

Purification of Cas9 Nuclease with a Two-Step Chromatographic Process

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Abstract

The clustered regularly interspaced short palindromic repeats with associated protein 9 (CRISPR-Cas9) system is an exciting technology for targeted genome editing that has been employed in a multitude of organisms and cells. While early applications relied on transfection of plasmid encoding Cas9, more recent work utilizes purified Cas9. Purified Cas9 is combined with chemically synthesized guide RNA (gRNA) *in vitro* to form a Cas9-gRNA complex, which is then delivered into cells via chemical or physical transfection methods. We have developed a fast, robust, and economical two-step chromatography workflow using prepacked EconoFit Columns for the purification of histidine-tagged Cas9. The EconoFit Nuvia IMAC Column provided efficient capture of the target protein, yielding excellent recovery at high flow rates. Further clearance of residual host cell proteins was achieved via cation exchange chromatography performed with an EconoFit UNOsphere S Column, which enabled minimal sample adjustment for efficient Cas9 binding. We provide detailed procedures to obtain Cas9 preparations that match the purity and the *in vitro* and *in vivo* activity of commercially available Cas9 products. Our study provides a comprehensive guide to purify large quantities of Cas9, or variants, in a cost-effective manner.

1. Introduction

CRISPR-Cas9 genome engineering is hailed as a revolution in molecular biology. The CRISPR-Cas9 technology utilizes the Cas9 nuclease, derived from bacteria, in conjunction with a gRNA to target and edit precise locations within the genome. This novel genome-editing technology (Ledford and Callaway 2020) has countless applications in life and biomedical sciences, earning Emmanuelle Charpentier, PhD and Jennifer Doudna, PhD the 2020 Nobel Prize in Chemistry.

While the early application of CRISPR-Cas9 relied on Cas9 nuclease expressed from plasmids, researchers are increasingly utilizing recombinant Cas9 nuclease overexpressed and purified chromatographically for genome engineering experiments. The use of recombinant Cas9 enables improved control of Cas9 activity, preventing off-target effects *in vivo* and reducing unintended cytotoxicity (Liu et al. 2015). To increase efficiency and target the desired genomic location for editing, Cas9 is combined with chemically synthesized gRNA *in vitro* to form a Cas9-gRNA

complex. This Cas9-gRNA complex is then delivered into cells via chemical or physical transfection methods (Fajrial et al. 2020).

Purified Cas9 proteins are commercially available from numerous vendors; however, the cost may be prohibitive to many researchers conducting large-scale genome editing studies. In addition, researchers may wish to conduct studies using their unique Cas9 variants and thus need to create and optimize specialized Cas9 purification strategies. Several protocols for the heterologous expression and purification of Cas9 have been published (Liu et al. 2015, Rajagopalan et al. 2018). The most common purification strategy (Lin et al. 2014) includes three chromatography steps: histidine-tagged affinity purification, cation exchange (CEX), and final polishing purification by size exclusion chromatography (SEC).

Here we developed a fast, robust, and cost-effective two-step purification strategy for Cas9 nuclease (Figure 1). This protocol requires little up-front investment, avoids high technical requirements and knowledge, and enables efficient Cas9 preparations of high quality and purity. We demonstrate the

functionality of our two-step Cas9 preparations by both in vitro and in vivo assays, comparing the activity of our nuclease against commercially available Cas9 as a benchmark.

2. Materials and Methods

2.1. Protein Expression and Sample Preparation

Wild-type Cas9 from *Streptococcus pyogenes* was expressed in *Escherichia coli* strain BL21 (DE3). The pET-NLS-Cas9-6xHis vector with an N-terminal nuclear localization signal (NLS), a 6xHis affinity tag at the C-terminus, and ampicillin resistance was obtained from Nova Lifetech Pte Ltd (#PVT10639). Vector maintenance and propagation were performed with One Shot Mach1 T1 Phage-Resistant Chemically Competent *E. coli* (Thermo Fisher Scientific Inc., #C862003), and the Aurum Plasmid Mini Kit (Bio-Rad Laboratories, Inc., catalog #7326400) was used for plasmid purification. Plasmid DNA transformation into competent *E. coli* BL21 (DE3) cells was performed according to a standard heat-shock protocol (Froger and Hall 2007). The *E. coli* expression culture (4 x 125 ml) was grown in LB media in 500 ml baffled flasks at 37°C to an optical density of 0.6–0.8 at 600 nm (OD 600). Prior to induction of protein expression with 0.4 mM isopropyl β -D-1-thiogalactopyranoside (IPTG), the temperature of the cell culture was reduced to 20°C. The induced cells were further incubated for 20 hr at 20°C. Cell harvest was performed by centrifugation at 4,000 x g at 4°C. Cell pellets were carefully washed with ice-cold phosphate buffered saline (PBS), centrifuged again, and stored at –80°C.

Frozen cell pellets were thawed on ice and resuspended in ice-cold lysis buffer at a ratio of 20 ml buffer to 1 g cell wet weight. The lysis buffer consisted of 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 300 mM NaCl, 25 mM imidazole, 2 mM MgCl₂, and 10% (v/v) glycerol, pH 7.5. Immediately before use in pellet resuspension, the lysis buffer was supplemented with Tris (2-carboxyethyl) phosphine (TCEP), Pefabloc SC, and Benzonase endonuclease to yield final concentrations of 0.5 mM, 1 mM, and 5 U/ml lysis buffer, respectively. The addition of lysozyme (~1 mg/ml) is optional and may improve protein extraction efficiency. The cells were lysed by several rounds of sonication on ice and then centrifuged at 40,000 x g for 10 min at 4°C. The efficiency of cell lysis was monitored by calculation of cell wet weight reduction. The supernatant protein concentration was determined by the DC Protein Assay Kit I (Bio-Rad, #5000111). The supernatant was then adjusted to 1 μ g/ μ l with lysis buffer containing TCEP and Pefabloc SC.

2.2. Chromatography

Each purification was performed with 250 ml of cell lysate adjusted to 1 mg total protein/ml. An NGC Chromatography System outfitted with prepacked EconoFit Columns (Bio-Rad, #12009286 and 12009305) was used for the following purifications. Samples were monitored at absorbances of 260 and 280 nm. Purifications were performed at ambient temperature, but using prechilled, sterile filtered through 0.45 μ m polyethersulfone (PES) membranes, and degassed chromatography buffers at 4°C. The four-tier NGC

