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Expression and radiolabeling of Cas9 protein

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Abstract As a robust platform for genome editing, CRISPR/Cas9 is currently being explored for engineering biology or therapeutics, yet means for quantitative detection of Cas9 proteins remain to be fully realized. Here, we expressed Cas9 proteins and developed a novel detection method that traced Cas9 based on radiolabeled iodine. Through optimizing the reaction conditions of reaction time, temperature and cycles, we obtained ^{125}I-Cas9 of high labeling yield. The prepared ^{125}I-Cas9 was stable in various media and preserved excellent genome editing efficiency. Thus, our strategy provides a convenient and efficient tool for further tracing biological behaviors of Cas9 proteins in living systems.

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1 Introduction

The clustered, regularly interspaced, short palindromic repeat (CRISPR) technology is a novel genome editing approach [1]. Cas9, a key component of CRISPR systems, is a unitary factor capable of colocalizing single-guide RNA (sgRNA) and target DNA. Many new tools and technologies based on Cas9 protein have been developed to produce an unparalleled control over cellular regulation, organization and behavior [2, 3]. For investigating CRISPR/Cas9-mediated engineering biology or therapeutics, it is vital to know the biological behaviors of Cas9 proteins in vitro and in vivo. At present, the mainstream method for tracing proteins in living systems is fluorescent labeling, which is disadvantageous in some aspects. The biggest problem is instability and quenching of fluorescent signals would result in poor accuracy of quantitative detection of Cas9 proteins in living systems [4, 5]. Also, preparing fluorescently labeled protein costs a lot [6–9]. Therefore, development of reliable methods is needed to overcome these disadvantages.

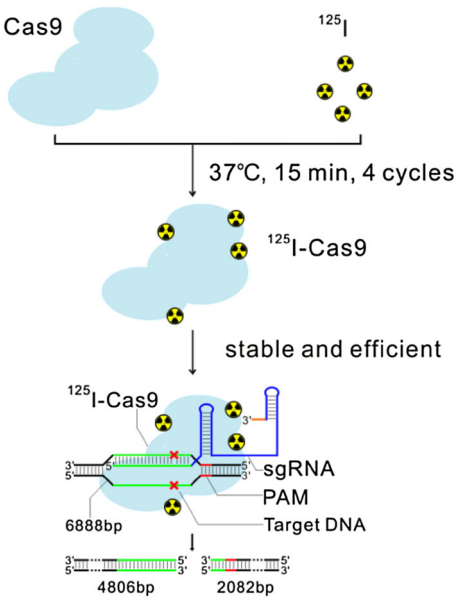
Radiolabeling technique, with high sensitivity and accuracy and fast analysis and interference-free, has been widely used in determining biological behavior of proteins in vitro and in vivo [10–15]. In particular, radiolabeled iodine covalently bound to proteins permits sensitive radioimmunoassays for protein identification and tracing [16, 17]. However, to our knowledge, no reports are available so far on radioiodination of Cas9 protein.

In this work, we selected the well-characterized and extensively applied *Streptococcus pyogenes* Cas9 (hereon referred to as Cas9) [18, 19] and purified the recombinant Cas9 protein fused with a C-terminal nuclear localization signal (NLS) following overexpression in *Escherichia coli*. Especially, an effective ^{125}I labeling method for Cas9 protein detection was established. By optimizing the radiolabeling conditions, we obtained Cas9 protein with high labeling yield, perfect stability and excellent cleavage activity (Scheme 1). The work is important for biomedical applications of CRISPR/Cas9 systems.

2 Materials and methods

2.1 Materials

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified and were used as received. Double-stranded DNA was purchased from Invitrogen Life Technologies (Shanghai, China). cOmplete^{TM}, EDTA-free Protease Inhibitor Cocktail was purchased from Roche Diagnostics Ltd (Indianapolis, USA). Ni–NTA Superflow Columns were obtained from QIAGEN GmbH (Hilden, Germany). Plasmids pET-Cas9-NLS-6xHis encoding the Cas9 protein was a gift from David Liu (Addgene plasmid # 62933).



