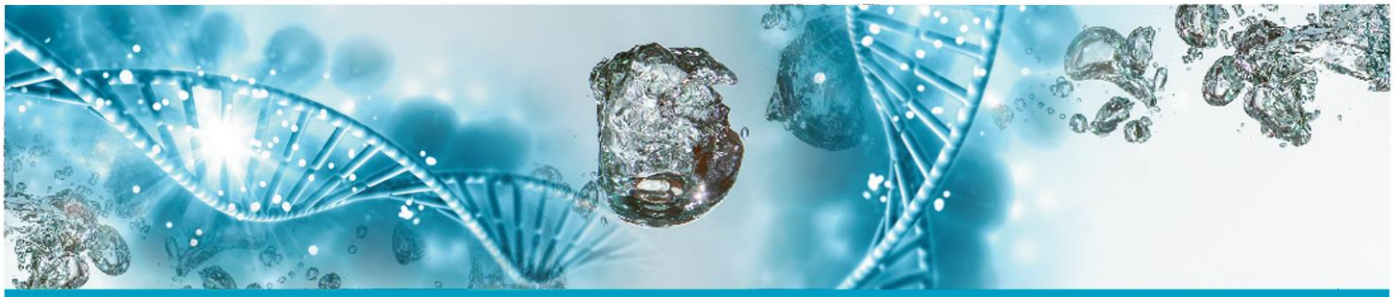


CIMmultus™ HiP^2 Plasmid Process Pack™

Contents of the pack:

- 1 CIMmultus™ DEAE Column
- 1 CIMmultus™ C4 HLD Column

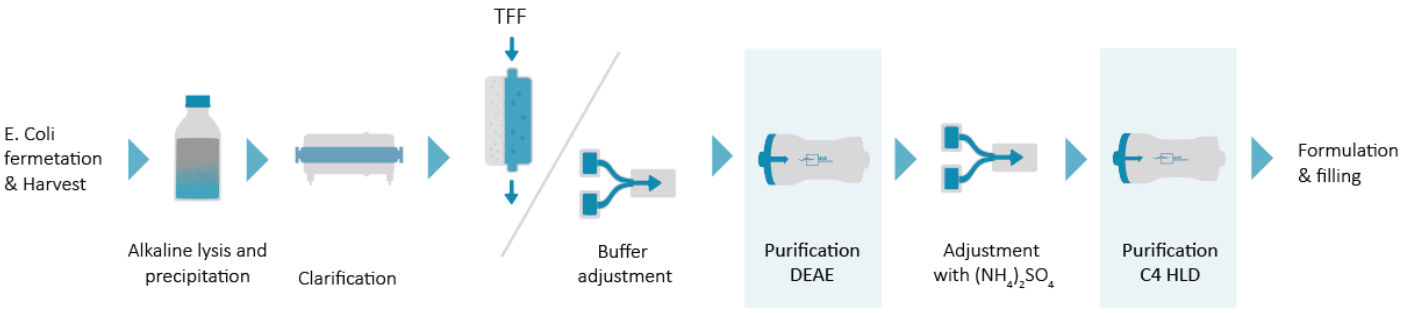
Before you begin

Use this Instruction manual in conjunction with the Product sheet of the columns.

Introduction

HiP2 Plasmid Process Pack™ produces low endotoxin, highly homogeneous supercoiled plasmid DNA (pDNA) of clinical grade. The process is applicable for bench scale purification and can be scaled up for manufacturing of pDNA as raw material or drug substance. The advantage of monolithic columns allows high flow rates and short processing times.

Contaminants are removed without the use of enzymatic treatment. Potassium acetate and calcium chloride ($CaCl_2$) neutralisation and precipitation alongside two chromatography steps are used for removal of RNA, endotoxin, genomic DNA, protein, and other contaminants. The first chromatography step, capture on anion-exchange (AEX) column (CIMmultus™ DEAE) separates contaminant RNA and proteins from the plasmid DNA. The polishing step on hydrophobic interaction chromatography (HIC) with a high ligand density butyl-modified (CIMmultus™ C4 HLD) monolithic support eliminates remains of genomic DNA, endotoxin, open circular (oc) and linear pDNA isoforms, resulting in the isolation of the supercoiled (sc) pDNA.



