Off to the races: rapid buffer exchange, protein quantification and stability screening with Junior, Lunatic and Uncle

Introduction

The conformational, chemical and colloidal stability of a protein are strongly influenced by the buffer solution. Altering buffer salts, pH, ionic strength, excipients and surfactants may increase or decrease the stability of a molecule. The process of screening formulation buffers to optimize stability is a time consuming process. The time required to perform buffer exchange is often a limiting factor in biologics development. Junior with buffer exchange removes the buffer exchange bottleneck by automating buffer exchange and sample concentration, which reduces required hands-on time and increases the number of samples that can be prepared in a day.

Figure 1: Junior with buffer exchange automates buffer exchange with the 96-well Unfilter. Up to 96 unique samples from 100 to 450 µL can be exchanged in a single run.

Junior automates buffer exchange with a pressure-based ultrafiltration/diafiltration (UF/DF) method for up to 96 samples in a single run (Figure 1). Junior uses the 96-well Unfilter with a 10 kDa molecular weight cutoff membrane to perform buffer exchange by applying pressure at 60 psi (Figure 2). The Unfilter is transferred

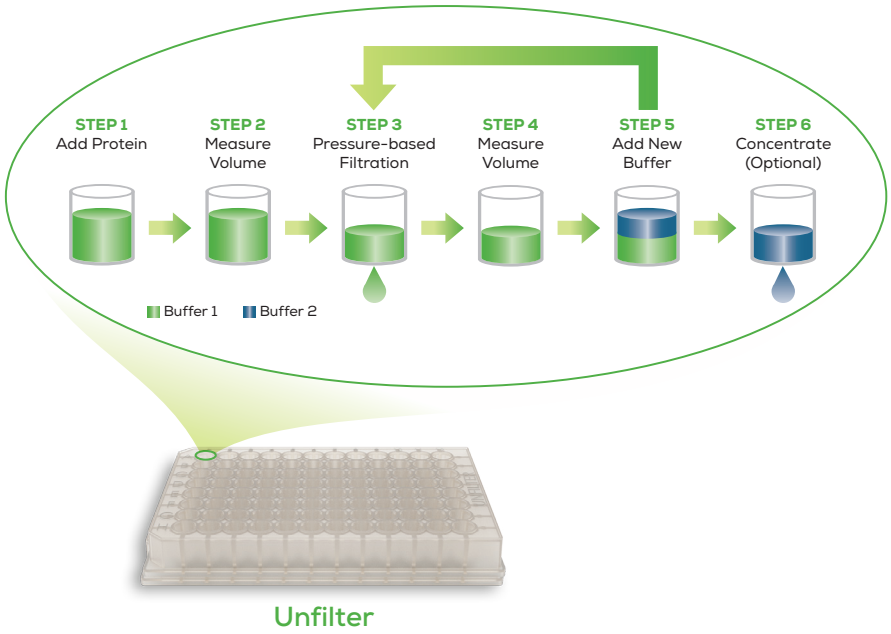

Figure 2: Junior uses a pressure based UF/DF method with gentle orbital mixing to buffer exchange proteins.

