High-throughput process development for the chromatographic purification of viral antigens

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Running Head: HTPD purification of viral antigens

ABSTRACT

Chromatography is a widely used method in the biotechnology industry, it functions to separate the desired product from process and product related impurities. There is a multitude of resins available based on different modalities (such as charge, hydrophobicity and affinity) to provide a spectrum of approaches to meet the separation challenges of the diverse products. The challenge of developing viral antigen purification processes is addressed in this method. A unique feature of this product class is that in order to protect against more than one strain of an antigen, vaccines are often multivalent. This entails multiple production processes for each antigen, all of which will require separate development and validation. Ideally, a universal purification method is sought, but differences in the protein subunits (frequently used as the antigens) makes this challenging and often bespoke purification steps are required. This means process development for the chromatographic stages of these products can be particularly challenging and labour intensive. With the numerous choices available, making critical process decisions that are usually unique to each product, process and strain, can be costly and time-consuming. To address this, scale down purification at < 1.0 mL column volume and automation approaches are increasingly applied to increase throughput. In this work, a method is described wherein a Tecan Freedom EVO® automated liquid handler is deployed for the evaluation of different resin chemistries and buffer conditions to find a suitable purification strategy. This method allows for the rapid evaluation of the separation viral antigens where limited information on chromatography behaviour is known at the early stages of process development. Here, we demonstrate the methodology firstly by explaining the automated purification script and secondly by applying the script for an efficient purification development for different serotypes of rotavirus antigens.

Keywords: Process development, High-throughput process development (HTPD), Rotavirus antigens, Purification, Chromatography

49 **1. INTRODUCTION**

50 High-Throughput (HT) development activities are currently performed in both upstream and
51 downstream unit operations and benefit by the advancements of automated liquid handlers [1,
52 2, 15]. Such screening activities, even from the early stages of process development, can lead
53 to optimal or near optimal conditions in a systematic and efficient fashion [8, 9, 13, 14]. In
54 downstream applications, and in particular in the development of chromatographic
55 separations, the techniques employed are separated into batch and packed bed column
56 chromatography [4, 10]. Recently, applications have been developed that employ both
57 diffusive and convective media, but the latter are still missing a commercially available flow
58 mode based HT technology. While batch HT methods allow the evaluation of multiple
59 conditions in parallel, including binding capacity measurements from small product masses
60 due to the low volumes of stationary phases employed. Moreover, the miniature packed bed
61 column technique (i.e., RoboColumns) can be easily scaled-up or –down [7]. It can also
62 return an efficient way of evaluating different stationary phases due to the advantage of
63 packing any beaded based resin at a cost. This is an application that is traditionally regarded
64 to be better accomplished with batch methods, requiring however sophisticated processes for
65 preparing resin slurry multi-well plates [11]. Jacob and co-workers [5] have demonstrated
66 that it is possible to design and synthesise affinity resins for high recovery of novel influenza
67 antigens and various recombinant proteins (i.e., erythropoietin, immunoglobulins, etc.) [3, 6].
68 Therefore, such HT techniques can be employed to evaluate their performance.

69 In this instance, the scale-down performance of the miniature columns was leveraged to guide
70 development efforts in a multivariate input space, including stationary phase type and
71 operating conditions, for recombinant vaccine antigen purification. The approach relies
72 heavily on the performance of the miniature columns and as such it employs custom solutions
73 and in-house developed tools that deliver full walk-away automation across the different parts