Getting started

To choose the right column, it is important to consider the following parameters:

Channel size

pDNA < 8 kbp	CIMmultus™ DEAE 2 µm	CIMmultus™ C4 HLD 2 µm
pDNA > 8 kbp	CIMmultus™ DEAE 6 µm	CIMmultus™ C4 HLD 2 µm

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Choosing the right channel size depends on the size of pDNA. Larger plasmids generate more viscous solutions, sometimes limiting the operating flow rates. In addition, open-circular isoforms of large plasmid DNA can become reversibly entrapped in the chromatographic column. Large channel (6 µm) monoliths successfully prevent pressure increase and entrapment of oc isoform.

Column capacity

CIMmultus™ columns are pre-packed and ready to use. Select a suitable column size based on the dynamic binding capacity (DBC) of the column shown below. When designing an experiment, the column capacity dictates the selection the column size.

CIMmultus™ DEAE 2 µm	Up to 6 mg of pDNA/mL of monolith
CIMmultus™ DEAE 6 µm	Up to 2.5 mg of pDNA/mL of monolith
CIMmultus™ C4 HLD 2 µm	Up to 2 mg of pDNA/ mL of monolith in sc polishing step

For optimal process implementation, evaluate the process at laboratory scale on CIMmultus™ 1 mL | 4 mL | 8 mL columns before scale-up.

Analytics with CIMac™ pDNA

CIMac™ pDNA column is used as an analytical column for pDNA analysis. It allows the quantification of all pDNA isoforms as well as monitoring degradation products and the removal of impurities (RNA). Additionally, if used in parallel with the downstream process it ensures that each production step is yielding the amount of supercoiled pDNA anticipated.

REFERENCE Quick Start methods for pDNA isoform monitoring with CIMac™ pDNA

IMPORTANT

Optimisation of crucial steps is recommended for best performance. Follow the optimisation points along the process steps. Process optimization steps might be required if the characteristics of the sample change (plasmid, ratio between open circular and supercoiled isoform and the loading amount).

Sample preparation

Before starting with the chromatography steps, the sample must be prepared, starting with an alkaline cell lysis, and followed by clarification phase which consist of three steps: precipitation, centrifugation, and filtration. Precipitation is done by $CaCl_2$, which is an ionic compound that precipitates large molecules of RNA and genomic DNA. Centrifugation or depth filtration is used to remove large particles such as cell debris and precipitated complexes from the solution. Fine filtration is performed before chromatography.

Conditions

Resuspension buffer	50 mM Tris-HCl, 10 mM EDTA, pH 8.0
Cell lysis buffer	0.2 M NaOH, 1% sodium dodecyl sulphate (SDS)
Neutralization buffer	3 M potassium acetate (CH_3COOK), pH 5.5, chilled
Stock $CaCl_2$ solution	5 M $CaCl_2$

Materials

Cell biomass (pellet)

Primary clarification: Centrifuge or Filtration media with 25-100 µm pores (Sartorius Sartopure® PP3, 50 µm)

Fine filtration: Filtration media with 0.45-2 µm pores (e.g., Sartorius Sartopore® 2, 0.45µm membrane filter)

Peristaltic or membrane pump enabling flow rates between 5 and 1000 mL/min - depending on the column size.

Procedure

1. Weight desired amount of frozen biomass containing pDNA into a beaker or reagent flask.
2. Add resuspension buffer in a ratio of 10 mL of buffer per g of frozen biomass. Mix until pellet is completely dissolved.
3. Add an equal volume of cell lysis buffer to the dissolved biomass, mix gently and incubate for 3-6 minutes.
4. To lower the pH of the sample, add chilled neutralization buffer while gently mixing the suspension. The volume added is equal to the volume of suspended cells.
5. Add stock $CaCl_2$ solution to a final concentration of 0.5–1 M $CaCl_2$ with gentle mixing.
6. Incubate the sample at room temperature for 10 min.
7. Proceed with clarification, starting with centrifugation or depth filtration, following with fine filtration.

IMPORTANT

Before starting with the chromatographic part of the process and to ensure reproducibility of the procedure, observe the following recommendations:

- Maintain loading of plasmid DNA consistent between runs and when scaling up. Always load the same amount of DNA per mL of column volume.
- To scale up the process consider pressure specifications of LC systems and evaluate the maximum flow rate at final scale. Speed up optimization work with high flow rates and adjust the flow rate before scaling up.

