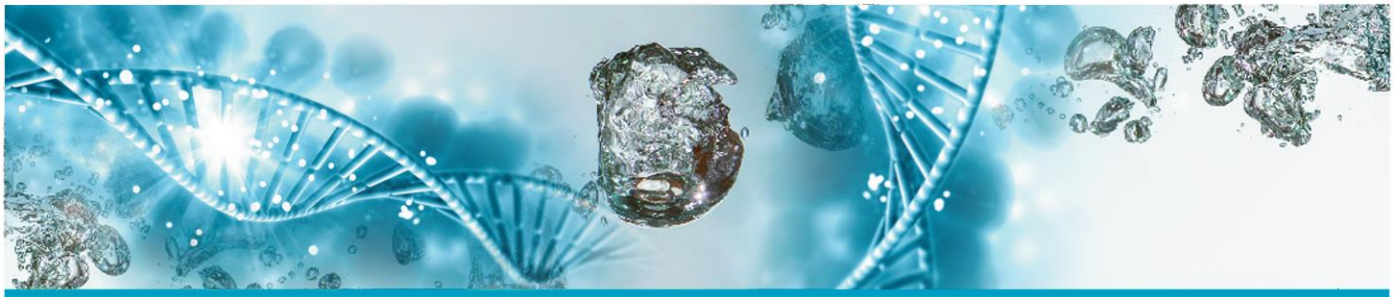


CIMmultus™ HiP^2 Plasmid Process Pack™

Contents of the pack:

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

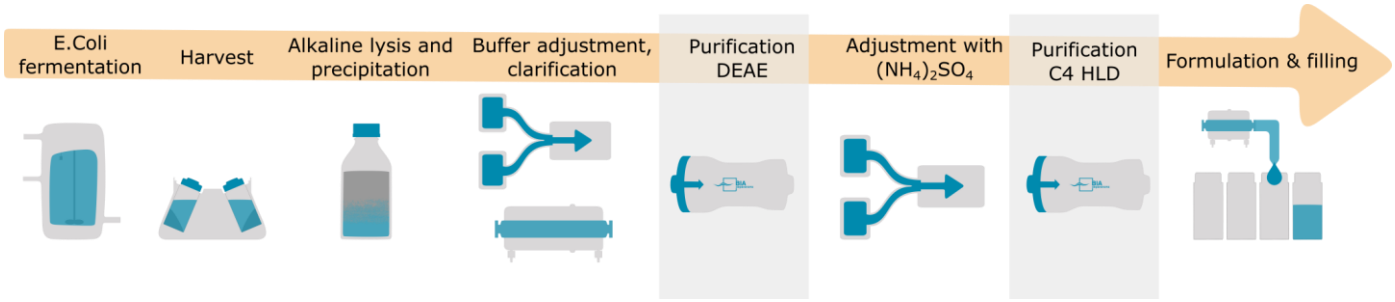
Before you begin

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

Introduction

HiP^2 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 precipitation alongside two separate chromatography steps are used for removal of RNA, endotoxin, genomic DNA, protein and other contaminants. The first chromatography step separates contaminant RNA and proteins from the plasmid DNA. The polishing step eliminates remains of genomic DNA, endotoxin and open circular (oc) and linear pDNA isoforms, resulting in the isolation of the supercoiled (sc) pDNA isoform.



Scheme 1: Purification process

Important notes

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.
- Optimisation of certain steps is recommended for best performance. Follow the **Notes** 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).

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- Prepare the chromatography system and equilibrate the column before performing sample preparation. This will avoid unnecessary hold up time.
- To scale up the process consider pressure specifications of LC systems and evaluate the maximum flow rate at final scale. Speed up optimisation work with high flow rates and adjust the flow rate before scaling up.

Suggested protocol

Sample preparation

The starting material is prepared by alkaline lysis. Start by suspending bacterial cells (typically cell biomass or cell pellet) in 50 mM Tris-HCl, 10 mM EDTA, pH 8.0. Perform lysis by addition of an equal volume of 0.1–0.4 M NaOH, 1 % SDS.

