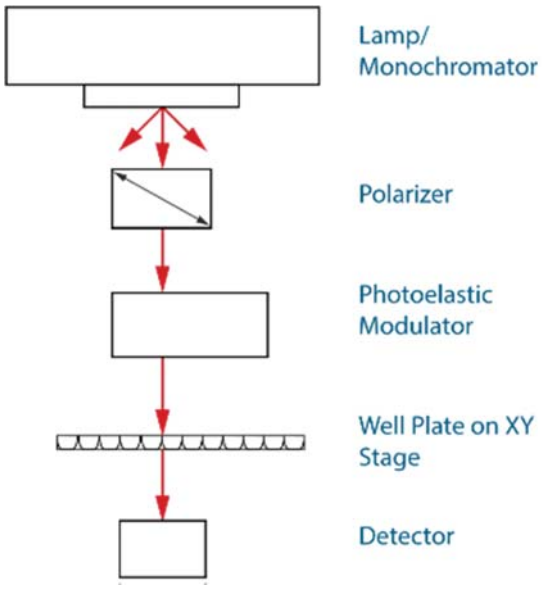
EKKO™ CD Microplate Reader
Reproducibility of CD Measurements

I – INTRODUCTION

The differential absorption between left and right circularly polarized light is a commonly used technique. Circular dichroism (CD) is often used for assigning the secondary structures of proteins and determining enantiomeric purities in asymmetric syntheses, both of which benefit from the ability to do the measurements in a high-throughput fashion [1,2].

The primary advantage of the EKKO™ CD Microplate Reader is its speed resulting from the use of well plates allowing for the highest throughput possible. It accomplishes this by turning the light path from the horizontal to vertical, allowing for the use of a computer-controlled XY stage so that CD signals are read directly from a well plate.



Block Diagram of the EKKO™ CD Microplate Reader

This removes the time-consuming steps of transferring the contents from each well of a well plate into a cuvette and cleaning the cuvette between measurements. As a result, it takes only two minutes to measure the CD signal in all 96 wells of a standard well plate at any given single wavelength and needs less than 90 minutes to measure all 96 CD spectra over 50 wavelengths in a standard well plate. This results in a significant increase in productivity, as much as 100-fold with respect to conventional CD systems coupled to liquid handling robotics[1,2,3].

However, unlike conventional CD systems, the path length, volume and optical characteristics of the glass or silica is fixed. As such, reproducibility between replicates is generally very high unless there has been a catastrophic failure in the system. This is not the apriori situation with measurements based upon the well plate format. not guaranteed to be identical between each of the wells. Variations in the replicate measurements can come from multiple sources: potential heterogeneity of the glass making up the bottom of the wells in a 96 well plate, reproducibility of positioning the beam through the well and path length effects from either sample loading or evaporation.

In this technical note, we address the possible reproducibility concerns that arise from consecutive positionings of the light beam through any given well and volume effects on the data

obtained with the EKKO™ CD Microplate Reader with CD measurements of (+) and (-) - camphorsulfonic acid (CSA) with multiple types of well plates and pipette-men.

II – RESULTS & DISCUSSION

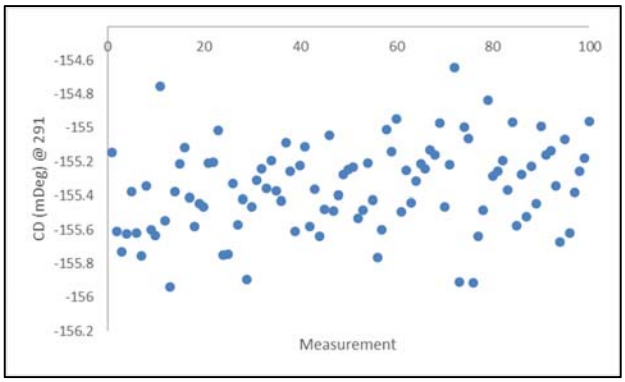


Fig. 1. Consecutive measurements in in wells A10 & F2 of (-) CSA as a function of measurement. Successive measurements of 200 µl of (-) CSA (Sigma) at 1 mg/ml in wells #A10 & #F2 of a solid fused silica 96 well plate (Hellma) taken at 291 nm. Data was collected with no effort to minimize evaporation (samples were not protected with an optically clear cover) or reduce the noise (data were collected with the shortest integration times possible) at a room temperature of 25°C.

Figure 1 illustrates the intra-well reproducibility of the EKKO™ CD Microplate Reader. For the consecutive measurements, a standard deviation of only 0.2 mDeg was observed at 291 nm. This represents a less than 1% variation for 100 measurements taken within a given well. To determine if moving to a new well would influence the reproducibility of the system, multiple plates were completely loaded and the CD at 291 nm was determined for each well.