Plasmid DNA capture on AEX DEAE Column

Chromatographic capture uses a weak anion exchange to separate plasmid DNA from contaminants, including remaining RNA and proteins. The sample is applied at low conductivity and eluted by increasing concentration of sodium chloride.

NOTICE

Omission of the $CaCl_2$ precipitation step may result in reduced performance of the chromatographic capture. The procedures described below start with a sample prepared according to this Instruction manual.

Direct chromatographic capture on CIMmultus™ DEAE

After precipitation and filtration, the sample can be captured directly on CIMmultus™ DEAE. This procedure may generate high processing volumes, which may not be suitable for large scale manufacturing. Tangential flow filtration can be implemented when required (see following chapter).

Materials

CIMmultus™ DEAE

Chromatography system suited for column size. Recommended multi-wavelength UV (260 nm and 280 nm), conductivity, pH monitors.

Conditions

Equilibration buffer A1:	50 mM TRIS, 10 mM EDTA, pH 7.2
Wash buffer A2:	50 mM TRIS, 10 mM EDTA, 0.6 M NaCl, pH 7.2
Elution buffer A3:	50 mM TRIS, 10 mM EDTA, 0.85 M NaCl, pH 7.2
Stripping buffer A4:	50 mM TRIS, 10 mM EDTA, 1 M NaCl, pH 7.2

Procedure

Preparation of CIMmultus™ DEAE column

1. Connect CIMmultus™ DEAE column to the chromatography system.
2. Perform the Column integrity test.

REFERENCE Guideline: Column Integrity Test

3. Equilibrate the CIMmultus™ DEAE column:
    a. Flush 20 column volumes (CV) of buffer A1.
    b. Flush 10 CV of buffer A4.
    c. Flush 20 CV of buffer A1.

Sample preparation

1. Dilute bacterial lysate with deionized water to conductivity of 35–40 mS/cm.
2. Filter the sample again before applying on the column.
3. Load the cleared diluted bacterial lysate to the column. Continue loading until pDNA breakthrough is observed. Note: if the pressure increases exponentially during loading, stop the run.

Sample elution

1. Wash the column with 20 CV of buffer A1.
2. Wash the column with 20 CV of buffer A2.
3. Elute and collect pDNA with 20 CV of buffer A3 (use half the working flow rate). If pressure approaches the specified limit for the column, reduce the flow rate further.
4. Strip the column with 20 CV stripping buffer A4 to remove remaining impurities.
5. Clean the column before the next run, refer to the cleaning procedure in the Product Sheet.

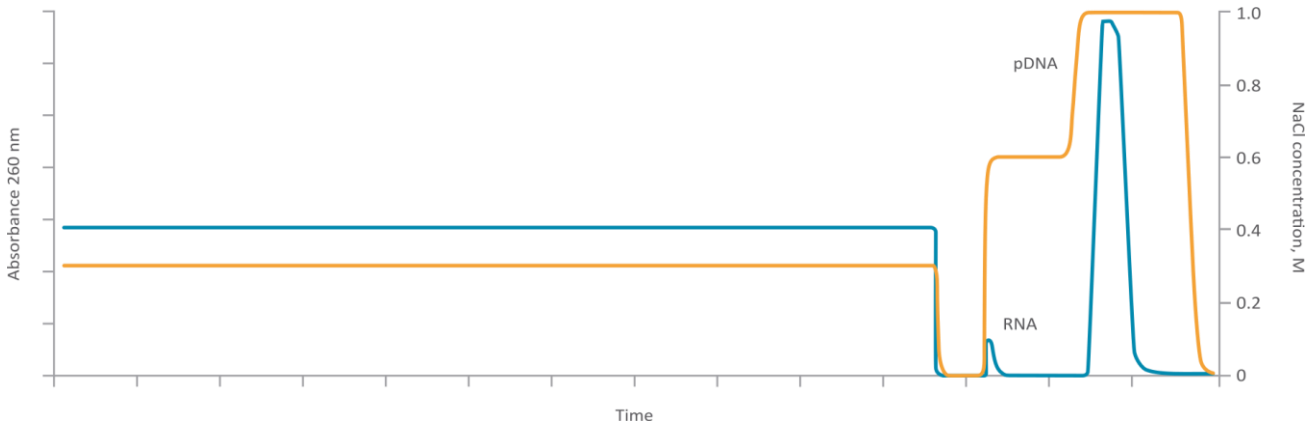

Figure 1) Preparative chromatogram of pDNA loading onto CIMmultus™ DEAE after lysate dilution to 35 mS/cm (without TFF step) and pDNA elution from previously loaded column DEAE column

Tangential flow filtration and chromatographic capture on CIMmultus™ DEAE

Tangential flow filtration (TFF) before chromatography will reduce working volumes and facilitate scalability of the process.

