

Nov 21, 2019

CasX Cleavage Assay

DOI

dx.doi.org/10.17504/protocols.io.8wshxee



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DOI: dx.doi.org/10.17504/protocols.io.8wshxee

Protocol Citation: Liz O'Brien, Connor Tsuchida 2019. CasX Cleavage Assay. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.8wshxee>

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Protocol status: Working

We use this protocol and it's working

Created: October 31, 2019

Last Modified: November 21, 2019

Protocol Integer ID: 29362

Keywords: CasX, Cleavage, Quenching

Abstract

This protocol describes a CasX Cleavage Assay.

Attachments



CasX Cleavage Assay .

106KB

Materials

MATERIALS

- ⊗ Magnesium Chloride **Fisher Scientific Catalog #AC223210010**
- ⊗ HEPES **Sigma Aldrich Catalog #H6147**
- ⊗ NaCl **Sigma-aldrich Catalog #S-3014**
- ⊗ DEPC (Diethyl pyrocarbonate) **Bio Basic Inc. Catalog #DB0154.SIZE.5ml**
- ⊗ Potassium Chloride **Sigma Aldrich Catalog #P9541**
- ⊗ Glycerol **Sigma Aldrich Catalog #G5516**
- ⊗ EDTA **Thermo Fisher Catalog #17892**
- ⊗ Tris Hydrochloride (Tris-HCl) **Sigma Aldrich Catalog #RES3098T-B7**
- ⊗ Tris(2-carboxyethyl)phosphine hydrochloride (TCEP) **Sigma Aldrich Catalog #C4706**
- ⊗ Heparin sodium **Sigma Aldrich Catalog #H0200000**

Safety warnings






! Please refer to the Safety Data Sheets (SDS) for health and environmental hazards.

Before start


Either purchase ribonucleoprotein from a vendor or express and purify the protein beforehand.







Prepare Buffers and Solutions




- 1 Prepare *CasX reaction buffer*.
 - 1.1 Mix together [M] 20 millimolar (mM) HEPES (pH 7.5) , [M] 10 millimolar (mM) magnesium chloride , [M] 150 millimolar (mM) potassium chloride , [M] 1 % volume glycerol , and [M] 0.5 millimolar (mM) TCEP . 
- 2 Prepare *CasX dilution buffer*.
 - 2.1 Mix together [M] 500 millimolar (mM) NaCl , [M] 10 % volume glycerol , [M] 20 millimolar (mM) Tris-HCl (pH 7.5) , [M] 1 millimolar (mM) magnesium chloride , and [M] 0.5 millimolar (mM) TCEP . 
- 3 Prepare *quencher*.
 - 3.1 Mix together [M] 0.5 Mass Percent heparin and [M] 25 millimolar (mM) EDTA . 
- 4 Prepare Formamide Loading Dye (2x concentrated).
 - 4.1 Add  20 mL formamide .
 - 4.2 Add EDTA to a final concentration of [M] 10 millimolar (mM) ( 2.2 mL of [M] 100 millimolar (mM) stock).
 - 4.3 Add a spatula tip-ful of powdered xylene cyanol.
 - 4.4 Add a spatula tip-ful of powdered bromophenol blue.





Annealing Duplex DNA

5 Prepare stock of labelled target strand DNA to be  100 μL of [M] 100 nanomolar (nM) .

6 Mix **1:1.2 molar ratio** of ^{32}P labelled target strand (TS) to unlabelled nontarget strand (NTS) of duplex substrate: 






Stock of unlabelled TS at  50 μL , [M] 120 nanomolar (nM) : dilute  4.48 μL TS stock in  45.42 μL DEPC.

Stock of unlabelled NTS at  50 μL , [M] 120 nanomolar (nM) : dilute  3.56 μL TS stock in  46.44 μL DEPC.

7 Mix  50 μL of [M] 100 nanomolar (nM) labelled target strand with  50 μL of [M] 120 nanomolar (nM) unlabelled nontarget strand to make a  100 μL stock of duplex substrate. 

Note

Reverse concentrations for labelled nontarget, unlabelled target.

8 Place on  95 $^{\circ}\text{C}$ heat block for  00:02:00 -  00:05:00 , then slow cool for about  02:00:00 . This is the **Duplex DNA stock** for cleavage assays. 

Note

DNA stock was diluted to 120nM with CasX reaction buffer.



CasX Activity Assays

- 9 Dilute CasX to [M] 4 micromolar (μM) in *CasX dilution buffer* (components shown above in go to step #2).
- 10 Dilute sgRNA to [M] 6 micromolar (μM) in *CasX reaction buffer* (components shown above in go to step #1).
- 11 Mix an equimolar ratio of CasX and sgRNA (2 μL sgRNA stock, 3 μL CasX stock, 1 μL *CasX reaction buffer*).
- 12 Allow samples to mix for 00:30:00 at Room temperature .
- 13 Dilute annealed, labelled duplex DNA into *CasX reaction buffer* (2 μL Duplex DNA stock, 43 μL *reaction buffer*).
- 14 Add RNP stock (5 μL , [M] 2 micromolar (μM) RNP) to the DNA:buffer mixture. Total volume should now be 50 μL .
- 15 Incubate at 37 $^{\circ}\text{C}$. For each timepoint (0, 1, 5, 10, 30, 60, 120 minutes), remove 5 μL and add to a tube filled with 5 μL *quencher* (CasX mixture and quencher solution should have equal volumes).
- 16 Incubate with *quencher* solution at Room temperature for 00:05:00 .
- 17 Add 5 μL formamide loading dye and incubate at 95 $^{\circ}\text{C}$ for 00:05:00 .
- 18 After quenching, load samples (4 μL /well, i.e. approximately 200 cpm/well was loaded per sample) on a 12 % PAGE gel and run at 40 – 45 W for 00:45:00 .

Note

Large gel was pre-warmed at 25W for ~45minutes.

Note

Loading less (50-100cpm/well) sample works fine. You may need to expose overnight.