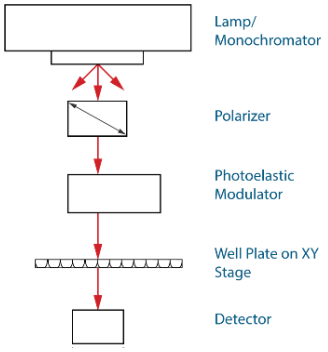
EKKO™ CD Microplate Reader
Global Concerns for CD Measurements using Well Plates

I – INTRODUCTION

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

Traditional CD spectrometers use a horizontal light path which require transferring sample into a cuvette for measurement. Even though robotic liquid handling systems are coupled to a conventional CD spectrometer to expedite this, the cuvette must be cleaned between measurements, a time-consuming laborious process.



The EKKO™ CD Microplate Reader is a vertical CD spectrometer that turns the measuring light beam from the horizontal direction to vertical allowing for the use of a computer-controlled XY stage so that CD signals are read from a well plate directly.

The EKKO™ CD Microplate Reader thus eliminates the time-consuming processes of transferring the contents from each well of a well plate into a cuvette and cleaning the cuvette between measurements, significantly increasing productivity, as much as 100-fold with respect to conventional CD systems coupled to a robot[1,2,3].

Multiple well plates exist for use with the EKKO™ CD Microplate Reader. Well plates with a glass bottom can be used in the visible spectral region. Well plates with a fused silica bottom can be used in the ultraviolet and visible spectral regions. Well plates made from solid fused silica provide the best durability and performance.

Even though the use of well plates significantly increases the potential of data collection, concerns with the type of data and data reproducibility arise from the detection of signals through the meniscus regardless of which well plate is used.

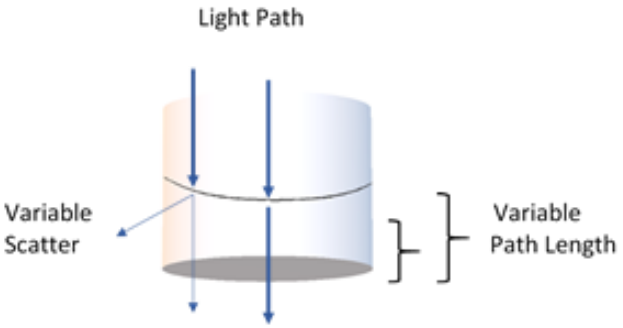


Fig. 1. Presumed effects on the light path as a function of the meniscus in a well.

For any given volume in a well plate, the light path through that solution will be dependent upon its

location in the well due to the meniscus. At a minimum, this position will determine both the effective path length and the degree of scatter of the light as it passes through the meniscus, both of which meaningfully affect the signal observed when measuring CD.

The significant concerns and/or issues that arise regardless of plate selection include, but not limited to:

A) Allowed Sample Types
B) Optical Alignment Effects
C) Reproducibility of the Measurements
D) Sensitivity
E) Short Wavelength Effects
F) Solvent Evaporation
G) Well Plate Variations

In this paper, we provide several summary measurements of multiple studies to address each of the above concerns for the EKKO™ CD Microplate Reader. For more in depth discussions, see the listed technical note.

II – Technical Studies Results

A – Allowed Sample Types (Technical Note 1)

In this technical note, we demonstrate measurements for several CD standards covering the various classes of molecules that circular dichroism is used to study. Enantiomeric excess measurements for CSA and the unique in plate assays not available for traditional CD systems are also demonstrated.

