Chapter 1

# Robust Design and Taguchi Method Application

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Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/56580

## 1. Introduction

The objective of this chapter is to present an application of Taguchi Experimental Design Method. A healthcare case was chosen for this purpose. It is specifically applied to Molecular Assays in Clinical Laboratories and the main target is to determine the best parameters adjustment of a Molecular Assays Process in order to obtain the best diagnostic result for Venous Thromboembolism investigation [1].

### 1.1. Venous thromboembolism investigation by molecular assays process

Disorders of haemostatic mechanisms that predispose a person to thrombotic episodes are generally referred to as thrombophilia [2]. The incidence of one such disorder, Venous Thromboembolism, increases with age, ranging from 1 in 10,000 among children to 1 in 100 among elderly. Its most common clinical manifestations are deep vein thrombosis of the lower limbs and pulmonary embolism. Origins of thrombophilia risk factors can be both acquired and genetic, making the thromboembolic disease a complex, multifactorial trait [3].

The acquired risk factors include pregnancy, surgery, trauma, immobilization, advanced age, as well as previous episodes of thrombosis. The genetic risk factors most relevant are two mutations: the Factor V Leiden mutation (1691G>A) and the 20210G>A point mutation in the 3′ untranslated region (UTR) of the Prothrombin gene. The first causes resistance to activated protein C and the second is associated with increased plasma factor II levels [4]. Both mutations act as gain of function, causing hypercoagulability.

There are two basic methods for investigating Factor V Leiden: functional (Activated Protein C Resistance) and molecular (such as PCR - Polymerase Chain Reaction - followed by restriction digestion, or real-time PCR). However, only the second method can be used to detect Prothrombin mutation (the functional methods are unacceptable due to overlapping between normal and carrier levels). Among molecular methods, real-time PCR offers many advantages over conventional PCR: higher sensitivity, quicker turn-around-time, better uniformity and objectivity in the analysis, and lower probability of contamination in the laboratory.

Real-time PCR with Fluorescence Resonance Energy Transfer (FRET) probes (also called hybridization probes) employ two labeled probes, commonly called FRET and anchor probes. These probes hybridize to the PCR product in head-to-tail fashion, at close proximity. Because the acceptor fluorophore emits light in a longer wavelength, signal detection is possible. One of these probes is labeled with a donor dye at the 3′ end and the other is labeled with an acceptor dye at the 5′ end. Quenched-FRET assays are similar to FRET assays, except in what they measure. Instead of measuring the increase in energy of the acceptor fluorophore, quenched-FRET assays measure, during amplification, the decrease in energy of the donor fluorophore. Use of a quencher molecule, such as black-hole quenchers, in place of an acceptor fluorophore enables multiplexing of more than one fluorophore. This permits the analysis of a greater number of mutations in the same tube. By performing a melting analysis after PCR to find the amplicon-probe melting temperatures, genotyping is achieved. Variation may be shown depending on the number of mismatches, the length of the mismatched duplex, the position of the mismatch and neighboring base pairs. Studies previously published have confirmed the efficiency of FRET real-time PCR for SNP detection and allelic discrimination of Factor V Leiden and Prothrombin (factor II) [5-10].

The FRET system requires four oligonucleotides. Therefore to conduct a successful experiment with reliable results two steps are necessary: careful design of the probes and primers and assays optimization.

### 1.2. Taguchi experimental design

The experimental design is widely used to optimize process parameter values in order to improve the quality properties of a product or a process. Full Factorial and One-Factor-at-the-time (OFTA) experiments are design methods that can possibly be used but requires a large number of experiments when the number of process parameters increases. Taguchi developed the foundations of Robust Design introduced in the 1950s and 1960s and the application of his method in electronics, automotive, photographic and many others industries has been an important factor in the rapid industrial growth of Japanese industries [11].

Among the various approaches to quality engineering products and processes, the method of Taguchi is identified for robust design [14]. The method deviates from the quality engineering concerns when it considers the objective to ensure good quality products and good process performance deliveries during the life cycle of these projects [12]. Taguchi methods are distinguished from other approaches to quality engineering by some specific concepts, as follow:

http://dx.doi.org/10.5772/56580

- Minimization of a quality loss function
- Maximization the signal to noise ratio
- Orthogonal Arrays

The strategy of experimental design used in the Taguchi method is based on orthogonal arrays and fractional factorial, in which not all possible combinations of factors and levels are tested. It is useful to estimate the effects of main factors on the process. The primary goal of this type of strategy is to obtain as much information about the effect of the parameters on the process with minimal experimental runs. In addition to the fact of requiring a smaller number of experiments, the orthogonal arrays still allow to test the factors using a mixing of number of levels.

Taguchi method uses a special design of Orthogonal Arrays that allows to study the whole parameter space with a limited number of experiments [12]. Besides, this method provides other advantages: it reduces economically the variability of the response variable, shows the best way to find out the optimum process conditions during laboratory experiments, it is an important tool for improving the productivity of the R&D activity and it can be applied to any process.

The usual steps to apply Taguchi experimental design [13] are: (a) to select the output variable(s) (response(s)) to be optimized; (b) to identify the factors (input variables) affecting output variable(s) and to choose the levels of these factors; (c) to select the appropriate Orthogonal Array; the arrays are found in literature [14]; (d) to assign factors and interactions to the columns of the array; (e) to perform experiments; at this step it is important to randomize the trials in order to minimize the systematic error; (f) to analyze the results using signal-to-noise ratio (S/N) analysis and analysis of variance (ANOVA); (g) to determine the optimal process parameters; (h) to perform confirmatory experiments, if it is necessary.

