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A universal indicator dye pH assay for crystallization solutions and other high-throughput applications

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In protein crystallization, as well as in many other fields, it is known that the pH at which experiments are performed is often the key factor in the success or failure of the trials. With the trend towards plate-based high-throughput experimental techniques, measuring the pH values of solutions one by one becomes prohibitively time- and reagent-expensive. As part of an HT crystallization facility, a colour-based pH assay that is rapid, uses very little reagent and is suitable for 96-well or higher density plates has been developed.

1. Introduction

The value of obtaining the atomic coordinates of a protein come from the insight that they provide into the biological function of the protein. The overall structure of a protein can be highly pH-dependent; for example, the influenza surface protein haemagglutinin undergoes a massive structural re-arrangement at low pH into the fusion-capable form (Russell *et al.*, 2008). Even without major structural changes, the pH of the formulation (or native environment) of a protein is likely to affect its binding and enzymatic functionality; knowledge of the ionization states of groups on the surface or in the active site of a protein can have a profound influence in, for example, drug discovery (Bethel *et al.*, 2009). Ultimately, the structure of a protein will show the effects of the pH of the crystallization experiment which was used to obtain the structure (see, for example, Alexov *et al.*, 2011).

The CSIRO Collaborative Crystallization Centre (C3) is a medium- to high-throughput crystallization service that provides both initial screening and optimization tools for macromolecular crystallization for a wide scientific community in Australia (Newman, 2011). Much of the initial screening provided by C3 is based on commercially available crystallization solutions. These solutions usually consist of 3–5 different chemicals, often a salt, a buffer and a polymer mixed in some specified ratio. Even though it is well appreciated that pH is one of the more important factors in a protein crystallization experiment (McPherson, 1990), around a third of the commercially available crystallization solutions are provided without any associated pH information (Newman *et al.*, 2010). Even when some pH information is forthcoming, it is often only the pH of one of the components (usually the buffer chemical) that is reported rather than the pH of the final mixture.

Arguably, if crystallization screening of a given protein could reliably generate crystals of sufficient quality to obtain a structure of that protein, the lack of pH information would not really matter: one could easily measure the pH of the one or two conditions that gave the crystals using a standard pH

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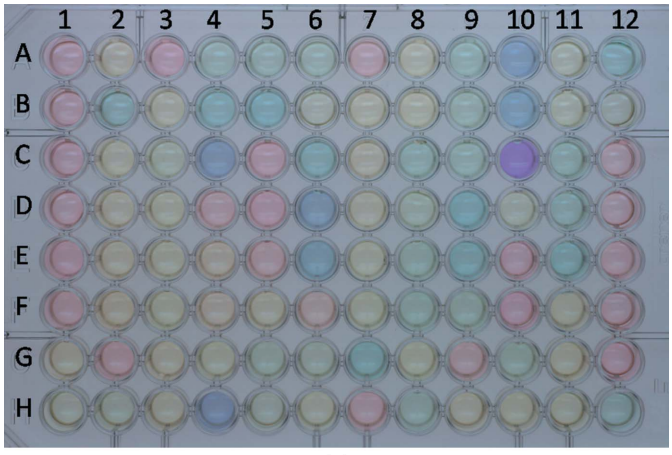
meter. However, it is rare that screening results in crystals, let alone crystals of high enough quality to be used in a diffraction experiment (Derewenda & Vekilov, 2006). Crystallization screening is most often followed by cycles of optimization (Luft *et al.*, 2003, 2011), in which the most successful crystallization experiments from screening are tweaked until better crystals are obtained or until resources (money, students, protein or time) dry up. In this case, knowing as much as possible about all your screening experiments, including the pH, becomes much more critical.

In 2012 there were over 14 000 crystallization solutions that could be purchased (Newman *et al.*, 2010) and even this is only an infinitesimal fraction of the possible crystallization solutions that could be made. Assuming that there are 500 chemicals found in crystallization solutions (Peat *et al.*, 2005) and that three of these are combined at one of three concentrations or pH levels in any one condition, this explodes into billions of possible combinatorial mixtures. Clearly, it is unlikely that any laboratory would have the ability to create or store even a small fraction of all possible conditions, but it is not implausible that there would be of the order of 10^3–10^5 crystallization solutions in an active crystallization laboratory. In our laboratory we can create thousands of new crystallization solutions in a day, routinely create hundreds every week and have approximately 20 000 solutions in-house at any time.