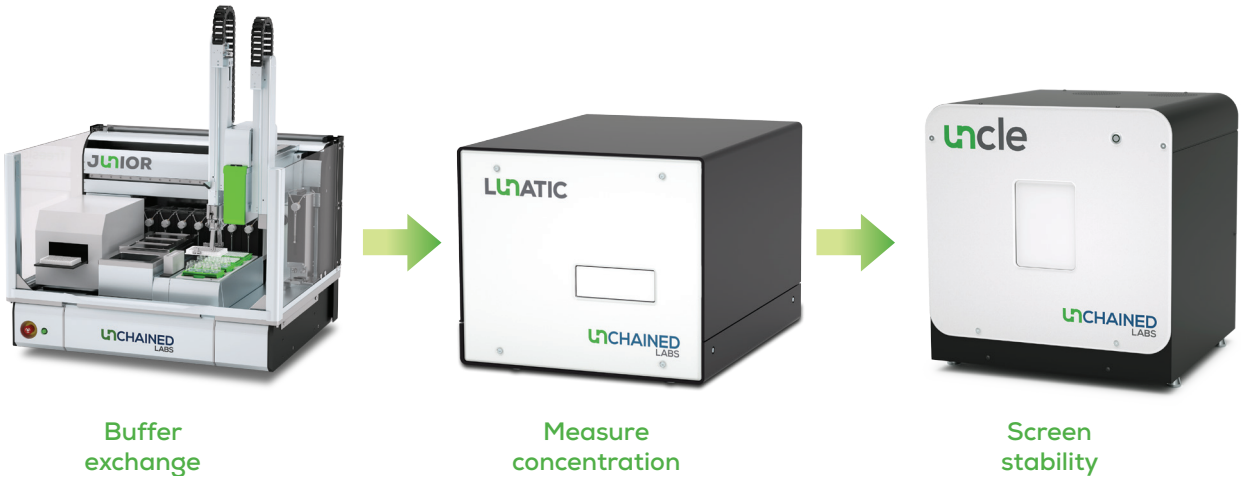

Figure 3: Buffer exchange, concentration quantification and thermal ramp stability screening can be rapidly accomplished with low volume requirements when Junior is paired with Lunatic and Uncle.

Buffer	Excipient	Samples	pH range (0.1 pH steps)	# of Final formulations
20 mM Histidine	None	1–12	6.3–7.4	12
20 mM Histidine	20 mM Sucrose	13–24	6.3–7.4	12
20 mM Histidine	100 mM NaCl	25–36	6.3–7.4	12
20 mM Histidine	25 mM Arginine	37–48	6.3–7.4	12

Table 1: Four sets of histidine buffers were prepared with different excipients at pH 6.3–7.4.

automatically between a pressure chamber, a volume check device and onboard liquid handling to perform a buffer exchange cycle. During this process, software takes data from the volume check to calculate the volume of buffer removed and the pressurization time for each cycle, optimizing buffer exchange in real time.

Buffer exchange experiments are highly customizable, making Junior a fit throughout the biologic development process. Junior can buffer exchange up to 96 unique proteins and formulations simultaneously, which complements Lunatic and Uncle, two higher throughput analytical devices that can quickly determine protein concentrations and stability, respectively. In this application note, Junior, Lunatic and Uncle are used to create a high-throughput buffer exchange, protein quantitation and stability screening process (Figure 3). Junior was used to buffer exchange 48 unique monoclonal antibody (mAb) formulations in one day. Protein concentration was quantified with a Lunatic and protein stability was assessed with a T_m and T_{agg} stability screen on Uncle. The combination of the three platforms allowed for the rapid determination of promising formulation conditions for the mAb.

Methods

A stock mAb was prepared at 25 mg/mL in 10 mM sodium succinate pH 5.3 and 450 µL was manually pipetted into each well of an Unfilter. Four sets of histidine buffers with varying excipients and pH were prepared (Table 1). The 48 buffers were pipetted in duplicate into a 2 mL, 96-well plate. The buffer exchange experiment was set up using the LEA software suite. An experimental design was created from a template in Library Studio and executed