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Materials:

TFF module (mPES, 300 kDa cut-off, hollow fibre or cassette, approximate surface area 25 L post-filtration solution/m^2)

CIMmultus™ DEAE column

Chromatography system suited for column size. Recommended multi-wavelength UV (260 nm and 280 nm), conductivity, pH monitors.

Conditions

Equilibration buffer A1:	50 mM TRIS, 10 mM EDTA, pH 7.2
Wash buffer A2:	50 mM TRIS, 10 mM EDTA, 0.6 M NaCl, pH 7.2
Elution buffer A3:	50 mM TRIS, 10 mM EDTA, 0.85 M NaCl, pH 7.2
Stripping buffer A4:	50 mM TRIS, 10 mM EDTA, 1 M NaCl, pH 7.2

Procedure

Tangential flow filtration

1. Prepare the TFF module according to manufacturer protocol.
2. Equilibrate the TFF membrane with buffer A1.
3. Recirculate the sample for 10 min without permeate flux, to allow for membrane equilibration.
4. Perform ultrafiltration until retentate concentration is close to 0.3 mg pDNA/mL.
5. Perform diafiltration (buffer exchange) into buffer A1 using 6 diafiltration volumes of buffer. If the sample is used directly for loading onto DEAE column, then A2 buffer could be used in diafiltration cycles. If it is not known exactly, what NaCl concentration will be used in the following step, then buffer A1 is preferable, because it enables easy NaCl adjustment.
6. Collect retentate.

Final adjustment

1. If precipitation is visible in the retentate, perform 0.45 µm filtration
2. Dilute with 2 equivalent volumes of buffer A2, if sample is already in buffer A2 after diafiltration. If not, first dilute the sample with buffer A4. For one part of sample use 1.5 part of buffer A4. Now dilute with 2 equivalent volumes of buffer A2.

Sample application on CIMmultus™ DEAE

1. Connect CIMmultus™ DEAE to the chromatography system.
2. Perform the Column integrity test.

REFERENCE Guideline: Column Integrity Test

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3. Equilibrate the column:
    a. Flush 20 column volumes (CV) of buffer A2.
    b. Flush 10 CV of buffer A4.
    c. Flush 20 CV of buffer A2.
4. Load the sample on the column. Stop loading when sample break-through is observed.
5. Note: if the pressure increases exponentially during loading, stop the run.

