ELSEVIER

Journal of Pharmaceutical and Biomedical Analysis
25 (2001) 131–142

JOURNAL OF
PHARMACEUTICAL
AND BIOMEDICAL
ANALYSIS

www.elsevier.com/locate/jpba

Screening of an enterovirus specific RT-PCR ELISA method for the quantification of enterovirus genomes in human body fluids by means of a three-level experimental design

S. Lauwers [a], Y. Vander Heyden [b], B. Rombaut [a,*]

[a] *Department of Microbiology and Hygiene, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium*
[b] *Department of Pharmaceutical and Biomedical Analysis, Vrije Universiteit Brussel, Laarbeeklaan 103, B-1090 Brussels, Belgium*

Received 1 August 2000; received in revised form 14 September 2000; accepted 21 September 2000

Abstract

In order to obtain a detection limit as low as possible for a quantitative enterovirus specific RT-PCR ELISA assay, optimal reaction conditions, which give rise to the highest response, need to be determined. This was done by investigating the influence of 13 factors, selected from RT and PCR, in a multivariate approach by means of a well-balanced three-level screening design, derived from a three-level Plackett–Burman design. Optimal reaction conditions could be determined by calculation and evaluation of the effects of the different factors on the response, i.e. the measured absorbance of the ELISA detection. The method will be used to study a possible longitudinal relationship between enteroviruses and the development of multiple sclerosis and juvenile diabetes.

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

1. Introduction

Multiple sclerosis (MS) and insulin-dependent diabetes mellitus (IDDM) or type I diabetes are diseases with a diverse aetiology. It is generally believed that the combination of a genetic predisposition, an autoimmune response and an environmental factor are necessary to initiate these diseases and to play an important role in their pathogenesis. Animal models exist and suggest that a virus can trigger the diseases [1,2]. In both cases enteroviruses, and in particular coxsackieviruses B, are important candidates [3–5]. Longitudinal follow-up studies, in which not only the presence of viral genome sequences will be determined but also their concentration (quantitative aspect), might help in the elucidation of a possible relationship between enteroviruses and these diseases.

To perform these longitudinal studies, a quantitative RT-PCR ELISA (reverse transcription-polymerase chain reaction enzyme linked

* Corresponding author. Tel.: +32-2-4774497; fax: +32-2-4774495.
E-mail address: brombaut@mich.vub.ac.be (B. Rombaut).

0731-7085/01/$ - see front matter
PII: S0731-7085(00)00486-6

immunosorbent assay) method will be used. In order to obtain a detection limit as low as possible, i.e. the ability to determine the lowest number of viral particles in a given sample, optimal reaction conditions for RT and PCR have to be established. Determining optimal conditions for PCR is commonly performed by optimising the different reaction parameters one by one (one-variable-at-the-time procedure). From this univariate approach, valuable conclusions can already be drawn, but one has to take into account that one variable can influence several others, i.e. interactions occur, e.g. the concentration of free Mg^{2+} ions in a PCR affects the dNTP (deoxynucleotide) concentration, the DNA polymerase activity and the melting temperature (T_m) of double stranded DNA and the primer/template interaction [6]. Secondly, one also has to be aware of the fact that the one-variable-at-the-time procedure examines only a limited part of the experimental domain, which means that on the one hand, the finally found 'best' conditions will depend on the starting conditions that were chosen and on the other, that the real optimal conditions will not necessarily be found. The global optimum reaction conditions would only be attained by investigating all possible combinations of factors and their possible levels in the experimental domain. This means, for example, that for the evaluation of n factors at three levels, there are 3^n possible combinations of variables, which is in practice not feasible to perform when the number of factors increases (in our study 13 factors were examined). This limitation can be overcome by using a multivariate approach in which all variables are changed simultaneously according to a well-considered experimental methodology. Plackett–Burman based designs have been proposed for the screening of a procedure in order to determine the influence of experimental factors at three levels on the responses of the method [7]. The application of experimental design for the optimisation of the polymerase chain reaction is not entirely new. Cobb and Clarkson [8] used a similar strategy, namely modified Taguchi methods, to determine reaction components that affected the PCR-product yield. There is, however, an important difference between their method and our method. They used a semi-quantitative approach (gel electrophoresis/ethidium bromide staining and densitometry) to detect the PCR-products instead of a quantitative ELISA format. For our study, a well-balanced three-level screening design, derived from an original Plackett–Burman design was used. The factors and their levels were selected based on a literature study [6,9–12] and on our own experience. For each factor examined, three levels were considered: a nominal (level 0) and two extreme levels (levels -1 and $+1$). The extreme levels were chosen based on the extreme values described in the literature. The nominal level was defined as being centrally situated in the interval between the two extreme levels. The aim of this study is to determine the most important factors on the formation of PCR reaction products, in order to be able to define experimental conditions leading to the highest yield of these products. The influence of the factors on the response of the method was evaluated by calculating their effects and drawing the corresponding effect plots. The best conditions predicted from the effect plots then permits the determination of a detection limit that is as low as possible. Cobb and Clarkson [8], in contrast, were evaluating Taguchi's signal-to-noise ratios.

All experiments were performed with poliovirus strain Mahoney, since this virus has been the most extensively studied within the enterovirus genus. The enterovirus specific primer pair and probe that were used are able to detect all available enterovirus prototypes (65 serotypes), since they are situated within a segment of the genome with absolute sequence conservation among all sequenced enteroviruses [13].

