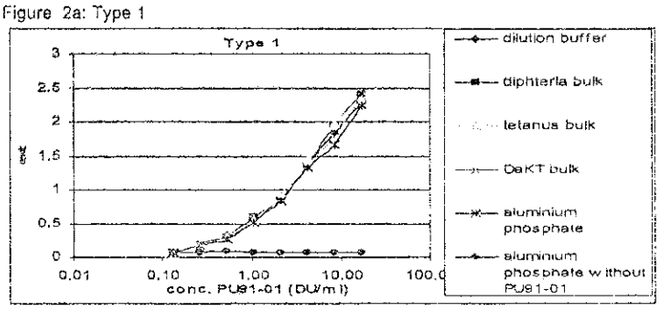


Figure 2b: Type 2

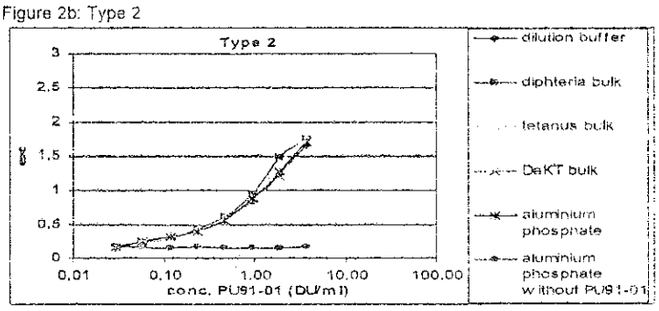


Figure 2c: Type 3

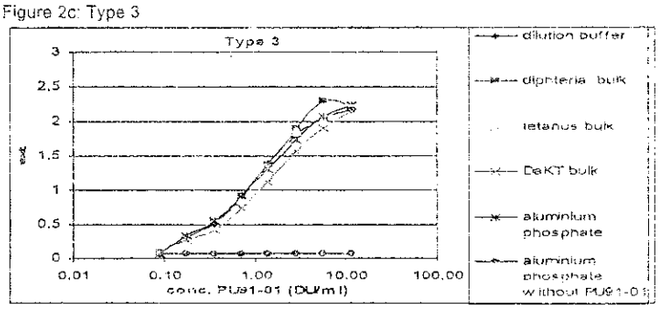
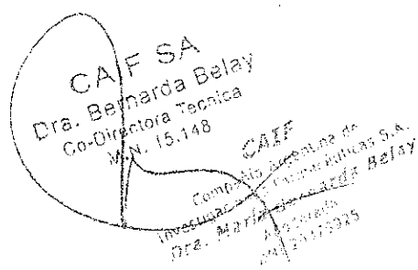


Figure 2: The antigen curves from the individual components spiked with PU91-01 are shown in the above graphs. The concentration of PU91-01 is represented on the horizontal axis and the extinctions on the vertical axis.



All the components are spiked with the same concentration PU91-01 and titrated out in the plate. The antigen curves of the components show the same reaction curve as that of the antigen dilution buffer and show no effect on the test.

7.3 Discussion

In this test the individual components of the vaccines were tested to determine if they have an effect on the test. Diphteria bulk, tetanus bulk, DaKT and aluminium phosphate were spiked with PU91-01 and titrated out in an ELISA plate in order to obtain a complete reaction curve. Antigen dilution buffer spiked with PU91-01 was used as a positive control and unspiked aluminium phosphate was used as a negative control.

The results of the test are shown in figure 2a – 2c. The graphs for the different components show the same rate of increase in concentration as the positive control and show no negative effect. The negative control shows no increase which indicates that aluminium phosphate has no effect on the test. This confirms the findings from the previous test (Specificity B) where it was also observed that aluminium phosphate has no interfering effect on the D-antigen ELISA test

7.4 Conclusion

This test shows that the individual vaccine components do not have an interfering effect on the ELISA test.

8 Linearity

8.1 Criteria

1. The linearity of the reference (PU91-01), the internal standard (785B), DTP, DaKTP and the monovalent samples is determined.

2. The correlation coefficient (R^2) value must be at least 0.990.

3. The residuals (extinctions of the reference calculated back to the fitted curve) may not deviate more than 10% with a maximum spread of 20%. Dilution ranges that fall outside these criteria shall not be included in the linear range.

8.2 Results

The linearity of PU91-01, 785B, DTP, DaKTP and the monovalent samples were determined by testing a dilution series and determining the linear region of the curve with the use of the 4-parameter fit method. The dilution series (starting dilution 6.25) was performed in eleven steps of a two-times dilution. A second dilution series (starting dilution 4.69) was also performed to increase the accuracy of the test. The second dilution series was performed using the between dilutions in the first dilution series.

