pAM_dCas9 Vector

Catalog No.: 53122
Format: 10 µg

Description

The pAM_dCas9 Vector is designed for use with Active Motif's enChIP Kit (Catalog No. 53125). The enChIP method relies on the CRISPR/Cas9 system to direct a guide RNA (gRNA) to a specific genomic locus for immunoprecipitation. The gRNA is designed as the complementary sequence of the desired target locus. An enzymatically inactive form of the *Streptococcus pyogenes* Cas9 protein, which contains Active Motif's unique AM-tag sequence, is co-expressed with the gRNA. The gRNA directs the dCas9 protein to its target sequence, immediately upstream of a Protospacer Adjacent Motif (PAM) (5´- NGG). Recognition of a PAM site leads to unwinding of the DNA and formation of an RNA-DNA heteroduplex. Cells are then formaldehyde fixed and chromatin is prepared. An antibody directed against the AM-tag is used to enrich for genomic sequences bound by the gRNA/dCas9 complex. DNA can be analyzed by qPCR or NGS to identify the enriched genomic regions. The pAM_dCas9 Vector can be used for cloning of the gRNA sequence for single vector transfections, or it can be used in combination with Active Motif's pAM_gRNA Vector (Catalog No. 53121) or any other suitable gRNA vector for dual transfections.

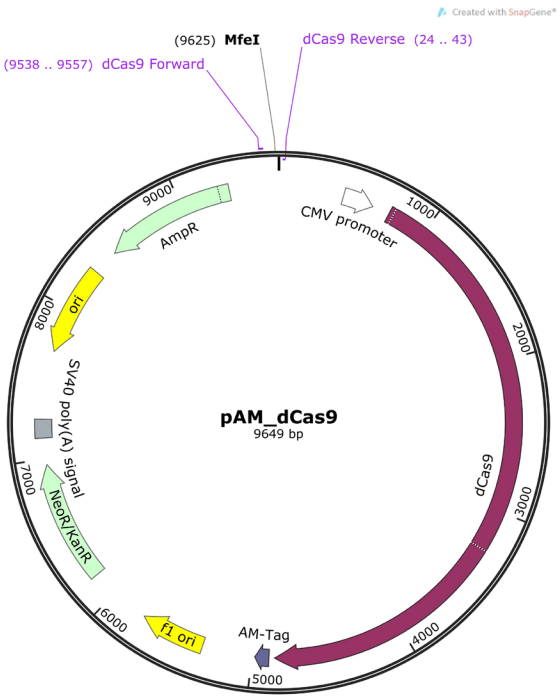
Contents

- 10 µg of pAM_dCas9 Vector provided at a concentration of 100 ng/µl.
- 250 pmol dCas9 Forward primer for DNA sequencing provided a concentration of 10 µM.
- 250 pmol dCas9 Reverse primer for DNA sequencing provided a concentration of 10 µM.

pAM_dCas9 Vector Features and Circle Map

The following features are present in the pAM_dCas9 Vector based on nucleotide sequence.

dCas9 reverse primer	24-43
CMV promoter	430-633
dCas9 coding region	748-4851
AM-Tag	4888-4980
Herpes simplex virus TK poly (A) signal	5079-5127
f1 origin of replication	5329-5757
Neomycin^R/Kanamycin^R coding region	6167-6961
SV40 polyadenylation signal	7137-7258
*Col*E1-derived plasmid origin of replication	7709-8297
β-lactamase (Amp^r) coding region	8468-9328
dCas9 forward primer	9538-9557
Universal cloning site (MfeI)	9625



Note: Using the gRNA insert template, a gRNA can be cloned into pAM_dCas9 at the MfeI site for single vector transfection. Alternatively, the pAM_dCas9 vector can be used with pAM_gRNA for dual vector transfection, or used alone as a no gRNA negative control.

Quality Control

Plasmid construct has been confirmed by restriction analysis and sequence verified. For the complete pAM_dCas9 Vector sequence, please visit the Documents tab at www.activemotif.com/enchip.

Shipping & Storage

Products are shipped on dry ice.
Resuspended DNA is stable for 6 months when stored at -20°C. Avoid repeated freeze/thaw cycles.

