Using Phenol Red to Assess pH in Long-Term Proliferation Assays

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Abstract

Media formulations for the propagation of *in vitro* tissue culture often contain the pH indicator dye, phenol red. Phenol red is a dye that exhibits a gradual transition from yellow to red over a pH range of 6.2 to 8.2. Above 8.2, the dye turns a bright fuchsia color. This dye has long been used to quickly assess the status of bulk cultures being propagated for subsequent experimental analysis. However, once an experiment has been initiated, the state of phenol red in the media is typically no longer utilized, particularly with image-based proliferation assays. Here we describe the use of this dye to monitor pH changes in live cell proliferation assays.

Phenol red spectral scans at different pH levels indicate an absorbance peak at 560 nm with significant changes that can be utilized to monitor pH changes in long-term mammalian cell proliferation studies. Measurements at this wavelength obey Beer's Law and are linear with respect to pathlength. Changes downward in pH result in a decreased absorbance at 560 nm while an increase in pH results in an increased 560 nm absorbance.

In these studies, we use a cell imaging multi-mode reader to make both absorbance measurements as well as digital microscopic images of long-term mammalian cell proliferation experiments. These experiments employ HCT116 cells that express a nuclear H2B-GFP chimera protein which fluorescently tags nuclei, allowing them to be counted using imaged-based analysis. Concurrent absorbance determinations are made using absorbance. In cell cultures seeded at low density, cells can grow and multiply unfettered for as long as 5 days. During this interval, the pH of the media decreased from 7.3 to approximately 7.0. In cultures seeded at higher density, cell growth ceased after 3.5 days and media pH decreased to 6.83.

Monitoring phenol red indicator absorbance can also be used to identify environmental changes. Bacterial contamination results in a rapid decrease in pH as bacteria rapidly consume nutrients. The loss of CO_2 gas supply results in a rapid increase in pH with carbon dioxide outgassing. Media formulations are designed to provide a pH 7.4 in conjunction with a defined percentage of augmented CO_2 gas. The interruption eventually results in the loss of acidic CO_2 causing a dramatic increase in pH.

Assay Basis

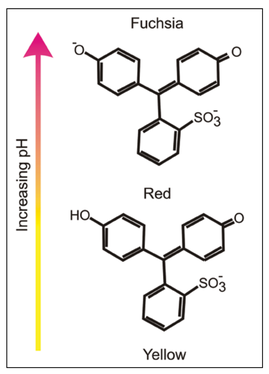


Figure 1. Structure of phenol red and different pH levels. With increasing pH, phenol red changes from a zwitter ion to an anion structure and eventually a di-anion. In doing so, the color of the compound changes from yellow to red to fuchsia.

Media vs Phenol Spectrum

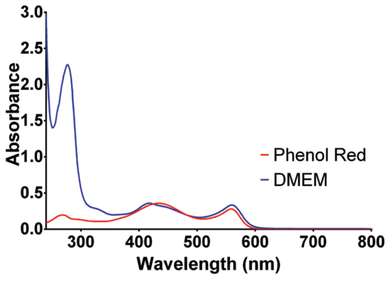


Figure 2. Comparison of phenol red and complete DMEM media absorbance spectra. The absorbance spectra of phenol red (15 mg/L) dissolved in PBS (pH 7.4) and complete DMEM containing 10% FBS were determined. Data represents the mean of three spectral curves after background subtraction.

Effect of pH on Media Absorbance

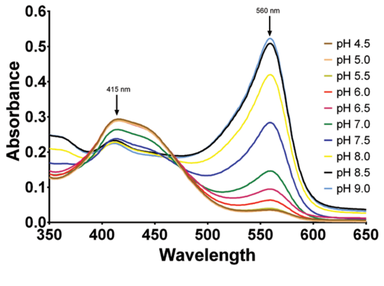


Figure 3. Absorbance spectra of complete DMEM culture media at different pH levels. Wavelengths with changing peak values with pH are indicated with arrows. Data represents the average of three spectral curves for each pH.

