Random DNA Binding Selection Assay (RDSA)

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[Abstract] Protein-DNA interaction is a very important cellular process, by which regulation of DNA biological function, usually gene expression, is exerted. The method of random DNA binding selection assay (RDSA) can be used to identify DNA elements bound by proteins with DNA-binding activities. This method is based on the enrichment of the target DNA element by the immobilized recombinant protein on special beads and repeated PCR amplification.

Materials and Reagents

1. The prokaryotic expression vectors: pGEX-6P-1 (GE Healthcare) or pTXB3 (New England Biolabs)
2. The expression strain [*Escherichia coli* (*E.coli*) BL21 (DE3) or others]
3. T-Vector pGEMT-easy and T4-DNA ligase (Promega Corporation, catalog number: A1360)
4. DNA polymerase (Takara Bio Company, catalog number: DR001)
5. Sterile ddH_2O (double distilled water)
6. Oligonucleotides (Pitzschke *et al.*, 2009; Wang *et al.*, 2014)
7. Chitin resin (New England Biolabs, catalog number: S6651S)
8. PBS buffer (see Recipes)
9. Column buffer (see Recipes)
10. RDSA buffer (Pitzschke *et al.*, 2009) (see Recipes)

Table 1. Oligonucleotides for RDSA

Oligonucleotides for RDSA	
RDSA_1	TAGTTGAGTCTCACAAACGAACAC(N20)CATTCCAAAATCCATGGCTGATA
RDSA_1 fo	TAGTTGAGTCTCACAAACGAACAC
RDSA_1 re	TATCAGCCATGGATTTTGGAATG
RDSA_2	AATGGATCCAAGCTTAAGC(N18)CGTTGAATTCCCATGGACA
RDSA_2 fo	AATGGATCCAAGCTTAAGC
RDSA_2 re	TGTCCATGGGAATTCAACG

Equipment

1. Glutathione sepharose beads (GE Healthcare)
2. The empty chromatography columns (Bio-Rad Laboratories)
3. 4 °C refrigerator
4. Centrifuge
5. Whirling shaker

Procedure

A. Random DNA preparation

1. PCR amplification.

   The double strand DNA fragments RDSA1 and RDSA2 with 20- and 18-bp random sequence regions flanked by independent sets of adaptors were amplified on the templates of RDSA_1 and RDSA_2 by PCR, using RDSA_1/2fo and RDSA_1/2re primers, respectively (Table 1). Conditions for PCR amplification are: 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, and 30 sec at 72 °C; 10 min at 72 °C.
2. Purification of PCR products.
   a. Take at least 200 µl PCR products; add equal volume of 24:1 (chloroform: isoamyl alcohol) to sample tubes; shake tubes vigorously by hand for at least 30 sec, centrifuge the sample at 15,000 *x g* for 10 min at 4 °C.
   b. Transfer the aqueous phase to fresh tubes; add equal volume of isopropanol and mix; place in -20 °C freezer for 1 h.
   c. Centrifuge the samples at 15,000 *x g* for 10 min at 4 °C.

d. Discard the supernatants and wash the precipitates with 70% alcohol.

e. Air dry the precipitates for at least 10 min; dissolve the precipitates in sterile ddH_2O and store at -20 °C until use.

B. Prokaryotic expression of target protein tagged with GST or CBD (chitin binding domain)

1. Construct expression vector.

   The coding region of the target gene is ligated in frame into the expression vector pGEX-6P-1 (for GST fusion protein) or pTXB3 (for CBD fusion protein).

