PCR cloning for in situ hybridization probes

By Akiya Watakabe 10/2/02
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<overview>

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0) General reagents for molecular biology

NOTE:

For the following protocol, ug and ul means microgram and microliter respectively. The letter u is the substitute for micro.

*MilliQ water: highly purified water

*3 M NaOAc (pH7.0): Dissolve sodium acetate (CH3COONa.3H2O: MW136.08) to small amount of MilliQ water, adjust pH to 7.0 with acetic acid. Autoclave

*DEPC-water: Add DEPC to 0.1 % to MilliQ water, keep RT overnight, autoclave with the cap loosened for 40 min.

*TE: 10 mM Tris (pH 8.0), 1 mM EDTA

*75% ethanol

*Ethanol

*Phenol: Melt crystal phenol @65C. Add hydroxyquinoline to 0.1 % (w/w). Equilibrate once with equal volume of 0.5M Tris (pH 8.0), twice with 0.1M Tris (pH 8.0), twice with TE. Keep @ 4C. #Equilibrated phenol is also commercially.

*Chloroform

*PC: Phenol/Chloroform: Mix equal volume of phenol and chloroform.

1) RT-PCR

>> Reagents

*RNA: Total RNA of tissues of interest. Prepare yourself or commercially available from Clontech etc.

*Reverse transcriptase: Superscript II (Gibco/Invitrogen) or others.

*10 mM 4dNTP: 10 mM each of dATP, dGTP, dCTP, dTTP mixed. Diluted from 100 mM stock reagents (Amerciam pharmacia).

*pdN9: random nonamer (Takara)

*PCR primers: I usually dissolve the powder to 100 uM and make working solution containing 5 uM each of primer pairs.

*PCR enzymes

KOD polymerase (TOYOBO): with strong proof reading activity

ExTaq polymerase (Takara): Used for TA cloning

*DMSO: Used for PCR enhancement. Keep aliquoted @ -20C.

*10xloading buffer:

*1xloading buffer: dilute 10x buffer with TE

*1% agarose gel: melt 1g of agarose (for molecular biology) to 100 ml of TAE buffer by electric oven. Add 5 ul of 10 mg/ml Ethidium bromide.

*TAE buffer:

>> Protocols

*Mix the following

total RNA	2 ug
pdN9	0.25 ug
DEPC-water	up to 12 ul

*Set the tube to PCR machine. Run the following program.

70C 10 min > 25C 5 min > 42C 60 min > 70C 10 min > 4C forever

*After 10 min incubation @ 70C, take the tube out and immediately put on ice. Pause the program

*Add the following to the tube

5xFirst strand buffer	4 ul	(comes with the enzyme)
0.1M DTT	2 ul	(comes with the enzyme)
10 mM 4dNTP	1 ul	
Superscript II	1 ul	

*Resume the program.

*After the reaction is over, add 20 ul of MilliQ water to the tube, mix and keep frozen until use.

#If the cDNA is used for quantitative RT-PCR, keep cDNA @ -80C and avoid repeated freeze-thaw cycles.

*Set PCR reaction as following. Prepare master mix for multiple samples.

	per sample	half scale	
10XKOD Buffer	2	1	(comes with the enzyme)
2.5 mM 4dNTP	1.6	0.8	(comes with the enzyme)
25 mM MgSO4	0.8	0.4	(comes with the enzyme)
5 uM PCR primer pair	2	1	
MilliQ water	10.2	5.1	
DMSO	1	0.5	
cDNA	2	1	
KOD polymerase	0.4	0.2	

*Run the following program.

95C 5min

(94C 30 sec, 55C 30 sec, 68C 2 min)x30 cycles

4C forever

*Mix an aliquot (1-2 ul is usually enough) with 1x loading buffer. Run on 1% agarose gel to check the size of PCR product. Save the rest on ice.

#This program needs to be adjusted depending on the PCR reactions. If the gene is abundant, 20-25 cycles are generally sufficient. If you did not get the reaction product or reaction products are scarce, adding 5 to 10 cycles could help. Just set the reaction tube to the machine and run additional cycles. If you still don't get the product, lower the annealing temperature to 50 C might help. If you get too many bands, increase annealing temperature and see if you get a specific band.

2) **Cloning of cDNA fragment**

>> Reagents

* PCR enzymes

ExTaq polymerase (Takara): Used for TA cloning

*100 mM dATP (Amerciam Pharmacia)

*QIAEXII (Quiagen) DNA extraction kit

*T-overhang vector (commercially available, or make yourself)

*Ligation Kit (ver.2) (Takara)

*Competent cells (commercially available, or make yourself)

*LB broth

*LB plate with Ampiciline (or other antibiotics)

>> Protocols

Adding A-overhang to the PCR product

*Add the following to the rest of the PCR reactions

(Make a master mix and add an aliquot)

	per sample	half scale
10XExTaq	1	0.5
100 mM dATP	0.4	0.2
MilliQ water	8.4	4.2
ExTaq	0.2	0.1
	10	5

*Incubate @ 72C for 30 min to 2 hr

purifying DNA fragment

*Run the reaction mix on 1% agarose gel.