2. Materials and methods

2.1. Virus

Poliovirus strain Mahoney (type 1) was used as a representative of the enterovirus genus. The virus was grown, collected and purified by sucrose gradient ultracentrifugation [14]. The concentration was determined spectrophotometrically, assuming $A_{260}^{1\%}$ for virus being 81.6 [15].

2.2. RNA isolation

Viral RNA (ribonucleic acid) was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method [16]. 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 $A^{1\%}_{260}$ for RNA being 250 [15]. The genome number was then calculated considering the molecular weight of poliovirus [17]. Prior to RT-PCR, the 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 enterovirus serotypes in the 5′ untranslated region of the viral genome [18]. The downstream primer (5′-TTGTCACCATAAGCAGCCA-3′) and the 3′ DIG (digoxigenin)-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 (the examined factors and their levels can be found in Table 1; all values correspond with final concentrations in the reaction mixture): RNasin ([factor A] Units (U); Promega, Madison), RT-buffer ([factor B]; 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 AMV (avian myeloblastosis virus) reverse transcriptase ([factor C] U; Roche Molecular Biochemicals). The mixture was adjusted to a volume of 10 μl with sterile RNase-

Table 1
Factors examined and their levels

Factors		Levels		
		−1	0	1
A	RNasin concentration (U/reaction)	10	25	40
B	Batch of RT buffer[a]	RT(a)	RT(b)	RT(c)
C	AMV-RT concentration (U/reaction)	2	6	10
D	RT temperature (°C)	37	42	47
E	RT incubation time (min)	30	60	90
F	Batch of PCR buffer[b]	PCR(a)	PCR(b)	PCR(a)
G	$MgCl_2$ concentration in PCR buffer (mM)	1.5	2.5	3.5
H	dNTP concentration (μM)	0	100	200
I	DNA polymerase concentration (U/reaction)	1.00	1.75	2.50
J	Primer1-PCR // primer2-PCR (μM)	0.0–0.2	0.375–0.6	0.75–1.0
K	Pre-PCR heating incubation time (min)	9	12	15
L	Primer annealing temperature (°C)	50	60	70
M	Cycle number	25	33	40

[a] Batches (a), (b) and (c) correspond with different manufacturing dates.
[b] Batches (a), (b) and (c) correspond with different manufacturing dates.

and DNase-free water and incubated for [factor E] min at [factor D]°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 ([factor F]; Perkin–Elmer, Foster City), $MgCl_2$ ([factor G] mM; Perkin–Elmer), dNTP-mix ([factor H] μM; Roche Molecular Biochemicals), AmpliTaq Gold® DNA polymerase ([factor I] U; Perkin–Elmer) and the primers ([factor J] 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: [factor K] min at 95°C (pre-PCR heat step to activate the enzyme), [factor M] cycles at 95°C for 30 s, [factor L]°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 Corp., Dubuque) thermocycler. In each analysis, a no template control was taken along with the samples to detect any form of contamination.

2.5. ELISA

All reagents were pre-incubated to the appropriate temperature. Incubation at 37°C was performed under constant shaking, protected from light. The detection of the 148 bp biotin-labelled PCR products was performed according to Ossewaarde et al. [18] and adapted as follows: to 20 μl of undiluted PCR product 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 MTP (microtiter plate)-wells (Roche Molecular Biochemicals). The plate was incubated at 37°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 37°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 25 pmol/ml DIG-labelled probe in SSC solution with 0.5% Tween-20 and incubating at 37°C for 30 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 37°C for 5 min and twice with double concentrated SSC solution at 37°C for 5 min. Then, 200 μl of 35 mU/ml anti-DIG-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 37°C for 30 min. After washing four times with 0.05% Tween-20 in PBS, 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 37°C for 30 min. The signals were quantified with an automated MTP-reader El_x800 (Bio-Tek® Instruments, Winooski) 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 well-balanced three-level design [7], derived from an original Plackett–Burman design [19], is presented in Table 2. In this particular design, 13 factors can be investigated at three levels in 27 experiments. These 13 factors and their levels were selected considering several literature sources [6,9–12] and personal experience in the field of RT-PCR. The factors investigated were for the RT part of the procedure: RNasin concentration (factor A), batch of buffer (factor B), AMV-RT concentration (factor C), RT temperature (factor D) and RT duration (factor E) and for the PCR part of the procedure: batch of buffer (factor F), $MgC1_2$ concentration (factor G), dNTP concentration (factor H), DNA polymerase concentration (factor I), primer concentration (factor J), pre-PCR duration (factor K), primer annealing temperature (factor L) and cycle number (factor

Table 2
Three-level design for 13 factors derived from the original 27 experiments, three-level Plackett-Burman design[a]

