The optimization of a laboratory protocol through Design of Experiment statistical methodology

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Abstract

The Design of Experiment (DoE) is a statistical methodology, very useful in the case of multivariable assays, that permits to evaluate simultaneously the influence of different factors on a specific output and to analyse the interactions among them in order to identify their optimal combinations. Moreover, DoE allows to select from the huge numbers of combinations only a limited number, to cover the whole frame, in order to save both time and money. DoE was mostly used in industrial field for maximizing robust processes, but recently it has been also applied in biomedical research field. Different studies have demonstrated the advantages of using a DoE approach compared to the classical one (a single parameter is tested in each assay) in the context of automated experiments, determination of cell media compositions [1] or HPLC tuning [2].

In the present study, we utilize for the first time the DoE methodology to optimize the transfection protocol of neural cells, as an example of DoE application to a laboratory procedure. Neural precursor cells are hard to transfect and refractory to lipidic reagents [3], for this reason we choose as transfectant reagent the cationic not-lipidic Poliethylenimine (PEI). The DoE approach allowed us to identify the main variables (factors) affecting the transfection efficiency and to discover their optimal combinations, developing a protocol that let us to triplicate the transfection efficiency compared to the initial conditions. Moreover, the covariance analysis unmasked significant variable interactions impossible to calculate in one factor-variation tests used in normal laboratory practices. Our results indicate that DoE might be very helpful also in research for the identification of the better experimental conditions and the analysis of interactions between different variables.

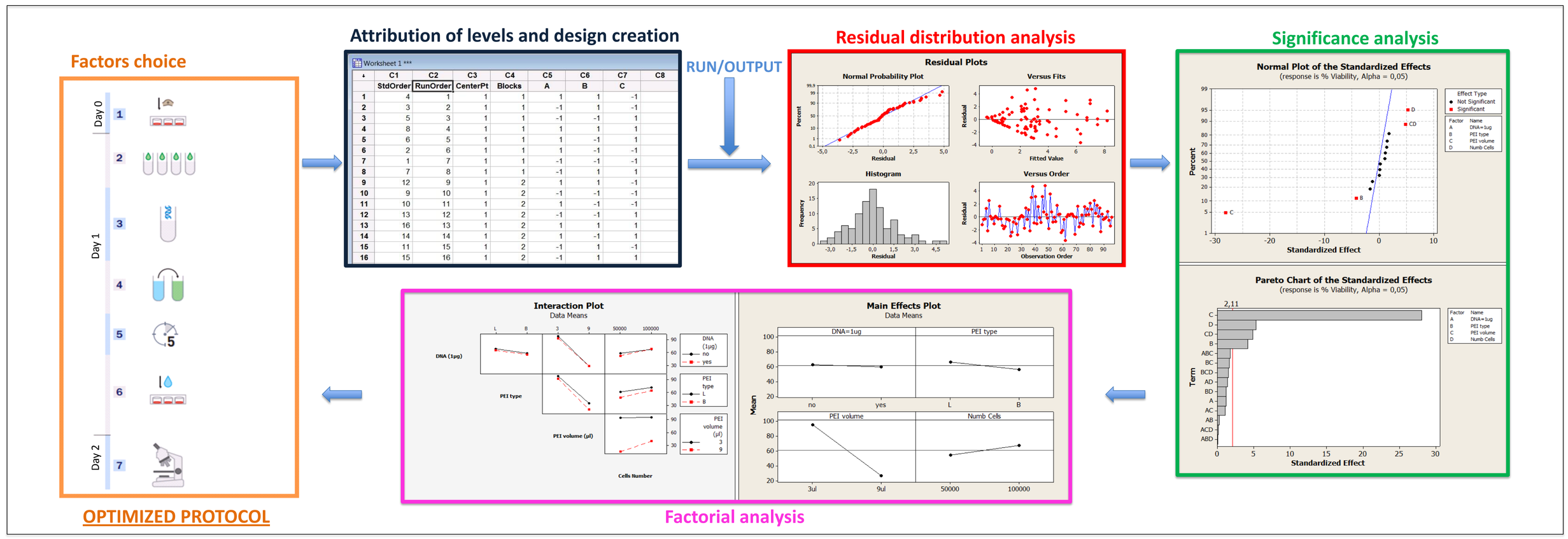

Figure 1. Experimental Scheme of DoE experiment for transient transfection protocol optimization. The DoE was used in these experiments as an essential tool to improve transfection efficiency of neural cell line, A1, using an home-made cationinc non-lipidic transfection reagent: Polyethylenimine, PEI. Minitab statistical software was used to create a design of experiment and analyze the results. The main steps of DoE experiments were: (i) choice of supposed main factors; (ii) attribution of adequate levels for each factor and design creation; (iii) run of the experiment and adequate output calculation; (iv) residual distribution analysis; (v) significance analysis; (vi) factorial analysis.

