SLIC CLONING MANUAL

Aleksei Lulla and Marko Hyvonen
Department of Biochemistry, University of Cambridge
https://hyvonen.bioc.cam.ac.uk
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SLIC VS RESTRICTION CLONING

Restriction cloning	SLI cloning	
PCR (2 h)	PCR (2 h)	
Gel analysis (0.5 h)	Gel Purification (1 h)	
PCR purification (0.5 h)	T4 DNA polymerase treatment (0.5 h)	
Restriction digest (2 h)		Day 1
Gel purification (1 h)		
Ligation (0.5 h)		
Transformation (0.5 h)	Transformation (0.5 h)	
Colony PCR (2 h)	Colony PCR (2 h)	Day 2
Gel analysis (0.5 h)	Gel analysis (0.5 h)	
Miniprep (1 h)	Miniprep (1 h)	Day 3
Send for sequencing	Send for sequencing	
Expression test	Expression test	Day 4

DESIGN OF OLIGONUCLEOTIDES FOR PCR WITH SLIC CLONING

Once you have decided on the exact construct to be cloned, you should design oligonucleotides for the PCR (some advice on construct design can be found in Appendix 1). In the most simplistic model, the oligos will contain SLIC overhang in the 5' end followed by sequence that anneals to the template DNA.

Below are several SLIC overhangs that work for the vectors we use regularly. These are typically 18-20nt in length and have melting temperature of 50°C or little bit over to ensure the fragments do not fall apart from the plasmids in heat shock transformation at 42°C. In the example oligos below the sequence is in frame with the intended open reading frame either at the 5' end (the linker includes the initiation ATG codon when needed) or at the 3' end (with a stop codon or in frame with C-terminal fusion tag).

One server for calculating oligo annealing temperature can be found here:
http://mcb.berkeley.edu/labs/krantz/tools/oligocalc.html

5' OVERHANGS

For un-tagged N-terminus (pOP1, pOP2, pBAT4: opened with *Nco*I. GOI start codon in green))

GAAGGAGATATAT**CCATG**

For mini-cistron (pOP1b, pExp-cis-Chis: opened with *Nco*I at 5' end. MInicistron stop codon in red)
TAAGGAGGAA**TAA**A**CCATG**

For N-terminal His_6-tag (pHAT2: opened with *Nco*I at 5' end)
CCATCACCATCACT**CCATG**

For TEV cleavage site (pExp vectors opened with *Bsa*I at 5' end)
GAAAACCTGTACTTCCAG
E N L Y F Q

For TEV cleavage site (pOP3/5 "T" vectors opened with *BamH*I at 5' end)
CTGTACTTCCAGGGATCC
L Y F Q G S

3' OVERHANGS
For C-terminal stop codon (pBAT, pOP, pExp vectors opened with *Hind*III at 3'end)
CACTATAGAATACTC**AAGCTTA**

For C-terminal 8xHis tag (pOP2H, pExp-cis-Chis opened with *Xho*I at 3'end)
GTGATGGTGATGAGGG**CTCGA**

For C-terminal Strep-tag (pOP2S, pExp vectors opened with *Xho*I at 3'end)
GGGTGGCTCCATGCG**CTCGA**

PCR

We use Phusion polymerase for all PCRs that are for amplifying an insert for cloning. In addition to being very fast enzyme, it is also one of the most accurate ones around and we very seldom see mutations in our constructs.

Phusion DNA polymerase can either be used with the High Fidelity (HF) buffer or the GC buffer (for GC-rich sequences), depending on the GC content of the template. DMSO can be added to the buffer to prevent the formation of DNA secondary structures and HF buffer + DMSO acts very similarly to GC buffer. Try HF buffer and HF buffer + DMSO first and see which one works better.

As template, prepare a 50 ng/ul stock of a plasmid or 10 ng/ml of liner DNA (from DNA synthesis).

Solubilise the PCR primers to 100 μM in TE.