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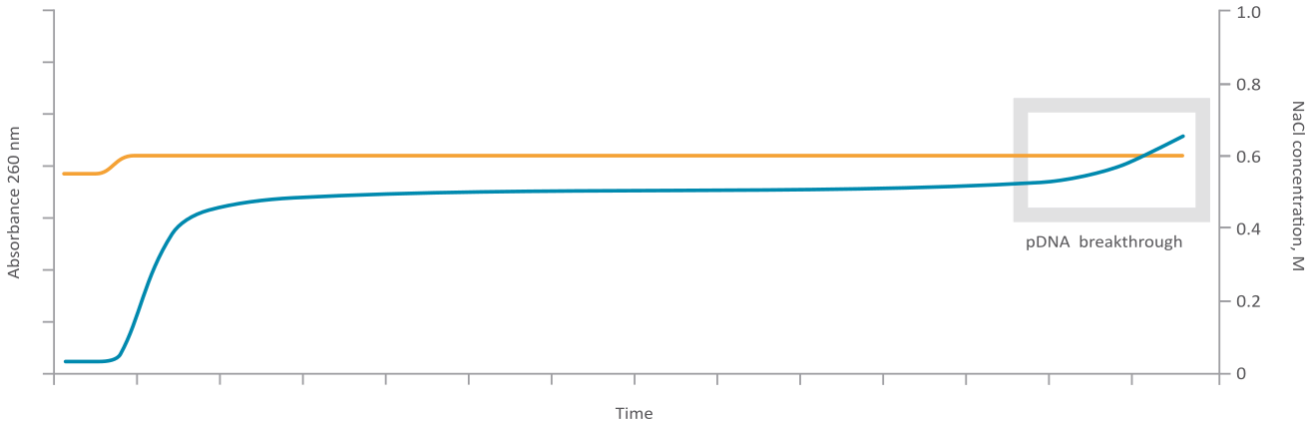
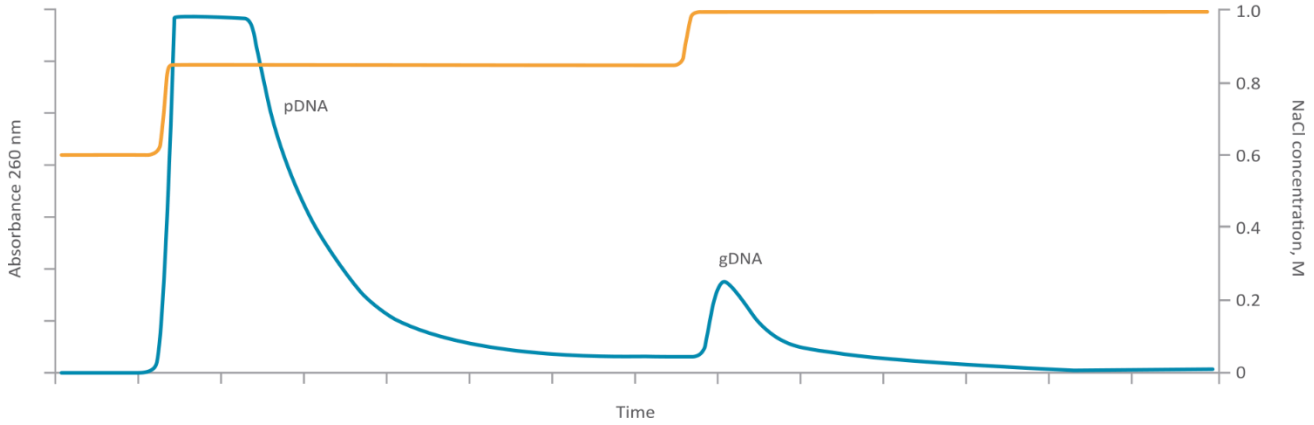


Graphic 2: A representative preparative chromatogram of pDNA capture in 0.6 M NaCl after TFF buffer exchange and concentration, showing typical breakthrough. High UV signal is observed due to RNA in flow through fraction.

Sample elution

1. Wash with 20 CV of buffer A2.
2. Reduce the flow rate to half the working flow rate and elute and collect the product with 20 CV of elution buffer A3.



Graphic 3: pDNA elution from previously loaded column DEAE column

3. Strip the column with 10 CV of stripping buffer A4.
4. Clean the column before the next run, refer to the cleaning procedure in the Product Sheet.

Separation of supercoiled plasmid DNA on HIC C4 HLD column

Chromatographic polishing step on CIMmultus™ C4 HLD removes other isoforms from supercoiled pDNA. The procedure below is the same regardless the previous capture step protocol. It is performed at lower ammonium sulphate concentrations than classical bind-elute approach. It exploits different relative binding affinities of components in a sample mixture and separates pDNA isoforms under conditions allowing for sc binding while oc flows through the column.

This approach increases yield of sc isoform, enables for 60% reduction in chemicals consumption and a 20% reduction in processing time per gram of product in comparison to classical bine-elute approach.

NOTICE

Loading and eluting conditions used in sample displacement mode are crucial to achieve high supercoiled isoform purity, since we are trying to separate two very similar entities. Preliminary experiments should be performed before preparative pDNA isolation in order to achieve desired results.

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Starting point conditions

Equilibration buffer B1:	50 mM TRIS, 10 mM EDTA, 1.65 M ammonium sulphate, pH 7.2
Elution buffer B2:	50 mM TRIS, 10 mM EDTA, 0.9 M ammonium sulphate, pH 7.2
Stripping buffer B3:	50 mM TRIS, 10 mM EDTA, pH 7.2
Adjustment solution B4:	4 M ammonium sulphate

Materials

CIMmultus™ C4 HLD column

Filtration media with 0.45 µm pores (Sartorius MiniSart PES or Sartorius Sartopore® 2, 0.45µm membrane filter)

Chromatography system suited for column size. Recommended multi-wavelength UV (260 nm and 280 nm), conductivity, pH monitors.

