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Screening and optimisation of an ELISA method for the quantitative detection of enterovirus specific RT-PCR products by means of a two-level experimental design

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Abstract

In a previous paper, optimal reaction conditions were determined for the RT-PCR part of a quantitative enterovirus specific RT-PCR ELISA method (J. Pharm. Biomed. Anal., 25 (2001) 131–142). In order to obtain a detection limit as low as possible, the ELISA part of the procedure was optimised as well. This was done by investigating the influence of seven factors at three levels in a multivariate approach. A reflected two-level screening design, derived from a Plackett–Burman design, was used. Optimal reaction conditions were established by calculation and by evaluation of the effects of the factors on the measured absorbance of the ELISA detection. Under these conditions, the linear range and detection limit of the test were determined and compared with the ELISA conditions before optimisation. The optimised RT-PCR ELISA will be used to study a possible longitudinal relationship between enteroviruses and the development of multiple sclerosis and juvenile diabetes. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: RT-PCR ELISA; Quantitative PCR; Enterovirus PCR; Experimental design; Plackett–Burman design

1. Introduction

Although the exact aetiologies of multiple sclerosis (MS) and insulin-dependent diabetes mellitus (IDDM) or type 1 diabetes are still unknown, it is generally assumed that the development of both diseases is dependent on the interaction of a genetic predisposition, an autoimmune response and an environmental factor [1,2]. Animal models exist and suggest that viruses play an important role in the pathogenesis of these diseases [1–4]. In both cases coxsackieviruses B, which belong to the enterovirus genus, are important candidates [4–8]. A possible relationship between enteroviruses and these diseases can be investigated by means of longitudinal follow-up studies, in which the presence and concentration (quantitative aspect) of viral genome sequences can be determined.

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The most suitable tool to perform such longitudinal studies is a quantitative RT-PCR ELISA (reverse transcription-polymerase chain reaction enzyme linked immunosorbent assay). In a previous paper [9], optimal reaction conditions were established for the RT and PCR parts of the enterovirus specific assay by means of a three-level experimental design. This enabled to assess the linear range and to improve the detection limit. However, in order to decrease the detection limit, i.e. to establish the ability to determine the lowest number of viral particles in a given sample, it is also necessary to optimise the detection part (i.e. ELISA) of the procedure. In this ELISA method, biotin-labelled PCR products are immobilised on streptavidin-coated microtiter plate wells and detected by consecutive addition of an enterovirus specific digoxigenin-labelled probe, anti-digoxigenin-peroxidase conjugate and a colourimetric substrate [10]. The absorbance in the finally obtained solution is then measured.

A choice was made for a multivariate Plackett–Burman (P–B) design, since these designs were already used for the optimisation of ELISA methods [11]. P–B designs, which are screening designs, allow to examine a relatively high number of factors in a limited number of experiments, while the experimental domain is mapped/covered as uniformly as possible. Indeed, one-variable-at-the-time procedures, which are also frequently applied, can lead to incomplete understanding of the behaviour of the assay [11], because they only examine a limited part of the experimental domain. To optimise the ELISA, a reflected two-level P–B design was performed [12] in order to examine the effects of each factor at three levels. This design, in fact, consists of two complementary two-level P–B designs with extremes at either side of a nominal value. The factors and their levels were selected based on several literature sources [11,13–16].

The influence of the factors on the response of the method was evaluated by calculating their effects and drawing the corresponding effect-plots [12], from which the best conditions then can be predicted.

Thus, our aim is to determine the most important factors on the detection of the enterovirus specific RT-PCR products, in order to establish the conditions leading to the highest absorbance for a given concentration of the products and therefore a possible lower detection limit of the assay.

2. Materials and methods

2.1. Virus

All experiments were performed with poliovirus strain Mahoney (type 1) as representative of the enteroviruses, since this virus has been the most extensively studied within the enterovirus genus. The virus was grown, collected and purified by sucrose gradient ultracentrifugation [17]. The concentration was determined spectrophotometrically, assuming $A_{260}^{1\%}$ for virus being 81.6 [18].

