Evaluation of plate edge effects in in-vitro cell based assay

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Abstract- Invitro cell-based assays are widely used in the biopharma industry to determine the relative potency of drug substance and drug product. As bioassays are performed on 96 well plate formats, one of the fundamental and experimental issues is edge effects. Abnormal cell response curve is observed when wells are located at the edge of the plate. In this paper, an experiment was performed to evaluate the edge effects of the 96 well plates. The experiment results were used to confirm the suitable layout of the 96 well plates. This framework provides a systematic approach of sample location in 96 well plate formats.
Keywords – Relative potency, 96 well plates, cell response

I. INTRODUCTION

To evaluate the biopharmaceuticals, animal tests are generally adopted by the Globally Harmonized System (GHS) classification and human health risk assessments. [1, 2] However, the cost of animal experiments is high and the understanding of the mechanism of action remains limited. Currently, regulatory guidelines also considered the testing of drugs on animals unethical and advised drug manufacturing organizations to switch for cell based assays. Cell-based in vitro assay is an alternative to animal testing in safety/hazard assessments.

A popular technique in cell-based in vitro assay is the plate-based techniques, which provide a reliable assessment for sample relative potency in comparison to the reference standard. The cell response throughout an experiment leads to the determination of the potency of the drug. It has been widely used in many different research fields, such as drug discovery, and toxicology investigations. [8-12] However, there are many practical challenges concerning the variability of the assay due to many factors involved such as the variable response of the cells, cell growth, and cell passage. One of the major challenges is concerning to the plate positions effects called as edge effects during the course of long experimentation.[7] One goal during development is to find operating conditions for the assay so that cell response to the analyte is free of systematic effects across the plate. These gradients may occur across rows, across columns, or from the edge to the center of the plate and are termed as plate effects [13]

In the experiment, the cellular response in the edge well of the plate is usually different from that of the inner well, especially in the plate ×96 formats, which is described as the edge effect. There are two main reasons why the edge effect appears: one reason is that the evaporation of long-term incubation causes the edge effect (evaporation effect), because the evaporation efficiency of water in the edge well is higher than that in the inner well; and the other reason is that the temperature of the edge well reaches the desired incubation temperature faster than that of the inner well owing to plate stacking (temperature effect) [13].To evaluate the edge effect, the whole plate was filled with the cells only (no drug compound): each well filled with 20000 cells in suspension in complete culture medium and a plate effect is observed.

The rest of the paper is organized as follows. Material and Methods are explained in section II. Experimental results are presented in section III. Concluding remarks are given in section IV.

II. MATERIAL AND METHODS

2.1 Problem statement –

In this study, the Plate × 96 well formats were selected as the experimental equipment and the schematic diagram of a typical 96 well format is shown in Figure 1. The basic principle of this experiment is to evaluate and ascertain the edge effect by monitoring the cell population changes at different location of the plate and monitor the cell number indirectly by detection dye termed as Alamar blue

12 × 8 = 96 well format												
	1	2	3	4	5	6	7	8	9	10	11	12
A	A1	A2	A3	A4	A5	A6	A7	A8	A9	A10	A11	A12

B	B1	B2	B3	B4	B5	B6	B7	B8	B9	B10	B11	B12
C	C1	C2	C3	C4	C5	C6	C7	C8	C9	C10	C11	C12
D	D1	D2	D3	D4	D5	D6	D7	D8	D9	D10	D11	D12
E	E1	E2	E3	E4	E5	E6	E7	E8	E9	E10	E11	E12
F	F1	F2	F3	F4	F5	F6	F7	F8	F9	F10	F11	F12
G	G1	G2	G3	G4	G5	G6	G7	G8	G9	G10	G11	G12
H	H1	H2	H3	H4	H5	H6	H7	H8	H9	H10	H11	H12