Exp	Factors													Response $A_{405/490}$
	A	B	C	D	E	F	G	H	I	J	K	L	M	
1	−1	−1	0	−1	0	1	0	0	1	−1	0	0	0	0.968
2	1	−1	−1	0	−1	0	1	0	0	1	−1	0	0	0.230
3	1	1	−1	−1	0	−1	0	1	0	0	1	−1	0	0.392
4	1	1	1	−1	−1	0	−1	0	1	0	0	1	−1	−0.002
5	−1	1	1	1	−1	1	0	−1	0	1	0	0	1	0.313
6	0	−1	1	1	1	−1	−1	0	−1	0	1	0	0	0.600
7	1	0	−1	1	1	1	−1	−1	0	−1	0	1	0	−0.004
8	1	1	0	−1	1	1	1	−1	−1	0	−1	0	1	0.296
9	0	1	1	0	−1	1	1	1	−1	−1	0	−1	0	0.760
10	1	0	1	1	0	−1	1	1	1	−1	−1	0	−1	0.119
11	−1	1	0	1	1	0	−1	1	1	1	−1	−1	0	0.413
12	1	−1	1	0	1	1	0	−1	1	1	1	−1	−1	−0.003
13	−1	1	−1	1	0	1	1	0	−1	1	1	1	−1	−0.004
14	−1	−1	1	−1	1	0	1	1	0	−1	1	1	1	0.066
15	0	−1	−1	1	−1	1	0	1	1	0	−1	1	1	0.280
16	0	0	−1	−1	1	−1	1	0	1	1	0	−1	1	0.232
17	0	0	0	−1	−1	1	−1	1	0	1	1	0	−1	0.174
18	−1	0	0	0	−1	−1	1	−1	1	0	1	1	0	0.043
19	1	−1	0	0	0	−1	−1	1	−1	1	0	1	1	0.001
20	0	1	−1	0	0	0	−1	−1	1	−1	1	0	1	1.428
21	0	0	1	−1	0	0	0	−1	−1	1	−1	1	0	−0.005
22	1	0	0	1	−1	0	0	0	−1	−1	1	−1	1	1.122
23	0	1	0	0	1	−1	0	0	0	−1	−1	1	−1	−0.006
24	−1	0	1	0	0	1	−1	0	0	0	−1	−1	1	0.740
25	0	−1	0	1	0	0	1	−1	0	0	0	−1	−1	0.273
26	−1	0	−1	0	1	0	0	1	−1	0	0	0	−1	0.440
27	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	−1	0.862

[a] The results of the design, after correction for negative detection and no template, are also shown.

M). The different factor levels can be found in Table 1. The situation of the factors within the RT-PCR procedure is already described higher.

The following effects were calculated for each factor:

$$E_{x(-1,0)} = \frac{\sum R(0)}{N/3} - \frac{\sum R(-1)}{N/3}$$

$$E_{x(0,1)} = \frac{\sum R(1)}{N/3} - \frac{\sum R(0)}{N/3}$$

$$E_{x(-1,1)} = \frac{\sum R(1)}{N/3} - \frac{\sum R(-1)}{N/3}$$

where E_x is the effect on the measured response (R) for the change of a factor x from one level to another; $\Sigma R(i)$ ($i = 0, -1, 1$) is the sum of the measured values associated with level i and N is the number of design experiments. These calculated effects, which show the magnitude of the influence of the change of a factor, can be visualised in effect–plots (Fig. 1), where the behaviour of the response is estimated as a function of the factor levels [20]. It can be remarked that only two of the three above effects are independent. The third effect is namely the sum of the first two. However, this third effect was also calculated for reasons mentioned further in the text. A graphical interpretation to define important effects was also performed, namely the draw-