Procedure

Sample preparation

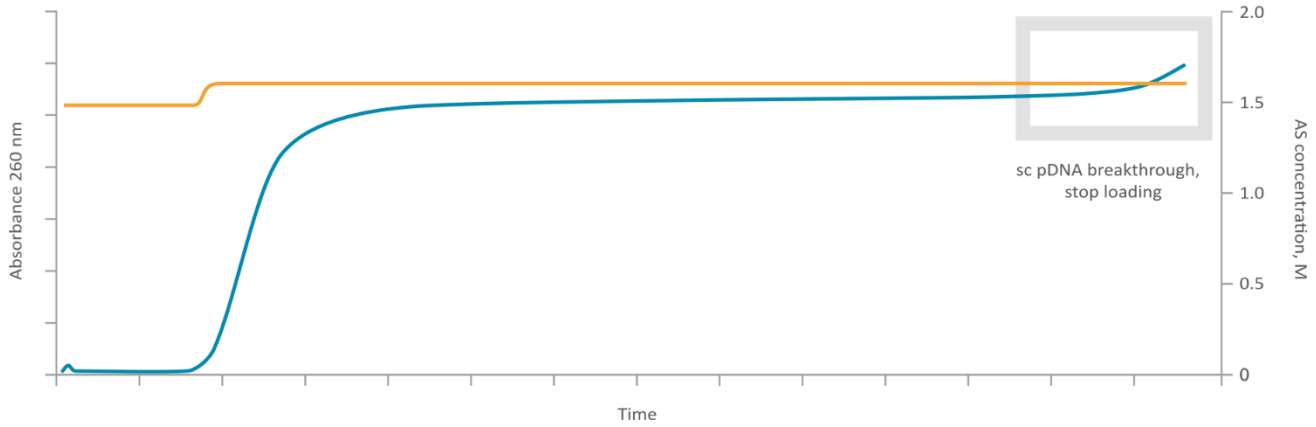
1. Dilute elution fraction from DEAE with adjustment solution B4. Use 1.67 volumes of B4 per 1 volume equivalent of eluate. The final concentration of ammonium sulphate will be 2.5 M.
2. Filter the solution through 0.45 µm filter.
3. Dilute the filtrate with stripping buffer B3. Use 0.56 volumes of B3 per 1 volume equivalent of filtrate. The final concentration of ammonium sulphate will be 1.6 M.
4. Dilute the solution with equilibration buffer B1. Use 0.65 volumes of B1 per 1 volume equivalent of your sample solution.

Sample application on CIMmultus™ C4 HLD

1. Connect CIMmultus™ C4 HLD to the chromatography system.
2. Perform the Column integrity test.

REFERENCE Guideline: Column Integrity Test

3. Equilibrate the column:
    a. Flush 20 column volumes (CV) of buffer B1.
    b. Flush 20 CV of B3.
    c. Flush 20 CV of buffer B1.
4. Load the sample on the column. Stop the loading when breakthrough of sc isoform is observed, typically between 1 and 1.5 mg of loaded sc pDNA per mL of column.



