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UNIVERSITÄT
MÜNCHEN

BIOZENTRUM DER LMU
DEPARTMENT BIOLOGIE II
HUMAN BIOLOGY & BIOIMAGING

# Protocol for Rapid and Versatile Genome Engineering Using The MIN (Multifunctional Integrase) Strategy

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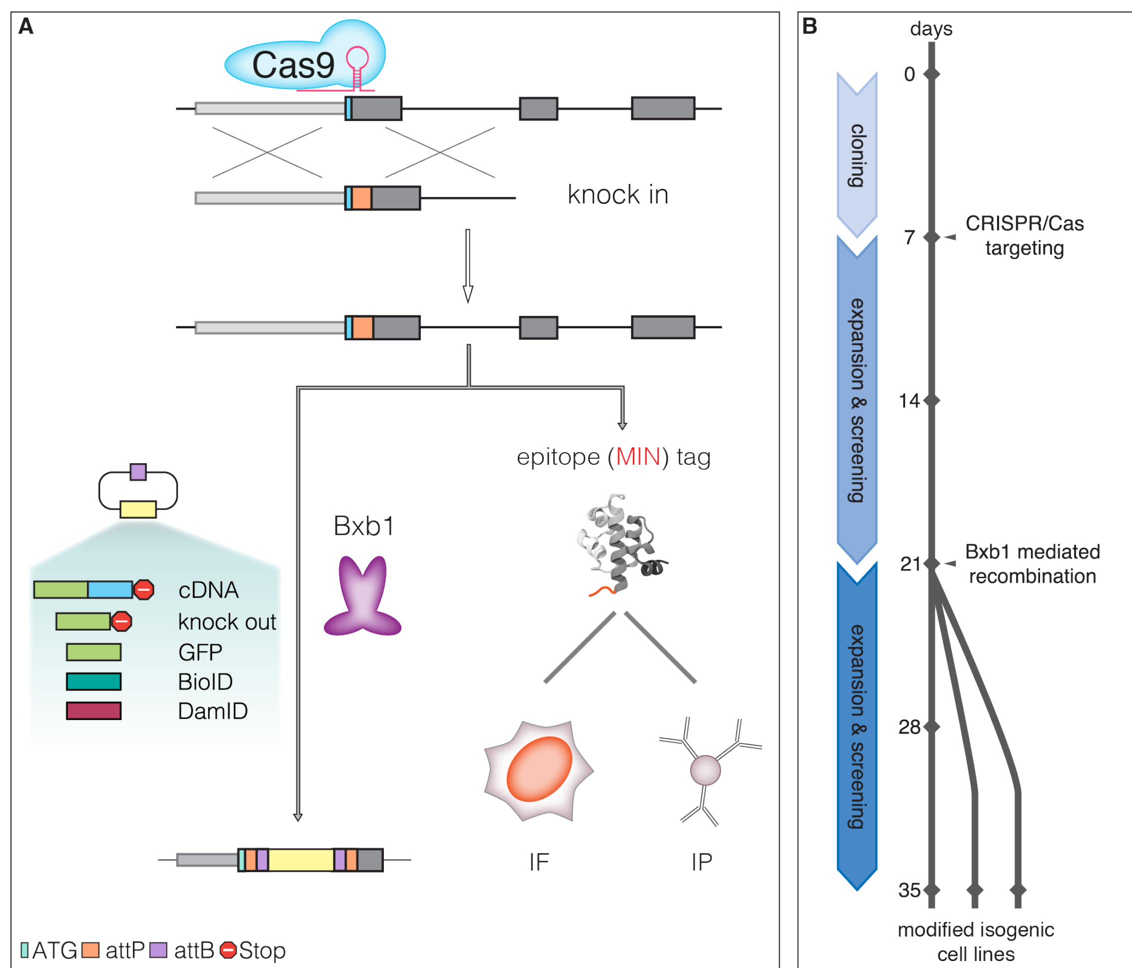
## Overview

MIN (Multifunctional Integrase) Tagging allows the rapid and versatile manipulation of a gene of interest through the use of an inserted MIN (Bxb1 *attP*) sequence either immediately following the first codon or before the stop codon of a gene of interest. This site acts not only as a genetic entry site for the phage serine integrase Bxb1but also as an epitope tag for a highly specific rat monoclonal antibody suitable for use in immunoprecipitation and immunofluorescence.  In a second step, Bxb1 can be used to recombine in any of the library of openly available pre-fabricated cassettes into the MIN site, allowing the efficient generation of numerous modified cell lines from a single entry cell line.

For more information see Mulholland et al. 2015




Mulholland et al. 2015

## Using the MINtool to Design Your MIN-tagging Strategy

### *Background*

The MINtool assists users in applying the MIN tag strategy to a given gene of interest (GOI). The MIN tag strategy relies on the presence of the MIN tag within the coding sequence of a GOI, either immediately following the start codon or preceding the stop codon.
To accomplish this, we employ the CRISPR/Cas9 system (Cong et al. 2013). This system uses a guide RNA (gRNA) to sequence-specifically target the bacterial Cas9 nuclease to create a double stranded break (DSB). We use the system to induce the DSB proximal to the site where the MIN-tag is to be inserted. This break can then be repaired with a MIN –tag donor, harboring the MIN-tag sequence and is homologous to the sequences 5' and 3' of the DSB.

