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We use this protocol and it's working

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Abstract

This protocol details the molecular analyses of assembly of a serine integrase-based platform for functional validation of genetic switch controllers in eukaryotic cells

Attachments



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83KB

Primer design ● Timing 1d

- 1 Select approximately 20 nucleotides both upstream and downstream of the core region of each att site formed in the reporter plasmid after recombination takes place.



Note

▲ CRITICAL STEP forward primer must anneal to attL, while the reverse primer will anneal to the attR sequence.

- 2 Use an online oligo design tool to define the best forward primers annealing to promoter sequence and reverse primers annealing to terminator sequence present in the reporter plasmid.

- 3 Define oligo pairs to obtain two amplicons for each reporter plasmid.



Note

▲ CRITICAL STEP: Primer pairs must consist of a forward oligo annealing to the attL site and a reverse oligo annealing to the terminator region for sequencing proper attR site formation in amplicon I and a forward oligo annealing to the promoter region and a reverse oligo annealing to the attR site for sequencing proper attL site formation in amplicon II. (Figure 5).

The primers used in our studies are presented in **Table 7**.

A	B	C	D
Oligonucleotides used for amplification of Amplicon I and sequencing of attL sites			
Promoter	Forward primer (5' → 3')	nt	Model
EFa_966F	TTCTCGAGCTTTT GGAGTACGTCGTC TTTAGGTTG	35	Mammal
35S_282F	ATTGATGTGATATC TCCACTGACGTAA GGGATGACGCAC	39	Plant

A	B	C	D
attR	Reverse primer (5'→ 3')	nt	Model
attR_Int2_R	GTGTCTACGCGAG ATTCTCGCCGGAC CGTCGACATACTG C	40	All models used
attR_Int4_R	AGTTTTCAACCCT TGATTTGAATAAG ACTGCTGCTTGTG T	40	
attR_Int5_R	ATAACTCTCCTGG GAGCGCTACACG CTGTGGCTG	34	
attR_Int7_R	CTGTGTGAGAGTT AAGTTTACATGGG CAAAGTTGATGAC	39	
attR_Int9_R	TGGAAGTGTGTAT CAGGTAAGTGGAT ACCTCATC	34	
attR_Int13_R	GTAGAACTTGACC AGTTGGTCCTGTA AATATAAGCAATC C	40	
attR_phiC_R	CCAACTGGGGTA ACCTTTGGGCTC C	25	
attR_Bxb1_R	CTGGTCAACCAC CGCGGTCTCCGT CGTCAGGATC	34	
Oligonucleotides used for amplification of Amplicon II and sequencing of attR sites			



A	B	C	D
attL	Forward primer (5' → 3')	nt	Model
attL_Int2_F	GGAGTAGCTCTTC GCCCGAGAACTT CTGCAAG	32	All models used
attL_Int4_F	CGACCTGAAATTT GAATTAGCGGTCA AATAATTTGTA	37	
attL_Int5_F	GACGGCCTGGGA GCGTTGACAACTT GCGCACC	32	
attL_Int7_F	GTCCGTCTGGGTC AGTTGCCTAACCT TAACTTTTAC	36	
attL_Int9_F	ATAATTGGCGAAC GAGGTATCTGCAT AGTTATTCCGAAC	39	
attL_Int13_F	TCCAGATCCAGTT GTTTTAGTAACAT AAATACA	33	
attL_phiC_F	TGCCAGGGCGTG CCCTTGAGTTCTC TCAGT	30	
attL_Bxb1_F	TGTCGACGACGG CGGTCTCAGTGGT GTACGGT	32	
Terminator	Reverse primer (5' → 3')	nt	Model
TermiAni_205R	AATGATTTGCCCT CCCATATGTCCTT	33	Mammal

A	B	C	D
	CCGAGTG		
NOS _t _283R	ATAACAATTTTCAC ACAGGAAACAGC TATGACATGATTAC G	40	Plant

Target sequence amplification by PCR ● Timing 5h

- 4
- Use a high-fidelity polymerase with non-template-dependent terminal transferase activity to insert a deoxyadenosine and the ends of generated amplicons.



Note

▲ CRITICAL STEP Amplicon modification is important for cloning into pGEM-t-Easy to be sequenced.

- 5
- Prepare a PCR mix for all reactions plus one (n+1) to account for pipetting errors. Include a negative control with water instead of DNA; positive control will require a previous synthesis of the expected recombined reporter plasmid.
- 6
- Combine the reagents in the order shown below in **Table 8**, mix well by vortexing and spin briefly:



TABLE 8. PCR reaction mix components

A	B
Component	Volume to add (μl)
dH ₂ O nuclease free	18.65
Buffer 10x	2.5
MgCl ₂ [50 mM]	1.5
PCR Fw primer [10 μM]	0.75

	A	B
	PCR Rev primer [10 µM]	0.75
	dNTP [10 mM]	0.75
	Taq DNA polymerase	0.1

7 Add  24.5 µL of the PCR mix to 0.2 mL PCR tubes.. 

8 To each respective tube, add  20 ng of template DNA and adjust the final volume to 25 µl if the DNA is too concentrated. 