Scheme 1 Schematics of preparing the radiolabeled Cas9 protein, with high labeling yield, perfect stability and excellent biological activity

2.2 Expression and purification of Cas9 protein

Escherichia coli BL21 (DE3) competent cells was transformed with pET-Cas9-NLS-6xHis and cultured for Cas9 expression. Briefly, a fresh clone among the *E. coli* transformants from the plate was inoculated into 2 mL LB medium containing ampicillin (100 μg/mL) and cultured at 37 °C overnight. The cell culture was then diluted with fresh LB medium by 500-fold and continued to culture for another 4–5 h until the OD600 reached 0.6–0.8. Isopropyl β-D-1-thiogalactopy-ranoside (IPTG) at a final concentration of 1 mM was then added to induce Cas9 expression at 18 °C for 16 h [20]. Cas9 protein purification was performed following the protocols described by D'Astolfo et al. [21]. The purified Cas9 was analyzed by SDS-PAGE and Western blot.

2.3 Transcription and purification of single-guide RNA (sgRNA)

The sgRNA was transcribed with in vitro T7 Transcription Kit (Takara) according to the manufacturer's instructions. Transcription templates encoding a T7 promoter followed by the sgRNA were synthesized by Invitrogen with the sgRNA containing a 20 bp EGFP targeting sequence and a control guiding RNA (cgRNA) that does not target EGFP or any genes in human genome [22] (Fig. 1). The transcribed RNA was extracted by TRIzol (Invitrogen): chloroform to separate the RNA containing water phase. The RNA solution was isopropanol precipitated, ethanol washed and re-suspended in RNase-free water. Purified RNA was analyzed by SDS-PAGE and quantified by measuring optical density at 260 nm (OD260).

2.4 Linearized plasmid DNA cleavage assay to detect Cas9 activity

Plasmid containing the EGFP gene was linearized with Sma1 (NEB), purified by MiniBEST Agarose Gel DNA Extraction Kit (Takara) and used as the substrate for Cas9 activity assay. Following pre-incubation of the purified Cas9 (300 nM, corresponding to 330 ng) with sgRNA (360 nM, corresponding to 150 ng) in 10 μL Cas9 buffer (50 mM $NaH_2PO_4{\cdot}H_2O$ at pH 7.4, 1 M NaCl, 1 mM $MgCl_2{\cdot}6H_2O$, 30% glycerol, 1 mM DTT) for 10 min at 25 °C, linearized plasmid (150 ng) was added in this