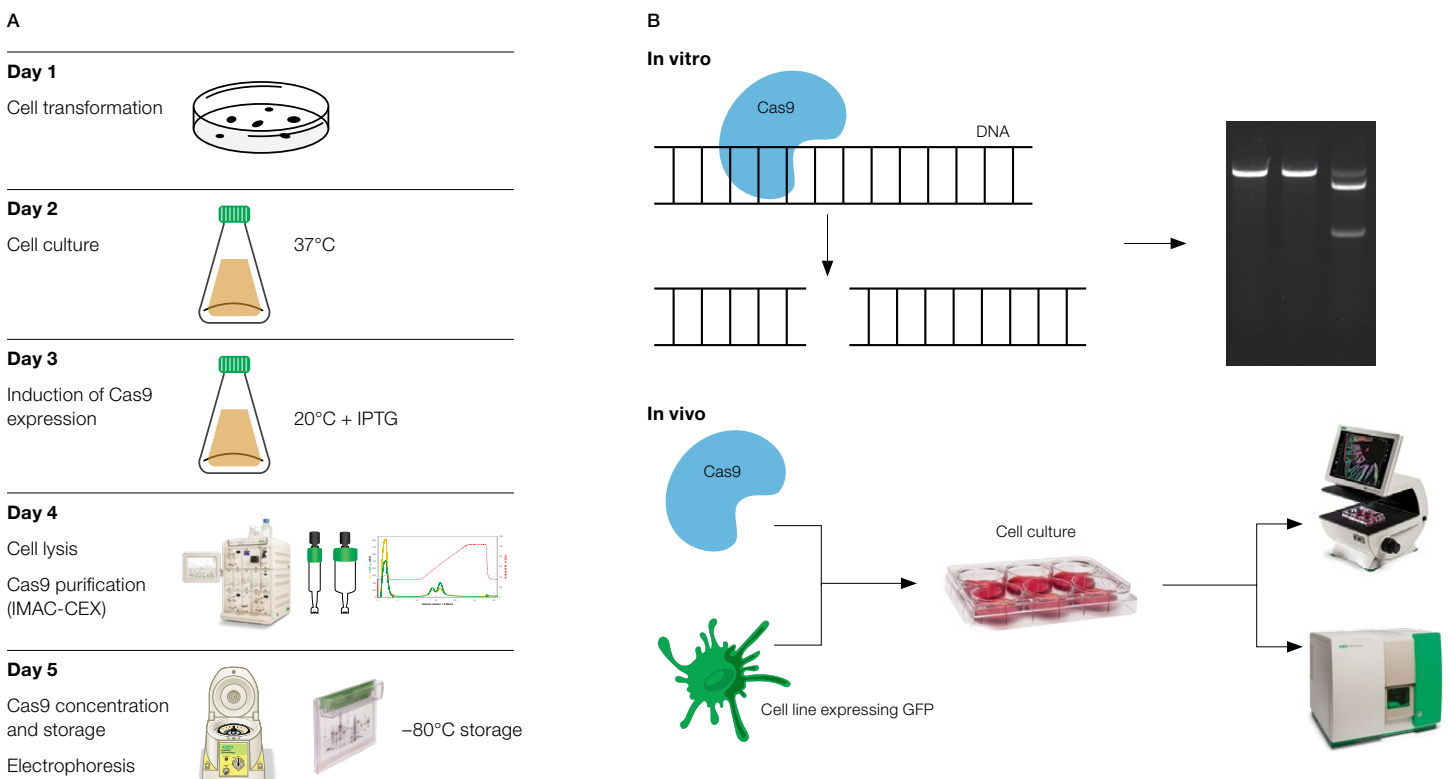


Fig. 1. Timeline and overview for the bacterial expression and fast two-step chromatographic purification of wild-type Cas9 from *Streptococcus pyogenes* (A) and subsequent in vitro and in vivo validation analysis (B).

Discover Pro Chromatography System was configured with a sample pump including sample inlet valve, two buffer inlet valves, column switching valve, multi-wavelength UV/Vis detector, pH and conductivity meter, outlet valve, and NGC Fraction Collector. NGC System sanitization using sodium hydroxide prior to purification, as detailed by Posch et al. 2019, was conducted to ensure no endotoxin contamination. Information on chromatography columns and respective buffer compositions are presented in Table 1 while the recommended operating conditions are detailed in Figure 2. After the final purification step, Cas9 eluate fractions were pooled, buffer-exchanged, and concentrated with ultrafiltration spin columns (100 kD cutoff). The concentration of pure Cas9 preparations was measured spectroscopically at 280 nm: a 1 mg/ml solution has an absorbance of ~0.76. Cas9 in storage buffer (20 mM HEPES, 150 mM KCl, 10% [v/v] glycerol, and 1 mM TCEP, pH 7.5) was aliquoted (50 μ l), flash-frozen in liquid nitrogen, and stored at -80°C .

Table 1. Overview of all columns and their operating conditions utilized in this study.

| 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, and 0.5 mM TCEP, pH 7.5 | Buffer A with 0.5 M imidazole | 4 |
| EconoFit Macro-Prep High S (5 ml) | Strong cation exchanger | 20 mM HEPES, 10% (v/v) glycerol, and 0.5 mM TCEP, pH 7.5 | Buffer A with 1 M NaCl or KCl | 2 |
| EconoFit UNOsphere S (5 ml) | Strong cation exchanger | 20 mM HEPES, 10% (v/v) glycerol, and 0.5 mM TCEP, pH 7.5 | Buffer A with 1 M NaCl or KCl | 2 |
| ENrich SEC 650 10 x 300 mm (24 ml) | Size exclusion | 20 mM HEPES, 10% (v/v) glycerol, 300 mM NaCl or KCl, and 0.5 mM TCEP, pH 7.5 | N/A | 0.75–1 |

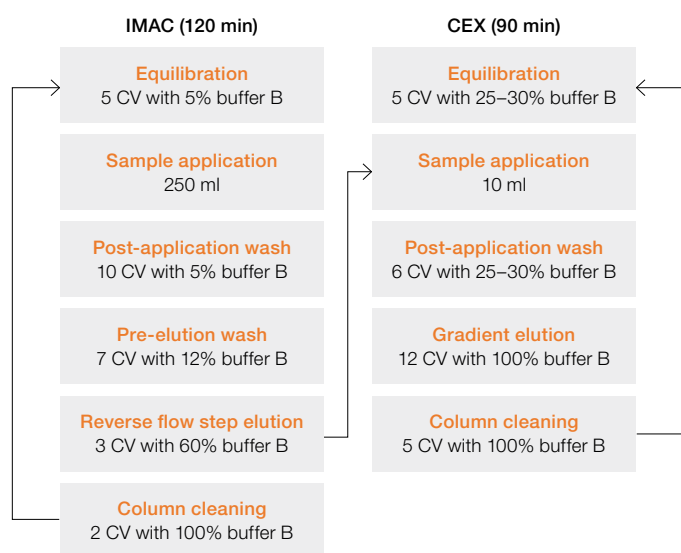


Fig. 2. Two-step purification strategy (IMAC-CEX) of Cas9 and proposed gradient segments for each method. Column and buffer information can be found in Table 1. CV, column volume.

2.3. Protein Electrophoresis and Western Blotting

All equipment and materials were from Bio-Rad unless specified otherwise. The quality of the chromatographic purification steps was monitored by SDS-PAGE with precast 4–20% gradient Criterion TGX Stain-Free Gels (#5678093). SDS-PAGE gels were UV activated after the electrophoresis, 45 sec for subsequent western blotting or 2.5 min for gel imaging only, on a ChemiDoc MP Imaging System. After imaging, the gels were either subjected to western blotting or counterstained with Oriole Fluorescent Gel Stain (#1610496), followed by overnight staining with QC Colloidal Coomassie Stain (#1610803).

