Protocol

SELEX to Identify Protein-Binding Sites on RNA

James L. Manley

SELEX (systematic evolution of ligands by exponential enrichment) is a very powerful method for determining the binding site of a protein on RNA. It relies on the ability of an RNA-binding protein to select high-affinity RNA ligands from a randomized pool of RNAs. SELEX experiments are performed over several “rounds,” with each round resulting in increased enrichment of RNAs capable of binding to the protein. There are many variations of SELEX strategies, but they all rely on the ability to separate bound RNA from unbound RNA. The method presented here uses tagged proteins; however, it is also possible to use other separation techniques (e.g., filter binding or antibodies).

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution’s Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this article for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

ATP, CTP, GTP, UTP (100 mM each)

BamHI (200 µL)

DNase I (RNase-free; 2 units/µL)

dNTP mix (10 mM each of dATP, dCTP, dGTP, dTTP)

Ethanol (70%, 100%)

Klenow buffer (5×) <R>

Klenow enzyme

NT2 buffer <R>

Phenol:chloroform:isoamyl alcohol (PCA) (25:24:1)

Primers:

- “Forward” primer sequence: 5′-CGCGAATTCTAATACGACTCACTATAGGGGCCACC AACGACATT-3′

The forward primer must contain a T7 promoter sequence and a restriction enzyme site for cloning. Here, the EcoRI restriction site is nucleotides 4–9, and the T7 promoter is nucleotides 10–29.

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- “Reverse” primer sequence: 5′-CCCGACACCGCGGGATCCATGGGCACTATTTATA TCAA-3′
The reverse primer must contain a restriction enzyme site for cloning and hybridization sequence for reverse transcription. Here, the BamHI restriction site is nucleotides 13–18.
- “Library” primer sequence: 5′-TTACAGCAACCACCGGGGATCCATGGGCACTATTT ATATCAAC (N)₂₅ AATGTCGTTGGTGGCCC-3′
The library primer must contain a region of randomized sequence as well as sequences that anneal to the forward and reverse primers.

Proteinase K (10 mg/mL)

Proteinase K buffer for SELEX (2×) <R>

Protein of interest fused to a tag for purification (such as GST or histidine)

Reverse transcriptase (M-MuLV 200 units/μL)

Reverse transcriptase buffer for SELEX (10×) <R>

RNase inhibitor (40 units/μL)

SDS extraction buffer <R>

Sepharose or agarose beads for purification of complexes

Sodium acetate (3 M, pH 5.2)

T7 RNA polymerase

T7 transcription buffer (10×) <R>

Taq DNA polymerase (1 unit/μL) and buffer

Polyacrylamide gel (15%; 19:1 acrylamide:bis-acrylamide) containing 8 M urea, prepared in 1× TBE

Polyacrylamide gel (15%; 19:1 acrylamide:bis-acrylamide), prepared in 1× TBE

TE buffer (pH 8.0)

Vector for cloning and bacteria for transformation (see Step 46)

Yeast tRNA (10 mg/mL)

Beaker with 50 mL of H₂O at 75°C

Conical centrifuge tube (15 mL)

Dry ice

Ice

Microcentrifuge

Microcentrifuge tubes (1.5 mL)

PCR tubes

Polypropylene tubes (12 mL)

Shaker

Spectrophotometer

Thermal cycler

Tube rocker

Ultraviolet (UV) light source (254 nm, handheld)

Vortex mixer

Water baths (37°C, 42°C, 55°C, and 75°C)

X-ray intensifying screen for UV shadowing

Equipment

METHOD

Convert Library of Oligonucleotides into Double-Stranded DNA Transcription Templates

Before beginning this protocol, it is necessary to understand the experimental flow (see Fig. 1).

