

Basic Protocol

(v. 2.0, May, 2003)

Preparation of RNA:DNA Handles

For the two handles (called **A** and **B**), you will need the following oligos:

Product 1

: Name=B_reverse
: Synthesis=1 umole
: Purification=HPLC (2 OD min)
: Sequence= L gcattaggaagcagcccagtagtagg
: Modifications= L = aminolinker + DIGOXIGENIN
: Quantity=1

Product 2

: Name=B_forward
: Synthesis=0.2 umole
: Purification=none
: Sequence= gctttaatgcggtagtttatcacag
: Modifications=none
: Quantity=1

Product 3

: Name=A_forward
: Synthesis=0.2 umole
: Purification=none
: Sequence= GGAATTCGactggtgagtactcaaccaagtc
: Modifications=none
: Quantity=1

Product 4

: Name=A_reverse
: Synthesis=0.2 umole
: Purification=none
: Sequence= attcttgaagacgaaagggc
: Modifications=none
: Quantity=1

Add a few drops of oil to the PCR tubes (say 150 μ l), and mix the following in a 15 ml falcon tube that is on ice:

1 ml reaction (scale as needed):

6 μ l Vent DNA polymerase (2000 u/ml, NEB)
10 μ l Primer mix (50 pmoles/ μ l) in Tris pH 7.7, 10 mM
100 μ l Thermopol buffer
16.4 μ l dNTP mix (25 mM)
40 μ l Template (pBR322, NEB) in H₂O (1 ng/ μ l)
828 μ l H₂O

Add 200 μ l reaction mix per tube, put on hot PCR block (95 °C). React for 30 cycles on a Perkin Elmer 9600 PCR machine. (By the way, this recipe works just fine for volumes between 50 - 200 μ l). Cycling parameters: 95°C 45 sec, 56°C 1 min, 72°C 1.5 min. For handle **A**, perform a 4 ml PCR reaction and purify the finished reaction using 4 Qiagen PCR purification columns. Then biotinylate using the exchange reaction. Mix on ice:

5 μ l T4 DNA polymerase (NEB)
50 μ l DNA (~ 10 μ g from PCR reaction)
1 μ l BSA
10 μ l T4 buffer (NEB)
biotin 16 dUTP (Roche) to a final conc. of 100 μ M

and incubate 20 min at RT. Purify using Qiagen column. For handle **B**, perform a 3 ml PCR reaction and purify using 3 Qiagen PCR purification columns. Check concentration of both handles on gel and mix **A** and **B** in a ratio of 1:1.

Cloning

First, you will need to prepare the vector. Digest pBR322 with EcoRI and HindIII and Calf Intestinal Alkaline phosphatase (CIP) in a total volume of 200 μ l:

2.5 μ l EcoRI
2.5 μ l HindIII
4.0 μ l CIP
40 μ l pBR322 (at 0.25 μ g/ μ l)
20 μ l 10x NEB EcoRI buffer
131 μ l H₂O

incubate 1 hr at 37°C. Gel purify (0.8% agarose gel) and Qiagen extract using the QIAquick Spin Kit. Gel purification is described in the QIAquick handbook on pages 24-25. Next, design your desired RNA sequence and order two complementary DNA oligos, with the following ends:

5' AATTC-ACAC-(5') RNA sequence(3') -ACAC-A 3'
3' G-TGTG- Complement -TGTG-TTCGA 5'

Phosphorylate the oligos using NEB kinase:

20 μ l oligo A (100 pmoles/ μ l in 10 mM pH 7.0 Tris)
20 μ l oligo B
8 μ l kinase (NEB 10 units per μ l)
10 μ l **ligase** buffer (do **NOT** use kinase buffer)
42 μ l H₂O

incubate 1 hr at 37°C. Then, add some oil, and anneal oligos using a temperature gradient (95°C to 4°C in 2 h, PCR machine, file 74).