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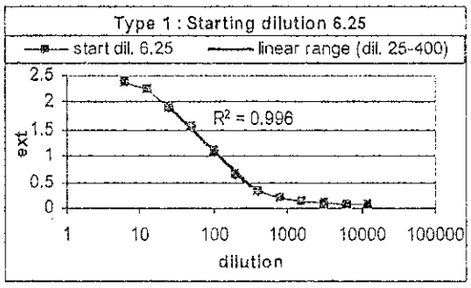
The graphs below represent the test sample curve (with the linear range) of PU91-01.

First Dilution series (Starting dilution 6.25)

Figure 3a: Linear range Type 1 (dilution 6.25)



Second Dilution series (Starting dilution 4.69)

Figure 3b: Linear range Type 1 (dilution 4.69)

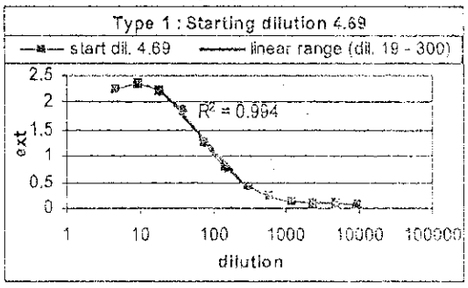


Figure 3c: Linear range Type 2 (dilution 6.25)

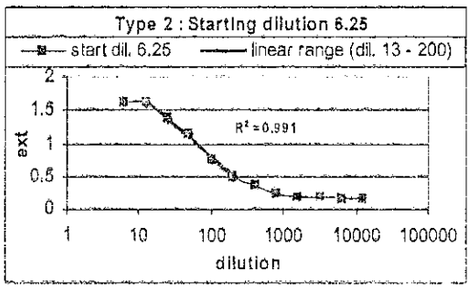


Figure 3d: Linear range Type 2 (dilution 4.69)

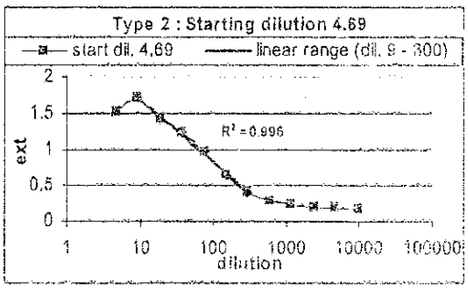


Figure 3e: Linear range Type 3 (dilution 6.25)

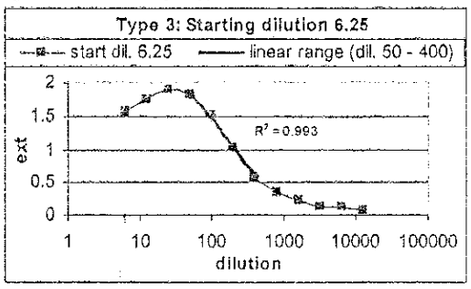


Figure 3f: Linear range Type 3 (dilution 4.69)

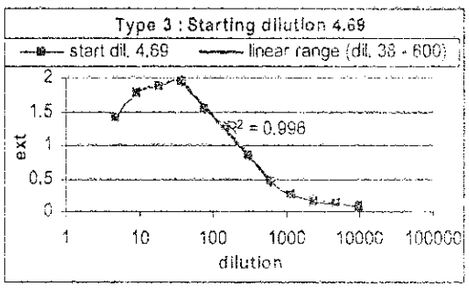


Table 4: Linear range and residuals PU91-01

Type	Linear Range		R^2 (≥ 0.990)	Residuals (≤ 10%)	Residual Spread (≤ 20%)
	Dilution range	Conc. range (DU/ml)			
1	19 – 400	23 - 1	≥ 0.990	0.06	4.9
2	13 – 300	7.3 – 0.3	≥ 0.990	0.83	10.5
3	75 – 600	3.8 – 0.8	≥ 0.990	-4.76	10.3

The graphs in figure 3 show the reaction curve of PU91-01 with the linear range.
Table 4 shows the backfitting and spread within that range.

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The following tables give the linear range with backfitting of the different vaccines. The accompanying graphs are shown in Appendix 2.

Table 5: Linear range and backfitting 785B

Type	Linear Range		R^2 (≥ 0.990)	Residuals (≤ 10%)	Residual Spread (≤ 20%)
	Dilution range	Conc. range (DU/ml)			
1	2 - 16	19 – 2.4	≥ 0.990	5.5	14.4
2	2 - 16	4 – 0.5	≥ 0.990	2.0	9.6
3	8 - 64	4 – 0.4	≥ 0.990	1.1	10.5

Table 6: Linear range and backfitting DaKTP

Type	Linear Range		R^2 (≥ 0.990)	Residuals (≤ 10%)	Residual Spread (≤ 20%)
	Dilution range	Conc. range (DU/ml)			
1	6 - 64	13 – 1.3	≥ 0.990	-1.6	5.5
2	6 - 48	3 – 0.3	≥ 0.990	2.1	9.6
3	12 - 96	5 – 0.7	≥ 0.990	5.9	18.0