For the S/N ratio analysis, the appropriate S/N ratio function must be chosen: smaller-the-better, larger-the-better, nominal-the-better. The S/N ratio is a logarithmic function used to optimize the process or product design, minimizing the variability, as shown by Equation 1.

$$\eta = -10\log_{10}[\frac{1}{n}\sum_{i=1}^{n}\frac{1}{y_i^2}] \quad (1)$$

In the equation (1), $\eta$ is the signal to noise ratio, $y_i$ is the Quality Function Deviation, problem type "larger-the-better", which is the case of this application and, $n$ corresponds the number of experiments runs.

The S/N ratio can be also understood as the inverse of variance and the maximization of S/N ratio allows reduction of the variability of the process against undesirable changes in neighbouring environment (also named uncontrollable factors or factors of noise). To minimize variability, the level of factor which produces the greatest value of S/N ratio must be chosen.

The analysis of variance (ANOVA) is applied in order to test the equality of several means, resulting in what process parameters (factors) are statistically significant.

In the methodology of experimental design, the test used to evaluate the significance of the levels changes of a factor or an interaction is a hypothesis test. In the case of full factorial, this test is an analysis of variance (ANOVA) [18]. When two levels of a factor generating have equal statistically mean responses, it is assumed that the factor does not affect the response of interest. When, instead, a significant difference is detected, the factor is important. For a full factorial with two factors A and B and two levels (+1, -1), the correspondent model can be shown in equation (2).

$$Y_{ijk} = \mu + A_i + B_j + AB_{ij} + \varepsilon_{ijk} \tag{2}$$

Where:

$i$ is the number of levels of the fator A;

$j$ is the number of levels of the fator B and $k$, the number of replicas;

$Y_{ijk}$ is the (ijk)th observation obtained in the experiment;

μ is the overall mean;

$A_i$ is the effect of the ith treatment of Factor A;

$B_j$ is the effect of the jth treatment of Factor B;

$AB_{ij}$ is the effect of the ij-th AB interaction between factors;

$\varepsilon_{ijk}$ is the component of random error.

The results of ANOVA are presented in a table that displays for each factor (or interaction) the values of the sum of squared (SS) deviations from the mean, the mean of squares (MS) and the ratio between the mean of squares effect and the mean of squares error (F). For background information many introductory texts on elementary statistical theory are available in literature and can also be found in most of the statistical packages for microcomputers [16-18].

In this chapter the Taguchi L27 Orthogonal Array employed for experimental design and data analysis, considers the search for the best conditions of operation, the effects of the main factors over the process, and the interactions among the factors. The Taguchi method was applied by Ballantyne et al. [15] for the optimization of conventional PCR assays using an L16 Orthogonal Array with four variables at two different levels each. The present research, however, is considered a more complex Taguchi´s method application once it optimizes a process that uses real-time PCR using FRET probes with six three-levels factors.

## 2. Material and methods

Distinct DNA pools for Factor V Leiden and Prothrombin were employed. Each was constituted of samples from quality control programs whose genotypes were already known. The mutation detection was performed by real-time PCR, followed by melting curve analysis with adjacent fluorescent probes using the quenched-FRET principle.

With the exception of the reverse primers (Prothrombin: 5′- ATTACTGGCTCTTCCTGAGC3′; Factor V Leiden:5′TGCCCAGTGCTTAACAAGAC-3′), the primer and probe sequences for both Prothrombin and Factor V Leiden genotyping were the same as those described by von Ahsen et al. [6] and Ameziane et al. [10], respectively. The Factor V Leiden detection probe, which was specific for the mutated allele, was 3′-labeled with 6-carboxyfluorescein (FAM). The adjacent probe, which functioned as an anchor, was 5′-labeled with Cy5 and phosphorylated at its 3′ end; this was to prevent probe elongation by *Taq* polymerase [5-8].

The Prothrombin detection probe, which was complementary to the wild-type allele, was 3′-labeled with 6-carboxy-4′,5′-dichloro-2′, 7′-dimethoxyfluorescein (JOE). The anchor probe was 5′-labeled with 6-carboxytetramethylrhodamine (TAMRA) and phosphorylated at its 3′ end. About 240 ng of genomic DNA in a final PCR volume of 25 μL were amplified and detected in the Rotor-Gene 3000 (Corbett Research, Australia), as shown in Figure 1.


**Figure 1.** Experiments Environment: Sample Preparation Chapel and Rotor Gene 3000

For standardization, PCR reactions were performed using distinct Master Mixes: MMA, Promega PCR Master Mix (1.5 mM $MgCl_2$); MMB, Promega PCR Master Mix (3.0 mM $MgCl_2$); MMC, QIAGEN PCR Master Mix (1.5 mM $MgCl_2$), different concentrations of primers (forward and reverse) and probes (FRET and anchor) and different PCR cycle numbers to test for the best combination.

All six factors selected as "input variables" for standardization (Table 1) were investigated at three different levels. These factors were selected for being essential components of a PCR. Also, the levels tested cover the range suggested in the literature. Although a standard real-time PCR usually uses a maximum of 45 to 50 cycles, some protocols, such as asymmetric PCR, may require more.