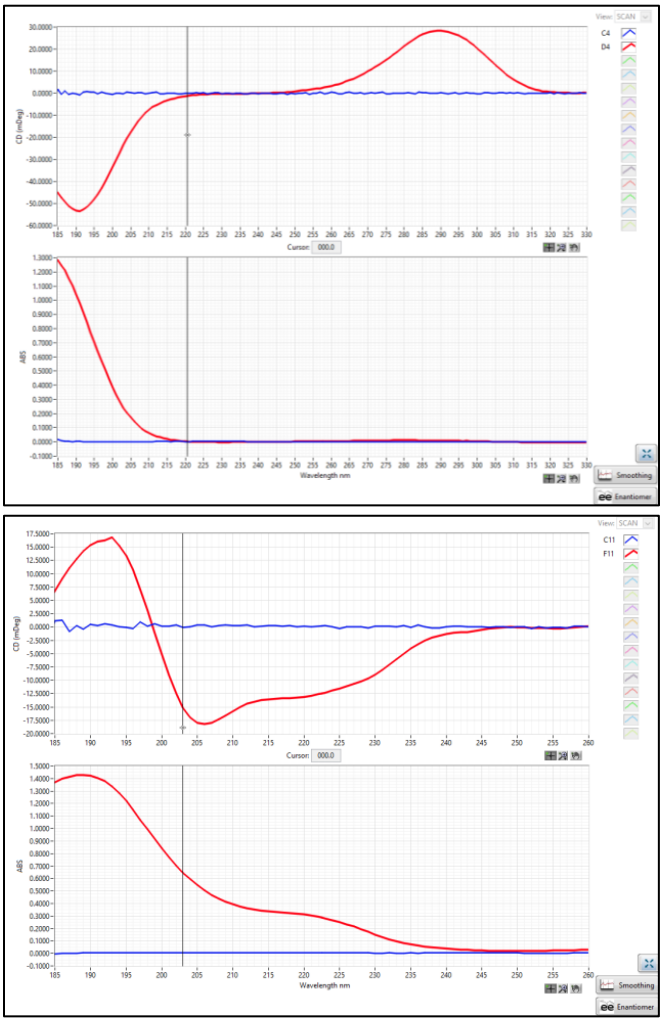


Fig. 2. Raw CD and Abs (top) spectra of CSA and smoothed CD and Abs spectra of Lysozyme. C. Top~ 200 µl of CSA (Sigma) at 0.2 mg/ml in well #D4 of a solid fused silica 96 well plate (Hellma). Raw spectra were collected from 185 – 330 nm in 1 nm steps with an integration time of 3 seconds at a room temperature of 25°C. Bottom ~ 80 µl of Hen Egg White Lysozyme (Sigma) at 0.1 mg/ml in well #F11 of a solid fused silica 96 well plate (Hellma). Raw spectra were collected from 185 – 260 nm in 1 nm steps with an integration time of 3 seconds at a room temperature of 25°C and smoothed with 3 point 3rd order Savitzky-Golay function.

Figure 2 illustrates the EKKO™ CD Microplate Reader can be used to measure small chiral molecules as well as large biological molecules, such as proteins, with performance similar to traditional CD Spectrometers. The EKKO™ CD Microplate Reader also has built in Enantiomeric Excess functions and is uniquely capable measurements of asymmetric chiral synthesis in the reaction well plate.

B - Optical Alignment Effects (Technical Note 2)

In this technical note, we present CD measurements of (+)-camphorsulfonic acid (CSA) with purposely displaced beam positions from the optical center of the well to address the concern of the effects of optical alignment in the presence of a meniscus.

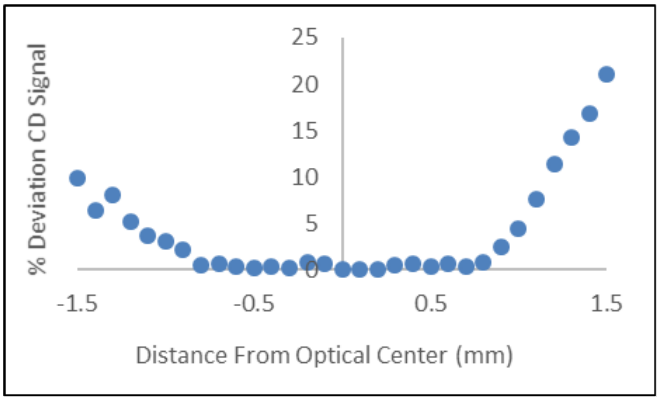


Fig. 3. % signal deviation as a function of distance from the optical center of the well. 200 µl of CSA (Sigma) at 0.2 mg/ml was in well #F10 of a solid fused silica 96 well plate (Hellma). The CD signal was collected for 30 displacements of the beam path along the X-axis at 290 nm. Each position was 0.1 mm incrementally further away from the optical center of the well. Data is plotted as the % deviation from the mean.

Figure 3 illustrates that the EKKO™ CD Microplate Reader tolerates misalignments of up to 0.8 mm in the beam position without effecting the results. Under normal operations, the meniscus and small misalignments between the measuring beam and the center of a well have no adverse effect on CD measurements using EKKO™ CD Microplate Reader given that it has typical positioning errors below 100 ± 5 µm.

C – Reproducibility of the Measurements (Technical Note 3)

In this technical note, we address this possible reproducibility concerns that arise from consecutive positionings of the light beam through any given well. We also tackle the sample volume loading effects on the data obtained with the EKKO™ CD Microplate Reader. This was accomplished by obtaining CD measurements of (+) & (-) camphorsulfonic acid (CSA) with multiple types of well plates and pipette-men.