Note: The optimal NaOH concentration and contact time should be investigated for the plasmid sample.

After lysis the suspension becomes viscous. Cellular debris and SDS complexes are precipitated by addition of chilled (4–8 °C) 3 M CH_3COOK, pH 5.5 (same volume as suspension buffer). Low temperature facilitates precipitation, and mixing should be gentle to prevent degradation of DNA. With gentle mixing, slowly add $CaCl_2$ (as concentrated 5 M solution) to a final concentration of 0.5–1 M and incubate for 15 minutes.

Note: Calcium chloride is used as precipitation agent to remove RNA, genomic DNA, and other impurities. Higher concentration of impurities will require $CaCl_2$ concentrations up to 1 M. Consider testing multiple concentrations and evaluate efficient removal of impurities and unaffected pDNA yield in the product. Addition should be slow to prevent local temperature spikes.

After incubation, perform a series of clarification steps, starting with centrifugation or coarse filtration, such as 80 µm depth filtration, and ending with 1–5 µm filtration. The sample will be filtered again before applying on the column.

System preparation

Connect the column as indicated in the Product sheet & Instruction manual. Flush the column with water to remove storage solution.

IMPORTANT: Adjust the pressure relief valve of the system (pump) to the maximum working system pressure of the column.

Plasmid DNA capture on AEX DEAE Column

Plasmid DNA is captured on a weak anion exchange column, where remaining RNA and proteins are removed. Sample binding requires low conductivity, achieved by dilution. Elution with increasing NaCl steps unbinds RNA first, followed by elution of plasmid DNA.

Conditions:
For suggested flow rate, consult the DEAE column Product sheet.

Equilibration buffer A1: 50 mM Tris, 10 mM EDTA, pH 7.2

Washing buffer A2: 50 mM Tris, 10 mM EDTA, 0.6 M NaCl, pH 7.2

Elution buffer A3: 50 mM Tris, 10 mM EDTA, 1 M NaCl, pH 7.2

Method:

1. Dilute bacterial lysate with deionised water to conductivity of 35–40 mS/cm. Dilution depends on the concentration of $CaCl_2$ added during sample preparation. The volumetric ratio of deionised water:sample should be 3:1, 4:1 or 5:1 for 0.5 M, 0.75 M or 1 M $CaCl_2$ respectively.
   Note: The conductivity of the loading sample will affect the column capacity for plasmid DNA as well as RNA removal efficiency. Loss of plasmid in the flow through or RNA contamination of plasmid elution indicate too