Figure 1. 96 well format

2.2 Experiment design –

As shown in Figure 2d to evaluate the edge well effect, The procedure of the assay is as follows: As shown in Figure 2b, a certain cell line of 20,000 cells/well was added to each well of the × 96 -plate and incubated in the culture medium about 24 h; second, compounds with different concentrations were added to the plate, highest from the top and bottom and then plate was incubated for 72 h. After Incubation plate was taken After 70 ± 2 hrs of incubation, 30 µL of pre-warmed Alamar blue was added to all the wells, shaken for 5 min at 300 rpm on plate shaker for proper mixing and incubated the plate for 17± 3 hrs at 37 °C and 5% CO2 in a humidified CO2 incubator. The plate was removed from the incubator, cooled to room temperature by shaking on a plate shaker at 300 rpm for 30 minutes. Plate was bottom read at excitation 530 nm and emission 590 nm in fluorescence mode using micro plate reader. Raw data was analyzed using SoftMax Pro software with 4 parametric logistics. Fluorescence raw data value is directly proportional to the cell proliferation index as shown in Figure.3c

As shown in Figure.3d, the observed fluorescence raw data values were lesser at edge wells compared to the inner wells; this was named as the edge effect.

To further ascertain the plate effect as shown in Figure 2c, 96 well plates was divided in zone 3, zone 1 was kept at the vertical left side of the plate; Zone 2 is exposed at the horizontal top of the plate. While zone 3 is considered as inner wells and also considered as a reference standard to evaluate the differences between the three zones.

As shown in Figure.3c and 3d, the time-dependent cellular response curves (TCRCs) of zone 1 and zone 2 (edge wells) were significantly different from the TCRCs of inner wells. The raw fluorescence values were observed to be very less from A1 to A12 while the same type of effect was observed in column 1. This confirms the edge effect, Also the % CV was observed to be more than 20 %, sequentially A1 to 12 wells values were lesser than Row B 1-12 and C1-12 values, this was named the edge effect. While at column 1 also observed to lesser value compared to 2 and 3 columns. To describe the differences, the standard deviations and % CV was taken as shown in Figure 4.The mean NCIs of the inner wells were set as standard.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Edge wells											
B	Edge wells	INNER WELLS OF THE PLATE										Edge wells
C												
D												
E												
G												
H	Edge wells											

(a)

	1	2	3	4	5	6	7	8	9	10	11	12
A	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well
B	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well
C	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well
D	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well

E	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well
F	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well
G	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well
H	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well	20K c/well

(b)

	1	2	3	4	5	6	7	8	9	10	11	12
A	01	Zone-1	01	01	02	03	04	Zone-2	06	07	08	Blank
B	02	02	02	01	02	03	04	05	06	07	08	01
C	03	03	03	01	02	03	04	05	06	07	08	01
D	04	04	04	08	07	06	05	Zone-3	03	02	01	01
E	05	05	05	08	07	06	05	04	03	02	01	01
F	06	06	06	08	07	06	05	04	03	02	01	01
G	07	07	07	01	01	01	01	01	01	01	01	01
H	08	08	08	01	01	01	01	01	01	01	01	01

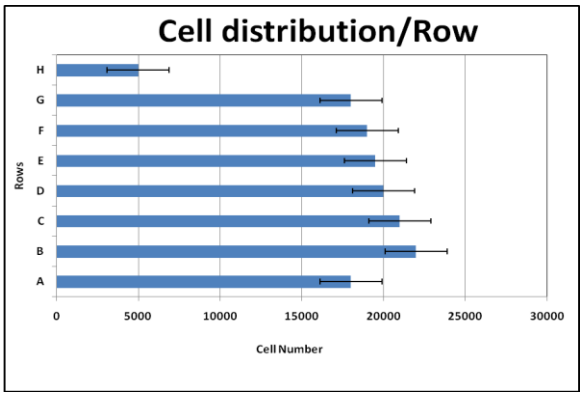
(C)

Figure 2. (a) Plate showing Edge wells (b) Plate design showing 20K cells/well (c) Plate design to evaluate edge effects