74 of the HT study, i.e., buffer preparation, method definition, experiment completion, and
75 results reporting. The followed workflow employs a Tecan Freedom EVO® 200 automated
76 liquid handling station (Tecan Group Ltd., Männedorf, Switzerland) and MATLAB® (The
77 MathWorks, MA, USA) codes, compiled into executables, which convert user-defined inputs
78 into robotic commands. These are then executed within generic Tecan Freedom EVOware®
79 (Tecan Group Ltd.) based scripts that implement all necessary actions to complete a study
80 with no end-user intervention [7]. Here, both 0.2 and 0.6 mL columns, packed with various
81 types of resin, can be used in flowthrough or bind and elute mode, with the latter employing
82 isocratic, multi-step and multi-slope gradient elution. The workflow is demonstrated by
83 implementing an early purification strategy in scouting for multimodal resins and conditions
84 for two different strains of rotavirus antigen. A small number of well-planned experiments
85 were sufficient to elucidate a lead resin candidate and separation conditions which were then
86 found to be scalable to a larger pilot scale. Hence, the combination of HT techniques,
87 automation and a systematic screening approach can lead to attractive and feasible
88 purification process conditions in a rapid fashion.

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96 # 2. MATERIALS

97 ## 2.1 Viral Antigen

98 Rotavirus antigens are expressed in *Pichia pastoris*.

99 ## 2.2 Miniature columns

100 Miniature columns (0.2 mL and 0.6 mL) are purchased from Repligen® (Waltham,
101 Massachussets, USA). These are packed with the commercially available resins (Table 1).
102 For custom designed resins, bulk resin can be sent to Repligen which can then be packed at a
103 desired bed volume in RoboColumns.

104 ***[Table 1 near here]***

105 ## 2.3 Automated workstation

106 The employed automated liquid handling station comprises of a Tecan Freedom Evo 200
107 robot. The station is controlled by Freedom EVOware v2.6 (Tecan Group Ltd.) on an Intel i5
108 4670 CPU machine with 4 GB of RAM running Windows 7 (Microsoft Corporation, WA,
109 USA). The robot's layout is depicted schematically in Figure 1A (see Notes 1 – 3). It includes
110 an eight-channel liquid handling (LiHa) arm, using short uncoated stainless steel tips, and an
111 eccentric robot manipulator (RoMa) arm. Integrated devices include the Te-Shuttle™ and Te-
112 Chrom™ (fraction collection system) modules and an Infinite® M200 Pro plate reader
113 (Tecan Group Ltd.), capable of UV/Vis and fluorescence measurements, and operated by i-
114 control™ software (Tecan Group Ltd.). An in-depth description of the layout is found in [7].
115 Briefly, all carriers are supplied by Tecan and are responsible for holding plates and troughs
116 filled with buffers. Nine site hotel carriers are also present and these are responsible for
117 storing plates containing collected fractions and newly prepared buffers. These are used to
118 blank correct the plate reader measurements of the collected fractions (see Note 4). All carrier

119 and labware definitions are edited in EVOware in order to implement robotic operations,
120 including plate transfers and liquid handling, robustly and reliably (See Note 5). Finally, the
121 deployment of the robotic station made use of standard liquid classes which also included
122 those supplied with the Te-Chrom and Te-Shuttle modules for the dispensing into the
123 RoboColumns. Custom liquid classes are employed only for the sanitization of the stainless
124 steel tips and during the implementation of the robot's liquid detect function (See Notes 6 –
125 8).

126 **[Figure 1 near here]**

127 ## 3. METHODS

128 ### 3.1 Miniature column chromatography

129 The implementation of the miniature column technique on the aforementioned robotic station
130 follows closely the operation of bench/large scale chromatography throughout eight main
131 steps (i.e., [i] removal of storage solution, [ii] equilibration, [iii] loading, [iv] wash, [v]
132 elution, [vi] strip, [vii] regeneration, and [viii] storage). Hardware differences between HT
133 RoboColumn and conventional chromatography means that analogies need to be made. In HT
134 chromatography, solutions are transferred to the RoboColumns discretely, as opposed to
135 continuously, and they are aspirated across different locations within and between labwares.
136 In the case of gradient based separations, a gradient is first broken into a series of small steps
137 with each step being a buffer with a given composition (See Note 9). Hence, all buffers in HT
138 column experiments need to be prepared in advance at the correct volume and composition
139 and be placed in specific labwares and locations within the robotic station. Since a robot is
140 usually equipped with 8 channels, and it is not possible to mix liquids continuously, each of
141 these channels play the role of a simple inlet/outlet pump delivering liquids to, up to 8
142 columns in parallel. Moreover, in HT column chromatography, the role of a fractionator is