For western blotting with fluorescent signal detection, the activated gel was transferred to a low fluorescence polyvinylidene difluoride (LF PVDF) membrane (#1620262) for 7 min using the Trans-Blot Turbo Transfer System (#1704150) with Trans-Blot Turbo Midi PVDF Transfer Packs (#1704157). The PVDF blotting membrane was blocked for 5 min at room temperature with gentle agitation using EveryBlot Blocking Buffer (#12010020). Primary rabbit polyclonal antibody against Anti-TurboGFP(d) Antibody (Evrogen JSC, #AB513) was diluted 1:1,000 and secondary goat-anti rabbit IgG conjugated with Starbright Blue 700 (#12004161) 1:20,000 in blocking buffer. After incubation with primary antibody for 1 hr, the blotting membrane was washed 4 x 10 min with Tris buffered saline with Tween 20 (TBST) and further incubated with secondary antibody solution. Before imaging with the ChemiDoc MP Imaging System, the blotting membrane was washed again 4 x 10 min with TBST. Gel and blot analyses were performed with Image Lab Software.

2.4. Analytical and Functional Assays

Aggregate levels in Cas9 preparations were analyzed using a high-resolution, ENrich SEC 650 10 x 300 mm Column (24 ml; Bio-Rad, #7801650) at a flow rate of 0.7–1 ml/min. A static loop was used to inject 250 μ l of Cas9 sample into the chromatography system and the sample was monitored at 260 and 280 nm. Aggregates were defined as the front eluting peak of the SEC profile. Fractions (0.9 ml) were collected and analyzed via SDS-PAGE as described in section 2.3.

In vitro Cas9 activity was examined via two methods: an RNA-independent and RNA-dependent assay. RNA-independent in vitro single-stranded DNA cleavage assays were performed in a total volume of 10 μ l of cleavage buffer (150 mM KCl, 20 mM HEPES, 1% glycerol, 5 mM MnCl_2 , and 1 mM dithiothreitol, pH 7.2) in the presence of 1 μ M Cas9 and 50 mM M13mp18 Single-Stranded DNA (New England Biolabs, Inc., #N4040S).

For RNA-dependent in vitro double-stranded DNA cleavage assays, the pGreenPuro (CMV) shRNA Cloning and Expression Lentivector (System Biosciences, #SI505A-1) was used as the DNA substrate and *copGFP* was the cleavage target. Prior to assays, the pGreenPuro vector (7,861 bp) was linearized with EcoRI (New England Biolabs) using standard digestion conditions. gRNA design was performed by CRISPOR software (Concordet and Haeussler 2018) and revealed the following sequence: 5' CACCCGCAUCGAGAAGUACG 3'. The cleavage buffer consisted

of 20 mM HEPES, 100 mM NaCl, 5 mM MnCl₂, and 1 mM ethylenediaminetetraacetic acid (EDTA), pH 6.5. Reaction volumes were 20 µl and contained 160 nM ribonucleoprotein (RNP) complex and 3.5 nM DNA substrate at a molar ratio of 50:1.

Reactions were incubated at 37°C, stopped by adding 1 µl Proteinase K (20 mg/ml), and heated at 56°C for 10 min. Cleavage products were separated on 0.5% agarose gels (Tris/acetic acid/EDTA buffer system) and stained with ROTI GelStain Red (Carl Roth, #0984.1). Agarose gels were imaged with the ChemiDoc MP Imaging System.

In vivo assays were performed with the pancreatic tumor cell line T3M-4, which was lentiviral transduced with pGreenPuro vector according to Anastasov et al. (2016). In brief, cultured cells were plated in 6- or 96-well format in RPMI media with 10% fetal bovine serum at a cell density necessary to reach ~70% confluence the next day. For Cas9 delivery, two reagent mixtures were prepared ex vivo: gRNA was incubated with Cas9 protein in Opti-MEM I Reduced Serum Medium (Thermo Fisher Scientific, #31985062) for 5 min before mixing with the TransFectin Lipid Reagent (Bio-Rad, #1703351) in Opti-MEM I Reduced Serum Medium. The resulting Cas9 nuclease/gRNA/transfection reagent mixture was incubated for 5–10 minutes at room temperature and added to the T3M-4 cells (10 µl or 250 µl of mixture per well). Approximately 6.2 µg Cas9 protein is needed for each well of a 6-well plate. The molar ratio of Cas9/gRNA was 1:1. Cells were visually inspected and assayed for changes in CopGFP expression with the ZOE Fluorescent Cell Imager (Bio-Rad, #1450031) and by flow cytometry with the ZE5 Cell Analyzer (Bio-Rad, #12004279) at different time points. Any samples analyzed more than 24 hours after transfection were subjected to daily media changes until analysis.

For analysis on the ZE5 Cell Analyzer, the cells were trypsinized and stained with either PureBlu DAPI Nuclear Staining Dye or VivaFix 410/450 Cell Viability Assay (Bio-Rad, #1351303 or 1351112, respectively) to assess cell health in flow cytometry experiments. In addition, cells were fixed with paraformaldehyde for 15 min at room temperature and stored in 1% bovine serum albumin prepared with PBS at 4°C until analysis using the ZE5 Cell Analyzer. The flow rate of the ZE5 Cell Analyzer was set to 2.5 µl/sec and 200,000 cells per sample were acquired. Data analysis was performed with the instrument software package Everest or FCS Express (De Novo Software).

3. Results and Discussion

Protein expression in *E. coli* is a well-proven technology platform and has been successfully used to produce numerous proteins over the last few decades (Rosano and Ceccarelli 2014). Successful protocol development for heterologous expression and purification of proteins requires the careful evaluation of important steps along the production path, such as cloning, expression conditions, cell lysis, purification, and storage. Although it seems that the laboratory-scale production process for Cas9 is well understood, there are some major pitfalls that can lead to incorrectly folded protein, solubility

issues, and protein aggregation. Protein stability is greatly enhanced during protein expression and purification through a few simple measures that were implemented in our protocol:

- Carry out protein expression at lowered temperatures
- Prevent sample overheating and foaming during cell lysis
- Perform chromatographic separations at low temperatures and maintain integrity of samples with a fraction collector that has a cooling option
- Optimize overall process time and circumvent lengthy buffer exchange procedures
- Avoid exceptionally high protein concentrations on chromatography columns and after elution
- Consider buffer additives to improve protein stability and integrity

3.1. Protein Expression and Sample Preparation Considerations

Here we overexpressed wild-type Cas9 protein from *S. pyogenes* in *E. coli* BL21 (DE3) cells. The C-terminal 6xHis affinity tag allows for the easy enrichment of expressed Cas9 protein after cell lysis by immobilized metal affinity chromatography (IMAC) and the NLS sequence enables Cas9 translocation to nucleus. Affinity tags can sometimes interfere with the activity of proteins and a number of solutions exist for the enzymatic removal of tags, including the Profinity eXact Fusion-Tag System from Bio-Rad. However, the 6xHis affinity tag of Cas9 has not been reported to interfere with its activity and commercially available Cas9 products (referred to as CC9 here) are offered as either N or C terminally tagged fusion proteins. Zuris et al. (2015) have tested the effect of a C-terminal histidine-tag on Cas9 in vivo delivery as a function of both gRNA and Cas9 concentration and reported no negative effects compared to native Cas9 protein.

The expression strains Rosetta 2 (DE3) or BL21 (DE3) and derivatives are the preferred strains for the overexpression of Cas9 in *E. coli* (Ma et al. 2015, Rajagopalan et al. 2018). The respective Cas9 expression protocols recommend cell incubation at 16–20°C for 16–20 hours after induction with IPTG at concentrations ranging from 0.1 to 0.5 mM. Reducing incubation temperature before and during induction slows protein synthesis and diminishes the risk of aggregation and improper protein folding of a given target protein.