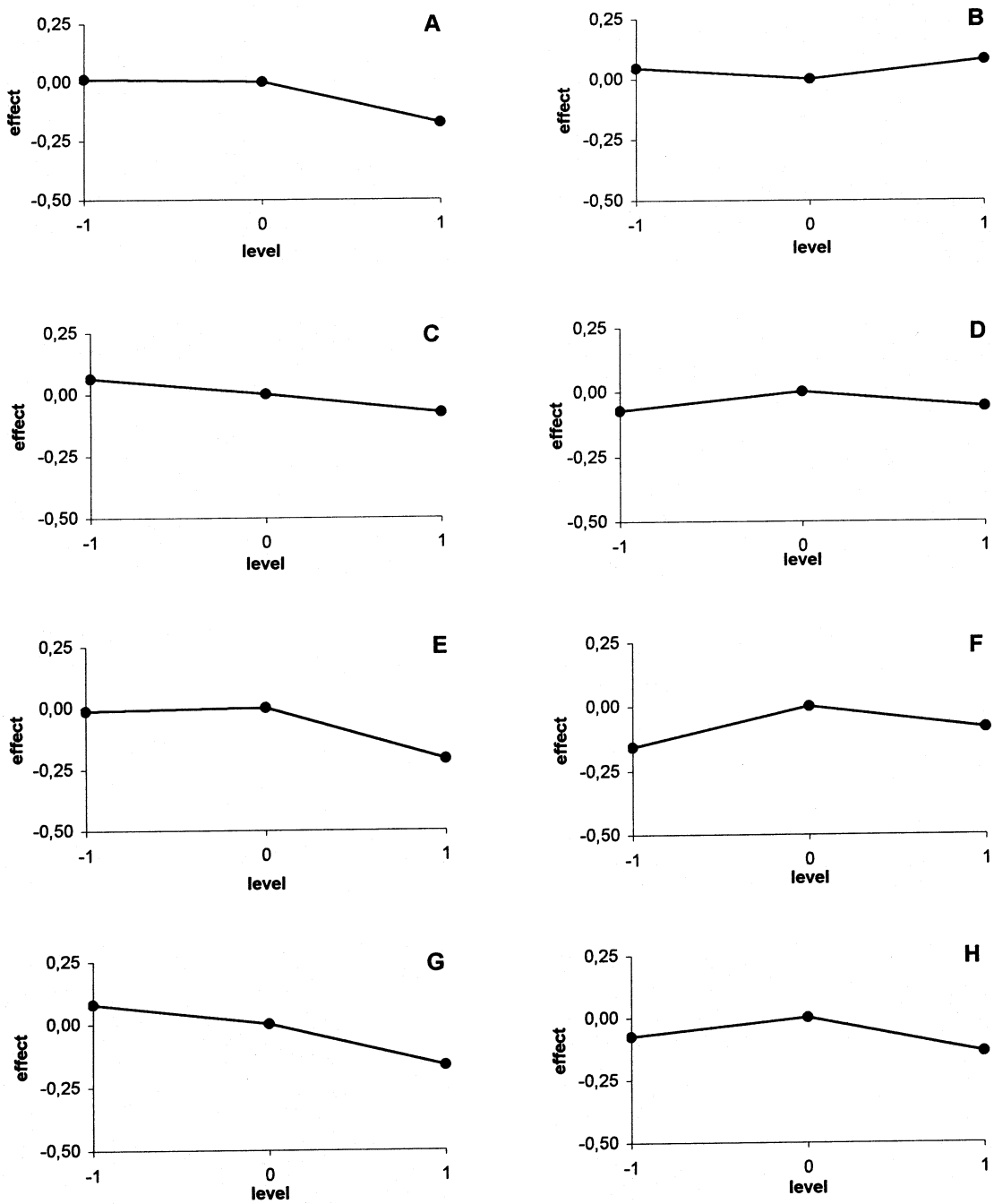

Fig. 1. Effect–plots of the 13 factors, selected from RT and PCR (Table 1), on the response $A_{405/490}$ of the ELISA detection method: (A) RNasin concentration; (B) batch of RT buffer; (C) AMV-RT concentration; (D) RT temperature; (E) RT duration; (F) batch of PCR buffer; (G) $MgCl_2$ concentration; (H) dNTP concentration; (I) DNA polymerase concentration; (J) primer concentration; (K) pre-PCR duration; (L) primer annealing temperature; (M) cycle number. The effects $E_{x(-1,0)}$ and $E_{x(0,1)}$ are shown.

ing of a normal probability plot [21]. In such a plot (Fig. 2), non-significant effects, which belong to a normal distribution with mean equal to zero (i.e. no effect), tend to fall on a straight line through the origin of the plot, while significant effects that do not belong to this distribution (i.e. effect $\neq 0$) deviate from it.

3. Results and discussion

3.1. Experimental design

In order to assure a positive detection of the PCR products, the design was performed by amplifying 10^6 enterovirus genomes and a corre-