Ligation

Ligate linearized and dephosphorylated vector (obtained from the gel purification step described earlier) with double-stranded and phosphorylated insert with T4 DNA ligase, at 19°C for 3 h to overnight. The obtained product corresponds to a plasmid containing the DNA insert, which is the template DNA of the desired RNA molecule. Use a Vector: insert ratio of about 1:100.

0.4 μ l vector (100 ng/ μ l)
1 μ l T4 DNA ligase
1 μ l 10X T4 ligase buffer
3 μ l insert (1 μ g/ μ l)
4.6 μ l H₂O

Transformation

Introduce your recombinant plasmid into bacteria (so-called “transformation”). Possible cells are *e.g.* Epicurian Coli XL-10-GOLD ultracompetent cells from Stratagene, or ultracompetent DH5 α from GibcoBRL. Follow the manufacturer’s protocol. For the XL-10-GOLD cells:

- Thaw the XL10-Gold cells on ice
- Gently mix the cell by hand. Aliquot 100 μ l of the cells into prechilled Falcon 2059 tubes.
- Add 4 μ l of the β -mercaptoethanol mix provide with the kit to the 100 μ l of cells.
- Swirl the contents of the tube gently. Incubate the cell on ice for 10 min .
- Add 0.1-50 ng of DNA (1-3 μ l of your ligation reaction) to one of the 100 μ l aliquots of cells and swirl gently.
- Incubate the tubes on ice for 30 min.
- Heat-pulse the tubes in at 42°C water bath for 45 sec.
- Incubate the tubes on ice for 2 min.
- Add 900 μ l of LB-agar medium to each tube and incubate the tubes at 37°C for 1h with shaking.
- Plate 100 μ l of the transformation reaction on ampicillin agar plates using a sterile spreader. Spin down remaining 900 μ l and plate remainder on another plate.
- Incubate the plates overnight at 37°C (12-16h)

If you got some colonies, then you are in good shape. Otherwise, you will need to redo your transformation and ligation, and perform in parallel some kind of control. A good control is a “known good” pair of oligos, such as the “SixStrong” pair.

Pick 5 colonies, streak for backup, and grow overnight in 3 ml LB + ampicillin. This means that you should carefully transfer some cells from a *single(!)* colony into a falcon

tube containing 3 ml of LB + ampicillin using a pipette tip or a sterile toothpick. Repeat this with five colonies. Then, you should let your cultures grow overnight with shaking at 37°C. Recover the plasmid DNA from the cells with the QIAprep Spin Miniprep kit protocol, handbook pg. 20-21. Next, you will need to identify the bacterial colonies that contain the recombinant plasmid by e.g. digestion with *Hind* III and *Hinc* II (for 10 digests):

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20 µl 10x NEB buffer 2
10 µl Hind III
8 µl Hinc II
2 µl BSA
110 µl H2O
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Digestion: 5 µl of DNA + 15 µl of premix, at 37°C for 1.5 h. Check on 1% agarose gel. There will be 3 bands : a large constant band (> 3000 nt), a constant band at about 622 nt., and another band that increases in length with your insert. This band is 456 nt. long in the unmodified pBR322, and in a “good” clone will be (456 + length of your insert) nt. So if you are inserting a 75 nt. hairpin, the expected length of this band is 531 nt. Finally, you should send some material to the sequencing facility to verify the sequence.

PCR of T7 template

Isolate and amplify the T7 template DNA by PCR from the recombinant plasmid, using the T7 promoter primer:

T7-forward

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Product 5
: Name=T7-forward
: Synthesis=0.2 umole
: Purification=unpurified
: Sequence =TAATACGACTCACTATAGGGActggtgagtactcaaccaagtc
: Modifications=none
Quantity : 1
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T7-reverse

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Product 6
: Name=T7-reverse
: Synthesis=0.2 umole
: Purification=unpurified
: Sequence =taggaagcagcccagtagtagg
: Modifications=none
Quantity : 1
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Add a few drops of oil to the PCR tubes (say 150 µl). For 1 ml PCR mix, use:

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250 µl enzyme premix:
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6 μ l Vent DNA polymerase (2000 u/ml, NEB)
25 μ l Thermopol buffer (10x)
219 μ l H₂O

750 μ l reaction:

10 μ l Primer mix (50 pmoles/ μ l) in Tris pH 7.7, 10 mM
75 μ l Thermopol buffer
16.4 μ l dNTP mix (25 mM)
40 μ l Template (pBR322 based) in H₂O (1 ng/ μ l)
608.6 μ l H₂O

add 150 μ l reaction mix per tube, put on hot PCR block (95 °C), and add 50 μ l enzyme mix. React for 30 cycles on a Perkin Elmer 9600 PCR machine. Cycling parameters: 95°C 45 sec, 52°C 1 min, 72°C 2 min. By the way, the cycling parameters used for the handles (95°C 45 sec, 56°C 1 min, 72°C 1.5 min) work just as well. You will need to do 5 x 200 μ l reactions to have enough DNA for your transcriptions. [General note on PCR: The key variable for the PCR conditions are the annealing temperature and the extension time. A good starting point for the annealing temperature is 50°C, but the optimal temperature may be anywhere from 50 to 60°C. The extension time should be at least 1 min, and should be extended according to the length of the product (about 1 min per kb)]. Purify the PCR reaction with QIAGEN PCR prep kit columns (2 columns per ml of PCR). You should end up with 50 μ l of template. You will need 16 μ l for the transcriptions.

To verify the insert, send some of the PCR DNA to the sequencing facility, if you did not already do that earlier. Sample: 200 ng of DNA (PCR product) + 0.8 pmol of primer in 13 μ l of H₂O or Tris pH 7, no TE!!

Product 1 : Name=SeqOligo322EcoRI
: Synthesis=0.2 umole
: Purification=unpurified
: Sequence= gacattaacctataaaaaataggcg
: Modifications=none
: Quantity=1

T7 Transcription

Use the Ambion T7 Megascript kit as directed. Normally you will only need to do a 40 – 60 μ l reaction. Incubate for 3 hours, and precipitate using lithium chloride as described in the Ambion protocol.

6 μ l 10x reaction buffer
24 μ l NTP mix
6 μ l Enzyme mix
24 μ l template DNA (3-5 μ g)
60 μ l at 37°C for 3 h (hot room)

Purify by LiCl precipitation: add 90 μ l nuclease-free water and 75 μ l LiCl mix provided by Ambion, incubate 1 h at -20°C , and finally spin down for 15 min, max speed. Resuspend the pellet in nuclease-free water. Check RNA on 4% denaturing acrylamide gel.

Annealing

The final annealing reaction conditions are:

80% formamide (Ambion)
1 mM EDTA pH 8.0
40 mM Pipes pH 6.3
0.4 M NaCl

To make the annealing buffer, mix:

800 μ l formamide (Ambion)
2 μ l 0.5 M EDTA pH 8.0
40 μ l 1 M Pipes pH 6.3
80 μ l 5 M NaCl
final volume: 922 μ l.

Addition of the handles/RNA will bring the concentrations to the desired (final) values. In general, mix:

8 μ l handle A
8 μ l handle B
1.5 μ l RNA (at 10–20 $\mu\text{g}/\mu\text{l}$)
80 μ l annealing mix

anneal using PCR machine:

85 $^{\circ}\text{C}$ 10 min
62 $^{\circ}\text{C}$ 1.5 hours
52 $^{\circ}\text{C}$ 1.5 hours
ramp to 10 $^{\circ}\text{C}$ over 10 min

add 3 volumes ETOH, put in freezer -20°C (or -80°C) for several hours (>3) to overnight, spin in microcentrifuge (max rpm, 10 min). The pellet should be tiny but visible and brown or white. Take off supernatant (careful – the pellet is easy to dislodge and loose) and resuspend pellet in Tris/NaCl buffer as desired (e.g. 100 mM NaCl, 10 mM Tris pH 7.0). Check on 1.4% agarose gel (if you really want to – we're still not quite sure what a "good" annealing reaction looks like, so running a gel will not be too informative).