2.2. RNA isolation

Viral RNA (ribonucleic acid) was isolated by the acid guanidinium thiocyanate–phenol–chloroform extraction method [19]. The RNA pellet was dissolved in 20 μl of sterile RNase- and DNase-free water (Sigma, St. Louis, MO) and RNA concentrations were again measured spectrophotometrically, assuming $A_{260}^{1\%}$ for RNA being 250 [18]. The genome number was then calculated considering the molecular weight of poliovirus [20]. Prior to RT-PCR, the vRNA (viral RNA) was diluted to the appropriate concentration(s).

2.3. Primers and probe

The sequences of the enterovirus specific primers and probe are situated in a 148 bp (base-pairs) segment with absolute sequence conservation among all sequenced enteroviruses in the 5′ untranslated region of the viral genome. The primers and probe are therefore able to detect all available enterovirus prototypes (65 serotypes) [21]. The downstream primer (5′-TTGTCACCATAAGCAGCCA-3′) and the 3′ digoxigenin (DIG)-labelled capture probe (5′-CCAAAGTAGTCGGTTCCGC-3′) are antisense to genomic viral RNA. The 5′ biotin-labelled upstream

primer (5′-CGGCCCCTGAATGCGGCTAAT-3′) is sense to genomic RNA. Both primers and probe were purchased at Pharmacia Biotech (Uppsala, Sweden).

2.4. RT-PCR

Before reverse transcription, 1 μl of RNA of the appropriate concentration was incubated at 65 °C for 5 min, followed by 5 min at 4 °C (= pre-RT step). The following components were then added to the incubated RNA (all values correspond with final concentrations in the reaction mixture): RNasin (25 U); Promega, Madison, USA), RT-buffer (50 mM Tris–HCl pH 8.5, 8 mM $MgCl_2$, 30 mM KCl, 1 mM DTT (dithiothreitol); Roche Molecular Biochemicals, Mannheim, Germany), dNTP mix (0.4 mM of each dATP, dCTP, dGTP and dTTP (deoxy-adenosine, -cytidine, -guanosine, -thymidine triphosphate); Roche Molecular Biochemicals), downstream PCR primer (1 μM) and avian myeloblastosis virus (AMV) reverse transcriptase (2 U; Roche Molecular Biochemicals). The mixture was adjusted to a volume of 10 μl with sterile RNase- and DNase-free water and incubated for 60 min at 42 °C, 5 min at 95 °C and 5 min at 4 °C. To the complete reverse transcription mixture the following components were added to perform a Hot-Start PCR: PCR-buffer (10 mM Tris–HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$; Applied Biosystems, Foster City, USA), AmpliTaq Gold® DNA polymerase (2.5 U; Applied Biosystems) and upstream biotin-labelled primer (0.2 μM). The mixture was adjusted to a volume of 40 μl with sterile RNase- and DNase-free water and submitted to the following thermal profile: 15 min at 95 °C (pre-PCR heat step to activate the enzyme), 40 cycles at 95 °C for 30 s, 60 °C for 30 s and 72 °C for 30 s, finally followed by an elongation period at 72 °C for 7 min (final elongation). All thermal profiles were performed using an Amplitron II® (Barnstead/Thermolyne Corporation, Dubuque, USA) thermocycler. In each analysis, a no template control was taken along with the samples to detect any form of contamination.

2.5. ELISA

A schematic overview of the subsequent steps of the ELISA method is given in Fig. 1. The examined factors and their levels can be found in Table 1 (when a concentration is concerned, all values correspond to final concentrations in the reaction mixture). For most factors, the nominal level was defined to be approximately centrally situated in the interval between the two extreme levels. All reagents were pre-incubated to the appropriate temperature. Incubation at the different temperatures was performed under constant shaking, protected from light. The detection of the 148 bp biotin-labelled PCR products was mainly based on Ossewaarde et al. [10] and adapted as