2. The constructs are transformed into *E.coli* expression strain BL21 (DE3) to express fusion proteins.

C. RDSA cycles

1. Harvest cells (100 ml bacterial cultures) and resuspend them in PBS buffer (for GST fusion protein) or column buffer (for CBD fusion protein).
2. Break cells by sonication (950 W x 20%, 2-10 min according to the solubility of the expressed protein and with 2 sec interruption after every 2 sec sonication. The tube with sample should be placed in ice-water during manipulation) and centrifuge at 1,000 *x g* for 10 min at 4 °C.
3. Prepare beads columns (load at least 3 ml beads in each chromatography column); equilibrate the glutathione sepharose beads column (for GST fusion protein) with PBS buffer (ten times of the bead volume), and the chitin beads column (for CBD fusion protein) with column buffer (ten times of the bead volume). In the following steps, the beads columns should be placed on ice.
4. Slowly load the clarified lysate on to the columns.
5. Wash the columns with at least 20 times of bed volume PBS buffer for GST fusion protein or column buffer for CBD fusion protein to thoroughly remove the unbound proteins; wash the beads at least three times with RDSA buffer; resuspend the beads in 3 ml RDSA buffer. Now the recombinant target protein is immobilized on beads.
6. Divide the beads into 5-10 tubes (1.5 ml tube).
7. Add the random DNA fragment RDSA1 or RDSA2 (10 μl, about 5-10 μg) into one tube; incubate for more than 2 h at 4 °C with gentle rolling.
8. Centrifuge at 500 *x g* for 5 min at 4 °C, discard the supernatant completely.
9. Wash the beads with RDSA buffer for at least four times to remove unbound DNA fragments; add 500 μl sterile ddH_2O and boil the beads for 5 min to release the bound DNA; centrifuge at 500 *x g* for 5 min at 4 °C.
10. Transfer the supernatant to a fresh tube; purify and precipitate the released DNA as described in step A2 (do not need to dissolve the DNA).

11. Add PCR reagents to the tube:
    74 µl ddH_2O
    10 µl 10x PCR buffer
    10 µl 2.5 mM dNTP
    2.5 µl 20 µM RDSA1 fo or RDSA2 fo primer
    2.5 µl 20 µM RDSA1 re or RDSA2 re primer
    1.0 µl rTaq DNA polymerase
    Perform PCR amplification using the following parameters: 5 min at 94 °C; 30 cycles of 30 sec at 94 °C, 30 sec at 50 °C, and 30 sec at 72 °C; 10 min at 72 °C.
12. Purify the PCR products as described in A2; dissolve the DNA in 10 µl sterile ddH_2O and transfer the DNA to another tube in C6 for the next RDSA cycle (repeat steps C7-12).
13. After 5 to 10 cycles, clone the purified PCR products into pGEM-T easy vector and sequence.

Notes

1. By increasing the amounts of the non-specific competitor poly (dIdC) (0, 50, 100, 150, 200, 250, 300 and 350 ng) and reducing the amounts of protein in the binding reactions (700 µl, 650 µl, 600 µl, 550 µl, 500 µl, 400 µl, 300 µl, 200 µl protein bound beads) the stringency can be increased in each RDSA cycle (Pitzschke *et al*., 2009; Wang *et al*., 2014).
2. To exclude effects from the nonrandom flanking adaptors, the RDSAs must be performed in duplicates with two different sets of input random DNA fragments (RDSA1 and RDSA2) (Pitzschke *et al*., 2009).
3. The number of total cycles may be different for different proteins. To our experience, at least 5 cycles should be performed to exclude the non-specific binding.
4. The results of RDSAs should be verified by other methods, such as electrophoretic mobility shift assay (EMSA) and yeast one-hybrid system.
5. The annealing temperature should be adjusted by different random primers.

Recipes

1. PBS buffer (pH 7.3)
   140 mM NaCl
   2.7 mM KCl
   10 mM Na_2HPO_4
   1.8 mM KH_2PO_4
   5 mM DTT

1 mM PMSF

2. Column buffer

   20 mM Tris-HCl (pH 8.5)

   500 mM NaCl

   1 mM EDTA

3. RDSA buffer

   5 mM Tris (pH 8.0)

   75 mM NaCl

   2.5 mM $MgCl_2$

   0.5 mM EDTA

   5% glycerol

   1% Tween

   1 mM DTT

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References

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