If amplifying multiple samples at the same time, it is easier to make a master mix of all the components apart from template and the primers, add those into each tube separately and then add the master mix in one go. If doing this, it can be helpful to pipette the primers and template (small volume) into the side of the tube where you can see the small drops and then rinse the primers and template with the master mix.

Component	For one sample	Master mix for 6	Master mix for 10
5x HF buffer	10 μl	60 μl	100 μl
8 mM dNTP mix	5 μl	30 μl	50 μl
Forward primer	0.3 μl		
Reverse primer	0.3 μl		
Template DNA	0.3 μl		

DMSO (optional)	0 µl / 1 µl	0 µl / 6 µl	0 µl / 10 µl
Milli Q water	33.5 / 32.5 µl	207 µl /195 µl	335 µl / 325 µl
Phusion polymerase	0.5 µl	3 µl	5 µl

Phusion becomes active at 98°C and works preferably at 72°C; many "traditional" thermostable polymerases like Taq do not like temperatures above 95°C.

Here is a typical thermocycler protocol for a Phusion PCR with up to 1 kb fragment to amplify.

Stage	Temperature	Time	Number of cycles
Initial denaturation	98°C	1 min	1
Denaturation	98°C	10 s	
Annealing	59°C	15 s	30
Elongation	72°C	20 s	
Final elongation	72°C	5 mins	1
Hold	4°C	∞	Hold

In case you have for some reason very short primers or primers with mismatches, you can improve this by running a few cycles at lower annealing temperature and then increasing the annealing temperature for later cycles when there is enough of template with oligo sequences incorporated as these extend the sequence to which the primers can anneal to.

Stage	Temperature	Time	Number of cycles
Initial denaturation	98°C	1 min	1
Denaturation	98°C	10 s	
Annealing	50°C	15 s	5
Elongation	72°C	20 s	
Denaturation	98°C	10 s	
Annealing	59°C	15 s	30
Elongation	72°C	20 s	
Final elongation	72°C	5 mins	1
Final hold	4°C	∞	Hold

The extension time depends on the length of the PCR product and the speed of the polymerase. As a rule of thumb, for Phusion polymerase needs 15 second per kilobase.

After the PCR, if the template used was a plasmid, you can destroy that by digestion with *Dnp*I enzyme. Add 0.5 µl of *Dnp*I to the PCR sample and incubate for 30 min at 37°C. This assumes you have used an *E. coli* strain that methylates DNA as *Dnp*I digests only DNA that is methylated on cytosines. Your PCR product is not methylated and hence not digested.

Add 10 µl of 6x gel loading dye to each 50 µl PCR product and run it on a preparative agarose gel.

Cut the band from the gel and extract the DNA with a gel extraction kit following manufacturers instructions, with the exception that you should elute the DNA with Milli Q water instead of their elution buffer. Typical elution volume is 50 µl but if your PCR product was not well amplified, elute in smaller volume.

Sequence and Ligation Independent Cloning (SLIC)

SLIC cloning relies on generation of matching single-stranded DNA overhangs in the plasmid and the insert using T4 DNA polymerase. In the absence of dNTP, T4 DNA polymerase has 3'→5' exonuclease activity and eats away one of the strands of double stranded DNA from the free 3' end and creates a single-stranded overhang. If this treatment is only for a short time, the ends of a PCR product or digested vector will be only partially eaten away by T4 DNA polymerase. Once any one of the four dNTPs has been added to the reaction, the enzyme stops the 3'-5' reaction at the position where that nucleotide can be added to the 3' end of the DNA. The matching overhangs in vector and PCR product can be annealed by raising the temperature and allowing the matching DNA strands to find each other. This non-covalently held, nicked plasmid product will endure transformation and is repaired by *E. coli* DNA repair systems. The beauty of SLIC cloning is that you do not need to digest your insert with restriction enzymes and therefore you are more free in the way you design your constructs.

Preparation of the plasmid

While the PCR is running, it is time to prepare the plasmid DNA. Typically this is done by restriction digestion, but you could also amplify the plasmid with oligoes that are complementary to your SLIC overhangs in PCR primers.