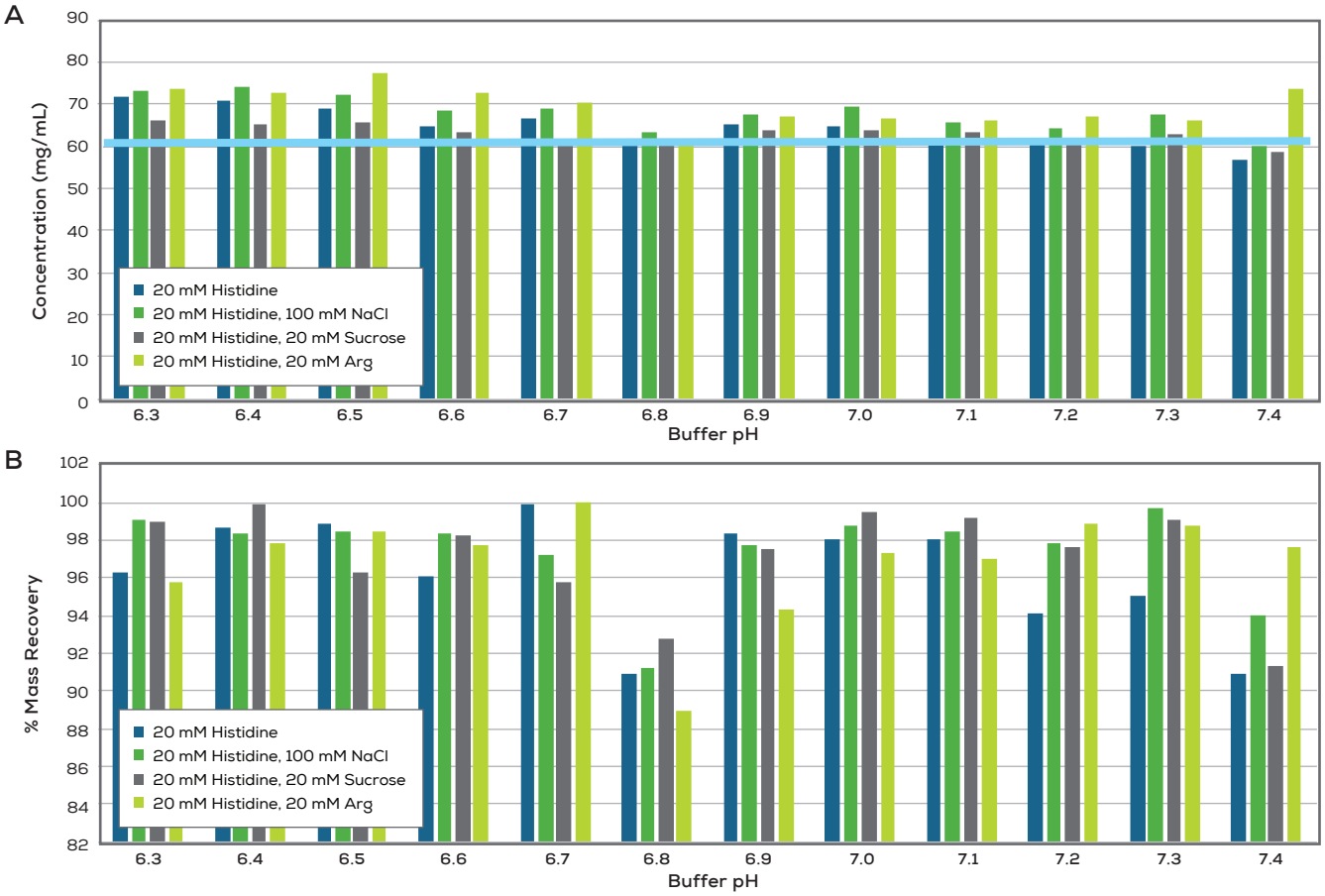

Figure 4: **A:** The final concentrations for 48 mAb formulations were measured with 2 µL of each sample on Lunatic. **B:** The protein concentrations were paired with final volumes measured by Junior to calculate protein mass recovery. All recoveries were above 88%.

in Automation Studio. Each formulation was exchanged in duplicate at ambient temperatures. Filtration pressure was set at 60 psi, buffer was removed to 66% per cycle while the Unfilter was orbitally mixed at 875 rpm. The buffer exchange target was 99%, with a final mAb concentration step set to 60 mg/mL. Once Junior completed buffer exchange, each formulation was pooled from their respective duplicate wells and stored at 4 °C.

Protein concentrations were quantitated by pipetting 2 µL of each sample into a 96-well Lunatic Plate and running the protein A280 application on Lunatic. Protein mass recovery was calculated using the A280 measurements and the final volumes reported by Junior. The pH of each formulation was determined after buffer exchange on 200 µL aliquots using a bench top Mettler-Toledo InLab Micro pH electrode. To determine the thermal stability and aggregation of the mAb in 48 formulations, a single thermal ramp experiment was performed on Uncle that simultaneously measured fluorescence and static light scattering (SLS). Each sample was diluted to 10 mg/mL with the corresponding formulation buffer. Three Unis were loaded with 9 µL of each formulation and a thermal ramp from 20–95 °C was run with a 0.25 °C/minute ramp rate. The resulting fluorescence emission spectra were analyzed with Uncle Analysis software to calculate T_m and T_{agg}.