Table: 7: Linear range and backfitting Monovalent IPV

Type	Linear Range		R^2 (≥ 0.990)	Residuals (≤ 10%)	Residual Spread (≤ 20%)
	Dilution range	Conc. range (DU/ml)			
1	160 – 2560	17 - 1	≥ 0.990	1.05	5.7
2	160 – 2560	8 – 0.5	≥ 0.990	-1.04	7.4
3	320 – 2560	4 – 0.5	≥ 0.990	2.59	8.6

Table 8: Linear range and backfitting DTP

Type	Linear Range		R^2 (≥ 0.990)	Residuals (≤ 10%)	Residual Spread (≤ 20%)
	Dilution range	Conc. range (DU/ml)			
1	3 – 32	14 – 1	≥ 0.990	1.0	10.5
2	3 - 16	1 – 0.3	≥ 0.990	-9.9	12.0
3	3 - 16	3 – 0.5	≥ 0.990	-1.5	11.0

Table 5-8: The dilution and concentration range given here represent the linear range of the different vaccines. The R^2 is above the criteria of 0.990, the residuals is not more than 10% and the backfit spread is smaller than 20%.

8.3 Discussion

The linear range and subsequently the start concentration of the reference and internal standard can be determined on the basis of the linearity results. It is important that the linear range of the test sample is similar to the linear range of the reference in order to determine the concentration of the test sample.

Figures 3a – 3f show the linear range of PU91-01 calculated by means of the 4-parameter fit method and residuals. Tables 9-11 shows the dilutions and the concentrations of the linear range for the reference, the internal standard and the D-antigen test samples.

Table 9: Linear range Type 1

Test Sample	Dilution	Concentration (DU/ml)
PU91-01 (reference)	19 – 400	23 – 1
785B (internal standard)	2 – 16	19 – 2.4
Monovalent IPV	160 – 2560	17 – 1
DTP	3 – 32	14 – 1.3
DaKTP	6 - 64	13 – 1.3
Average		17 – 1.5

Table 10: Linear range Type 2

Test Sample	Dilution	Concentration (DU/ml)
PU91-01 (reference)	13 – 300	7 – 0.5
785B (internal standard)	2 – 16	4 – 0.5
Monovalent IPV	160 – 2560	7.5 – 0.5
DTP	3 – 16	1.3 – 0.3
DaKTP	6 - 48	3 – 0.3
Average		4.5 – 0.4

Table 11: Linear range Type 3

Test Sample	Dilution	Concentration (DU/ml)
PU91-01 (reference)	75 – 600	4 – 1
785B (internal standard)	8 – 64	4 – 0.5
Monovalent IPV	320 – 2560	4 – 0.5
DTP	3 – 16	3 – 0.5
DaKTP	12 - 96	5 – 0.7
Average		4 – 0.7

The values reported in table 9 – 11 represent the linear range for the different test samples for the 3 different types.The concentration range per type for the different test samples is the same. This indicates the test sample dose-response curve increase in the same rate as the reference and the internal standard. (This is also shown in the specificity B test). The dosis-response curve of the test sample should be the same as the reference curve and their linear ranges should correspond in order to determine the concentration of the test sample. This is the case for all products tested in this study.

The start concentration and thus the initial dilution for the reference are calculated based on the linear range of the reference. The start concentration of the reference is chosen that produces a dosis-response curve containing a plateau by the lowest and the highest concentrations and the linear range by 25% - 75% of the curve.

8.4 Conclusion

The linearity was determined for the D-antigen ELISA test. The D-antigen test is linear in 25% - 75% of the curve for all three polio types

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9 Accuracy

9.1 Criteria

IPV 785B, DaKTP, Monovalent IPV and DTP vaccines are spiked with 25%, 50% en 75% of the nominal value with the reference PU 91-01. The recovery is reported as a percentage. This percentage must be within 10% of the expected value.

9.2 Results

The accuracy is determined by spiking the vaccine (785B, monovalent IPV, DaKTP and DTP) with PU91-01 and recovering this value in the result. Each vaccine was tested in duplicate without spike and with a spike of 25%, 50% and 75% of the nominal value. The following table represent the mean results of the duplicates. The unspiked test sample is shown as 100%. (The individual results are illustrated in graphs in Appendix 3.)

Table 14: The table demonstrates the 25%, 50% and 75% spike. The unspiked sample is assumed to be 100% and subsequently +25%, +50% and +75% spike. The concentrations are expressed in whole numbers however the deviation is calculated on the basis of one decimal place.