Given these numbers, it is untenable to measure the pH of each solution using a traditional glass pH probe and pH meter. Furthermore, each crystallization solution is likely to be available only in relatively small quantities: 0.5–1 ml. Even with the microprobes commonly found in biological laboratories, sample volumes of 0.2–0.5 ml would normally be required to acquire a pH reading from the sample.

We have combined a well known universal indicator dye (Yamada Universal Indicator; Foster & Gruntfest, 1937) with the automation available in a crystallization laboratory to give a rapid low-sample-volume pH assay which has good correlation with the pH measured using a standard laboratory pH meter and glass electrode. The assay consists of mixing the dye solution with a small aliquot of the crystallization solution, measuring the hue of the resulting solution and comparing it with the hue of a standard curve.



(*a*)

(*b*)

Figure 1
(*a*) The crystallization screen NPCF_4 with colour developed by the addition of a dilute solution of Yamada Universal Indicator dye (1:2:17 volume ratio of dye:screen:water). (*b*) A set of standard pH curves treated with the same dye. The standard curves were prepared by mixing a pH 4 and a pH 10 stock of the same three-component buffer system in varying ratios from 10:0 to 0:10 in steps of 1. Row A shows a curve made with the CHC buffer system, row B with the MIB buffer system, row C with the SPG system and row E with an equivolume mixture of all three buffer systems. The pH values obtained using a solid-state pH sensor (H135; Hach, USA) for the equivolume mixture are shown below the wells containing the dyed solutions.

2. Materials and methods

2.1. Preparation of universal dye

Yamada Universal Indicator dye was prepared by dissolving 25 mg thymol blue, 62 mg methyl red, 250 mg bromothymol blue and 500 mg phenolphthalein (all from BDH) in 500 ml ethanol. The pH was adjusted to 7 with sodium hydroxide and the volume was adjusted to 1 l with H_2O. The resulting dark green solution was stored in the dark at room temperature. This method follows the recipe provided by the Instructional Support Lab (ISL) of the California State University, Stanislaus (http://cabernet.csustan.edu/stkrm/RECIPES/Recipes-Yamada.htm). Dilutions of the concentrated dye stock were made just before use.

2.2. Colour-plate preparation

The experimental colour plates were prepared by adding 5 µl Yamada Universal Indicator dye, 10 µl crystallization solution and 85 µl water to each of the wells in a 96-well microtitre plate (Greiner 655101; see Fig. 1). As the crystallization solutions are stored in 96-well blocks (a collection of 96 crystallization solutions is called a *crystallization screen*), the 96-syringe dispenser of a Phoenix crystallization robot (Art Robbins Industries, USA) was used for this step. The colour plates were heat-sealed with an optically clear seal (Agilent 16985-001).

2.3. Test of dilution levels

A set of 17 solutions at increasing pH values from pH 3 to pH 11 was created using a three-part buffer system containing sodium citrate, HEPES and CHES (CHC; Newman, 2004). The buffers were prepared as described in the literature (Newman, 2004), which gives a nominal concentration of 1 *M*. The pH 3 and pH 3.5 points were prepared by adding HCl to the three-part pH 4 buffer system stock solution, the points from pH 4 to pH 10 were prepared by mixing the three-part pH 4 and pH 10 buffer system stock solutions and the pH points above pH 10 were prepared by adding NaOH to the pH 10 buffer system stock solution. The pH values of the 17 buffer solutions were measured using a PMH93 pH meter (Radiometer Copenhagen) with a pHC2401 KCl-filled glass electrode. 50 µl of these solutions was dispensed into a 96-well microplate four times, yielding four replicates of a 17-point pH curve. 50 µl of diluted Yamada Universal Indicator dye in water was added and the resulting wells were imaged and their hues were compared. The sets of duplicate wells were developed with a 1:5, 1:10, 1:20 or a 1:50 dilution of the dye (Fig. 2*a*). This same plate was imaged on two independent systems: one which images at 293 K and one which images at 281 K (Fig. 2*b*).