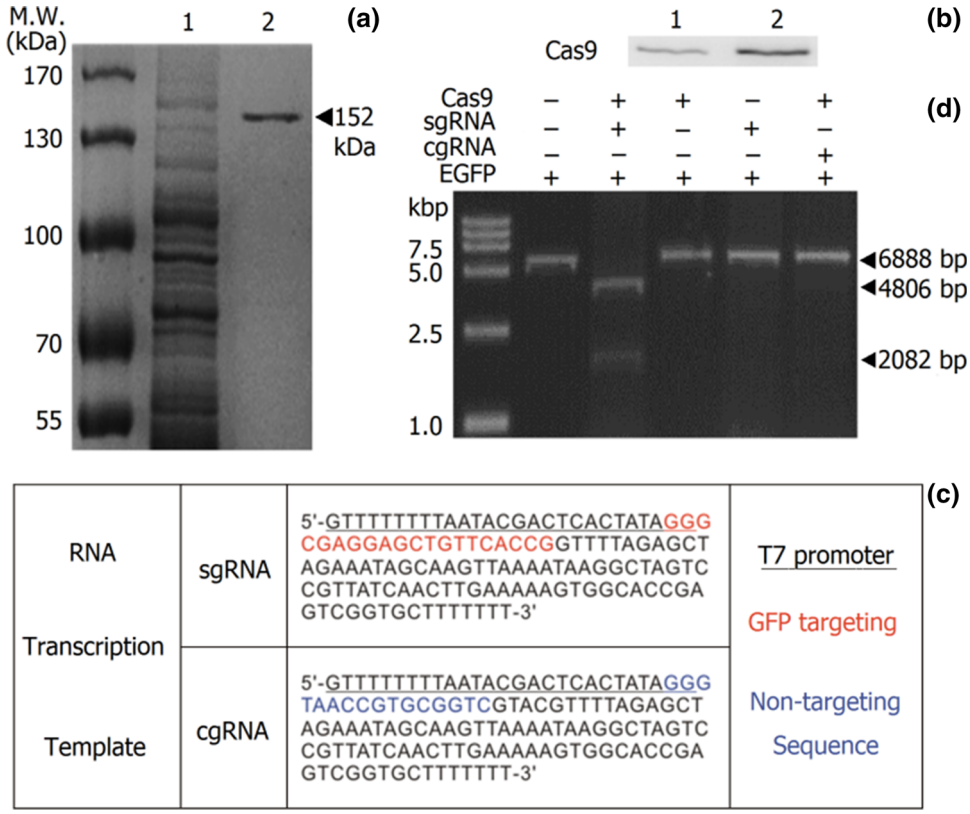


Fig. 1 Expression and activity determination of Cas9 Protein. **a** SDS-PAGE of *E. coli* (BL21) total protein extracts (*lane 1*) and purified Cas9 (*lane 2*). The purified Cas9 showed molecular weight of 152 kDa. **b** Representative immunoblot of Cas9 protein in purified Cas9 stock solution (*lane 2*). Immunoblot of Cas9 protein in commercial Cas9 nuclease solution (New England Biolabs Ltd, lane 1) was used as standard control. **c** Sequence of DNA oligos [22]. **d** Cas9 activity assessment using linearized plasmid encoding the EGFP gene as substrate. Only Cas9 complexed with sgRNA can digest the plasmid DNA

reaction system. After digestion for 90 min at 37 °C, the DNA fragmentation was analyzed using 1% agarose gel electrophoresis.

2.5 ^{125}I labeling of Cas9 protein and its condition optimization

Conventional Iodogen method was used to radiolabel the Cas9 protein [23]. In short, the Cas9 protein solution at a final concentration of 2 mg/mL was transferred into a vial coated internally with Iodogen (1,3,4,6-tetrachloro-3α, 6α-diphenylglycoluril, Chengdu Gaotong Isotope Co., China). We initiated the radiolabeling of Cas9 protein through adding 18 MBq (≈500 μCi) Na^{125}I and gently mixed and incubated the mixture. Finally, we added $Na_2S_2O_5$ solution to stop the labeling reaction. After the reaction, the mixture (10 μL) was used to determine the labeling yields by paper chromatograph (PC) with Whatman NO. 1 (1 × 13 cm^2). The ^{125}I distribution on PC was measured with a γ-ray counter. The radioactivity of ^{125}I-Cas9 was at 0.73 on the chromatography paper developed by n-butyl alcohol:pyridine:water = 6:4:1 (retardation faction, $Rf = 0.73$), with *Rf* value for free iodine ions being at 0.9–1.

To optimize the labeling procedures, we examined the effects of reaction conditions on the yield of ^{125}I-Cas9, including the reaction time, incubation temperature and reaction cycle.

2.6 In vitro stability and activity analysis of ^{125}I-Cas9

The in vitro stability of ^{125}I-Cas9 was determined by incubating the labeled protein stock solution (1 mg/mL in Cas9 buffer) in MilliQ water, phosphate-buffered saline (PBS), RPMI-1640 cell culture medium with 10% fetal bovine serum (FBS) and normal saline (NS) at 37 °C. The aliquots (10 μL) were then analyzed at time points of 1, 3, 6, 12, 24 and 48 h in the manner mentioned above. ^{125}I-Cas9 activity was detected by the same method used for Cas9.

3 Results and discussion

3.1 Expression and activity determination of Cas9 protein

To obtain Cas9 protein, we first chose the well-characterized and extensively applied *Streptococcus pyogenes* Cas9 [18, 19]. We successfully purified recombinant *Streptococcus pyogenes* Cas9 nucleases carrying a nuclear localization signal sequences (NLS) fused at the C-terminal following over expression in *Escherichia coli* (Fig. 1a). Western blot analysis further confirmed that the purified protein was Cas9 (Fig. 1b). We then co-incubated Cas9