We first conducted a small-scale Cas9 expression study to optimize the inducer concentration. An IPTG concentration of 0.4 mM yielded a significant Cas9 band with our expression vector as monitored by SDS-PAGE. For the production of Cas9 in the lower 3–5 mg range, 4 x 125 ml *E. coli* cell culture volume with a cell density of 0.6–0.8 (OD 600) at the time of induction is a good starting point. After cell harvest by centrifugation at 4°C, the cell pellets can be either used for immediate cell lysis or stored at –80°C for several months without loss of Cas9 activity.

The composition of the lysis buffer is critical for a successful cell lysis protocol. The lysis should guarantee the stability, integrity, and solubility of the target protein, so appropriate pH and buffer conditions are critical. In addition, the lysis buffer should be

compatible with the first downstream application, here IMAC, to avoid an extra buffer exchange step. The chosen IMAC buffer consists of 20 mM HEPES, 25 mM imidazole, and 300 mM NaCl, pH 7.5–8.0, and is a good starting point for the design of a suitable cell lysis buffer. To avoid common obstacles during sample preparation, such as enzymatic protein degradation, oxidation, and aggregation, additional buffer additives are recommended (Table 2).

Typical methods for the mechanical disruption of *E. coli* cells are sonication or high-pressure homogenization, for example, French press, which usually lyses cells in one passage. In contrast to high-pressure homogenization instruments, ultrasonic processors tend to be more widely found in research laboratories but require more time and attention for protocol development. Important parameters to consider are sample volume, probe size, pulsing, and amplitude (Islam et al. 2017). The perfect balance varies for different cell types and sample volumes and may take some optimization. Of utmost importance is always to keep the samples on ice and avoid overheating of the solution, which may lead to protein denaturation or even fragmentation. Usually, DNA is sheared during sonication, but since Cas9 can bind nonspecifically to nucleic acids, it is recommended to add a nonspecific endonuclease to the lysis buffer. In our study, 1 g cell mass in 20 ml lysis buffer was the preferred cell density for the sonication process. The reduction in cell wet weight is a simple indicator of sonication efficiency and should be in the range of 50–70% after a few sonication cycles.

Table 2. Buffer additives for effective lysis and maintaining the integrity and solubility of Cas9 during sample preparation.

| Lysis Buffer Additives | | | |
|------------------------|-------------|-----------------------|---|
| Additive Type | Example | Initial Concentration | Purpose |
| Salts | KCl | 200 mM | Reduction of electrostatic interaction |
| | NaCl | 300 mM | |
| Osmolytes | Glycerol | 10% (v/v) | Stabilization of folding and prevention of aggregation |
| Reducing agents | TCEP | 0.5 mM | Reduction of oxidation damage |
| Endonucleases | Benzonase | 5 U/ml | DNA/RNA cleavage: prevention of cell clumping and viscosity reduction |
| Proteinase inhibitors | Pefabloc SC | 1 mM | Prevention of enzymatic degradation |

3.2. Chromatography

Bio-Rad offers a wide selection of chromatography resins and columns for a range of application types. Here we utilized prepacked EconoFit Columns that are ideally suited for protein purification at analytical to preparative scale, as shown by the production of research grade Cas9 nuclease described in sections 3.2.1 and 3.2.2.

3.2.1. Immobilized Metal Affinity Chromatography

IMAC is a well proven technology for the purification of histidine-tagged proteins and can be applied under both native and denaturing conditions. Nuvia IMAC, a rigid macroporous high-capacity resin (>40 mg protein/ml resin) designed for process

production at high flow rates, was used to capture histidine-tagged Cas9 from *E. coli* lysate. As with other affinity chromatography techniques, protein enrichment via IMAC is robust and requires minimal optimization of conditions. However, the most important workflow element in IMAC for enhanced product purity is the optimization of the pre-elution column wash. In initial IMAC experiments (data not shown), a 12%B pre-elution column wash step (60 mM imidazole final) was determined to be optimal for strong Cas9 binding in conjunction with removal of most lightly bound contaminating proteins. A typical IMAC chromatogram of the optimized workflow is shown in Figure 3. Cas9 was eluted from a 5 ml EconoFit Nuvia IMAC Column in reverse flow with 300 mM imidazole. Here, the flow travels from the bottom to the top of the column and this is especially useful for applications that require elution of proteins at higher concentration. The Cas9-containing peak fractions (~10 ml) were pooled, kept on ice, and subjected to CEX chromatography. However, the eluates can be stored for at least 18 hours at 4°C if it is not possible to continue with the CEX purification step on the same day.

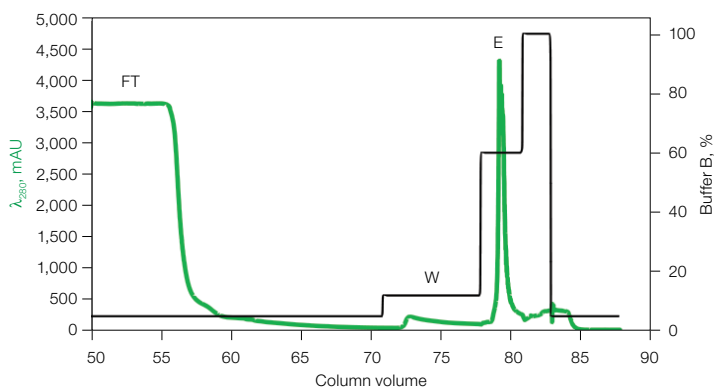


Fig. 3. Representative chromatogram of the capture of histidine-tagged Cas9 on EconoFit Nuvia IMAC Column (5 ml). E, eluted target; FT, flowthrough; W, pre-elution wash.

3.2.2. Cation Exchange Chromatography

Cas9 (158 kD) has a theoretical isoelectric point of 8.98. At physiological pH conditions, Cas9 is expected to carry a positive charge and CEX chromatography is thus ideally suited for further purification of the IMAC eluate pool containing Cas9. In most Cas9 purification protocols, it is common practice to equilibrate the respective CEX columns with 20 mM HEPES and 100–150 mM NaCl or KCl, pH 7.5. The Cas9 IMAC elution pool is a high-conductivity sample (300 mM NaCl and 300 mM imidazole) and needs to be buffer-exchanged or diluted to match the proposed CEX equilibration conditions. Different resins can yield varied results for a given protein due to differences in bead chemistries and sizes. We tested the performance of two different 5 ml EconoFit CEX Columns (Macro-Prep High S and UNOsphere S) under low-conductivity buffer conditions (Figure 4). The Macro-Prep High S Column showed a two-peak separation with the main peak exhibiting a shoulder. In contrast, the UNOsphere

S Column was able to resolve the main peak, resulting in a clear three-peak separation. The corresponding fractions of both CEX runs were analyzed by SDS-PAGE for Cas9 content.