143 fulfilled by the Te-Shuttle module which collects effluent, or fractions, from each
144 RoboColumn to different wells in 96 well collection plates. Finally, the plate reader
145 integrated with the robot, plays the role of the detector since it reads the plates containing the
146 collected fractions at particular wavelengths and modes compatible with the capabilities of
147 the reader (See Notes 10 – 11). Figure 2 details how the different components of the robotic
148 station are used during a typical experiment with 8 RoboColumns whereas Figure 3 depicts
149 an illustrative example of collecting the first 12 elution fractions in a collection plate.

150 **[Figure 2 near here]**

151

152 **[Figure 3 near here]**

153 **3.2 Robotic buffer preparation**

154 Buffer preparation is implemented on the aforementioned robotic station and it includes both
155 stock preparation and elution buffer preparation in the case of HT column experiments
156 employing gradient elution. This employs custom written MATLAB (The MathWorks)
157 codes, compiled into executables, which are launched through in-house developed VBA tools
158 providing an interface for end-user input definition. For buffer stock preparation, the tools
159 require the specification of their conjugate acid-base pairs and salt, along with their desired
160 composition (e.g., buffer concentration, pH and salt concentration), and generate robotic
161 instructions that are loaded and executed in generic Tecan Freedom EVOware scripts and
162 result in the preparation of stocks in troughs (i.e. 100 mL). The same tools are implemented
163 to prepare buffers corresponding to the steps in elution gradients in multiple 96 well plates.
164 This involves the end-user specification of up to 8 Buffer A/B pairs (Figure 1B), the duration
165 of the gradients in column volumes, the %B at the beginning and end of the gradients, and the
166 desired volume for preparing each buffer/step in the gradients. Here, gradients for up to 8
167 miniature columns are prepared at a time by mixing together pairs from up to 16 buffer stocks
168 at different ratios in order to obtain the desired step compositions per gradient and column. In
169 each of these pairs one stock plays the role of Buffer A whereas the other the role of Buffer
170 B. Upon input definition, the tools result in the generation of robotic instructions to prepare
171 miniature column gradients in an automated fashion through generic Tecan Freedom
172 EVOware scripts (See Notes 12 and 13).

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176 **3.3 Implementation of HT column chromatography**

177 The step-by-step methodology below describes the implementation of HT column
178 chromatography for testing various buffered conditions against rotavirus antigens using
179 different multimodal chromatography (MMC) resins.

180 1. Fill the Reagents plate (Figure 1A) with 4 mL of the listed buffers (Table 2) (See Note
181 14).

182 ***[Table 2 near here]***

183 2. The elution plates (ElPlates1 – ElPlates4 in Figure 1A) are 96-well deep square well
184 plates. Fill the plates with 2 mL of buffers. These buffers are prepared to return mobile
185 phase conditions (e.g., pH, buffer concentration and species, salt concentration and type,
186 additives) that are believed to affect the separation and from which an optimal condition
187 will be identified. Listed in Table 3 are the selected buffered conditions used for the
188 MMC columns.

189 ***[Table 3 near here]***

190 3. Once all of the plates are in their positions as shown in Figure 1A. Place the 96-well
191 microplates (Collection plates) in the hotels. Fill the tip sanitization trough (Figure 1A)
192 with 0.5 M sodium hydroxide.
193 4. Launch the EVOware software and select the purification script for 0.6 mL miniature
194 column purification (Appendix 1). The choice of column size, residence time (See Note
195 15), and column volume (CV) for each buffered step is decided (Table 4). This can be
196 selected depending on your requirements.

197 ***[Table 4 near here]***

198 **3.4 Application of HT column chromatography for Multimodal resins (MMC)**

199 Each resin in Table 1 was tested for a different set of buffered conditions and they included
200 changes in pH, sodium chloride concentration and buffer species (Table 3).