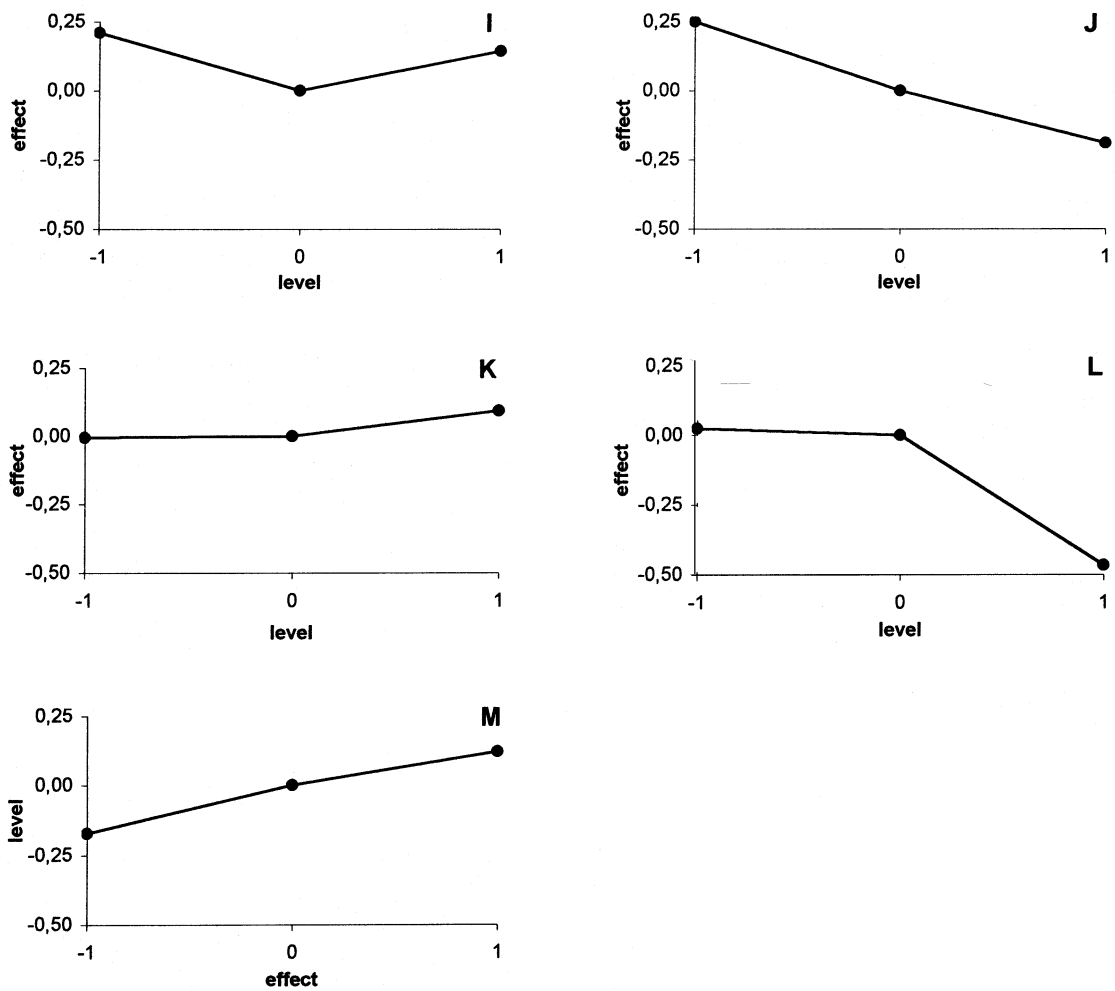

Fig. 1. (*Continued*)

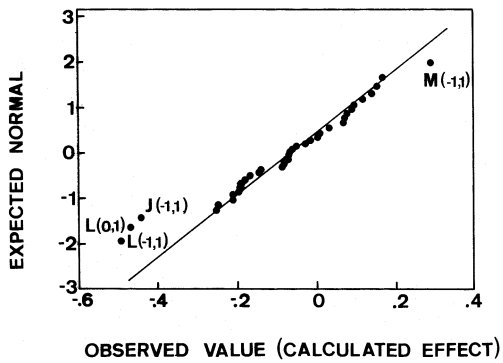

Fig. 2. Normal probability plot of all effects $E_{x(-1,0)}$, $E_{x(0,1)}$ and $E_{x(-1,1)}$, calculated from the performed design. Important effects, deviating from the straight line, are identified in the figure.

sponding no template for each of the 27 experiments. The results are shown in Table 2 (measured responses) and Table 3 (calculated effects) and in Fig. 1 (effect–plots) and Fig. 2 (normal probability plot). The factors that have an important effect were determined graphically by interpretation of the effect–plots and the normal probability plot. Intermediately important effects in the interval $(-1, 1)$ between the levels, can be considered negligible when considering only the effects $E_{x(-1,0)}$ and $E_{x(0,1)}$. Therefore, effect $E_{x(-1,1)}$ is also given. From the normal probability plot four effects are clearly important, namely the effect of factor L (primer annealing temperature) in the intervals $(-1, 1)$ and $(0, 1)$, factor J (primer concentrations) in the interval $(-1, 1)$ and factor M (cycle number) in the interval $(-1, 1)$. However, in addition for the less important factors, we tried to find, if possible, a physical explanation for the observed effects.

3.1.1. RNasin concentration (factor A, Fig. 1A)

Lower RNasin concentrations (level -1 (10 U) and level 0 (25 U)) produce higher yields than a high concentration (40 U). This can be explained by a possible inhibition of the PCR at higher RNasin concentrations. Taking into consideration the cost of this reagent, the lowest concentration being 10 U/reaction is preferable.

3.1.2. RT and PCR buffer batches (factors B and F, Fig. 1B,F)

Both RT and PCR buffer batches have no meaningful effect. This was to be expected, since the response of the PCR should not be dependent on the lot of a certain reagent.

3.1.3. AMV-RT concentration (factor C, Fig. 1C)

The optimum level of the RT enzyme is at level -1, corresponding with the lowest concentration

Table 3
Calculated effects[a]

Factors		Effects		
		$E_{x(-1,0)}$	$E_{x(0,1)}$	$E_{x(-1,1)}$
A	RNasin concentration	−0.012	−0.176	−0.188
B	Batch of RT buffer	−0.046	0.081	0.035
C	AMV-RT concentration	−0.064	−0.077	−0.141
D	RT temperature	0.072	−0.058	0.014
E	RT incubation time	0.014	−0.209	−0.194
F	Batch of PCR buffer	0.157	−0.084	0.072
G	$MgCl_2$ concentration in PCR buffer	−0.079	−0.165	−0.244
H	dNTP concentration	0.075	−0.137	−0.062
I	DNA polymerase concentration	−0.210	0.144	−0.066
J	Primer1-PCR//primer2-PCR	−250.0	−0.190	**−0.440**
K	Pre-PCR heating incubation time	0.006	0.093	0.099
L	Primer annealing temperature	−0.025	**−0.467**	**−0.491**
M	Cycle number	0.172	0.120	0.292

[a] Bold numbers indicate most important effects.

(2 U/reaction). Higher concentrations produce lower responses (though not strikingly important). As with the RNasin concentration, this effect is not so important and could be explained in the same way, namely by a possible inhibition of the PCR. However, it is also possible that higher enzyme concentrations cause enzyme inhibition of the RT itself.

3.1.4. RT temperature (factor D, Fig. 1D)

Contrary to our expectation and to the literature [9,22], the results do not show a clear temperature optimum of the AMV-RT at 42°C. The RT temperature does not seem to have an important effect in the examined interval. No valuable explanation for the difference with the literature can be given.