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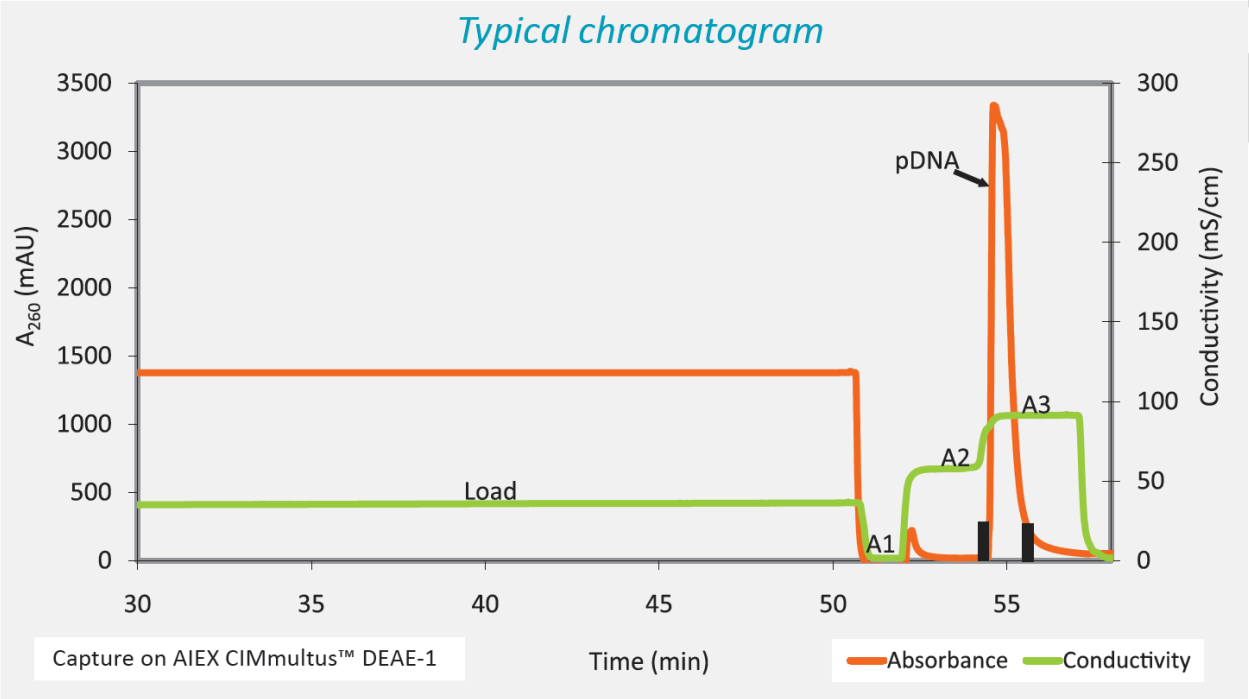
high or too low conductivity respectively. Consider testing multiple conductivities within the range and evaluate DNA and RNA recoveries in the flow through and elution fractions.

2. Filter the diluted sample through 0.45 µm filter.
3. Equilibrate the DEAE column by applying 20 CV of buffer A1 (pH at the outlet should match the pH of buffer A1).
4. Load the cleared dilute bacterial lysate to the column.
   Note: Max. 6 mg of pDNA/mL of monolith can be loaded on the DEAE column. Recommended load is 2/3 of the maximum capacity.
5. Wash the column with 20 CV of buffer A1.
6. Wash the column with 20 CV of buffer A2.
7. 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 further reduce the flow rate.



Separation of supercoiled plasmid DNA on HIC C4 HLD column

To enrich the supercoiled isoform of plasmid DNA, the DEAE eluate is loaded onto a high ligand density butyl column (C4 HLD). In addition, this step further improves impurity clearance.

Conditions:
For suggested flow rate, consult the C4 HLD column Product sheet.

Equilibration buffer B0: 50 mM Tris, 10 mM EDTA, 3 M $(NH_4)_2SO_4$, pH 7.2

Washing buffer B1: 50 mM Tris, 10 mM EDTA, 1.7 M $(NH_4)_2SO_4$, pH 7.2

Adjustment buffer: 4 M $(NH_4)_2SO_4$

Elution buffer B2: 50 mM Tris, 10 mM EDTA, 0.4 M $(NH_4)_2SO_4$, pH 7.2

Regeneration buffer A1: 50 mM Tris, 10 mM EDTA, pH 7.2

Method:

1. Adjust the pDNA-containing eluate from the DEAE column by adding 3 volumes (V) of 4 M $(NH_4)_2SO_4$ per 1 V of eluate.
2. Equilibrate the C4 HLD Column by applying 20 CV of buffer B0.