2.4. Preparation of standard curves

Standard curves were prepared for three broad-range buffer systems, sodium citrate, HEPES and CHES (CHC), malate, imidazole and borate (MIB), and succinic acid, phosphate and glycine (SPG), or an equivolume mixture of all three systems (Newman, 2004). The standard curves consisted of points taken at 17 different pH values from pH 3 to pH 11 in steps of 0.5 pH units, with the points less than pH 4 or greater than pH 10 being prepared by the addition of HCl or NaOH as above. The pH of each of the solutions that make up the standard curves was measured using a PMH93 pH meter (Radiometer Copenhagen) with a pHC2401 KCl-filled electrode. 50 µl of each of the 17 pH points of the four systems was dispensed into a well of a 96-well flat-bottomed microtitre plate and 50 µl of a 1:10 dilution of the Yamada dye was added to each well. After imaging, four standard curves were created: one for each of the buffer systems (CHC, MIB, SPG or the combination of all three; Fig. 2*c*).

2.5. Dilution tests of the sample crystallization conditions

Three dilutions (no dilution, 1:1 and 1:5) were made of an in-house crystallization screen (NPCF_4) which contained six intrinsically coloured solutions (three of these coloured solutions are quite intense; Fig. 3*a*) and the three different dilutions of the screen were treated with the dye (1:10 dilution) to observe the effect of the screen dilution on subsequent colour development (Fig. 3*b*).

2.6. Estimation of pH

One of the more recent additions to crystallization automation options has been the development of *imagers*, which automatically collect an image of each well (or subwell) of crystallization experiments set up in specialized SBS footprint plastic plates (Berry *et al.*, 2006). This technology was very suitable for automatically collecting the colour information of the dyed crystallization solutions. As the crystallization solutions are stored in 96-well deep storage blocks (as shown in Fig. 3*a*), a convenient way of obtaining the colour images was to make up a set of 96 dyed crystallization solutions in a standard 96-well flat-bottomed assay plate (as shown in Fig. 1*a*). The hue of each well was measured by placing the colour plate into a crystallization imaging station (Rigaku Minstrel HT), where a photograph of each well was recorded with a 5 megapixel colour camera (QImaging Micropublisher 5.0 RTV). The crystallization imagers in C3 were set up to capture a rectangular region within each well of the microtitre plate containing the dye solution, which resulted in images of a

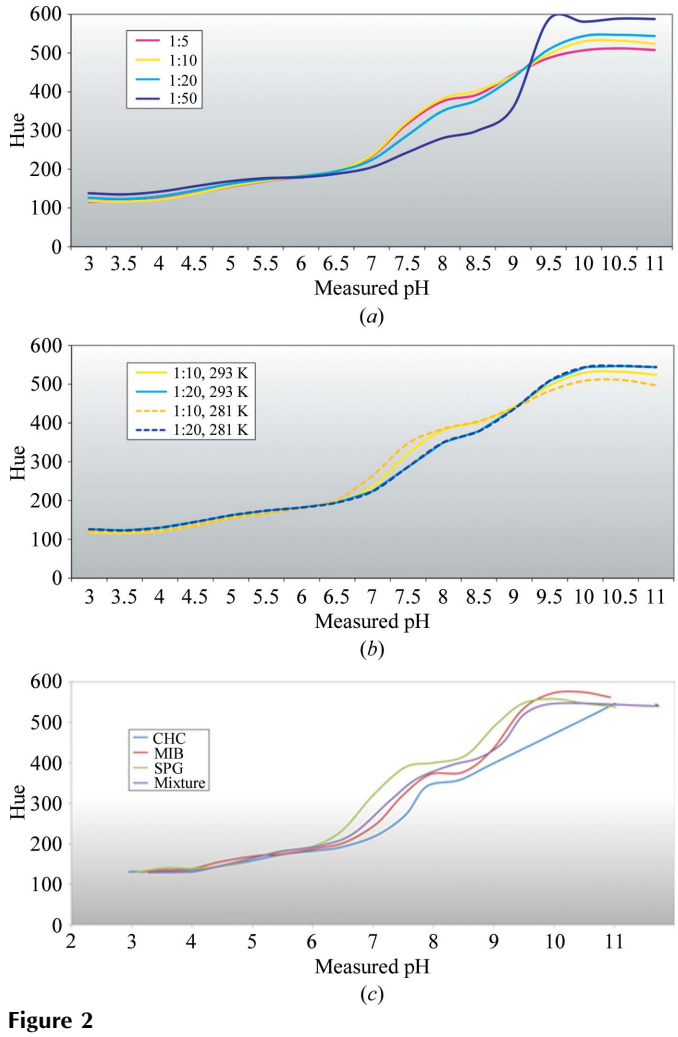
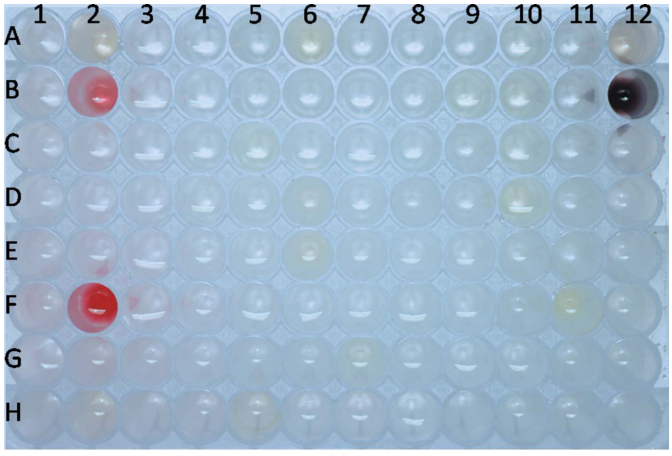
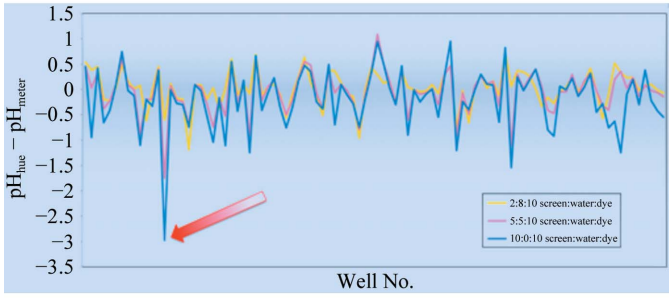