Therefore, the insertion of the MIN-tag at a GOI requires:

- Cas9
- GOI-specific MIN tag donor
- GOI-specific gRNA

The MINtool facilitates the identification of gRNA targets proximal to the start or stop codon of a GOI. Once a gRNA target is chosen, the MINtool will produce a file containing the oligos needed for cloning the chosen gRNA into Zhang Lab (www.genome-engineering.org) plasmids, as well as the necessary GOI-specific MIN-tag donor.

### *Step I: Choose Gene and Organism*

Choose the gene you would like to MIN-tag as well as the organism you are working with in the top bar.

- ✓ Click on the box with correct gene information
- ➢ If gene is not found, make sure proper GenBank name was used.

### *Step II: Choose an isoform*

Genes often have numerous isoforms due to the use of alternative transcriptional start sites and splicing. Thus a gene might have isoforms using different upstream or downstream start or stop codons. Therefore, it is important to carefully consider which isoform to choose when applying the MIN tag strategy.

- ✓ If your GOI has multiple isoforms, click on the schematic representation of the one you would like to MIN-tag

### *Step III: N- or C-terminal Tagging*

The choice of N or C terminal MIN tagging is dependent on the downstream applications desired by the user. Both options can be used to create fluorescent and enzymatic fusions. However, N-terminal targeting allows the most flexibility, as it offers the possibility to knockout the gene using the “knockout cassette” as well as the replacement of endogenous gene products with cDNAs. This type of tagging is necessary for studying point mutants or domain deletions.

- ✓ Click on “N-terminal tagging” (green button) for N-terminal MIN tag insertion or on “C-terminal tagging” (red button) for C-terminal MIN-tag insertion

### *Step IV: Choosing a gRNA*

The CRISPR/Cas9 system has a sequence requirement of $N_{21}$GG for gRNA targeting. All of the potential targets on either strand within 100 bp 5’ and 3’ of the ATG (red) are depicted. If possible, we recommend choosing a gRNA target sequence, which will be disrupted upon MIN-tag insertion. Failure to do this can result in additional, unwanted mutations at the gRNA target sequence.

- ✓ Hold the cursor over each graphical depiction of a gRNA to see its target sequence in the lower box containing the sequence containing and surrounding the start or stop codon
- ✓ Click on your favorite gRNA

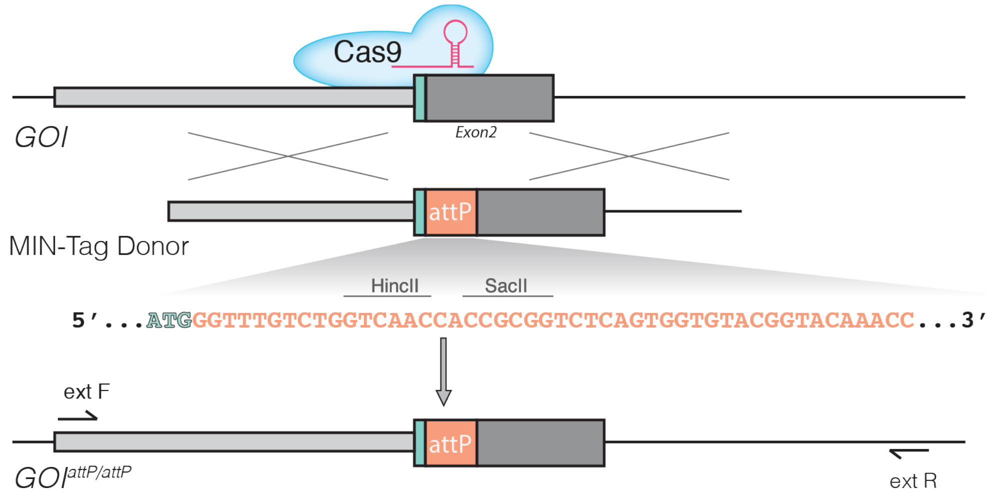
### *Step V: Export Sequences*

The sequences can now be exported as a spreadsheet by clicking on the “generate oligo” button.
The MIN-tag donor can be ordered as a ready-to-use 150bp ssDNA oligo.
The oligos for the gRNA are designed to be used in one of the Zhang Lab (MIT, www.genome-engineering.org ) plasmids (px330, px335, px458 available at Addgene http://www.addgene.org/Feng_Zhang/ ) which contain two expression cassettes, Cas9 and the gRNA.

- ✓ We recommend using px458, on which Cas9 fused to GFP, useful for downstream FACS sorting.

## 1. Using CRISPR/Cas to Insert the MIN-tag

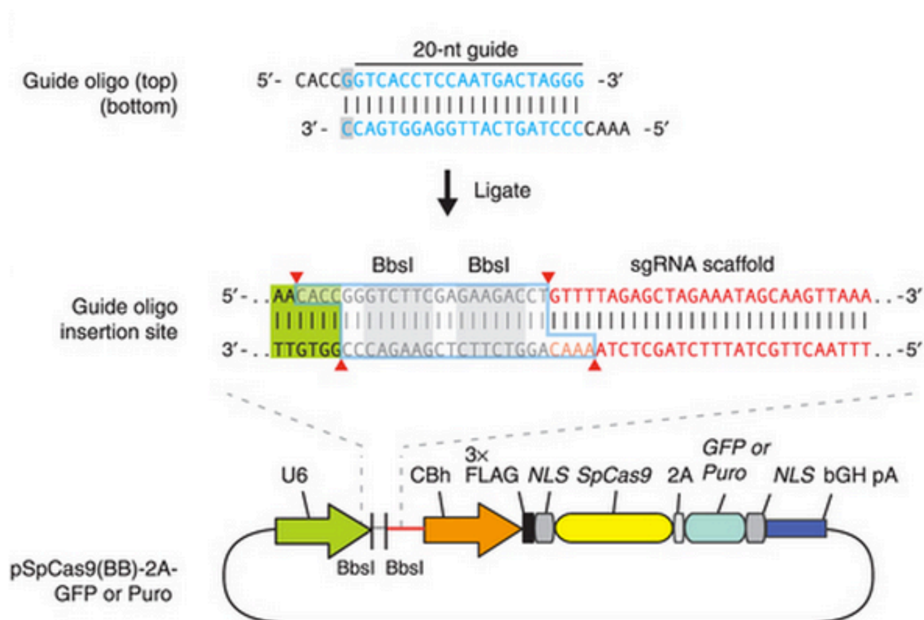
In this section, we describe how to produce and use the CRISPR/Cas system to integrate the MIN-tag into a locus of interest. The gRNA and MIN-tag donor oligonucleotides required for this step can be generated using the MINtool (mentioned above).