Cycling and melting profiles were performed according to the following protocol: 95°C for 4 min as the initial denaturation step. This was followed by n cycles (n = 50, 65 or 85) of 95°C for 10 s, 53°C for 20 s and 72°C for 20 s. Thereafter, melting curve analysis of the duplex amplicon-probe was performed. Analysis started at 49°C and proceeded until to 88°C, at a linear rate of 1°C every 5 s.

| **Factor name** | **Column allocated to the factor in L27 Taguchi Orthogonal Array** | **Level 1** | **Level 2** | **Level 3** | **Unit** |
|---|---|---|---|---|---|
| Master Mix | A | MMA | MMB | MMC | Composition/ supplier |
| Primer forward concentration (P1) | B | 0.1 | 0.5 | 1.0 | µM |
| Primer reverse concentration (P2) | E | 0.1 | 0.5 | 1.0 | µM |
| FRET probe concentration (S1) | J | 0.2 | 0.3 | 0.4 | µM |
| Anchor probe concentration (S2) | K | 0.2 | 0.3 | 0.4 | µM |
| Number of PCR cycles | L | 45 | 65 | 85 | cycles |

**Table 1.** Experiments Factors and Levels

The Rotor-Gene software calculated the negative derivative of the fluorescence ratio with respect to temperature (-dF/dT), which was plotted against temperature (T°C). The melting curves were then converted to melting peaks. The "output variable" was the melting peak height measured after the melting analysis, which could be visualized in the negative derivative plot (-dF/dT x T°C). This variable was chosen as the output because it reflected the efficiency of the whole process, including both amplification and melting analysis.

PCR mix preparation, sample loading, and amplification reactions were carried out in three separate rooms. All rooms were subject to temperature and humidity control. In order to minimize the effect of pipetting errors between runs, amplification mix was prepared once and then divided into aliquots. These were stored, protected from light, at -20°C. Each aliquot was thawed only once at the time of use. In addition, in order to minimize the effect of inter-operator variation, a single person was responsible for the execution of the whole process. In order to maintain reagents and sample stability, each of the 27 experiments was performed over consecutive days in quadruplicate.

Tubes were randomly loaded in the 36 carousel rotor. These procedures, performed independently, were adopted for both Factor V Leiden and Prothrombin genotyping. The research equipment outputs fluorescence values at the origin point of the fluorescence curve. This constraint may lead to low output values for curves of high resolution and high values for

curves of low resolution. This restriction was overcome by employing the derivative of the curve for Fluorescence and defining the output as the peak mean values or the peak values. Thus meaningful values for both Prothrombin genotyping and V Leiden were achieved.

The curve for Melting was generated with the use of Rotor-Gene 3000 equipment by using the curve for Fluorescence of each sample. In order to estimate the individual and interaction effects among the factors, a Taguchi L27 Orthogonal Array was employed, with four replicates for each experiment.

The Taguchi method differs from other quality engineering tools in terms of some specifics concepts, once it includes the minimization of the quality loss function, the maximization of the noise-to-signal ratio, a quadratic loss function [14], and the usage of Orthogonal Arrays [16-18]. The results were later compiled and analyzed through statistical methods using Statistica9 software.

## 3. Results

Tables 2 and 3 show the L27 Orthogonal Array results considering four replicates per run. Table 3 shows the experimental factors and the levels considered by Taguchi method and used to determine the optimal adjustments for the best final results.

The Factors A, B, E, J, K AND L are assigned to columns 1, 2, 5, 9, 10 and 11 in the L27. The Factors C, D, F, G, H, I, M and N are assigned to columns 3, 4, 6, 7, 8, 12, and 13 in L27 and represent the interaction effects being treated as dummy factors.

The output variability is reduced when the signal-to-noise ratio is maximized. In this Taguchi method application the design condition to reach this goal is larger is better.

In the sequence, Tables 2 and 3 are shown.

The same procedure is equally done for Factor V Leiden genotyping.

Figures 2 and 3 show the main effects for means and signal-to-noise ratio for Prothrombin genotyping, as follow.

### 3.1. Results from prothrombin genotyping analysis

By distributing the mean output values among experimental setups, it was possible to evaluate each experimental factor's impact on the output. In this case, the experimental factors major impact results in a descending order are E, B, A, H, L, K, G, J, F, N, M, C and D, varying from 60.29% to 1.83% respectively.

Statistical analysis was performed by applying Analysis of Variance (ANOVA) to the obtained results. Each ANOVA factor adopted two degrees of freedom, which corresponds to the number of levels adopted to a given experimental factor less one. Square sums of the two less