Figure 2
(*a*) Standard curves of the CHC buffer system measured with different dilutions of dye. (*b*) Standard curves of the CHC buffer system with different dilutions of dye with images collected on two systems: one where the plate was held at 293 K and one where the plate was held at 281 K. (*c*) Standard curves obtained for three different three-part buffer systems or an equivolume mixture of the three different buffer systems. The pH values were obtained by measuring the pH of the solutions with a conventional pH meter and a glass (KCl-containing) pH electrode before the addition of dye.

single colour with no features for each well. Clear flat-bottomed microtitre plates were used to minimize the variation in colour over the resulting colour image. The resulting images were processed using the in-house analysis program *pH-Hue-ristic*. This program was written in Microsoft Visual C# 2008 (Microsoft, USA) and allows the user to choose which standard curve to use and which set of images to analyse, and provides the option of either writing the resulting RGB and derived pH values to a text file or directly inserting the pH values into a database table.



(*a*)



(*b*)

Figure 3
(*a*) The photograph shows a 96-well block containing the in-house crystallization screen NPCF_4; there are six solutions that appear coloured (wells A2, B2, B10, B12, E6 and F2). Wells B2, B12 and F2 have the most intense colour by visual inspection. (*b*) The effect of the intrinsic colour can be mitigated by diluting the screen before adding the dye: the graph shows the difference in pH value estimated from the dye assay (at various dilutions of the screen) compared with the pH value measured using a standard pH meter (*i.e.* electrode measurement of pH). The differences in the pH values obtained from each of the undiluted crystallization solutions (1:1 ratio of crystallization solution:diluted dye) and the pH from the same solutions from an electrode pH measurement are shown by the blue trace [average pH difference −0.19 pH units, root-mean-squared average deviation (r.m.s.d.) 0.61 pH units]. The pink line charts the pH difference of the dye pH estimate from the electrode-derived measurement of pH using a 1:2 dilution of the screen in water before mixing with the diluted dye (average pH difference −0.04 pH units, r.m.s.d. 0.42 pH units). The yellow line shows the pH difference of the dye pH estimate from the electrode-measured pH with a 1:5 dilution of the crystallization screen in water before combining with the diluted dye (average pH deviation −0.03 pH units, r.m.s.d. 0.34 pH units). The largest pH difference observed was in the 10:10 dilution in well *B2* (3 pH units, shown by a red arrow); this outlier was resolved by dilution of the screen. For each of the dye-based pH estimates the combined standard curve (the standard curve created by averaging the CHC, MIB and SPG standard curves) was used. For the electrode pH measurements a PMH93 pH meter (Radiometer Copenhagen) with a pHC2401 KCl-filled glass electrode was used to measure the pH of 30 µl of screen diluted in water (final total volume 200 µl) in duplicate.