Effect of pH on 560 nm Absorbance

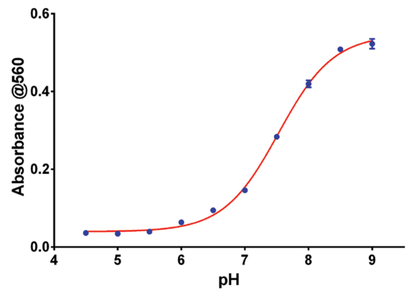


Figure 4. Absorbance at 560 nm for complete media at various pH levels. The absorbance of complete DMEM media was measured at 560 nm at various pH levels and plotted as a function of pH. Data represents the mean and standard deviation of three data points.

Effect of pH on 415/560 Ratio

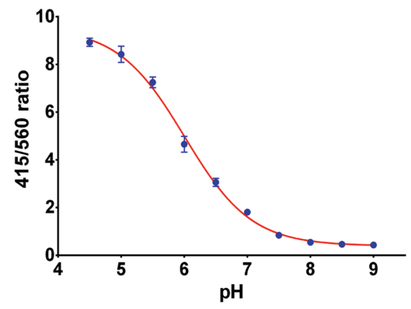


Figure 5. Ratio of 415 and 560 nm absorbance values for complete DMEM media at various pH levels. The absorbance of complete DMEM media was measured at 415 nm and 560 nm at various pH levels and the ratio of the two values plotted as a function of pH. Data represents the mean and standard deviation of three data points.

Effect of CO_2 Loss

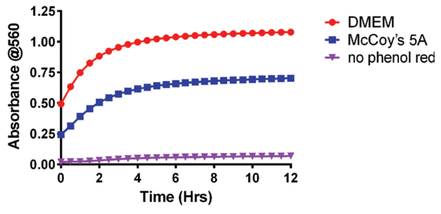


Figure 6. Change in absorbance after cessation of CO_2 supply with different media formulations. Deferent media formulations were aliquoted (200 µL) into wells of a microplate and equilibrated in a CO_2 incubator at 37 °C for 6 hours. The microplate was then placed in the microplate reader and the absorbance at 560 nm measured every 30 minutes at 37 °C without CO_2. Data points represent the mean of 24 determinations.

Calculated Media pH with CO_2 Loss

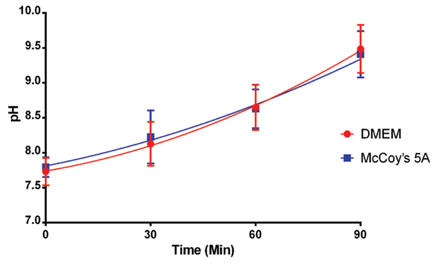


Figure 7. Change in pH levels with DMEM after loss of CO_2 supply. DMEM and McCoy's 5A media were aliquoted (200 µL) into wells of a microplate and equilibrated in a CO_2 incubator at 37 °C for 6 hours. The microplate was then placed in the microplate reader and the absorbance at 560 nm measured every 30 minutes at 37 °C without CO_2. pH values were determined by interpolating the 560 nm absorbance values with a previously generated calibration curve. Data points represent the mean of 24 determinations.

Effect of Media Contamination

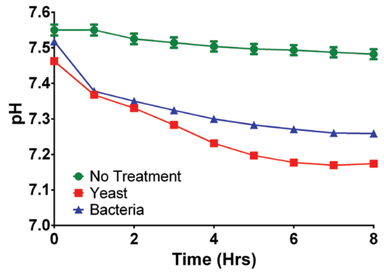


Figure 8. Change in pH with culture contamination. NIH3T3 cells were seeded at a density of 4000 cells per well. After 24 hours to allow for attachment, cells were inoculated with either yeast or bacterial cells. Absorbance measurements were performed on 96-well plate cultures every hour for 8 hours and the results plotted. pH was determined by interpolating the 560 nm absorbance values with a previously generated calibration curve. Data represents the mean and SEM for 96 determinations at each data point.