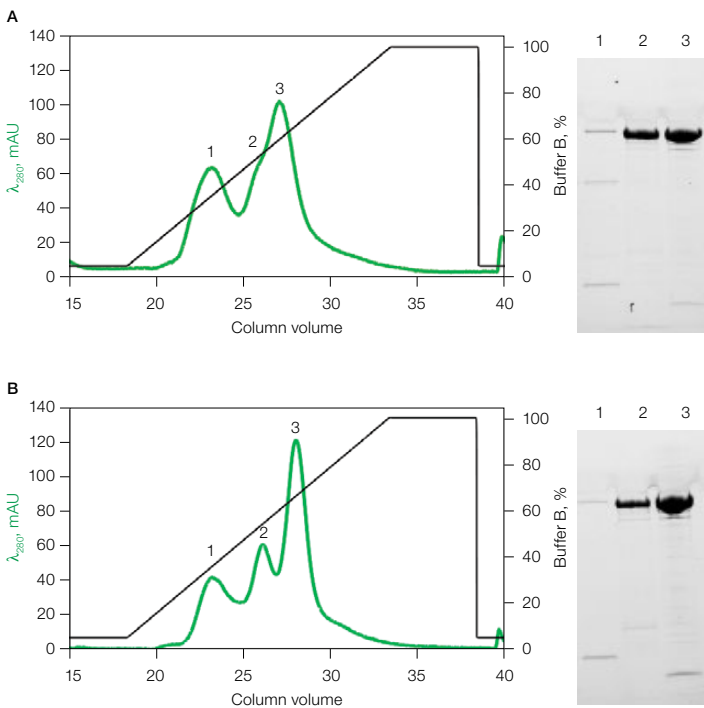


Fig. 4. Scouting of cation exchange resins for the purification of Cas9 following IMAC purification step. **A**, Macro-Prep High S Column; **B**, UNOsphere S Column. For both resins, 5 ml EconoFit Columns were used and eluate fractions 1–3 from each column type were analyzed by Stain-Free SDS-PAGE for Cas9 content.

SDS-PAGE analysis of both CEX chromatograms suggests that the elution of Cas9 starts at about 400 mM salt concentration. The tight binding of Cas9 to both CEX resins at relatively high salt concentrations enables direct loading of the Cas9 IMAC elution pool. This binding strategy at elevated conductivity buffer conditions was evaluated with the UNOsphere S Column, which has shown better selectivity for Cas9 compared to the Macro-Prep High S Resin. In Figure 5, a significant flow-through (FT) peak can be observed, followed by the already described two-peak elution profile for Cas9. The high absorbance of the FT peak is partly caused by imidazole and confirmed by applying 10 ml of IMAC elution buffer (60% of buffer B) to CEX (data not shown). The three peaks were evaluated for Cas9 content by Stain-Free SDS-PAGE and, as anticipated, only a negligible amount (about 1%) of Cas9 nuclease could be found in the CEX FT fraction (Figure 6). In addition, the purity of the two Cas9 eluate peaks (E2 and E3) were individually analyzed and compared to Cas9 products (CC9-1 and CC9-2) from two different vendors, which showed purity of 94 and 96%, respectively. A similar purity level of ~94% was calculated for E2 and E3 by our two-step purification protocol at a total recovery rate of 92%. A protein purity level of 94% for a two-column approach is remarkable and due to the excellent CEX flow-through clearing of residual host proteins at high-conductivity binding conditions.

According to Anders and Jinek (2014), Cas9 typically elutes in two peaks with different ratios of the absorbances at 260 and 280 nm. Cas9 has a moderate nonspecific affinity for nonspecific RNA and DNA. Nucleic acids bound to Cas9 reduce the overall positive net charge of Cas9, leading to a heterogeneous protein elution in CEX. For the CEX run presented in Figure 5, the 260/280 nm ratio of both elution peaks was calculated to be 0.76 for E2 and 0.6 for E3. In protein purification, the 260/280 nm ratio can be a useful tool to determine the purity of an isolated protein. An ideal 260/280 nm ratio for most proteins is 0.6 or lower. Higher ratios may indicate the contamination of isolated proteins with nucleic acids (Wilfinger et al. 1997). However, a modest contamination of Cas9 with nucleic acids does not necessarily lead to protein malfunction. It is very likely that these nonspecifically bound nucleic acids are displaced by the cognate guide RNA during Cas9-mediated gene editing experiments.

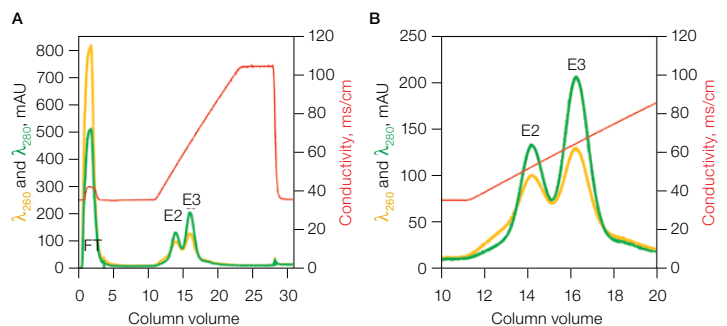


Fig. 5. UNOsphere S cation exchange chromatography with high conductivity binding conditions and direct sample loading of IMAC eluate pool on a 5 ml EconoFit Column. **A**, full-scale chromatogram; **B**, enlargement of elution area. Cation exchange chromatography flow-through (FT) and eluate fractions (E2 and E3) were analyzed by Stain-Free SDS-PAGE for Cas9 content (Figure 6).

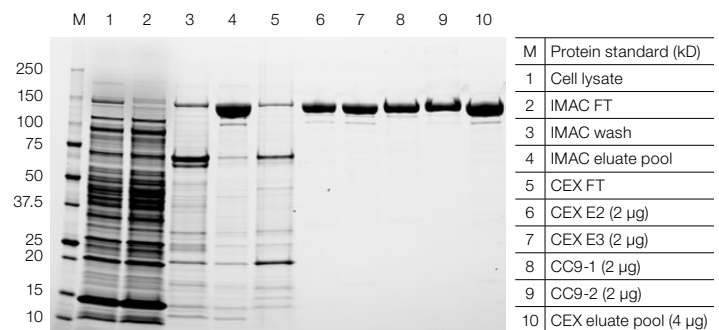


Fig. 6. Purity confirmation of two-step chromatographic purification (IMAC-CEX) of heterologous Cas9 from *E. coli* lysate. Stain-Free SDS-PAGE analysis of fractions and purity comparison against two commercial Cas9 samples (CC9-1 and CC9-2).

3.3. Qualitative, Quantitative, and Functional Assays

3.3.1 Cas9 Product Analysis with Size Exclusion Chromatography

A general problem leading to multiple protein species is the formation of protein aggregates during recombinant protein production. During purification, both intrinsic protein properties and physicochemical conditions surrounding a protein, including protein concentration, may contribute to unwanted protein aggregation (Lebendiker and Danieli 2014). Aggregate detection and removal are among the most important aspects in the purification process of aggregation-prone proteins, such as monoclonal antibodies.

Cas9 is not known for extensive aggregation formation during expression, purification, and storage. However, it is common practice to use SEC as a quality control tool in combination with SDS-PAGE, especially for final product analysis. In this study, possible Cas9 aggregates were analyzed in samples prepared for storage at -80°C . The fractions of the two Cas9 CEX elution peaks (E2 and E3) were individually pooled and concentrated to approximately 5 mg/ml using ultrafiltration spin columns. Figure 7 provides typical examples of the corresponding SEC and SDS-PAGE profiles. Data evaluation of the Stain-Free images indicates that Cas9 aggregation levels in samples obtained after IMAC-CEX and concentration by ultrafiltration were at an acceptable level of 5–7% of total protein.