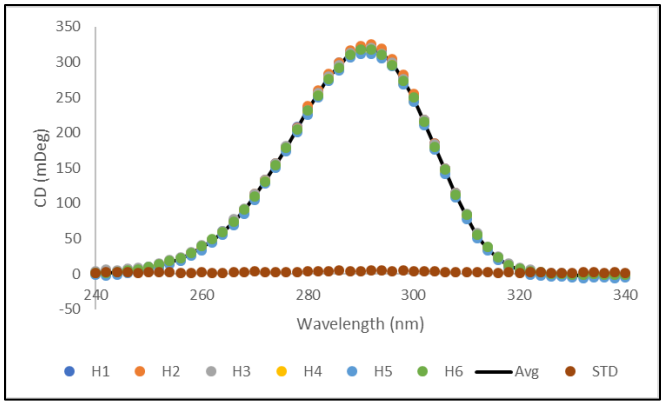


Fig. 4. Raw CD spectra of CSA from the final row of wells of an unskilled technician. The CD spectra of 200 µl (+) CSA (Sigma) at 1 mg/ml in wells #A1-H12 of a solid silica bottom 96 well plate with black sidewalls (Porvair) from 240 - 340 nm were taken. Raw data were collected with no efforts to minimize 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 4 illustrates the reproducibility of the measurements from an unskilled technician at the end of loading a single 96 well plate using the EKKO™ CD Microplate Reader. This study strongly suggests that any observed errors in reproducibility observed with the EKKO™ CD Microplate Reader are a function of sample loading.

D – Sensitivity (Technical Note 4)

In this technical note, we address the sensitivity of the EKKO™ CD Microplate Reader with CD measurements of measurements of multiple

proteins. (α)-lactoglobulin, Cytochrome C, Hemoglobin and Lysozyme with variable well volumes and protein concentrations are demonstrated.

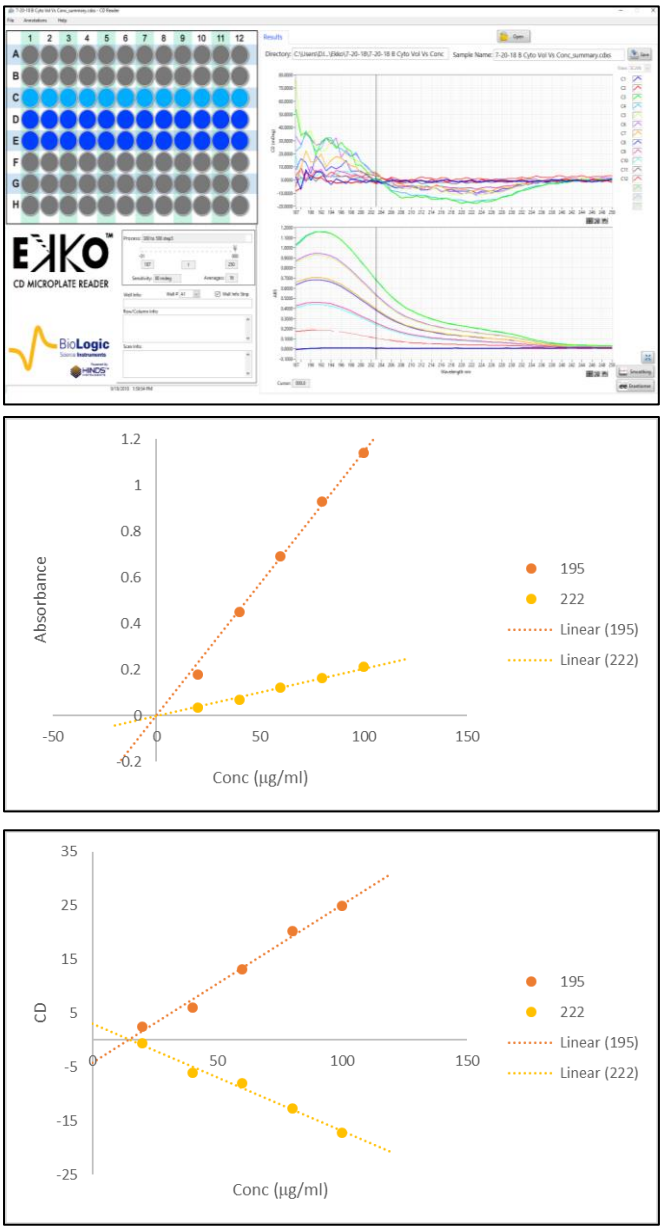


Fig. 5. Signal verses Concentration for Cytochrome C Matrix. 100 µl of Cytochrome C (Sigma) at 20 to 100 µg/ml was in wells #C1 to #C12 of a solid fused silica 96 well plate (Hellma). No effort to reduce the noise were used to collect the raw data (top). Replot of the Absorbance 100 µl of Cytochrome C as a function of concentration for 20 to 100 µg/ml at 195 and 222 nm (middle). Replots of the CD 100 µl of Cytochrome C as a function of concentration for 20 to 100 µg/ml at 195 and 222 nm (bottom).

