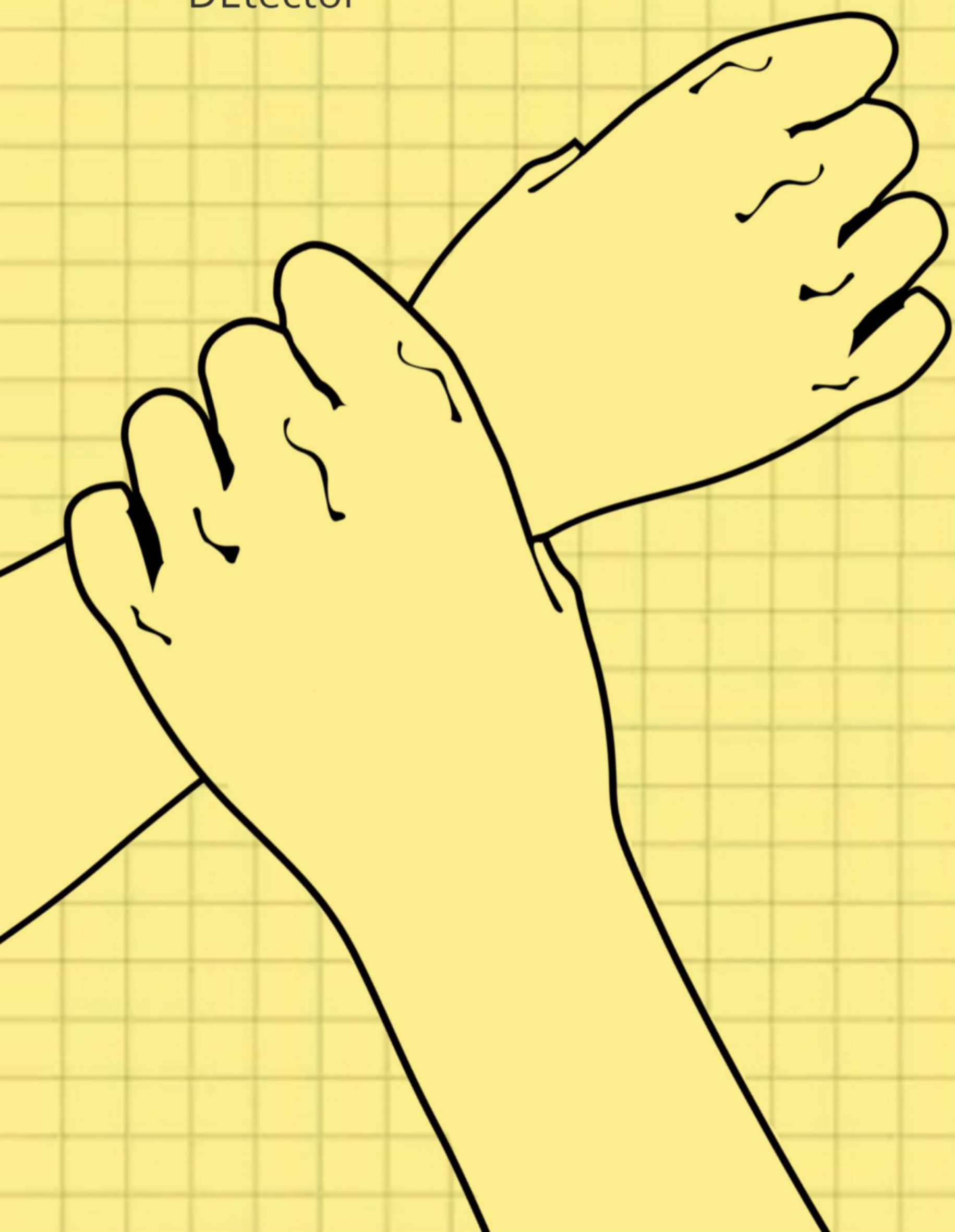
iGEM 2020

PROTOCOL

ECUST_China

——DEtector



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DNA walker

Sequence and reagents:

Oligonucleotides used in this work were custom-synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). Streptavidin magnetic beads used in this work were Beijing Zhongke Leiming technology Co, Ltd.