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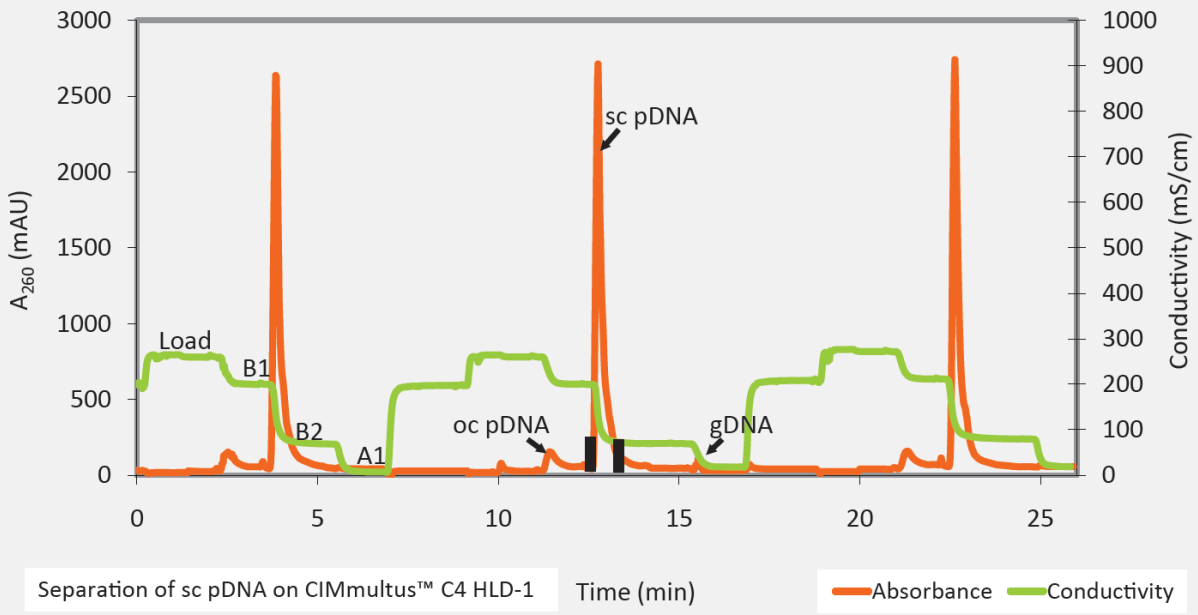
3. Load adjusted pDNA fraction eluted from the DEAE column.
   Note: Max. 3 mg of pDNA/ mL of monolith can be loaded on the C4 HLD column. Recommended load is 2/3 of the maximum capacity.
4. Wash the column with 20 CV of buffer B1.
5. Elute and collect pDNA with buffer B2 (at half working flow rate).
6. Regenerate the column with 30 CV of buffer A1.
7. Repeat the method two more times to process the remaining material.
   Note: If all the plasmid can be loaded in a single run, step 7 can be omitted.
8. Sanitise the column after loading sample 3 times.
   Note: Clean the column (CIP) between consecutive cycles if changes in performance (capacity, purity or recovery), or change in pressure are observed. Cleaning and sanitisation instructions located in product sheet.

Note: If the final sample contains excess oc isoform or if sc pDNA is lost in the B1 wash, the concentration of ammonium sulphate in the buffers can be optimised. To do this, load the plasmid as indicated up to step 3 and perform stepwise elution by incremental decrease (or linear gradient) of ammonium sulphate concentration. Collect and analyse the fractions to determine the optimal salt concentration in the wash step (elimination of oc isoform).

Note: If the final sample contains excess RNA, the concentration of ammonium sulphate in the elution buffer can be increased. Consider testing multiple concentrations up to 1 M.

Typical chromatogram



Separation of sc pDNA on CIMmultus™ C4 HLD-1

Polishing & buffer exchange

Supercoiled pDNA fraction eluted from the C4 HLD column contains ammonium sulphate, which must be removed prior to biological application of the plasmid. Buffer exchange can be performed with e.g. diafiltration or size exclusion chromatography. Additional processing may be required for formulation and filling.

Sanitisation and storage

The columns can be sanitised and re-used. Consult the Product Sheet of each column for sanitisation procedures.

Refer to the Product Sheet for storage instructions.

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Ordering information

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

100.#### standard version, 101.### cGMP compliant version

Catalog number	Product	Volume
150.8501-1.4	CIMac™ pDNA-0.3 Analytical Column (Pores 1.4 µm)	300 µL

For any additional information or further enquiries please contact us:

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