### *1.1 Cloning the GOI-specific gRNA*

Here we summarize the cloning process for incorporating gRNA oligos produced by the MINtool into one of the Zhang Lab vectors. For more details see:

Ran, F. A., et al. (2013). "Genome engineering using the CRISPR-Cas9 system." Nat Protoc 8(11): 2281-2308.




Ran et al. Nat. Protoc. (2013). 8, 2281-2308

### *1.1.1 Annealing gRNA Oligos*

The two oligos need to be annealed in order to use them as dsDNA fragment in the subsequent step

| Reagents | Stock Concentration | Volume (µl) |
|---|---|---|
| gRNA F | 100 µM | 20 |
| gRNA R | 100 µM | 20 |
| NEB 4 Buffer | 10x | 5 |
| $H_2O$ | 1x | 5 |
| TOTAL | | 50 |

- Incubate mixture at 95°C for 5 min

- Slowly cool mixture to room temp (If using heating block, simply switch it off)

### *1.1.2 Cloning gRNA into pSpCas9 (Backbone)*

This reaction consists of a simultaneous digestion and ligation. Make sure to check that gRNA oligos do not contain a BpiI site

- Prepare the following reaction mix:

| Reagents | Volume |
|---|---|
| pSpCas9 (BB) | 40 fmol |
| Annealed gRNA fragment | 120 fmol |
| Fast Digest *BpiI* (ThermoFisher) | 1 µl |
| T4 Ligase (30 U) | 1 µl |
| T4 Ligase Buffer | 2 µl |
| $H_2O$ | X µl |
| TOTAL | 20 µl |

- Incubate the reaction in a thermal cycler:

| Temperature | Time | Cycle |
|---|---|---|
| 37°C | 5 min | 55x |
| 20°C | 5 min | |
| 37°C | 60 min | 1x |
| 65°C | 10 min | 1x |

- After run is finished add additional 1 µl BpiI and incubate for 1 h @ 37°C
- Transform *E.coli* with 5 µl

## *1.2. Transfection: CRISPR/Cas Components with MIN-tag Donor*

- For embryonic stem cells, seed $5x10^5$ cells in a p60 or one well of a six-well plate
- ✓ For convenience, we recommend resuspending the ssDNA, MIN-tag donor oligo to a concentration of 2 µg/µl
- Transfect the following (using Lipofectamine 3000 according to manufacturer’s instructions):

| Reagent | Amount |
|---|---|
| MIN-tag Donor ssDNA oligo | 2 µg |
| pSpCas9-gRNA construct | 0.5 µg |

- ✓ Harvest cells for FACS sorting 48h after transfection

### *1.3 FACS Sorting*

- Sort $5x10^5$ GFP-positive cells into a p100 cell culture dish.
- Allow colonies to grow for 5-7 days (depending on growth rate) at 37°C and 5% $CO_2$

### *1.4 Consolidating Colonies*

- Prepare 96-well plates (with 50 µl/well of 0.2% gelatin for mESCs)
- In advance, add 30 µl trypsin to a column of 8-wells
- Examine colonies under the microscope
- Transfer a single colony using a p10 micropipette and transfer it into a well-pretreated with trypsin
- Repeat for the entire column
- Incubate the plate at 37°C for 3 min
- Thoroughly resuspend cells using 170 µl media per well
- Repeat previous steps for the entire plate

### *1.5 Duplicating the Plate*

- Prepare 96-well plates (with 50 µl/well of 0.2% gelatin for mESCs)
- Aspirate/Decant the medium from the wells
- Wash the wells with 100 µl of cell culture grade PBS
- Aspirate/Decant the PBS from the wells
- Add 30 ml of trypsin to the wells
- Incubate the plate at for 5 min at 37°C and 5% $CO_2$
- Resuspend (vigorously) the cells in each well with 170 µl of fresh media
- Transfer portion of the cells (for 1:2 split use 100 µl) of the resuspended cells into the freshly prepared plate
- Incubate plate at 37°C and 5% $CO_2$ until cells are roughly 70% confluent
- One plate will be used for keeping the potential clones in culture while the other plate's clones will be lysed and used for a screening PCR

### *1.6 Screening Clones*

For simplicity, we recommend using the following protocol in which cells are lysed and the lysate is directly used for the PCR. This eliminates the need for isolating and purifying genomic DNA from 96 separate wells.

- ✓ After trying many DNA polymerases, we have found Bioline MyTaq to be the most robust and compatible with the lysate.