| L27 Orthogonal Array and Replicates Results for Factor V Leiden | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Run | A | B | C | D | E | F | G | H | J | K | L | M | N | Replicas | | | |
| | | | | | | | | | | | | | | 1 | 2 | 3 | 4 |
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 2 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0.09000 | 0.08500 | 0.08500 | 0.07500 |
| 3 | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0.20000 | 0.16500 | 0.19000 | 0.18500 |
| 4 | 1 | 2 | 2 | 2 | 1 | 1 | 1 | 2 | 2 | 2 | 3 | 3 | 3 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 5 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 1 | 1 | 1 | 0.02000 | 0.00001 | 0.01500 | 0.00001 |
| 6 | 1 | 2 | 2 | 2 | 3 | 3 | 3 | 1 | 1 | 1 | 2 | 2 | 2 | 0.05500 | 0.04500 | 0.05000 | 0.05000 |
| 7 | 1 | 3 | 3 | 3 | 1 | 1 | 1 | 3 | 3 | 3 | 2 | 2 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 8 | 1 | 3 | 3 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 3 | 3 | 3 | 0.00500 | 0.00001 | 0.00001 | 0.00500 |
| 9 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 0.05500 | 0.06000 | 0.05000 | 0.06000 |
| 10 | 2 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 11 | 2 | 1 | 2 | 3 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 0.04500 | 0.00001 | 0.00001 | 0.00001 |
| 12 | 2 | 1 | 2 | 3 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 0.08000 | 0.06000 | 0.06000 | 0.08500 |
| 13 | 2 | 2 | 3 | 1 | 1 | 2 | 3 | 2 | 3 | 1 | 3 | 1 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 14 | 2 | 2 | 3 | 1 | 2 | 3 | 1 | 3 | 1 | 2 | 1 | 2 | 3 | 0.00500 | 0.00500 | 0.02000 | 0.00001 |
| 15 | 2 | 2 | 3 | 1 | 3 | 1 | 2 | 1 | 2 | 3 | 2 | 3 | 1 | 0.02000 | 0.00001 | 0.02000 | 0.01000 |
| 16 | 2 | 3 | 1 | 2 | 1 | 2 | 3 | 3 | 1 | 2 | 2 | 3 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 17 | 2 | 3 | 1 | 2 | 2 | 3 | 1 | 1 | 2 | 3 | 3 | 1 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 18 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 2 | 3 | 1 | 1 | 2 | 3 | 0.02000 | 0.02000 | 0.00001 | 0.02000 |
| 19 | 3 | 1 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 20 | 3 | 1 | 3 | 2 | 2 | 1 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 0.07000 | 0.06500 | 0.05500 | 0.06500 |
| 21 | 3 | 1 | 3 | 2 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 2 | 1 | 0.10000 | 0.09000 | 0.08500 | 0.09500 |
| 22 | 3 | 2 | 1 | 3 | 1 | 3 | 2 | 2 | 1 | 3 | 3 | 2 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 23 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 3 | 2 | 1 | 1 | 3 | 2 | 0.00001 | 0.02000 | 0.00001 | 0.02500 |
| 24 | 3 | 2 | 1 | 3 | 3 | 2 | 1 | 1 | 3 | 2 | 2 | 1 | 3 | 0.04500 | 0.03500 | 0.04000 | 0.03500 |
| 25 | 3 | 3 | 2 | 1 | 1 | 3 | 2 | 3 | 2 | 1 | 2 | 1 | 3 | 0.01000 | 0.00001 | 0.00001 | 0.00001 |
| 26 | 3 | 3 | 2 | 1 | 2 | 1 | 3 | 1 | 3 | 2 | 3 | 2 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 27 | 3 | 3 | 2 | 1 | 3 | 2 | 1 | 2 | 1 | 3 | 1 | 3 | 2 | 0.04000 | 0.03000 | 0.04000 | 0.02500 |

**Table 2.** Factor Prothrombin Gene L27 Orthogonal Array Results

influential factors were employed to estimate errors, due to the fact that a saturated Taguchi design was employed.

| L27 Orthogonal Array and Replicates Results for Factor V Leiden | | | | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Run | A | B | C | D | E | F | G | H | J | K | L | M | N | Replicas | | | |
| | | | | | | | | | | | | | | 1 | 2 | 3 | 4 |
| 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 2 | 1 | 1 | 1 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 3 | 1 | 1 | 1 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 3 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 4 | 1 | 2 | 2 | 2 | 1 | 1 | 1 | 2 | 2 | 2 | 3 | 3 | 3 | 0.54000 | 0.46000 | 0.51500 | 0.48000 |
| 5 | 1 | 2 | 2 | 2 | 2 | 2 | 2 | 3 | 3 | 3 | 1 | 1 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 6 | 1 | 2 | 2 | 2 | 3 | 3 | 3 | 1 | 1 | 1 | 2 | 2 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 7 | 1 | 3 | 3 | 3 | 1 | 1 | 1 | 3 | 3 | 3 | 2 | 2 | 2 | 0.43000 | 0.43500 | 0.35500 | 0.39000 |
| 8 | 1 | 3 | 3 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 3 | 3 | 3 | 0.08000 | 0.10500 | 0.08500 | 0.07500 |
| 9 | 1 | 3 | 3 | 3 | 3 | 3 | 3 | 2 | 2 | 2 | 1 | 1 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 10 | 2 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 11 | 2 | 1 | 2 | 3 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 12 | 2 | 1 | 2 | 3 | 3 | 1 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 13 | 2 | 2 | 3 | 1 | 1 | 2 | 3 | 2 | 3 | 1 | 3 | 1 | 2 | 0.26000 | 0.29000 | 0.25000 | 0.24000 |
| 14 | 2 | 2 | 3 | 1 | 2 | 3 | 1 | 3 | 1 | 2 | 1 | 2 | 3 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 15 | 2 | 2 | 3 | 1 | 3 | 1 | 2 | 1 | 2 | 3 | 2 | 3 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 16 | 2 | 3 | 1 | 2 | 1 | 2 | 3 | 3 | 1 | 2 | 2 | 3 | 1 | 0.25500 | 0.25000 | 0.25500 | 0.24500 |
| 17 | 2 | 3 | 1 | 2 | 2 | 3 | 1 | 1 | 2 | 3 | 3 | 1 | 2 | 0.10000 | 0.10000 | 0.07500 | 0.06500 |
| 18 | 2 | 3 | 1 | 2 | 3 | 1 | 2 | 2 | 3 | 1 | 1 | 2 | 3 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 19 | 3 | 1 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 20 | 3 | 1 | 3 | 2 | 2 | 1 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 21 | 3 | 1 | 3 | 2 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 2 | 1 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 22 | 3 | 2 | 1 | 3 | 1 | 3 | 2 | 2 | 1 | 3 | 3 | 2 | 1 | 0.39500 | 0.43500 | 0.41000 | 0.42500 |
| 23 | 3 | 2 | 1 | 3 | 2 | 1 | 3 | 3 | 2 | 1 | 1 | 3 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 24 | 3 | 2 | 1 | 3 | 3 | 2 | 1 | 1 | 3 | 2 | 2 | 1 | 3 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |
| 25 | 3 | 3 | 2 | 1 | 1 | 3 | 2 | 3 | 2 | 1 | 2 | 1 | 3 | 0.55000 | 0.53500 | 0.53500 | 0.58000 |
| 26 | 3 | 3 | 2 | 1 | 2 | 1 | 3 | 1 | 3 | 2 | 3 | 2 | 1 | 0.15500 | 0.18000 | 0.14000 | 0.12500 |
| 27 | 3 | 3 | 2 | 1 | 3 | 2 | 1 | 2 | 1 | 3 | 1 | 3 | 2 | 0.00001 | 0.00001 | 0.00001 | 0.00001 |