Buffer I (Suitable for binding biotinylated nucleic acids): 10mM Tris-HCl (pH7.5), 1mM EDTA, 1M NaCl, 0.01%~0.1% Tween-20

PBS: 15mM Phosphate buffer, 0.1% Tween-20

PBS I: 15mM Phosphate buffer, 0.1% Tween-20, 300mM NaCl

Table 1. Sequence information for oligonucleotides used in DNA walker

Oligo Name	Sequence (5'-3')	Length	Purification	Modification
Line-A	5'- TTT TTT TTT TTC TCA CTA ACT TAC GG -3'	26	HPLC	5' Biotin
Line-B	5'- CCC TAG TCC TAT ATG CGA C -3'	19	ULTRAPAGE	
Line-C	5'- GAC ATG TCG CAT ATA GGA CTA GGG CCG TAA GTT AGT GAG A -3'	40	ULTRAPAGE	
Line-L	5'- TTT TTT TTT TTT TTT TTT TTT TTT TGG TTC AAT CTG TCA A -3'	40	HPLC	5'Biotin
Line-T	5'- TTG CTT TGC TGC TGC TTG ACA GAT TGA ACC -3'	30	ULTRAPAGE	
Line-W	5'- GCA GCA GCA AAG CAA TTT TTT TTT TTT TTT TTT TTT TTT TTT TTT GTC CTA TAT GCG ACA TGT C -3'	64	ULTRAPAGE	
Line-F	5'- TAA CTT ACG GCC CTA GTC CTA TAT GCG AC -3'	29	ULTRAPAGE	

Instruments and equipment:

Magnetic rack, SDS-PAGE slot, power supply, gel imager, micropipette, shaker.

1 Assemble the magnetic beads LABC

1.1 Assemble the magnetic beads LABC

1. After shaking and mixing, take 100ul of 5mg/ml magnetic beads into Eppendorf tube, magnetically separate, and discard the supernatant;
2. Add 1mL Buffer I to wash, shake and mix for 15s, magnetically separate, and discard the supernatant;
3. Repeat step 2;
4. Add 24.5uL A + 2.5 uL L + 473uL Buffer I;
5. Fully shake and mix, shaker 30min (300r/min);
6. Magnetic separation, keep 'the supernatant AL';
7. Wash three times with Buffer I, add 500uL Buffer I each time, shake for 15s, magnetically separate, and discard the supernatant;
8. Add 51ul PBS I and resuspend;
9. Take 24.5ul C + 24.5ul B into the PCR tube, and run the program on the PCR machine.
   Step 1: 95C° 10min
   Step 2: 4C°
10. Add 49uL C:B pairing to the magnetic beads;
11. Heat in a water bath at 60°C for 10 minutes, then place on ice to cool down.
12. Magnetic separation, keep 'the supernatant CB';
13. Wash three times with PBS I. After the third magnetic separation, save 'the supernatant assemble the magnetic beads LABC';
14. Resuspend in 500ul PBS I;

The 500uL magnetic beads have a concentration of 1 mg/mL and are fully loaded with LABC sequences. Try to store the magnetic beads at 4°C. (Named as assembled magnetic beads LABC)

1.2 polyacrylamide gel electrophoresis

1. Take 1uL 6x Loading and 5 uL specimen into PCR tube, pipette and mix well;
2. Take 6uL sample and load;
3. Electrophoresis 180V, 70min;
4. dyeing. GelRED 1 uL + 10 mL deionized water for 10 minutes;
5. Gel imaging;

Table 2. Gel electrophoresis spotting sequence

Lane	specimen	expected outcome
1	the supernatant AL	If there are AL bands, it proves that AL remains and the surface modification of the magnetic beads is saturated.
2	the supernatant CB	If there are CB bands, it proves that the ACB sandwich structure is saturated.
3	the supernatant assemble the magnetic beads LABC	If there is no band, it proves that the magnetic beads are washed clean.
4	AL	Indication (target strip)
5	CB	Indication (target strip)
6	L	Indication (target strip)
7	ABC	Indication (target strip)
8	Marker	Indication (target strip)

2 Reaction

2.1 Reaction

General outline:
1. Shake and mix well, take 73uL magnetic beads LABC, save 'the supernatant before the reaction magnetic beads LABC' ;
2. Shake and mix well, add reagent;
   PC: add 2.5uL W, 5uL T → 24.5uL F
   NC: add 2.5uL W, 5uL ddH_2O → 24.5uL F
3. React at 37°C for 5, 10, 15, 20, 30 minutes, magnetically separate, and take 'the supernatant 1, 2, 3, 4, 5' ;
4. Wash with PBS I and resuspend the magnetic beads.

Overview of Experimental Design:

Number	reagent
PC-12	T、W -> F
PC-34	T2、W -> F
PC-2	Tα、W -> F
PC-3	Tβ、W -> F
PC-4	Tαβ、W -> F
NC	ddH20、W -> F

2.2 polyacrylamide gel electrophoresis

1. Take 1uL 6x Loading and 5 uL specimen into PCR tube, pipette and mix well;
2. Take 6uL sample and load;
3. Electrophoresis 180V, 70min;
4. dyeing. GelRED 1 uL + 10 mL deionized water for 10 minutes;
5. Gel imaging.

