Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Glycerol concentrations required for the successful vitrification of cocktail conditions in a high-throughput crystallization screen

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Received 14 November 2007
Accepted 18 December 2007

The Hauptman–Woodward Medical Research Institute runs a high-throughput crystallization screening service in which macromolecules are screened against 1536 potential crystallization cocktails. Typically, multiple crystallization leads are identified. With a limited amount of sample, the question becomes 'How many leads can be optimized and which leads are most likely to produce X-ray diffraction data?'. In order to prioritize the hits for optimization, the amount of glycerol required to successfully cryocool each cocktail has been determined for the cocktails used in the high-throughput screen. Those hit conditions that require the minimum amount of cryoprotectant for successful vitrification will be closer in chemical make-up to the mother liquor. Hence, if the physical properties of the crystals are similar, one could logically prioritize leads that are more likely to produce diffraction based upon the chemical similarity of the native to the cryopreserved mother liquor.

1. Introduction

High-throughput crystallization is a highly automated process; hundreds of experiments can be conducted with a few milligrams of the macromolecule of interest. A high-throughput screening service is currently offered at the Hauptman–Woodward Medical Research Institute (HWI). Samples solicited from the biological community are screened against 1536 different biochemical cocktails (Luft *et al.*, 2003) using the microbatch-under-oil crystallization method (Chayen *et al.*, 1992). Individual experiments are composed of 200 nl macromolecule solution ($\sim$10 mg ml^{-1}) and 200 nl of a crystallization cocktail. Experiments are incubated at 296 K and the outcomes are imaged for four weeks. The images are archived and are immediately available to the investigator providing the sample. The cocktails used are broken down into three different groups: highly soluble salts, different molecular-weight PEG combinations and commercially available screens that complement the previous groups. Currently, the success rate is $\sim$50%, *i.e.* half of the screened samples result in a lead: a likely crystallization condition that can be optimized. Frequently, leads are observed from several chemically distinct cocktails. With a limited supply of macromolecule available for crystallization, can we devise a strategy to rationally prioritize these leads for optimization?

For X-ray structural data collection, the majority of samples are cryocooled in order to reduce radiation damage (Garman & Owen, 2006). Cryoprotective agents (cryoprotectants) are typically required to eliminate crystalline ice formation. One of these cryoprotectants is glycerol. The amounts of glycerol needed to successfully vitrify the Hampton Research Crystal

Screen (50 different biochemical cocktails) were determined by Garman & Mitchell (1996). A similar study expanded these data with the addition of 48 cocktails (adding Hampton Research Crystal Screen II) using glycerol and also PEG 400, ethylene glycol and 1,2-propanediol as cryoprotectants for all 98 (50 + 48) cocktails (McFerrin & Snell, 2002). In both studies, solutions were tested for successful vitrification using X-ray diffraction. McFerrin and Snell noted that 73% of the glycerol concentrations required to produce a visually clear solution were successfully vitrified as determined by X-ray diffraction. The remaining solutions required a 5% increase (the minimum glycerol concentration step used) to be successfully vitrified. Simple visual observation provided a good guide to the initial cryoprotectant condition within the sampling constraints.

We have expanded on previous studies and visually determined the concentrations of glycerol required to vitrify the first two groups of cocktails used in the HWI high-throughput screening laboratory. The introduction of any non-native component into a crystal, *e.g.* a cryoprotective agent, has the potential to cause damage (Mitchell & Garman, 1994). By determining the minimum concentration of glycerol required for successful vitrification of a lead condition, we can use this information as one of the criteria to prioritize the leads that are subsequently optimized, *i.e.* those where minimal additional of cryoprotection would be needed for data collection.

2. Experimental

The 1536-condition HWI crystallization screen can be divided into three groups. Groups 1 and 2 were constructed using an incomplete factorial design (Audic *et al.*, 1997) and are buffered with 100 m*M* concentrations of CAPS (pH 10.0), TAPS (pH 9.0), Tris (pH 8.0), HEPES (pH 7.5), MOPS (pH 7.0), MES (pH 6.0), sodium acetate (pH 5.0) and sodium citrate (pH 4.0). Group 1 cocktails are highly soluble salts (262 cocktails). They include 36 different salts (11 cations and 14 anions) at ~30, 60 and 90% saturation, buffered as described. Group 2, PEG/salt (722 cocktails), includes five different molecular-weight PEGs (20, 8, 4, 1 kDa and 400 Da), combined with 35 salts at 100 m*M* concentration and buffered as described. Group 3 are the commercial screens (552 cocktails). This group is comprised of Hampton Research Natrix, Quick, PEG/Ion, PEG Grid, Ammonium Sulfate Grid, Sodium Chloride Grid, Crystal Screen HT, Index and SaltRx screens. For historical reasons, the first 22 cocktails from Hampton Research Crystal Screen Cryo are distributed within groups 1 and 2. These and other occurrences of Hampton Research cryocondition cocktails serve as a control during the experimental process. The first two groups were studied by the addition of 2.5%(*w*/*v*) increments of glycerol concentration to identify cryoprotectant conditions. For the third group, glycerol concentrations for Crystal Screen HT have been described elsewhere (McFerrin & Snell, 2002). Grid Screen Ammonium Sulfate and Grid Screen PEG/LiCl were used to investigate the fine sampling of chemical space, complementing the incomplete factorial sampling of the first two groups. The remainder were studied in somewhat less detail.

The instrumentation used consists of an offline goniometer system with an Oxford 700 cryostream positioned to cool the sample from directly above (Fig. 1*a*). Each sample was imaged with a Navitar zoom lens coupled to a Pixelink color firewire-linked CCD camera. Each component could be precisely translated. A Fibre-Lite metal halide machine-vision illuminator was used to illuminate the sample from the front. To bias the experiment towards the worst case, large 0.7–1.0 mm Hampton Research cryoloops mounted on magnetic heads were used to hold the solutions. Multiple loops were used, all of a similar measured size. They were washed and dried between each experiment.

In the high-throughput crystallization screening laboratory, the crystallization cocktail is mixed in a 1:1 ratio with the macromolecule in buffer. For the vitrification studies described here, all the cocktails were studied at full strength and then diluted in a 1:1 ratio with double-distilled water (ddH_2O). At full strength, the data provide an indication of the initial cryoprotectant properties of the cocktail. As solutes lower the vapor pressure of a solvent and decrease the freezing point, the data from the 1:1 dilution with ddH_2O

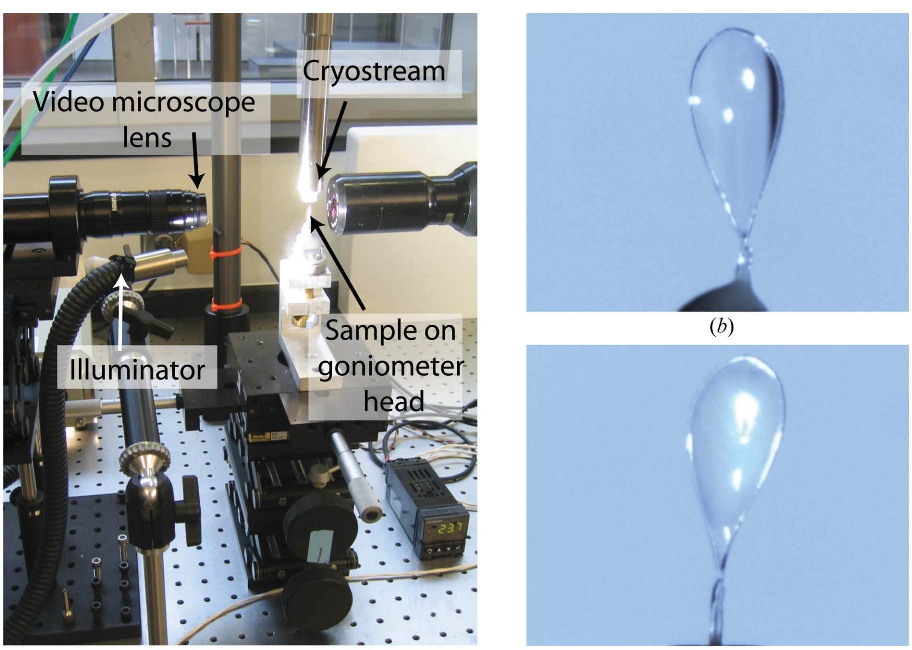

Figure 1
(*a*) Photograph of the experimental setup showing the video microscope lens, the fiber-optic illuminator, cryostream and goniometer mount. The instrument focused on the sample to the right-hand side is a thermal imaging camera used for other studies (Snell *et al.*, 2002). Examples of (*b*) a successful vitrification and (*c*) a poor flash-cooling result are also shown.

(having no solutes) represent a worst-case scenario. A total of 10 µl solution was pipetted onto a glass microscope slide and the loop was used to pick up solution and place it on the goniometer with the gas stream blocked. Once on the goniometer, the gas stream was swiftly unblocked to cool the cryoloop and the solution it contained. Magnified images of the loops were examined to determine whether the solution had vitrified successfully (Fig. 1*b*) or whether crystalline ice was present (Fig. 1*c*).

The first experiment, with the cocktail at full strength, identified conditions that already had cryoprotectant properties and the second with 50% ddH_2O was used as the starting point to study the glycerol concentrations needed for vitrification. If the 50% cocktail solution did not show successful vitrification, further investigation of the cocktail took place. Glycerol solutions containing 60, 55, 50, 45, 40, 35, 30, 25, 20, 15, 10 and 5%(*v*/*v*) glycerol were prepared by volumetric dilution with ddH_2O. The glycerol solution was warmed in a water bath to reduce its viscosity and increase pipetting accuracy. For the first 984 cocktails, each solution was pipetted in equal volumes onto a glass microscope slide, aspirating and dispensing the mixed drop several times. The effective percentage of cryoprotectant was therefore from 30% to 0% glycerol in 2.5%(*v*/*v*) steps. Starting from the highest concentration of cryoprotectant, each solution was loaded in a loop and cooled and then imaged until evidence of crystalline ice was seen. The cryoprotectant concentration that remained clear was then recorded. The initial 984 cocktails (excluding the 22 Crystal Screen Cryo cocktails) provide an incomplete factorial sampling of crystallization space.

A similar procedure was followed for the Hampton Research Grid Screen Ammonium Sulfate and Grid Screen PEG/LiCl cocktails. Sample volume can be an important factor in cryoprotectant concentration and successful vitrification (Chinte *et al.*, 2005). Therefore, a single cocktail that required a larger than average amount of cryoprotectant was selected from the high-salt cocktails group (1.14 *M* ammonium sulfate pH 6) and from each of five different molecular-weight PEGs in the PEG group (lithium chloride pH 10, calcium acetate pH 6.0, ammonium sulfate pH 7.5, potassium phosphate pH 7.0 and ammonium phosphate dibasic pH 4.0 all at 0.1 *M* concentration for PEGs 20, 8, 4, 1 kDa and 400 Da, respectively). The experimental procedure was repeated in 5% steps (rather than the previous 2.5% steps) with these cocktails, using a succession of smaller loops ranging from 1.0 to 0.05 mm across. Each loop was independently measured using a light microscope to confirm its size.

The remaining cocktails were studied with 1:1 dilutions of the cocktails with 20, 10 and 5%(*v*/*v*) glycerol solutions. These cocktails (and the Ammonium Sulfate and PEG/LiCl Grid Screens) are used as reference points with the HWI crystallization screen in order to understand the behavior of the macromolecules over fine-sampled chemical shifts, to pinpoint the best category of potential crystallization chemicals and as a means to sample outliers in chemical space not covered by the 962-cocktail incomplete factorial sampled cocktails. Note that the Crystal Screen HT has been studied in detail elsewhere (Garman & Mitchell, 1996; McFerrin & Snell, 2002).