*Cut the DNA band as small as possible.

*Add 3 vol QX1 buffer and 10 ul QuiaexII resin

*melt the gel piece @ 50C for 10 min (mix from time to time)

*centrifuge (cfg) at top speed for 30 sec

*discard the sup. (preferably by aspiration)

*wash the resin in 500 ul Qx1 buffer once

(suspend the resin by vortex, centrifuge, aspirate the supernatant)

*wash the resin in 500 ul PE buffer twice.

*remove the buffer as much as possible, air dry completely (RT 10 min).

#Don't vacuum dry.

* Suspend the resin in 20 ul TE. Elute for 5 min @ RT.

*cfg and save the sup.

*Run 2 ul for check.

I usually do this in half scale: 5 ul resin, and 250 ul wash, 10 ul elute.

Ligation to TA-cloning vector

*Mix the following.

T-overhang vector	0.5 ul
purified cDNA	2 ul
Ligation kit sol I	2.5 ul

*incubate the reaction @16C for 2 hr to overnight.

Transformation

*Add 50 ul of competent cells to the reaction tube.

*Mix and keep the tuber on ice for 20 min-1 hr

*Heat shock the tube @ 42C for 1min and immediately return the tube on ice.

*Add 500 ul LB.

*cfg for 1 min.

*Discard the sup with approximately 50 ul left.

*Resuspend the bacterial pellet.

*Spread the bacteria on LB Amp plate.

*Incubate the plate @ 37 C overnight.

3) **Check for correct inserts**

Each colony on the LB-plate stems from a single bacteria harboring a single kind of plasmid. Thus, it is needed to select the colony that contains the plasmid with the correct insert DNA fragment. Confirmation of the insert sequence can be done conveniently by colony-PCR protocol (adapted from Dr. Yuriko Komine's protocol) or can be done from mini-prep DNA.

Conoly-PCR

*Prepare the PCR reaction mixture for the number of colonies you want to check.

	Per sample
10xExTaq buffer	2
2.5 mM 4dNTP	1.6
MilliQ water	13.3
Primer mix	2
(5uM each for M13 forward and reverse primers)	
DMSO	1
ExTaq	0.1
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	20

*Aliquot 20 ul each to PCR tubes on ice.
*Pick a colony by a sterile pipet tip, touch it to the master plate, and put the tip into the PCR tube with reaction mixture. Repeat for the number of colonies you desire.

*Run PCR
95C 5min
(94C 30 sec, 55C 30 sec, 72C 1 min)x25-30 cycles
4C forever
*Run 5 ul by 1% agarose gel

*Add 15 ul of PEG-NaCl (20% PEG6000, 2.5 M NaCl) to each tube, mix and keep on ice while waiting.

*When the presence of insert is confirmed, proceed for purification.

>>Purification

*Cfg at top speed for 15 min.

*Discard the supernatent.

*Rinse the pellet with 75% ethanol once.

*Dry the pellet.

*Dissolve DNA into 10 ul TE.

*1ul is usually enough for sequencing (Run the gel for confirmation if necessary).

Mini-prep

Can be done by mini-prep machine, or by commercial kit. When I have only a small number of clones to be checked, I do a simplified mini-prep protocol as follows.

>> Reagents

Solution I	50 mM Glucose, 25 mM Tris.HCl (pH8.0), 10 mM EDTA (pH 8.0)
Solution II	0.2 N NaOH, 1 % SDS
Solution III	Dissolve potassium acetate 58.9 g into MilliQ water, add glacial acetic acid 23 ml, then add MilliQ water to 200 ml

1) Inoculate a single colony in 2 ml of LB broth containing ampicilin @37C overnight vigorously shaking.
2) Pour the culture into 1.5 ml microtube.
3) Collect the bacreria by cfg at top speed for 1min.
4) Remove the sup as much as possible.
5) Suspend the pellet into 100 ul of Sol.I by pipetting (dislodge the pellet completely).
6) Add 200 ul of Sol II, immediately shake several times to evenly disperse the solution (it becomes viscous due to release of bacterial genomic DNA).
7) Add 150 ul of Sol III. Shake several times to mix the solution.

