Automated Enzyme Assay Design and Optimization

James D. Batchelor, Ph.D., Laurel M. Provencher, Ph.D., Abbie L. Esterman, Ph.D., and Seth P. Cohen, Ph.D. Caliper Life Sciences, Hopkinton, MA 01748

Abstract

Optimizing enzymatic assays involves determining the right buffer, detergent, salt, and cation combination. The process of finding the best conditions is very tedious, requiring many individual experiments to test each combination of the reagents. Design of Experiment software can help eliminate some of the experiments by selecting reagent combinations that will result in optimal conditions, while at the same time, check for interferences between reagents. Several mobility-shift kinase assays were selected to evaluate the utility of this approach for assay development using a 384-well microtiter plate format. Six different buffer conditions, 5 different detergents, several salt and cation concentrations were chosen as variables. The Caliper Sciclone 3000 Liquid Handler was used to prepare the 384 different conditions created in the DesignExpert design of experiment software. The subsequent addition of the substrate and enzyme to the plate, incubation and termination of the reaction by the addition of EDTA, was completed on the Caliper Sciclone 3000. The amount of product formed was determined using the LabChip 3000. Data, from the LabChip 3000 system was entered into the Design of Experiment software and analyzed. Interactions between the various buffer components were determined and the optimal conditions selected. The presence of different detergents and their interactions with different buffers had a profound effect on the enzyme turnover. The results from these experiments will be discussed.

Materials

- Caliper Sciclone ALH 3000 equipped with Z-8, Low Volume Head and 96-Mandrel Array
- LabChip 3000
- Stat-Ease Design Expert Design of Experiment Software
- Corning #3656 384-well plates
- MJ Research MSP-9601 96-well PCR plates
- Innovative Microplates S30018 and S30028 reservoirs
- 100 µL 96 format tips (Caliper #109079)
- 25 µL 384 format tips (Caliper #108849)

Caliper LabChip 3000

Methods

- Design Expert was used to create a D-optimized Factorial design of experiment (DoE) table. (Table 1)
- The Sciclone deck was populated as shown. (Figure 1) Each reagent was placed in a column of one of the three 12-column reservoirs. Water was placed in an open reservoir for volume normalization.
- The Sciclone application launched a custom user interface to map each reagent of the DoE table to locations on the deck.
- The Sciclone Z-8 stepped through the DoE table, distributing reagents to the appropriate wells of a 384-well plate. Water was used to bring the total volume of each well to 60 µL. Enzyme (5 µL) was then distributed using the Z-8. To initiate the reaction the main head was used to stamp and mix peptide/ATP (15 µL) in the assay plate. After incubation, the reaction was stopped by adding 5 µL of a 400 mM EDTA stock solution using the main head giving a final volume of 80 µL.
- The plates were analyzed by the LC3000 off-chip mobility shift assay format using a 12-sipper chip and the appropriate separation for each peptide. (Table 2)
- From the LC3000 data the ratio of product/product + substrate (P/P+S) was imported back into the Design Expert spreadsheet for analysis.

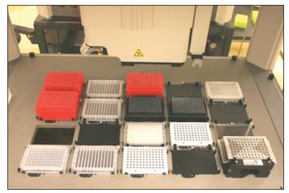

Figure 1. Sciclone Deck Layout

Factors	Levels (Values in parentheses are labels used in the design of experiment software)					
BSA	**0 mg/ml** 8 µL water (0)	**0.1 mg/ml** 8 µL 1mg/mL stock (8)				
NaCl	**0 mM NaCl** 8 µL water (None)	**25 mM NaCl** 8 µL 0.25 M stock (Lo)	**100 mM NaCl** 8 µL 1 M stock (Hi)			
Cation	**5 mM $MgCl_2$** 8 µL 50 mM stock (LoMg)	**20 mM $MgCl_2$** 8 µL 200 mM stock (HiMg)	**20 mM $MnCl_2$** 8 µL 200 mM stock (HiMn)			
Detergent	**0.004% Triton X-100** 8 µL 0,04% stock (Triton)	**0.002% Brij-35** 8 µL 0,02% stock (Brij)	**0.002% Tween-20** 8 µL 0,02% stock (Tween)	**0.15% CHAPS** 8 µL 1.5% stock (CHAPS)		
Buffer	**50 mM HEPES pH 7.0** 8 µL 0.5 M stock (HEPES7.0)	**50 mM HEPES pH 7.5** 8 µL 0,5 M stock (HEPES7.5)	**50 mM HEPES pH 8.0** 8 µL 0,5 M stock (HEPES8.0)	**50 mM MOPS pH 6.5** 8 µL 0,5 M stock (MOPS6.5)	**50 mM MOPS pH 7.0** 8 µL 0,5 M stock (MOPS7.0)	**50 mM MOPS pH 7.5** 8 µL 0,5 M stock (MOPS7.5)