Table 14.1: 785B

Type	D-antigen concentration (DU/ml)				Deviation from expected value (%)		
	100%	+ 25%	+ 50%	+ 75%	+ 25%	+ 50%	+ 75%
1	37	49	56	65	5.6	0.7	0.6
2	8	10	12	15	-3.2	-4.0	1.7
3	26	32	39	46	-1.6	0	0
					Average deviation (%)		0.62

Table 14.2: Monovalent

Type	D-antigen concentration (DU/ml)				Deviation from expected value (%)		
	100%	+ 25%	+ 50%	+ 75%	+ 25%	+ 50%	+ 75%
1	2686	3423	4178	5091	1.6	-3.3	-8.0
2	1036	1259	1588	1705	-2.4	-3.3	5.1
3	1242	1606	2061	2246	4.0	-10.7	-3.4
					Average deviation (%)		2.3

Table 14.3: DTP

Type	D-antigen concentration (DU/ml)				Deviation from expected value (%)		
	100%	+ 25%	+ 50%	+ 75%	+ 25%	+ 50%	+ 75%
1	30	38	45	53	1.6	2.0	1.7
2	3	4	5	6	-3.2	12.7	11.4
3	7	9	11	12	0	2.7	-0.6
					Average deviation (%)		3.1

Table 14.4:DaKTP

Type	D-antigen concentration (DU/ml)				Deviation from expected value (%)		
	100%	+ 25%	+ 50%	+ 75%	+ 25%	+ 50%	+ 75%
1	67	84	101	114	0.8	0	-2.4
2	16	20	23	24	-4.0	-8.0	-14.9
3	64	76	94	105	-5.6	-1.3	-5.7
					Average deviation (%)		4.7

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The recovery of all the samples is good. All the spikes show an increase in value. Only DaKTP shows a lower recovery by the higher spikes. DTP shows a good recovery except for type 2. This shows a higher result than expected for the higher spikes.

9.3 Discussion

Table 14 reports the results obtained for the unpiked and spiked test samples. It also illustrates the variation between these results and the expected result for the spikes. The expected result is based on the result obtained for the unspiked test sample (100%). All the results show an increase in value for all spikes. The average deviation per product is within the 10% criteria.
Table 14 shows a good recovery for most test samples with little variation. The deviation per product ranges from 0.02% to 4.7%. This is the average deviation per product. The deviation per spike was within the 10% criteria with the exception of three spikes.
DTP shows a higher deviation than 10% (between 10% and 15%) by the 50% and 75% spike for type 2 and DaKTP also shows a higher deviation for spike 50% type 2 (deviation between 10% and 15%). These deviations occur only by the higher spikes and not in the standard range of measurement for the products. These deviations are not evident in both samples of the duplicate as shown in the graphs in Appendix 3. The plain polio vaccine shows a good recovery and neither the DaKTP bulk nor the DTP have a interfering effect on the test, as shown in the specificity tests.

9.4 Conclusion

This test proves that the accuracy of the D-antigen test is acceptable. The recovery is good for most samples with less than 10% variation. Some spikes show a higher variation (between 10% and 15%). However since this does not occur standard and not within the measurement range of the products it may be seen as a deviation. Such a result would lead to an underestimation and not an overestimation of polio in the final product.

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10 Precision

The precision of the D-antigen test is determined by testing the repeatability and the intermediate precision.

10.1 Repeatability

10.1.1 Criteria

The maximum coefficient of variation (CoV) per product is 10%.

10.1.2 Results

Three ELISA tests (three times the same test samples) were performed by one analist in one day. The three results from each test sample were compared and the CoV was calculated. The CoV must be ≤ 10%. The results are shown in the following table.

Table 15: Repeatability results

Test sample	Test (DU/ml).			average	CoV %
Type 1	1	2	3		
PV-06-127-6.1	2439	2347	2561	2449	4.4
DTP 135	29	27	31	29	6.9
DaKTP 006	60	62	63	62	2.5
IPV05-129	399	382	444	408	7.8
785B	36	36	38	37	3.1
Type 2					
PV04-211-6.1	738	819	836	798	6.6
DTP 135	3	4	4	4	4.1
DaKTP 006	13	14	14	14	4.2
IPV05-129	75	85	85	82	7.1
785B	7	7	8	7	4.4
Type 3					
PV-06-316-3.1	1215	1206	1219	1213	0.5
DTP 135	7	6	7	7	5.7
DaKTP 006	57	58	57	57	1.0
IPV05-129	309	296	334	313	6.2
785B	26	25	26	26	2.2

10.1.3 Discussion

To test the repeatability three tests were performed by one technician on one day. Sufficient samples were tested to give a reliable picture of the repeatablilty of the D-antigen test. The results illustrate that the D-antigen test has a good repeatability.The results for the three tests are comparable and have a CoV% between 0.5% and 7.8% (table 15). This is within the criteria of 10%.

10.1.4 Conclusion

Since all the results are within the criteria it may be concluded that the repeatability of the D-antigen test is good.