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202 **3.4.1 Elution buffer plate preparation for step gradients**

203 1. Use the script dedicated to elution buffer preparation for step gradients (Appendix 2) to
204 find the optimal desorption conditions. Its layout is shown in Figure 1B. Dual elution step
205 gradients are defined to optimise multimodal effectiveness by decreasing the
206 concentration of sodium chloride (NaCl) and increasing the pH (i.e. Table 3, Column 7 &
207 8 for MMC conditions).
208 2. In the buffer preparation script, the gradients are prepared in a step-wise fashion and the
209 salt concentration changes in a step-wise fashion. However, the pH change will not show
210 such a simple trend; instead it will follow a non-linear trend common for titration curves.
211 Once prepared, determine the pH of all prepared solution buffers experimentally using an
212 off-line pH probe (See Note 16).
213 3. To prepare the elution gradients described in Table 3, prepare the buffer stocks of the
214 starting and ending buffers and input the steps into the script (Table 5A).
215 ***[Table 5A near here]***
216 4. Table 5B demonstrates the inputs used to create step gradient elution buffers in 96 well
217 deep square well plates using the automated protocol. In addition flow rates can be
218 calculated relating the larger scale to the smaller scale or vice versa.
219 ***[Table 5B near here]***

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223 **3.5 A case study: Nuvia™ cPrime™ and CMM Hypercel ™**

224 Resins Nuvia cPrime and CMM Hypercel were superior compared to the other commercial
225 multimodal resins in terms of achieved product yield and purity. The chemistry of ligands in
226 both resins includes hydrophobic (benzene) and cation exchange (carboxylic acid) groups
227 (Figure 4). Multiple conditions were evaluated rapidly (pH, salt and buffer) and these two
228 resins were selected for scale-up using the ÄKTA™ pure (GE Healthcare, Uppsala Sweden).

229 **[Figure 4 near here]**

230 Initial experiments on the Tecan at 0.6 mL scale resulted in Nuvia cPrime outperforming
231 CMM Hypercel (Table 6) under multimodal buffered conditions to purify rotavirus antigen
232 A. Nevertheless, both antigens (A and B) were purified at smaller (0.6 mL) and larger scale
233 (>1mL) in order to assess the reproducibility of the HT method. The deployment of CMM
234 Hypercel at HT scale is shown in Figure 5 as an example of method application (Columns 7
235 & 8 MMC conditions in Table 3). Further work was undertaken using rotavirus antigen B and
236 the evaluation of both resins in order to develop a purification process.

237 ***[Table 6 near here]***

238 The chromatogram in Figure 5 shows the purification of rotavirus antigen A using CMM
239 Hypercel. The employed conditions exploit the multimodal capability of the resin as the
240 equilibration buffer has 0.8 M ammonium sulphate for hydrophobic binding and a pH of 4 to
241 induce attraction between negatively charged ligand and positively charged product. Elution
242 is achieved by reducing the ammonium sulphate concentration and increasing the pH. This
243 sets a dual gradient (Figure 5) and resulted in 97% purity and a yield of 49% based on
244 densitometry (Table 6). The separation between the impurities ($1E_2$ and $2E_2$) and the rotavirus
245 antigen A ($3E_2$) can also be observed in Figure 5. The high molecular weight (HMW)
246 impurities are eluting from the column in elution fraction $1E_2$. These are well separated from

the smaller molecular weight (LMW) impurities which elute at the end of the gradient and are followed by the elution of the antigen in the column strip phase. While this method is highly capable of clearing one of the main impurity species, the mixing between the LMW impurities and the antigen would require further optimization. For example, the starting ammonium sulphate concentration and pH can be reduced and increased respectively to bind antigen and flow through HMW impurities. This can then be followed by a shallower gradient with the aim of resolving the LMW impurities from the antigen resulting to an even higher purity and, more importantly, a higher yield by increasing the volume of the product pool. To assess the scalability of these initial results, the elution gradients were applied at a 5 mL scale and the ternary elution peaks were observed (data not shown). Therefore, there is reproducibility from 0.6 mL to 5 mL given the scale up parameters (Table 5B) remain consistent and any further improvements with the HT scale columns would also be scalable.