Beads/Protein G/Ab

Materials

Activation buffer

0.1 M MES (Sigma M-8250)

0.5 M NaCl

Adjust the pH to 6.0 with NaOH

1% Tween20 (Sigma P-7949)

PBS (7.0)

0.14 M NaCl

2.7 mM KCl

61 mM K_2HPO_4

39 mM KH_2PO_4

Adjust the pH to 7.0 with HCl

PBS (7.4)

0.14 M NaCl

2.7mM KCl

80.2 mM K_2HPO_4

20 mM KH_2PO_4

Adjust the pH to 7.4 with HCl

Sodium phosphate

100 mM Na_2HPO_4

Adjust the pH to 8.5 with HCl

Ab X-linking

100 mM Na_2HPO_4 pH 8.5

100 mM NaCl (or other non-amine containing buffer pH 7-9).

B. Spherotech polystyrene carboxylated beads (2.36 micron), 5% w/v.

C. Protein G (Pierce 5mg 21193) Dissolve in 1ml PBS pH 7.4.

D. Sulfo-NHS (Pierce 24510)

E. EDC (Pierce 22980)

F. 2-mercaptoethanol (Sigma M3148)

G. Hydroxylamine•HCl (Sigma H2391)

H. Crosslinker: DMP (add 50 mg to 1 ml of crosslinking buffer, dissolve, and immediately add 30 μ l to 1 ml reaction) The desired final concentration is 10 mM.

J. Sheep polyclonal anti-dig antibody (Roche 1333 089). Dissolve in 200 μ l PBS pH 7.4.

Method

1. Spin down beads at 3000 rpm for 3 min. Resuspend beads in 1 ml MES (activation) buffer.
2. Dissolve 10 mg EDC and 22 mg sulfo-NHS in 1 ml deionized water and immediately add 100 μ l to the beads. This corresponds to about \sim 5 mM EDC and \sim 10 mM sulfo-NHS. The molarities are doubled with respect to the standard Pierce protocol. React for 15 min at room temperature.
3. Add 2.8 μ l of 2-mercaptoethanol (final concentration 40 mM) to quench the reaction. Spin down and resuspend in 1 ml MES activation buffer.
4. Add 1 mg of protein G (200 μ l). Allow the protein and the beads to react for 3 hours at room temperature.
5. Spin down and wash with PBS 7.4. Spin down and resuspend beads/protein G in 1 ml PBS 7.0 (for storage) OR in 1 ml of Ab X-linking buffer.
6. Add 60 μ l of the Roche polyclonal antibody and 30 μ l of dissolved DMP. Tumble at RT for 60 min. Spin down and resuspend in PBS pH 7.0. The final product should be kept at neutral to acidic pH since imidoamides slowly hydrolyze at high pH's.

Notes:

Imidoester reactions are carried out at 0°C or room temperature as it has been shown that elevated temperatures are principle causes of poor yields with these reactions.

The DMP reaction can be terminated with Glacial Acetic Acid at a 1:4. Alternatively, an amine containing solution like Tris or glycine can be added to quench the reaction.

Store the crosslinkers desiccated at temperature indicated on the vial label.

Allow the vial to warm completely to room temperature before opening.

Unopened, these crosslinkers are stable for at least one year. After opening, they should be stored protected from moisture and used within 6 months.

Phosphate, carbonate, Hepes, Triethylamine, N-ethyl morpholineacetic acid buffers, free of primary amines, between pH 7-9.0 (8-9 optimal), may be used for the DMP step.

A 10-30 fold molar excess of DMP crosslinker over primary amine containing molecules is recommended.

When crosslinking two different proteins, mix the proteins in a 1:1 ratio then add the crosslinker.

If a stock solution of the crosslinker is used it should be prepared immediately before use.