Design of gRNA

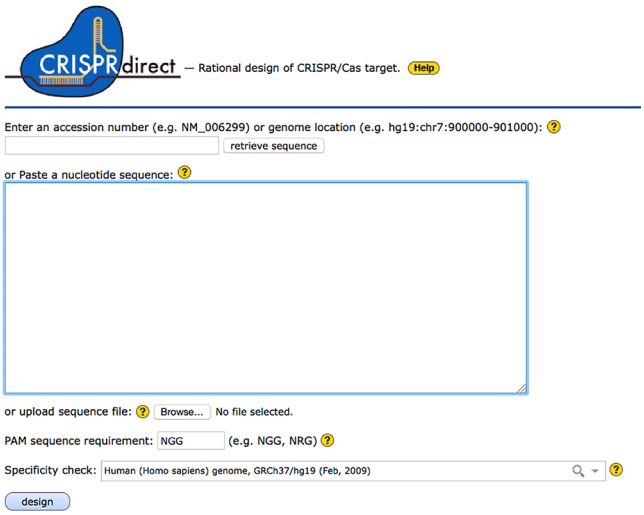
In the enChIP Kit, a guide RNA (gRNA) is designed to target a specific genomic locus. Online software programs are available to assist in the design of a 23 bp gRNA in the form of 5´-N_{20}NGG-3´, where the NGG represents the Protospacer Adjacent Motif (PAM) sequence immediately following the target sequence that is required for successful binding of Cas9 protein. The identified gRNA sites may reside on the + or - strand. We recommend designing multiple gRNAs to a specific locus and then experimentally testing each gRNA for specificity using the enChIP Kit. If possible, try to avoid target sequences that overlap with known recognition sites for DNA-binding proteins as this may block access of the gRNA to its target location.

1. Determine the genomic region of interest for gRNA targeting. Retrieve the DNA sequence of approximately 500 bp surrounding this target locus. This sequence will be analyzed using online software programs and gRNA selection tools to identify the best gRNA sequences.
2. There are several online programs available to assist with gRNA design. A few are listed below. Select a program and follow the software provider's instructions for use.

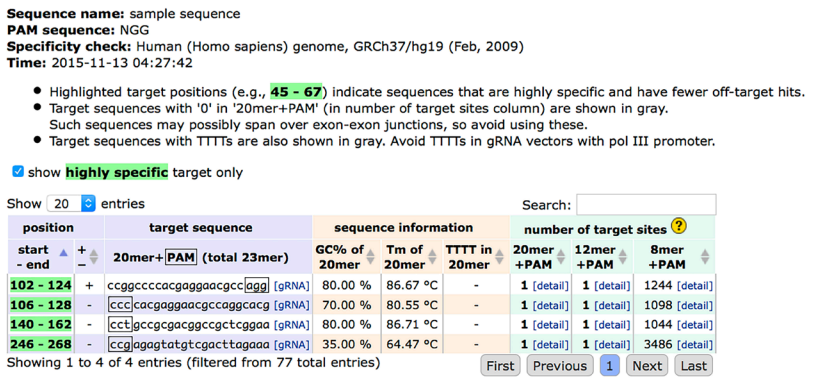
   CRISPRdirect - http://crispr.dbcls.jp

   Michael Boutros Lab - http://www.e-crisp.org/E-CRISP/designcrispr.html

   Feng Zhang Lab - http://crispr.mit.edu
3. Below is an example of gRNA design using CRISPRdirect. Software instructions may change over time. Please follow the recommendations of the software provider for use.
   a. Paste the genomic coordinates or genomic sequence from Step 1 above into the CRISPRdirect site.



   b. Check the box for "show highly-specific targets only".
   c. The output box will show potential gRNA sequencing for the genomic target.



d. Click the "gRNA" link to the right of the sequence and copy the **20 bp non-PAM** sequence.

e. Paste the 20 bp non-PAM sequence from step 3d above into the appropriate gRNA insert template for either single vector or dual vector transfection. Downloadable versions gRNA templates can be found online at www.activemotif.com/enchip. Please note that each cloning vector has unique overlapping ends and gRNA inserts are not interchangeable between the different vectors.

Dual Vector Transfection: The gRNA is cloned into a vector designed specifically to express the gRNA. The dCas9 protein is expressed in a separate expression vector. This option requires co-transfection of two vectors (gRNA vector + dCas9 vector) into your cell line of interest. Perform cloning using the pAM_gRNA vector (Catalog No. 53121).

Single Vector Transfection: The gRNA sequence of interest is cloned directly into the dCas9 expression vector. Both the gRNA and the dCas9 protein will be expressed from the single vector. This option requires only a single transfection, but the vector size is extremely large (>10 kb) and may prove challenging to transfect in some cell lines. Perform cloning using the pAM_dCas9 vector (Catalog No. 53122).

