

Use of Statistical Design and Automation in Optimizing a Protein Tyrosine Phosphatase-1B Activity Assay

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ABSTRACT

Protein tyrosine phosphatase-1B is a potential therapeutic target for the treatment of type 2 diabetes. Analogs of this enzyme may inhibit deployment of the insulin receptor, and consequently, maintain it in an activated state leading to increased insulin sensitivity. To evaluate the activity of potential antagonists of this enzyme, a fluorescence-diphosphate-based activity assay was developed. Performance of this assay was variable; furthermore, both enzyme and substrate activity varied with buffer conditions. Therefore, the assay was reoptimized using the SigmapTM Automated Assay Optimization system on a Biomek i2000. Test buffer factors were evaluated in an iterative fashion for their effects on assay performance as assessed in terms of total activity, background activity, inhibition by antagonists, and signal-to-noise ratio. Enzyme and substrate concentrations were then optimized in the refined buffer system. Statistical design proved to be a powerful tool for efficient optimization of this assay, and automation greatly facilitated its application.

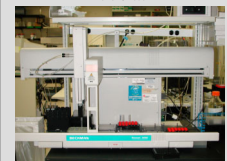
INTRODUCTION

Protein Tyrosine Phosphatase-1B (PTP-1B)

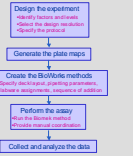
PTP-1B is a key modulator of insulin sensitivity; it phosphorylates the insulin receptor, and consequently, maintains it in an inactive state. Inhibitors of this enzyme may serve to maintain the insulin receptor in an activated state, and therefore, may have therapeutic potential in the treatment of type 2 diabetes. Efforts were underway to identify peptides which bind to PTP-1B and also to generate small molecule inhibitors using structure based design. Assays which had been developed to support this project include a fluorescence-diphosphate (FDP)-based activity assay, a peptide-based activity assay, an insulin receptor kinase receptor activation (IRKA) assay, and a fluorescence-polarization-peptide binding assay. In the FDP-based activity assay, we used optical variability in both the enzyme activity level and the level of inhibition by antagonists. Furthermore, the assay was very sensitive to buffer conditions. Therefore, we considered this assay a good candidate for reoptimization using the SigmapTM Automated Assay System.

SigmapTM Automated Assay Optimization (AAO)

AAO is an integrated system of hardware and software for optimizing assay conditions. It combines statistical design of experiments based on 2-level designs with the automated pipetting of the Biomek i2000 in a single-chamber liquid handling workstation. The software employs a step-by-step wizard-like interface to set up the experimental design and establish the protocol parameters for the Biomek i2000.



AAO involves 5 main steps:

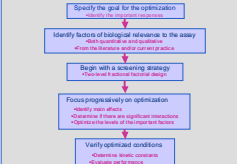


INTRODUCTION (continued)

Statistical Design of Experiments (DOE) in Assay Optimization

DOE facilitates assay optimization by allowing the examination of a large number of variables in an efficient manner. The use of statistics is critical in choosing appropriate design of the experiments and in interpreting the data.

An integrated strategy for assay optimization follows:



Two-Level Factorial Designs

Designs in which each factor is examined at 2 levels. **Screening**: experimentation and analysis. **Response**: Z' factor, where Z' is the number of factors. **Diagnose**: major trends. **Assess**: blind to curvature. **Call**: direct interactions. **Represent**: balanced designs. **Half**: of the experiments are run at each level of each factor.

Factorial Fractional Designs

Designs which are only a fraction of the experiments required for a full factorial design. **Allow**: to reduce the number of experiments. **Allow**: to distinguish the important factors from the trivial. **Factor**: Plots. **1/2**: denotes the level of fractionation. **Number**: of experiments = 2ⁿ. **Ability**: to detect interactions decreases as the extent of fractionation increases. **Design**: resolution refers to the ability to distinguish main effects from each other and from multi-factor interactions. **Not**: important to balance the ability to resolve interactions with the number of experiments required.

Main Effects

Main: variations in the response produced by individual factors. **Effect**: estimate: the difference between the means of the high level responses and the low level responses for a given factor.

Interactions

Result: when the response to the combination of two factors is not the expected sum of their individual effects. **Effect**: estimate: generally, decrease in magnitude as the number of factors involved increases, so two-factor interactions are the primary concern.