8) Add 400 ul of phenol/chloroform, mix well, cfg at 15000rpm for 3 min.
9) Save the upper layer.
10) Add 1 ml of ethanol, shake, cfg at 15000rpm for 2min.
11) Remove supernatant.
12) Rinse the pellet once with 75% ethanol.
13) Dry the pellet.
14) Dissolve the pellet into 50 ul of TE containing RNase A (20 ug/ml)

This mini-prep DNA contains genomic DNA but is suitable for most purposes of checking such as restriction enzyme digestion and cycle sequencing. There is no need for further purification if you just want to confirm the sequence. If you need high quality long read, it may be better to further purify the plasmid.

Sequencing

Following is the protocol I routinely use. But this will probably change often according to the change of available reagents. Especially, it should be remembered that Bigdye reagents may be diluted further.

Make a master mix when possible

	Per sample
5xSequenase buffer	1.2
BigDye terminator ver 1.1 (Perkin-Elmer)	0.5
Sequence primer (4~50 uM)	1.2
DMSO	0.4
Template DNA(approx 0.2 ug) +MilliQ water	3.7
	7

When the sequence is GC-rich, the above formulation may result in premature termination of the sequence reaction. In such cases, the following protocols may be used.

	Per sample
BigDye terminator ver 1.1	
(Perkin-Elmer)	2
Sequence primer (50 uM)	0.2
DMSO	0.4
Template DNA(approx 0.2 ug)	
+MilliQ water	4.4
	7

*Run PCR

95C 5min

(96C 10 sec, 50C 5 sec, 60C 4 min)x25 cycles

4C forever

*Add 0.7 ul 3M NaOAc, 20 ul 100 % Ethanol.

*Keep on ice for 10 min.

*Spin at 14000 rpm for 15 min.

*Rinse with 100 ul 75% ethanol.

*Vacuum dry for 10 min.

*Dissolve in 13 ul formamide and run the sample on Genetic Analzyzer 310

4) Maxi-prep for template plasmids

The following protocol is for Qiagen plasmid purification kit, which is one of the most popular method of plasmid purification. However, I routinely use alkaline-lysis method combined with PEG precipitation for preparation of probe plasmids.

One point I don't like about Qiagen Kit is that it uses RNase A in the relatively early phase. I strongly recommend using this kit in a restricted area with extra care, if you have to work with RNA in other experiments.

>> Reagents

STE: 0.1 M NaCl, 10 mM Tris.Cl (pH8.0), 1 mM EDTA
Qiagen Plasmid Maxi Kit.
Chill Buffer P3 on ice before starting

1. Pick a single colony and inoculate it in 100 ml of LB medium containing ampiciline (100 ul of 100 mg/ml stock solution) @ 37C overnight vigorously shaking.
2. Harvest bacteria by centrifugation at 5000 rpm for 10 min.
3. Suspend the pellet in 5 ml STE and move it to disposable 50 ml tube.
4. Harvest bacteria by centrifugation at 3000 rpm for 20 min.

Step 3 and 4 is actually not necessary. I simply hate to reuse RNase-contaminated centrifuge bottle.

5. Resuspend the bacterial pellet in 10 ml Buffer P1 (watch out!! contains RNase A)
6. Add 10 ml of Buffer P2, mix gently but thoroughly by inverting several times and incubate at room temp for 5 min.
7. Add chilled Buffer P3 mix immediately and incubate on ice for 20 min.
8. centrifuge at 3000 rpm for 30 min at 4C. Remove supernatant containing plasmid DNA.
9. Equilibrate Qiagen-tip 500 by applying 10 ml Buffer QBT
10. Apply the sup from 8 to the column.

11. Wash the tip with 2x30 ml of Buffer QC
12. Elute DNA with 15 ml Buffer QF.
13. Precipitate DNA by adding 10.5 ml of isopropanol, mix and keep @room temp for 15 min, and spin for 20 min at 3000 rpm.
14. Suspend the DNA pellet in 500 ul 75% ethanol, move it to 1.5 ml microtube.
15. cfg and remove sup as much as possible.
16. Air dry briefly.
17. Dissolve pellet in 200 ul TE and measure OD260 for quantification.

5) Restriction digestion for template linearization

1) Select appropriate restriction enzymes for linearization of the probe plasmid.
   When sequence information is available, check that there is no internal site.

2) Digest 10 ug of plasmids @ 37 C (or other temp depending on enzymes) for 2 hr to overnight.