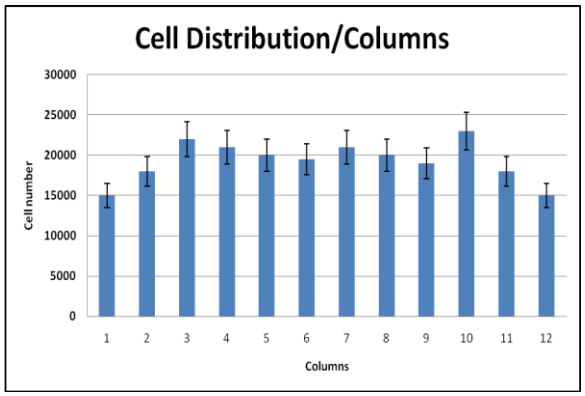
III. EXPERIMENT AND RESULT

3.1 Results:

The bar graph for the cell distribution/Row and columns is as shown in figures 3 and 4. It is evident that the cell recovery at H and A row were lesser than B to G. with this, it can be confirmed that there is an edge effect at the highest and lowest rows of the plates. Similarly, it has also been observed that column 1 and column 12 were observed to be lesser cell values compared to inner columns of the plate. Therefore it is regarded as a confirmed edge effect at all four corners of the plates as shown below in Figures 3a and 3b.



(a)



(b)

Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.4e4	5.6e4	5.2e4	4.9e4	2.5e4	1.3e4	6705.0	3540.0	986.00	432.00	276.00	178.00
B	2.5e4	7.3e4	7.6e4	7.0e4	3.5e4	1.8e4	8970.0	4765.0	1987.0	1076.0	567.00	190.00
C	1.3e4	3.7e4	3.7e4	7.4e4	3.7e4	1.9e4	9876.0	4276.0	1876.0	987.00	760.00	186.00
D	6075.0	1.9e4	1.7e4	789.00	952.00	2507.0	4789.0	1.1e4	2.0e4	3.6e4	7.3e4	175.00
E	4050.0	9493.5	9874.0	675.00	1086.0	1900.0	3987.0	9987.0	2.0e4	3.6e4	7.2e4	185.00
F	3564.0	4746.8	3789.0	766.00	1034.0	2376.0	4652.0	1.1e4	1.7e4	3.0e4	7.6e4	174.00
G	942.00	1876.0	1879.0	253.00	220.00	240.00	240.00	254.00	276.00	290.00	300.00	145.00
H	300.00	938.00	1476.0	190.00	187.00	167.00	185.00	187.00	174.00	179.00	174.00	147.00

(c)

Plate1

	1	2	3	4	5	6	7	8	9	10	11	12
A	2.4e4	5.6e4	5.2e4	4.9e4	2.5e4	1.3e4	6705.0	3540.0	986.00	432.00	276.00	178.00
B	2.5e4	7.3e4	7.6e4	7.0e4	3.5e4	1.8e4	8970.0	4765.0	1987.0	1076.0	567.00	190.00
C	1.3e4	3.7e4	3.7e4	7.4e4	3.7e4	1.9e4	9876.0	4276.0	1876.0	987.00	760.00	186.00
D	6075.0	1.9e4	1.7e4	789.00	952.00	2507.0	4789.0	1.1e4	2.0e4	3.6e4	7.3e4	175.00
E	4050.0	9493.5	9874.0	675.00	1086.0	1900.0	3987.0	9987.0	2.0e4	3.6e4	7.2e4	185.00
F	3564.0	4746.8	3789.0	766.00	1034.0	2376.0	4652.0	1.1e4	1.7e4	3.0e4	7.6e4	174.00
G	942.00	1876.0	1879.0	253.00	220.00	240.00	240.00	254.00	276.00	290.00	300.00	145.00
H	300.00	938.00	1476.0	190.00	187.00	167.00	185.00	187.00	174.00	179.00	174.00	147.00

(d)

Figure 3. (a) Cell distribution rows wise (b) Cell distribution column wise (c) Plate raw data (d) Plate masked data