3. Results

Figs. 2–7 list the cocktails and the concentrations of glycerol required to successfully vitrify the solution. The percentage column is divided into three sections, with the first identifying whether or not the cocktail was successfully vitrified at 100% concentration (without added cryoprotectant), the second if it was successful at 50%(*v*/*v*) concentration and the third the percentage of glycerol needed needed to vitrify a solution containing a 1:1 dilution of the cocktail with ddH_2O. In Figs. 8 and 9 the Hampton Research Grid Screen Ammonium Sulfate and Grid Screen PEG/LiCl results are displayed in a similar manner. Figs. 10–15 show the effect of loop size on the amount of cryoprotectant needed for successful vitrification. Fig. 16 provides a listing of the remaining screens. For brevity, only those conditions that displayed natural cryoprotectant qualities or that were cryoprotected with 20%(*v*/*v*) glycerol or less are displayed. Finally, Tables 1 and 2 summarize the results.

The highly soluble salts (223 cocktails; Fig. 2) required on average the highest concentrations of cryoprotectant [22.5%(*v*/*v*)], with the exceptions of lithium chloride, magnesium acetate and magnesium chloride hexahydrate at high concentration and pH. This was also observed in the data for 100% concentration cocktail conditions, *i.e.* no glycerol. In general, a higher initial salt concentration required a lower cryoprotectant concentration, as observed by Garman (1999). High salt concentrations as cryoprotectant agents have been observed elsewhere (Holyoak *et al.*, 2003; Rubinson *et al.*, 2000).

The PEG 20K results are shown in Fig. 3; for all the PEGs the salt concentration was 0.1 *M*. For 81 cocktails containing 20.0%(*v*/*v*) PEG 20K there was little variation in the required cryoprotectant concentration; that for glycerol averaged 23.9%. At 40%(*v*/*v*) PEG 20K (61 cocktails) the average cryoprotectant concentration was 16.0%(*v*/*v*). Two conditions required no cryoprotectant: ammonium bromide pH 7 and magnesium acetate pH 9. In the case of magnesium acetate, as the pH decreased the required concentration of cryoprotectant increased [0%(*v*/*v*) at pH 9, 10%(*v*/*v*) at pH 6 and 15%(*v*/*v*) at pH 5]. Ammonium bromide was only sampled once at 40%(*v*/*v*) PEG, so the extent of any pH trends are unknown. For PEG 8K (Fig. 4) at 20%(*v*/*v*) concentration (83 conditions), the average required cryoprotectant was 24.1%(*v*/*v*), similar to that for PEG 20K. PEG 8K at 40%(*v*/*v*) (70 conditions) reduced the average cryoprotectant to 16.2%. Again, there were a number of samples that needed no cryoprotectant. These were ammonium chloride pH 4, ammonium nitrate pH 7, magnesium acetate pH 7, sodium nitrate pH 4, lithium sulfate monohydrate pH 5 and manganese sulfate monohydrate pH 6. These cocktails included only single occurrences of magnesium, sodium and manganese salts and so no pH trends could be observed. PEG 4K (Fig. 5) at 20%(*v*/*v*) (75 conditions) required an average of 24.7% cryoprotectant and for 40%(*v*/*v*) (73 conditions) a concen-

No.	Salt (*M*)		pH	%		
1	Ammonium bromide	3.56	8	0	0	30.0
2		3.56	10	0	0	27.5
3		2.38	4	0	0	30.0
4		2.38	7.5	0	0	Fail
5		1.19	5	0	0	Fail
6		1.19	7	0	0	30.0
7		1.19	9	0	0	Fail
8	Ammonium chloride	3.74	4	0	0	30.0
9		3.74	7	0	0	27.5
10		3.74	7.5	0	0	30.0
11		2.5	5	0	0	30.0
12		2.5	9	0	0	Fail
13		2.5	10	0	0	Fail
14		1.25	6	0	0	30.0
15		1.25	8	0	0	30.0
16	Ammonium nitrate	6.46	4	0	0	27.5
17		6.46	9	0	0	25.0
18		6.46	10	0	0	22.5
19		3.23	6	0	0	30.0
20		3.23	7	0	0	30.0
21		3.23	8	0	0	30.0
22	Ammonium phosphate, monobasic	0.87	8	0	0	30.0
23		1.74	4	0	0	30.0
24		1.74	6	0	0	27.5
25		1.74	10	0	0	22.5
26		0.87	5	0	0	30.0
27		0.87	7	0	0	Fail
28		0.87	9	0	0	Fail
29	Ammonium phosphate, dibasic	2.13	7	0	0	22.5
30		3.2	8	1	0	22.5
31		3.2	9	0	0	22.5
32		2.13	6	0	0	22.5
33		2.13	7.5	0	0	22.5
34		1.07	4	0	0	Fail
35		1.07	5	0	0	30.0
36		1.07	9	0	0	Fail
37	Ammonium sulfate	3.42	4	0	0	22.5
38		3.42	5	0	0	22.5
39		3.42	7	0	0	22.5
40		2.28	7.5	0	0	22.5
41		2.28	8	0	0	22.5
42		1.14	6	0	0	30.0
43		1.14	9	0	0	25.0
44		1.14	10	0	0	30.0
45	$Ca(C_2H_3O_2)_2$	1.09	7	0	0	27.5
46		0.55	6	0	0	30.0
47		0.55	5	0	0	Fail
48	$CaCl_2.2H_2O$	3.57	5	0	0	17.5
49	HR-Cryo-1			1	0	15.0
50	HR-Cryo-2			0	0	12.5
51	HR-Cryo-3			1	0	17.5
52	Lithium bromide	5.6	5	1	0	17.5
53		5.6	4	1	0	12.5
54		3.73	6	0	0	22.5
55		3.73	7	1	0	22.5
56		1.87	4	0	0	22.5
57		1.87	8	0	0	27.5
58		1.87	9	0	0	27.5
59	Lithium chloride	6.62	5	1	0	15.0
60		6.62	7.5	1	0	12.5
61		6.62	10	1	1	0
62		4.42	6	1	0	7.5
63		4.42	7	1	0	20.0
64		4.42	8	1	0	20.0
65		2.21	4	0	0	27.5
66		2.21	9	0	0	27.5
67	Magnesium acetate	2.69	7	1	1	0
68		2.69	9	1	1	0
69		1.79	4	0	0	12.5
70		1.79	6	1	1	0
71		1.79	8	1	1	0
72		0.9	5	0	0	12.5
73		0.9	7	0	0	15
74	Magnesium chloride hexahydrate	3.73	5	1	1	0
75		3.73	4	1	1	0
76		2.48	7.5	1	0	20.0
77		2.48	8	1	0	15.0
78		1.24	4	0	0	27.5
79		1.24	6	0	0	25.0
80		1.24	7	0	0	22.5

No.	Salt (*M*)		pH	%		
81	Magnesium sulfate heptahydrate	1.59	9	0	0	22.5
82		1.59	8	0	0	22.5
83		1.06	7	0	0	25.0
84		0.53	5	0	0	27.5
85		0.53	7.5	0	0	27.5
86		0.53	8	0	0	27.5
87	Manganese chloride	3.82	4	1	1	0
88		3.82	8	1	1	0
89		3.82	5	1	0	7.5
90		2.54	5	1	0	17.5
91		2.54	4	0	0	22.5
92		1.27	4	0	0	22.5
93		1.27	5	0	0	22.5
94	Potassium acetate	8.64	8	1	1	0
95		8.64	9	1	1	0
96		5.76	4	1	1	0
97		5.76	5	0	0	22.5
98		5.76	7.5	1	1	0
99		2.88	6	0	0	22.5
100		2.88	7	0	0	22.5
101		2.88	10	1	1	0
102	Potassium bromide	4	5	0	0	25.0
103		4	8	0	0	22.5
104		2.66	6	0	0	27.5
105		2.66	7	0	0	27.5
106		2.66	10	0	0	27.5
107		1.33	4	0	0	25.0
108		1.33	7.5	0	0	27.5
109		1.33	9	0	0	30.0
110	Potassium carbonate	2.54	4	0	0	22.5
111		2.54	7	0	0	22.5
112		2.54	8	0	0	22.5
113		1.69	5	0	0	25.0
114		1.69	7.5	0	0	27.5
115		1.69	9	0	0	22.5
116		0.85	6	0	0	25.0
117		0.85	10	0	0	27.5
118	Potassium chloride	2.32	4	0	0	22.5
119		2.32	7	0	0	22.5
120		2.32	7.5	0	0	25.0
121		1.55	6	0	0	30.0
122		1.55	8	0	0	30.0
123		1.55	9	0	0	30.0
124		0.77	5	0	0	30.0
125		0.77	10	0	0	30.0
126	Potassium nitrate	0.88	5	0	0	30.0
127		0.88	9	0	0	30.0
128		0.88	8	0	0	30.0
129		1.77	5	0	0	30.0
130		0.88	4	0	0	30.0
131		0.88	7.5	0	0	30.0
132		0.88	10	0	0	20.0
133	Potassium phosphate, monobasic	1.28	8	0	0	25.0
134		0.85	7	0	0	30.0
135		0.85	7.5	0	0	25.0
136		0.85	10	0	0	27.5
137		0.43	4	0	0	30.0
138		0.43	6	0	0	30.0
139		0.43	9	0	0	Fail
140	Potassium thiocyanate	9.5	4	1	0	20.0
141		9.5	6	0	0	22.5
142		6.34	5	0	0	22.5
143		6.34	8	0	0	27.5
144		6.34	10	0	0	27.5
145		3.17	7	0	0	30.0
146		3.17	7.5	0	0	Fail
147		3.17	9	0	0	30.0
148	RbCl	2.85	5	0	0	30.0
149		1.9	4	0	0	25.0
150		1.9	6	0	0	30.0
151	Sodium bromide	5.14	4	0	0	22.5
152		5.14	5	0	0	22.5
154		5.14	6	0	0	22.5
154		3.43	7	0	0	22.5
155		3.43	7.5	0	0	25.0
156		3.43	8	0	0	25.0
157		1.71	9	0	0	25.0
158		1.71	10	0	0	25.0