The average 260/280 nm ratio of all Cas9-containing SEC fractions is about 0.85 for E2 and 0.65 for E3, indicating a higher nucleic acid contamination level for E2. The predominant Cas9 peak in SEC for both the E2 and E3 fractions shows a 260/280 nm ratio of 0.5, indicating improved purity. Nucleic acid contamination seems to be more pronounced and very problematic in the purification of Cas9 orthologs from *Streptococcus thermophilus*, *Campylobacter jejuni*, and *Neisseria meningitidis* (Jinek et al. 2012). These results support the already outlined hypothesis that the heterogeneous elution behavior in CEX can be caused by nonspecific binding of Cas9 to nucleic acids. If more stringent removal of copurifying nucleic acids is desired, it is recommended to apply heparin chromatography after the CEX step for removal, should this be required.

3.3.2. Cas9 In Vitro Activity

Two types of in vitro assays were performed to assess the nuclease activity of Cas9 after chromatography and concentration. Without gRNA, Cas9 is able to cleave single-stranded DNA (ssDNA) in a sequence-independent manner when Mn^{2+} is present (Sundaresan et al. 2017). The second, more common assay requires a double-stranded DNA substrate, Mg^{2+} , and the formation of an RNP.

The RNA-independent assay was used to test the general nuclease activity of the purified Cas9 protein during workflow optimization. The assay is easy to perform and does not require gRNA optimization for functionality. Figure 8 demonstrates the activity of Cas9 CEX eluate pools E2 and E3 during method development in comparison to the commercial product CC9-1. Here, a time course assay was performed and the two Cas9 samples degrade the DNA target (circular ssDNA) at an acceptable speed.

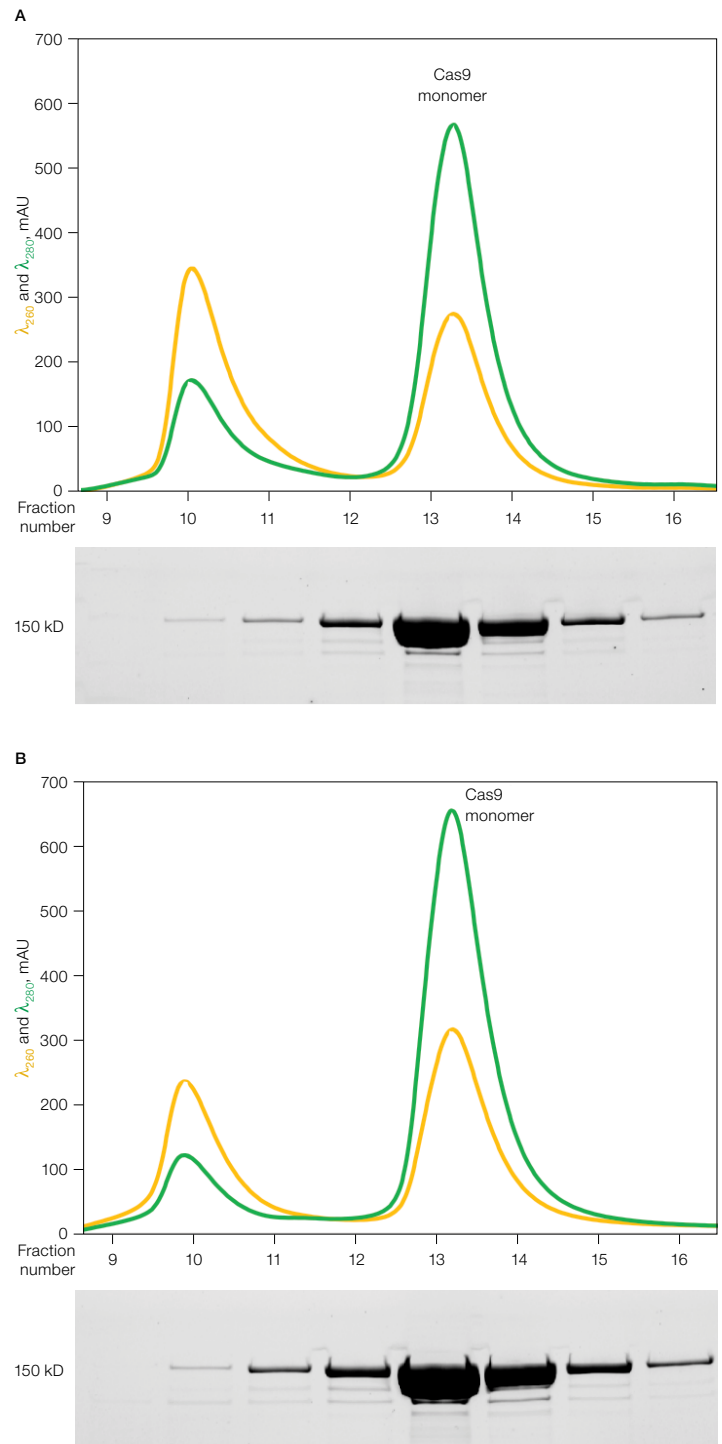


Fig. 7. Size exclusion analysis of the two Cas9 elution peaks (E2 and E3) after IMAC-CEX and concentration by ultrafiltration. A, E2 elution peak; B, E3 elution peak. Individual size exclusion fractions (0.9 ml) were analyzed by Stain-Free SDS-PAGE.

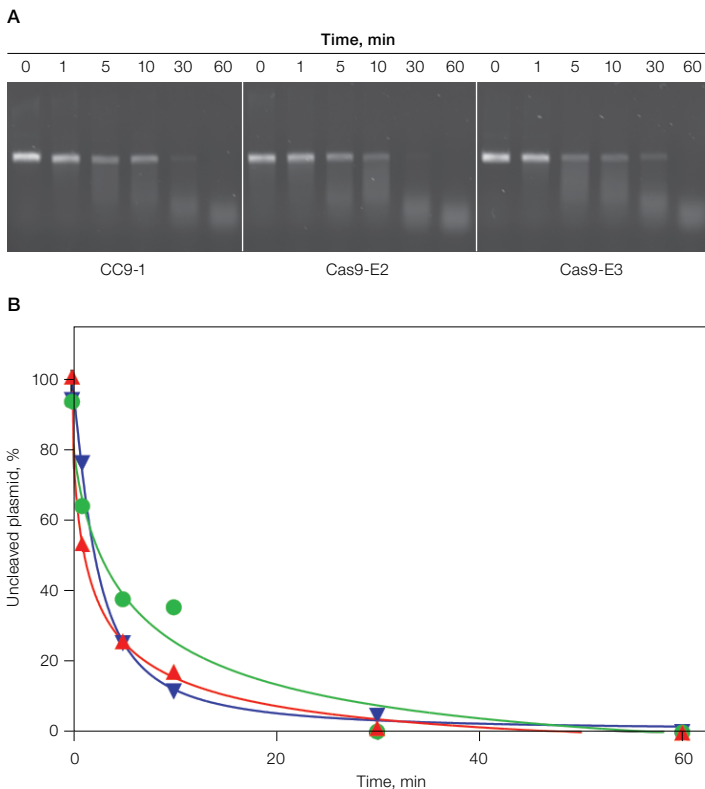


Fig. 8. RNA-independent DNA cleavage activity of IMAC-CEX purified Cas9 (peaks E2 and E3) against a commercial Cas9 product (CC9-1) over 1 hour. Target: M13mp18 circular ssDNA, which is degraded during the reaction. **A**, representative image of samples analyzed on 0.5% native agarose gel and stained with ROTI-GelStain Red; **B**, uncleaved plasmid percentage plotted against time for each Cas9 preparation. CC9-1 (▲); Cas9-E2 (●); Cas9-E3 (▼).