Figure 5 illustrates that the EKKO™ CD Microplate Reader is linear for the determinations of absorbance and CD up to a value of ~ 1.2, equating to a protein concentration of 50 to 100 µg/ml for a helical protein, performance similar to standard CD Technologies.

E – Short Wavelength Effects of Volume and Solution Components (Technical Note 4)

In this note, we present circular dichroism measurements of water, buffer, (α)-lactoglobulin and Cytochrome C to address the volume and solution dependent effects on the UV cut off wavelength for the EKKO™ CD Microplate Reader apart from buffers known to inhibit CD measurements.

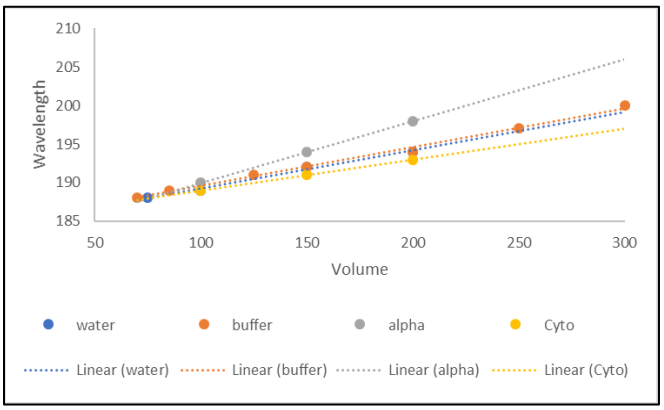


Fig. 6. Cut Off Wavelength as a Function of Volume. Replots of the cut off wavelength as a function of volume for H_20, K_2PO_4 (10 mM, pH 7.4 Sigma), α-lactoglobulin (40 µg/ml Sigma) and Cytochrome C (40 µg/ml Sigma). Each point data point represents the STD of n ≥ 12 individual spectra.

Figure 6 demonstrates that solution composition affect the UV wavelength cut off for CD measurements. And, unlike conventional technologies, the wavelength cut off must be empirically determined for each concentration and well plate type used with the EKKO™ CD Microplate Reader.

F – Solvent Evaporation (Technical Note 6)

In this technical note, we address the effects of solvent evaporation in well plate applications using the EKKO™ CD Microplate Reader with CD measurements of (+)-camphorsulfonic acid (CSA), (-)-pantolactone (PL), and bovine serum albumin (BSA) with purposely extended exposure to and protection from the environment to ensure the presence and absence of the evaporative loss of solvent.