[Figure 5 near here]

These initial screens (Table 3, Figure 5) are beneficial in determining where the product, product related impurities, host cell DNA and host cell proteins elute and the difficulty of the separation. In this instance, HMW impurities eluted first and followed by a close elution of LMW impurities and the product at the end of the gradient. This information can then be used to optimize the separation further with steps at a systematically chosen pH and salt values. Taking into account that a RoboColumn run can be typically completed within a day (the method in Figure 5 had a duration of <8) with no end-user intervention, other than setting up the robot and the method, demonstrates the power of the RoboColumn technique as a tool for generating valuable process information in an efficient and effective fashion. The benefit of adopting this High Throughput method is further compounded by the fact that viral antigen products are characterized by even larger screening spaces due to the existence of multiple

strains and/or serotypes of viruses (i.e. influenza, lentivirus, HPV). Here, the offered parallelization and walk-away automation, can lead to a rapid development of purification processes, which would be impossible to achieve with conventional workflows, and more importantly to assess the potential of establishing a platform process for all different strains of a given virus. This would allow for significant process development simplification and return significant time and cost savings.

4. NOTES

1. We use two MP3 pos carriers from Tecan making it possible to store up to 6 plates on the robot's deck. It is possible to use two MP4 pos carriers allowing the storage of up to 8 plates instead.
2. It is possible to place the Te-Shuttle carrier to the front of three MP3 pos carriers resulting in space saving on the robot's deck in the case of EVO® 150 or EVO® 100 instruments. To implement this, the worktable will have to be edited in EVOware by changing the two carrier definitions:
    a. Locate the MP3 pos carrier in EVOware's CARRIERS tab
    b. Right click and select Edit
    c. Make note of carrier definitions (take and save a screen capture)
    d. Change the X and Y Dimensions to 1 and 1 mm
    e. Change to Y Reference Offset to 100 mm
    f. Add three carriers, for example, grid locations 2, 8 and 14 (leave 6 grids spacing between each carrier)
    g. Locate the Te-Shuttle carrier in EVOware's CARRIERS tab
    h. Right click and select Edit
    i. Make note of carrier definitions (take and save a screen capture)

296 j. Change the X and Y Dimensions to 1 and 1 mm

297 k. Add carriers to grid location 3

298 l. Change carrier definitions to their original values

299 3. If using MP4 pos carriers or MP3 pos carriers placed behind the Te-Shuttle as opposed to
300 next to it, beware that the liquid handling will be compromised due to the physical
301 dimensions of the robot. Channel 8 of the LiHa will not be able to reach the first row of a
302 plate in the first site of a MP4 pos carrier and Channel 1 of the LiHa will not be able to
303 reach the last row of a plate in the fourth site of a MP4 pos carrier. In this case, liquid
304 handling commands will need to take such limitations into consideration.

305 4. Depending on the installed EVOware version, it may not be possible to define variables
306 for grid numbers in Transfer Labware commands. This is circumvented by joining two 9-
307 site Hotel Carriers to a single 18-site hotel carrier. In this carrier the first nine sites will
308 have exactly the same X-Offsets as in a conventional 9-site Hotel carrier, whereas the
309 next 9 sites will have increased X-Offsets by ~ 128 mm.

310 5. Labware transfers are trained via carrier definitions. All such training is to be made using
311 a single location as a reference point and by using a single plate as a reference labware.

312 6. The sanitization protocol washes the tips with 10 mL in the waste and cleaner using the
313 Fast-Wash module. This is followed with two cycles of aspirating and dispensing 900 µL
314 of 0.5 M NaOH to a single trough and using a custom liquid class. Finally, the tips are
315 then washed again with 10 mL in the waste and cleaner using the Fast-Wash module and
316 then with 1 mL in the waste and cleaner while not using the Fast-Wash module so as to
317 regenerate the air gap.