**Table 3.** Factor V Leiden L27 Orthogonal Array Results

Therefore, error term was considered to have four degrees of freedom. Table 4 shows ANOVA results for output average value in experiments involving Prothrombin genotyping. The ANOVA analysis revealed which experimental factors were significant to the process output.

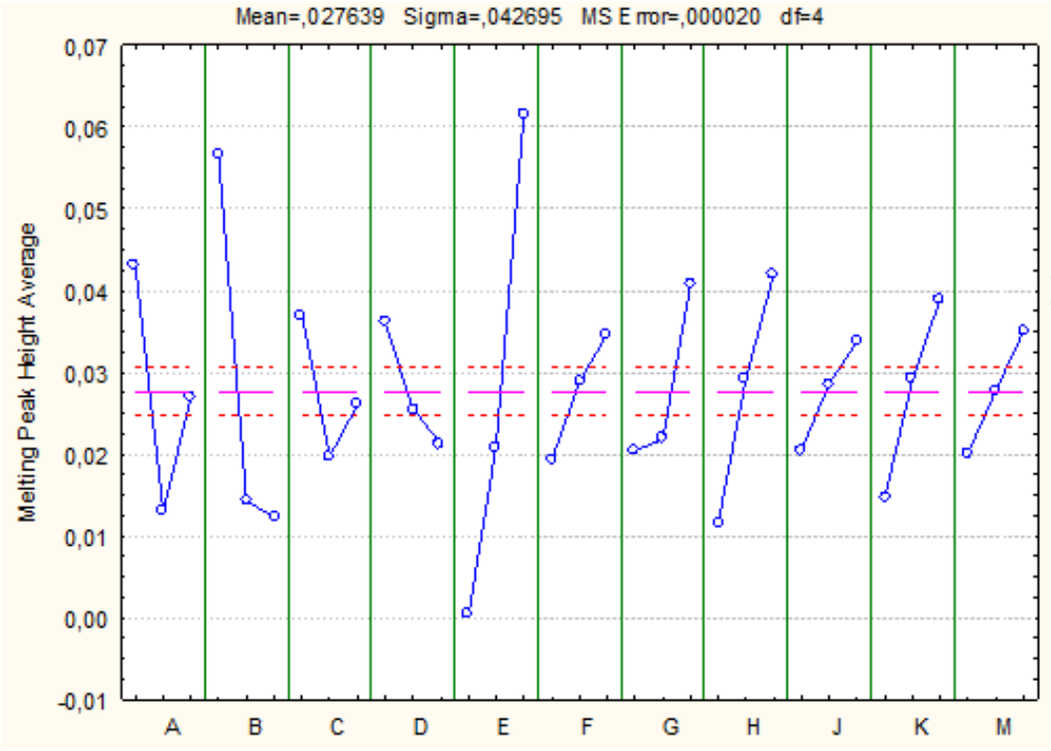


**Figure 2.** Means Main Effects for Prothrombin Genotyping Experiments

The decreasing order of significance for maximization of output for Prothrombin genotyping is as follows: E (concentration of primer reverse); B (concentration of primer forward); H, A (Master Mix), L (Number of PCR cycles), G, C, F, D, N and K (Anchor probe concentration).

By observing the interaction diagram of the L27 Taguchi method employed, it is evident that columns H, G, C, F, D and N contain information about interaction between physical factors. This suggests that interactions between the levels of physical factors significantly influence the outcome of the process. The levels of factors that will maximize the output for Prothrombin genotyping are displayed in the Table 5.

In order to estimate experimental conditions that maximize the robustness of the process, the ANOVA test was carried out on experimental results obtained for signal-to-noise ratio, as shown in Table 6.