Results

Buffer exchange

An Unfilter and a buffer plate were prepared manually offline. They were loaded onto Junior and an experimental design was created with sample and experimental conditions to run the

pH target	ΔpH 20 mM His	ΔpH 20 mM His 20 mM sucrose	ΔpH 20 mM His 100 mM NaCl	ΔpH 20 mM His 25 mM Arg
6.3	0.04	0.04	0.02	0.01
6.4	0.01	0.00	0.00	0.02
6.5	−0.01	0.02	−0.01	0.00
6.6	−0.03	0.00	0.00	0.05
6.7	−0.02	0.03	0.02	−0.01
6.8	−0.02	0.00	0.01	−0.06
6.9	−0.02	0.02	−0.03	0.03
7.0	−0.05	0.00	−0.05	−0.01
7.1	−0.05	−0.05	−0.04	−0.04
7.2	−0.12	−0.06	−0.10	−0.04
7.3	−0.16	−0.15	−0.14	−0.10
7.4	−0.19	−0.21	−0.22	−0.21

Table 2: The final pH accuracy was determined by calculating ΔpH = pH final – pH target for each final formulation.

automated buffer exchange method. Junior performed seven buffer exchange cycles to reach the 99% buffer exchange target in less than seven hours. Junior exchanged 48 unique formulations in a single work day, significantly increasing buffer exchange throughput over comparable manual methods.

Concentration and recovery

Protein concentrations were rapidly determined in a single experiment in less than 5 minutes by collecting A280 measurements on a Lunatic. Final concentrations were 56.7–77.5 mg/mL (Figure 4A). Mass recoveries were calculated using the mAb stock concentration, the final volume measurements from Junior and the final concentration readings from Lunatic. Calculated mass recoveries for all formulations were between 89% and 99% (Figure 4B). Overall, Junior exchanged 48 formulations at once within 6% of the concentration target of 60 mg/mL, while maintaining high mass recoveries.

pH

The pH of each sample was measured with a Mettler Toledo InLab Pro electrode. The difference in pH (ΔpH) between the final formulation and pH target were calculated to determine pH accuracy (Table 2). All formulations in the pH range of 6.3–7.1 were within 0.05 pH units of the target. Larger deviations from target were measured at pH 7.2–7.4, possibly caused by a change in the net charge of the mAb at higher pH.

T_m & T_{agg}

The 48 formulations were screened for stability and aggregation on Uncle. A single thermal ramp experiment was run, and intrinsic fluorescence and SLS data were simultaneously collected on 9 µL of each sample formulation. All formulations yielded a similar melting curve profile with two transitions at T_{m1} 66–69 °C and T_{m2} 74–80 °C. There was a small dip in the barycentric mean (BCM) that corresponded to

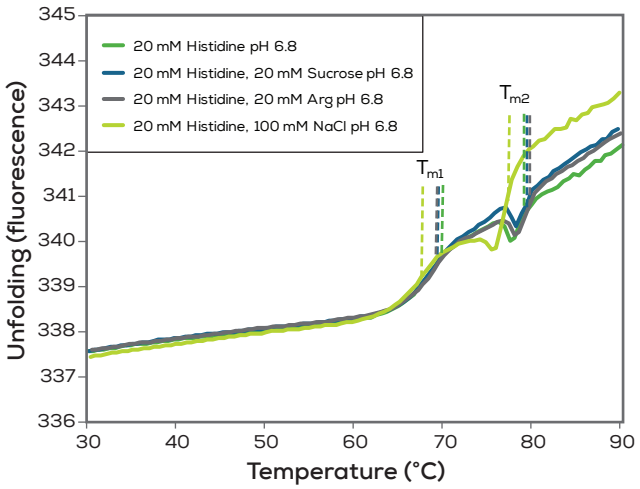

Figure 5: A representative overlay of the thermal ramp fluorescence experiments performed on Uncle. Each pH 6.8 formulation is overlaid and T_{m1}, and T_{m2} temperatures are represented by dotted lines.