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10.2 Intermediate Precision

10.2.1 Criteria

The maximum CoV per product is 10%.

10.2.2 Results

Six tests were performed by two technicians on different days, three tests per technician. The results of the six tests were compared and the coefficient of variation was calculated. The maximum CoV must be ≤ 10%.

Table 16: Intermediate Precision results (DU/ml)

Test sample	Technician 1			Technician 2				
type 1	1	2	3	1	2	3	average	CoV %
PV06-127-6.1	2553	2561	2539	2678	2583	2762	2613	3.4
DTP 135	31	31	30	31	30	33	31	3.8
DaKTP 006	58	63	61	58	70	64	62	7.0
IPV05-129	405	444	416	430	403	469	428	6.0
785B	35	38	34	40	37	39	37	6.2
type 2								
PV04-211-6.1	797	836	809	858	920	861	847	5.2
DTP 135	3	4	4	3	4	4	4	4.4
DaKTP 006	13	14	15	14	15	13	14	4.7
IPV05-129	79	85	84	82	85	92	84	4.9
785B	7	8	8	8	8	9	8	7.6
type 3								
PV06-316-3.1	1282	1219	1165	1473	1330	1441	1318	9.2
DTP 135	6	7	7	7	7	8	7	8.4
DaKTP 006	54	57	54	65	64	65	60	9.0
IPV05-129	323	334	319	334	366	380	343	7.2
785B	28	26	26	26	29	31	28	7.4

10.2.3 Discussion

To determine the intermediate precision six ELISA tests were performed on six different days, three by technician 1 and three by technician 2. The results show a good intermediate precision. The CoV% varies between 3.4% and 9.2% (Table 16). This falls within the criteria of 10%.

10.2.4 Conclusion

Since all the results are within the criteria and the variation within the internal standard is for both technicians within the criteria of the trendanalysis for the internal standard (Appendix 4), it may be concluded that the intermediate precision of the D-antigen test is sufficient.

11 Comparison with current method

The old method and the new method were compared to determine the impact, if any, the new method will have on the D-antigen determination of the routine test samples. The difference between the two methods were examined for two aspects; test samples and internal standard.

11.1 Test Samples

The test samples used for the precision tests were already routinely tested according to the old method. Table 17 gives the results of the old method compared to the results of the precision test during this validation (new method). There is a time interval of about three months between the two tests. This may lead to a slight decrease in value over time.

Table 17: Comparison of D-antigen amount determined with the old method and with the new method

Test sample	Polio Type	Old method (DU/ml)	New method (DU/ml)	% difference
PV06-127-6.1		2511	2613	-4
DTP 135		33	31	+6
DaKTP 006	1	64	62	+3
IPV05-129		401	428	-7
785B		38	37	+3
PV04-211-6.1		863	847	+2
DTP 135		4	4	0
DaKTP 006	2	16	14	+13
IPV05-129		90	85	+6
785B		8	8	0
PV06-316-6.1		1396	1318	+6
DTP 135		8	7	+13
DaKTP 006	3	64	60	+6
IPV05-129		325	343	-6
785B		28	26	0

Table 17: When the old method and the new method are compared the difference is between -7% and +13%. The results from both methods are within the same range. Therefore this shall not lead to a trendbreak in the test samples.

11.2 Internal standard

The internal standard is tested with the new method as for the old method. The results obtained during the validation study were compared with the most recent twenty results from the old method. Figure 4 illustrates the difference between the two methods for the three types.

Figure 4a: Comparison the new method and the old method: Type 1

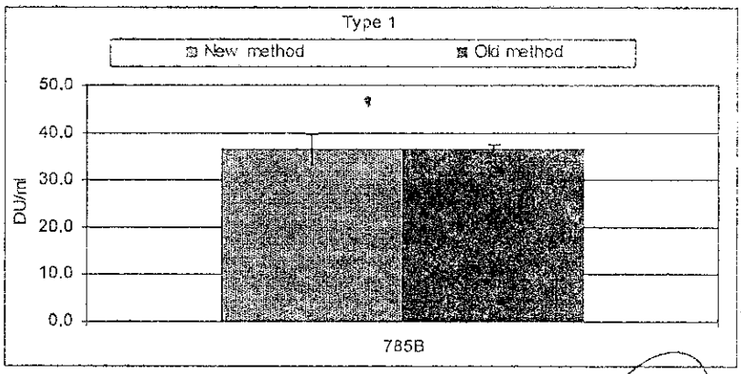

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Figure 4b: Comparison between the new method and the old method: Type 2