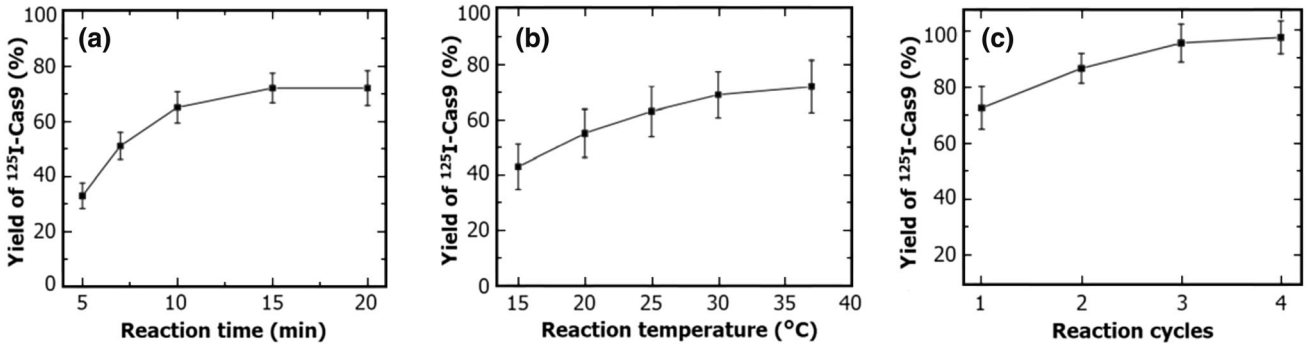


Fig. 2 Labeling yield of ^{125}I-Cas9 as function of reaction time (**a**) temperature (**b**) and cycles (**c**). *Data* are represented as mean ± SD

