PROTOCOL: TA-CLONING AND SEQUENCING PCR PRODUCT

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This is a method to separate the multiple fragments (alleles or different genes) that are being amplified in a PCR reaction via cloning, so they can then be sequenced separately. This protocol assumes you know how to do PCR. The excel templates in file PCR-SEQ-Template.xlxs can be used with this protocol.

Overview of Steps

1) Generate PCR product
2) Clone the PCR product
3) Screening the clones for the product of interest
4) Sequence the clones

Materials

- *PCR reagent: dNTPs (10mM, PCR buffer, MgCl2, NON-Proofreading Taq, primers specific to gene of interest)*
- *Topo TA-Cloning Kit with Chemically Competent Cells (Invitrogen)*
- *LB plates with antibiotic (1% triptone, 0.5% yeast extract, 1% NaCl, 1.5% agar, 50 ug/ml antibiotic [either ampicillin or kanamycin], pH 7.0.*
  - For 500 ml of plates: 5 g tryptone, 2.5 g yeast extract, 5 g NaCl, 7.5 g bacteriological grade agar, up to500 ml with water
  - Autoclave. When cooled (around 55C) add 500ul 50mg/ml ampicillin or kanamycin.
  - Pour plates—makes 25-30. Date and store in refrigerator. Good for approximately 1 month.
- *Ampicillin: 50mg/m*l
  - For a bottle containing 200mg of ampicillin, add 4 ml sterile water, to obtain a 50mg/ml concentration.
  - Make 500 ul aliquots and store in -20C freezer.
- *X-gal: 40mg/ml*
  - For 100 mg X-gal add 2.5 ml DMF (dimethyl formamide). Wrap bottle in foil (X-gal is light-sensitive) and store in -20C in freezer.
  - Spread 40 ul on to plates prior to spreading cells.
- *Petri Plates, plate spreaders, ice & ice bucket, water bath or heat block.*

Procedure

1) Generate PCR product and check on a gel. This assumes you have attempted to optimize your PCR product. You can use the PCR-SEQ-Template.xlsx. Do the PCR reaction as you normal, but make sure of the to NOT use a proofreading *Taq* as the cloning reaction is dependent upon the additional A that is added to the end of the PCR fragment. See below for an example for 8 samples.

Master Mix **Samples**

Reagents	**[stock]**	**[final]**	**ul/rxt**	**8.5**
dh2o			10.875	**92.4375**
dNTPs mM	10	0.2	0.3	**2.55**
Buffer	10	1	1.5	**12.75**
MgCl2 (mM)	50	2.5	0.75	**6.375**
Forward primer (uM)	10	0.5	0.75	6.375
Reverse primer (uM)	10	0.5	0.75	6.375
Hotstart Taq (units)	5	0.025	0.075	**0.6375**
DNA (ng)	30		1	**aliquot vol.**
Total rxt vol. =			15	**14**

Thermal cycle		
95C	2 min	Hot start
95c	30 sec	
55C	30 sec	35 cycles
72C	45 sec	
72C	30 min	Extention for atleast 30 minutes to ensure the addition of the extra A

2) Cloning PCR product using a Topo TA-Cloning Kit (Invitrogen).

Read the complete manual for the kit. These instructions make reference to the manual for *Invitrogen pCR 2.1-Topo Vector w/ Chemically Competent Cells*

a. Ligation (page 4 of manual).

In this step, each plasmid vector takes in a single PCR product insert.
It works to do ½ reactions.

0.5-2 ul fresh PCR product, dependent on strength of PCR reaction (typically 1 ul)
0-1.5 ul water adjust based on PCR product to make a total volume of 3 ul (typically 1 ul)
0.5 ul salt solution,
0.5 ul Topo Vector (linearized plasmid with overhanging T's)
3 ul reaction

Incubate at room temperature (22-23C) for 5 minutes.

b. Transformation.

In this step, each bacterial cell takes in one plasmid with an insert.

- Warm LB-Ampicillin plates at 37C for at least 30 minutes prior to starting
- Take competent cells from -80C freezer and keep on ice.
- When thawed, split into two reactions – take 25 ul and put in a new 1.5ml tube (be gentle)
- Add 1 ul of ligation reaction to 25 ul of chemically competent *E.coli* cells and swirl gently.
- Mix by flicking a few times, and incubate on ice for 15 minutes to weaken the cell membrane of the bacteria.
- Heat shock cells for 30 sec at 42C to force the vector into the cells.
- Immediately put the tubes back on ice..
- Add 125 ul room temperature SOC medium, and incubate at 37C for 1 hour, shaking at 200rpm. This starts the cells replicating.

c. Plating/Growing Cells.

