Cas9 Nuclease Preparation Using EconoFit Columns and the NGC Chromatography System

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Introduction

The clustered regularly interspaced short palindromic repeats with associated protein 9 (CRISPR-Cas9) system has revolutionized the genetic engineering of cells. Central to this technology is the Cas9 nuclease which, in conjunction with a specific guide RNA (gRNA), targets and edits precise locations within a genome. Purified Cas9 can be delivered directly into cells, as Cas9-gRNA ribonuclease enables highly efficient genome editing. The use of recombinant Cas9 enables improved control of Cas9 activity, limits off-target effects in vivo, and reduces unintended cytotoxicity. We have developed a fast, robust, and cost-effective two-step chromatography workflow using prepacked EconoFit Columns for the purification of histidine-tagged Cas9 on the NGC Chromatography System. Although Cas9 proteins are commercially available from numerous vendors, the cost may be prohibitive to some researchers, especially if large amounts are needed. Here we provide a quick guide to produce histidine-tagged recombinant Cas9 protein that matches the purity and the in vitro and in vivo activity of commercially available Cas9 products. The EconoFit Nuvia IMAC Column provides efficient capture of the histidine-tagged Cas9, yielding excellent recovery at high flow rates. Further clearance of residual host cell proteins is achieved via cation exchange (CEX) chromatography performed with an EconoFit UNOsphere S Column, which requires minimal sample adjustment for efficient Cas9 binding after immobilized metal affinity chromatography (IMAC) column elution.

For a full protocol describing Cas9 production and validation, and a list of materials used, refer to our application note (bulletin 3211).

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Cas9 Expression and Purification Workflow

A timeline and overview for the bacterial expression and fast two-step chromatographic purification of histidine-tagged wild-type Cas9 are shown.

Day 1 to 2 *Escherichia coli* Transformation and Preparation of –80°C Glycerol Stocks

Day 1
Cell transformation

Day 2
Cell culture

37°C

- ***E. coli* strain:** BL21(DE3)
- **Cas9 expression plasmid:** pET-NLS-Cas9-6xHis (Nova Lifetech Pte Ltd, #PVT10639) was used for this protocol. Other expression plasmids are commercially available
- **Expression strain creation:** introduce the Cas9 expression plasmid into *E. coli* using standard transformation protocols, select for transformants on Luria-Bertani (LB) agar plates with appropriate antibiotic, and prepare glycerol stocks for storage at –80°C

Day 2 to 3 Cell Cultivation

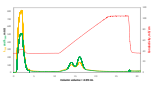
Day 3
Induction of Cas9 expression

20°C

- **Cell cultivation:** use four 500 ml baffled flasks, each containing 125 ml of LB medium and appropriate antibiotic, and inoculate to an optical density of 0.05 at 600 nm (OD 600) with culture grown overnight at 37°C. Incubate cultures with shaking (200–250 rpm) at 37°C until an OD 600 of 0.6–0.8 is achieved
- **Induction of Cas9 expression:** reduce the temperature of the culture and incubator to 20°C and induce expression with 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG). Continue incubation for 20 hr at 20°C
- **Cell harvest:** chill flasks in an ice bath and transfer to prechilled centrifugal flasks. Harvest cells by centrifugation at 4,000 x g at 4°C. Wash cell pellets carefully with ice-cold phosphate buffered saline and centrifuge again. Pellets can be lysed immediately or stored at –80°C

Day 4 Cell Lysis

Day 4
Cell lysis
Cas9 purification (IMAC-CEX)



- **Cell lysis buffer:** prepare lysis buffer without Tris(2-carboxyethyl) phosphine (TCEP), Pefabloc SC, and Benzonase and adjust pH at the temperature that will be used during chromatography (4°C is recommended). Filter, sterilize, and degas all buffers. Immediately before use in cell suspension, supplement the base buffer with TCEP, Pefabloc SC, and Benzonase (see bulletin 3211 for details)
- **Sonication:** resuspend cells in ice-cold lysis buffer at a ratio of 20 ml buffer to 1 g cell wet weight. Lyse by several rounds of sonication on ice
- **Removal of cell debris:** centrifuge the lysate at 40,000 x g for 10 min at 4°C. Decant supernatant and keep on ice. Determine total protein concentration on an aliquot of lysate using the *DC* Protein Assay Kit I and adjust to 1 mg/ml with cold lysis buffer

Day 4 IMAC and CEX Chromatography

This protocol utilizes an NGC Quest Chromatography System with a Sample Pump and Column Switching Valve.