For the same reasons presented before, square sums of the two less influential factors for robustness, C and D, were employed to estimate errors. Once more the number of degrees of the error was set to four, which corresponds to the sum of degrees of freedom of the factor employed to estimate the error term.

The ANOVA analysis also revealed the order of significance for maximizing the process's robustness. The decreasing order of significance is as follows: E (concentration of primer reverse); B (concentration of primer forward); A (Master Mix), H, L (Number of PCR cycles),

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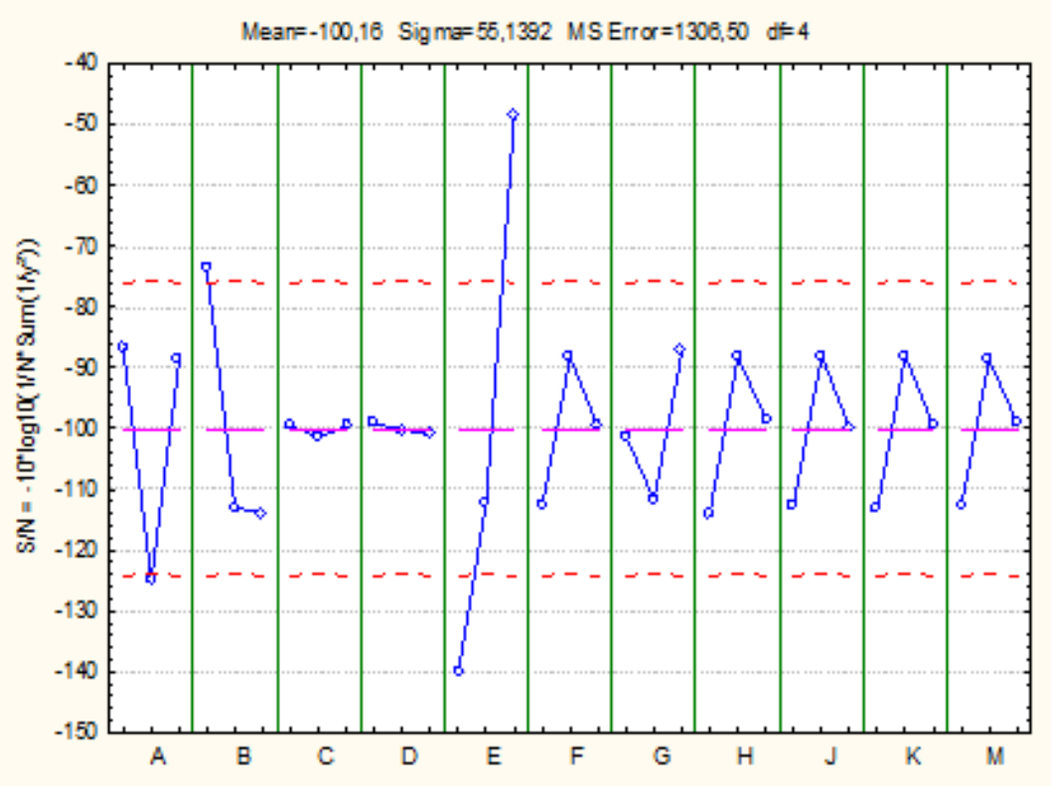


**Figure 3.** Signal-To-Noise Ratio Main Effects for Prothrombin Genotyping Experiments

K (Anchor probe concentration), G, J (FRET probe concentration), N, F and M. By observing the interaction diagram of the L27 Taguchi method employed, it's apparent that columns H, G, N, F and M contain information about interaction between physical factors. This suggests that interactions play a significant role in process robustness.

The levels of factors that will maximize robustness are also displayed in Table 5. For the factors Master Mix, Primer Forward Concentration, and Primer Reverse Concentration, let us compare their recommended levels for output optimization and robustness maximization. Levels that maximize Prothrombin genotyping output are the same as those that maximize process robustness. The remaining factors (Anchor probe concentration and number of PCR cycles) have distinct levels maximize output and robustness.

### 3.2. Results from factor V leiden analysis

Analysis performed on results obtained for Prothrombin genotyping were integrally repeated on results obtained for Factor V Leiden genotyping and the recommended values are shown in Table 7.

In this case, the experimental factors major impact results in descending order were E, B, L, H, A, F, C, N, J, M, D, G and K, varying from 61.49% to 0.41% respectively.

| Analysis of Variance on Melting Peak Height Average | | | | | | |
|---|---|---|---|---|---|---|
| **Mean =0 .027639** | | | | **Sigma =0 .042695** | | |
| Factors (S – Source) | SS Sum of Squares | df Degree of Freedom | MS Mean Square | F F-Ratio | P P-Value | Result |
| A | 0.004019 | 2 | 0.002010 | 101.5351 | 0.000 | Significant |
| B | 0.011178 | 2 | 0.005589 | 282.3947 | 0.000 | Significant |
| C | 0.001388 | 2 | 0.000694 | 35.0614 | 0.003 | Significant |
| D | 0.001052 | 2 | 0.000526 | 26.5877 | 0.005 | Significant |
| E | 0.017559 | 2 | 0.008779 | 443.5877 | 0.000 | Significant |
| F | 0.001091 | 2 | 0.000545 | 27.5526 | 0.005 | Significant |
| G | 0.002310 | 2 | 0.001155 | 58.3509 | 0.001 | Significant |
| H | 0.004239 | 2 | 0.002119 | 107.0877 | 0.000 | Significant |
| K | 0.000796 | 2 | 0.000398 | 20.1140 | 0.008 | Significant |
| L | 0.002690 | 2 | 0.001345 | 67.9561 | 0.001 | Significant |
| N | 0.000994 | 2 | 0.000497 | 25.1140 | 0.005 | Significant |
| Residual Error | 0.000079 | 4 | 0.000020 | | | |

**Table 4.** ANOVA table for output values for Prothrombin Genotyping Experiments

The maximization of output for coagulation Factor V Leiden, in descending order of significance, is as follows: E (Primer reverse Concentration); B (Primer forward Concentration) and L (Number of PCR cycles), H, A (Master Mix), F, C, G, N, J (FRET probe concentration).