No.	Salt (*M*)		pH	%		
159	Sodium chloride	4.48	6	0	0	22.5
160		4.48	7.5	0	0	22.5
161		4.48	10	0	0	22.5
162		2.99	4	0	0	22.5
163		2.99	5	0	0	22.5
164		1.49	7	0	0	25.0
165		1.49	8	0	0	27.5
166		1.49	9	0	0	30.0
167	Sodium molybdate dihydrate	2.03	6	0	0	27.5
168		2.03	7.5	0	0	22.5
169		1.35	7	0	0	25.0
170		1.35	9	0	0	25.0
171		0.68	4	0	0	22.5
172		0.68	8	0	0	25.0
173		0.68	10	0	0	22.5
174	Sodium nitrate	3.9	6	0	0	22.5
175		3.9	7.5	0	0	22.5
176		2.6	5	0	0	22.5
177		2.6	8	0	0	25.0
178		2.6	9	0	0	25.0
179		1.3	4	0	0	22.5
180		1.3	7	0	0	22.5
181	Sodium phosphate, monobasic	3.32	4	1	0	17.5
182		3.32	7	1	0	17.5
183		3.32	9	1	1	0
184		2.21	6	0	0	22.5
185		2.21	7.5	0	0	22.5
186		1.11	5	0	0	27.5
187		1.11	8	0	0	22.5
188		1.11	10	0	0	22.5
189	Sodium thiosulfate pentahydrate	2.83	6	1	0	22.5
190		2.83	8	1	0	17.5
191		2.83	9	1	0	15.0
192		1.88	7	0	0	25.0
193		1.88	9	0	0	22.5
194		0.94	6	0	0	25.0
195		0.94	10	0	0	22.5
196		0.94	7.5	0	0	25.0
197	Zinc acetate	1.18	7.5	0	0	25.0
198		1.18	8	0	0	22.5
199		0.79	5	0	0	22.5
200		0.79	8	0	0	27.5
201		0.39	5	0	0	25.0
202		0.39	6	0	0	25.0
203	Potassium phosphate, dibasic	3.46	6	1	0	15.0
204		3.46	7.5	0	0	22.5
205		2.3	7	0	0	25.0
206		2.3	9	0	0	25.0
207		2.3	5	0	0	22.5
208		1.15	4	0	0	22.5
209		1.15	5	0	0	22.5
210		1.15	8	0	0	22.5
211	Cobalt sulfate heptahydrate	0.53	8	0	0	25.0
212		1.06	9	0	0	22.5
213		0.53	5	0	0	25.0
214		1.06	4	0	0	22.5
215		1.06	5	0	0	22.5
216		1.06	8	0	0	22.5
217		0.53	6	0	0	25.0
218		0.53	7	0	0	27.5
219	$Li_2SO_4.H_2O$	2.03	6	0	0	22.5
220		1.35	4	0	0	22.5
221		1.35	7.5	0	0	22.5
222		0.68	5	0	0	25.0
223	K_3PO_4	3.05	4	1	1	0
224		3.05	8	1	0	22.5
225		3.05	9	1	0	22.5
226		2.03	5	1	0	25.0
227		2.03	7	1	0	20.0
228	Ammonium thiocyanate	8.1	4	0	0	20.0
229		8.1	5	0	0	22.5
230		5.4	6	0	0	25.0
231		5.4	8	0	0	25.0
232		5.4	9	0	0	27.5
233		2.7	7	0	0	25.0
234		2.7	7.5	0	0	30.0
235		2.7	10	0	0	22.5
236	$MnSO_4.H_2O$	2.05	4	0	0	22.5
237	HR Cryo-4			1	1	0

Figure 2
The first 237 crystallization cocktails representing 46 highly soluble salts, 11 different cations and 14 distinct anions (conditions 49–51 and 237 are from Hampton Research Crystal Screen Cryo). The % column shows 1 in the left column if vitrified with no cryoprotectant and 1 in the middle column if vitrified without cryoprotectant when diluted 1:1 with ddH_2O; the third column shows the percentage (*v*/*v*) of glycerol in ddH_2O needed to vitrify a solution cocktail at a 1:1 ratio. Each salt is present at 0.1 *M* concentration with the buffer at 0.01 *M*.

No.	Salt	pH	PEG	%		
238	Ammonium bromide	10	20% PEG 20 000	0	0	22.5
239		9		0	0	27.5
240		7		0	0	22.5
241		7.5		0	0	22.5
242	Ammonium chloride	6		0	0	22.5
243		4		0	0	22.5
244	Ammonium nitrate	6		0	0	22.5
245		9		0	0	22.5
246	Ammonium phosphate, monobasic	10		0	0	22.5
247		7.5		0	0	22.5
248	Ammonium phosphate, dibasic	10		0	0	22.5
249	Ammonium sulfate	7		0	0	22.5
250	HR Cryo-5			1	1	0
251	HR-Cryo-6			1	0	12.5
252	HR-Cryo-7			1	0	15.0
253	Lithium bromide	7	20% PEG 20 000	0	0	22.5
254		9		0	0	22.5
255	Lithium chloride	9		0	0	22.5
256		5		0	0	22.5
257		10		0	0	27.5
258		6		0	0	25.0
259	Magnesium acetate	9		0	0	22.5
260	Magnesium chloride hexahydrate	8		0	0	22.5
261		4		0	0	25.0
262		7		0	0	22.5
263	HR-Cryo-8			1	0	12.5
264	Manganese chloride	6	20% PEG 20 000	0	0	22.5
265		4		0	0	22.5
266	Potassium acetate	7.5		0	0	22.5
267		7		0	0	25.0
268		8		0	0	25.0
269		6		0	0	22.5
270	Potassium bromide	7.5		0	0	22.5
271		5		0	0	25.0
272		8		0	0	25.0
273	Potassium carbonate	9		0	0	27.5
274		5		0	0	27.5
275		7.5		0	0	22.5
276	Potassium chloride	10		0	0	22.5
277		7.5		0	0	25.0
278	Potassium nitrate	6		0	0	22.5
279		8		0	0	27.5
280		4		0	0	22.5
281	Potassium phosphate, monobasic	8		0	0	22.5
282		5		0	0	25.0
283		6		0	0	25.0
284		9		0	0	22.5
284	Potassium thiocyanate	7		0	0	27.5
286	Rubidium chloride	4		0	0	22.5
287		10		0	0	25.0
288		9		0	0	25.0
289	Sodium bromide	4		0	0	25.0
290		10		0	0	25.0
291	Sodium chloride	7.5		0	0	22.5
292		8		0	0	22.5
293	Sodium molybdate dihydrate	7		0	0	25.0
294		8		0	0	22.5
295	Sodium nitrate	7		0	0	27.5
296	Sodium phosphate, monobasic	4		0	0	22.5
297		6		0	0	22.5
298	Sodium thiosulfate pentahydrate	8		0	0	22.5
299	Zinc acetate	6		0	0	25.0
300		5		0	0	25.0
301	Potassium phosphate, dibasic	10		0	0	22.5
302	Cobalt sulfate heptahydrate	7		0	0	25.0
303	Lithium sulfate monohydrate	6		0	0	25.0
304		4		0	0	22.5
305	Potassium phosphate, tribasic	9		0	0	22.5
306		10		0	0	25.0
307	Ammonium thiocyanate	7.5		0	0	25.0
308	Manganese sulfate monohydrate	5		0	0	22.5
309		6		0	0	22.5
310		7		0	0	22.5
311	Magnesium nitrate hexahydrate	5		0	0	22.5
312		8		0	0	22.5
313		7		0	0	25.0

No.	Salt	pH	PEG	%		
314	Ammonium bromide	7	40% PEG 20 000	1	1	0
315	Ammonium chloride	6		1	0	15.0
316		7		1	0	15.0
317	Ammonium nitrate	8		1	0	15.0
318		5		1	0	17.5
319	Ammonium phosphate, monobasic	4		1	0	15.0
320		8		1	0	15.0
321	Ammonium phosphate, dibasic	7	20%	0	0	25.0
322	Ammonium sulfate	4	40%	1	0	15.0
323		8		1	0	17.5
324		5		1	0	17.5
325	HR Cryo-9			1	1	0
326	Calcium acetate	7	40% PEG 20 000	1	0	15.0
327	Lithium bromide	10		1	0	15.0
328		7.5		1	0	17.5
329		6		1	0	15.0
330	Lithium chloride	8		1	0	17.5
331	Magnesium acetate	6		1	0	10.0
332		9		1	1	0
333		5		1	0	15.0
334	Magnesium chloride hexahydrate	5		1	0	15.0
335	Magnesium sulfate heptahydrate	5	20%	0	0	27.5
336		6		0	0	27.5
337		9		0	0	22.5
338	Potassium acetate	9	40% PEG 20 000	1	0	15.0
339	Potassium bromide	10		1	0	15.0
340		6		1	0	15.0
341	Potassium carbonate	6		1	0	15.0
342		9		1	0	15.0
343	Potassium chloride	8		1	0	15.0
344		7		1	0	17.5
345		4		1	0	15.0
346	Potassium nitrate	7.5		1	0	17.5
347		5		1	0	17.5
348		9		1	0	17.5
349	Potassium phosphate, monobasic	9		1	0	12.5
350	Potassium thiocyanate	6		1	0	22.5
351		10		1	0	22.5
352		5		0	0	22.5
353	Rubidium chloride	10		0	0	22.5
354		8		0	0	22.5
355		7.5		1	0	15.0
356	Sodium bromide	6		1	0	22.5
357		8		1	0	17.5
358	Sodium chloride	4		1	0	17.5
359		8		1	0	15.0
360	Sodium molybdate dihydrate	10		1	0	12.5
361		7.5		1	0	10.0
362		7		1	0	17.5
363		9		1	0	17.5
364	Sodium nitrate	4		1	0	17.5
365		7.5		1	0	15.0
366		9		0	0	22.5
367		5			0	22.5
368	Sodium phosphate, monobasic	5		1	0	12.5
369		10		1	0	17.5
370	Sodium thiosulfate pentahydrate	8		1	0	17.5
371	Zinc acetate	6		1	0	15.0
372	HR-Cryo-10			1	0	15.0
373	HR-Cryo-11			1	0	22.5
374	HR-Cryo-12			0	0	22.5
375	Hr-Cryo-13			1	1	0
376	Cobalt sulfate heptahydrate	8	20%	0	0	25.0
377		5		0	0	22.5
378		4		0	0	25.0
379	Lithium sulfate monohydrate	7.5	40%	1	0	15.0
380		7		1	0	12.5
381	HR-Cryo14			1	0	15.0
382	Potassium phosphate, tribasic	5	20%	0	0	22.5
383		4		0	0	30.0
384	HR-Cryo-15			1	0	15.0
385	Ammonium thiocyanate	8	40%	1	0	15.0
386		9		0	0	15.0
387	HR-Cryo-16			1	0	12.5
388	Magnesium nitrate hexahydrate	5	40%	1	0	17.5
389		8		1	0	15.0
390		7		1	0	22.5

Figure 3
PEG 20 000 cocktail conditions (Nos. 238–390) shown in a similar manner to Fig. 2. Within these conditions several Hampton Research Crystal Screen Cryo condition screens are also included; Nos. 250–252, 263, 325, 372–375, 381, 384 and 387.