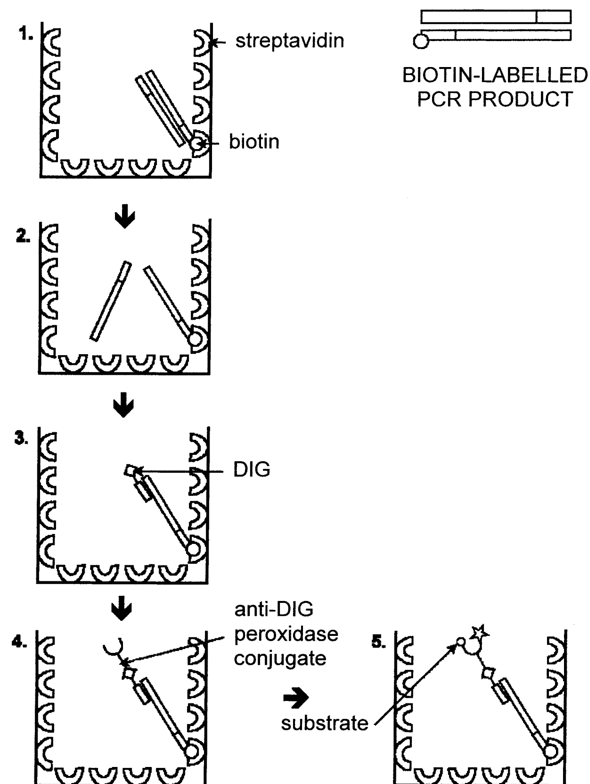

Fig. 1. Schematic overview of the subsequent steps of the ELISA detection: 1, binding biotin-streptavidin; 2, denaturation of double stranded PCR product; 3, hybridisation of DIG-labelled capture probe; 4, binding of anti-DIG-POD conjugate; and 5, addition of substrate ABTS®, resulting in a green-coloured end product ($A_{405/490}$).

Table 1
The two-level Plackett–Burman design (design 1) that was reflected (design 2) with the seven factors examined and their levels (the results of the design, after correction both for negative detection and no template are shown)

Factors		Levels −1	0	+1
A	Dilution of PCR product	100×	10×	Undiluted
B	Probe concentration (pmol/ml)	1	25	50
C	Hybridisation temperature (°C)	37	46	55
D	Hybridisation time (min)	30	105	180
E	Anti-DIG-POD conjugate concentration (mU/ml)	5	30	50
F	Incubation time anti-DIG-POD conjugate (min)	10	30	50
G	Incubation time ABTS® (min)	10	30	50

Exp	Factors A	B	C	D	E	F	G	Response $A_{405/490}$ No dummy factor	With dummy factor
Design 1									
1	−1	−1	−1	0	−1	0	0	0.000	0.192
2	0	−1	−1	−1	0	−1	0	0.025	0.316
3	0	0	−1	−1	−1	0	−1	0.078	0.080
4	−1	0	0	−1	−1	−1	0	0.004	0.317
5	0	−1	0	0	−1	−1	−1	0.033	0.083
6	−1	0	−1	0	0	−1	−1	0.002	0.843
7	−1	−1	0	−1	0	0	−1	0.022	1.064
8	0	0	0	0	0	0	0	0.771	2.509
Design 2									
1	+1	+1	+1	0	+1	0	0	2.182	0.900
2	0	+1	+1	+1	0	+1	0	0.452	0.474
3	0	0	+1	+1	+1	0	+1	0.763	0.798
4	+1	0	0	+1	+1	+1	0	1.365	1.308
5	0	+1	0	0	+1	+1	+1	1.487	2.009
6	+1	0	+1	0	0	+1	+1	2.089	0.494
7	+1	+1	0	+1	0	0	+1	1.284	0.893
8	0	0	0	0	0	0	0	0.595	2.529

follows: to 20 µl of PCR product [factor A], 200 µl SSC solution (saline sodium citrate = 0.15 M NaCl plus 0.015 M sodium citrate; both Merck, Darmstadt, Germany) with 0.5% Tween 20 (Sigma) was added and the samples were mixed. Duplicate analysis was performed by transferring twice 100 µl of this mixture to different streptavidin-coated microtiter plate (MTP)-wells (Roche Molecular Biochemicals). The plate was incubated at [factor C] ° for 30 min allowing immobilisation of the biotin-labelled PCR products on the streptavidin-coated wells. The plate was then washed four times with 200 µl SSC solution at [factor C] °C, filled with 100 µl 0.1 N NaOH (Merck) and incubated for 10 min at room temperature to allow denaturation of the double stranded PCR product. After washing twice with 200 µl SSC solution at room temperature, hybridisation was performed by adding 100 µl of [factor B] pmol/ml DIG-labelled probe in SSC solution with 0.5% Tween 20 and incubating at [factor C] °C for [factor D] min. The plate was then washed twice with double concentrated SSC solution with 0.1% SDS (sodium dodecyl sulphate,