It is recommended to perform chromatographic separations at low temperatures to maintain sample integrity. The buffers described in Table 1 should be prepared, refrigerated, and pH adjusted at 4°C before filter sterilization and degassing under vacuum. See Figure 1 for purification strategy.

Table 1. Columns and buffers.

Column Type	Resin Type	Buffer A	Buffer B	Flow Rate, ml/min
EconoFit Nuvia IMAC (5 ml)	Affinity	20 mM HEPES 300 mM NaCl 25 mM imidazole 10% (v/v) glycerol 0.5 mM TCEP, pH 7.5	Buffer A with 0.5 M imidazole	4
EconoFit UNOsphere S (5 ml)	Strong cation exchanger	20 mM HEPES 10% (v/v) glycerol 0.5 mM TCEP, pH 7.5	Buffer A with 1 M NaCl or KCl	2

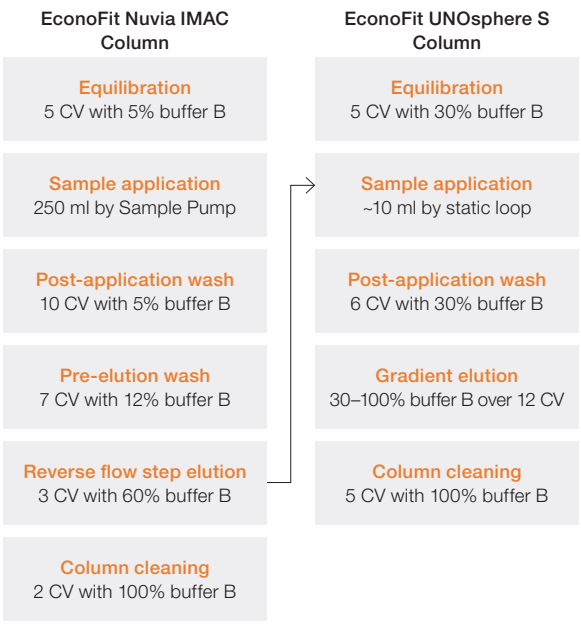


Fig. 1. Two-step purification strategy (IMAC-CEX) of Cas9. Proposed conditions of each phase are shown for each method. Column and buffer information are in Table 1. CV, column volume.

Note: The Cas9-containing peak fractions (~10 ml) that eluted from the EconoFit Nuvia IMAC Column were pooled, kept on ice, and then subjected to CEX chromatography (Figure 2). However, IMAC eluates can be stored for up to 18 hours at 4°C if it is not possible to continue with the CEX purification step on the same day.