No.	Salt	pH	PEG	%		
391	Ammonium bromide	5	20% PEG 8000	0	0	27.5
392		8		0	0	27.5
393	Ammonium chloride	7		0	0	25.0
394	Ammonium nitrate	8		0	0	25.0
395		10		0	0	22.5
396	Ammonium phosphate, monobasic	7		0	0	27.5
397		8		0	0	25.0
398		9		0	0	22.5
399		5		0	0	25.0
400	Ammonium phosphate, dibasic	9		0	0	22.5
401		6		0	0	22.5
402	Ammonium sulfate	7.5		0	0	22.5
403		10		0	0	25.0
404	Calcium acetate	6		0	0	27.5
405		7.5		0	0	22.5
406	Calcium chloride dihydrate	5		0	0	25.0
407		7		0	0	27.5
408	Lithium bromide	5		0	0	22.5
409		10		0	0	22.5
410		7.5		0		22.5
411	Lithium chloride	8		0	0	22.5
412		9		0	0	25.0
413	Magnesium acetate	6		0	0	22.5
414	Magnesium chloride hexahydrate	7.5		0	0	22.5
415		8		0	0	25.0
416		7		0	0	22.5
417	Magnesium sulfate heptahydrate	4		0	0	22.5
418		7		0	0	25.0
419	Potassium acetate	10		0	0	22.5
420	Potassium bromide	9		0	0	22.5
421		10		0	0	25.0
422	Potassium carbonate	10		0	0	25.0
423		6		0	0	22.5
424	Potassium chloride	5		0	0	25.0
425		4		0	0	22.5
426	Potassium nitrate	10		0	0	22.5
427		7		0	0	22.5
428	Potassium phosphate, monobasic	5		0	0	22.5
429		7.5		0	0	22.5
430	Potassium thiocyanate	5		0	0	27.5
431	Rubidium chloride	7.5		0	0	25.0
432		6		0	0	27.5
433	Sodium bromide	6		0	0	25.0
434	Sodium chloride	7.5		0	0	25.0
435		6		0	0	22.5
436	Sodium molybdate dihydrate	6		0	0	25.0
437		7		0	0	25.0
438		9		0	0	25.0
439		8		0	0	25.0
440	Sodium nitrate	6		0	0	27.5
441		7		0	0	22.5
442		4		0	0	27.5
443		7.5		0	0	27.5
444	Sodium thiosulfate pentahydrate	10		0	0	22.5
445	Zinc acetate	4		0	0	25.0
446		5		0	0	30.0
447	HR-Cryo-17			1	1	22.5
448	Potassium phosphate, dibasic	7.5	20% PEG 8000	0	0	22.5
449		4		0	0	22.5
450		7		0	0	22.5
451		8		0	0	22.5
452	Cobalt sulfate heptahydrate	4		0	0	25.0
453	Lithium sulfate monohydrate	9		0	0	25.0
454		4		0	0	25.0
455		8		0	0	22.5
456	Potassium phosphate, tribasic	7.5		0	0	22.5
457		7		0	0	25.0
458		4		0	0	22.5
459	Ammonium thiocyanate	8		0	0	25.0
460		5		0	0	22.5
461		10		0	0	22.5
462	Manganese sulfate monohydrate	5		0	0	25.0
463		7		0	0	22.5
464	Magnesium nitrate hexahydrate	4		0	0	22.5
465		9		0	0	25.0
466		6		0	0	22.5
467		7.5		0	0	22.5

No.	Salt	pH	PEG	%		
468	Ammonium bromide	4	40% PEG 8000	1	0	15.0
469		7		0	0	25.0
470		5		1	0	17.5
471	Ammonium chloride	5		1	0	17.5
472		4		1	1	0
473		8		1	0	17.5
474	Ammonium nitrate	4		1	0	17.5
475		7.5		0	0	22.5
476		7		1	1	0
477	Ammonium phosphate, monobasic	5		1	0	15.0
478		6		1	1	0
479	Ammonium phosphate, dibasic	5	20%	0	0	25.0
480		7.5		0	0	22.5
481	Ammonium sulfate	5	40% PEG 8000	1	0	15.0
482		6		1	0	15.0
483		9		1	0	20.0
484	HR-Cryo-18			1	1	0
485	Calcium chloride dihydrate	6	40% PEG 8000	1	0	17.5
486		5		1	0	17.5
487	Lithium bromide	4		0	0	22.5
488		9		1	0	17.5
489	Lithium chloride	6		1	0	15.0
490		5		1	0	17.5
491		7.5		0	0	22.5
492	Magnesium acetate	7		1	1	0
493	Magnesium chloride hexahydrate	5		1	0	15.0
494	Magnesium sulfate heptahydrate	10	20%	0	0	22.5
495		7.5		0	0	25.0
496		5		0	0	25.0
497	Manganese chloride	7	40% PEG 8000	1	0	10.0
498		5		1	0	22.5
499		6		1	0	17.5
500	Potassium acetate	5		1	0	22.5
501		7		1	0	17.5
502	Potassium bromide	7.5		1	0	12.5
503		7		1	0	17.5
504		8		0	0	22.5
505	Potassium carbonate	5		1	0	15.0
506		8		1	0	15.0
507		7.5		1	0	17.5
508		10		1	0	20.0
509	Potassium chloride	9		0	0	22.5
510		10		0	0	22.5
511		6		1	0	15.0
512	Potassium nitrate	9		1	0	15.0
513		7.5		1	0	15.0
514		8		1	0	15.0
515	Potassium phosphate, monobasic	7.5		1	0	17.5
516		7		1	0	20.0
517		4		0	0	22.5
518		10		1	0	17.5
519	Potassium thiocyanate	5		1	0	12.5
520		8		1	0	22.5
521	Rubidium chloride	9		0	0	22.5
522		10		0	0	22.5
523	Sodium bromide	7.5		0	0	22.5
524		9		1	0	15.0
525		10		1	0	17.5
526	Sodium chloride	8		1	0	17.5
527		9		1	0	15.0
528		6		1	0	15.0
529	Sodium nitrate	4		1	1	0
530	Sodium phosphate, monobasic	8		1	0	17.5
531		6		1	0	15.0
532		7		1	0	17.5
533	Sodium thiosulfate pentahydrate	10		1	0	15.0
534		9		1	0	15.0
535		8		1	0	17.5
536	Zinc acetate	6		1	0	17.5
537		8		0	0	22.5
538	Potassium phosphate, dibasic	5	20%	1	0	22.5
539	Lithium sulfate monohydrate	5	40%	1	1	0
540		7		1	0	22.5
541		9		1	0	15.0
542	HR-Cryo-19			0	0	17.5
543	Potassium phosphate, tribasic	5	20%	0	0	25.0
544	Ammonium thiocyanate	4	40%	0	0	22.5
545		6		1	0	15.0
546	Manganese sulfate monohydrate	6		1	1	0

Figure 4
PEG 8000 cocktail conditions (Nos. 391–546), similar to Fig. 2. Within these conditions several Hampton Research Crystal Screen Cryo condition screens are also included: Nos. 447, 484 and 542.

No.	Salt	pH	PEG	%		
547	Ammonium bromide	10	20% PEG 4000	0	0	25.0
548		8		0	0	25.0
549	Ammonium chloride	10		0	0	25.0
550		5		0	0	25.0
551	Ammonium nitrate	8		0	0	25.0
552	Ammonium phosphate, monobasic	9		0	0	25.0
553		4		0	0	25.0
554		7		0	0	22.5
555	Ammonium phosphate, dibasic	4		0	0	25.0
556		9		0	0	25.0
557		7		0	0	22.5
558	Ammonium sulfate	7.5		0	0	27.5
559		9		0	0	25.0
560	Calcium acetate	7		0	0	25.0
561	Calcium chloride dihydrate	5		0	0	25.0
562		7.5		0	0	25.0
563	Lithium bromide	5		0	0	25.0
564		8		0	0	27.5
565	Lithium chloride	8		0	0	25.0
566		10		0	0	25.0
567		5		0	0	25.0
568		7.5		0	0	27.5
569	Magnesium chloride hexahydrate	6		0	0	25.0
570		7.5		0	0	22.5
571	Magnesium sulfate heptahydrate	7		0	0	22.5
572		8		0	0	22.5
573		9		0	0	25.0
574		6		0	0	25.0
575		4		0	0	25.0
576	Manganese chloride	5		0	0	22.5
577		7		0	0	27.5
578		6		0	0	25.0
579	Potassium acetate	4		0	0	25.0
580		6		0	0	22.5
581	Potassium bromide	4		0	0	22.5
582		8		0	0	27.5
583		7.5		0	0	22.5
584	Potassium carbonate	7		0	0	27.5
585		8		0	0	25.0
586	Potassium chloride	10		0	0	22.5
587		8		0	0	27.5
588	Potassium nitrate	7		0	0	27.5
589	Potassium phosphate, monobasic	4		0	0	25.0
590	Potassium thiocyanate	4		0	0	25.0
591		9		0	0	22.5
592		7.5		0	0	22.5
593	Rubidium chloride	7		0	0	27.5
594	Sodium bromide	7		0	0	25.0
595		5		0	0	22.5
596	Sodium chloride	5		0	0	25.0
597		7		0	0	25.0
598		9		0	0	25.0
599		7.5		0	0	27.5
600	Sodium molybdate dihydrate	10		0	0	22.5
601		4		0	0	22.5
602	Sodium nitrate	8		0	0	27.5
603		6		0	0	25.0
604	Sodium phosphate, monobasic	6		0	0	25.0
605		4		0	0	25.0
606	Sodium thiosulfate pentahydrate	8		0	0	25.0
607	Potassium phosphate, dibasic	4		0	0	25.0
608		9		0	0	22.5
609	Cobalt sulfate heptahydrate	5		0	0	25.0
610		8		0	0	22.5
611		4		0	0	27.5
612	Lithium sulfate monohydrate	5		0	0	27.5
613		4		0	0	22.5
614	Potassium phosphate, tribasic	8		0	0	25.0
615		10		0	0	22.5
616		4		0	0	22.5
617	Ammonium thiocyanate	5		0	0	22.5
618		6		0	0	25.0
619	Manganese sulfate monohydrate	6		0	0	25.0
620	Magnesium nitrate hexahydrate	9		0	0	22.5

No.	Salt	pH	PEG	%		
621	Ammonium bromide	4	PEG 4000 40%	0	0	22.5
622		6		1	0	12.5
623	Ammonium chloride	10		1	0	17.5
624		7		1	0	12.5
625	Ammonium nitrate	7.5		1	0	12.5
626		6		1	0	17.5
627		9		1	0	22.5
628		10		1	0	12.5
629	Ammonium phosphate, monobasic	7.5		1	1	0
630		10		0	0	22.5
631	Ammonium phosphate, dibasic	10	20%	0	0	27.5
632	Ammonium sulfate	10	40% PEG 4000	1	0	17.5
633		4		0	0	22.5
634		6		1	0	15.0
635	Calcium chloride dihydrate	7		1	0	17.5
636		5		1	0	17.5
637	Lithium bromide	4		1	0	17.5
638		10		1	0	15.0
639	Magnesium acetate	7.5		0	0	22.5
640	Magnesium chloride hexahydrate	5		1	0	22.5
641		7.5		1	1	0
642	Manganese chloride	6		1	0	22.5
643	Potassium acetate	5		1	0	20.0
644		8		1	1	0
645		7.5		1	1	0
646	Potassium bromide	7		1	0	20.0
647		9		1	0	17.5
648		6		0	0	22.5
649	Potassium carbonate	7.5		1	0	15.0
650		8		0	0	22.5
651		10		1	0	20.0
652	Potassium chloride	9		1	0	15.0
653		6		1	1	0
654	Potassium nitrate	6		1	0	10.0
655		10		1	1	22.5
656		4		1	0	17.5
657	Potassium phosphate, monobasic	8		1	0	17.5
658		4		1	0	17.5
659		6		1	0	12.5
660	Potassium thiocyanate	7		1	0	17.5
661		8		1	1	0
662	Rubidium chloride	4		1	0	17.5
663		7.5		1	0	22.5
664		8		1	0	15.0
665		5		1	0	15.0
666		7		1	0	12.5
667	Sodium bromide	5		1	0	15.0
668		7.5		1	0	12.5
669	Sodium chloride	5		1	1	22.5
670	Sodium molybdate dihydrate	10		1	0	15.0
671		8		1	0	15.0
672	Sodium nitrate	9		1	0	17.5
673		5		1	0	12.5
674	Sodium thiosulfate pentahydrate	7.5		1	1	0
675		9		1	0	10.0
676		10		1	1	0
677	Zinc acetate	5		1	0	12.5
678		6		1	0	12.5
679	Potassium phosphate dibasic	8		0	0	22.5
680	Lithium sulfate monohydrate	7		1	1	0
581		10		1	1	0
682	Potassium phosphate, tribasic	9		0	0	25.0
683		8		0	0	22.5
684	Ammonium thiocyanate	9		1	0	17.5
685		4		1	0	12.5
686		6		1	0	22.5
687	Manganese sulfate monohydrate	6		0	0	22.5
688		7		1	0	12.5
689		7		1	0	15.0
690		6		1	0	12.5
691	Magnesium nitrate hexahydrate	7		1	0	12.5
692		6		1	0	22.5
693		5		1	0	12.5
694		9		1	0	15.0

Figure 5
PEG 4000 cocktail conditions (Nos. 547–694), similar to Fig. 2.