The DNA substrate for the RNA-dependent *in vitro* activity assay was EcoRI linearized pGreenPuro vector containing *copGFP* target gene. Vector cleavage with Cas9 will result in two DNA fragments with 2,095 and 5,766 bp. Such an assay is commonly utilized to experimentally validate the functionality of different gRNAs before genome editing experiments. Here, a single gRNA was used for an *in vitro* assay investigating multiple Cas9 chromatographic preparations that were regularly tested in comparison to each other and against two commercial Cas9 formulations (CC9-1 and CC9-2). As outlined in section 3.2.2, Cas9 preparations elute heterogeneously with comparable protein purity, but differ in their 260/280 nm ratios. Both Cas9 eluate samples (E2 and E3) were individually assayed for RNA-dependent *in vitro* activity and the reaction mixtures were analyzed by native agarose gel electrophoresis (Figure 9). This endpoint assay is a common tool in Cas9 quality control and our results showed similar performance for Cas9 samples E2 and E3. In addition, the relative cleavage rate of both Cas9 samples is highly comparable to CC9-1 and CC9-2 products. Taken together, these data suggest that peak pooling is possible, but for highly demanding gene editing experiments, the usage of a Cas9 eluate sample with a 260/280 nm ratio of about 0.6 is recommended (Anders and Jinek 2014).

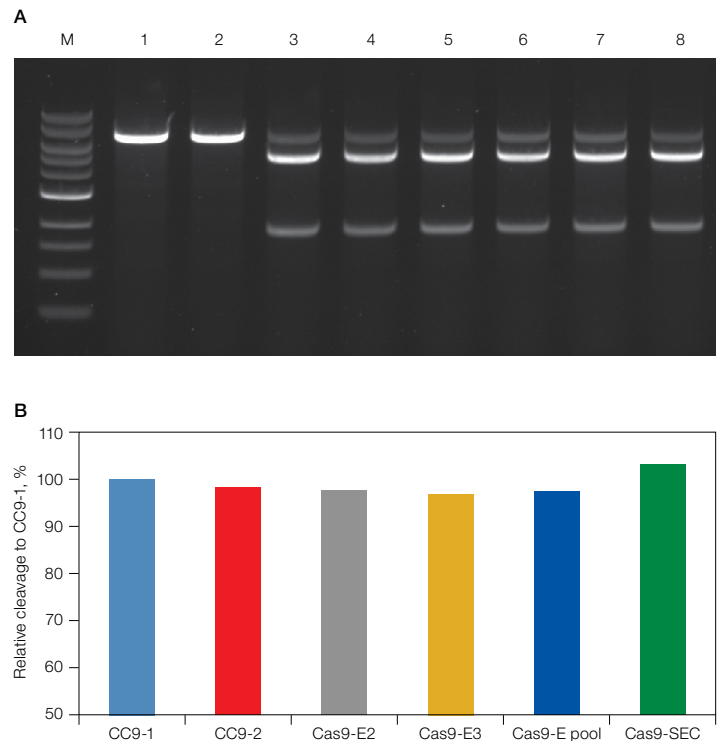


Fig. 9. RNA-dependent DNA cleavage activity of IMAC-CEX purified Cas9 compared to two commercial Cas9 products. Endpoint assay after 60 min. Target: linearized dsDNA (pGreenPuro vector: 7,861 bp). **A**, representative image of cleavage products (5,766 and 2,095 bp) analyzed on native 0.5% agarose gel with M, 1 kb DNA ladder; 1 and 2, control without gRNA; 3, CC9-2; 4, CC9-1; 5, Cas9-E2; 6, Cas9-E3; 7, Cas9 pool of E2 and E3; 8, IMAC-CEX-SEC-purified Cas9. **B**, relative cleavage comparison of Cas9 samples. Absolute cleavage performance of CC9-1 was 91% and set to 100% for relative sample comparison.

In order to estimate a possible performance advantage of a three-step purification protocol, IMAC-CEX-SEC-purified Cas9 was assayed as well and demonstrated a moderate increase in relative cleavage rate compared to our presented standard two-step purification protocol. Overall, our data show that a two-step purification protocol using EconoFit Columns is highly effective and results in a rapid purification workflow.

3.3.3. Cas9 *In Vivo* Activity

The successful functional validation of purified Cas9 by *in vitro* nuclease assays is an important achievement, but it needs to be confirmed by the positive delivery of CRISPR RNP complexes into mammalian cells. The intention of our gene editing experiments was not to answer a specific biological question, but to evaluate the *in vivo* efficacy of purified Cas9. To this end, we chose a pancreatic tumor cell line (T3M-4), transduced with vector pGreenPuro encoding CopGFP, as a model system for RNP delivery. In this cell line, loss of CopGFP functionality can easily be detected by the decline or absence of fluorescent signal, either qualitatively by fluorescence microscopy or quantitatively by flow cytometry. Here, a Cas9 preparation with a 260/280 nm ratio of 0.6 was used in transfection experiments and CC9-1 served as the positive control.

It is important to note that a well assembled and fully functional Cas9 RNP complex is an important prerequisite for successful gene editing. However, many other factors such as cell passage number, transfection conditions, and RNP concentration may be worthwhile to optimize.

Proof-of-principle transfection experiments were performed with T3M-4 cells grown in 96-well plates and qualitatively inspected for the decline of CopGFP intensity with the ZOE Fluorescent Cell Imager. The images (Figures 10A and 10B) obtained at day 2 showed a significant reduction in CopGFP intensity in samples treated with both Cas9 nucleases compared to the control cells. Two independent delivery tests were performed, and the harvested cells were analyzed in duplicate using a ZE5 Cell Analyzer at day 2 posttransfection (Figure 11). Untreated cells and cells incubated with Cas9 in the absence of gRNA served as control samples. After gating for the cells in the forward scatter (FSC) vs. side scatter (SSC) plot (data not shown), a gate for doublet exclusion was set on a FSC area vs. FSC height plot (Figure 11A). Live/dead discrimination was performed by gating on the cells that are 4',6'-diamidino-2-phenylindole (DAPI) negative (Figure 11B). CopGFP data are shown in Figure 11C. The mean fluorescence intensity (MFI) of GFP was determined for all living single cells in a histogram (Figure 11D). Flow cytometry data showed that CopGFP expression declined at day 2 after transfection for both samples: 55% for CC9-1 and 52% for Cas9 compared to the untreated

control and control that was incubated with only Cas9 in the absence of gRNA (Figure 11E). The flow cytometry data indicate that the lab-scale Cas9 preparation has an effective gene editing potential as measured at day 2 after transfection. Taken together, these data demonstrate that Cas9 purified using our two-step protocol is effective at *in vivo* gene editing, without an obvious disadvantage over commercial Cas9 products.

Another useful instrument in this context is the S3e Cell Sorter, which has been already successfully applied for CRISPR-Cas9 experiments (see [bulletin 6961](#)). The S3e Cell Sorter is temperature controlled and easy to use. It can analyze cells and sort them into multiple types of collection vessels, including 8-well PCR strips, in one step.