Zone-1

Sample	Well	Concentration	Values	MeanValue	Std.Dev.	CV%
01	A1	400.000	23762.000	44124.667	17739.668	40
	A2		56234.000			
	A3		52378.000			
02	B1	200.000	25462.000	58225.667	28406.479	49
	B2		73253.000			
	B3		75962.000			
03	C1	100.000	12731.000	28634.833	13773.181	48
	C2		36626.500			
	C3		36547.000			
04	D1	50.000	6075.000	13938.667	6901.052	50
	D2		18987.000			
	D3		16754.000			
05	E1	25.000	4050.000	7805.833	3258.206	42
	E2		9493.500			
	E3		9874.000			
06	F1	12.500	3564.000	4033.267	628.095	16
	F2		4746.800			
	F3		3789.000			
07	G1	6.250	942.000	1565.667	540.113	34
	G2		1876.000			
	G3		1879.000			
08	H1	3.125	300.000	904.667	588.708	65
	H2		938.000			
	H3		1476.000			

(a)

Zone-1

Sample	Well	Concentration	Values	MeanValue	Std.Dev.	CV%
01	A1	400.000	Masked	54306.000	2726.604	5
	A2		56234.000			
	A3		52378.000			
02	B1	200.000	Masked	74607.500	1915.552	3
	B2		73253.000			
	B3		75962.000			
03	C1	100.000	Masked	36586.750	56.215	0
	C2		36626.500			
	C3		36547.000			
04	D1	50.000	Masked	17870.500	1578.969	9
	D2		18987.000			
	D3		16754.000			
05	E1	25.000	Masked	9683.750	269.054	3
	E2		9493.500			
	E3		9874.000			
06	F1	12.500	Masked	4267.900	677.267	16
	F2		4746.800			
	F3		3789.000			
07	G1	6.250	Masked	1877.500	2.121	0
	G2		1876.000			
	G3		1879.000			
08	H1	3.125	Masked	Masked	Masked	Masked
	H2		Masked			
	H3		Masked			

(b)

Zone-2

Sample	Well	Concentration	Values	MeanValue	Std.Dev.	CV%
01	A4	400.000	48769.000	64057.333	13368.086	21
	B4		69856.000			
	C4		73547.000			
02	A5	200.000	25472.000	32238.000	5954.101	18
	B5		34564.000			
	C5		36678.000			
03	A6	100.000	13456.000	16767.000	2921.104	17
	B6		17865.000			
	C6		18980.000			
04	A7	50.000	6705.000	8517.000	1633.315	19
	B7		8970.000			
	C7		9876.000			
05	A8	25.000	3540.000	4193.667	616.636	15
	B8		4765.000			
	C8		4276.000			
06	A9	12.500	986.000	1616.333	548.699	34
	B9		1987.000			
	C9		1876.000			
07	A10	6.250	432.000	831.667	348.970	42
	B10		1076.000			
	C10		987.000			
08	A11	3.125	276.000	534.333	243.648	46
	B11		567.000			
	C11		760.000			

(c)

Zone-2

Sample	Well	Concentration	Values	MeanValue	Std.Dev.	CV%
01	A4	400.000	Masked	71701.500	2609.931	4
	B4		69856.000			
	C4		73547.000			
02	A5	200.000	Masked	35621.000	1494.824	4
	B5		34564.000			
	C5		36678.000			
03	A6	100.000	Masked	18422.500	788.424	4
	B6		17865.000			
	C6		18980.000			
04	A7	50.000	Masked	9423.000	640.639	7
	B7		8970.000			
	C7		9876.000			
05	A8	25.000	Masked	4520.500	345.775	8
	B8		4765.000			
	C8		4276.000			
06	A9	12.500	Masked	1931.500	78.489	4
	B9		1987.000			
	C9		1876.000			
07	A10	6.250	Masked	1031.500	62.933	6
	B10		1076.000			
	C10		987.000			
08	A11	3.125	Masked	663.500	136.472	21
	B11		567.000			
	C11		760.000			