318 7. The custom liquid class used for the sanitization has an 8 mm offset in the aspiration so as
319 to submerge the tips further in the sanitization solution.

320 8. The sanitization protocol is effective in cleaning the tips for most crude and purified
321 proteinaceous solutions. It needs to be tested in a new laboratory for its effectiveness by
322 liquid handling a sample, applying the sanitization protocol, liquid handling a buffer or
323 DI water and testing the lastly liquid handled solution for its content in contaminants via a
324 sensitive assay (e.g., ELISA).
325 9. The number of steps in a gradient will affect the total number of collected fractions and
326 prepared elution buffers and also the volumes of the collected fractions. For example, a
327 10 CV gradient with 100 steps will result in steps with a size of 0.1 of a CV. In the case
328 of both 0.2 mL and 0.6 mL RoboColumns, such fractions are too small to be measured
329 reliably in a plate reader with full area collection plates and offer too little volume for
330 further analytical steps. Maintain fraction volumes of at least 100 μL to obtain separations
331 with a sufficient number of steps and with enough volume for further analysis.
332 Conversely, in the case of small number of steps, do not select them in a fashion giving
333 fraction volumes greater than the volumetric capacity of a collection plate (typically ~ 0.3
334 mL for most 96 well full area microplates) as this will cause the plates to flood and will
335 lead to cross contamination of samples and robot contamination.
336 10. To measure fractions in the UV spectrum, use UV transparent plates (Corning Inc., NY,
337 USA).
338 11. To determine fraction volumes, also measure each collected fraction at 900nm and 990nm
339 and determine volumes, and then pathlength (by dividing the volume by the cross
340 sectional surface area of the wells in a collection plate), as described in [12].
341 12. When preparing buffers ensure to prepare them at a volume that includes at least 150 μL
342 of excess for 96 well deep square well plates (Fisher Scientific, Loughborough, U.K.) and
343 an additional of 150 μL for the aliquoting of the buffers in plates to blank the measured
344 fractions.

13. When defining gradients in the HT scale, define them on a basis of mM CV^{-1} or %B CV^{-1} and not as mM or %B per unit time, since, when scaling down or up using RoboColumns this takes place on a constant residence time basis. In this case, the flowrate is adjusted so as to match the residence time, as opposed to the linear velocity that is typically implemented with conventional chromatography.
14. Include at least ~ 500 μL of excess to account for dead volumes in the wells of 48 well deep well square plates (Elkay Ltd., Hampshire, U.K.).
15. Residence times of up to 4 min and 12 min for the 0.2 mL and 0.6 mL RoboColumns respectively are possible with the provided liquid classes; On EVO robots it is not possible to implement different liquid classes per channel and hence RoboColumn screening experiments are grouped based on residence times.
16. A probe capable of fitting into wells of 96 well plates needs to be used (~ 4 mm shaft diameter).

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417 # 6. APPENDICES

418 **Appendix 1: EVOware script for preparation of gradient elution buffers**

419 **Appendix 2: EVOware script for performing RoboColumn chromatography using 0.6**
420 **mL columns**

421 **Figure captions:**

422 **Figure 1:** A) Robotic layout for performing RoboColumn experiments. The Sanitizer is a 100
423 mL trough containing sanitization solution (typically 0.5 M NaOH) to decontaminate the tips.
424 plates ElPlate1 – ElPlate4 are 96-well deep square well plates and contain elution buffers for
425 each RoboColumn in each of their rows respectively (i.e., up to 48 buffers per column). The
426 Reagents plate is a 48-well deep square well plate containing solutions to dispense into the
427 RoboColumns during the Equilibration, Load, Wash, Strip, CIP and Storage phases (each row
428 of the plate corresponds to the respective RoboColumn). Fill the reagents plate appropriately
429 with the selected buffers and solutions. Place the ElPlate1 - 4 and Reagents plates onto 2
430 plate carriers. The second plate carrier also includes a vacant spot. This is used either as a
431 position to transfer an empty microplate from the hotel, to fill it up with buffers from the
432 Reagents and ElPlates1 – 4 plates during a Blank Plate preparation or to transfer and then
433 liquid detect a filled Collection plate to determine the volume of the collected fractions. Store
434 the Collection and Blank plates in two 9-site hotels. The Collection plates from these hotels
435 can be transferred to the Transfer position of the Te-Shuttle module which will move them to
436 the start position and eventually to a final position once 12 fractions have been collected. At
437 the start position, the first column of a Collection plate is aligned with the RoboColumns
438 which are held in position on the Te-Chrom module. Finally, Collection and Blank plates are
439 measured in a Plate reader to determine absorbances and also the volumes of their well
440 contents if volume determination occurs with near infrared measurements instead of using the

