Protocol for cloning MHC ligand minigenes into PresentER vector

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Reagents:

- Phusion High Fidelity Polymerase (NEB)
- SfiI restriction enzyme 20,000 units/ml (NEB)
- Calf Intestinal Phosphatase 10,000 units/ml (NEB)
- T4 DNA Ligase 400,000 units/ml (NEB)
- Primers:
  - T7_SfiI: CGACTCACTATAGGGCCGTATTGGCC
  - T3_SfiI: AGTGATTTCCGGCCTGTTTGGCC
- Qiagen MinElute Kit (to PCR purify amplicons < 100nt)
- Gel purification kit (Qiagen or other)
- PresentER Cassette (GFP or mCherry version)

Oligonucleotide design:

Order standard desalted oligonucleotide(s) with the following design:
GGCCGTATT**GGCC**CCGCCACCTGTGAGCGGG + Peptide DNA + TAA**GGCC**AAACA**GGCC**

For example:

RMFPNAPYL:

GGCCGTATTGGCCCCGCCACCTGTGAGCGGGAGGATGTTTCCTAACGCGCCCTACCTGTAAGGCCAAACAGGCC

ALYVDSLFFL:

GGCCGTATTGGCCCCGCCACCTGTGAGCGGGGCTCTCTATGTGGACTCTTTATTTTCCTTTAAGGCCAAACAGGCC

1. Resuspend oligonucleotides at 100µM stock.
2. Make 1:10,000 dilutions of the oligo (~1.5 ng/ul):
3. Amplify oligos in 50µl reactions:
   10 µl Phusion HF Buffer
   1 µl dNTPs (freshly thawed from an aliquot)
   2.5 µl of 10µM T7_SfiI primer
   2.5 µl of 10µM T3_SfiI primer
   0.5 µl Phusion DNA polymerase
   32.5 µl water
   1 µl oligo DNA

   33 cycles w/ 72ºC annealing and run 5µl out on a 2.2% gel to check for amplification.
   (10s denaturation; 30s annealing; 30s extension; 5 minute final extension)
   Amplicon is ~100nt
4. PCR purify with MinElute kit. Elute in 10-20µl EB.
5. Digest amplicon and PresentER Cassette with SfiI at 50ºC for 2h - o/n
6.

Amplicon restriction digest	**PresentER Cassette (Vector) restriction digest**
8µl amplicon DNA 2µl cutsmart 1µl SfiI 9µl water --- 20µl total reaction volume Check amplicon digest on a 2.2% gel. Digest should result in a faint band at ~70nt (for a 8-9aa MHC-I ligand) and elimination or reduction in the ~100nt amplicon band. It helps to use ethidium bromide gels because it is more sensitive than SYBR safe. Purify digested amplicon with MinElute kit and elute in 10-20µl of water. Technically some digestion products will be smaller than the lower limit of the MinElute kit, but in practice this hasn't been a problem.	5µg PresentER Cassette Plasmid DNA 5µl Cutsmart 1µl SfiI X µl water --- 50µl total reaction volume Digest 20-50µg of DNA total in reactions of 5µg (50µl) each. Check digest on a gel to verify it has gone to completion. If digest is incomplete, add 1µl SfiI + 1µl cutsmart + 8µl water to each reaction and digest for another hour. Add 1µl of Calf intestinal phosphatase to each reaction and incubate 1h at 37ºC Gel purify digested vector.

6. Ligate digested+purified amplicon and digested+CIP treated+gel purified vector at 16ºC overnight
   x µl MinEluted insert
   X µl backbone
   2µl 10x T4 buffer
   1µl T4 ligase (400 units)
   X µl water

   20µl total volume

   5 moles Insert : 1 mole Vector

9. Transform 10µl of NEB STABLE cells with 2µl of ligation product, pick colonies; miniprep;
10. Sanger sequence with custom primer CATCAGTCCGGGTCACCTAC or universal primer GAG (GTTCGACCCCGCCTCGATCC)