### *1.6.1 Lysis on the 96-well plate*

- Wash the wells 2x with 100 µl of cell culture grade PBS
- Decant the PBS by inverting the plate
- To each well add 50 µl of PCR lysis buffer consisting of the following:

| Reagents | Volume |
|---|---|
| 1x PCR Buffer (500 mM Tris, 100 mM $CaCl_2$) | 5 µl |
| Proteinase K (50 µg/ml) | 0.25 µl |
| 1.7 mM SDS | 0.05 µl |
| $H_2O$ | 44.7 µl |
| TOTAL | 50 µl |

- Seal plate with aluminum 96-well plate sticker
- Incubate the plate at -20°C for 30 min
- Incubate the plate at 55°C (to activate Proteinase K) for 3 h
  - ✓ We recommend placing the plate in a 96-well plate shaker for 3 min (500 rpm)
- Incubate the plate at 85°C (to inactivate the Proteinase K)

### *1.6.2 Screening PCR*

For this step, the user must design primers for a screening PCR. We recommend primers which will produce an amplicon between 300-450 bp. Take into consideration that the MIN site contains a HincII and SacII site for convenient genotyping. Try to avoid primers producing amplicons containing these sites endogenously.

- Prepare the following master mix for the PCR:

| Reagents | Volume (per well) | Volume (100 rxns) |
|---|---|---|
| Forward Screening Primer (10 µM) | 1 µl | 100 µl |
| Reverse Screening Primer (10 µM) | 1 µl | 100 µl |
| 5x MyTaq Buffer | 5 µl | 500 µl |
| MyTaq | 0.1 µl | 10 µl |
| $H_2O$ | 15.4 µl | 1540 µl |
| TOTAL | 22.5 µl | 2250 µl |

- Dispense 22.5 µl master mix into each well of 96-well PCR plate
- Add 2.5 µl of each lysate to the wells
- Use the following cycling conditions:

| Temperature | Time | Cycle |
|---|---|---|
| 95°C | 5 min | 1x |
| 95°C | 0:30 min | 45x |
| 60°C (may differ) | 0:30 min | |
| 72°C | 0:30 min | |
| 72°C | 0:40 min | 1x |

- Run 10 µl of the finished PCR product over a 1.5% agarose gel
  - ✓ Keep the remaining PCRs as 7.5 µl will be used in the following restriction digest.

### *1.6.3 Restriction Digest*

The HincII or SacII site contained within the MIN-tag sequence can be exploited to accurately assess the success of MIN-tag incorporation at the locus of interest.

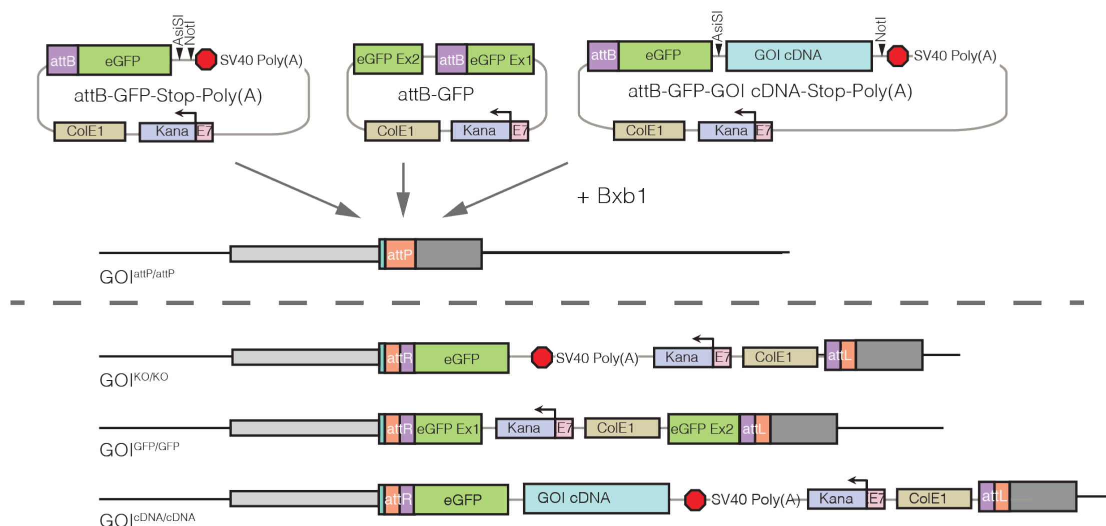
- Prepare the following master mix for the restriction digest:

| Reagents | Volume (per well) | Volume (100 rxns) |
|---|---|---|
| 10x FastDigest Buffer Green(Thermo) | 1 µl | 100 µl |
| HincII or SacII | 0.5 µl | 50 µl |
| $H_2O$ | 11 µl | 1100 µl |
| TOTAL | 12.5 µl | 1250 µl |

- Dispense 12.5 µl master mix into each well of a 96-well PCR plate
- Add 7.5 µl of each PCR to the wells
- Incubate at 37°C for at least 1 h
- Run 10 µl of the finished PCR product over a 1.5% agarose gel
  - ✓ Positive clones should show a lower band. Homozygous clones should have one lower band. Heterozygotes should show both bands.
  - ✓ Compare restriction digest banding pattern with the original PCR to determine if difference in banding patterns is due to indels resulting from non-homologous end joining.