Sample Design Matrix: 2⁵ (One-Quarter Fraction)

Run	A	B	C	D	E
1	PEG	glycerol	NaCl	CHAPS	DTT
2	+	+	+	+	+
3	-	-	-	-	-
4	+	-	+	-	+
5	-	+	-	+	-
6	+	+	-	-	+
7	-	-	+	+	-
8	+	+	+	-	+

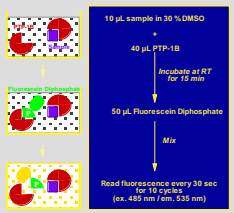
of Factors: 5
of Experiments: 8
Resolution: III
Design Generators: D = ABC, E = BC
Two-Factor Aliases: A-D, B-C, C-E, D-A, E-A, AB-CD, AC-BD

MATERIALS & METHODS

Key Reagents

Enzyme: PTP-1B, recombinant human, amino acids 1-321, produced in E. coli (Genentech)
Substrate: Fluorescein diphosphate (Molecular Probes)
PTP-1B Inhibitor: Suramin (Calbiochem)

Fluorescein Diphosphate-Based Activity Assay



The kinetic activity assay uses FDP as substrate. The assay protocol was modified slightly for use within the AAO system. A combination of manual and automated procedures were employed to facilitate the assay runs. The experiments were designed and randomized in AAO; this resulted in the creation of a BioWell's method for operating the Biomek i2000. Generally, these buffers were prepared on the Biomek i2000. Many mixtures of each of the test buffers were added to the appropriate wells of duplicate, polypropylene, 96-well bottom plates. One of these plates was used for dilution of substrate, 12.5 µL of 60% DMSO (total of background) or 50 µM suramin in 60% DMSO (standard) and 12.5 µL of enzyme (total of standard) or of buffer (background) were added to the wells of the first dilution plate, and 10 µL of fluorescein diphosphate were added to the wells of the second plate. The FDP concentration was constant, the FDP was added to the wells, manually with a multi-channel pipet. All other additions to these plates were made on the Biomek i2000. The contents of each plate were mixed gently by rotation with a multi-channel pipet.

The enzyme and sample (60% DMSO + suramin) were preincubated for a minimum of 15 minutes. To start the reaction, 50 µL of the enzyme sample solution followed by 50 µL of the diluted substrate were manually added to the corresponding wells of a black 96-well assay plate (Costar). The contents of the plates were mixed for 15 seconds on a rotating mixer, and the plates were read kinetically on a Victor V90 (excitation: 485 nm, emission: 535 nm; read every 30sec for a total of 60 cycles).

The following table summarizes the 5 experiments runs that were used to optimize the assay conditions:

Run #	1	2	3	4	5
# of Factors	10	5	3	3	2
# of Buffer Factors	10	5	3	3	0
# of Levels/Factor	2	2	2	2	3
# of Wells	192	192	192	162	150
# of Repeats	1	2	8	2	3
Design Resolution	IV	Full	Full	Full	Full
Protocol	T, S, B	T, S, B	T, S, B	T, S, B	T, S, B

The first four runs were used to optimize the buffer components, and the fifth run was used to optimize the concentrations of enzyme and substrate. Runs # 1 and 5 were designed in JMP 4.0 and imported into AAO. All runs include both T, S, B standard (5, 10 µM suramin) and background (0) wells. For runs # 1-4, enzyme and substrate concentrations were 2 nM and 40 µM, respectively. The data were processed in either AAO (runs # 1-3) or JMP 4.0 (runs # 4, 5). The responses for runs # 1-5 are displayed as Pareto plots which indicate direction and magnitude of main effects and/or two-factor interactions.

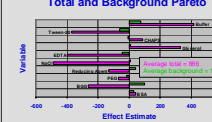
RESULTS & DISCUSSION

Assay Optimization Run #1: Buffer Component Screen

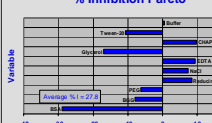
Goal: to screen a large number of buffer factors to identify those which are important for optimal activity and inhibition.

ID	Factor	-	+	Total
A	BSA	0	0.1	%
B	PEG	0	0.1	%
C	DTT	0	0.1	%
D	Substrate Agent	0	5	fold
E	NaCl	0	100	fold
F	EDTA	0	5	fold
G	Glycerol	0	5	%
H	CHAPS	0	0.03	%
I	Tween-20	0	0.02	%
J	Base Buffer	470 µM T, S, B	100 µM T, S, B	4.6

Total and Background Pareto



% Inhibition Pareto



Buffer Factors to Eliminate from Further Consideration

BSA, Minimal effect on signal response; strong negative effect on % inhibition.

