JOUNG LAB gRNA CLONING PROTOCOL

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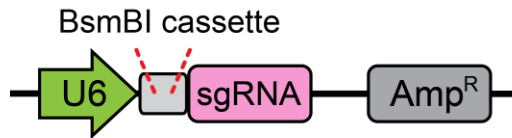
MASSACHUSETTS GENERAL HOSPITAL

HARVARD MEDICAL SCHOOL

For cloning the spacer (target site) sequence onto the 5' end of single guide RNAs (sgRNAs) for human and bacterial expression vectors:

1) Human sgRNA expression vectors (U6 promoter)



Plasmid	Addgene ID	Description	Components	Cut with	spacer
BPK1520 & MLM3636*	65777 & 43860	*S.pyogenes* sgRNA cloning	U6-BsmBIcassette-Sp.sgRNA	BsmBI	17-20
BPK2301	65778	*S.thermophilus1* sgRNA cloning	U6-BsmBIcassette-St1.sgRNA	BsmBI	20-23
BPK2660 & VVT1**	70709 & 65779	*S.aureus* sgRNA cloning	U6-BsmBIcassette-Sa.sgRNA	BsmBI	21-23

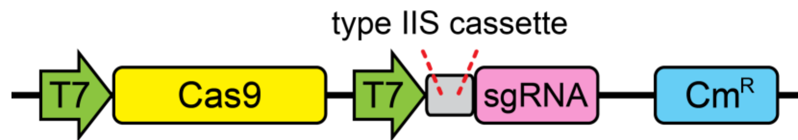
Ordering oligonucleotides to generate the spacer (target) sequence for human sgRNA vectors:

For BPK1520, BPK2301, BPK2660, & VVT1: *For MLM3636:

* The N_{20} sequence can be of variable length, as indicated in the table above

** VVT1 contains the full crRNA/tracrRNA sequence, whereas BPK2660 is a truncated and more active sgRNA

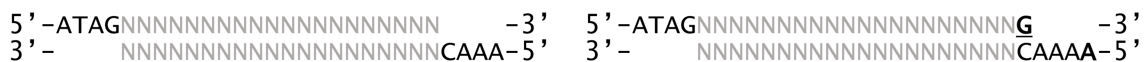
2) Bacterial sgRNA/Cas9 expression vectors (T7 promoter)



Plasmid	Addgene ID	Description	Components	Cut vector with
BPK764	65767	*S.pyogenes* Cas9/sgRNA cloning vector	T7-SpCas9-T7-BsaI-Sp.sgRNA	BsaI
MSP712*	65768	*S.pyogenes* **d**Cas9/sgRNA cloning vector	T7-Sp**d**Cas9-T7-BsaI-Sp.sgRNA	BsaI
MSP1673	65769	*S.thermophilus1* Cas9/sgRNA cloning vector	T7-St1Cas9-T7-BspMI-St1.sgRNA	BspMI
BPK2101	65770	*S.aureus* Cas9/sgRNA cloning vector	T7-SaCas9-T7-BsaI-Sa.sgRNA	BsaI

Ordering oligonucleotides to generate the spacer (target) sequence for bacterial sgRNA vectors:

For BPK764, MSP1673, and BPK2101: *For MSP712:



Cloning steps (*see following page for protocol***):**

1) **Order oligos** with indicated overhangs (*note*: ***1.*** *N_{20} corresponds to your target of interest for the top strand oligo, use the reverse complement N_{20} for the bottom strand oligo.* ***2.*** *The N_{20} sequence can be extended or truncated, should different spacer lengths be desired.* ***3.*** *Please note the overhang differences for MLM3636 & MSP712 vs. the other vectors*)
2) **Anneal** and **phosphorylate (optional)** the top & bottom strand oligos for a given spacer pair
3) **Digest** and **dephosphorylate (optional)** the vector backbone, into which annealed oligos will be cloned (*note: different IIS enzymes depending on vector of choice*)
4) **Ligate** the phosphorylated oligo duplexes into the corresponding cut backbone
5) **Transform** the ligation product into *E.coli* (*note: human sgRNA vector cloning should be plated on ampicillin or carbenicillin, while bacterial sgRNA cloning should be plated on chloramphenicol*)
6) **Prep** and **sequence** the resulting clones

PARTNERS HealthCare System Member

Cloning protocol:

1) **Suspend oligos**: to 100 μM in 0.1xTE buffer, dilute 1:10 to 10 μM in H_2O

2) **Generate phosphorylated (optional) oligo duplex**:

   1 μL top strand oligo (10 μM)
   1 μL bottom strand oligo (10 μM)
   2 μL 10x T4 DNA Ligase buffer (NEB)
   0.5 μL T4 PNK (NEB) **(optional)**
   16 or 15.5 μL H_2O
   20 μL reaction volume

   Reaction conditions
   - Incubate at 37°C for 60 minutes (only if phosphorylating)
   - Heat to 95°C for 5 minutes
   - Cool to 10°C at -5°C/minute

3) **Digest & dephosphorylate (optional) the vector backbone**:

   x μL plasmid (2-3 μg plasmid DNA)
   4 μL 10x buffer (corresponding to enzyme)
   y μL restriction enzyme
   0.5 μL CIP (NEB) **(optional)**
   z μL H_2O (to sum to 40)
   40 μL reaction volume

   Reaction conditions
   - 37°C or 55°C for 4-16 hours

Enzyme	Buffer	Temp
BsmBI	NEB 3.1	55°C
BsaI-HF	Cutsmart	37°C
BspMI	NEB 3.1	37°C

   - Isolate cut plasmid

4) **Ligation**:

   x μL digested plasmid from step 3 (10-40 ng)
   1 μL of oligo duplex from step 2 (0.5 μM)
   1 μL 10x T4 DNA ligase buffer (NEB)
   0.5 μL T4 DNA ligase (NEB)
   y μL H_2O
   10 μL reaction volume

   Reaction conditions
   - Incubate at 24°C for 10 minutes
   - Chill on ice prior to transformation

5) **Transform the ligation into *E.coli* and plate on appropriate selective media**:
   - **ampicillin** or **carbenicillin** for human sgRNA expression vectors
     - ***note***: *high copy number vectors*
   - **chloramphenicol** for bacterial Cas9/sgRNA expression vectors
     - ***note***: *low copy number vectors – appropriate liquid media recommended when starting O/N cultures*

6) **Sequence prepped plasmids:**
   - For human sgRNA vectors, use a primer 5' of the U6 promoter in the pUC19 backbone
     - For example, the primer oS280: 5'-CAGGGTTATTGTCTCATGAGCGG-3'
   - For bacterial Cas9/sgRNA expression vectors, use a reverse primer 3' of the sgRNA
     - For example, the T7 terminator primer: 5'-GCTAGTTATTGCTCAGCGG-3'