441 robot's liquid detect function. B) Robotic layout for preparing buffers for up to 8
442 RoboColumn experiments. Same as layout A with the addition of troughs containing 8 pairs
443 of Buffers A and B (i.e., BufferA1, BufferB1, BufferA2, BufferB2, …, BufferA8, BufferB8)
444 and two more plates containing elution buffers (i.e., ElPlate5, 6). Here, each row in each of
445 these plates also corresponds to the respective RoboColumn (i.e., row A cotains buffers for
446 RoboColumn 1, row B for RoboColumn B, …, row H for RoboColumn 8). Hence, a gradient
447 with 6×12 = 72 steps can be prepared for each RoboColumn across 6 ElPlates.
448 **Figure 2:** Flowchart of actions taken during the deployment of RoboColumns on an
449 automated liquid handler.

450 **Figure 3:** Depiction of first 12 fraction collections using an automated liquid handler and the
451 Te-Shuttle module which ensures that fraction collection occurs in a different column of a
452 Collection plate for each new fraction.
453 **Figure 4:** The structure of multimodal resins Nuvia cPrime and CMM Hypercel.
454 **Figure 5:** Purification of rotavirus antigen A using 0.6 mL CMM Hypercel RoboColumn
455 under multimodal buffered conditions performed on the Tecan EVO 200. Left y-axis depicts
456 blank corrected and pathlength normalized fraction absorbances at 280 nm (fraction at 15
457 CVs has increased normalized absorbance due to spuriously low pathlength). The
458 chromatogram shows the purification of two columns under the same conditions to
459 demonstrate reproducibility at a small scale. SDS-PAGE of the ternary elution of the
460 impurities and rotavirus antigen A is also shown on the right.
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464 **Table Captions:**

465 Table 1. List of commercially available multimodal chromatography resins tested at 0.6mL
466 scale.

467 Table 2. Contents of 48 well deep-well square Reagents plate. Each row of the plate (A – H)
468 corresponds to RoboColumn (RC1 – RC8).

469 Table 3. List of different buffered conditions trialled for each of the resins.

470 Table 4. List of chromatography steps entered in the automated script. In this instance, the
471 column size was 0.6 mL and the residence time 2 minutes (i.e., flow rate of 5 μL/s).

472 Table 5A. Details of tested elution conditions.

473 Table 5B. The table below shows the inputs used to create the elution buffer steps per
474 RoboColumn (RC).

475 Table 6. Purification of rotavirus antigen A using 0.6 mL Nuvia^{TM} cPrime^{TM} and CMM
476 Hypercel^{TM} under MMC buffered conditions performed on the Tecan EVO$^{®}$ 200.