10XBuffer	5 ul	(comes with the enzyme)
DNA	10 ug	
MilliQ water	to make the reaction to 50 ul	
Restriction enzyme	3-5 ul (20 unit-30 unit)	

3) Check for complete digestion by agaraose gel electrophoresis.
   #If the digestion is not complete, add some more enzyme and continue incubation.
4) Add 130 ul D3W, 20 ul 3M NaOAc, 200 ul phenol/chloroform, mix well.
5) cfg for 5min
6) Save the upper layer and add 200 ul chloroform to it.
7) Save the upper layer and add 500 ul ethanol to it.
8) Cool @-20C for 15min, spin at 15000rpm for 15min.
9) Discard the supernatnant. Rinse once with 75% ethanol.
10) Air dry the pellet.
11) Dissolve the pellet in 15 ul TE (TE for RNA): this would be 0.5-0.7 ug/ul

#Note:
For restriction enzyme digestion, avoid 3' overhang enzymes (such as KpnI, SacI and PstI) because they make inefficient templates for in vitro transcription. For KpnI, use Asp718 (Roche), which is a 5'-overhang enzyme recognizing the same sequence.

6) In vitro transcription

>> General notes

1: Work in RNase-free space. Make it a custom to ensure RNase-free environment. Wear gloves when working with RNA-grade reagents. Wipe benches with Cleaners such as RNAse-zap (Promega) or Absolve (NEN).

2:

>> Reagents

DIG labeling mix (Roche)

T3/T7 RNA polymerase (Roche)

10xTranscription buffer comes with the enzyme

RNasin ribonuclease inhibitor (Promega)

DEPC-water

RQ1 RNase-free DNase (Promega)

ProbeQuant G50 spin column (Amerciam pharmacia)

STE-RNA (150mM): Add 30ul of 5 M NaCl to 970 ul of TE saved for RNA use.

>> protocol

*Mix the following

10xTranscription buffer	2 ul
DIG labeling mix (10x)	2 ul
DEPC-water	11 ul
Template plasmid	2 ul
RNasin	1 ul
T3/T7 RNA polymerase	2 ul

*Incubate @ 37C for 2 hr-5 hr

*Add 1 ul RQ1 DNase to the reaction mix and incubate @37C for further 20 min

*Add 30 ul RNA-STE and mix

*Run 1 ul mixed with RNA loading buffer on agarose gel for check

*Prepare ProbeQuant G50 spin column by cfg @ 3000 rpm for 1 min.
*Apply the reaction mixture to the column and spin 2 min @3000 rpm
*Add 50 ul NH4OAc, 250 ul Ethanol, -20C15 min, spin 15 min
*rinse with 75% ethanol, dry for 10 min
*Dissolve in DEPC-water 50 ul
*Measure OD 260/280/320 for quantitation.

##The yield should be around 5-10 ug of RNA/20 ul reaction. The OD measure is inaccurate due to wierd absorbance without purification by ethanol precipitation. But if you do not care, the probe solution can be used right after the column purification.

7) Further notes

<Specificity of hybiridization>
Specificity of the hybiridization pattern is always a matter of concern. The "must" control is the sense probe. But bear in mind that the absence of hybiridization signals with the sense probe does not ensure the specificity of the antisense probe. Making two kinds of antisense probes with non-overlapping sequence would be a good confirmation. If they are identical, you can mix the probes to make the signal stronger. Avoid GC-rich sequences, because they tend to produce non-specific hybridization.
In my opinion, the best evidence for the specificity is the pattern itself. If you see a characteristic pattern, it is easier to believe the specificity of ISH. If not, you need to be very careful about the specificity. The non-specific hybridization is not necessarily uniform. Some structures tend to produce higher background signals such as the dendate gyrus of hippocampus.

<DIG-probe>

Contamination of free DIG-nucleotides can be a cause of high background. Ethanol precipitation alone cannot efficiently remove the free nucleotides. We usually use a spun column (Probe quant G-50: amerciam-pharmacia) before ethanol precipitation. The standard length of RNA probe in my experiments are 500-1000 nt. For a longer probe (>800 nt), hydrolysis may improve the signal intensity. When I used a cDNA

probe spanning 2.6kb, hydrolysis made a big difference in hybridization intensity. Below is the protocol for hydrolysis.

Reagents:

0.25M $NaHCO_3$

0.25M Na_2CO_3

Hydrolysis buffer

Mix 2 ml of $NaHCO_3$, 3 ml of Na_2CO_3 and 5.75 ml DEPC-treated water.

(This is expected to be 0.1M $Na\text{-}CO_3$ buffer, pH 10.2)

Protocol>

>In vitro transcription in 20 ul.

>Add 180 ul Hydrolysis buffer and incubate at 60C for appropriate time.

Probe length (kb)	0.8	1.0	1.2	1.5	2	2.5
Time (min)	7	9	11	12	14	15

> Immediately place the tube on ice and add 1 ul of glacial acetic acid.

> Add 20 ul of 3M NaOAC, 500 ul EtOH and mix well.

> Immediately spin at 14000 rpm for 15 min.

> Discard the sup. Dissolve the pellet to 50 ul STE.

> ProbeQuant G-50 spun column.

> Ethanol precipitate.