- Spread LB/Amp plates with 40 ul of 40mg/ml of X-gal using sterle technique.
- Warm plates at 37C.
- Spread desired amount of cells on the plates using sterile technique. I recommend 25 ul or 10 ul cells + 40ul SOC.
- Incubate plates at 37C for 15-18 hours.

3. Screening Clones using PCR with M13 primers.

This is one method to screen the clones to ensure they contain the PCR fragment of interest. This also allows you to decide which samples you want to sequence.

a. Pick white colonies using a toothpick (white with blue center are also OK). It is a good idea to include one blue colony as a negative control (it would have an empty vector)

b. Touch/smear toothpick on designated spot on master plate, then swirl the toothpick in PCR tube containing 30ul of water (or TE)

c. Put the tubes with the water/cells at 95C for10 minutes to lyse the cells.

d. Use this as template for PCR reaction using M13 primers specific to the vector. Do a 15 ul reaction with 1 ul of lysed cells from chosen bacterial colonies.

Master Mix **Samples**

Reagents	**[stock]**	**[final]**	**ul/rxt**	**10**
dh2o			12.087	**120.87**
dNTPs mM	10	0.2	0.3	**3**
buffer	10	1	1.5	**15**
MgCl2 (mM)	50	2.5	0.75	**7.5**
M13 (forward) uM	10	**0.1**	0.15	**1.5**
M13 (reverse) uM	10	**0.1**	0.15	**1.5**
Platinum Taq	5	0.021	0.063	**0.63**
Boiled clone			1	**aliquote vol.**
Total rxt vol. =			15	**14**

Thermal cycle program:		CLONES	
94C	2 min		Initial denature
94C	30 sec		denature
55C	30 sec	35cycles	anneal
72C	45 sec		extension
72C	30 min		final extension

e. Run 10ul of the sample on a 1.5% agarose gel
f. An empty vector would produce a band of 203bp. The clones with your band of interest would produce a PCR product of your fragment size + 203 bp. Take note of which samples (i.e. which colonies) have a band at the size you are looking for. These are the samples you will go on to sequence.

You can either send this PCR product to the DNA Facility for them to do the sequencing reaction, or you can do the reaction and clean-up yourself following the protocol below. If you have more than 30 samples to sequence, it is cost effective to submit the samples as high through put and have the sequencing facility to the sequencing reaction. That is, it cost the same to either have the facility to the sequencing reaction and run 96 high-through put samples (in a 96 well plate), as to do the sequencing reaction yourself for individual 32 samples and just have them run them.

4. Sequencing Reaction.

a. Purify the PCR product from the cloning reaction.

The easy way to do this is an ExoSap Reaction.
2.5ul of PCR product + 1 ul of ExoSap.
On Thermal cycler: 37C for 15min; 80C for 15 min

b. Sequencing Reaction

Sequencing Master Mix

Reagents	**ul/rxt**	**5**
dh2o	3	**15**
Big Dye v. 3.1	1.4	**7**
buffer 5X	1.4	**7**
M13 (forward) 5 pmol/ul	0.7	**3.5**
ExoSaped PCR product	0.5	**aliquot vol.**
Total rxt vol. =	7	**6.5**

Thermal cycle program:			
96C	1 min		Initial denature
96c	10 sec		denature
55C	5 sec	35cycles	anneal
60C	4 min		extension
10C			hold

c. Sequencing Clean-Up

- Bring volume of tubes from sequencing reaction up to 20 ul with dH20
- Transfer to a 1.5 ml tube.
- Add 2 ul 0.125 M EDTA. Do not mix with pipette
- Add 50 ul of 95-99% EtOH. Do not mix with pipette.
- Vortex gently to ensure complete mixing.
- Incubate at RT for 15 minutes.
- Place in temperature controlled centrifuge, ensuring the hinges of the tubes are facing outwards so that the pellet will form at the outside edge. Spin at 4C at 13200 rpm for 20 min.
- Carefully remove the tubes from the centrifuge, and remove the supernatant with the pipette being careful not to touch the invisible pellet.
- Add 250 ul 70% ethanol, running it over the pellet. Spin at 4C for 5 minute. Remove supernatant carefully with pipet, not touching the invisible pellet.
- Dry in the vacuum centrifuge (high heat, high spin) with the lights out for 5 minutes.
- Rehydrate with 20 ul H20.
- Send to Sequencing Facility.