1. Anneal the “forward” and “library” primers by mixing the following in a 1.5-mL tube:

Klenow buffer (5×)	200 μ L
100 μ M “forward” primer	85 μ L
100 μ M “library” primer	85 μ L
H ₂ O	590 μ L

2. Heat the mixture for 15 min to 75°C and then transfer it to a beaker containing 50 mL of H₂O at 75°C. Allow the tube to cool slowly to room temperature. Centrifuge briefly for 5 sec at room temperature.
3. Extend the annealed primers by adding 20 μ L of dNTP mix (10 mM each of dATP, dCTP, dGTP, and dTTP) and 20 μ L of Klenow enzyme. Incubate for 1 h at 37°C.
4. Add 100 μ L of 3 M sodium acetate (pH 5.2).
5. Divide the sample into four 275- μ L aliquots and add 600 μ L of 100% ethanol to each.
6. Freeze on dry ice for 10 min and then centrifuge at $\geq 12,000g$ for 15 min at room temperature.
7. Decant the supernatant and wash the pellet with 70% ethanol. Centrifuge at $\geq 12,000g$ for 5 min at room temperature. Decant the supernatant and air-dry the pellet.

1. Convert library of oligonucleotides into double-stranded DNA transcription templates

FIGURE 1. Schematic representation of a “round” of SELEX. Each “round” of SELEX consists of the same sequence of steps. First, it is necessary to construct DNA templates for synthesizing the randomized RNA pool. The pool is then synthesized, labeled, and allowed to bind to the protein of interest. Bound RNAs are then converted back into DNA templates by RT-PCR. This process is reiterated until the bulk ($\geq 90\%$) of the pool is capable of binding to the protein. At this point, the “final” pool is converted back to double-stranded DNA, cloned, and sequenced.

8. Resuspend the double-stranded DNA library from Step 7 and gel-purify using a 15% non-denaturing TBE polyacrylamide (19:1 acrylamide:bis-acrylamide) gel. Electrophorese until the xylene cyanol dye is close to the bottom of a 15-cm-long gel.
9. Visualize the nucleic acids by UV shadowing.
10. Excise the band containing the full-length double-stranded library. Crush the excised gel band and elute the DNA using 1–2 mL of 0.5 M sodium acetate overnight with shaking.
11. Collect supernatant, precipitate the DNA with ethanol, and wash the precipitate with 70% ethanol. Repeat the elution once to increase recovery if desired.
12. Dissolve the DNA in ~100 μ L of TE buffer (pH 8.0).
13. Determine the OD₂₆₀ and convert the value to molar concentration.
For example, for 50 μ g/mL per OD₂₆₀, the 95-nucleotide product has a molecular weight of 62,440 g/mol.
14. Dilute the DNA to 1.7 nmol/100 μ L and divide it into 100- μ L aliquots.

Transcribe the Double-Stranded DNA Template for First Round

15. Set up the transcription reaction in a 15-mL conical centrifuge tube as follows:

1.7-nmol double-stranded DNA library (from Step 14)	100 μ L
10 $\times$ T7 transcription buffer	1 mL
100 mM ATP	100 μ L
100 mM CTP	100 μ L
100 mM GTP	100 μ L
100 mM UTP	100 μ L
T7 RNA polymerase	1 mL
H ₂ O	7.5 mL

16. Mix the reagents gently by inverting and then incubate for 2 h at 37°C.
The solution should become cloudy.
17. Add 100 μ L of RNase-free DNase I to the transcription reaction and incubate for a further 15 min at 37°C.
18. Split into 2.5-mL aliquots in 12-mL polypropylene tubes and precipitate the RNA with ethanol.
19. Purify the full-length RNA transcripts on an 8 M urea, 15% polyacrylamide gel.
20. Visualize the nucleic acids by UV shadowing.
21. Excise the band containing full-length RNA transcripts. Elute RNA overnight as in Step 10. Collect the supernatant, precipitate with ethanol, and wash the precipitate with 70% ethanol.
22. Dissolve the RNA in ~100 μ L of TE buffer (pH 8.0) or in SDS extraction buffer.
23. Determine the OD₂₆₀ and convert the value to molar concentration.
For example, for 40 μ g/mL per OD₂₆₀, the 69-nucleotide, single-stranded RNA has a molecular weight of 23,630 g/mol.
24. Dilute the RNA to 17 nmol/100 μ L and store in 100- μ L aliquots at –80°C.
Each aliquot on average contains 10 RNA copies of each of the 1×10^{15} DNA templates.