No.	Salt	pH	PEG	%		
695	Ammonium bromide	7.5	20% PEG 1000	0	0	25.0
696	Ammonium chloride	4		0	0	27.5
697		5		0	0	22.5
698		9		0	0	22.5
699		8		0	0	25.0
700	Ammonium nitrate	7.5		0	0	22.5
701		5		0	0	22.5
702		4		0	0	25.0
703	Ammonium phosphate, monobasic	7		0	0	25.0
704		6		0	0	25.0
705	Ammonium phosphate, dibasic	4		0	0	22.5
706		9		0	0	22.5
707	Ammonium sulfate	6		0	0	22.5
708		7		0	0	22.5
709	Calcium chloride dihydrate	5		0	0	22.5
710	Lithium bromide	7		0	0	25.0
711	Lithium chloride	8		0	0	25.0
712		6		0	0	25.0
713	Magnesium chloride hexahydrate	9		0	0	27.5
714		8		0	0	22.5
715		6		0	0	22.5
716	Magnesium sulfate heptahydrate	7.5		0	0	25.0
717		9		0	0	25.0
718		5		0	0	25.0
719	Manganese chloride	5		0	0	25.0
720		4		0	0	25.0
721		6		0	0	25.0
722	Potassium acetate	4		0	0	22.5
723	Potassium bromide	4		0	0	22.5
724	Potassium carbonate	5		0	0	22.5
725		4		0	0	22.5
726	Potassium chloride	5		0	0	25.0
727		7		0	0	25.0
728	Potassium nitrate	5		0	0	25.0
729		10		0	0	22.5
730		6		0	0	22.5
731	Potassium phosphate, monobasic	7		0	0	22.5
732		6		0	0	27.5
733	Potassium thiocyanate	10		0	0	25.0
734		8		0	0	27.5
735	Rubidium chloride	6		0	0	25.0
736		9		0	0	25.0
737	Sodium bromide	7		0	0	22.5
738		4		0	0	25.0
739	Sodium chloride	10		0	0	25.0
740	Sodium molybdate dihydrate	9		0	0	25.0
741		7.5		0	0	25.0
742	Sodium nitrate	7		0	0	27.5
743		8		0	0	27.5
744	Sodium phosphate, monobasic	7.5		0	0	22.5
745	Sodium thiosulfate pentahydrate	8		0	0	22.5
746	Zinc acetate	5		0	0	25.0
747	Potassium phosphate dibasic	8		0	0	25.0
748		4		0	0	25.0
749		10		0	0	22.5
750	Cobalt sulfate heptahydrate	8	10%	0	0	27.5
751		7	20% PEG 1000	0	0	22.5
752		6		0	0	27.5
753		5		0	0	25.0
754	Lithium sulfate monohydrate	6		0	0	25.0
755		7		0	0	25.0
756		9		0	0	27.5
757	Potassium phosphate, tribasic	9		0	0	22.5
758		7		0	0	27.5
759		7.5		0	0	25.0
760	Ammonium thiocyanate	10		0	0	22.5
761		8		0	0	25.0
762		7.5		0	0	25.0
763	Manganese sulfate monohydrate	4		0	0	27.5
764		5		0	0	27.5
765	Magnesium nitrate hexahydrate	8		0	0	25.0
766		5		0	0	22.5
767		7		0	0	25.0

No.	Salt	pH	PEG	%		
768	Ammonium bromide	8	40% PEG 1000	0	0	22.5
769		5		0	0	22.5
770	Ammonium chloride	8		1	0	17.5
771	Ammonium nitrate	7		1	0	15.0
772	Ammonium phosphate, monobasic	10		1	0	12.5
773		4		1	0	17.5
774		7.5		1	1	0
775		7		1	0	15.0
776	Ammonium phosphate, dibasic	4		1	0	10.0
777	Ammonium sulfate	7		1	1	0
778		10		1	0	15.0
779		8		1	0	25.0
780	Calcium acetate	5		1	0	17.5
781	Calcium chloride dihydrate	7		1	0	12.5
782	Lithium bromide	6		1	0	12.5
783		4		1	0	17.5
784		8		1	0	17.5
785		9		1	0	12.5
786	Lithium chloride	5		1	0	12.5
787		9		0	0	12.5
788		4		1	1	0
789		10		0	1	0
790	Magnesium acetate	8		1	0	17.5
791	Magnesium chloride hexahydrate	9		1	0	17.5
792	Magnesium sulfate heptahydrate	9		1	0	17.5
793	Manganese chloride	5		0	0	22.5
794		6		1	0	17.5
795	Potassium acetate	7		0	0	22.5
796		6		1	1	0
797	Potassium bromide	5		1	1	0
798		8		1	0	17.5
799		4		0	0	25.0
800	Potassium carbonate	10		1	0	17.5
801		4		1	0	17.5
802		5		1	0	15.0
803		9		1	0	20.0
804	Potassium chloride	10		1	0	15.0
805		9		1	0	17.5
806		7.5		1	0	17.5
807	Potassium nitrate	7.5		0	0	22.5
808		4		1	0	17.5
809	Potassium phosphate, monobasic	8		1	0	17.5
810		4		1	0	17.5
811	Potassium thiocyanate	7.5		1	0	15.0
812		6		1	0	17.5
813	Rubidium chloride	4		1	0	10.0
814		8		1	0	15.0
815		6		1	0	17.5
816	Sodium bromide	10		1	0	17.5
817		9		1	0	10.0
818	Sodium chloride	8		1	0	17.5
819		10		1	0	10.0
820	Sodium molybdate dihydrate	7.5		1	0	17.5
821		8	20%	1	0	22.5
822	Sodium nitrate	4	40% PEG 1000	0	0	12.5
823		10		1	0	17.5
824	Sodium phosphate, monobasic	6		1	0	15.0
825	Zinc acetate	5		1	0	15.0
826		8		1	0	10.0
827		5		1	0	15.0
828	Potassium phosphate, dibasic	7		0	0	22.5
829	Lithium sulfate monohydrate	8		1	0	12.5
830		6		0	0	17.5
831		10		0	0	17.5
832	Potassium phosphate-tribasic	7.5		0	0	17.5
833		6		0	0	25.0
834	Ammonium thiocyanate	9		1	0	17.5
835		10		1	0	15.0
836		7		1	0	17.5
837	Manganese sulfate monohydrate	6		1	0	17.5
838		7		1	0	17.5
839	Magnesium nitrate hexahydrate	9		1	0	12.5

Figure 6
PEG 1000 cocktail conditions (Nos. 695–839), similar to Fig. 2.

#	Salt	pH	PEG	%		
840	Ammonium bromide	8.0	40% PEG 400	1	1	0
841		4.0		1	0	17.5
842	Ammonium chloride	6.0		1	0	20.0
843		10.0		0	0	22.5
844	Ammonium nitrate	7.0		0	0	22.5
845		5.0		1	0	17.5
846	Ammonium phosphate, monobasic	5.0		1	0	17.5
847		7.5		1	0	12.5
848	Ammonium phosphate, dibasic	8.0		1	0	15.0
849	Ammonium sulfate	7.0		1	0	17.5
850		7.5		1	0	17.5
851	Calcium acetate	5.0		1	0	10.0
852		7.5		1	0	17.5
853		6.0		1	1	0
854	Calcium chloride dihydrate	7.5		1	0	17.5
855	HR-Cryo-20			1	1	0
856	Lithium bromide	6.0	40% PEG 400	1	0	15.0
857		4.0		0	0	22.5
858		8.0		1	0	20.0
859		9.0		1	0	15.0
860	Lithium chloride	7.0		1	0	20.0
861	Magnesium acetate	5.0		1	0	17.5
862	Magnesium chloride hexahydrate	6.0		1	0	17.5
863	Magnesium sulfate heptahydrate	6.0		1	0	17.5
864		7.0		1	0	17.5
865	Potassium acetate	10.0		1	0	12.5
866	Potassium bromide	7.0		1	0	17.5
867		7.5		1	1	0
868		6.0		1	0	12.5
869		4.0		1	0	20.0
870	Potassium carbonate	9.0		1	0	12.5
871		5.0		1	0	12.5
872		7.0		1	1	0
873	Potassium chloride	4.0		1	0	10.0
874		6.0		1	1	0
875	Potassium nitrate	7.5		1	1	0
876		9.0		1	0	0
877		6.0		1	1	0
878	Potassium phosphate, monobasic	7.0		1	1	0
879		10.0		1	0	12.5
880		6.0		1	1	0
881		9.0		1	1	0
882	Potassium thiocyanate	10.0		1	1	0
883		4.0		1	1	0
884		9.0		1	1	0
885	Rubidium chloride	4.0		1	0	20.0
886		7.0		1	0	20.0
887		5.0		1	0	12.5
888	Sodium bromide	8.0		1	0	22.5
889		7.0		0	0	12.5
890	Sodium chloride	7.5		1	1	0
891	Sodium molybdate dihydrate	8.0		1	0	25.0
892	Sodium nitrate	6.0		1	1	0
893		8.0		1	0	17.5
894	Sodium phosphate, monobasic	5.0		1	1	0
895		6.0		0	0	22.5
896		8.0		1	0	22.5
897	Sodium thiosulfate pentahydrate	8.0		0	0	22.5
898		9.0		1	1	0
899		10.0		1	0	7.5
900	Zinc acetate	8.0.		1	1	0
901		6.0		1	1	0
902		5.0		1	1	0
903	Potassium phosphate, dibasic	5.0		1	1	0
904		10.0		1	1	0
905	Lithium sulfate monohydrate	7.5		1	1	0
906		10.0		1	0	22.5
907	Potassium phosphate, tribasic	5.0		1	1	0
908		7.0		1	1	0
909	Ammonium thiocyanate	9.0		1	0	12.5
910		7.5		1	1	0
911		8.0		1	0	10.0
912		4.0		1	0	22.5
913	Magnesium nitrate hexahydrate	7.5		1	0	12.5
914		9.0		1	1	0
915	Ammonium bromide	5.0	80%	1	1	0
916		7.0		1	1	0
917	Ammonium chloride	5.0		1	1	0
918		8.0		1	1	0
919	Ammonium nitrate	7.5	60%	1	1	0
920	Ammonium phosphate, monobasic	8.0	20% PEG 400	0	0	30.0
921		9.0		0	0	25.0
922	Ammonium phosphate, dibasic	5.0		0	0	25.0
923		7.0		0	0	27.5
924		4.0		0	0	27.5
925		8.0		0	0	25.0
926	Ammonium sulfate	9.0		0	0	25.0
927	Calcium acetate	5.0		0	0	25.0
928	HR-Cryo-21			1	1	0
929	Calcium chloride dihydrate	7.5	80%	1	1	0
930	Lithium bromide	8.0		1	1	0
931		10.0	20%	0	0	25.0
932	Lithium chloride	7.0	80%	1	1	0
933	Magnesium acetate	7.0		1	1	0
934	Magnesium chloride hexahydrate	8.0		1	1	0
935		7.0		1	1	0
936	Magnesium sulfate heptahydrate	5.0	20%	0	0	25.0
937		7.5		0	0	27.5
938	Manganese chloride	7.0	80%	1	1	0
939	Potassium acetate	9.0		1	1	0
940	Manganese chloride	8.0		1	1	0
941	Potassium acetate	5.0		1	1	0
942		10.0	20%	0	0	22.5
943		6.0	80%	1	1	0
944		7.0		1	1	0
945	Potassium bromide	9.0		1	1	0
946	Potassium carbonate	7.0	60%	1	1	0
947	Potassium chloride	8.0	80%	1	1	0
948		7.0		1	1	0
949	Potassium nitrate	10.0	20%	0	0	27.5
950		7.0	80%	0	0	22.5
951	Potassium phosphate, monobasic	5.0	20%	0	0	25.0
952	Potassium thiocyanate	7.5	80%	1	1	0
953		10.0	20%	1	0	27.5
954	Rubidium chloride	7.5	80%	1	1	0
955	Sodium bromide	5.0		1	1	0
956		7.5		1	1	0
957	Sodium chloride	5.0		1	1	0
958		9.0		1	1	0
959		4.0	60%	1	1	0
960	Sodium molybdate dihydrate	8.0		1	0	7.5
961		9.0		1	1	0
962	Sodium nitrate	10.0	20%	0	0	25
963		5.0	80%	1	1	0
964		7.0		1	1	0
965	Sodium phosphate, monobasic	10.0	20%	0	0	25
966	HR-Cryo-22			1	0	17.5
967	Sodium thiosulfate pentahydrate	8.0	80%	1	1	0
968		6.0		1	1	0
969	Zinc acetate	8.0		1	1	0
970	Potassium phosphate, dibasic	6.0	20%	0	0	27.5
971		5.0		0	0	27.5
972	Cobalt sulfate heptahydrate	5.0		0	0	27.5
973		5.0	40%	1	1	0
974		8.0	20%	0	0	27.5
975		4.0		0	0	25.0
976	Lithium sulfate monohydrate	8.0	60%	1	1	0
977	Potassium phosphate, tribasic	4.0	20% PEG 400	0	0	27.5
978		8.0		0	0	22.5
979		10.0		0	0	25.0
980	Manganese sulfate monohydrate	4.0		0	0	27.5
981		6.0		0	0	30.0
982		5.0		0	0	30.0
983	Magnesium nitrate hexahydrate	6.0	60%	1	1	0
984		7.5	80%	1	1	0