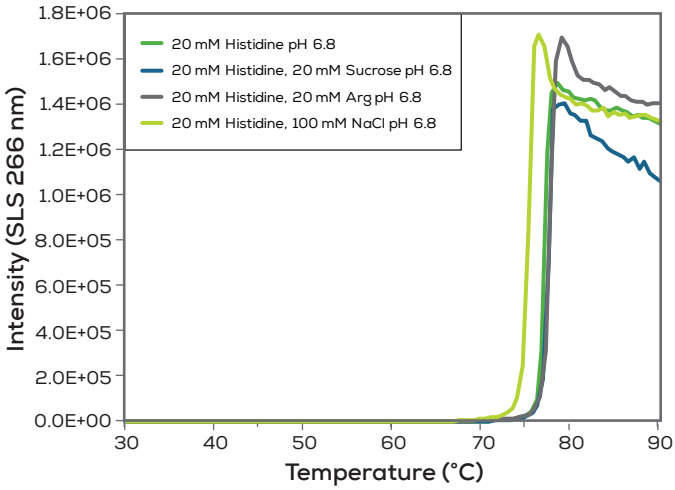

Figure 6: A representative overlay of SLS thermal ramp experiments performed on Uncle. Each pH 6.8 formulation is overlaid demonstrating the earlier onset of aggregation for formulations containing NaCl.