## 2. Bxb1-mediated Recombination using the MIN-tag

Here we describe the procedure for using Bxb1 to integrate a pre-fabricated cassette into a MIN-tag locus as well as how to screen for positive recombinations. This step requires a cassette harboring an *attB* sequence. This can be done using one of the many already available constructs from the MIN Toolbox (available via the MINtool website or www.addgene.com). For designing and producing your own *attB* constructs, see the section on page 15 and 17.




Schematic representation of Bxb1-mediated recombination of functional cassettes into the MIN (*attP)* site. Cassettes containing an attB site are linearlized at the genomic locus, resulting in the inclusion of the cassette between a new *attR* and *attL* site.

### *2.1 Transfection: CRISPR/Cas Components with MIN-tag Donor*

- For embryonic stem cells, seed 5x10$^5$ cells in a p60 or one well of a six-well plate
- ✓ There are two options for enriching for successfully transfected cells in the following FACS step. We have developed a ***Bxb1-surrogate reporter*** to allow for the identification of cells in which all components necessary for recombination are present. Additionally, we have made a construct expressing an ***RFP-Bxb1*** fusion.
- For recombination using the Bxb1-surrogate reporter for enrichment, transfect the following (using Lipofectamine 3000 according to manufacturer's instructions):

| Reagent | Amount |
|---|---|
| pCAG-NLS-HA-RFP-Bxb1 | 1 µg |
| *attB* Construct | 1 µg |
| pCAG-attP (Bxb1 surrogate reporter) | 0.5 µg |

- For recombination using only the RFP-Bxb1 for enrichment, transfect the following (using Lipofectamine 3000 according to manufacturer's instructions):

| Reagent | Amount |
|---|---|
| pCAG-RFP-NLS-HA-Bxb1 | 1.25 µg |
| *attB* Construct | 1.25 µg |

- ✓ Harvest cells for FACS sorting 48h after transfection

### *2.2 FACS Sorting*

- o When using ***Bxb1-surrogate reporter*** for enrichment, sort $5x10^5$ GFP positive cells into a p100 cell culture dish.
- o When using ***RFP-Bxb1*** for enrichment, sort $5x10^5$ GFP positive cells into a p100 cell culture dish.
- o Allow colonies to grow for 5-7 days (depending on growth rate) at 37°C and 5% $CO_2$

✓ Don't worry, this is not a mistake! The following cell culture steps are nearly identical to those mentioned for MIN-tag integration. They are repeated for simplicity

### *2.3 Consolidating Colonies*

- o Prepare 96-well plates (with 50 µl/well of 0.2% gelatin for mESCs)
- o In advance, add 30 µl trypsin to a column of 8-wells
- o Examine colonies under the microscope
- o Wash plate with the media already on the plate, to detach as many colonies as possible from the plate
  - ✓ This will make the colonies to pipette
- o Find a single colony under the microscope
- o Transfer the single colony using a p10 micropipette and transfer it into a well-pretreated with trypsin
- o Repeat for the entire column
- o Incubate the plate at 37°C for 3 min
- o Thoroughly resuspend cells using 170 µl media per well
- o Repeat previous steps for the entire plate

### *2.4 Duplicating the Plate*

- o Prepare 96-well plates (with 50 µl/well of 0.2% gelatin for mESCs)
- o Aspirate/Decant the medium from the wells
- o Wash the wells with 100 µl of cell culture grade PBS
- o Aspirate/Decant the PBS from the wells
- o Add 30 ml of trypsin to the wells
- o Incubate the plate at for 5 min at 37°C and 5% $CO_2$
- o Resuspend (vigorously) the cells in each well with 170 µl of fresh media
- o Transfer portion of the cells (for 1:2 split use 100 µl) of the resuspended cells into the freshly prepared plate
- o Incubate plate at 37°C and 5% $CO_2$ until cells are roughly 70% confluent
- o One plate will be used for keeping the potential clones in culture while the other plate's clones will be lysed and used for a screening PCR

### *2.5 Screening Clones*

As with the MIN-tag integration, we recommend using the following protocol in which cells are lysed and the lysate is directly used for the PCR. This eliminates the need for isolating and purifying genomic DNA from 96 separate wells.

### 2.5.1 Lysis on the 96-well plate

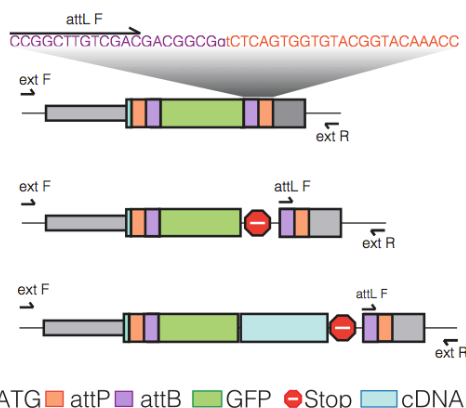
- Wash the wells 2x with 100 µl of cell culture grade PBS
- Decant the PBS by inverting the plate
- To each well add 50 µl of PCR lysis buffer consisting of the following:

| Reagents | Volume |
|---|---|
| 10x PCR Buffer (500 mM Tris, 100 mM $CaCl_2$) | 5 µl |
| Proteinase K (50 µg/ml) | 0.25 µl |
| 1.7 mM SDS | 0.05 µl |
| $H_2O$ | 44.7 µl |
| TOTAL | 50 µl |

- Seal plate with Thermowell sealing tape for 96-well plates
- Incubate the plate at -20°C for 30 min
- Incubate the plate at 55°C (to activate Proteinase K) for 3 h
  - ✓ We recommend placing the plate in a 96-well plate shaker for 3 min (500 rpm)
- Incubate the plate at 85°C (to inactivate the Proteinase K)

### 2.5.2 Screening PCR

To screen for positive recombinations, we employ a multiplex PCR strategy using three primers. This approach takes advantage of the unique attL site created by successful integrations, and can be used to screen for the proper integration of any of the constructs in the MIN toolbox.