Figure 7
PEG 400 cocktail conditions (Nos. 840–984), similar to Fig. 2. Note that within these conditions several Hampton Research Crystal Screen Cryo screens are also included: Nos. 855, 928 and 966.

Table 1
Summary of the cryoprotection needed for the different components of the first two groups of the HWI crystallization cocktails as described in Figs. 1–7.

The data are tabulated excluding results from the Crystal Screen Cryo cocktails distributed through the first 984 cocktails. The cryoprotectant concentrations are final concentrations (*v*/*v*).

			Cocktail and ddH$_2$O		Cocktail solution with 1:1 cryoprotectant					
		No. of cocktails	0%	50%	30%	25%	20%	15%	10%	5%
Salts (1–237)	All	233	16.9%	5.5%	94.0%	75.5%	16.3%	10.7%	7.7%	6.9%
PEG 20K (238–390)	All	141	35.3%	1.3%	100%	92.9%	36.2%	24.8%	2.8%	1.4%
	20%	81	0%	0%	100%	87.7%	0%	0%	0%	0%
	40%	60	93.3%	3.3%	100%	100%	85.0%	58.3%	6.7%	3.3%
PEG 8K (391–546)	All	153	37.9%	4.6%	100%	92.8%	34.6%	19.6%	5.2%	4.6%
	20%	83	1.2%	0%	100%	86.7%	0%	0%	0%	0%
	40%	70	81.4%	10.0%	100%	100%	74.3%	42.9%	11.4%	10.0%
PEG 4K (547–694)	All	148	42.6%	6.8%	100%	90.5%	37.2%	25.7%	8.1%	6.8%
	20%	75	0%	0%	100%	82.4%	0%	0%	0%	0%
	40%	73	86.3%	13.7%	100%	100%	75.3%	52.1%	16.4%	13.7%
PEG 1K (695–839)	All	145†	39.6%	4.1%	100%	91.7%	42.8%	21.4%	7.6%	4.1%
	20%	72	0%	0%	100%	84.9%	0%	0%	0%	0%
	40%	72	80.3%	8.5%	100%	100%	87.3%	43.7%	15.5%	8.5%
PEG 400 (840–984)	All	142	76.0%	46.5%	100%	90.1%	72.5%	59.2%	50.0%	46.5%
	20%	28	0%	0%	100%	51.9%	0%	0%	0%	0%
	40%	75	90.7%	39.5%	100%	100%	86.7%	61.3%	44.0%	38.7%
	60%	7	100%	85.7%	100%	100%	100%	100%	85.7%	85.7%
	80%	32	100%	100%	100%	100%	100%	100%	100%	100%
1–984 (962 excluding Crystal Screen Cryo)		962	39.0%	11.1%	98.8%	85.4%	37.8%	25.4%	12.9%	11.1%

† One condition is 10% PEG 1K.

tration of 14.9% was required. PEG 1K (Fig. 6) was similar; at 20%(*v*/*v*) PEG (72 conditions) the average cryoprotectant concentration was 24.5% and for 40%(*v*/*v*) PEG (72 conditions) it was 15.3%. The reduction in cryoprotectant concentration required for vitrification of 20% and 40% PEG for the 20K, 8K, 4K and 1K PEGs were similar.

The PEG 400 group (Fig. 7) was more complex in composition and sampled 20% (28 conditions), 40% (75 conditions), 60% (seven conditions) and 80%(*v*/*v*) (32 conditions) PEG 400 with glycerol. The concentrations of glycerol required averaged 26.3, 10.43, 1.1 and 0%(*v*/*v*), respectively. Unfortunately, comparison with the other PEGs in the screen is difficult as PEG 400 is sampled at a larger number of concentrations but at a reduced number of chemical conditions. We can say that the 20% PEG 400 conditions required cryoprotectant concentrations comparable to similar conditions in the other PEG screens. It is also noticeable that at 40% PEG 400 the concentration of cryoprotectant required is significantly less than that of the higher molecular-weight PEGs.

In the case of the Ammonium Sulfate Grid Screen (Fig. 8) there was a small decrease in the cryoprotectant required with an increasing concentration of ammonium sulfate and no apparent pH effect. Similarly, as shown in Fig. 9, as the PEG 6000 concentration increased there is a slight decrease in the cryoprotectant needed. The most dramatic effect arises from

#	Chemical (*M*)		Buffer (0.1 *M*)	pH	%		
1201	Ammonium sulfate	0.8	Citric acid	4	0	0	30.0
1202			Citric acid	5	0	0	30.0
1203			MES	6	0	0	27.5
1204			HEPES	7	0	0	30.0
1205			Tris	8	0	0	30.0
1206			Bicine	9	0	0	27.5
1207		1.6	Citric acid	4	0	0	27.5
1208			Citric acid	5	0	0	25.0
1209			MES	6	0	0	25.0
1210			HEPES	7	0	0	27.5
1211			Tris	8	0	0	25.0
1212			Bicine	9	0	0	27.5
1213		2.4	Citric acid	4	0	0	22.5
1214			Bitric acid	5	0	0	22.5
1215			MES	6	0	0	22.5
1216			HEPES	7	0	0	22.5
1217			Tris	8	0	0	22.5
1218			Bicine	9	0	0	22.5
1219		3.2	Citric acid	4	0	0	25.0
1220			Citric acid	5	0	0	22.5
1221			MES	6	0	0	22.5
1222			HEPES	7	0	0	22.5
1223			Tris	8	0	0	22.5
1224			Bicine	9	0	0	22.5

Figure 8
Hampton Research Grid Screen Ammonium Sulfate, cocktail Nos. 1201–1224.

No.	Chemicals (0.1 *M*)		pH	Chemicals		%		
1105	1.0 *M* Lithium chloride	Citric acid	4			0	0	30.0
1106			5			0	0	30.0
1107		MES	6			0	0	30.0
1108		HEPES	7			0	0	30.0
1109		Tris	8			0	0	27.5
1110		Bicine	9			0	0	27.5
1111		Citric acid	4	PEG 6000	10%(*w*/*v*)	0	0	25.0
1112		Citric acid	5			0	0	25.0
1113		MES	6			0	0	27.5
1114		HEPES	7			0	0	27.5
1115		Tris	8			0	0	30.0
1116		Bicine	9			0	0	22.5
1117		Citric acid	4		20%(*w*/*v*)	0	0	25.0
1118		Citric acid	5			0	0	22.5
1119		MES	6			0	0	22.5
1120		HEPES	7			0	0	22.5
1121		Tris	8			0	0	22.5
1122		Bicine	9			0	0	22.5
1123		Citric acid	4		30%(*w*/*v*)	0	0	22.5
1124		Citric acid	5			0	0	22.5
1125		MES	6			0	0	22.5
1126		HEPES	7			1	0	20.0
1127		Tris	8			0	0	22.5
1128		Bicine	9			0	0	22.5

Figure 9
Hampton Research Grid Screen PEG/LiCl, cocktail Nos. 1105–1128. With the exception of PEG and LiCl, all chemicals are at 0.1 *M* concentration.

Table 2
The percentage of cocktails from commercial screens used in the 1536-condition HWI high-throughput screening laboratory that show cryoprotectant properties without dilution, diluted 1:1 with ddH_2O and diluted 1:1 with 20, 10 and 5% glycerol solution.

Note that those conditions that did not require cryoprotectant at 1:1 dilution with ddH_2O are not counted in the figures for those requiring cryoprotectant. The cryoprotectant numbers are cumulative, *i.e.* the 20% cryoprotectant numbers also encompass those that were successful with 10% and 5% cryoprotectant.

		Conditions successfully cryoprotected				
		Cocktail solution and ddH_2O		Glycerol concentration		
Hampton Research Screen name	No. of conditions	0%	50%	20%	10%	5%
Natrix	48	10.4%	4.2%	18.8%	0.0%	0.0%
Quick Screen	24	0.0%	0.0%	0.0%	0.0%	0.0%
Nucleic Acid	24	25.0%	0.0%	0.0%	0.0%	0.0%
Sodium Malonate	24	33.3%	4.2%	33.3%	4.2%	0.0%
PEG/LiCl	24	4.2%	0.0%	4.2%	0.0%	0.0%
PEG/Ion	48	0.0%	0.0%	0.0%	0.0%	0.0%
PEG 6000	24	0.0%	0.0%	20.8%	0.0%	0.0%
Ammonium Sulfate	24	0.0%	0.0%	0.0%	0.0%	0.0%
Sodium chloride	24	0.0%	0.0%	4.2%	0.0%	0.0%
Crystal Screen HT	96	21.8%	6.2%	57.3%	11.0%	5.2%
Index HT	96	20.8%	6.2%	45.8%	5.2%	0.1%
Salt RX	96	19.8%	3.1%	9.4%	0.0%	0.0%
All	552	14.5%	3.2%	23.9%	3.0%	0.9%

the reduction of solution volume (cryoloop size). In each case (Figs. 10–15) there is a clear trend in the reduction of cryoprotectant required as a function of the cryoloop size. All of the cocktails studied still required cryoprotectant, even for the smallest cryoloop size.