The interaction diagram of the L27 Taguchi method reveals that columns H, F, C, G and N contain information about interaction between physical factors. This suggests that interactions between the levels of physical factors significantly influence the outcome of the process [11].

### 3.3. Confirmation experiments

Confirmation experiments were conducted using six samples, each of them in two replicates and are shown in the table 8. Also included were the factors adjusted to the recommended levels for process optimization and control samples.

| Factor Index | Factor Name | Taguchi Factor Levels recommended for output optmization | Physical Value linked to Factor Level | Taguchi Factor Levels recommended for robustness maximization | Physical Value linked to Factor Level | Unit |
|---|---|---|---|---|---|---|
| A | Master Mix | 1 | MMA | 1 | MMA | Composition /supplier |
| B | Primer forward Concentration (P1) | 1 | 0.1 | 1 | 0.1 | µM |
| E | Primer reverse Concentration (P2) | 3 | 1 | 3 | 1 | µM |
| J | FRET probe concentration (S1) | Not significant | Not significant | 1 | 0.2 | µM |
| K | Anchor probe concentration (S2) | 3 | 0.4 | 2 | 0.3 | µM |
| L | Number of PCR cycles | 3 | 85 | 2 | 65 | cycles |

**Table 5.** Factor levels recommended for output optimization or robustness maximization for Prothrombin Genotyping Experiments

The expected results are positive values and as much higher as possible to be considered good results for clinical significance and diagnostics qualitative analysis. The reproducibility is another important result for this application and the lowest standard deviation among replicates is also desirable which is also shown in the table 8. The results disclosed significant "responses," with values above zero. Such a finding clearly demonstrates that the recommendations about the conditions for the best process adjustments obtained by the Taguchi method meet the requirements and goals of this study.

A significant advantage to using the Taguchi method is the time and cost saved. Using the standard factorial design (or a non-formal method), will produce a much higher number of assays than will a fractional factorial as the Taguchi method uses. Extensively used to optimize engineering processes, the method incorporates one primary experiment to study the main effects of each factor, modeling some of the important interactions. Secondary Taguchi arrays can then be designed from the primary results, to narrow the optimal windows for each factor.

The method's strength lies in its Orthogonal Array design; each level of each factor occurs in an equal number of times across the entire array. Its potential savings are apparent when compared to factorial design. With the Taguchi method only 27 experiments were needed. For the same number of factors and levels examined, full factorial design requires 729 experiments [15, 16, 18].

| Analysis of Variance on S/N Ratio (Larger- the – Better) | | | | | | |
|---|---|---|---|---|---|---|
| **Mean = -100.16** | | | **Sigma = 55.1392** | | | |
| Factors (S - Source) | SS Sum of Squares | df Degree of Freedom | MS Mean Square | F F-Ratio | P P-Value | Result |
| A | 8271.62 | 2 | 4135.81 | 406.00 | 0.000 | Significant |
| B | 9470.42 | 2 | 4735.21 | 464.84 | 0.000 | Significant |
| E | 39531.31 | 2 | 19765.66 | 1940.33 | 0.000 | Significant |
| F | 2633.31 | 2 | 1316.65 | 129.25 | 0.000 | Significant |
| G | 2674.61 | 2 | 1337.31 | 131.28 | 0.000 | Significant |
| H | 3034.27 | 2 | 1517.13 | 148.93 | 0.000 | Significant |
| J | 2655.79 | 2 | 1327.89 | 130.35 | 0.000 | Significant |
| K | 2688.69 | 2 | 1344.34 | 131.97 | 0.000 | Significant |
| L | 2828.24 | 2 | 1414.12 | 138.82 | 0.000 | Significant |
| M | 2570.21 | 2 | 1285.10 | 126.15 | 0.000 | Significant |
| N | 2649.50 | 2 | 1324.75 | 130.05 | 0.000 | Significant |
| Residual Error | 40.75 | 4 | 10.19 | | | |

**Table 6.** ANOVA results for Signal-To-Noise ratios for Prothrombin Genotyping Experiments

This work suggests new studies of similar processes employing the full factorial DOE technique [16] or RSM - Response Surface Method [20] using the Taguchi method. Such studies should yield a better, more accurate estimation of the significance of experimental factors and interactions among factor levels on process outputs. It must be emphasized that the results obtained in this research should not be extrapolated to other clinical processes.

## 4. Conclusions

The most relevant genetic risk factors associated with thrombophilia are the Factor V Leiden and the 20210G>A point mutation in the Prothrombin gene. Employing the Taguchi method, the study successfully optimized a screening method for Prothrombin genotyping and for Factor V Leiden mutation detection. The Taguchi Experimental Design method [16] proved an efficient tool in determining the different levels that maximize the process output. Here that is defined as the melting peaks at the derivative plot (-dF/dT x T°C) and the relevance of factors obtained by Taguchi method.