Schematic representation of multiplex PCR. External primers flanking the recombination site are used in combination with a primer specific for the *attL* site created only after successful recombination.

- Prepare the following master mix for the PCR:

| Reagents | Volume (per well) | Volume (100 rxns) |
|---|---|---|
| Forward Screening Primer (10 µM) | 1 µl | 100 µl |
| Reverse Screening Primer (10 µM) | 1 µl | 100 µl |
| attL Forward Primer (10 µM) | 1 µl | 100 µl |
| 5x MyTaq Buffer | 2 µl | 20 µl |
| MyTaq | 0.08 µl | 8 µl |
| $H_2O$ | 3.42 µl | 342 µl |
| TOTAL | 8.5 µl | 1000 µl |

- Dispense 8.5 µl master mix into each well of 96-well PCR plate
- Add 2.5 µl of each lysate to the wells
- Use the following cycling conditions:

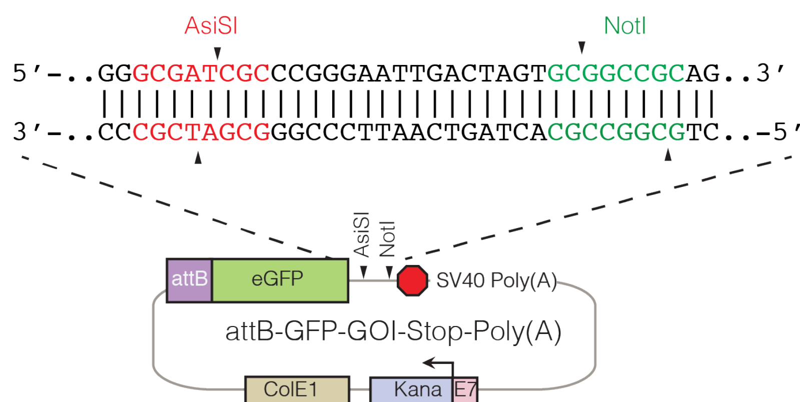
| Temperature | Time | Cycle |
|---|---|---|
| 95°C | 5 min | 1x |
| 95°C | 0:30 min | 45x |
| 60°C (may differ) | 0:30 min | |
| 72°C | 0:30 min | |
| 72°C | 0:40 min | 1x |

- Run 10 µl of the finished PCR product over a 1.5% agarose gel
- Analyze the gel:
  - ✓ Homozygous remcomination: one lower band (attL F-screening R amplicon)
  - ✓ No recombination: one higher band (screening F -screening R amplicon)
  - ✓ Heterozygous recombination: two bands (both amplicons)

# 3. Cut and Paste Cloning: cDNAs into attB-GFP/mCh-Poly(A) Vector

In order to investigate specific mutations and domain deletions at endogenous levels, the MIN-tag strategy can be applied to replace the expression of an endogenous gene with any desired cDNA, while maintaining physiological expression levels. To accomplish this, we designed our knockout cassettes to include inframe AsiSI and NotI restriction enzyme recognition sites directly following either GFP and mCherry. These sites can be exploited to open the knockout cassette backbone and ligate in the appropriate cDNA with AsiSI NotI overhangs. The following MINtoolbox plasmids can be used for creating fluorescent cDNA fusions: Addgene #65524, #65525, #65526, #65528 and #65529.

Once a cDNA is cloned into one of these backbones, it can be used to further simply generate cDNA cassettes harboring domain deletions or point mutations using a simple circular amplification strategy (See Section 4)




Schematic representation of the AsiSI and NotI sites following eGFP (mCherry is also available).

## *3.1 Open attB Plasmid Backbone*

- Digest 4 µg of a pCAG- fluorophore backbone to remove unwanted cDNA.
  - ✓ Make sure the virtual digest yields differential bands, far enough apart in size to isolate from gel. If not, use another enzyme to cut in the cDNA to assist in distinguishing and extracting the correct band
- Use the following amounts of reagents:

| Reagents | Amount |
|---|---|
| attB-GFP/mCh-Poly(A) (eventual BB) | 4 µg |
| 10x FastDigest Buffer (ThermoFisher) | 4 µl |
| FastDigest AsiSI | 2 µl |
| FastDigest NotI | 2 µl |
| $H_2O$ | Up to 40 µl |
| TOTAL | 40 µl |

- Incubate at 37°C overnight
- Run over 1% agarose gel
- Cut out backbone band
- Purify the DNA from the extracted gel fragment
- Measure concentration of DNA

### *3.2 Isolate cDNA from vector*

We mostly work with cDNAs in pCAG-GFP/mCherry overexpression constructs which cDNAs are flanked with AsiSI/NotI sites. This allows the simple removal of cDNAs for cloning into the attB-GFP/mCh-Poly(A) backbone. The procedure is described below. Alternatively, cDNAs can be cloned by PCR amplification. For this we recommend using overhang primers with nested AsiSI and NotI sites. Make sure to maintain the correct frame!