Output calculation: new ImageJ plug-in

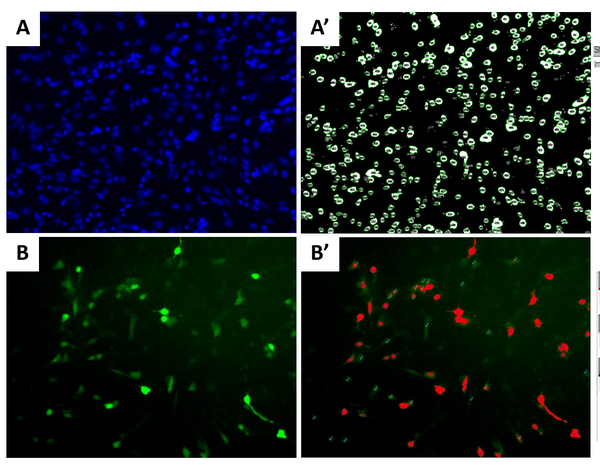

Figure 2. Transfection efficiency calculation trough ImageJ software. A plasmid containing the enhanced green fluorescent protein (EGFP) reporter, pIRES2-EGFP, was used for transfection. Cells expressing EGFP (transfected cells, B) were visualized directly by fluorescence microscope after PFA 4% fixation and Hoechst counterstaining (A); Image J java-based image processing program was used for images processing. In order to save time and avoid worker-dependent variability a new plug-in was created to automate and standardize cell counting of total cells (A-A''). EGFP$^+$ cells were impossible to count with the above mentioned plug-in because of the extreme variability of the fluorescence emitted by the cells, for this reason transfected cell were counted by summing the number of cells captured by the threshold (red cells, B') and the cells presenting a weak signal undetectable for the threshold calculated by the software (B-B'').

References

1. Alimperti S. et al., Biotechnol. Prog. (2014) 30, 4, 974-983.
2. Zheng H. et al 2013, Anal. Chem. (2013) 85, 7109-7116.
3. D. Karra, R. Dahm. J.Neurosci. (2010), 30,6171.

Screening DoE

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Table 1. Analysis of Variance for Transfection Efficiency (%)

Source	DF	SeqSS	AdjSS	AdjMS	F	P
Blocks	1	11,348	11,348	11,348	3,57	0,063
DNA	1	85,093	85,093	85,093	26,78	**0,000**
PEIvol	2	109,844	109,844	54,922	17,28	**0,000**
PEItype	1	9,858	9,858	9,858	3,1	0,082
Cellnumb	1	6,732	6,732	6,732	2,12	0,150
DNA*PEIvol	2	50,851	50,851	25,426	8	**0,001**
DNA*PEItype	1	4,302	4,302	4,302	1,35	0,248
DNA*Cellnumb	1	2,737	2,737	2,737	0,86	0,356
PEIvol*PEItype	2	142,799	142,799	71,4	22,47	**0,000**
PEIvol*Cellnumb	2	16,086	16,086	8,043	2,53	0,087
PEItype*Cellnumb	1	2,448	2,448	2,448	0,77	0,383
DNA*PEIvol*PEItype	2	10,471	10,471	5,236	1,65	0,200
DNA*PEIvol*Cellnumb	2	2,53	2,53	1,265	0,4	0,673
DNA*PEItype*Cellnumb	1	2,135	2,135	2,135	0,67	0,415
PEIvol*PEItype*Cellnumb	2	2,381	2,381	1,191	0,37	0,689
Error	73	231,963	231,963	3,178		
Total	95	691,578				