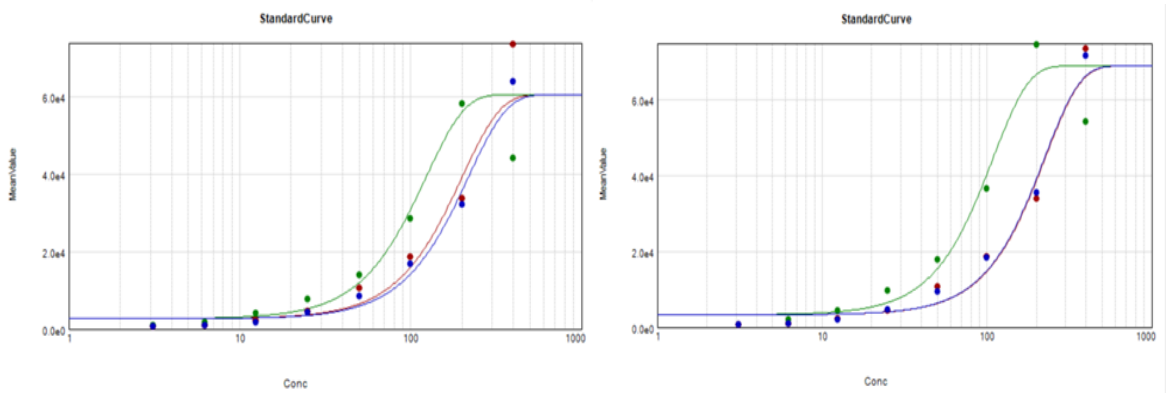
(d)

Zone-3

Sample	Well	Concentration	Values	MeanValue	Std.Dev.	CV%
01	D11 E11 F11	400.000	73456.000 71690.000 75643.000	73596.333	1980.233	3
02	D10 E10 F10	200.000	35897.000 35678.000 29873.000	33816.000	3416.493	10
03	D9 E9 F9	100.000	19853.000 19643.000 16754.000	18750.000	1731.773	9
04	D8 E8 F8	50.000	10980.000 9987.000 10870.000	10612.333	544.340	5
05	D7 E7 F7	25.000	4789.000 3987.000 4652.000	4476.000	428.991	10
06	D6 E6 F6	12.500	2507.000 1900.000 2376.000	2261.000	319.423	14
07	D5 E5 F5	6.250	952.000 1086.000 1034.000	1024.000	67.557	7
08	D4 E4 F4	3.125	789.000 675.000 766.000	743.333	60.285	8

(e)

Figure 4. (a) Zone-1 calculative data (b) Zone-1 calculative masked data (c) Zone-2 calculative data (d) Zone-2 calculative masked data (e) Zone-3 calculative reference data



(a) (b)

Figure 5. (a) 4PL graph between raw data (b) 4PL graph between masked raw data

3.2 Discussion:

The raw plate data obtained as shown in Figure 3(c) was identified as a plate position effect on vertical left and top horizontal of the 96 well format of the plate. The % RSD obtained at zone-1 and zone -2 is more than 20 % higher than zone -3 which is placed at the inner side of the plates. When the Zone-1 1st column was masked, the % relative standard deviation was observed to be less than 20 % at all the doses. Similarly when zone-2 upper horizontal row from A4 to A11 was masked the % relative standard deviation was observed to be less than 20 % at all the doses.

Since all the masked value as shown in Figure 3(d) was observed to be lesser than other replicates, it indicates edge effect due to evaporation, leads to a reduction in lower value compared to other replicates, which upon masking bring the reading under precision conditions. Since the zone-3 was sandwiched and placed in the inner side of the wells, there are no edge effects observed in the plate. This indicates that zone-3 is the best position in the plate to evaluate the results.

As shown in Figures 5a and 5b, there is an evident observation that analysis of the plate is more appropriate in a horizontal manner compared to vertical, As zone-1 raw data value is quite different from zone 2 and zone-3, while zone-2 values/curve are similar to the zone -3 which indicates that zone-2 position is suitable in between the inner side of the wells.

IV. CONCLUSION

The primary goal of this work was to develop an effective approach to detect/evaluate the edge effect, which can help technicians rapidly screen plate layouts which are fit for the Invitro assay development and reduce the biases in the assay values. A lot of statistical software was used to determine this. The statistical method effectively determined the edge effect and reduced the risk of error by manual screening.

Although the results demonstrated the effectiveness of the proposed method, future research should include the following:

- The choice of significant levels should be discussed based on the statistical data,
- Edge wells should be tested in addition to the negative control;
- Certain screening strategies should be discussed.

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