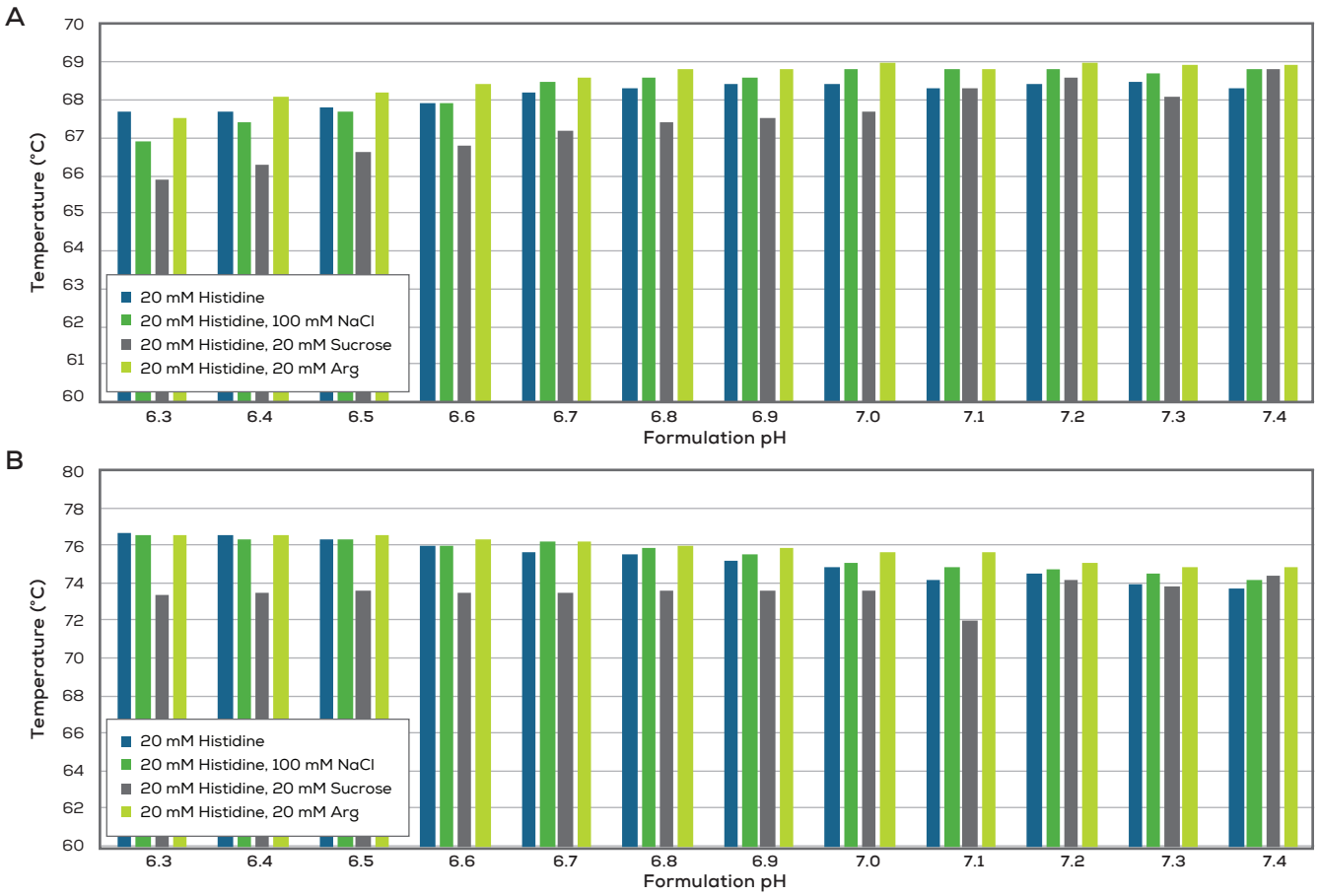

Figure 7: **A:** T_{m1} plotted by pH shows an increase in thermal stability at higher pH. **B:** T_{agg} at 266 nm indicates that aggregation also occurs earlier at higher pH.

the formation of small aggregates after the first unfolding event, as detected by SLS at 266 nm (Figures 5 & 6).

A few general trends appear when T_{m1} and T_{agg} at 266 nm are analyzed together (Figure 7). First, formulations with 100 mM NaCl have lower

Formulation	T_{m1} (°C)	T_{agg} @ 266 nm (°C)
20 mM His, pH 6.3	67.7	76.7
20 mM His, pH 6.4	67.7	76.5
20 mM His, pH 6.5	67.8	76.2
20 mM His, pH 6.6	67.9	76.0
20 mM His, pH 6.7	68.2	75.7
20 mM His, 20 mM Sucrose, pH 6.5	67.7	76.3
20 mM His, 20 mM Sucrose, pH 6.6	67.9	76.0
20 mM His, 20 mM Sucrose, pH 6.7	68.5	76.2
20 mM His, 20 mM Sucrose, pH 6.8	68.6	75.8
20 mM His, 20 mM Arg, pH 6.4	68.1	76.5
20 mM His, 20 mM Arg, pH 6.5	68.2	76.5
20 mM His, 20 mM Arg, pH 6.6	68.4	76.4
20 mM His, 20 mM Arg, pH 6.7	68.6	76.2
20 mM His, 20 mM Arg, pH 6.8	68.8	76.0
20 mM His, 20 mM Arg, pH 6.9	68.8	75.8

Table 3: The formulations with T_{m1} > 67.5 °C and T_{agg} > 75.5 °C were identified.

thermal stability and a higher propensity to aggregate than other conditions at the same pH. This indicates that NaCl is a poor excipient for the mAb. Second, melting temperatures generally increased at higher pH, while aggregation temperatures decreased at higher pH. The same trends were observed when T_{m2} and T_{agg} at 473 nm are analyzed (data not shown). A good formulation balances the risk between conformational stability and colloidal stability. To identify formulations which strike a balance between stability and aggregation formulations with T_{m1} > 67.5 °C and T_{agg} > 75.5 °C were identified (Table 3). The cutoff reduced the list of formulation candidates to 15.

Conclusion

The combination of high-throughput automated buffer exchange by Junior and the higher throughput, low-volume analytics of Lunatic and Uncle makes it possible to both rapidly prepare and assess up to 96 formulations in as little as 1.5 working days. Junior exchanged 48 unique mAb formulations, in duplicate, in less than a working day. Absorbance measurements from Lunatic confirmed the final concentration accuracy and protein mass recovery on Junior. The stability and aggregation properties of every mAb formulation were screened by T_m and T_{agg} measurements on Uncle in a single experiment, narrowing down the list of formulation candidates for further consideration. The high throughput of Junior along with the low volume requirements of Lunatic and Uncle make it easier than ever to study a wider range of conditions for biologic formulation development and optimization.

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