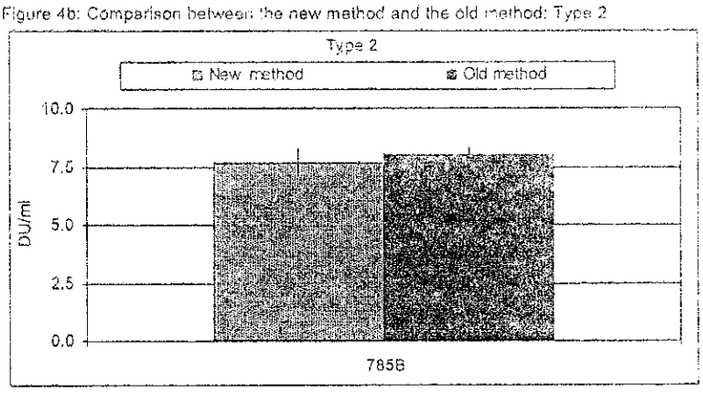

Figure 4c: Comparison between the new method and the old method: Type 3

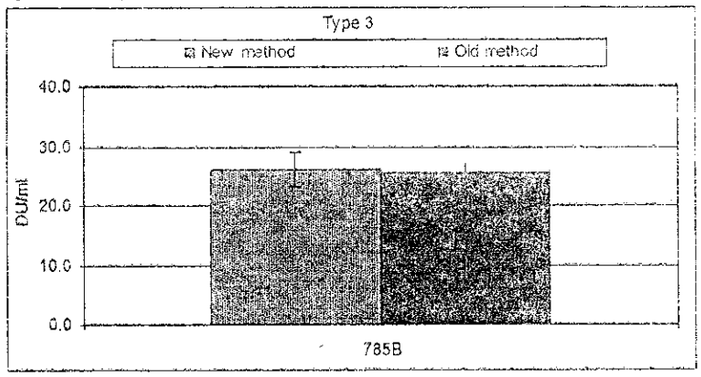

Figure 4: The graphs show comparable results for the new method and the old method. The difference in value between the two methods is not significant (T test ≥ 0.05 – Appendix 5). This means that no great difference can be expected between the two methods.
The variation within the new method (VC% between 7.6% and 11.0% - Appendix 5) is greater than the variation with the old method (VC% between 2.8% and 5.1% - Appendix 5). This variation however is minimal and is tracked by the trendanalysis of the internal standard (Appendix 4).

12 Risk Analysis for Reagents

There are a few critical reagents used in the D-antigen test. These are the anti-polio serum used for the coating of the ELISA plates, the type specific monoclonal anti-polio antibody and the enzyme labeled anti-mouse conjugate. The D-antigen test will be revalidated if there is a change in article number of one of the critical reagents. By a change in batch number of a critical reagent, the reagent will be titrated to determine the optimal dilution for use.

13 Conclusion

On the basis of the results obtained from the validation study it may be concluded that the new D-antigen test conforms to the criteria and may be adopted for use as the D-antigen test for routine samples use.

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14 Appendices

Appendix 1: Extinctions Specificity A: Cross-reaction

Coat:	Type 1											
Moab:	Type1		Type 2		Type 3		Type 2		Type 3		Type1	
Antigen:	Type 1		Type 2		Type 3		Type 1		Type 1		PU91-01	
	1.9	2.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	2.0	1.9
	1.6	1.6	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	1.5	1.5
	1.0	1.1	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	1.1	1.0
	0.6	0.6	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.6	0.6
	0.4	0.4	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.3	0.3
	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.2	0.2
	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
Background:	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Coat:	Type 2											
Moab:	Type1		Type 2		Type 3		Type 1		Type 3		Type 2	
Antigen:	Type 1		Type 2		Type 3		Type 2		Type 2		PU91-01	
	0.3	0.2	1.2	1.2	0.2	0.1	0.1	0.1	0.1	0.1	1.3	1.3
	0.2	0.2	0.9	0.9	0.2	0.2	0.1	0.1	0.1	0.1	1.0	1.0
	0.2	0.2	0.7	0.6	0.1	0.2	0.1	0.1	0.1	0.1	0.6	0.7
	0.2	0.2	0.5	0.5	0.2	0.2	0.1	0.1	0.1	0.1	0.5	0.5
	0.1	0.2	0.3	0.3	0.1	0.1	0.1	0.1	0.2	0.1	0.3	0.3
	0.1	0.2	0.3	0.3	0.2	0.2	0.1	0.2	0.1	0.1	0.3	0.2
	0.1	0.1	0.2	0.2	0.1	0.2	0.1	0.2	0.1	0.1	0.2	0.2
Background:	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2	0.2