Qualitative verification:
1. From the polyacrylamide gel electrophoresis diagrams of the PC-12 and NC groups, if CF bands are observed and the brightness of the CF bands of the PC-12 group is higher than that of the NC group, it proves that the DNA walker we designed can work successfully, namely , After identifying the T chain, the CF double chain can be released.
2. From the polyacrylamide gel electrophoresis diagram of the NC group, if a CF band is observed, it proves that the DNA walker is leaking.
3. From the polyacrylamide gel electrophoresis of the PC-34 and NC groups, if CF bands are observed and the brightness of the CF bands in the PC-34 group is higher than that in the NC group, it proves that the detection limit of our designed DNA Walker is pmol, That is, after

identifying the T2 chain, the CF duplex can be released.

4. In PC-34, the CF band is observed at a certain time, which proves that the DNA walker can complete the observable strand displacement reaction in the vicinity.

E-CRISPR

Sequences and reagents:

Oligonucleotides used in this work were custom synthesized by Sangon Biological Engineering Technology & Services Co., Ltd. (Shanghai, China). The sgRNA used in this work is custom synthesized by Genscript Biotech Corporation. Cas9 nickase (D10A) and buffer 3.1 used in this work are all bought from New England Biolabs (Beijing) LTD. RNase free water used in this work is bought from Takara Biomedical Technology (Beijing) Co., Ltd.

Table 3. Sequence information for oligonucleotides used in E-CRISPR

Oligo Name	Sequence (5'-3')	Length	Purification	Modification
Line-C	5'- GAC ATG TCG CAT ATA GGA CTA GGG CCG TAA GTT AGT GAG A -3'	40	HPLC	
Line-F	5'- TAA CTT ACG GCC CTA GTC CTA TAT GCG AC -3'	29	HPLC	
Line-G	5'- ATT CTC ACT AAC TTA CGG CCC TAG TCC TAT ATG CGA CAT GTC TAT -3'	45	HPLC	5' HS-SH C6 3' Methylene blue
Line-G-pure	5'- ATT CTC ACT AAC TTA CGG CCC TAG TCC TAT ATG CGA CAT GTC TAT -3'	45	HPLC	
sgRNA-AGG	5'- GACAUGUCGCAUAUAGGACUGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU-3'	100	HPLC	
sgRNA-GGG	5'- ACAUGUCGCAUAUAGGACUAGUUUUAGAGCUAGAAAUAGCAAGUUAAAAUAAGGCUAGUCCGUUAUCAACUUGAAAAAGUGGCACCGAGUCGGUGCUUUU-3'	100	HPLC	

Instruments and equipment:

Buffer-alpha: 20 mM Tris-HCl, 100 mM NaCl, 10 mM $MgCl_2$

Buffer-beta: 2 mM MCH, 10 mM Tris, pH 8.0

Buffer-gama: 100 mM Tris-HCl, 25mM $MgCl_2$, 5 mM $CaCl_2$

1 validation on electrode

1.1 line G modification experiments on electrode

1. Centrifugate the tube containing power DNA at 10,000 rpm for 5 min;
2. Add ultrapure water to make the ultimate concentration at 10 μM;
3. Rinse the electrode with ultrapure water, then ultrasonic 5 min in ethanol, rinse again with ultrapure water, soak in 0.5 M sulfuric acid for 30 min, finally, rinse again with ultrapure water, soak in ethanol for 5 min;
4. Extract 5 μL line G (10 μM) into a new RNase free EP tube, add 5 μL TCEP (10μM) and wait 10 minutes or the reaction to take place;
5. Add 90 μL RNase free water to the above mentioned RNase free EP tube;
6. Add 5μl line G (1μM) to the electrode wafer, covering the wafer, and wait 2 hr for reaction to take place;
    a) Note: If the droplet diffuses to the reference electrode, completly coverage Line G added.
7. Add Buffer-beta to the electrode, and wait 0.5 hr for reaction to take place;
8. Put the electrode into 5 mL EP tube, soak in Buffer-alpha for 5 min and then measure the electrical signals;
9. Use ultrapure water to wash the electrode, and soak it in the Buffer-alpha for 10 min after measuring electrical signals;
10. Put it in 5 mL EP tube (including Buffer-alpha) and store in 4C° refrigerator.