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486 **Tables**

487 Table 1.

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Type	Resin	Manufacturer
Multimodal	Capto^{TM} MMC	GE Healthcare
	PPA Hypercel^{TM}	Pall
	Nuvia^{TM} cPrime^{TM}	Bio-Rad
	CMM HyperCel^{TM}	Pall
	Toyopearl® MX-Trp-650M	Tosoh Bioscience
	Eshmuno® HCX	Merck KgaA

489

490 Table 2.

491

Row	Column					
	1 (Equilibration)	*2 (Wash)*	*3 (Wash)*	*4 (Strip)*	*5 (CIP)*	*6 (Storage)*
A(RC1)	Equilibration	Load	Wash	Strip	1M NaCl, 0.5M NaOH	20% EtOH
B(RC2)	Equilibration	Load	Wash	Strip	1M NaCl, 0.5M NaOH	20% EtOH
C(RC3)	Equilibration	Load	Wash	Strip	1M NaCl, 0.5M NaOH	20% EtOH
D(RC4)	Equilibration	Load	Wash	Strip	1M NaCl, 0.5M NaOH	20% EtOH
E(RC5)	Equilibration	Load	Wash	Strip	1M NaCl, 0.5M NaOH	20% EtOH
F(RC6)	Equilibration	Load	Wash	Strip	1M NaCl, 0.5M NaOH	20% EtOH
G(RC7)	Equilibration	Load	Wash	Strip	1M NaCl, 0.5M NaOH	20% EtOH
H(RC8)	Equilibration	Load	Wash	Strip	1M NaCl, 0.5M NaOH	20% EtOH

492

493 Table 3.

494

Column	Buffered conditions	Start – Ending Elution buffers
1	CIEX	20 mM Sodium Citrate pH 5 – 20 mM Sodium Phosphate pH 7
2	CIEX	20 mM Sodium Citrate pH 5 – 20 mM Sodium Phosphate pH 7
3	MMC	20 mM Sodium Citrate pH 5, 1M NaCl – 20 mM Sodium Phosphate pH 7
4	MMC	20 mM Sodium Citrate pH 5, 1M NaCl – 20 mM Sodium Phosphate pH 7
5	CIEX	50 mM Sodium Citrate pH 4 – 50 mM Tris-HCl 0.8M NaCl pH 8
6	CIEX	50 mM Sodium Citrate pH 4 – 50 mM Tris-HCl 0.8M NaCl pH 8
7	MMC	50 mM Citrate pH 4, 0.8 M $(NH_4)_2SO_4$ – 50 mM Tris-HCl pH 8
8	MMC	50 mM Citrate pH 4, 0.8 M $(NH_4)_2SO_4$ – 50 mM Tris-HCl pH 8

495 Table 4.

496

Step	Length of step (CV)
Removal of storage solution	3
Equilibration	5
Fermentation Load	5
Wash	3
Elution	10
Strip	4
CIP	1
Storage	3

497

498 Table 5A.

499

Elution condition	Start salt concentration (M)	Ending salt concentration (M)	%B buffer change	pH change	No. Column volumes in elution	Fraction volume (mL)
CIEX	0	0.8	0 – 100	4 – 8	15	0.2
MMC	0.8	0	0 – 100	4 – 8	15	0.2

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501 Table 5B.

RC	Gradient CV	Fraction Volume (mL)	Buffer A trough	Buffer B trough	Start %B	End %B	Gradient slope (%B/CV)
1	**15**	**0.2**	**BufferA1**	**BufferB1**	**0**	**100**	**6.67**
2	**15**	**0.2**	**BufferA2**	**BufferB2**	**0**	**100**	**6.67**
3	**15**	**0.2**	**BufferA3**	**BufferB3**	**0**	**100**	**6.67**
4	**15**	**0.2**	**BufferA4**	**BufferB4**	**0**	**100**	**6.67**
5	**15**	**0.2**	**BufferA5**	**BufferB5**	**0**	**100**	**6.67**
6	**15**	**0.2**	**BufferA6**	**BufferB6**	**0**	**100**	**6.67**
7	**15**	**0.2**	**BufferA7**	**BufferB7**	**0**	**100**	**6.67**
8	**15**	**0.2**	**BufferA8**	**BufferB8**	**0**	**100**	**6.67**

502

503 Table 6.

Resin	Scale (mL)	Conditions used to purify	Purity (%)	Yield (%)	OD260/280
CMM Hypercel^{TM}	0.6	MM	97	49	0.59
Nuvia^{TM} cPrime^{TM}	0.6	MM	97	56	0.59

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