3.1.5. RT incubation time (factor E, Fig. 1E)

The incubation time of the RT reaction has some effect. At longer incubation time (level +1, 90 min) there is a decrease in response compared with the other two levels. For this effect we do not have an explanation. Since there is very little difference in result between 30 (level −1) and 60 min (level 0), 30 min could be preferred (gain of time).

3.1.6. Mg^{2+} concentration (factor G, Fig. 1G)

As expected, a meaningful effect can be seen for the $MgCl_2$ concentration, since the amount of free Mg^{2+} can, according to the literature [6,9,11,12], have a rather important effect on the specificity and yield of PCR. Magnesium ions form a soluble complex with dNTPs that is essential for dNTP incorporation. They also stimulate DNA polymerase activity and increase the T_m of the double-stranded DNA and the primer/template interaction. The best level for this factor was found at level −1, being 1.5 mM.

3.1.7. dNTP concentration (factor H, Fig. 1H)

The amount of dNTPs added during the PCR has no meaningful influence since the results are not clearly higher than in the situation where no dNTPs are further added (level −1). This can be explained by the fact that dNTPs were already added for the RT. From these results, we can conclude that the amount added for RT is sufficient for both steps of the procedure. The fact that a low concentration of dNTP gives a satisfactory response is beneficial, since high dNTP concentrations could lead to mispriming and misincorporation, resulting in a reduced yield of specific PCR product.

3.1.8. DNA polymerase concentration (factor I, Fig. 1I)

The AmpliTaq® Gold DNA polymerase concentration behaves rather strange. There is little difference in the PCR yield between levels −1 and +1, being 1 and 2.5 U/reaction. However, at level 0 (1.75 U/reaction), a considerably strong decrease in response is observed relative to the other two levels. For this phenomenon, no explanation could be given. For economical reasons, the lowest concentration is preferable.

3.1.9. Primer concentrations (factor J, Fig. 1J)

The effect of the primer concentrations is clearly significant. At the −1 level (lowest concentration), a maximal response is seen. This is in agreement with the fact that higher primer concentrations could lead to mispriming and the formation of primer–dimer artefacts that can interfere with the specific reaction, dramatically reducing the yield.

3.1.10. Incubation time of the pre-PCR heating step (factor K, Fig. 1K)

The incubation time of the pre-PCR heating step, which is necessary to activate the AmpliTaq® Gold DNA polymerase enzyme, has no significant effect, although 15 min (level +1) leads to a somewhat higher response than the other two levels. Since there is no difference between 9 min (level −1) and 12 min (level 0), we can conclude that after 9 min the enzyme is already sufficiently activated.

3.1.11. Primer annealing temperature (factor L, Fig. 1L)

The primer annealing temperature produces the most significant effect of all 13 factors studied. This could be expected since it is generally known that the annealing temperature is the most critical

factor in a PCR [6,11]. At a higher temperature (level +1, 70°C) an obvious decrease in response is seen compared with the other two levels, being 50°C (level −1) and 60°C (level 0). In order to achieve the highest specificity and a satisfactory product yield, the highest annealing temperature, i.e. 60°C is to be preferred [6,12].

3.1.12. Cycle number (factor M, Fig. 1M)

The cycle number appears also to be a clearly significant factor. An increasing cycle number leads to a higher response, which is quite logical as a result of the exponential character of the PCR. The best level in the examined interval corresponds with the highest cycle number (level +1, 40 cycles). At higher cycle numbers we have to keep in mind that in the response a plateau, which is independent of the initial amount of template added, can be reached. This is certainly to be avoided if one wants to perform quantitative PCR. However, from our results we could conclude that at a concentration of 10^6 enterovirus genomes the plateau-phase has not been reached yet.

In summary, it can be said that the above results clearly show meaningful effects for the following three factors (in descending order of importance): primer annealing temperature (optimum at 60°C, occasionally at 50°C since similar results are obtained), primer concentration added for PCR (optimum: primer 1: 0 μM, primer 2: 0.2 μM), cycle number (optimum at 40 cycles) and an intermediately important effect for the factor Mg^{2+} concentration (optimum 1.5 mM $MgCl_2$). All other investigated factors seem to have little or no effect. It has to be remarked that what is called optimal in the above is not necessarily the global optimal level of a factor, but it is the best one of those evaluated. From the 27 experiments of the executed design, only one was performed with the four most important factors at their optimal levels, namely experiment 20. It can be observed in Table 2 that this experiment also in practice gave the highest response. Five experiments of the design also had relative high response values, namely experiments 1, 9, 22, 24 and 27. These experiments were all performed at the optimal values for the most important factor, i.e. primer annealing temperature at 50 or 60°C, but one or two of the other important factors were at less favourite levels. Therefore, the subsequent experiments were performed according to the conditions of experiment 20. When considering economics and speed of the assay as being important, similar high responses can be predicted under some specific conditions differing from experiment 20. However, the two requirements following need to be fulfilled: the four important factors have to be at their optimal levels and less important factors can only be adjusted to a level yielding equal responses as the level used in experiment 20. In the case of our enterovirus specific RT-PCR ELISA method, the following adjustments can be made to reduce the cost of the assay, the RNasin concentration and the AmpliTaq® Gold DNA polymerase concentration can be reduced to the lowest concentration (level −1), being 10 U (instead of 25 U) and 1 U (instead of 2.5 U), respectively. Concerning the gain of time, the following two factors can be adjusted: RT duration (30 min (level −1) instead of 60 min (level 0)) and duration of the pre-PCR heating step (9 min (level −1) instead of 15 min (level +1)).