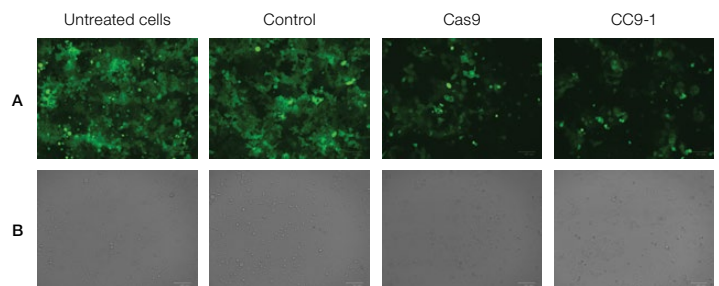


Fig. 10. Validation of CopGFP (26 kD) knockdown in T3M-4 cells at day 2 posttransfection using the ZOE Fluorescent Cell Imager. A, fluorescent cell imaging; **B,** brightfield cell imaging.

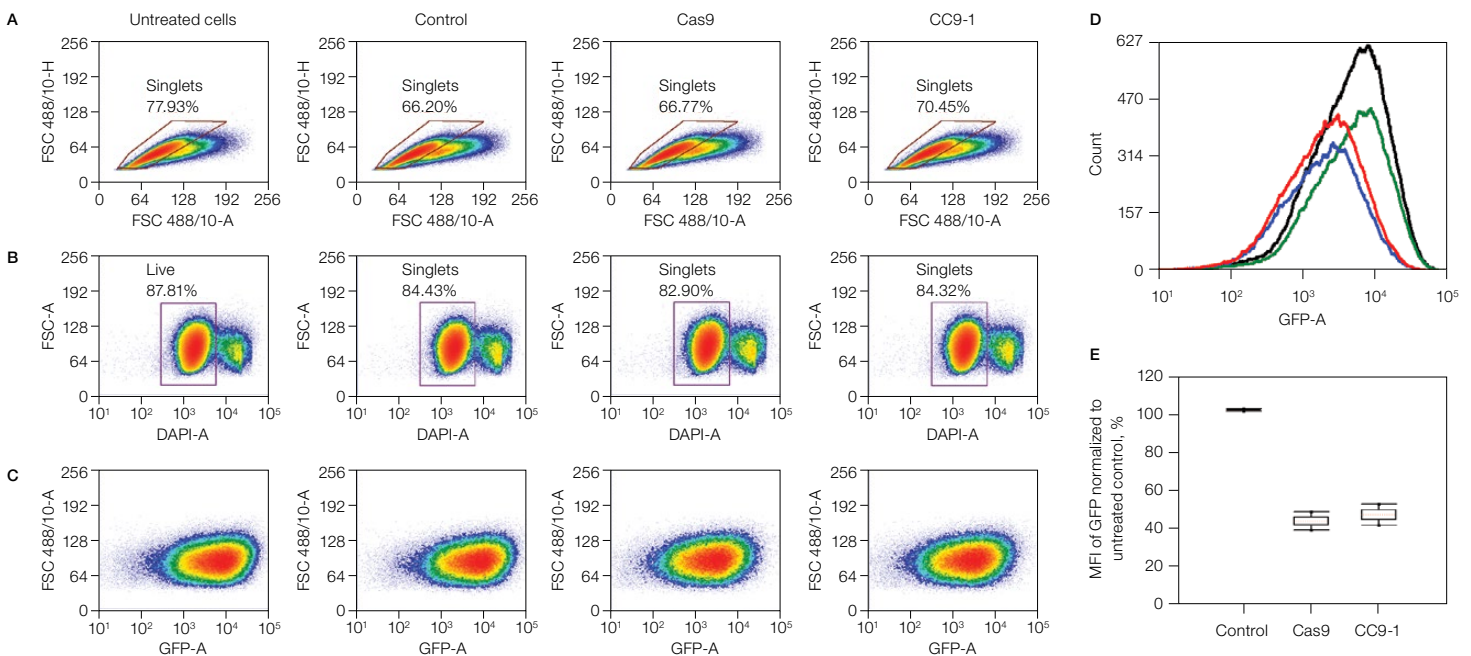


Fig. 11. Day 2 flow cytometry data of Cas9-treated T3M-4 cells on the ZE5 Cell Analyzer. Gating of flow cytometry data is shown for **A**, singlet analysis (doublet exclusion); **B**, live/dead discrimination by gating on the DAPI-negative cells; and **C**, GFP-positive live cells. **D**, the MFI of GFP was determined for all living single cells in a histogram, which is shown in an overlay of untreated control cells (—), control cells (—), cells treated with Cas9 (—), and cells treated with CC9-1 (—). **E**, the MFI of GFP signals normalized to the untreated control acquired by flow cytometry post-treatment is shown (n = 2).

Further mutation detection analysis and characterization was beyond the scope of this project, but in a proof-of-principle experiment, highly sensitive fluorescent western blotting was used to successfully detect CopGFP in RIPA-lysed T3M-4 cells. The quantitation of protein expression is a more definitive measure of gene knockdown (Estep et al. 2016) but requires highly specific primary antibodies and a very sensitive detection system. Fluorescently labeled secondary antibodies in combination with a sophisticated image acquisition system (Oh 2021) is an extremely successful combination for the relative quantitation of low-abundance targets (Gürtler et al. 2013). To demonstrate feasibility of this approach, cells were lysed at day 6 after transfection, assuming a very low CopGFP content. Total protein lysates were subjected to western blotting, incubation with anti-CopGFP antibody, and immunodetection with the Bio-Rad Starbright Blue 700 Secondary Antibody. The anti-CopGFP signals of two samples obtained after transfection with Cas9 and CC9-1 were compared to a control lysate and the reduction in CopGFP intensity in both samples is apparent (Figure 12).

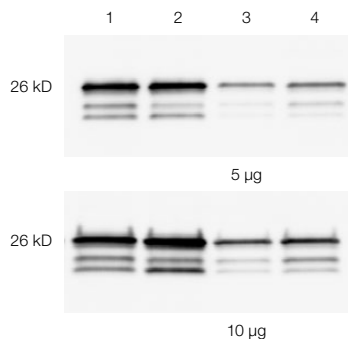


Fig 12. Validation of CopGFP (26 kD) knockdown in T3M-4 cells at day 6 post-transfection by fluorescent western blotting of cell lysates. Total protein load was 5 µg (top) or 10 µg (bottom) per lane. Lanes 1 and 2, control samples without gRNA; lane 3, transfection with CC9-1; lane 4, transfection with Cas9.

4. Conclusions

Since 2012, quite a few pioneering protocols have been published for the lab-scale preparation of Cas9 nuclease. Here, we have employed a fast and robust two-step chromatography approach (IMAC-CEX) to obtain highly purified histidine-tagged Cas9. While the EconoFit Nuvia IMAC Column provides efficient capture of the target protein from *E. coli* extract at high flow rates, the EconoFit UNOsphere S Column provides high selectivity for Cas9 at elevated conductivity conditions and, thus, unmatched potential for residual host cell protein clearance. In addition, the presented workflow provides the benefit of minimal sample conditioning, which greatly reduces purification complexity and allows reproducibility in other laboratories. Cas9 functionality was successfully demonstrated by in vitro cleavage assays, and in vivo performance was measured by flow cytometry in samples obtained with a human cell line transfected with a Cas9-gRNA complex.

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