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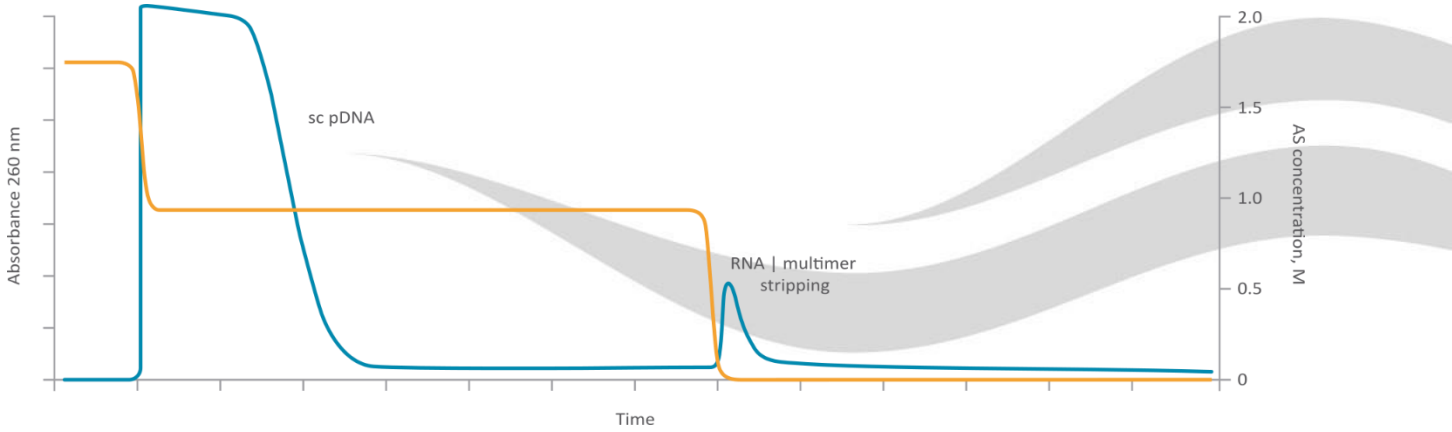
Graphic 4: A representative preparative chromatogram of pDNA polishing on CIMmultus™ C4 HLD 2, in 1.6 M AS after DEAE capture, AS concentration adjustment and filtration, showing typical breakthrough. High UV signal is observed due to oc isoform in flow through fraction.

Sample elution

1. Wash the column with 20 CV of B1.
2. Elute the sample with 20 CV of B2.
3. Strip the column with 20 CV of stripping buffer B3.
4. Clean the column before the next run, refer to the cleaning procedure in the Product Sheet



Graphic 5: pDNA elution and stripping from previously loaded column C4 HLD column.

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References: Guidelines and Optimisation points

Guideline: Column Integrity Test

Quick Start methods for pDNA isoform monitoring with CIMac™ pDNA

Ordering information

100.#### standard product

101.#### cGMP compliant product

Product	Volume	Catalog number*
CIMmultus™ HiP² Plasmid Process Pack™		
CIMmultus™ HiP² Plasmid Process Pack™ 1-1 (1× DEAE 311.5114-2, 1× C4 HLD 311.8136-2)	2 x 1mL	100.0011-2
CIMmultus™ HiP2 Plasmid Process Pack™ 4-4 (1× DEAE 414.5114-2, 1× C4 HLD 414.8136-2)	2 x 4mL	100.0016-2
CIMmultus™ HiP² Plasmid Process Pack™ 8-8 (1× DEAE 411.5114-2, 1× C4 HLD 411.8136-2)	2 x 8mL	100.0012-2
CIMmultus™ HiP² Plasmid Process Pack™ 40-40 (1× DEAE 614.5114-2, 1× C4 HLD 614.8136-2)	2 x 40mL	100.0017-2 101.0017-2
CIMmultus™ HiP² Plasmid Process Pack™ 80-80 (1× DEAE 611.5114-2, 1× C4 HLD 611.8136-2)	2 x 80mL	100.0013-2 101.0013-2
CIMmultus™ HiP² Plasmid Process Pack™ 400-400 (1× DEAE 814.5114-2, 1× C4 HLD 814.8136-2)	2 x 400mL	100.0018-2 101.0018-2
CIMmultus™ HiP² Plasmid Process Pack™ 800-800 (1× DEAE 811.5114-2, 1× C4 HLD 811.8136-2)	2 x 800mL	100.0014-2 101.0014-2
CIMmultus™ HiP² Plasmid Process Pack™ 4000-4000 (1× DEAE 1014.5114-2, 1× C4 HLD 1014.8136-2)	2 x 4000mL	100.0019-2 101.0019-2
CIMmultus™ HiP² Plasmid Process Pack™ 8000-8000 (1× DEAE 1011.5114-2, 1× C4 HLD 1011.8136-2)	2 x 8000mL	100.0015-2 101.0015-2
CIMac™		
CIMac™ pDNA-0.3 Analytical Column (Channels 1.4 µm)	300 µL	150.8501-1.4
CIMac™ pDNA-0.3 Analytical Column (Channels 6 µm)	300 µL	150.8501-6

CIMmultus™ HiP² Plasmid Process Pack™ with 6 µm channel size CIMmultus™ DEAE column is available on request.

For any additional information or further enquiries please contact us:

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