3.2. Linear range and detection limit

Since our aim is to perform quantitative analysis, it is important to determine the linear range of the test, where quantification is possible. This was done by analysing a $\log_{10}$ dilution series of enteroviral RNA over a broad range (10^0–10^8 enterovirus genomes) (Fig. 3A). Linearity is seen over an $\approx$4 log interval (10^3–10^7 genomes), suitable for quantification. This experiment already provided us with a first prediction of the detection limit. It has to be remarked that in the domain of PCR analysis, there is a lack of uniformity in the way detection limits are defined, determined and interpreted. In PCR ELISA different criteria for the detection limit are used [23,24] and in most cases, no criteria are even given on how reported detection limits were obtained. From our point of view, the definition of the detection limit must be based upon statistics. It should correspond with the lowest concentration level that can be deter-

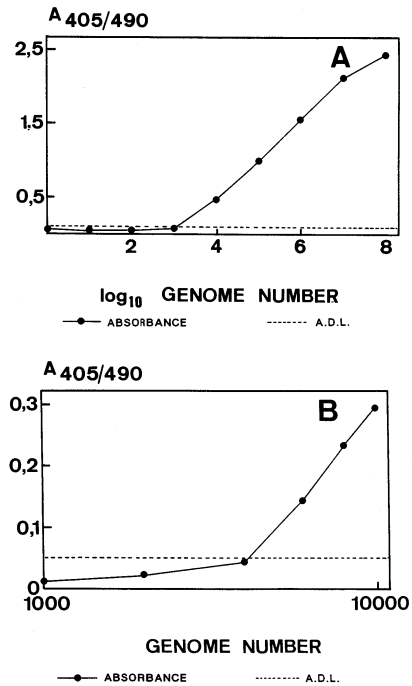

Fig. 3. Determination of the linear range and arbitrary detection limit (ADL) of the enterovirus RT-PCR ELISA. The dilution series of enteroviral RNA were amplified according to the conditions of experiment 20 of the design (Table 2). (A) 10^0–10^8 enterovirus genomes, (B) 10^3–10^4 enterovirus genomes.

mined to be statistically different from the no template control. In the first instance, the criterion for a positive detection was arbitrarily set at the concentration corresponding with a S/N (signal-to-noise) ratio equalling 3 ($S/N = 2H/h$, where h is the amplitude range of the no template controls and H the difference between the means of the samples and the no template controls. In our case, using this criterion, the detection limit would be situated somewhere between 10^3 and 10^4 genomes. To determine the detection limit more accurately, a new dilution series was prepared within the more narrow range of 10^3–10^4 genomes (Fig. 3B). Linearity is seen for concentrations ≥ 4000 genomes. From this experiment we observed that if the above predictive criterion is used, the detection limit is estimated for a too low genome copy number. This is due to the shape of the curve. Since there is no linear descendance over the whole area, inappropriate estimates for the detection limit are obtained. For this reason, the detection limit (≈ 100 genomes) was determined based on experimental results. Replicate experiments were performed and a t-test was used to determine whether a given concentration situated between 10^1 and 10^4 genomes is statistically different from the no template results. For both the positive samples and the no template, six samples were subjected to RT-PCR and were analysed in duplicate. At the $\alpha = 0.05$ significance level, the signals corresponding with concentrations ≥ 1000 genomes are statistically different from the no template ($t = 3.16$ for 1000 genomes). This was not the case anymore for concentrations ≤ 100 genomes ($t = 1.59$ for 100 genomes), $t_{critical} = 2.02$ (one-tailed test). Thus, we can state that our enterovirus specific RT-PCR ELISA can be used to detect concentrations above the detection limit of 1000 enterovirus genomes and quantify concentrations above 4000 genomes, since linearity is required for quantification. It has to be remarked that if we would use less stringent criteria, a lower detection limit could be predicted. Fini et al. [23] define the detection limit as the genome copy number that produces a signal 3 S.D. above background. Using this criterion, the detection limit for our enterovirus specific RT-PCR ELISA would be situated between 100 and 1000 genomes. According to Ylikoski et al. [24], where the detection limit is defined as twice the mean of the zero signal in the hybridisation assay, the detection limit could even be as low as one enterovirus genome. However, as was demonstrated these predictive values have no practical meaning.

In conclusion, we can say that the applied experimental design has proven to be effective for the investigation of the influence of the 13 factors examined. In this way, it enabled us to generate good reaction conditions for RT and PCR in a non-blood matrix. The developed method will result in the generation of quantitative and longitudinal data of enterovirus genomes in the blood of diabetes patients and their relatives and in blood and cerebrospinal fluid samples of MS patients, since tests (Lauwers et al., in preparation) have already indicated that similar results are obtained for these type of matrixes. These quantitative and longitudinal data might then help in the elucidation of the relationship between enteroviruses and

IDDM diabetes on the one hand and multiple sclerosis on the other.