Merck) and 3 M urea (Merck) at [factor C] °C for 5 min and twice with double concentrated SSC solution at [factor C] °C for 5 min. Then 200 μl of [factor E] mU/ml anti-DIG-POD (peroxidase) (poly) Fab immunoglobulin fragments conjugate (Roche Molecular Biochemicals) with 0.5% Tween 20, 1% BSA (Roche Molecular Biochemicals) and 0.5% gelatine (Merck) in PBS (phosphate buffered saline pH 7.4; Sigma) was added. The mixture was incubated at [factor C] °C for [factor F] min. After washing four times with 0.05% Tween 20 in PBS at room temperature, 100 μl of ABTS® (2,2′-Azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt) substrate solution (1 mg/ml in ABTS® substrate buffer, Roche Molecular Biochemicals) was incubated at [factor C] °C for [factor G] min. The signals were quantified with an automated MTP-reader El_x800 (Bio-Tek® Instruments, Winooski, USA) by measuring the absorbance at 405 nm (reference filter 490 nm). In each analysis a negative detection control (20 μl of sterile RNase- and DNase-free water) was taken along.

2.6. Experimental design

The reflected two-level P–B design [12], is presented in Table 1. In this particular design, seven factors can be investigated at three levels in 15 different experiments (in fact $(2 \times 8) = 16$ experiments, but experiment 8 is identical in both designs). This is done by executing the two-level design twice, namely once with the nominal level and a first extreme level ((+1), design 1), and once with the nominal level and the other extreme level ((−1), design 2). Both schemes are then treated individually and two sets of main effects (see Table 2) are calculated. Notice that in P–B designs, if the number of factors to be evaluated is less than the maximal possible one (here 7), dummy factors are included to complete the design. A dummy factor is an imaginary variable for which the change from one level to another does not cause any physical change in the method.

The seven factors and their levels were selected considering several literature sources [10,13–15] and the manufacturer's instruction manual for most of the important reagents used in the ELISA [16]. The factors investigated were: dilution of PCR product (factor A), probe concentration (factor B), hybridisation temperature (factor C), hybridisation time (factor D), anti-DIG-POD conjugate concentration (factor E), incubation time anti-DIG-POD conjugate (factor F) and incubation time ABTS® (factor G). The different factor levels can be found in Table 1. The situation of these factors within the ELISA procedure is already described higher.

The following effects were calculated for each factor:

$$E_{x(-1,0)} = \frac{\sum R(0)}{N/2} - \frac{\sum R(-1)}{N/2}$$

Table 2
Calculated effects of the investigated factors

Factors		Effects			
		$E_{x(-1,0)}$	$E_{x(0,+1)}$	$E_{x(-1,0)}$	$E_{x(0,+1)}$
		No dummy		With dummy	
A	Dilution of PCR product/dummy	0.220	0.906	0.143	−0.554
B	Probe concentration	0.194	0.149	0.524	−0.213
C	Hybridisation temperature	0.181	0.189	0.636	−1.018
D	Hybridisation time	0.169	−0.622	0.463	−0.615
E	Anti-DIG-POD conjugate concentration	0.176	0.344	1.015	0.156
F	Incubation time anti-DIG-POD conjugate	0.201	0.142	0.572	−0.209
G	Incubation time ABTS®	0.167	0.258	0.316	−0.254

$$E_{x(0,+1)} = \frac{\sum R(+1)}{N/2} - \frac{\sum R(0)}{N/2}$$

where E_x is the effect on the measured response (R) for the change of a factor x from one level to another; $\Sigma R(i)$ $(i = 0, -1, +1)$ represents the sums of the measured values associated with level i and N is the number of experiments in one design (the design is treated as if two separate designs with N experiments, here 8, were performed). These calculated effects, which show the magnitude of the influence of the change of a factor on the response, can be visualised in effect-plots [12], where the behaviour of the response is plotted as a function of the factor levels.