There are many ways of describing colour, but the system used in many image file formats is RGB, an additive colour system in which the primary colours red (R), green (G) and blue (B) are added to a black background (Tkalcic & Tasic, 2003). A black pixel has no added colour; a white pixel has the three primaries in equal amounts. The relative ratio of these three primaries gives the range of colour that is observed and the value for the colour of each pixel is given as an RGB value. In 24-bit RGB each primary hue can vary from 0 to 255. The *pH-Hue-ristic* program averages the R, G and B values for each of the five million pixels in an image and converts the RGB value to a hue value. The conversion from an RGB value to a hue value is based on the algorithm published by Smith (1978). For each estimate of pH, the (averaged) R, G and B values for a standard pH curve are also converted to hues. The hues from the image and the standard pH curve are compared and an estimate of the image pH is derived using linear interpolation. The resultant pH values may be exported as a text file or inserted into our local CrystalTrak crystallization database (Rigaku Automation, USA). In order to test whether a pH-value estimation would be affected by the colour of neighbouring wells, the pH estimation from images collected from clear polystyrene microtitre plates was compared with images of the same solutions from either black- or white-walled (clear-bottomed) microtitre plates. No clear difference was seen in the RGB values of the same solution measured in clear plates or in opaque plates (data not shown).

3. Results

The estimation of pH values is a three-part process which requires the preparation of a microtitre plate containing the crystallization solutions (10 µl), water (85 µl) and universal indicator solution (5 µl) (the 'colour plate'), the imaging of the colour plate and a translation of the resulting coloured images into an averaged RGB value per image and thus a numerical pH value by comparison with a standard pH curve. The preparation of a colour plate has been automated using the 96-syringe head of a Phoenix crystallization robot (Art Robbins Industries, Sunnyvale, USA) and using this system a colour plate can be generated in under 5 min, with much of this time being used in the syringe-washing steps at the beginning and end of the procedure. The collection of the coloured images from the colour plate is performed in an automatic imaging system designed for crystallization experiments (Rigaku Minstrel HT imaging system) and takes approximately 12 min. The analysis, which is performed by the in-house program *pH-Hue-ristic*, is a two-part procedure requiring first the translation of each coloured image into an averaged RGB value, which is then used to generate a pH value by comparison to a standard curve. This program takes less than 1 min to analyse 96 images. The imaging, which takes the majority of the time for this assay, requires no user

intervention after loading the colour plate in the imager; the real 'operator time' for the entire process is less than 10 min.

The Yamada Universal dye solution will vary slightly, depending on the sources and manufacturers of its constituent dyes as well as the exact recipe used to prepare the stock (Woods & Mellon, 1941; Foster & Gruntfest, 1937; King, 1952). Although we have not exhausted our original stock of concentrated dye, we would produce a new standard curve with each new preparation of dye. The feature that the user selects the appropriate standard curve for analysis in the *pH-Hue-ristic* program was included with this batch-to-batch variation in mind. Although the concentrated dye stock appears to be stable, diluted dye is not, and after two months we observed a loss of purple colouration for high pH values (data not shown). From dye-dilution studies (Figs. 2*a* and 2*b*) it appears that the results are not unduly sensitive to limited changes in dye concentration. The colour plates are prepared with a Phoenix robot; as the coefficient of variation (CV) for this robot is estimated by the manufacturer to be 5% or less, this should not be a major source of variation in the final pH readouts. The hues returned by two cameras of the same type measuring the colour plates at two temperatures showed little deviation (Fig. 2*b*), suggesting that the dye assay is neither sensitive to minor temperature variation nor to measurement on a specific camera. Of course, some buffers (particularly Tris) and other chemicals may change their pH in response to temperature change (Scorpio, 2000), but the dye system itself appears to be stable to moderate changes in temperature. The broad-range buffer systems were chosen for production of the standard curve for two reasons: firstly as these systems contain many of the buffering chemicals used in protein crystallization screening solutions (Newman, 2004) and secondly to ensure that if there were a specific (that is, not a simple pH) response of the dye to one of the chemicals in the buffer system then it would be consistent within the pH curve. The three different curves investigate the effect of the chemical nature of the buffering species on the dye response. There is some variation in the response that we mitigate by using an equivolume mixture of three three-part buffers: thus, the standard curve contains nine different chemicals. The colour plates have a finite lifetime: water will evaporate slowly through the polystyrene of the plate, which will certainly change the pH of the remaining solution even if no other changes occur. A test on a colour plate created from the crystallization screen NPCF_4 that was imaged 11 times over the course of 40 d shows a variation in the estimated pH of the wells of 0.06 on average (data not shown) over this time period. Thus, the colour plates are stable for weeks and the imaging can be scheduled for light usage times on the imager without perturbing the results of the assay.