- Digest 4 µg of your cDNA containing vector.
  - ✓ Make sure the virtual digest yields differential bands, far enough apart in size to isolate from gel. If not, use another enzyme to cut in the backbone to assist in distinguishing and extracting the correct band
- Use the following amounts of reagents:

| Reagents | Amount |
|---|---|
| Vector harboring cDNA | 4 µg |
| 10x FastDigest Buffer (ThermoFisher) | 4 µl |
| FastDigest AsiSI | 2 µl |
| FastDigest NotI | 2 µl |
| $H_2O$ | Up to 40 µl |
| TOTAL | **40 µl** |

- Incubate at 37°C overnight
- Run over 1% agarose gel
- Cut out backbone band
- Purify the DNA from the extracted gel fragment
- Measure concentration of DNA

### *3.3 Ligation*

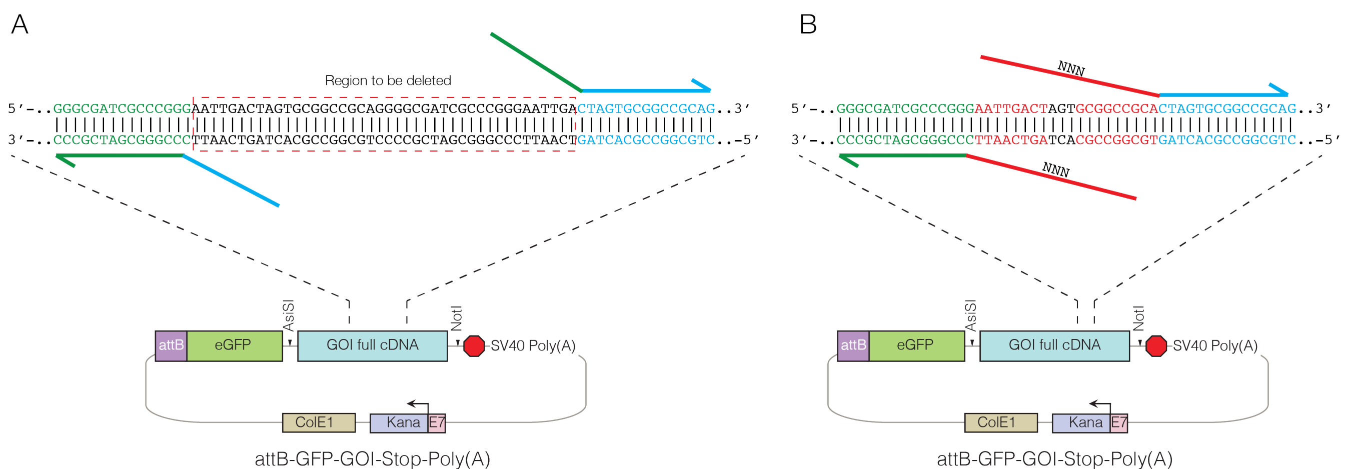
- We recommend cloning in inserts into the backbone in a 3:1 molar ratio, respectively, with 50 ng of backbone
- Prepare the following mix for your ligation:

| Reagents | Amount |
|---|---|
| backbone | 50 ng |
| cDNA fragment | 3x mol of BB |
| 10x T4 Ligase Buffer | 2 µl |
| T4 Ligase | 1 µl |
| $H_2O$ | Up to 20 µl |
| TOTAL | **20 µl** |

- Incubate at room temp. for 2 h
- Heat inactivate at 65°C for 10 min
- Directly transform JM109 *E. coli* with 5 µl of ligation

## 4. Generating Deletions and Point Mutations in cDNA Cassettes

In order to investigate protein function, point mutations and domain deletions of a protein of interest are often required. As the MIN strategy allows the replacement of an endogenous gene with a cDNA, here we offer a strategy for rapidly generating a library of deletion constructs and point-mutants without the need for subcloning. Once a wild-type attB-GFP/mCherry-GOI-Poly(A) construct is generated, the following circular amplification strategy with primer overhang can be performed to modify the cDNA construct as desired.




Schematic representation of the circular amplification strategy for generating (A) deletions and (B) point-mutations in already existing cDNA constructs.

### *4.1 Designing the Appropriate Primers*

As this strategy relies on the circular amplification of an entire plasmid into linear amplicons which are subsequently transformed, the homology of amplicon ends is critical. The following steps describe how to properly design primers to ensure bacterial recombinations of the linear PCR products back into a plasmid.

#### *4.1.1 For Deletion Constructs*

- First, choose the region to be deleted.
- Find sequences with similar melting temperatures immediately flanking the region to be deleted
  - ✓ For the forward primer, this must be downstream of the region to be deleted
  - ✓ For the reverse primer, this must be upstream of the regions to be deleted
- Design the forward and reverse primers such that the forward primer has a 5'overhang complementary to the annealing sequence of the reverse primer and the reverse primer has a 5' overhang complementary to the annealing sequence of the forward primer (See schematic A above).
  - ✓ We recommend a melting temp of 60-62°C for the annealing sequence of each primer.