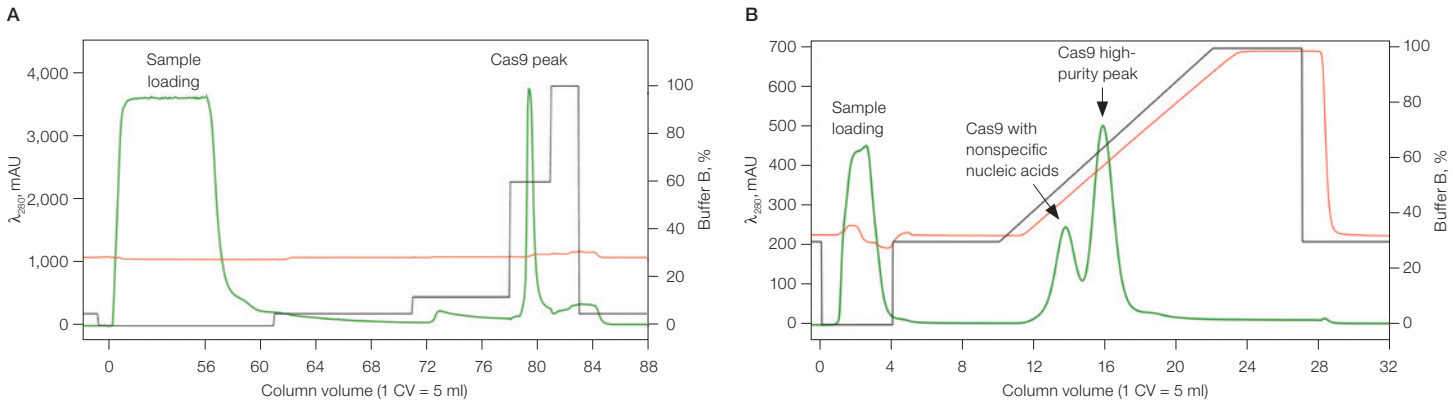


Fig. 2: Representative chromatograms of purification with EconoFit Nuvia IMAC Column followed by EconoFit UNOsphere S Column. A, EconoFit Nuvia IMAC Column; **B**, EconoFit UNOsphere S Column. λ 3 (280 nm) (—); conductivity (—); buffer B percentage (—).

Day 5 Electrophoresis and Storage

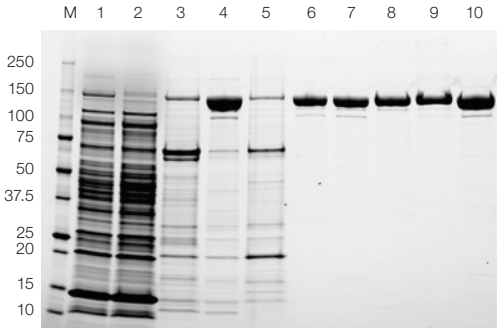
Day 5

Cas9 concentration and storage

Electrophoresis

–80°C storage

- **Electrophoresis:** check quality of the chromatographic purification steps by SDS-PAGE (Figure 3) on a Criterion TGX Stain-Free Protein Gel, 4–20% gradient
- **Storage:** pool Cas9 eluate fractions if needed, buffer-exchange into storage buffer (see bulletin 3211 for details), and concentrate with ultrafiltration spin columns (100 kD cutoff). Aliquot and flash-freeze in liquid nitrogen, then store at –80°C.



M	Protein standard (kD)
1	Cell lysate
2	IMAC FT
3	IMAC wash
4	IMAC eluate pool
5	CEX FT
6	CEX E2 (2 µg)
7	CEX E3 (2 µg)
8	CC9-1 (2 µg)
9	CC9-2 (2 µg)
10	CEX eluate pool (4 µg)

Fig. 3. Representative Stain-Free gel of chromatographic fractions. The purity of eluates was confirmed using Stain-Free SDS-PAGE gels activated for 2.5 min. Fractions from the two-step chromatographic purification (IMAC-CEX) of heterologous Cas9 from *E. coli* lysate (lanes 1–7 and 10) were compared against two commercially available Cas9 samples (lanes 8 and 9). CEX, cation exchange; E2, eluate 2; E3, eluate 3; FT, flowthrough; IMAC, immobilized metal affinity chromatography.

Ordering Information

Catalog #	Description
12009286	**EconoFit Nuvia IMAC Column**, Ni-charged, 1 x 5 ml
12009305	**EconoFit UNOsphere S Column**, 1 x 5 ml
5000111	***DC* Protein Assay Kit I**
5678093	**Criterion TGX Stain-Free Protein Gel**, 4–20% gradient
7880001	**NGC Quest 10 Chromatography System**
7884004	**NGC Sample Pump Module**
7884012	**NGC Column Switching Valve Module**
7410002	**BioFrac Fraction Collector**
17002070	**NGC Fraction Collector with Racks**

For a full protocol describing Cas9 production and validation, and a list of materials used, refer to our application note (bulletin 3211).

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