Coat:	Type 3											
Moab:	Type1		Type 2		Type 3		Type 1		Type 2		Type 3	
Antigen:	Type 1		Type 2		Type 3		Type 3		Type 3		PU91-01	
	0.1	0.1	0.1	0.1	2.1	1.9	0.1	0.1	0.1	0.1	2.1	1.9
	0.1	0.1	0.1	0.1	1.9	1.8	0.1	0.1	0.1	0.1	1.9	1.9
	0.1	0.1	0.1	0.1	1.5	1.6	0.1	0.1	0.1	0.1	1.5	1.5
	0.1	0.1	0.1	0.1	1.0	1.1	0.1	0.1	0.1	0.1	1.0	1.0
	0.1	0.1	0.1	0.1	0.6	0.6	0.1	0.1	0.1	0.1	0.6	0.6
	0.1	0.1	0.1	0.1	0.4	0.4	0.1	0.1	0.1	0.1	0.4	0.4
	0.1	0.1	0.1	0.1	0.2	0.2	0.1	0.1	0.1	0.1	0.2	0.2
Background:	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

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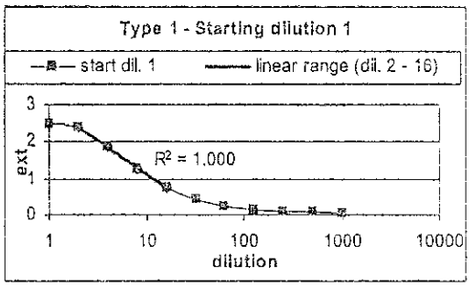
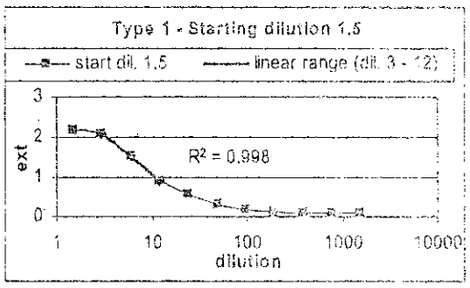
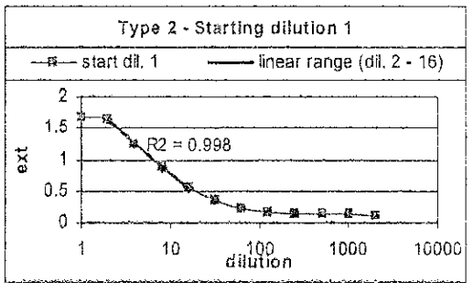
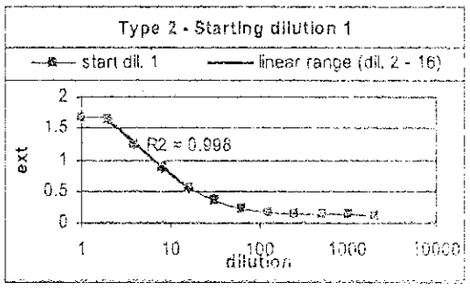
Appendix 2: Linearity Graphs

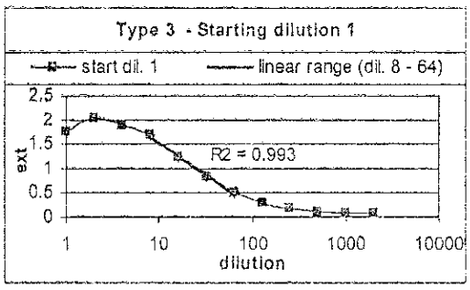
Linear range of 7858

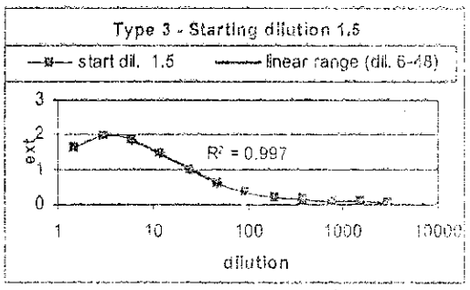












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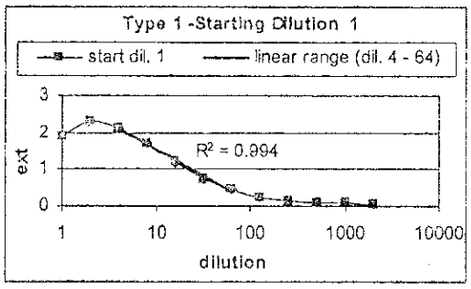
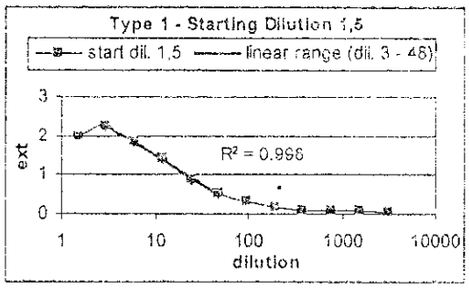
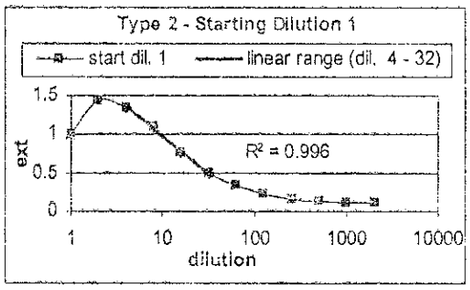
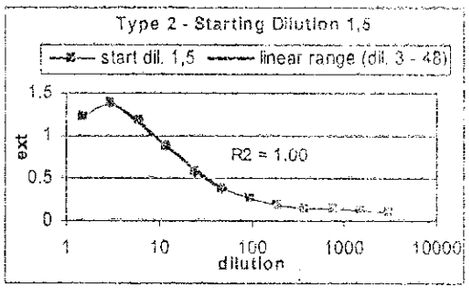
Appendix 2 (continued): Linearity Graphs