Loop size (mm)	Glycerol concentration [%(*v*/*v*)]						
	30	25	20	15	10	5	0
0.7–1.0	-	-	-	-	X	X	X
0.5–0.7	-	-	-	-	X	X	X
0.4–0.5	-	-	-	-	X	X	X
0.3–0.4	-	-	-	-	-	X	X
0.2–0.3	-	-	-	-	-	X	X
0.1–0.2	-	-	-	-	-	-	X
0.05–0.1	-	-	-	-	-	-	X

Figure 10
1.14 *M* ammonium sulfate pH 6. 'X' indicates the observation of ice, while '–' indicates that vitrification was visually successful.

Loop size (mm)	Glycerol concentration [%(*v*/*v*)]						
	30	25	20	15	10	5	0
0.7–1.0	-	-	-	X	X	X	X
0.5–0.7	-	-	-	-	X	X	X
0.4–0.5	-	-	-	-	X	X	X
0.3–0.4	-	-	-	-	X	X	X
0.2–0.3	-	-	-	-	X	X	X
0.1–0.2	-	-	-	-	-	X	X
0.05–0.1	-	-	-	-	-	-	X

Figure 11
20% PEG 20 000, 0.1 *M* lithium chloride pH 10. 'X' indicates the observation of ice, while '–' indicates that vitrification was visually successful.

The results for the 984-condition incomplete factorial screen are summarized in Fig. 16. They are broken down into the salt and PEG groups. Within the PEG group, the results are broken down as a function of the PEG concentration. Cryoprotectant conditions for the remaining cocktails (the Hampton Research Natrix, Quick Screen, Nucleic Acid Mini

Loop size (mm)	Glycerol concentration [%(*v*/*v*)]						
	30	25	20	15	10	5	0
0.7–1.0	-	X	X	X	X	X	X
0.5–0.7	-	-	X	X	X	X	X
0.4–0.5	-	-	X	X	X	X	X
0.3–0.4	-	-	-	-	-	X	X
0.2–0.3	-	-	-	-	-	X	X
0.1–0.2	-	-	-	-	-	X	X
0.05–0.1	-	-	-	-	-	-	X

Figure 12
20% PEG 8000, 0.1 *M* calcium acetate pH 6. 'X' indicates the observation of ice, while '–' indicates that vitrification was visually successful.

Loop size (mm)	Glycerol concentration [%(*v*/*v*)]						
	30	25	20	15	10	5	0
0.7–1.0	-	X	X	X	X	X	X
0.5–0.7	-	-	-	X	X	X	X
0.4–0.5	-	-	-	-	X	X	X
0.3–0.4	-	-	X	X	X	X	X
0.2–0.3	-	-	-	X	X	X	X
0.1–0.2	-	-	-	-	X	X	X
0.05–0.1	-	-	-	-	-	-	X

Figure 13
20% PEG 4000, 0.1 *M* ammonium sulfate pH 7.5. 'X' indicates the observation of ice, while '–' indicates that vitrification was visually successful.

Loop size (mm)	Glycerol concentration [%(*v*/*v*)]						
	30	25	20	15	10	5	0
0.7–1.0	-	-	X	X	X	X	X
0.5–0.7	-	-	X	X	X	X	X
0.4–0.5	-	-	-	X	X	X	X
0.3–0.4	-	-	X	-	X	X	X
0.2–0.3	-	-	-	-	X	X	X
0.1–0.2	-	-	-	-	X	X	X
0.05–0.1	-	-	-	-	-	-	X

Figure 14
20% PEG 1000, 0.1 *M* potassium phosphate tribasic pH 7.0. 'X' indicates the observation of ice, while '–' indicates that vitrification was visually successful.

Loop size (mm)	Glycerol concentration [%(*v*/*v*)]						
	30	25	20	15	10	5	0
0.7–1.0	-	-	X	X	X	X	X
0.5–0.7	-	-	X	X	X	X	X
0.4–0.5	-	-	-	X	X	X	X
0.3–0.4	-	-	-	-	X	X	X
0.2–0.3	-	-	-	-	X	X	X
0.1–0.2	-	-	-	-	X	X	X
0.05–0.1	-	-	-	-	-	-	X

Figure 15
20% PEG 400, 0.1 *M* ammonium phosphate dibasic pH 4.0. 'X' indicates the observation of ice, while '–' indicates that vitrification was visually successful.

Screen, Grid Screens Sodium Malonate, PEG/Ion Screen, PEG 6000, Sodium Chloride, Index and SaltRx) are shown in Table 1. For the Quick Screen and PEG/Ion screen no cocktails could be satisfactorily cryoprotected with 20% glycerol or less.

Table 1 breaks down the results into the percentages of cocktail groups 1 and 2 that were successfully vitrified without cryoprotectant, diluted 1:1 with ddH_2O and finally as a function of the 1:1 dilution with different concentrations (*v*/*v*) of glycerol in ddH_2O. The latter represents the final cryoprotectant concentration and is cumulative, *e.g.* a cocktail vitrified with 10% cryoprotectant is also counted as successful with higher concentrations. Of the 962 cocktails in groups 1 and 2 (excluding the Crystal Screen Cryo cocktails), ~40% were natively cryoprotected, ~38% were cryoprotected with 20% glycerol and almost all were cryoprotected with 30%

Hampton Research Natrix, cocktail Nos. 985–1032

No.	Salt (*M*)		Buffer	Precipitant		pH	Other	100%	50%	%
985	$MgCl_2$	0.01	MES	$Li_2SO_4.H_2O$	2.0 *M*	5.5		0	0	20
987	Magnesium acetate	0.1		MPD	20%(*v*/*v*)			0	0	20
996	Magnesium sulfate	0.01	Sodium cacodylate	$Li_2SO_4.H_2O$	1.8 *M*	6		0	0	20
1000	Magnesium acetate	0.04		MPD	30%(*v*/*v*)			1	1	0
1011	Ammonium acetate	0.2		PEG 8000	30%(*w*/*v*)	6.5	0.1*M* magnesium acetate	0	0	20
1013	Magnesium chloride	0.01	Sodium HEPES	LiCl	4.0 *M*	7		1	0	–
1015		0.005		PEG MME 550	25%(*v*/*v*)			1	0	20
1016	Potassium chloride	0.2		1,6-Hexanediol	20%(*w*/*v*)		0.01 *M* $MgCl_2$	0	0	20
1017	Ammonium chloride	0.2			30%(*w*/*v*)			0	0	20
1018	Potassium chloride	0.1		MPD	15%(*v*/*v*)		0.005 *M* $MgSO_4$ aqueous	1	0	20
1030	$MgSO_4$ aqueous	0.005	Tris hydrochloride	1,6-Hexanediol	35%(*w*/*v*)	8.5		1	1	0
1032	Ammonium chloride	0.2		PEG 4000	30%(*w*/*v*)		0.01 *M* $CaCl_2$	0	0	20

Hampton Research Quick Screen, cocktail Nos. 1033–1057

No conditions found at 20% or less cryoprotectant

Hampton Research Nucleic Acid Mini Screen cocktail Nos. 1058–1080

No.	Chemical (m*M*)		Buffer	pH	Chemical			100%	50%	%
1061	Potassium chloride	80	40 m*M* sodium cacodylate	6	10% MPD	12 m*M* spermine tetra-HCl	20 m*M* magnesium chloride	1	0	-
1066	Sodium chloride	12					80 m*M* potassium chloride	1	0	-
1071	Potassium chloride	80		7				1	0	-
1075	Sodium chloride	12					80 m*M* potassium chloride	1	0	-
1078	Lithium chloride	40					80 m*M* $SrCl_2$, 20 m*M* $MgCl_2$	1	0	-
1080	Strontium chloride	80					20 m*M* magnesium chloride	1	0	-

Hampton Research Grid Screen Sodium Malonate, conditions 1081–1104

No.	Chemical	*M*	pH	100%	50%	%	No.	*M*	pH	100%	50%	%
1086	Sodium malonate	3.4	4	1	0	12.5	1098	3.4	6	1	0	20.0
1090		2.4	5	0	0	20.0	1102	2.4	7	1	0	20.0
1091		2.9		1	0	20.0	1103	2.9		1	0	20.0
1092		3.4		1	0	12.5	1104	3.4		1	1	0
1097		2.9	6	1	0	10.0						

Hampton Research Grid Screen PEG/LiCl, cocktail Nos. 1105–1128

Data available in Fig. 9.

Hampton Research Grid screen PEG/Ion Screen, cocktail Nos. 1129–1176

No conditions found at 20% or less cryoprotectant

Hampton Research Grid Screen PEG 6000, cocktail Nos. 1177–1200

No.	Buffer (0.1 *M*)	pH	Chemicals		100%	50%	%
1195	Citric acid	4	PEG 6000	30%(*w*/*v*)	0	0	20.0
1196		5			0	0	20.0
1197	MES	6			0	0	20.0
1198	HEPES	7			0	0	20.0
1200	Bicine	9			0	0	20.0

Hampton Research Grid Screen Ammonium Sulfate, cocktail Nos. 1201–1224

Data available in Fig. 8

Hampton Research Grid Screen Sodium Chloride, cocktail Nos. 1225–1248

No.	Chemical (*M*)		Buffer (0.1 *M*)	pH	100%	50%	%
1243	Sodium chloride	4.0 *M*	Citric acid	4	0	0	20.0

Hampton Research, Crystal Screen HT, cocktail Nos. 1249–1344

Data available in McFerrin & Snell (2002)

Figure 16
Components of the commercial screens used in the 1536 cocktails that could be successfully cryocooled with 20% glycerol or less or showed cryoprotectant properties alone and after 1:1 dilution with H_2O. For brevity, the cocktails that were not successfully vitrified are omitted.

Hampton Research Index, cocktail Nos. 1345–1440								
No.	**Chemical (*M*)**		**Buffer (0.1 *M*)**	**pH**	**Chemical**	**100%**	**50%**	**%**
1345	Ammonium sulfate	2.0	Citric acid	3.5		0	0	20.0
1364	Tri-sodium citrate dihydrate	1.4	HEPES	7.5		0	0	20.0
1365	Tri-ammonium citrate pH 7.0	1.8				0	0	20.0
1367	DL-Malic acid pH 7.0	2.1				1	0	10.0
1368	Sodium acetate trihydrate pH 7.0	2.8				0	0	20.0
1369	Sodium formate pH 7.0	3.5				0	0	20.0
1370	Di-ammonium tartrate pH 7.0	1.1				1	0	20.0
1371	Sodium malonate pH 7.0	2.4				1	1	0
1380	Tacsimate pH 7.0	15%	HEPES	7.0	2%(*w*/*v*) PEG 3350	0	0	20.0
1381					25%(*w*/*v*) PEG 1500	0	0	20.0
1382					30%(*v*/*v*) Jeffamine M-600 reagent pH 7.0	1	0	10.0
1383					30%(*v*/*v*) Jeffamine ED-2001 reagent pH 7.0	0	0	20.0
1384			Citric acid	3.5	25%(*w*/*v*) PEG 3350	0	0	20.0
1386			Bis-tris	5.5		0	0	20.0
1388			HEPES	7.5		1	0	20.0
1389			Tris	8.5		0	0	20.0
1391					28%(*w*/*v*)	1	0	20.0
1393	Calcium chloride	0.2	Bis-tris	6.5	45%(*w*/*v*) MPD	1	1	0
1394	Ammonium acetate			5.5		1	1	0
1395				6.5		1	0	10.0
1396			HEPES	7.5		1	1	0
1397			Tris	8.5		1	1	0
1398	Calcium chloride	0.05	Bis-tris	6.5	30%(*v*/*v*) PEG MME 550	1	0	20.0
1399	Magnesium chloride	0.05	HEPES	7.5		1	1	0
1400	Potassium chloride	0.2		7.5	Pentaerythritol propoxylate (5/4 PO/OH)	1	0	10.0
1401	Ammonium sulfate	0.05	Bis-tris	6.5	Pentaerythritol ethoxylate (15/4 EO/OH)	1	0	20.0
1402				6.5	45%(*v*/*v*) polypropylene glycol P 400	1	0	10.0
1403	Magnesium chloride	0.02	HEPES	7.5	22%(*w*/*v*) polyacrylic acid 5100 sodium salt	1	0	10.0
1404	Cobalt chloride	0.1	Tris	8.5	20%(*w*/*v*) polyvinylpyrrolidone K15	1	0	20.0
1406	Trimethylamine *n*-oxide	0.20	Tris	8.5	20%(*w*/*v*) PEG MME 2000	0	0	20.0
1412	Ammonium sulfate		HEPES	7.5	25% (*w*/*v*) PEG 3350	0	0	20.0
1414	Sodium chloride		Bis-tris	5.5		0	0	20.0
1415				6.5		0	0	20.0
1416			HEPES	7.5		0	0	20.0
1417			Tris	8.5		0	0	20.0
1418	Lithium sulfate		Bis-tris	5.5		0	0	20.0
1419				6.5		0	0	20.0
1420			HEPES	7.5		0	0	20.0
1421			Tris	8.5		0	0	20.0
1422	Ammonium acetate		Bis-tris	5.5		0	0	20.0
1423				6.5		0	0	20.0
1424			HEPES	7.5		0	0	20.0
1426	Magnesium chloride		Bis-tris	5.5		0	0	20.0
1427				6.5		1	0	20.0
1428			HEPES	7.5		0	0	20.0
1429			Tris	8.5		1	0	20.0
1435	DL-Malic acid pH 7.0	0.15			20%(*w*/*v*) PEG 3350	0	0	20.0
1438	Tri-sodium citrate	0.20				0	0	20.0
1439	Potassium thiocyanate	0.10			30%(*w*/*v*) PEG MME 2000	0	0	20.0
1440	Potassium bromide	0.15				0	0	20.0