Using existing gRNA vectors: For researchers who already have their gRNA of interest cloned into an expression vector, the dual vector transfection system should be used. This involves co-transfection of the researchers' gRNA expression vector with Active Motif's pAM_dCas9 vector (Catalog No. 53122). No additional cloning is required. Proceed to the next section for Transfection of gRNA and dCas9 expression plasmids.

Dual Vector Transfection – Perform cloning with pAM_gRNA vector (Cat. No. 53121)

*GGCGGCCGTTACTAG*TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCG
ACTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTT
CATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGAC
TGTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCT
TGGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACC
GTAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAA
ACACCGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTA
AAATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCT
TTTTTTT*AGCTTGATGCATAGC*

Green:	20 bp of the selected target sequence
Yellow:	U6 promoter
Blue:	guide RNA scaffold
Red:	termination signal
Copper:	Overlapping ends for subcloning into pAM_gRNA expression vector

Single Vector Transfection – Perform cloning with pAM_dCas9 vector (Cat. No. 53122)

*GCTTGACCGACAATT*TGTACAAAAAAGCAGGCTTTAAAGGAACCAATTCAGTCGA
CTGGATCCGGTACCAAGGTCGGGCAGGAAGAGGGCCTATTTCCCATGATTCCTTC
ATATTTGCATATACGATACAAGGCTGTTAGAGAGATAATTAGAATTAATTTGACT
GTAAACACAAAGATATTAGTACAAAATACGTGACGTAGAAAGTAATAATTTCTT
GGGTAGTTTGCAGTTTTAAAATTATGTTTTAAAATGGACTATCATATGCTTACCG
TAACTTGAAAGTATTTCGATTTCTTGGCTTTATATATCTTGTGGAAAGGACGAAA
CACCGNNNNNNNNNNNNNNNNNNNNGTTTTAGAGCTAGAAATAGCAAGTTAA
AATAAGGCTAGTCCGTTATCAACTTGAAAAAGTGGCACCGAGTCGGTGCTT
TTTTTT*AATTGCATGAAGAAT*

Green:	20 bp of the selected target sequence
Yellow:	U6 promoter
Blue:	guide RNA scaffold
Red:	termination signal
Copper:	Overlapping ends for subcloning into pAM_dCas9 expression vector

Figure 3: gRNA insert templates should be used for the design of gBlocks® Gene Fragments for cloning into the appropriate expression vector for either single or dual vector transfections. Color images for the gRNA insert templates can be found online at www.activemotif.com/enchip.

4. Synthesize gBlocks® Gene Fragments (Integrated DNA Technologies, https://www.idtdna.com/site/order/gblockentry) representing the entire 453 bp sequence to be cloned into the gRNA expression plasmid. The gBlocks sequence should contain the overlapping ends needed for subcloning into the expression vector of choice, the 20 bp target sequence, the guide RNA scaffold and the termination signal.

5. Linearize the appropriate expression plasmid using restriction enzyme digestion.
   a. **pAM_gRNA vector:** Linearize the pAM_gRNA vector with a double digest using restriction enzymes SpeI and HindIII. Gel purify the 3891 bp digested backbone.
   b. **pAM_dCas9 vector:** Linearize the pAM_dCas9 vector with a single digest using restriction enzyme MfeI.
6. Clone each gBlocks gRNA insert into Active Motif's expression plasmid using directional cloning methods such as InFusion® (Clontech) or Gibson Assembly® (New England Biolabs). Follow the manufacturer's recommendations for cloning.
   a. For InFusion cloning, we recommend a 1:3 molar ratio of vector : insert. For example:
      Use 150 ng pAM_gRNA vector with 50 ng gRNA insert.
      Use 150 ng pAM_dCas9 vector with 22 ng gRNA insert.
7. Perform transformations of each gRNA construct into competent *E.coli*. We recommend the use of Active Motif's RapidTrans TAM1 Competent cells. (Catalog No. 11096). Plate transformation reactions onto LB agar plates containing the appropriate antibiotic selection for the cloning vector used. Grow overnight at 37°C.

   pAM_gRNA vector: Kanamycin selection is required
   pAM_dCas9 vector: Ampicillin selection is recommended
8. Select 2-4 colonies for each gRNA construct and isolate the plasmid DNA. The DNA should be free of nucleases or other contaminants prior to transfection. Sequence validate each plasmid to confirm the correct sequence. We suggest preparing a glycerol stock for each candidate construct.
9. Proceed to the next section for Transfection of gRNA and dCas9 expression plasmids.