The CSIRO in-house NPCF_4 crystallization screen was chosen for initial validation tests for two reasons. Firstly, this screen covers many of the types of reagents found in typical commercial crystallization screens. The formulations of this screen can be found in the Supplementary Material[1]. Secondly, this screen contained an unusually high number of coloured solutions (Fig. 3*a*): six of the 96 solutions could be seen by eye to have some colour associated with them. Any intrinsic colour of the solution could mask or alter the colour developed by the dye system.

Visual inspection suggested that the colour of the solutions in this screen could be diluted to the point of not being visible by eye in a 1:10 dilution, and the dye-assay pH values of a dilution series of this screen show that the 1:10 dilution gave results that seemed to be unperturbed by any intrinsic colour associated with the crystallization solution. This would be likely to depend on the 'extinction coefficient' of the solution; the more strongly absorbing the solution, the more it would need to be diluted to remove its effect.

The overall correlation of pH values obtained from colour plates and pH values obtained from a pH meter can be seen in Fig. 4. The correlation is reasonable, with an R^2 value of 0.93; as expected, the intercept of the straight-line fit of the scatter plot passes close to 0. There is a flat response of the dye system around pH 5.5–7, which we believe is a limitation of the Yamada Universal Dye system. The response of the system strays from the line of best fit above pH 9, where there is a marked tendency of the dye estimation of pH to be higher than the estimation generated by a glass electrode. This may in part arise from the alkaline error, which is the nonlinear response of pH electrodes at high pH (Cheng & Zhu, 2005); in this case, we would expect the glass electrode to systematically underestimate the true pH of high-pH solutions. We believe that the reproducibility of the pH assay is a consequence of the large number of pixels in each of the images and the averaging of extracted RGB values from all of the pixels, which means that the even small changes in colour can be extracted reliably and that local flaws in the imaging from the colour plates (for example, an air bubble) do not change the overall RGB value and the pH value unduly.

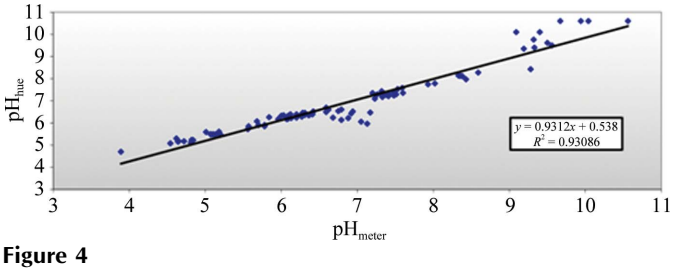


Figure 4
Scatter plot of the pH obtained for each of the crystallization solutions in the NPCF_4 crystallization screen from a standard colour plate (10 µl screen, 85 µl water, 5 µl dye) and by measurement with a pH meter.

4. Discussion

This work reinforces the observation that the pH of the buffer component of a crystallization solution does not necessarily indicate the pH of the complete solution (Bukrinsky & Poulsen, 2001; see Supplementary Material[1]). The buffer component is generally a minor part (0.05–0.1 *M*) of a crys-

[1] Supplementary material has been deposited in the IUCr electronic archive (Reference: BE5201). Services for accessing this material are described at the back of the journal.

tallization solution that can have very high concentrations of salts (3–7 *M*) or high concentrations of polymers [up to 50%(*w*/*v*)] which swamp the effect of the buffering species. In particular, the very widely used salt ammonium sulfate is well known to alter the pH of any crystallization solution containing it through the equilibrium of the ammonium ion with the volatile NH_3 species: ammonia gas leaves and the solution that remains becomes more acid (Mikol *et al.*, 1989). This is more pronounced at higher pH and happens rapidly (Rodeau *et al.*, 1991). Buffers are most effective when set to a pH value close to their pK_a value: generally, a chemical is considered to buffer if it is within one pH unit of a pK_a. Outside this range, the nonbuffer components of a crystallization solution will dominate the resultant pH. A case in point is the sodium citrate buffer often used at pH 5.5 in crystallization screens: this pH value is almost equidistant from the pK_a step 2 and pK_a step 3 values of 4.8 and 6.4, respectively (http://www.zirchrom.com/organic.htm) and the weakness of citrate as a buffer at this pH can be seen in the Supplementary Material, where many of the solutions showing large discrepancies between the measured pH and the buffer pH contain citrate buffer at pH 5.5.