3. Results and discussion

3.1. Experimental design

To assure a positive detection, the PCR was performed by amplifying 10^6 enterovirus genomes. The responses measured in the designs are shown in Table 1 and the calculated effects in Table 2. The factors that have an important effect were determined graphically by interpretation of the effect-plots (Fig. 2). However, note that focusing on the selected factors not necessarily implies that other factors are unimportant or do not affect the assay performance. Therefore these factors were maintained as constant as possible during the execution of the design.

From design 1 it appears that the response for experiment 8 is much higher than the responses of the rest of the experiments of this design. For the calculation of the effects this results in a comparable effect for all factors (Table 2), which implies that no valuable conclusions can be drawn from the results of design 1.

To ascertain which factors have an important effect in design 2, one can consider the magnitude of the effects (0, +1). From this it is evident that mainly factor A (dilution of PCR product) and factor D (hybridisation time) show an effect. The large effect of factor A can be explained by the fact that the absorbance is proportional to the concentration of PCR product. The above results show that examining factor A (dilution of PCR product) in the design was not the most lucky choice. Moreover, due to this selection (too low concentration of PCR products), almost all results in design 1 were close to zero, leading to meaningless effects. On the other hand, since factor A has a predominant effect in design 2, the possibility exists that the other effects, which are the average effects at both dilutions of the PCR product, are not representative for the effect at the (+1) level of factor A (= undiluted PCR product). This would be the case if the PCR product concentration interacts with other examined factors. The (+1) level for factor A namely seems to be the best one for an optimisation of the ELISA detection because of the high positive effect obtained (0.906). Therefore, it was decided to repeat both designs after eliminating factor A and replacing it by a dummy factor. In each experiment of both designs the ELISA is then performed on undiluted PCR product. Calculation and evaluation of the effects is done as before (Tables 1 and 2). The effect-plots are drawn in Fig. 2.

Table 3
Two-factor interaction effects confounded with the main effects in the designs of Table 1

Primary factor	Confounded two-factor effects		
A	BF	CD	EG
B	AF	CG	DE
C	AD	BG	EF
D	AC	BE	FG
E	AG	BD	CF
F	AB	CE	DG
G	AE	BC	DF

Factor A is now the dummy factor and the effect calculated for it is expected to be close to zero, since it only measures the experimental error of the system. Here it is evident that the effect calculated for factor A in the interval (0, +1) is considerable (Table 2, Fig. 2). This effect is probably due to one of the two-factor interactions that in the P–B design are confounded with the main effect. The two-factor interactions confounded with the main effects in the applied design are shown in Table 3. Factor A is confounded with