Media Formulation

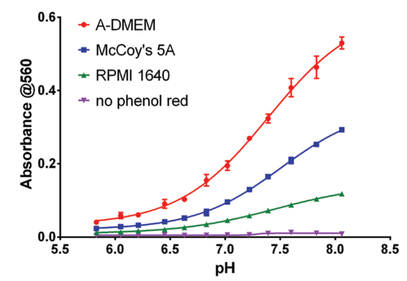
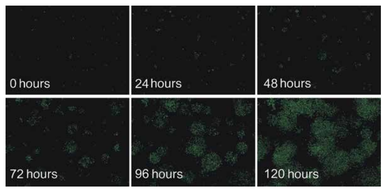


Figure 9. Absorbance at 560 nm for different media formulations at different pH levels. Different media formulations were treated to alter their pH, and equal volumes (200 µL) were aliquoted into a microplate and the absorbance at 560 nm determined. The absorbance values were plotted against pH. Data represents the mean and standard deviation of duplicates.

Long Term Cell Growth



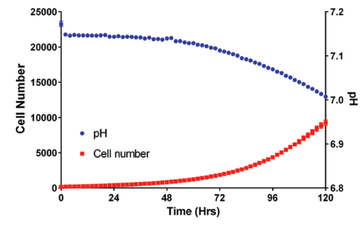
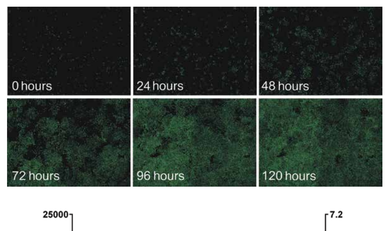


Figure 10. Change in HCT116 cell count and pH over time in non-confluent cultures. HCT116 cells were seeded at a density of 500 cells per well. After 24 hours to allow for attachment, image-based and absorbance analyses were performed on 96-well plate cultures every 2 hours for 5 days and the results plotted. pH was determined by interpolating the 560 nm absorbance values with a previously generated calibration curve. Data represents the mean and SEM for 96 determinations at each data point.



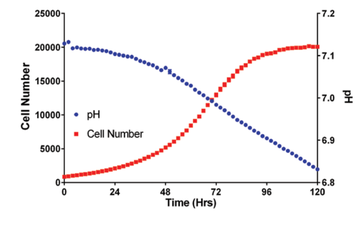


Figure 11. Change in HCT116 cell count and pH over time in confluent cultures. HCT116 cells were seeded at a density of 2000 cells per well. After 24 hours to allow for attachment, image-based and absorbance analysis performed on 96-well plate cultures every 2 hours for 5 days. pH was determined by interpolating the 560 absorbance values with a previously generated calibration curve. Data represents the mean and SEM for 96 determinations at each data point.

Phenol Red Containing Media

Figure 12. Culture flask with phenol red containing media.

BioTek Instrumentation

Figure 13. BioSpa™ 8 Automated Incubator. The BioSpa 8 is a microplate incubator that can interface a BioTek microplate liquid handing device with a BioTek microplate reader/imager. The BioSpa 8 maintains temperature and humidity, as well as provides CO_2 and O_2 gas control for up to 8 microplates.

Figure 14. Cytation™ 5 Cell Imaging Multi-Mode Reader. The modular, upgradable Cytation 5 combines automated digital microscopy and conventional microplate detection. Cytation 5 includes both filter- and monochromator-based detection; the microscopy module provides up to 60x magnification in fluorescence, brightfield, color brightfield and phase contrast. Incubation to 65 °C and plate shaking are standard features. The imaging module uses a turret to hold up to 6 objectives. Excitation and emission wavelengths for fluorescence microscopy are provided using LED light cubes in combination with specific band pass filters and dichroic mirrors. The imaging module holds up to 4 LED cubes. In conjunction with the multi-mode reader, Gen5™ Microplate Reader and Imager Software controls reader function, and also provides image analysis and data reduction.

Conclusions

1. Phenol red indicator dye has absorbance peaks that change with environment pH.
   - 415 nm peak decreases with an increase in pH
   - 560 nm peak increases with an increase in pH
2. pH of media can be monitored in real time using absorbance and interpolation of standard curve.
3. Loss of CO_2 supplementation can be identified with pH change.
4. Media contamination causes a substantial decrease in media pH.
5. Long-term growth experiments can be monitored using pH as a metric.
6. Spent media can be identified using changes in pH.