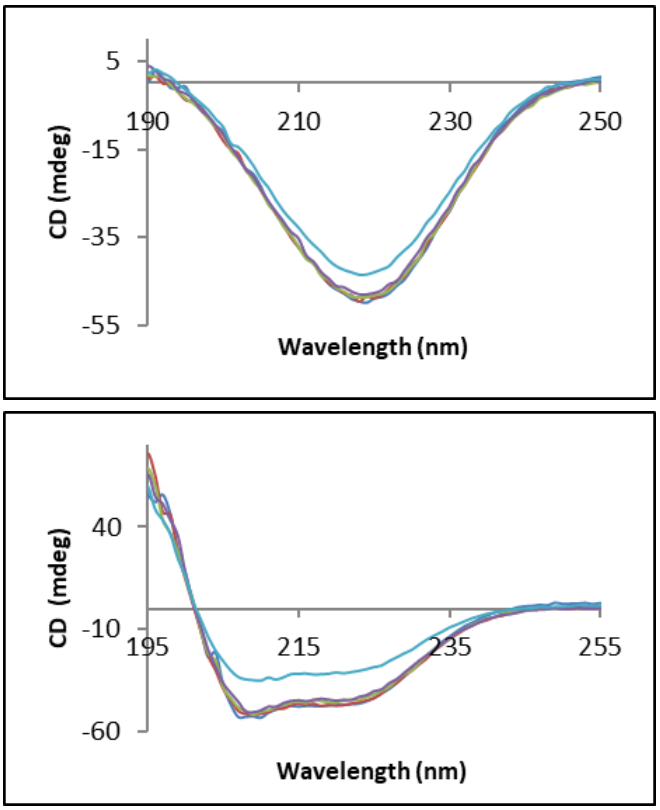


Fig. 7. Raw CD spectra of PL (top) and BSA (bottom) with moderate effective pathlengths as a function of time. 150 µl of PL (Sigma) at 0.1 mg/ml in well #D9 and 140 µl of BSA (Sigma) at 0.1 mg/ml in well #C10 of a solid fused silica 96 well plate (Hellma). Raw spectra were collected after 0, 2, 4, 6 and 23 hours of constant exposure to the environment of the sample chamber. No efforts to minimize evaporation at a room temperature of 25°C or noise were taken.

Figure 7 illustrates that evaporation will have a negligible effect on the CD results for most experiments using the EKKO™ CD Microplate Reader and a 96 well plate given that they will be completed in less than three hours. This holds for experiments that begin with volumes ≥140 µl simply due to the longer effective sample pathlength. When a low starting volume or extended measurement times is ideal empirically, a protective cover made of optical glass or fused silica, depending on wavelengths needed, should be used to minimize the effects of evaporation on CD measurements made with the EKKO™ CD Microplate Reader.

G – Well Plate Variations (Technical Note 7)

In this technical note, we address the effect of variations in the optical quality of fused silica plates on the data obtained with the EKKO™ CD Microplate Reader with CD measurements of Insulin in two different solid fused silica 96 well plates obtained from Hellma.

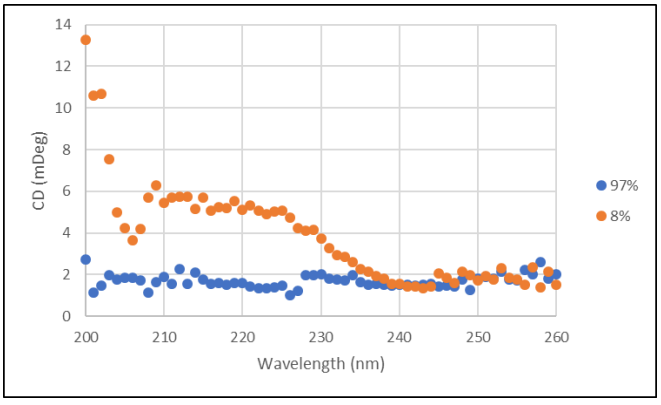


Fig. 8. Standard Deviation as a function of Wavelength for the Averaged CD spectra for the different Solid Fused Silica Well Plates (Hellma). The standard deviation of the n=30 CD spectrum of 200 µl of Insulin (Sigma) at 50 µg/ml in wells C2-E11 of two solid fused silica 96 well plates (Hellma) with different optical characteristics were recorded at 25°C with an integration time of 1 sec/nm.

Figure 8 illustrates that for experimental regimes at wavelengths > 240 nm, homogeneity in the optical characteristics of the well plate do not affect the results using the EKKO™ CD Microplate Reader. In situations ≤ 240 nm, it is recommended to have a plate that is as homogeneous as possible, ideally with < 1 nm/cm deviation across the entire plate when using the EKKO™ CD Microplate Reader.

III – DISCUSSION

We have purposely used extreme conditions to test the global concerns raised by potential users in these studies. For example, we used beam displacements of greater than a mm to test the effect of optical alignment in the presence of a meniscus to determine deviations from the normal operating conditions. We used an unskilled technician with limited experience using pipette-men to load a plate to test the well to well reproducibility of the measurements. We allowed the plate to sit open to for 24 hours to determine the effect of evaporation on the signal. In all cases, the EKKO™ CD Microplate Reader performed admirably. If care is taken in setting up the empirical protocols, it has performance similar to standard CD technologies with a through put that is in some cases ~100 fold higher.

IV – SUMMARY

1. The EKKO™ CD Microplate Reader can be used to measure small chiral molecules as well as large biological molecules such as proteins.
2. The EKKO™ CD Microplate Reader is ideal for studying combinatory mixing of reagents, catalysts, solvents, and various experimental conditions in micro-well plates.
3. At a desired wavelength, EKKO™ CD Microplate Reader can measure CD values from all 96 wells in less than 2 minutes. The user can easily select an integration time for lower noise measurements.
4. The EKKO™ CD Microplate Reader has performance similar to standard technologies.

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).
4) Hussain, R., Javorfi, T., Rudd, T. R., and Siligardi, G., High-throughput SRCD using multi-well plates and its applications. *Nature, Sci Reports* 6, 38028 (2016).