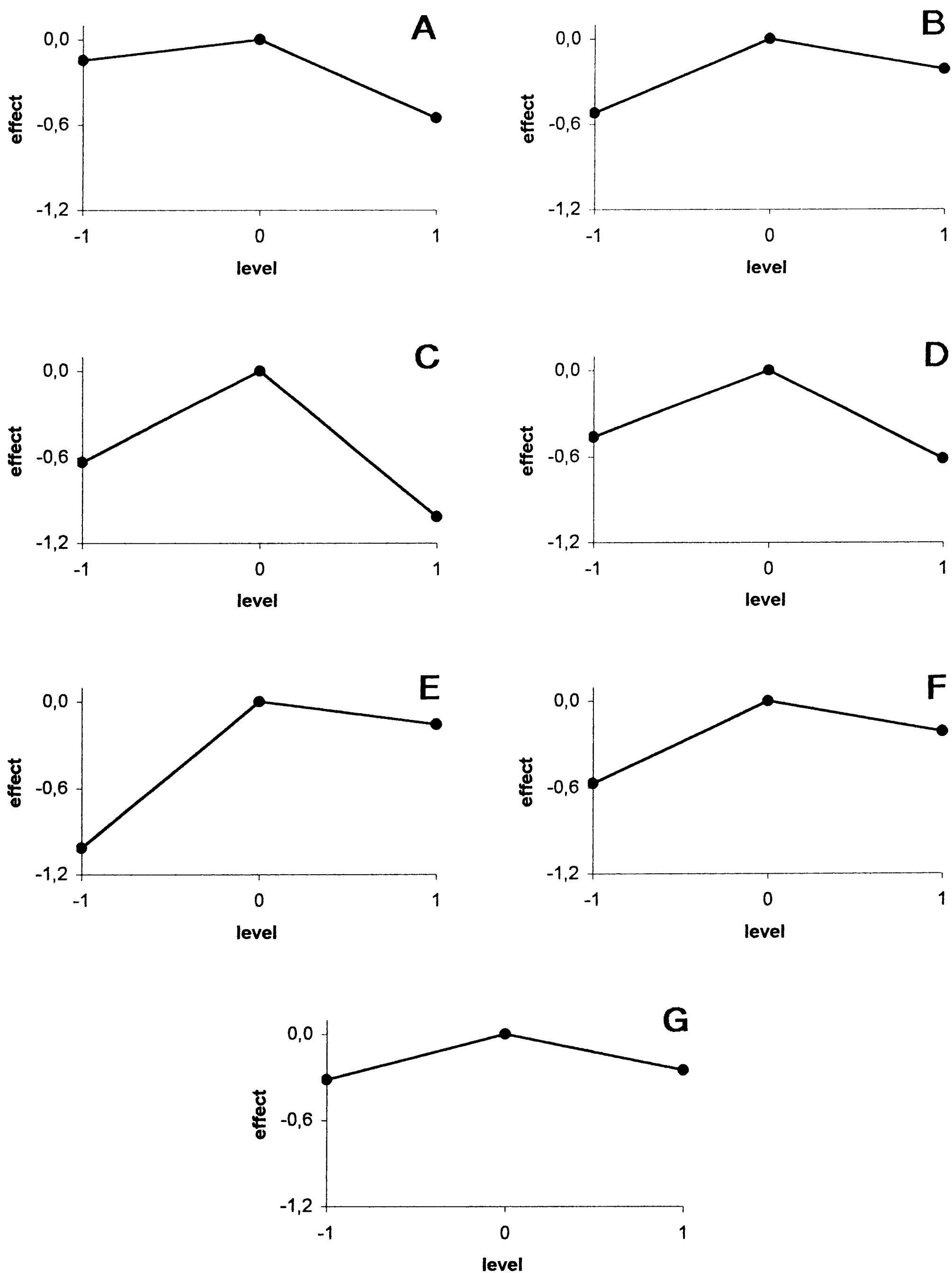

Fig. 2. Effect-plots of the seven examined factors (Table 1), on the response $A_{405/490}$ of the ELISA detection method: A, dummy factor; B, probe concentration; C, hybridisation temperature; D, hybridisation time; E, anti-DIG-POD conjugate concentration; F, incubation time anti-DIG-POD; and G, incubation time ABTS®. The effects $E_{x(-1,0)}$ and $E_{x(0,1)}$ are shown.

the interactions BF, CD and EG. The high value of the dummy factor effect in design (0, + 1) could possibly be explained by the interaction CD, since important effects are observed for factor C (hybridisation temperature) and D (hybridisation time), while the effects for factors B, E, F and G are much less pronounced. Also the nature of factors C and D, does allow to suspect such interaction. The effect-plots show that both factors have an optimum at the zero level, being a hybridisation temperature of 46 °C and a hybridisation time of 105 min respectively.

The conclusions that can be drawn for the remaining factors are the following.

Factor B (probe concentration) is a less important factor. A possible optimum can be defined at level 0 (25 pmol/ml). Factor E (anti-DIG-POD conjugate concentration) also has an optimum at the zero level (30 mU/ml). For both factors it is observed that higher concentrations do not improve the response anymore.

For factor F (incubation time anti-DIG-POD conjugate) it is observed that the response at level (− 1) is lower than at levels (0) and (+ 1). This indicates that an incubation time of 10 min is too short. An incubation time of 30 min seems to be appropriate. Factor G (incubation time ABTS®) does not have a large effect on the response. However not to risk that an incubation time of 10 min is rather too short, also here 30 min could be preferred.