Protein–RNA Binding and Selection

25. Select RNA from Step 24 using a matrix bound to the protein of interest (e.g., glutathione–Sepharose beads with a GST-tagged protein or Ni-NTA agarose with a histidine-tagged protein).
Immunoprecipitation, filter binding, or a mobility shift assay can also be used to separate protein–RNA complexes from free RNA.

26. Prepare sufficient beads for 2 mg of each protein of interest and an equal volume of beads to preclear the RNA library. Wash the beads in NT2 buffer.
27. Bind 1–10 μg of protein to Sepharose or agarose beads for at least 2 h at 4°C with rocking.
28. Pellet the beads by centrifugation at 8000g for 10 sec at room temperature. Then, wash the beads three times with 1 mL of NT2 buffer each time.
29. Preclear a 17-nmol aliquot of the RNA library from Step 24 by binding it to beads for 30 min at 4°C. Pellet the beads by centrifugation at 8000g for 10 sec at room temperature. Keep the supernatant, which contains the precleared RNA library.
30. Bind the precleared RNA library from Step 29 to the proteins from Step 28 by incubating for at least 2 h at 4°C with rocking.
31. Wash the beads five times with 1 mL of NT2 buffer each time. After the last wash, leave 100 μL of NT2 buffer on the beads.
32. Add 100 μL of H_2O , 200 μL of 2 $\times$ proteinase K buffer for SELEX, and 5 μL of proteinase K. Incubate for 15 min at 37°C.
33. Add 400 μL of PCA (25:24:1), vortex for 30 sec, and then centrifuge at 12,000g for 1 min at room temperature.
34. Transfer aqueous layer, which contains the RNA, to a clean tube. Add 2 μL of 10 mg/mL of yeast tRNA, 40 μL of 3 M sodium acetate (pH 5.2), and 1 mL of 100% ethanol. Mix thoroughly, freeze on dry ice for 10 min, and then centrifuge at $\geq 12,000\text{g}$ for 15 min at room temperature.
35. Decant the supernatant, wash the RNA pellet with 70% ethanol, and centrifuge at $\geq 12,000\text{g}$ for 5 min at room temperature. Decant the supernatant and air-dry the pellet.

Reverse Transcription PCR

36. Resuspend the RNA pellet from Step 35 in 13 μL of H_2O . Add the following:

“Reverse” primer	3 μL
Reverse transcriptase buffer for SELEX (10 $\times$)	2 μL
dNTP mix	2 μL
Reverse transcriptase	0.5 μL

Incubate the reaction for 5 min at 55°C and then for 1 h at 42°C.

37. Transfer 6 μL from the reverse transcription reaction into a PCR tube and add the following:

H_2O	75.5 μL
<i>Taq</i> buffer (10 $\times$)	10 μL
dNTP mix	2 μL
“Forward” primer	3 μL
“Reverse” primer	3 μL
<i>Taq</i> polymerase	0.5 μL

38. Denature for 5 min at 94°C. Then, amplify for 25 cycles of 30 sec at 94°C, 30 sec at 50°C, and 30 sec at 72°C followed by a final extension of 10 min at 72°C.
39. Add 1 μL of BamHI to the reaction and incubate at 37°C to regenerate the 3' ends of original library.
40. Extract with an equal volume of PCA. Precipitate the double-stranded DNA template with ethanol, air-dry the pellet, and resuspend it in 15 μL of H_2O .

Transcribe Double-Stranded DNA for Subsequent Rounds

41. Combine the following:

T7 transcription buffer (10×)	2 μL
NTP mix (2.5 mM each ATP, CTP, UTP, GTP)	8 μL
Double-stranded DNA template (from RT-PCR of previous round [Step 40])	3 μL
RNase inhibitor (40 units/μL)	1 μL
H ₂ O	4 μL
T7 RNA polymerase	2 μL
Incubate for 1 h at 37°C.	

42. Add 1.5 μL of RNase-free DNase I to the reaction and incubate for an additional 10 min at 37°C.
43. Add 78.5 μL of H₂O and 10 μL of 3 M sodium acetate (pH 5.2). Extract the reaction with an equal volume of PCA, precipitate with ethanol, air-dry the RNA pellet, and then resuspend RNA in 20 μL of H₂O.
44. Use one-quarter (5 μL) of the RNA from Step 43 to repeat the cycle for the binding to protein–RNA-binding step (Steps 25–35). Repeat the RT-PCR (Steps 36–40). Usually 4–10 cycles of enrichment are required.
45. Check for protein–RNA binding by electrophoretic mobility shift assay using radiolabeled RNA (either by incorporation of radioactive nucleotide during transcription or by end-labeling RNA transcripts) and the purified protein of interest.