Which enzymes to use for the digestion depends on how you designed your SLIC oligos as the overhang left after digestion must be complementary to the overlapping sequences in your insert. Typically that for us would be the very first and last restriction sites. While for ligation-based cloning it is advisable to use two different enzymes for the digest, to minimise vector self-ligation, in SLIC this is less of an issue as the overhangs generated by restriction enzymes are not stable and will not anneal sufficiently well.

For a restriction digest (which can be used for some 10 constructs), prepare the following:

Component	Volume
10x buffer	5 μl
Plasmid DNA (100 ng/μl	40 μl
Milli Q water	3 μl
Enzyme 1	1 μl
Enzyme 2	1 μl

Incubate the digest at 37°C for 1-2 hours (or rather, until the PCR has finished).

Add 10 ul of 6x DNA loading dye and load all of the sample on a preparative well in an agarose gel.

Cut the plasmid band from the gel and purify by gel extraction kit, elute the DNA with 50 ul of Millli Q water and store frozen for future clonings.

Overhang generation and plasmid assembly

Set either a PCR machine or a heat block to 65°C.

In a PCR or Eppendorf tube, mix:

4 μl of digested vector
5 μl of PCR insert
1 μl of NEB buffer 2.1
0.2 μl of T4 DNA polymerase

Incubate 10 min at room temperature (times from 1 min – 30 mins are reported in literature) Terminate the 3'->5' exonuclease activity by adding any single dNTP to a final concentration of 1-3mM. For example, for a 10 μl reaction, add 0.5 μl of 20 mM dGTP.

Incubate further 1 min on the bench followed by 5 min at 65°C to inactivate the T4 DNA polymerase.

Let the sample cool down to room temperature to allow for the vector and insert to anneal together.

TRANSFORMATION

Thaw the competent *E. coli* cells (50 μl) on ice for 10 min. For making constructs for *E. coli* expression, you cab use T7 Express cells which are highly competent and which support expression test without further transformation.

Take the LB plates with appropriate antibiotics from the fridge to warm them to room temperature.

Add all of the SLIC mix into the competent cells.

Incubate 10 min in ice.

Heat shock for 45 sec at 42°C.

Incubate 10 min in ice.

(Optional: add 50 ul LB and incubate 30 min on the shaker at 37°C)

Plate all the 100 ul culture and grow overnight at 37°C.

In the following morning, take the plates from the incubator. Put the plate(s) in the fridge if not analysed immediately.

SCREENING OF CORRECT CLONES BY PCR

3-10 colonies from the transformation are analysed by colony PCR using primers that anneal in the plasmid, outside the cloning site. Below are a few that are useful for our pBAT, pOP and pEXP series of plasmids.

pOP_up (annealing at T7 promoter, adds 81 nt before ATG)
`TACGACTCACTATAGGGAATTGTGAGC(27 nt, Tm=58°C)`

pOP_dn (annealing before T7 terminator, adds 48 nt to the product after stop codon)
`GCAGCCAACTCAGCTTCCTTTCG (23 nt,Tm=59°C)`

TEV_up (for pExp fusion vectors, adds 24 nt to the product)
`GGTACCGAAAACCTGTACTTCCAG (24 nt, Tm=57°C)`

lacO (for all T7lac promoter vectors, anneals on lac operator, adds ca. 60 nt to the product)
`ATTGTGAGCGGATAACAATTCC (22nt, Tm=51°C)`

lacO2 (for all T7lac promoter vectors, anneals on lac operator, adds ca. 60 nt to the product)
`GGGAATTGTGAGCGGATAACAATTCC (26 nt, Tm=58°C)`

We use a ready-made PCR solution (Bioline Biomix Red) that contains all the common components for PCR, including nucleotides and enzyme. There is even glycerol and gel dye to allow the samples to be loaded directly on agarose gel.

Prepared a master mix with the following components:

Component	**For 1 sample**	**For 5 samples**	**For 10 samples**
Biomix Red	5 μl	25 μl	50 μl
Forward primer	0.05 μl	0.25 μl	0.5 μl
Reverse primer	0.05 μl	0.25 μl	0.5 μl
MilliQ water	4.9 μl	24.5 μl	49 μl

Aliquot the master mix to PCR tubes with 10 μl per tube.