If we now combine the optimum levels for each factor, it then appears that the optimal factor-level combination corresponds with experiment 8 of both designs. From Table 1, it can also be observed that for this experiment by far the highest response was measured. These conditions were then further used to determine the linear range and detection limit of the method and were compared with those at the previously used ELISA conditions, namely the conditions before optimisation which are fully described and applied in a previous paper [9].

3.2. Linear range and detection limit

The final goal of our study is to perform quantitative analysis. Therefore the linear range of the test, where quantification is possible, should be determined. This was done in a first step by analysing a $\log_{10}$ dilution series of enterovirus RNA over a broad concentration range (10^0–10^8 enterovirus genomes) (Fig. 3A). Linearity is seen over an approximately 3 log interval (10^3–10^6 genomes). Higher concentrations ($\geq 10^7$ genomes) produce absorbances that are out of the measurement range of the MTP-reader. This is in contrast with the previously used ELISA conditions (see also Fig. 3A), where all tested concentrations in this range are measurable and a linear range is seen between 10^3 and 10^7 enterovirus genomes. Thus, we can already conclude here that the optimised ELISA method is more sensitive than the old one because the slope in the linear range is steeper and that the optimised conditions cause considerable higher absorbances for a given genome number. Therefore most probably a lower detection limit will be obtained.

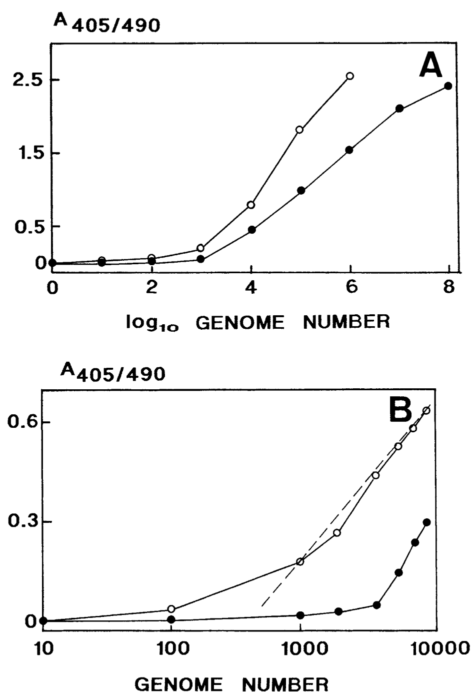

Fig. 3. Absorbances ($A_{405/490}$) of the ELISA detection as a function of the enterovirus genome concentration (non-optimised (–●–) and optimised (–○–) ELISA conditions). A, 10^0–10^8 enterovirus genomes; B, 10^1–10^4 enterovirus genomes.

In a second step, new dilutions were prepared within the more narrow range of 10^1–10^4 genomes (Fig. 3B). This in order to determine the lower limit of the linear range and compare it with the non-optimised ELISA conditions. With the optimised conditions linearity is seen for concentrations ≥ 1000 genomes. For the previously used conditions the linear range starts at 4000 genomes.

Finally, the detection limit for the optimised enterovirus RT-PCR ELISA method was determined. Replicate experiments were performed and a t-test was used to determine whether a given amount situated between 10^1 and 10^4 genomes is statistically higher than the no template results. For both the positive samples and the no template six RT-PCR replicates were performed and analysed in duplicate by ELISA. At the $\alpha = 0.05$ significance level the signals corresponding with concentrations above 1000 genomes are statistically different from the no template ($t = 18.04$ for 1000 genomes). This was not the case for concentrations ≤ 100 genomes ($t = 1.59$ for 100 genomes), $t_{\mathrm{critical\ (5\ d.f.)}} = 2.02$ (one-tailed test), while for 100 genomes borderline non-significance has been observed, indicating that the detection limit is close to 100 genomes. It can be stated that optimising the ELISA part of the procedure did not lead to a lower detection limit for the enterovirus specific RT-PCR ELISA, since with the previously used ELISA conditions a similar detection limit of 1000 enterovirus genomes was obtained ($t = 3.16$ for 1000 genomes) and a borderline non-significance for 100 genomes ($t = 1.59$ for 100 genomes) [9]. However, from Fig. 3B it can be concluded that the optimised ELISA conditions are much more sensitive for concentrations between 10^2 and 10^4 than the previously used conditions. For a concentration of 100 enterovirus genomes, the same t-value (i.e. $t = 1.59$) was obtained with both methods, although the optimised ELISA conditions produce a considerably higher absorbance. This is due to the fact that the optimised method is less repeatable, i.e. has less good precision, than the previously used method. Thus, evaluating the sources of variability by means of for instance a robustness test and standardising the main sources strictly should lead to a better precision and consequently a lower detection limit.