1.2 Oligo T modification experiments on electrode

1. Centrifugate the tube containing power DNA at 10,000 rpm for 5 min;
2. Add ultrapure water to make the ultimate concentration at 10 μM;
3. Rinse the electrode with ultrapure water, then ultrasonic 5 min in ethanol, rinse again with ultrapure water, soak in 0.5 mM sulfuric acid for 30 min, finally, rinse again with ultrapure water, soak in ethanol for 5 min;
4. Add 5μl Oligo T (diluted to 1μM) to the electrode wafer, covering the wafer, and wait 2 hr for reaction to take place;
    a) Note: If the droplet diffuses to the reference electrode, completly coverage Oligo T added.
5. Add Buffer-beta to the electrode and wait 0.5 hr for reaction to take place;
6. Put the electrode into 5 mL EP tube, soak in Buffer-alpha for 5 min and then measure the electrical signals;
7. Use ultrapure water to wash the electrode, and soak it in the Buffer-alpha for 10 min after measuring electrical signals;
8. Put it in 5 mL EP tube (including Buffer-alpha) and store in 4C° refrigerator.

1.3 Direct cut validation on electrode

1. Add line C on the electrode which has been modified by line G and wait 0.5 hr for reaction to take place and then measure the electrical signals;
2. Add sgRNA solution (0.04 μM) and Cas9 nickase solution (0.02 μM) to the electrode after the reaction;
3. Put the electrodes in the Petri dish and seal it with parafilm;
4. Place the Petri dish in the incubator (temperature set at 37C°) and wait 1 hr for reaction to take place and then measure the electrical signals;
5. Calculate the difference between the initial and final values of the electric signal, and the time it takes after adding samples to the signal no longer changes.

1.4 Electric signal decline validation by DNase I on electrode

1. Add 1 μL 10x Buffer-gama, 1 μL DNase I, 8 μL ultrapure water to the electrode;
2. Incubate the electrode modified by oligo T for 1 min and then measure the electrical signals;
3. Incubate the electrode modified by line G for 1 hr and then measure the electrical signals.

2 validation in PCR tube

2.1 Direct cut validation in PCR tube

1. Add 3.5μL line C and 3.5μL line G to 0.2ml PCR tube and wait 1 hr for reaction;
2. Add 1μL Cas9 nickase, 1μL sgRNA and 1μL 10xbuffer 3.1 to the PCR tube;
3. Place PCR tube in a 37C° water bath and wait 1 hr for reaction;
4. Heat it at 65C° for 10min after reaction;
5. Add an equal volume (10μL) of phenol chloroform to the PCR tube, mix and centrifuge;
6. Take the supernatant and put it into a new PCR tube;
7. Add glycerol to make the final concentration of glycerol at 10%;
8. Run the non-denatured polyacrylamide gel electrophoresis at 90V, 180min;
9.The non-denatured polyacrylamide gel electrophoresis procedure:

(1) The non-denatured polyacrylamide gel electrophoresis components:

20% non-denatured PAGE 6mL system	
30% Acr/Bis	4mL
10×TBE	0.6mL
ultrapure H_2O	1.4mL
10% APS	50μL
TEMED	4μL

(2) Prepare the non-denatured polyacrylamide gel electrophoresis and load the sample.
(3) Add 1×TBE to electrophoresis tank.
(4) Set the procedure of 90V and 180 min fo electrophoresis, and then start.

2.2 line G Competition experiment in PCR tube

1. Add 5μL line C and 5μL line F to 0.2ml PCR tube, and wait 1 hr for reaction;
2. Add 5μL line G to 0.2ml PCR tube, and wait 1 hr for reaction;
3.Add glycerol to make the final concentration of glycerol at 10%;
4.Run the non-denatured polyacrylamide gel electrophoresis at 90V, 180min as mentioned above at 2.1.9.

2.3 Competitive cut validation in PCR tube

1. Add 5μL line C and 5μL line F to 0.2ml PCR tube, and wait 1 hr for reaction;
2. Add 5μL line G to 0.2ml PCR tube, and wait 1 hr for reaction;
3.Add 2μL Cas9 nickase, 2μL sgRNA, 1μL 10xbuffer 3.1 to the PCR tube;
4. Place PCR tube in a 37C° water bath and wait 1 hr for reaction;
5. Heat it at 65C° for 10min after reaction;
6. Add an equal volume (10μL) of phenol chloroform to the PCR tube, mix and centrifuge;
7.Take the supernatant and put it into a new PCR tube;

8.Run the non-denatured polyacrylamide gel electrophoresis at 90V, 180min, as mentioned above at 2.1.9.