Transfection of gRNA and dCas9 expression plasmids

Following sequence validation of the expression constructs, cells can be transiently transfected with the gRNA and pAM_dCas9 expression plasmids. The following protocol provides recommendations for transfection using FuGENE® HD Transfection Reagent (Catalog No. 32042). Optimization may be required for each cell line and expression construct tested.

To determine the efficiency of the transfection, set up a duplicate transfection for each cell type used. Cell lysates can be prepared from the duplicate transfection reaction using Active Motif's Nuclear Extract Kit (Catalog No. 40010) for analysis by Western blot. Use the AM-Tag polyclonal antibody (Catalog No. 61677) at a 1:250 - 1:1,000 dilution for detection of the AM-tagged dCas9 protein. If the tagged protein is not detected, continue to optimize transfection conditions.

Calculate the number of transfections you will perform with each gRNA and cell line. Small scale reactions provide enough chromatin to perform one enChIP reaction. Large scale reactions provide enough chromatin to perform 5 enChIP reactions. We recommend running a no gRNA negative control reaction for each cell line tested. Active Motif also offers positive control vectors for both dual or single transfection. The positive control gRNA targets a locus corresponding to a CTCF binding site on chromosome 19 (5,804,115 – 5,804,290). These vectors are available as pAM_gRNA_CTCF vector (Catalog No. 53123) and pAM_dCas9_CTCF vector (Catalog No. 53124).

1. Seed cells in either a 6-well plate (small scale) or 10cm dish (large scale) using the appropriate growth medium. Incubate in a humidified incubator for 24 hours. Cells should be 70-80% confluent at the time of transfection.

	Small Scale Cell Culture (1 enChIP rxn)		Large Scale Cell Culture (5 enChIP rxns)	
	Single Vector	Dual Vector	Single Vector	Dual Vector
Cell culture plate	6-well plate	6-well plate	10 cm dish	10 cm dish
Cell seeding density*	5 x 10^5 cells	5 x 10^5 cells	3 x 10^6 cells	3 x 10^6 cells
Growth medium	10 ml	10 ml	20 ml	20 ml

*These conditions were established for cells with doubling times of 14-18 hours. Cell seeding densities may need to be optimized to ensure that cells are ~80% confluent at the time of transfection.

2. Prepare a separate microcentrifuge tube for each transfection reaction. To each tube add the recommended amount of DNA and Opti-MEM according to the table below.

		Small Scale Cell Culture		Large Scale Cell Culture	
		Single Vector	Dual Vector	Single Vector	Dual Vector
Test gRNA or Positive control CTCF gRNA	gRNA vector	–	1 µg	–	2 µg
	dCas9 vector	–	1 µg	–	2 µg
	(dCas9 + gRNA) vector	1 µg	–	2 µg	–
	Opti-MEM	Up to 275 µl	Up to 275 µl	Up to 550 µl	Up to 550 µl
No gRNA negative control	gRNA vector	–	–	–	–
	dCas9 vector	1 µg	1 µg	2 µg	2 µg
	(dCas9 + gRNA) vector	–	–	–	–
	Opti-MEM	Up to 275 µl	Up to 275 µl	Up to 550 µl	Up to 550 µl

3. Add FuGENE HD Transfection reagent drop wise directly to the DNA/media mixture. Do not allow FuGENE to come directly in contact with the plastic from the tube. Mix the solution by pipetting up and down and incubate at room temperature for 30 minutes.
   Small scale cell culture: Add 6 µl FuGENE to each transfection reaction.
   Large scale cell culture: Add 12 µl FuGENE to each transfection reaction.
4. Add the entire DNA/media/FuGENE mixture drop wise to each cell culture plate. Incubate on a shaking platform at 100 rpm for 2 minutes to evenly distribute the transfection mixture.
5. Return plate to humidified incubator for 24 hours.
6. 24 hours post-transfection, passage cells.
   Small scale cell culture: Transfer cells in each well of a 6-well plate to a 10 cm dish.
   Large scale cell culture: Transfer cells from a 10 cm dish to a 15 cm dish.
7. Return plate to humidified incubator for 24 hours.
8. Transfected cells are now ready to be processed for chromatin fixation using the enChIP Kit (Catalog No. 53125). Alternatively, if duplicate reactions were performed cell lysates can be prepared using Active Motif's Nuclear Extraction Kit (Catalog No. 40010) for Western blot analysis of transfection efficiency using the AM-Tag polyclonal antibody (Catalog No. 61677) to recognize the tagged dCas9 protein.

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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