Determine the Sequence Recognized by the Protein

46. After sufficient enrichment has been achieved ($\geq 90\%$ of the transcribed RNA is bound by the protein), clone the double-stranded DNA templates into a suitable vector and transform bacteria.
47. Pick a large number (50–100) of transformants and determine the nucleotide sequence of the inserts.

DISCUSSION

SELEX (Kenan and Keene 1999; Bouvet 2001) is extremely useful for determining preferred RNA-binding sites for specific RNA-binding proteins. Consensus sequences for binding sites should emerge from the analysis. It is often the case that suboptimal (but functional) sequence elements can be determined if the cloning and sequencing are performed at earlier “rounds” of selection. In general, the number of iterative selection rounds required is determined by the size of the starting pool (e.g., RNA containing seven randomized positions contains only $\sim 16,000$ individual molecules, whereas an RNA randomized at 10 positions has a pool size of $\sim 10^6$ individual molecules) and the stringency of selection. To ensure adequate coverage, it is important that ~ 10 times the theoretical pool size be synthesized for each reaction. It is essential that the investigator know and keep in mind the number of distinct molecules required for adequate representation of the pool and take steps to ensure that each molecule of distinct sequence is present in the starting pool.

RECIPES

Klenow Buffer (5×)

Reagent	Quantity (for 1 mL)	Final concentration
NaCl (5 M)	50 μ L	250 mM
Tris-HCl (1 M, pH 7.9)	50 μ L	50 mM
DTT (dithiothreitol; 1 M)	5 μ L	5 mM
H ₂ O	895 μ L	

Store for 6 mo at -20°C .

NT2 Buffer

Reagent	Quantity (for 100 mL)	Final concentration
Tris-HCl (1 M, pH 7.4)	5 mL	50 mM
NaCl (5 M)	3 mL	150 mM
MgCl ₂ (1 M)	0.1 mL	1 mM
Nonidet P-40 (NP-40; 10%)	0.5 mL	0.05%
H ₂ O	91.4 mL	

Store for 6 mo at 4°C .

Proteinase K Buffer for SELEX (2×)

Reagent	Quantity (for 50 mL)	Final concentration
SDS (10%)	2.5 mL	0.5%
EDTA (0.5 M, pH 8.0)	0.4 mL	4 mM
H ₂ O	47.1 mL	

Store indefinitely at room temperature.

Reverse Transcriptase Buffer for SELEX (10×)

Reagent	Quantity (for 1 mL)	Final concentration
Tris-HCl (1 M, pH 8.3)	500 μ L	500 mM
KCl (2 M)	200 μ L	400 mM
MgCl ₂ (1 M)	60 μ L	60 mM
DTT (dithiothreitol; 1 M)	10 μ L	10 mM
H ₂ O	230 μ L	

Store for 6 mo at -20°C .

SDS Extraction Buffer

Reagent	Quantity (for 500 mL)	Final concentration
Tris-HCl (1 M, pH 7.5)	10 mL	20 mM
EDTA (0.5 M, pH 8.0)	1 mL	1 mM
SDS (10% w/v)	25 mL	0.5% (w/v)
H ₂ O	464 mL	—

Store indefinitely at room temperature.

T7 Transcription Buffer (10×)

Reagent	Quantity (for 1 mL)	Final concentration
Tris-HCl (1 M, pH 8.0)	400 μ L	400 mM
MgCl ₂ (1 M)	200 μ L	200 mM
DTT (dithiothreitol; 1 M)	50 μ L	50 mM
H ₂ O	350 μ L	
Store for 6 mo at -20°C .		

REFERENCES

- Bouvet P. 2001. Determination of nucleic acid recognition sequences by SELEX. *Methods Mol Biol* **148**: 603–610.
- Kenan DJ, Keene JD. 1999. In vitro selection of aptamers from RNA libraries. *Methods Mol Biol* **118**: 217–231.

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