### *4.1.1 For Point Mutants Constructs*

- First, choose the mutation to be added.
- Choose approx. 20 bp of sequence surrounding the site where the mutation should occur.
- For the sense strand, replace the original coding seq. with the mutant seq. i.e. with a different codon for a point mutant.
- Also do this for the anti-sense strand, by determining the reverse-complement of the sequence generated in the previous step
  - ✓ These sequences will be the overhangs of the primers
- For the forward and reverse primers, find sequences with similar melting temperatures immediately downstream and upstream of the approx. 20 bp sequence already chosen (containing the mutation) respectively.
- To complete the overhang primers, append the appropriate sense 20 bp containing the mutation to each primer's 5' end

## *4.2 Circular Amplification PCR*

This PCR will use the overhang primers generated in the previous step to amplify most of the plasmid sequence excluding deleted regions or the site to be mutated. As plasmid sequences are frequently GC rich, we suggest the following PCR conditions:

- Prepare the following master mix for the PCR:
  - ✓ We suggesting performing at least 2 PCRs in parallel in order to have enough product for the subsequent steps

| Reagents | Volume (per well) | Volume 4 Rxns |
|---|---|---|
| Forward overhang Primer (10 µM) | 1 µl | 4 µl |
| Reverse overhang Primer (10 µM) | 1 µl | 4 µl |
| dNTPs (10 mM) | 1 µl | 4 µl |
| DMSO (10 µM) | 2.5 µl | 10 µl |
| 5x NEB GC Buffer ( | 10 µl | 40 µl |
| Template (attB-GFP/mCh-cDNA-Poly(A)) | 1 ng | |
| Phusion Polymerase | 0.5 µl | |
| $H_2O$ | Up to 50 µl | |
| TOTAL | 50 µl | |

- Use the following cycling conditions:

| Temperature | Time | Cycle |
|---|---|---|
| 95°C | 5 min | 1x |
| 95°C | 0:30 min | 30x |
| 60°C (may differ) | 0:30 min | |
| 72°C | 0:30 min/kb | |
| 72°C | 0:40 min | 1x |

- Pool the PCR products over a spin column (Machery-Nagel)
- Elute in 35 µl dd$H_2O$

### *4.2 DpnI Digest*

In contrast to the PCR products, the template plasmid contains methylated adenines. Therefore *DpnI* is used to degrade the original template in order to prevent false positives after transformation.

- Prepare the following

| Reagents | Volume (per well) |
|---|---|
| Eluate of pooled PCR products | 35 µl |
| 10x Fast Digest Buffer | 4 µl |
| FastDigest *DpnI* | 1 µl |
| TOTAL | 40 µl |

- Incubate at 37°C for 1 h
- Heat inactivate at 80°C for 20 min
- Transform 5 µl into competent bacteria

## 5. Using the MIN-tag antibody in Immunofluorescence

### *5.1 Immunostaining Samples with MIN-tag antibody*

- Seed cells on coverslips at least 24h prior to fixation
- Wash cells 2x with PBS (tissue culture grade, free of sodium azide)
- Fix cells in 2%-4% formaldehyde in PBS for 10 min at room temp.
- Exchange fixative stepwise with PBST
- Wash 2x with PBST
- Quench with ***freshly prepared*** 20 mM glycine dissolved in PBS for 10-20 min to block free aldehydes
- Wash 2x with PBST
- Permeabilize with 0.5% Triton X-100 in PBS for 10 min
- Block for 1 hr in blocking buffer (1x PBST, 2% BSA, 0.5% Fish skin gelatin)
- Wash 3x with PBST
- Incubate for 5-10 min in blocking buffer while preparing antibody solutions
- Incubate coverslips with primary antibody for 1 h in humidified chamber
  - ✓ Use the MIN-tag antibody in a 1:1000 dilution
- Wash 3x with PBST
- Incubate coverslips with appropriately diluted primary antibody for 1 h in humidified chamber
- Wash 3x with PBST
- Wash 2x with PBS
- Postfix with 4% Formaldehyde in PBS for 10 min at room temp.
- Exchange fixative stepwise with PBST
- Counterstain DNA with DAPI (2 µg $mL^{-1}$ in PBST) for 6 min at room temp.
- Wash 3x with PBS
- Rinse briefly with dd$H_2O$, only immediately prior to mounting
- Remove excess liquid by touching the coverslip edge to a Kimwipe tissue
- Mount the coverslip on a drop of Vectashield

## 6. Using the MIN-antibody for Immunoprecipitation (IP)

### *6.1 Couple the MIN-antibody to beads*

The monoclonal rat-anti-MIN antibody is provided as hybridoma culture supernatant. For coupling of the antibody to protein G beads (magnetic or sepharose), use a 10:1 ratio of antibody to bead volume, e.g. 500 µl antibody + 50 µl beads.

- incubate beads with appropriate amount of antibody for 1 h at 4 °C while rotating
- remove supernatant
- wash twice with TBS-T (20 mM TrisHCl pH 7.5, 150 mM NaCl, 0.02 % Tween), 10:1 ratio TBS-T:beads
- store antibody-coupled beads in TBS-T at 4 °C

### *6.2 Immunoprecipitation*

In principle, any standard cell lysis procedure should be compatible with anti-MIN-IP. Usually, 25 µl beads are sufficient to precipitate MIN-tagged protein from ~ 10 million ES cells.

Lyse ES cells on ice; e.g. 10 million cells in 100 µl lysis buffer (20 mM TrisHCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA, 0.5 % NP-40, 2 mM PMSF, protease inhibitor mix) for 30 min

*Dependent on lysis protocol and protein of interest:* precipitate cellular debris by centrifugation: 15 min, 14 000 g, 4 °C

Dilute cell lysate to 400 µl with IP buffer (20 mM TrisHCl pH 7.5, 150 mM NaCl, 0.5 mM EDTA) and incubate with 25 µl anti-MIN-coupled beads for 1 h at 4 °C while rotating

Remove supernatant

Wash 3 times with 500 µl wash buffer (20 mM TrisHCl pH 7.5, 250 mM NaCl, 0.5 mM EDTA)

*For subsequent Western blot analysis:* boil beads in 50 µl 2xLaemmli sample buffer at 95 °C for 10 min