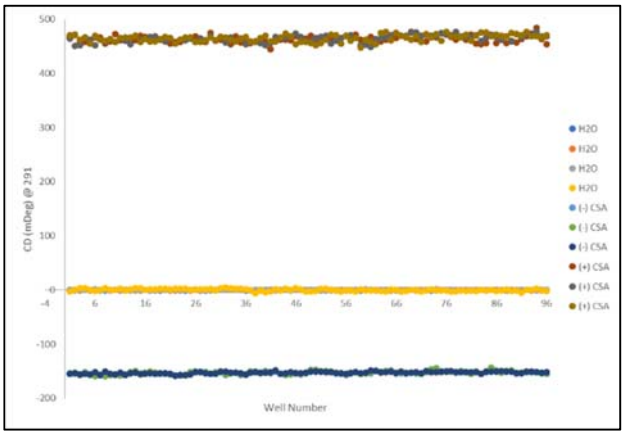


Fig. 2. CD Determinations of CSA in both a clear and black walled solid fused silica well plates. Determinations of the CD magnitude of 200 µl H_20 & (-) CSA (Sigma) at 1 mg/ml in wells #A1-H12 of a solid fused silica 96 well plate (Hellma) taken at 291 nm. CD Measurements of 200 µl H_20 & (+) CSA (Sigma) at 3 mg/ml in wells #A1-H12 of a solid fused silica 96 well plate with black sidewalls (Hellma) at 291 nm were also performed. Raw data was collected with no effort to minimize the noise or evaporation at a room temperature of 25°C.

Figure 2 demonstrates the deviation of the inter-well reproducibility was larger than the within-well deviation of the measurements. For the solid fused silica well plate, the standard deviation (STD) was 2.6 mDeg or 1.8 % of the total CD measured for (-) CSA with (n=405), while the fused silica plate with black walls possessed a STD of 6.2 mDeg or 1.2% of the magnitude of the CD for (+) CSA (n=384). The deviation for water was 2.1 mDeg (n=960). Next, we determined the reproducibility of the system's performance by performing multiple loads of the entire well plate and scanning from 240 to 340 nm to see if any motion in the optical path besides plate movement would have significant effects on the reproducibility of the measurements.

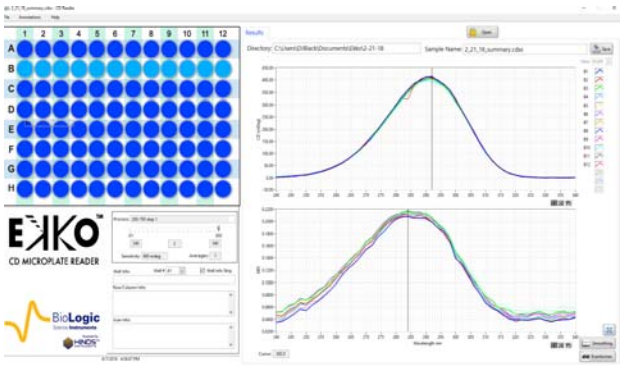


Fig. 3. Raw CD spectra of CSA. The CD spectra of 200 µl H_20 & (+) CSA (Sigma) at 3 mg/ml in wells #A1-H12 of a solid fused silica 96 well plate with black sidewalls (Hellma) from 240 - 340 nm were taken. Raw data was collected with no effort to minimize the noise or evaporation at a room temperature of 25°C. Representative data from cells B1-B12 are shown.

Figure 3 exemplifies the reproducibility of the EKKO™ across the entire spectrum of CSA. The data shown from wells B1-B12 are representative of the spectra obtained across the entire plate. The average standard deviation was 4.1 ± 1.1 mDeg across the entire spectrum or less than 1% of the magnitude of the observed CD signals. The standard deviation in the absorbance spectra was 0.004 ± 0.0006 or 1.1% of the peak absorbance.

The intra-well deviation of less than 1% and inter-well deviations of approximately 1% intimated the errors observed resulted from pipetting errors given the similarity to the systematic error observed when using pipette-men. As such, we had an unskilled technician load a plate with mis-calibrated pipettes at a fixed volume (~3-5% error in delivery volume) and then scan.