In conclusion, the applied experimental design enabled us to establish optimal reaction conditions for the enterovirus specific ELISA. This resulted in a more sensitive method (higher absorbances for equal genome numbers and a steeper slope in the linear range) and a reduction of the quantification limit with a factor four (i.e. 1000 enterovirus genomes in comparison with 4000 under previous conditions).

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References

[1] P. Sarchielli, A. Trequattrini, F. Usai, D. Murasecco, V. Gallai, Acta Neurol. Napoli 15 (1993) 363–381.
[2] J.W. Yoon, H.M. Eun, K. Essami, D.A. Roncari, L.E. Bryan, Clin. Invest. Med. 10 (1987) 450–456.
[3] J.W. Yoon, Diabetes Metab. Rev. 11 (1995) 83–107.
[4] R.B. Dessau, L.P. Nielsen, J.L. Frederiksen, Acta Neurol. Scand. 95 (1997) 284–286.
[5] M. King, A. Shaikh, D. Bidwell, A. Voller, J. Banatvala, Lancet 1 (1983) 1397–1399.
[6] D.R. Gamble, K.W. Taylor, H. Cumming, Br. Med. J. 4 (1973) 260–262.
[7] G.B. Clements, D.N. Galbraith, K.W. Taylor, Lancet 346 (1995) 221–223.
[8] R. Kaiser, R. Dorries, R. Martin, U. Fuhrmeister, K.F. Leonhardt, V. ter Meulen, J. Neurol. 236 (1989) 395–399.
[9] S. Lauwers, Y. Vander Heyden, B. Rombaut, J. Pharm. Biomed. Anal. 25 (2001) 131–142.
[10] J.M. Ossewaarde, M. Rieffe, G.J.J. van Doornum, C.J.M. Henquet, A.M. Van Loon, Eur. J. Clin. Microbiol. Infect. Dis. 13 (1994) 730–740.
[11] G.S. Sittampalam, W.C. Smith, T.W. Miyakawa, D.R. Smith, C. Mc Morris, J. Immunol. Methods 190 (1996) 151–161.
[12] S. Boonkerd, M.R. Detaevernier, Y. Vander Heyden, J. Vindevogel, Y. Michotte, J. Chromatogr. A 736 (1996) 281–289.
[13] C.P. Pollini, L. Giunchedi, R. Bissani, J. Virol. Methods

67 (1997) 127–133.
[14] M. Callens, K. De Clercq, J. Virol. Methods 77 (1999) 87–99.
[15] K.F. Karpinski, Biometrics 46 (1990) 381–390.
[16] Roche Molecular Biochemicals, PCR ELISA (DIG Detection) Instruction Manual, Roche Diagnostics GmbH, Mannheim, 2001.
[17] B. Rombaut, R. Vrijsen, A. Boeyé, J. Gen. Virol. 66 (1985) 303–307.
[18] B. Rombaut, B. Verheyden, K. Andries, A. Boeyé, J. Virol. 68 (1994) 6454–6457.
[19] P. Chomczynski, N. Sacchi, Anal. Biochem. 126 (1987) 156–159.
[20] N. Kitamura, B.L. Semler, P.G. Rothberg, G.R. Larsen, C.J. Adler, A.J. Dorner, E.A. Emini, R. Hanecak, J.L. Lee, S. van der Werf, C.W. Anderson, E. Wimmer, Nature 291 (1981) 547–553.
[21] P. Halonen, E. Rocha, J. Hierholzen, B. Holloway, T. Hyypiä, P. Hurskainen, M. Pollansch, J. Clin. Microbiol. 33 (1995) 648–653.