Table 1. Experiment Design Table

Results

KDR

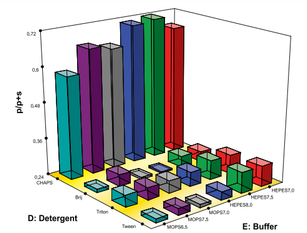

Figure 2a. Buffer vs Detergent. BSA=0, NaCl=0, Mg=20 mM

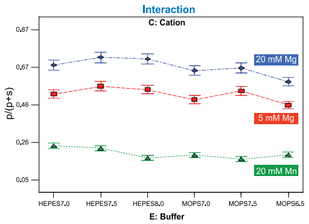

Figure 2b. Buffer vs Cation. BSA=0, NaCl=0, Detergent=CHAPS

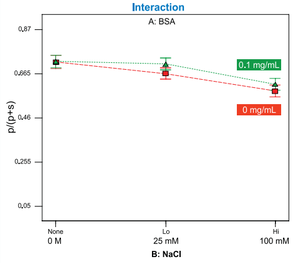

Figure 2c. BSA vs. NaCl. Mg=20 mM, Detergent=CHAPS, Buffer=HEPES pH7.5

AuroraB

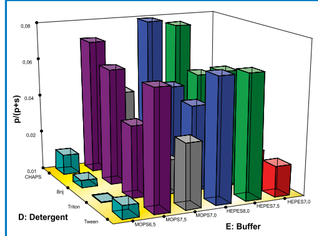

Figure 3a. Buffer vs Detergent, BSA=0,1 mg/mL, NaCl=0, Mg=20 mM

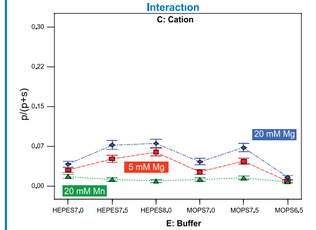

Figure 3b. Buffer vs Cation. BSA=0,1 mg/mL, NaCl=0, Detergent=CHAPS

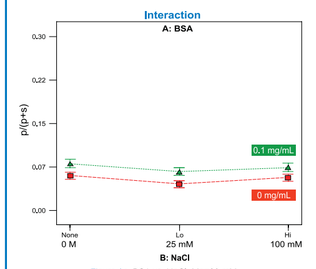

Figure 3c. BSA vs. NaCl. Mg=20 mM, Detergent=CHAPS, Buffer=HEPES pH8.0

ERK2

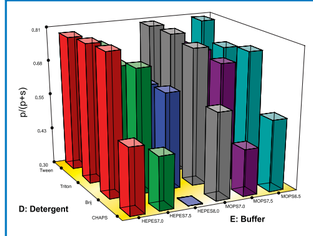

Figure 4a. Buffer vs Detergent, BSA=0, NaCl=0, Mg=20 mM

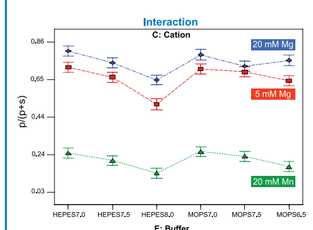

Figure 4b. Buffer vs Cation. BSA=0, NaCl=0, Detergent=Brij

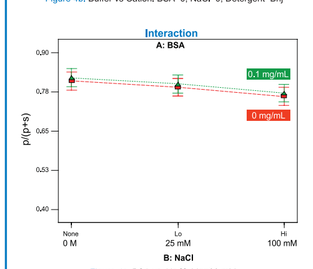

Figure 4c. BSA vs. NaCl. Mg=20 mM, Detergent=Brij, Buffer=HEPES pH7.0

insR

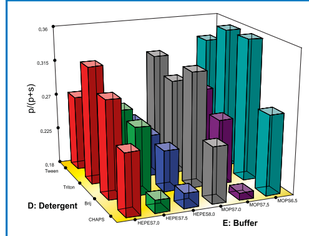

Figure 5a. Buffer vs Detergent, BSA=0, NaCl=0, Mn=20 mM

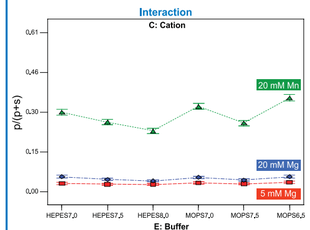

Figure 5b. Buffer vs Cation, BSA=0, NaCl=0, Detergent=Brij

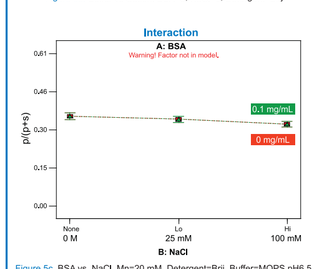

Figure 5c. BSA vs. NaCl. Mn=20 mM, Detergent=Brij, Buffer=MOPS pH6.5
Note: BSA was removed from the model because of its lack of significance

PKA

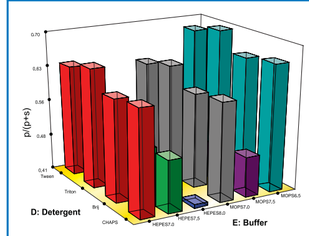

Figure 6a. Buffer vs Detergent, BSA=0,1 mg/mL, NaCl=0, Mg=5 mM

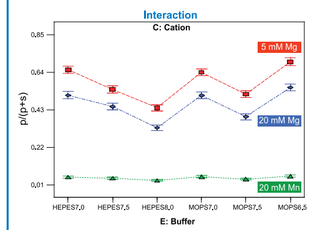