Acknowledgements

This work was supported by the Charcot Stichting (Belgium) and by OZR (Vrije Universiteit Brussel). Y. Vander Heyden is a postdoctoral fellow of the Fund for Scientific Research (FWO), Vlaanderen. The authors wish to thank M. De Pelsmaecker and A. De Rees for technical assistance.

References

[1] P. Sarchielli, A. Trequattrini, F. Usai, D. Murasecco, V. Gallai, Role of viruses in the etiopathogenesis of multiple sclerosis, Acta Neurol. Napoli 15 (1993) 363–381.
[2] J.W. Yoon, A new look at viruses in type I diabetes, Diabetes Metab. Rev. 11 (1995) 83–107.
[3] M. King, A. Shaikh, D. Bidwell, A. Voller, J. Banatvala, Coxsackie B-virus specific IgM responses in children with insulin-dependent diabetes mellitus, Lancet 1 (1983) 1397–1399.
[4] D.R. Gamble, K.W. Taylor, H. Cumming, Coxsackie viruses and diabetes mellitus, Br. Med. J. 4 (1973) 260–262.
[5] R.B. Dessau, L.P. Nielsen, J.L. Frederiksen, Absence of entero- and cardioviral RNA in multiple sclerosis brain tissue, Acta Neurol. Scand. 95 (1997) 284–286.
[6] C.R. Newton, A. Graham, in: C.R. Newton, A. Graham (Eds.), Instrumentation, Reagents and Consumables, PCR Bios Scientific, Oxford, 1994, pp. 9–25.
[7] Y. Vander Heyden, M.S. Khots, D.L. Massart, Three-level screening designs for the optimisation or the ruggedness testing of analytical procedures, Anal. Chim. Acta 276 (1993) 189–195.
[8] B.D. Cobb, J.M. Clarkson, Nucleic Acids Res. 22 (1994) 3801–3805.
[9] Boehringer Mannheim, Basic PCR: Protocols and optimisation strategies (Chapter 4) and Reverse transcription-PCR (Chapter 6), in: PCR Applications Manual, Boehringer Mannheim GmbH, Mannheim, 1995, pp. 26–53, 80–94.
[10] R.S. Cha, W.G. Thilly, Specificity, efficiency, and fidelity of PCR, in: C.W. Dieffenbach, G.S. Dveksler (Eds.), PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1995, pp. 37–51.
[11] M.A. Innis, D.H. Gelfand, Optimalisation of PCRs, in: M.A. Innis, D.H. Gelfand, J.J. Sninsky, T.J. White (Eds.), PCR Protocols: a Guide to Methods and Applications, Academic Press, San Diego, CA, 1990, pp. 3–11.
[12] K.H. Roux, Optimization and troubleshooting in PCR, in: C.W. Dieffenbach, G.S. Dveksler (Eds.), PCR Primer: A Laboratory Manual, Cold Spring Harbor Laboratory Press, New York, 1995, pp. 53–62.
[13] P. Halonen, E. Rocha, J. Hierholzen, B. Holloway, T. Hyypiä, P. Hurskainen, M. Pollansch, Detection of enteroviruses and rhinoviruses in clinical specimens by PCR and liquid-phase hybridization, J. Clin. Microbiol. 33 (1995) 648–653.
[14] B. Rombaut, R. Vrijsen, A. Boeyé, Stabilization by host cell components and Mg^{2+} of the neutralization epitopes of poliovirus, J. Gen. Virol. 66 (1985) 303–307.
[15] B. Rombaut, B. Verheyden, K. Andries, A. Boeyé, Thermal inactivation of oral polio vaccine: contribution of RNA and protein inactivation, J. Virol. 68 (1994) 6454–6457.
[16] P. Chomczynski, N. Sacchi, Single-step method of RNA isolation by acid guanidinium-phenol-chloroform-extraction, Anal. Biochem. 126 (1987) 156–159.
[17] 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, Primary structure, gene organization and polypeptide expression of poliovirus RNA, Nature 291 (1981) 547–553.
[18] J.M. Ossewaarde, M. Rieffe, G.J.J. van Doornum, C.J.M. Henquet, A.M. Van Loon, Detection of amplified Chlamydia trachomatis DNA using a microtiter plate-based enzyme immunoassay, Eur. J. Clin. Microbiol. Infect. Dis. 13 (1994) 730–740.
[19] R.L. Plackett, J.P. Burman, The design of optimum multifactorial experiments, Biometrika 33 (1946) 305–325.
[20] S. Boonkerd, M.R. Detaevernier, Y. Vander Heyden, J. Vindevogel, Y. Michotte, J. Chromatogr. A 736 (1996) 281–289.
[21] E. Morgan, Factorial design and analysis (Chapter 3) and fractional factorials (Chapter 4), in: Chemometrics, Experimental Design, Analytical Chemistry by Open Learning, Wiley, Chichester, 1991, pp. 81–133 pp. 151–177.
[22] Promega Corporation, Modifying enzymes, in: K. Doyle, J. Miles (Eds.), Promega Protocols and Applications Guide, third ed, Promega Corp., Madison, 1996, p. 31.
[23] F. Fini, G. Gallinella, S. Girotti, M. Zerbini, M. Musiani, Clin. Chem. 45 (1999) 1391–1396.
[24] A. Ylikoski, M. Sjoroos, A. Lundwall, M. Karp, T. Lovgren, H. Lilja, A. Iitiä, Clin. Chem. 45 (1999) 1397–1407.