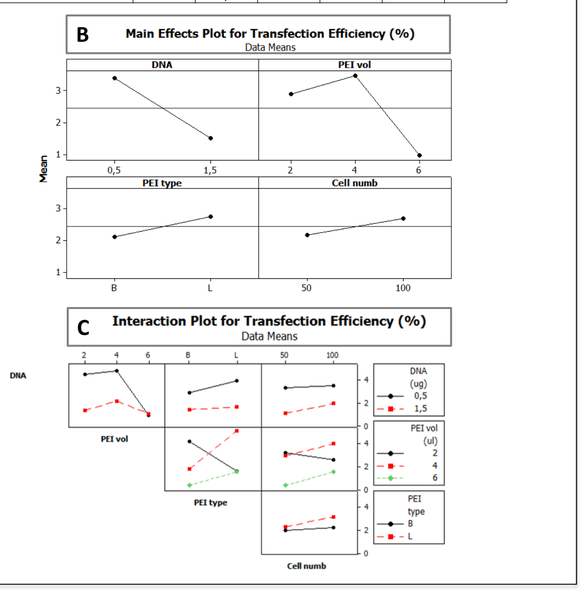

Figure 3. Screening of identified factors. *Analysis of variance*: column 'P' shows p-value calculated with ANOVA test (main factors and interactions are in red, **A**). *Main Effect plot* shows the effect (mean) of each factor: DNA amount ad PEI volume resulted to be the factors influencing the transfection efficiency output (**B**). *Interaction Plot* represents interaction between two factors (plot in which lines show a different trend): DNA*PEI volume and PEI type*PEI volume are the main interactions identified inside the interval analyzed in the experiment (**C**).

Optimization DoE

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Table 2. Analysis of Variance for Transfection Efficiency (%)

Source	DF	SeqSS	AdjSS	AdjMS	F	P
PEI Volume	2	12,308	12,308	6,154	4,34	**0,048**
Cell number	2	9,884	9,884	4,942	3,49	0,076
PEI Volume*Cell	4	61,884	61,884	15,471	10,92	**0,002**
Error	9	12,753	12,753	1,417		
Total	17	96,829				

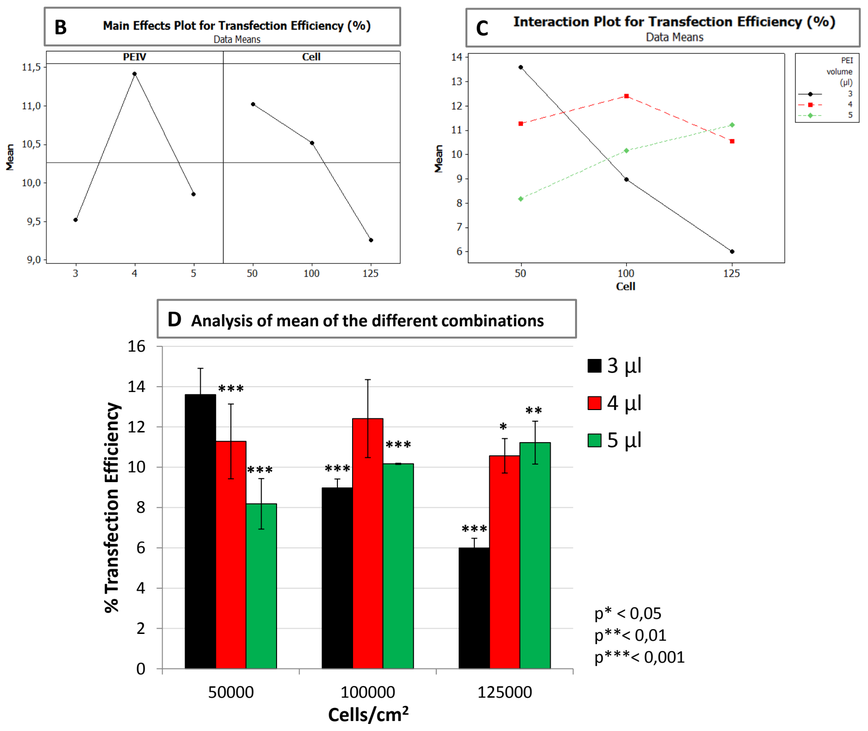

Figure 3. Optimization of the transfection protocol. The non influential factors and the ones already optimized have been fixed (DNA amount and PEI type), while the other factors have been changed to further optimize the protocol (PEI volume and Cell density). *Analysis of variance*: column 'P' shows p-value calculated with ANOVA test (main factors and interactions are in red, **A**). Levels corresponding to 4µl of PEI and 50.000 cells/cm^2, combined with fixed amount of DNA (0,5 µg) and PEI type (Linear), reached the highest transfection efficiency in our system (**B**). *Interaction Plot* (**C**) unmasks two different factor combinations that give a comparable transfection efficiency corresponding to PEI volume 3µl–cells/cm^2 50.000 and PEI volume 4µl–cells/cm^2 100.000 (**C**, **D**).