The Yamada Universal Indicator dye has a limited sensitivity range (six pH units), but it has the strong advantages of only requiring a few component dyes and undergoing a visible colour transition approximately every pH unit. The weakness of the Yamada dye in this assay is its poor response in the pH range 5.5–7, which is an important region of pH space in protein crystallization (Kantardjieff & Rupp, 2004). However, the precision of the pH values obtained from the dye system appears to be robust, which is perhaps more relevant than absolute accuracy of the values, particularly for use in protein-crystallization experiments, where reproducibility is a critical factor (Benvenuti & Mangani, 2007). Furthermore, for systems where the accuracy of the pH measurement is paramount, this assay provides a convenient method to select those solutions that need to be measured by a traditional pH meter. It is worth noting that the value obtained for the pH of a system using a glass electrode is still only an estimation of the true pH of the system: no electrode has a perfect response, even when new, and they do decay over time. The requirement that pH meters be calibrated regularly attests to this (Cheng & Zhu, 2005). Although measurements from the same apparatus (electrode and electronic meter) are most often self-consistent, there can be substantial differences between pH meters. It is nontrivial to construct a dye system which provides a good discrimination across a wide pH range: the constituent dyes have to change colour at appropriate pH values to ensure a full coverage, but more importantly the colours of the individual components have to be selected so that the two or more colours of one dye do not mask or are not masked by another dye in the dye mix. The complexity of this problem can be appreciated by the fact that the Yamada Dye was patented in 1933 (Japanese Patent 99664; Foster & Gruntfest, 1937).

The assay described here can produce 96 pH values in less than 30 min, with much of that time being taken up by imaging or washing steps, which require no operator intervention. As a comparison, measurement of the pH values for the NPCF_4 screen manually took several hours. Even greater speed-ups could be obtained by moving to higher density well colour plates.

One of the drawbacks of this assay is the need to use a colour imager to generate the RGB values used as the lookup values in the *pH-Hue-ristic* program: it would be a much more accessible assay if the colour plate could be read on a plate-capable spectrophotometer, for example. With this in mind, we tried using an UV–Vis spectrophotometer to measure absorbance curves for each of the dyed screening solutions and using a least-squares minimization algorithm to fit the dyed screens to standard colour curves. This did not work reliably and so was not continued as a viable alternative. The physics and biology of colour perception are complex and part of the failure of this approach could be that the spectral power density of the wells of the colour plate is simply ignored in a simple absorbance measurement, or it could be that the robustness obtained by the averaging of the RGB values over the five million pixels in the colour images obtained in the crystallization imager is required for the reliable interpolation of pH estimates from coloured solutions.

Even without translating the colour plate into images and pH estimates, they can be useful for quality control. A snapshot of the colour plate created from a given crystallization screen may be compared with other instances of the same crystallization screen (Newman, 2011). An extension of this idea would use colour plates to check the integrity of a crystallization screen over time, as it has been shown that the pH of the crystallization solutions changes over time (Bukrinsky & Poulsen, 2001). Conditions containing ammonium sulfate become more acid owing to loss of ammonia gas (described above) and conditions containing polyethyethylene glycols, particularly in the presence of metals, become more acid owing to the formation of formic acid and other products (Glastrup, 1996; Rossiter *et al.*, 1985).

5. Conclusion

A colour-based pH assay has been developed using the Yamada Universal Indicator dye system which requires only minutes of operator time and uses 10 µl of sample. This assay has been used to measure the pH of close to 300 96-condition crystallization screens in the C3 crystallization centre. The values obtained from these assays are available through the publicly accessible web-tool *C*6 (http://c6.csiro.au). Please contact the authors for access to the *pH-Hue-ristic* program and the protocols used to prepare the colour plates.

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