Mark as many well separated, evenly sized colonies you want to test on the original transformation plate. Mark positions on a separate replicate plate for the colonies. (Optional: label also sterile 1.5 ml Eppendorf tubes with the same labels and add 1 ml of LB media into each tube.

Using a sterile 10 μl tip, touch a marked colony to pick cells. Just touch, no more.

Touch the labelled position for that colony on the replica plate. Just touch, no more.

Rinse the tip inside a PCR tube that contains colony PCR master mix. Gentle swirl against the inside wall of the tube will do well or pipette up and down a couple of times.

(Optional: eject the tip into a sterile 1.5 ml Eppendorf tube with 1 ml of LB media and place the tubes in 37°C incubator until you get results from the colony PCR.)

Ideally, include a negative control of cells with a plasmid without an insert or a very different sized insert to ensure you can recognise a positive hit from a possible fragment arising from an empty plasmid you would amplify just as well. If testing different sized constructs in parallel, they act as controls (or, rather, comparators) for each other.

Run the PCR with following protocol and put the replicate bacterial plate in 37°C incubator.

(note: this uses Taq DNA polymerase, so the denaturation temperature is lower than with Phusion).

Stage	Temperature	Time	Number of cycles
Initial denaturation	95°C	1 min	1
Denaturation	95°C	15 s	
Annealing	51°C	15 s	25 cycles
Elongation	72°C	30 s	
Final elongation	72°C	5 min	1 cycle
Hold	4°C	hold	hold

Load all of the colony PCR sample onto agarose gel, alongside a suitable molecular weight marker and a control sample, if used.

(The speedy protocol: For 1 or 2 positive clones from each construct, divide the sample in 1.5 ml Eppendorf into two. In one 14 ml snap-cap tube add 2 ml of LB with antibiotics and add 100 μl of the media into this for overnight growth for miniprep. Add the remaining LB into another 14 ml snap-cap tube and add LB with 2x antibiotics to 1.8 ml final volume. Grow this until turbid and test for expression.)

Minipreps

By late afternoon (assuming you started the colony PCR first thing in the morning), the replica plate will have clearly grown bacterial "colonies". Start overnight miniprep cultures from these colonies in 2ml of LB media with appropriate antibiotics.

In the following morning, prepare the minipreps using kit manufacturer's instructions, with the only exception that you should elute the plasmid DNA using 100 μl of MilliQ (assuming you are using very high copy number plasmids, like our pBAT/pOP/pEXP vectors). This will give you, give or take, 100 ng/ul of DNA and you can safely send this for sequencing without checking the concentration ('cause life is too short).

With pBAT/pOP/pEXP plasmids, if the insert is less than 600-700bp, do only reverse sequencing with T7T(erminator) primer. Longer than that, do forward sequencing as well with appropriate primer.

Checking the sequences

Given that we are working with expression constructs, (almost) the only thing I am interested in is the sequence of the expressed protein. Therefore for the most of the time I only want to know that the protein sequence translated from the vector sequence is correct. That means automatically translating the DNA sequence in all three forward and all three reverse sequences and comparing these to expected target protein sequence. What you need to pay particular attention to, apart from making sure you have the complete sequence in a single frame (ie without frame shifts), are:

- Is my sequence in frame with any possible N-terminal fusion?
    - Is the initiation methionine there?
- Is my sequence in frame with any C-terminal elements?
    - Is there are stop codon as expected?
    - Is the C-terminal tag in the same frame with my target protein?
    - Are possible mutations all there. Always compare to wild type protein so the mutations are immediately immediate in the alignment.

Assuming all is ok, use the protein sequence from the sequencing data to generate a protein sequence file of this expression construct, with tags and all, so as to avoid making mistakes compiling it from your design. Missing for example one glycine from a linker will make the calculated mass of your protein 57 Da too small and that will throw a mass spectrometric analysis of the purified protein completely. Been there, done that - rather you did not repeat it.