Hampton Research SaltRX, cocktail Nos. 1441–1536.							
No.	**Salt (*M*)**		**Buffer (0.1 *M*)**	**pH**	**100%**	**50%**	**%**
1442	Sodium acetate	2.8	Bis-tris propane	7.0	1	0	-
1452	Sodium chloride	3.2	Sodium acetate	4.6	0	0	-
1453			Bis-tris propane	7.0	1	0	20.0
1458	Tri-ammonium citrate pH 7.0	2.0			1	0	20.0
1461	Tri-sodium citrate dihydrate	1.2			1	0	-
1467	Sodium formate	2.0	Sodium acetate	4.6	1	0	-
1468			Bis-tris propane	7.0	1	0	-
1470		3.5	Sodium acetate	4.6	1	0	20.0
1472			Tris	8.5	1	0	
1474	DL-Malic acid pH 7.0	2.2	Bis-tris propane	7.0	1	0	12.5
1476	Sodium malonate pH 7.0	2.4			1	0	20.0
1478	Ammonium nitrate	2.5			1	0	-
1488	Sodium nitrate	4.0	Tris	8.5	1	0	-
1512	Lithium sulfate monohydrate	1.5			0	0	20.0
1513	Magnesium sulfate hydrate	1.0	Sodium acetate	4.6	1	1	0
1514			Bis-tris propane	7.0	1	0	-
1523	Di-ammonium tartrate	1.3			1	0	-
1531	Potassium thiocyanate	0.5	Tris	8.5	0	0	20.0
1532	Ammonium acetate	4.0	Sodium acetate	4.6	1	0	20.0
1533			Bis-tris propane	7.0	1	1	0
1534			Tris	8.5	1	1	0
1536	Tacsimate	60%(*v*/*v*)	Bis-tris propane	7.0	1	0	20.0

Figure 16 (continued)

glycerol. There was a sharp increase in cryoprotection going from 20% to 30% glycerol. The commercial screens (Table 2) were not as well suited to cryoprotection, with only 14.5% natively cryoprotected and 24% protected with 20% glycerol. This should not be construed as a criticism of the commercial screens, since cryoprotection was not a factor in their design.

4. Discussion

Cryocooling for X-ray data collection requires transforming the crystal and any mother liquor surrounding it into an amorphous form, *i.e.* vitrification. Vitrifying pure water, even for the smallest volumes, requires cooling to below 136 K (Mayer, 1991) in less than 10^{-4} s (Bruggeller & Mayer, 1980; Mayer, 1988). Glycerol is thought to work as a cryoprotectant by causing bulk water depletion and hydrogen-bond linearization and by increasing alkyl backbone interactions within the macromolecule (Dashnau *et al.*, 2006). There are many cryoprotectants available, but in addition to its cryoprotective properties glycerol is an effective enhancer of both macromolecular structural order and stabilizes against noncovalent modification (Gekko & Timasheff, 1981; Priev *et al.*, 1996; Sousa, 1995). Practically, glycerol can be formulated as a component in the storage-buffer component and on crystallization it can be readily incorporated into the crystal lattice, effectively displacing water (Charron *et al.*, 2002). The Hetero-compound Information Centre (HIC-Up; Kleywegt, 2007) lists over 2280 macromolecules in the Protein Data Bank (PDB; Berman *et al.*, 2000) in which glycerol is observed within the structure. Ethylene glycol is the next most common cryoprotectant and is observed in over 700 structures. Similarly, a survey of crystallization reports published in *Acta Crystallographica Section D* in 2000 and 2001 showed that glycerol was used in 50% and ethylene glycol was used in 10% of cases (Garman & Doublié, 2003). This does not necessarily imply that glycerol is the best cryoprotectant to use. For reasons of convenience it is often the first; if it works, no further optimization is carried out (Garman & Doublié, 2003).

McFerrin & Snell (2002) determined the amounts of glycerol, PEG 400, ethylene glycol and 1,2-propanediol needed to successfully vitrify the 98 Hampton Research Crystal Screen I and II conditions. In comparing the concentration of glycerol required for vitrification *versus* other cryoprotectants, there were differences in a small number of samples, *e.g.* Crystal Screen I condition No. 44 (0.2 *M* magnesium formate) required 50% glycerol but only 35%, 30% and 30% PEG 400, ethylene glycol and 1,2-propanediol, respectively. However, the average magnitudes of the difference in cryoprotectant concentration when compared with glycerol were 4.0%, 3.2% and 5.9% for PEG 400, ethylene glycol and 1,2-propanediol, respectively. The data for glycerol can thus be used as a guide for the concentration of these cryoprotectants. McFerrin and Snell also used (2*R*,3*R*)-(−)-2,3-butanediol for the nine conditions under study that required the highest concentration of glycerol. On average, 10.6% less butanediol than glycerol was required for vitrification.

The cryoprotective properties of glycerol, methanol, 2-propanol, sucrose, xylitol, dextrose, trehalose, ethylene glycol, PEG 200, PEG 2K, PEG 20K, dimethyl sulfoxide (DMSO), 2-methyl-2,4-pentanediol (MPD) and salt (NaCl) with pure water have been systematically studied as a function of volume from 1 nl to 20 µl. Cryoprotectant conditions were determined for plunge-cooling into liquid nitrogen (Berejnov *et al.*, 2006). The concentration required for vitrification decreased with volume, especially in the range ∼5–0.1 µl. This range includes the typical volumes held in a sample loop and the observation is similar to previous observations that smaller loops require less cryoprotectant for vitrification (Chinte *et al.*, 2005) and is empirically well known. Berejnov *et al.* (2006) note the presence of three regimes in the cooling process: large volume and therefore slow cooling rate where the critical concentration is nearly constant, intermediate volumes where the concentration shows a sharp decrease with volume and small volumes where the cooling rate saturates and the critical cryoprotectant concentration levels off. From Figs. 10–15 it is clear that typical crystallographic samples are in the intermediate regime. The results of Berejnov and coworkers also illustrate that there are cryoprotectants, *i.e.* 2-propanol, MPD and dextrose, that successfully vitrify solutions at significantly lower concentrations than glycerol. Our results are in agreement with Berejnov *et al.* (2006) and Chinte *et al.* (2005): smaller volumes require less cryoprotectant. However, the crystal volumes required for X-ray diffraction coupled with currently available cooling technologies make it impossible to rapidly cool pure H_2O in the time required for vitrification, *i.e.* in less than 10^{-4} s, even for the smallest cases (Bruggeller & Mayer, 1980; Mayer, 1988). Unlike Chinte *et al.* (2005), we do not observe any evidence indicating that the concentration of cryoprotectant needed tends to be zero at the smallest loop size. This may be a consequence of the fact that we chose worst-case cocktails while Chinte *et al.* (2005) used a random sampling of conditions.

Cryocooling samples requires both a good cryoprotectant and good experimental technique and there are many excellent articles that cover these in detail (Pflugrath, 2004; Garman & Schneider, 1997; Garman & Owen, 2006; Garman, 1999; Rodgers, 1997; Garman & Doublié, 2003). Garman & Owen (2006) make a number of suggestions for the choice of cryoprotectant. For two-thirds of cases they suggest that 15–25% glycerol is appropriate. For conditions with PEGs less than 4K, increasing the PEG concentration or adding other low-molecular-weight PEGs is effective. PEGs greater than 4K can be cryoprotected with lower molecular-weight PEGs and crystallization conditions that already contain MPD can be cryoprotected by increasing the MPD concentration. Finally, those with salt that were not protected with glycerol can be cryoprotected with ethylene glycol, with a mixture of sugars, by increasing the salt concentration or by exchanging the salt for an organic solvent. While there are many cryoprotectants, given the ability of glycerol to form ordered conformations within the crystal structure (Charron *et al.*, 2002) and its stabilizing effect (Sousa, 1995) it seems prudent to incorporate at least a small amount during the crystallization step or earlier unless there is the potential for competition with a ligand of interest. For penetrating cryoprotectants, adding them before or during the crystallization step prevents possible disruption to the lattice by addition of the cryoprotectant after crystals have formed (Pflugrath, 2004).

5. Conclusion

In terms of high-throughput crystallization-condition screening, the data presented here provide a criterion for prioritizing subsequent optimization of crystallization conditions. However, it is important to note that the data represent a worst-case scenario for vitrification; a dilution of the cocktail with glycerol solution was used rather than replacement of the water with glycerol and larger than typical sample volumes were examined. Replacing water in the cocktail with the cryoprotectant agent maintains the original cocktail composition at the same concentration and thereby minimizes deleterious effects to the crystal (unlike the dilution used here). This is the optimum and recommended method to produce a good cryoprotectant solution (Garman, 1999). In terms of volume, a balance is required between the reduction in cryoprotectant needed owing to sample size and practical considerations for collecting X-ray data. The optimum concentration required for the collection of the best X-ray data may not be the same as that which is just sufficient for vitrification (Mitchell & Garman, 1994). Similarly, annealing techniques that could be used to improve crystal quality (Hanson *et al.*, 2003) have the potential to work well with a higher than required cryoprotectant concentration but will not work so well if the concentration is too low (Juers & Matthews, 2004). The data presented here provide a starting point for the optimization of cryoprotectant concentrations under similar biochemical conditions.

We would like to thank Erie County New York for their support of ES and KL in the HWI summer intern program. Joseph Luft is thanked for critical reading of the manuscript and the staff of the HWI high-throughput laboratory are thanked for all their help and comments. Dr Elspeth Garman is acknowledged for useful discussions. Support from the John R. Oishei Foundation and NIH U54 GM074899 is acknowledged.

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