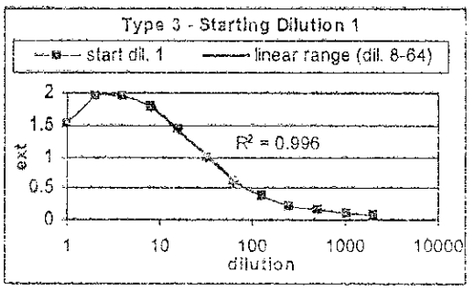
Linear range of DaKTP

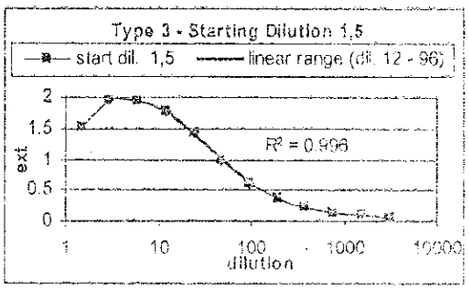












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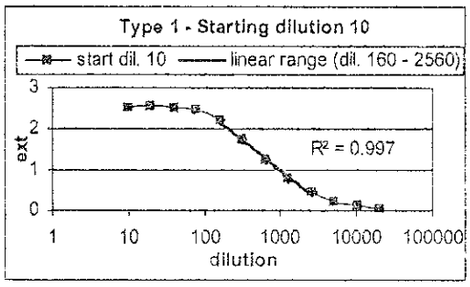
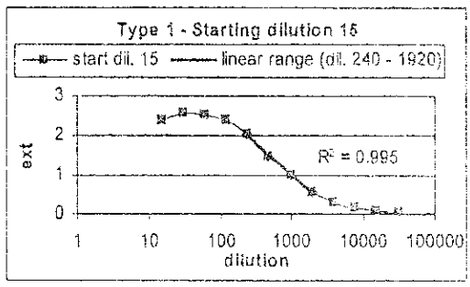
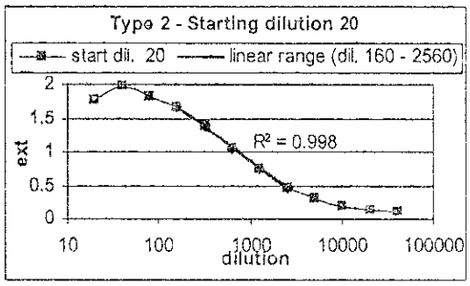
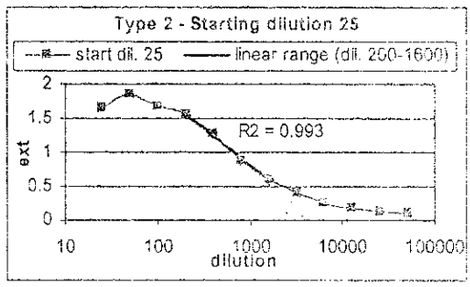
Appendix 2 (continued): Linearity Graphs

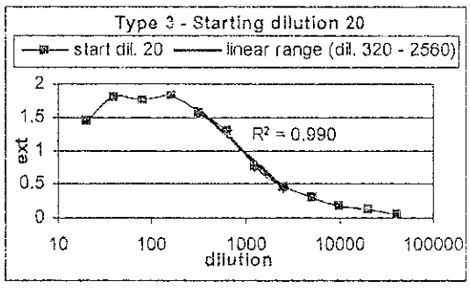
Linear range of Monovalent IPV samples

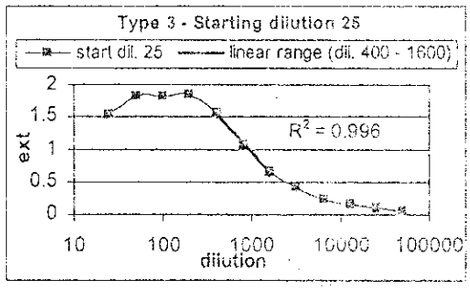












Appendix 2 (continued): Linearity Graphs

Linear range of DTP