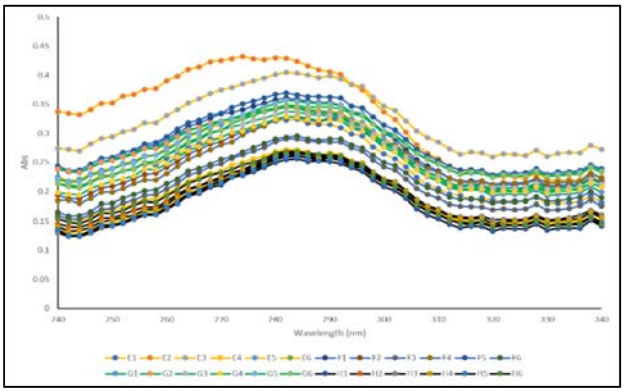


Fig. 4. Absorbance determinations of CSA by an unskilled technician. The Absorbance spectra of 200 µl (+) CSA (Sigma) at 1 mg/ml in wells #A1-H12 of a solid silica bottom 96 well plate with black sidewalls (Porvair) from 240 - 340 nm were taken. Raw data were collected with no effort to minimize the noise or evaporation at a room temperature of 25°C. Representative data is shown in groupings of six and are color coded.

Figure 4 illustrates the progression in the reproducibility for the absorbance spectra loaded by a novice technician. In the beginning, volumes and reproducibility are inconsistent. About half way through the plate, the inflection point in the learning curve for pipetting is reached. See traces E1-E6. After this, a clear progression is observed until the final row of the plate is reached. See traces H1-H6.

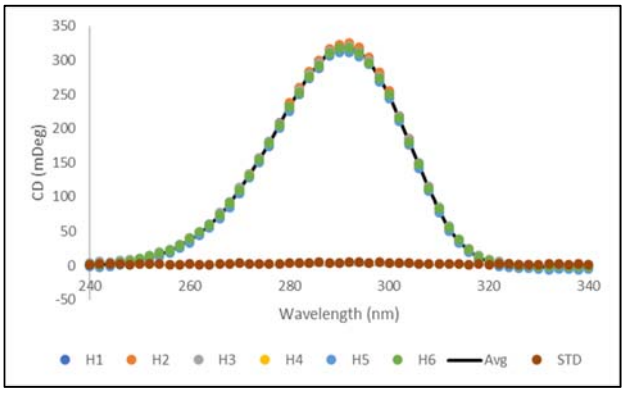


Fig. 5. Raw CD spectra of CSA from the final row of wells of an unskilled technician. The CD spectra of 200 µl (+) CSA (Sigma) at 1 mg/ml in wells #H1-H6 of a solid silica bottom 96 well plate with black sidewalls (Porvair) from 240 - 340 nm were taken. Raw data were collected with no effort to minimize the noise or evaporation at a room temperature of 25°C. Wells H1-H6 are shown in addition to the mean and standard deviation of those six.

Figure 5 illustrates that pipetting skills are learned within a single 96 well plate. Table 1 summarizes the progression of the technician and observed errors for the various pipette-men used in this study and strongly suggests that any observed errors in reproducibility observed with the EKKO™ CD Microplate Reader are a function of sample loading.

Variable Volume		
	CD	ABS
Knock Off	2.6 ± 0.7%	7.5 ± 0.8%
Gilsen	0.6 ± 0.2%	1.7 ± 0.4%
Fixed Volume		
	CD	ABS
Knock Off	1.4 ± 0.3%	4.1 ± 1.1%
Gilsen	0.4 ± 0.1%	0.8 ± 0.2%
Unskilled Technician with Locked Volume		
	CD	ABS
1st set 6	9.8 ± 2.6%	28.7 ± 4.1%
5th set 6	5.7 ± 1.4%	12.4 ± 1.9%
9th set 6	3.0 ± 0.5%	3.8 ± 0.7%
Final set 6	1.1 ± 0.3%	3.5 ± 0.6%

Table 1. Average Standard Deviation in % of Signal.

III – SUMMARY & RECOMMENDATIONS

1. The reproducibility of the EKKO™ CD Microplate Reader is comparable to the reproducibility of traditional CD technologies.
2. Errors in the reproducibility of the EKKO™ CD Microplate Reader are primarily associated with loading volume or well plate characteristics under normal operating conditions.
3. If large errors are observed under normal operating conditions, check sample loading, plate features or other mechanisms to alter the effective pathlength

REFERENCES

1) Metola, P., Nichols, S.M. Kahr, B., and Anslyn, E.V., Well plate circular dichroism reader for the determination of enantiomeric excess. *Chem. Science.* 5, 4278-4282 (2014).
2) Jo, H.H., Cao, X. You, L., Anslyn, E.V., and Krische, M.J., Application of high-throughput enantiomeric excess optical assay involving a dynamic covalent assembly: parallel asymmetric allylation and ee sensing of homoallylic alcohols. *Chem. Science.* 6, 6747-6753 (2015).
3) Fielder, S., Cole, L., and Keller, S., Automated Circular Dichroism spectroscopy for medium throughput analysis of protein conformation. *Anal. Chem.* 85, 1868-1872 (2013).