

Cloning Heterologous Constructs (TK-LUC)

Purpose: To test a regulatory element upstream of a heterologous promoter to see if it is functional out of its endogenous promoter.

1. Design an oligo (20-60bp in length) spanning the region of interest with approximately 10 bases on either side. Add BamHI overhangs on both strands by adding GATCC to each 5 prime end and a G on each 3 prime end. When ordering the oligos, specify that you would like them kinased otherwise you can kinase them after you anneal them (the less expensive option).

5' -GATCCgtcatcatgcatctaG
 GcagtagtacgtagatCCTAG-5'

2. Anneal 5ug of each oligo by putting them together in a total volume of 75ul TE buffer. Boil for 2 minutes in a heat block and allow the heat block to return to room temp slowly by pulling the heat block out of the base. If you still need to kinase the oligo:

To kinase: 25 uL total volume

1 uL annealed primer
 2.5 uL ATP (10 mM)
 5 uL 5X fwd rxn buffer
 1 uL T4 kinase (10 U)
 15.5 uL H₂O
 10 min at 37 C. Heat inactivate 10' at 65 C.

3. Run an aliquot (add loading dye to 5 ul of sample) of the annealed primer on a non-denaturing, non-SDS acrylamide gel to make sure that there is one tight band and not any contaminants. **USE HALF OF THE VOLUMES BELOW FOR A CRITERION SIZED GEL.**

	20% Acryl Gel	12% Acryl Gel	5% acryl Gel
Oligo size	2-100	50-200	100-500
Bromphenol Blue	12bp	20bp	65bp
Acryl/Bis (30%/0.8%)	26.7mls	16mls	6.6mls
10x TBE	4mls	4mls	4mls
Water	9.4mls	20.1mls	29.1mls
10% APS	250ul	250ul	250ul
TEMED	25ul	25ul	25ul

4. In the meantime, prepare the TK-LUC heterologous construct for insertion of your oligos. Digest 5ug of TK-LUC with BamHI in a 20ul volume. After the digest, heat inactivate the enzyme at 70C for 15 minutes. Then, to 8ul of the digest, add 1ul SAP buffer and 1ul of SAP (shrimp alkaline phosphatase) in order to phosphatase the vector. Incubate 37C for 10-15 minutes. Run SAP'd and UNSAP'd digested TKLUC on a gel and gel purify (see separate protocol).
5. After purification, estimate the yield by running 1ul of SAP'd and UNSAP'd on a gel with 5ul DNA LADDER. Compare this estimated concentration to the concentration of your oligos (10ug/75ul) in order to decide how much to use for the ligation reactions. See below.

1) Control for uncut vector in your vector prep	2) Ligation control
Bam cut Vector 20-50ng 1ul	Bam cut Vector 20-50ng 1ul
10x Ligase buffer 1ul	10x Ligase buffer 1ul
H2O 8ul	H2O 7ul
	High Conc Ligase 1ul
3) Control for uncut vector in your SAP'd prep	4) Control for SAP
Vector SAP'd 20-50ng 1ul	Vector SAP'd 20-50ng 1ul
10x Ligase buffer 1ul	10x Ligase buffer 1ul
H2O 8ul	H2O 7ul
	High Conc Ligase 1ul
5) Actual Ligation 1:10 (vector:insert)	6) Actual Ligation 1:100 (vector:insert)
Vector SAP'd (20-50ng) 1ul	Vector SAP'd (20-50ng) 1ul
insert (10x molar) 2.5ul	insert (100x molar) 5ul
10x Ligase buffer 1ul	10x Ligase buffer 1ul
H2O 4.5ul	H2O 2ul
High conc Ligase 1ul	High conc Ligase 1ul

Note: Approximately, use 1ul straight of the SAP'd insert to 50ng of TK-vector for #5 and 1ul of a 1:10 dilution for #6.

Calculate moles from ng:

$\text{Ng}/(315)(\# \text{ of bp})(2)=\text{nmoles} \times 1000=\text{pmol}$

Example: Vector is 20ng/ul and 4kb

$20/(315)(2)(4000)= 0.0000079\text{nmol/ul}$ or 0.0079pmol/ul

For 50x molar, need 0.397pmoles of oligo for ligation for every 20ng of vector.

NOTE: For all reactions, the amount of vector and insert should be kept constant so that you can directly compare the number of colonies per plate at the end.

6. Allow ligation reactions to incubate at 11-14°C overnight in the cold room water bath.
7. Using 5ul of the ligation reaction (freeze other 5ul) perform the transformation (see separate protocol). For large plasmids, over 10kb, use XL10 Gold cells (Stratagene) (don't forget to add 2ul of BME to 50ul cells). Otherwise, GC5 (PGC Scientific) cells, similar to DH5 alpha, are ok to use. Plate 150ul of transformants onto 10cm plate with appropriate antibiotics (ampicillin for TK-Luc constructs).
8. Incubate plates overnight at 37°C and assess growth.

Interpreting results:

Expected results:

Reaction #1) No colonies

Reaction #2) Lots of colonies

Reaction #3) No colonies

Reaction #4) No colonies

Reaction #5) Lots of colonies

Reaction #6) Lots of colonies

Notes:

- If you have colonies on plate #1, then you have incomplete digestion. If this is the case, reisolate the vector with complete cutting.
- If you do not have colonies on 1 or 2, then the ligase isn't working.
- If 4 has more colonies than 3, then the SAP did not work.
- If 1 has no colonies and 2 has colonies and 4 does not have colonies, then your phosphatase worked!
- If you have more colonies on 5 or 6 then either 3 or 4 then your ligation probably worked and you should pick colonies.
- If you have few colonies on #5 and #6 that are mostly positive for low copy number plasmids, this represents a problem with the kinase reaction allowing the non-kinased oligos to compete resulting in few colonies.

9. If results are as expected, pick colonies and inoculate first into 100ul water in a 1.5ml tube and then, with the same tip, into 3-5mls of LB with Ampicillin using a 14ml polypropylene tube with snap cap. Grow broth culture overnight.
10. For the inoculated water, boil for 20 minutes and then spin in microfuge at high speed for 5 minutes. Use 10ul of this sample for a PCR reaction to confirm your inserts. As a negative control, use 100ng of TK-luc backbone as a template (uncut).

PCR Program:

5 min 94 }
 4 min 55 } X 1
 4 min 72 }

1 min 94 }
 2min 55 } X 25
 4min 72 }

4C soak

TK Luc primers:

SENSE 5'-GTG AGC GGA TAA CAA TTT-3'

ANTISENSE 5'-TTA ATA TGC GAA GTG GAC-3'

11. Run on 5% acrylamide gel, as in #3 above (100 volts, approx 2 hours, until slow dye is 1cm from the bottom of the gel). Your TK-Luc negative control will show you the size of the amplicon with no insert (approx 190pb). Because of the high percentage of this gel, you should be able to differentiate between 1, 2 and more copies of the oligo.
12. Make glycerol stocks (700ul of culture + 300ul sterilized glycerol, vortex, freeze) from the overnight preps that you grew for any clones that have an insert.
13. Do minipreps (see separate protocol) on the positive clones and sequence (see separate protocol) a few at a time to check for correct orientation. Finding one in the sense and antisense direction would be useful. If the element is functional even in the antisense orientation, it can be categorized as an enhancer element.
14. Make a Map of the constructs that you have made and add it to the plasmid binder as well as the frozen storage list.