Note

Negative controls were prepared first by adding an equivalent volume of nuclease-free water and closing lids before pipetting templates to minimize contamination risk.

9 Gently pipette each sample up and down ten times to mix thoroughly. Place the PCR microtubes into a thermal cycler, and run the following program listed in **Table 9** (volume = 25 µL).   

TABLE 9. PCR cycling condition

	A	B	C	D
	Cycle no.	Denature	Anneal	Extend
	1	94°C, 3min		
	2-34	94°C, 30s	65°C, 30s	72°C, 60s
	35			72°C, 5min

**Note**

▲ **CRITICAL STEP** Given the need for primers to align to a defined att site sequence, some parameter adjustments, such as T_m, GC content and 3' end base composition, will be limited and can vary from one integrase reporter to another, requiring adjustments to PCR cycling conditions.

- 10 Resolve amplicons by electrophoresis in agarose gel following PCR. Run settings and gel density will depend on amplicon size according to the analyzed gene length and oligo pairs used.

**Note**

▲ **CRITICAL STEP** Load the same negative control in the every gel both technical (PCR without DNA) and biological (PCR using DNA from groups transformed with only either reporter plasmid or integrase plasmid) to ensure obtained bands indicate DNA inversion by Integrase activity. ? TROUBLESHOOTING

Amplicon purification ● Timing 2d

- 11 Excise the amplicon bands by cutting a square around them with the help of a scalpel on a UV light or blue light transilluminator.

**Note**

▲ **CRITICAL STEP** Use different scalpel blades for each band to avoid cross-contamination of samples. ! **CAUTION** UV light can damage DNA, nicking and possibly removing DNA strand ends and interfering with downstream cloning steps. When available, blue light is highly recommended. If using UV, proceed quickly, turning the transilluminator off after making the cuts in the gel.

- 12 Proceed with amplicon purification using commercial DNA Clean-Up and Concentration kits, following the manufacturer's recommendations.

- 13 Clone purified amplicons in an entry vector to ensure high-quality sequencing results. Although specifics may vary depending on the plasmid, we recommend a molar ratio of 1:3 (vector to amplicon) and 1.5 U of T4 ligase in 5 µl reactions with  Overnight incubation at  16 °C .



- 14 DH10b chemically competent cells were transformed with ligation products.

Heatshock transformation of DH10b chemically competent cell ● Timing 3d 2h 32m 45s

- 15 Add  5 μL of the ligation reaction to  200 μL of cells thawed  On ice . 
- 16 Incubate cells  On ice for  00:30:00 . 

- 17 Subject cells to heat shock at  42 $^{\circ}\text{C}$ for  00:00:45 and return to ice for  00:02:00 . 
- 18 Add  1 mL of LB or SOC medium. 
- 19 Incubate at  37 $^{\circ}\text{C}$ for  01:00:00 and then plate different dilutions on LB plates with appropriate selecting agents. Incubate  Overnight at  37 $^{\circ}\text{C}$. 

- 20 Screening for positive transformants by colony PCR. A polymerase with less fidelity can be used in this step. Combine the reagents in the order listed in **Table 10** below, mix well by vortexing and spin briefly: 


TABLE 10. PCR reaction mix components for colony screening.

	A	B
	Component	Volume to add (μL)
	dH2O nuclease free	18.65
	Buffer 10x	2.5
	MgCl2 [50 mM]	1.5
	PCR Fw primer [10 μM]	0.75
	PCR Rev primer [10 μM]	0.75

	A	B
	dNTP [10 mM]	0.75
	Taq DNA polymerase	0.1

21 Add  25 µL of the PCR mix to 0.2 mL PCR tubes.



22 With a sterile toothpick or 200 µl pipetting tip, pick approximately 1/3 of each colony and add it to their respective tubes containing the PCR mix.

23 Gently pipette each sample up and down ten times to mix thoroughly. Place the PCR microtubes into a thermal cycler, and run the following program listed in **Table 11** (volume = 25 µL)



TABLE 11. PCR cycling condition for colony screening

	Cycle no.	Denature	Anneal	Extend
	1	94°C, 10min		
	2-34	94°C, 30s	60°C, 30s	72°C, 90s
	35			72°C, 5min

24 Resolve amplicons by electrophoresis in agarose gel following PCR.

25 Select multiple confirmed clones to isolate plasmids using commercial kits following the manufacturer's recommendations and have the purified plasmids sequenced.



Note

▲ **CRITICAL STEP** Have your samples sequenced in both directions and in replicates to check for sequencing errors and identify possible mutations and DNA damage resulting from integrase activity.

26 Analyze sequencing electropherograms and alignment to expected sequences to confirm proper DNA recombination by Integrase activity.

Note

Troubleshooting

TABLE 12. Troubleshooting for the molecular analyses stage

A	B	C	D
Step	Problem	Possible reason	Solution
10	Unspecific amplification and unexpected bands on agarose gel	Oligos annealing at att sites has a 3' end complementarity to both original and recombined att sites	Increase the annealing temperature to more sselective conditions