Regarding Prothrombin genotyping, data analysis uncovered the most significant factors for maximizing the process's output and robustness. Those factors, in decreasing order, are: Primer Reverse Concentration, Primer Forward Concentration, Master Mix Type, and number of cycles in PCR. In addition, the adjustment levels for maximization of the process output are:

| Factor Index | Factor Name | Taguchi Factor Levels recommended for output optmization | Physical Value linked to Factor Level | Taguchi Factor Levels recommended for robustness maximization | Physical Value linked to Factor Level | Unit |
|---|---|---|---|---|---|---|
| A | Master Mix | 3 | MMC | 3 | MMC | Composition /supplier |
| B | Primer forward Concentration (P1) | 3 | 1 | 3 | 1 | μM |
| E | Primer reverse Concentration (P2) | 1 | 0.1 | 1 | 0.1 | μM |
| J | FRET probe concentration (S1) | 2 | 0.3 | Not Significant | Not Significant | μM |
| L | Number of PCR cycles | 3 | 85 | 3 | 85 | cycles |

**Table 7.** Factor levels recommended for output optimization or robustness maximization for Factor V Leiden Genotyping Experiments

primer reverse concentration = 1 μM, primer forward concentration = 0.1 μM, master mix type MMA (Promega PCR Master Mix) and number of PCR cycles = 85 cycles. FRET probe concentration was considered non-significant.

Data analysis for Factor V Leiden genotyping uncovered the most significant factors for maximizing the process's output. Those factors, in decreasing order, are: Primer Reverse Concentration, Primer Forward Concentration, Number of Cycles in the reaction, Master Mix Type and FRET probe concentration. The adjustment levels that lead to maximizing process output are: Primer Reverse Concentration = 0.1 μM, Primer Forward Concentration = 1 μM, number of cycles = 85, Master Mix Type MMC and FRET probe concentration equal to 0.2 μM. Anchor probe concentration was considered as non-significant.

The same kind of analysis was performed for process robustness for both Prothrombin genotyping and Factor V Leiden. The proper levels, as well as the recommended levels of the experimental factors, for maximizing robustness were pointed out and revealed as significant.

Analysis also showed that interactions among factor levels play a significant role in maximizing both process output and robustness for both Prothrombin genotyping and Factor V Leiden. The nature and intensity of this interaction should be further investigated. One other thing is worth mentioning. All reactions for Factor V Leiden and Prothrombin genotyping described in this paper were performed independently. However, multiplexing (simultaneously conducting both reactions in the same tube) may be enabled through the use of different fluorophores (FAM and JOE) for Factor V and Prothrombin probe labeling. This permits the operator to load more samples in the equipment. Usually, different conditions of an assay

| Run | Prothrombin (Factor II) | Standard Deviation | Fator V Leiden | Standard Deviation |
|---|---|---|---|---|
| 1 | 0.350 | 0.014 | 0.480 | 0.021 |
| 1 | 0.330 | | 0.450 | |
| 2 | 0.280 | 0.028 | 0.550 | 0.099 |
| 2 | 0.320 | | 0.690 | |
| 3 | 0.195 | 0.007 | 0.460 | 0.028 |
| 3 | 0.185 | | 0.500 | |
| 4 | 0.155 | 0.025 | x | x |
| 4 | 0.190 | | x | |
| 5 | x | x | 0.600 | 0.011 |
| 5 | x | | 0.615 | |
| 6 | 0.320 | 0.000 | 0.760 | 0.049 |
| 6 | 0.320 | | 0.830 | |
| am ctrl FII | 0.150 | 0.018 | x | x |
| am ctrl FII | 0.175 | | x | |
| am ctrl FV | x | x | 0.580 | 0.004 |
| am ctrl FV | x | | 0.585 | |

**Table 8.** Confirmation Results for Prothrombin and Factor V Leiden using the Taguchi recommended Factors Levels

optimization are tested independently, in different assays, in which all conditions are kept constant except for the one being tested.

This process is usually costly and time-consuming. Neither does it assure that all possible combinations are tested. Therefore, the Taguchi method offers several advantages over the traditional optimization process; it allows for the performing different experiments, testing different levels for each factor in just a few days. This saves and reduces the time needed for the complete optimization and standardization processes of new diagnostic tests. In addition, it provides a mathematical support not only for the choice of the condition that generates the best result but also for the determination of the significant factors in the reaction. Such a benefit allows researchers to eliminate the non-relevant experimental factors for posterior fine-scale adjustments using the full factorial DOE technique [16] or Response Surface Methodology [19], when necessary.

The DOE methods can also be applied to a variety of quantitative tests. In conclusion, in clinical analysis laboratories that develop in house diagnostic tests, especially in the R&D area, applying the Taguchi method is an alternative and efficient approach for fast, low-cost assays optimization. It is important to clarify that Taguchi Method will not supply the final diagnostics for the patient but allows the best and optimal clinical assays factors adjustments to support a subsequent qualitative analysis by the clinical technician.

## Acknowledgements

The authors are grateful to CAPES, Process PE024/2008 (Pro-Engineering Program - a Brazilian Government Program) and to Fleury Diagnostics.

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