Figure 6b. Buffer vs Cation. BSA=0,1 mg/mL, NaCl=0, Detergent=Triton

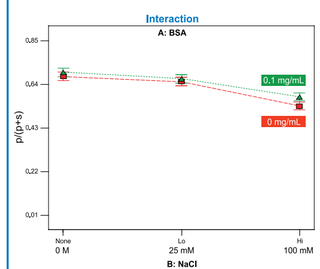

Figure 6c. BSA vs. NaCl. Mg=5 mM, Detergent=Triton, Buffer=MOPS pH6.5

Conclusions

	Peptide	BSA mg/mL	NaCl mM	Cation mM	Detergent	Buffer
AuroraB	5-FAM-**LRRASLG**-$CONH_2$	0,1 (8)	0	20 (HiMg)	CHAPS	HEPES 8.0
PKA	5-FAM-**LRRASLG**-$CONH_2$	0.1 (8)	0	5 (LoMg)	Triton	MOPS 6.5
ERK2	5-FAM-**IPTSPITTTYFFFKKK**-COOH	0**	0	20 (HiMg)	Brij	HEPES 7.0
insR	5-FAM-**KKSRGDYMTMQIG**-$CONH_2$	0**	0	20 (HiMn)	Brij	MOPS 6.5
KDR-1	5-FAM-**EEPLYWSFPAKKK**-$CONH_2$	0**	0	20 (HiMg)	CHAPS	HEPES 7.5
KDR-2*	5-FAM-**EEPLYWSFPAKKK**-$CONH_2$	0**	0	20 (HiMg)	CHAPS	HEPES 7.5
KDR-3*	5-FAM-**EEPLYWSFPAKKK**-$CONH_2$	0**	0	20 (HiMg)	CHAPS	HEPES 7.5

Table 2. Optimized Conditions

* KDR-2 was a replicate on a different day, KDR-3 was a replicate using a different design table run order
** BSA was found to not have a significant influence

- *Design of experiment software allows experiments to be built and modeled virtually prior to benchwork*
- *The Sciclone ALH3000 prepares 384 unique reactions in about 2.5 hours*
- *2-3 reagent plates can be prepared in an 8-hour day*
- *Reagent plates are stable for 24 hours when sealed and stored at 4°C*
- *Local and global maxima are easily identified, allowing optimization of reactions while meeting specific criteria*
- *Unexpected interactions between reagents are revealed*
- *Use of the optimization platform allows rapid development of mobility-shift kinase assays*
- *The process is easily adaptable to a plethora of assay formats and technologies*

Caliper LifeSciences