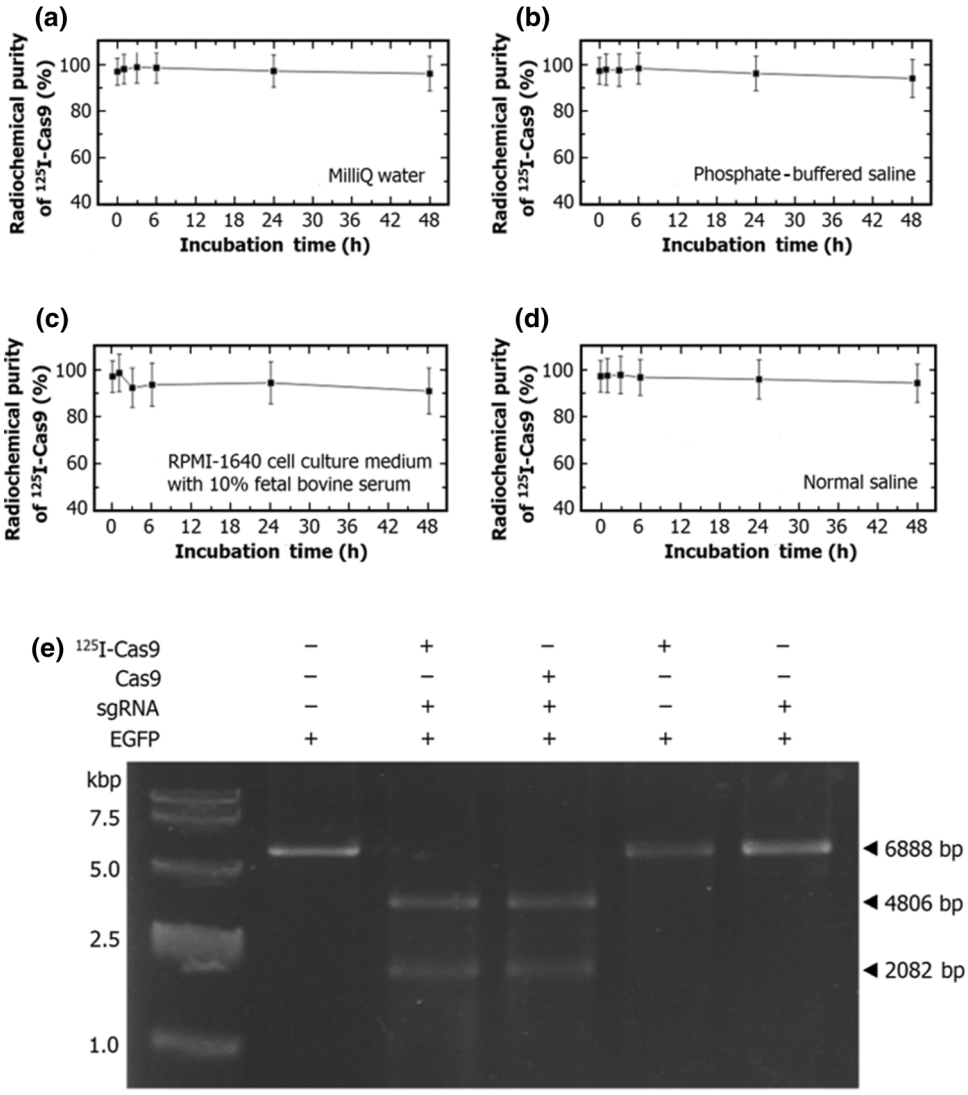


Fig. 3 In vitro stability and activity of ^{125}I-Cas9. **a**–**d** The radiochemical purity of ^{125}I-Cas9 in **a** MilliQ water, **b** PBS, **c** RPMI-1640 cell culture medium with 10% FBS and **d** normal saline at time points of 1, 2, 3, 6, 24 and 48 h. **e** ^{125}I-Cas9 activity assessment using linearized plasmid encoding the EGFP gene as substrate

protein and in vitro-transcribed sgRNA designed to specifically recognize the human enhanced green fluorescent protein (EGFP) genomic sequence [22] (Fig. 1c). We found that the resulting Cas9/sgRNA ribonucleoprotein (RNP) complex was active in vitro by cleavage of a EGFP gene sequence in a linearized plasmid, but only both Cas9 and the EGFP-targeting sgRNA existed simultaneously (Fig. 1d and Fig. S1).

3.2 ^{125}I labeling of Cas9 and its optimization conditions

Next, we prepared ^{125}I-labeling cas9 proteins by a conventional Iodogen method [23]. To optimize the labeling procedures, effects of reaction time, temperature and cycles on yield of ^{125}I-Cas9 were examined.

We found that at 37 °C, the labeling yield of ^{125}I-Cas9 increased with reaction time, reaching a stable value at 15 min (Fig. 2a). The temperature effects on labeling yield of ^{125}I-Cas9 were examined at 15–37 °C, so as to keep bioactivity of the radiolabeled Cas9 proteins. Similarly, at optimized reaction time of 15 min, the labeling yield of ^{125}I-Cas9 increased with temperature, with the highest labeling yield of approximately 72% (Fig. 2b). To further improve the labeling yield, we repeated this labeling process for several times at the optimized reaction time and temperature. After 1, 2, 3 and 4 reaction cycles, the labeling yield of ^{125}I-Cas9 was about 72, 86, 95 and 97%, respectively (Fig. 2c).

3.3 In vitro stability and activity of ^{125}I-Cas9

After obtaining ^{125}I-Cas9 of high labeling yield, we checked the in vitro stability of ^{125}I-Cas9 by their radiochemical purities at various elapsed times using PC analysis. At 37 °C, the ^{125}I-Cas9 was stable in MilliQ water, PBS, RPMI-1640 cell culture medium with 10% FBS and normal saline. The radiochemical purities in the media were still over 90% 48 h after labeling (Fig. 3a–d). By incubating the ^{125}I-Cas9 protein with EGFP-targeting sgRNA, we found that the genome editing efficiency of resultant ^{125}I-Cas9/sgRNA complex was comparable with that of Cas9/sgRNA complex, indicating labeled proteins preserved excellent cleavage activity (Fig. 3e).

Radioiodination of tyrosine residues is a common strategy for efficient radiolabeling of proteins and peptides [24]. In this work, Cas9 protein also has many tyrosine residues. Moreover, all the active sites of Cas9 do not contain tyrosine [25, 26]. Thus, we consider tyrosine residues as tagged sites of ^{125}I. Also, we noticed that the radiochemical purity of ^{125}I-Cas9 was about 96% and 80–85% after 15 and 30 days of storage at −80 °C, respectively. However, it was just 70–75% and 55–65% after 15 and 30 days of storage at −20 °C, respectively. This suggest that the labeled proteins should be stored at −80 °C and used as far as possible, best within 2 weeks.

4 Conclusion

In this paper, we successfully expressed and purified the Cas9 protein and confirmed the resulting Cas9/sgRNA complex showed high activity in vitro. More significantly, we developed a convenient and effective method for Cas9 protein labeling. By optimizing the reaction time, incubation temperature and reaction cycle, we obtained radioiodinated Cas9 with high labeling yield. Moreover, our prepared ^{125}I-Cas9 was stable in various media and preserved excellent genome editing efficiency. We also suggested the optimal storage temperature and time, −80 °C and within 2 weeks, beyond that the radioiodinated Cas9 was not stable. Our work provides an useful tool for further investigating the biological behaviors of Cas9 proteins in living systems.

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