DTT, Reduced the total signal, increased the background signal, and reduced % inhibition.

NaCl, DTT, Tween-20, Very strong negative effects on total signal.

Buffer Factors for Further Consideration

PEG, Slightly reduced total signal, background signal, and % inhibition; the least negative of the bulkings agents with respect to total and background signal but not % inhibition.

Repeating Agent, DTT was insufficient to S.M.E. with respect to total and background signal but not % inhibition.

CHAPS, Positive effects on both total signal and % inhibition.

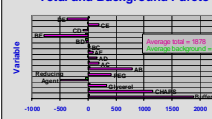
Base buffer, Surinate had a strong positive effect on total signal; little effect of base buffer on % inhibition.

Run #2: Buffer Component Optimization

Goal: to more fully characterize the main effects and interactions of the important buffer factors from an #1 in attempt to minimize total signal and percent inhibition.

ID	Factor	-	+	Total
A	BSA	0	0.03	%
B	CHAPS	0	5	%
C	Glycerol	0	5	%
D	DTT	0	0.02	%
E	PEG	0	0.1	%

Total and Background Pareto

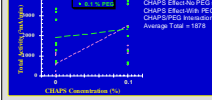


Effect Estimate

Surinate, CHAPS, glycerol, DTT, and PEG each have positive effects on assay signal. Nevertheless, interaction between factors need also be considered. There is a strong positive interaction between the buffer and CHAPS (A); however more importantly, there is a strong negative interaction between CHAPS and PEG (B).

Surinate and DTT were selected over Tween and S.M.E. for further studies.

Negative CHAPS*PEG Interaction



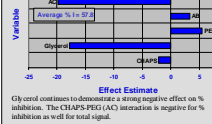
Run #3: Confirmed Buffer Component Optimization

Goal: to further evaluate the effects of CHAPS, glycerol, PEG, and the CHAPS-PEG interaction on total signal, % inhibition, and precision (n=8).

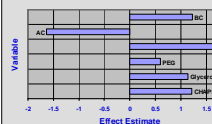
ID	Factor	-	+	Total
A	CHAPS	0	0.03	%
B	Glycerol	0	5	%
C	PEG	0	0.1	%

Base buffer contains 50 mM sucrose/0.6% CHAPS, 2 mM DTT, pH 6.

% Inhibition Pareto



SignalNoiseRatio Pareto



Run #4: 3-Level Buffer Factor Optimization

Goal: to use a 3-level design to determine whether intermediate concentrations of CHAPS, PEG, and/or glycerol may lead to more optimal responses.

Factor	1	2	3	4	5
CHAPS	0	0.01	0.03	%	
PEG	0	0.01	0.1	%	
Glycerol	0	1	2	%	

CHAPS	PEG	Glycerol	Total	Standard	Background	T/S
0.03	0	0	3311	1231	594	5.8
0.01	0	0	9551	1133	241	19.1
0	0.1	1	1047	1072	245	12.9
0.01	0.01	0	3824	927	429	11.5
0.01	0.01	1	3881	1338	278	24.5

The conditions resulting in the best set of responses in the previous run were 0.03% CHAPS without PEG or glycerol. The results from these conditions for this run are shown in gray. The results of the four other conditions yielding the best responses for this run are also included in the above table. All of these four conditions contain at least one of the factors at its intermediate level. The set of conditions yielding the best of responses is shown in gray. It corresponds to 0.01% CHAPS and demonstrates improvement across the board as compared to 0.03% CHAPS.

Run #5: Enzyme and Substrate Concentration Optimization

Goal: to use a 5-level design to optimize enzyme and substrate concentrations to yield the best combination of total activity and percent inhibition.

Enzyme Concentration (nM)	1	2	3	4	5
0	0	0.5	1	2	4
Substrate Concentration (µM)	0	20	40	60	80

Optimized Buffer: 50 mM sucrose, 0.6% CHAPS, 2 mM DTT, pH 6

Optimized Conditions

50 mM sucrose, 0.01% CHAPS, 2 mM DTT, pH 6

4 nM enzyme with 30 µM substrate is more favorable across the board: higher total activity, lower background, greater % inhibition, greater T/S, greater SN, and greater Z'-factor.

Optimized Conditions: 50 mM sucrose, 0.01% CHAPS, 2 mM DTT, pH 6

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Several sets of suramin titration curves were run at 2 nM enzyme in combination with 20 (0) µM substrate (1 - 2x the Km). The IC₅₀