REMI-SEQ HANDBOOK

For preparation of samples for next generation sequencing from GWDI-bank mutant cells.

'REMI-seq: generation of a genome wide mutant resource for Dictyostelium functional genomics' is funded by the Wellcome Trust, from a grant awarded to Professor Chris Thompson and Professor Adrian Harwood.

Please reference the following publication when publishing your data:

XXXXXXXXXXXXXXXXXXXXXXXXXXXX

The REMI-seq Project

REMI-seq combines restriction enzyme mediated integration with the power of next generation sequencing technology for functional genomic studies. The REMI-seq project generated both single gene mutants with defined insertion sites and large pools of mutants that can be used for genomic level screening strategies of population fitness and parallel phenotyping to probe sub-lethal sensitivity changes to developmental signals, toxins or drugs.

The collection of mutants generated by the REMI-seq project is known as the **G**enome **W**ide ***D**ictyostelium* **I**nsertion resource or GWDI-bank for short.

Contents

The GWDI-bank mutants

The GWDI-bank mutants were generated by the REMI-seq process. The GWDI-bank mutants are available both individually and pooled for functional genomic studies. The composition of these pools is known and searchable via our website, www.remi-seq.org. Pools can be requested from the Dicty Stock Centre. A planning tool is also available on the website (www.remi-seq.org) for pool selection experiments.

Each mutant contains a stable 1,589 bp insertion at a known location. Inserts were integrated at either GATC or CATG sites. The location and orientation of particular insertions can be viewed on our website (www.remi-seq.org). The GWDI insert encompasses the blasticidin resistance gene, *bsr*, and two defined borders. Each border incorporates:

- a I-SceI restriction enzyme recognition site - this sequence does not appear anywhere in the *Dictyostelium* genome;
- a MmeI recognition site. MmeI is an unusual Type II enzyme that cuts 20/18 bp outside the recognition sequence and captures a portion of genomic DNA;
- a unique 6 bp index that identifies not only which insert but also which arm, thereby defining the orientation of the insert;
- primer binding sites;
- either GATC- or -CATG sticky ends.

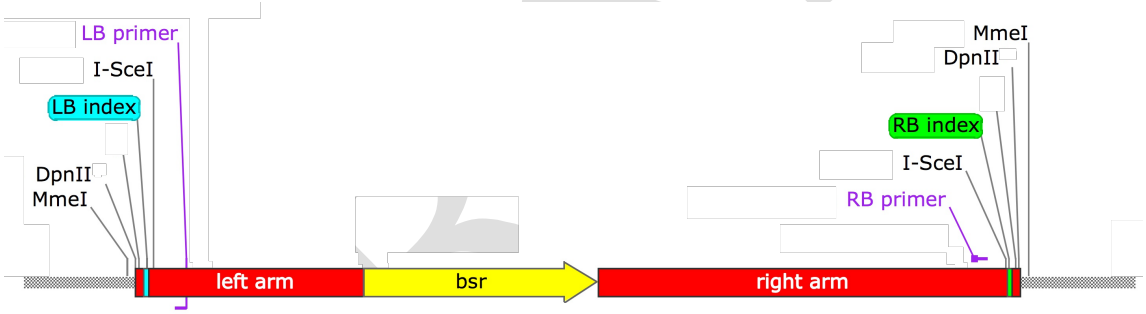
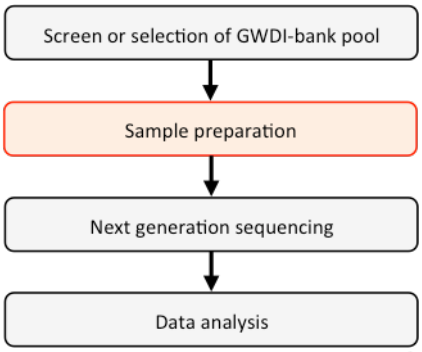


Figure 1. The GWDI insert.

***** All GWDI-bank mutants are in the AX4 background. *****

Introduction

This handbook contains the protocols that will allow you to prepare a sample for next generation sequencing from GWDI-bank cells. Next generation sequencing is essential for genome level screening strategies. Data analysis reveals the presence and abundance of variants.



Sample preparation involves the capture of DNA bordering the insertion. This flanking sequence is sufficient to define the location of the insertion in the genome in the majority of cases. Different samples are distinguished by labelling with different adapter combinations.

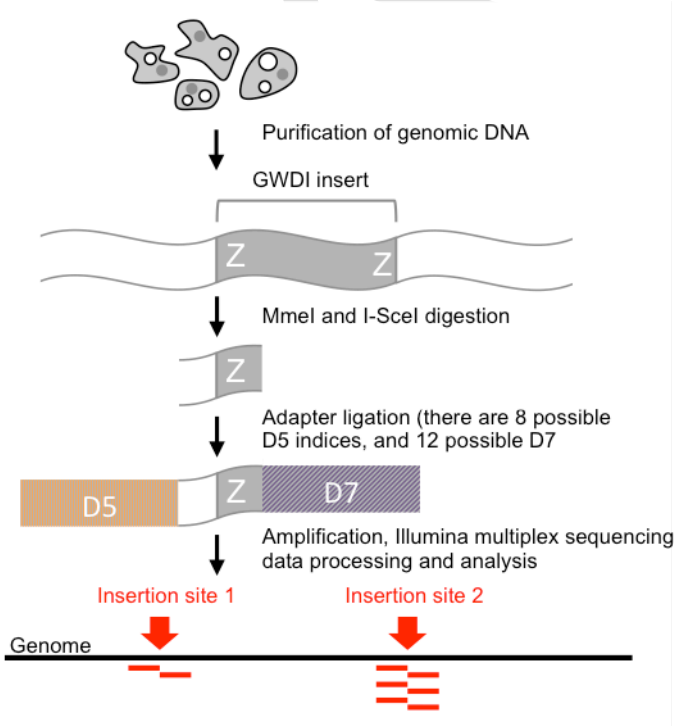


Figure 2. Purified genomic DNA is digested by MmeI and I-SceI. MmeI cuts 20/18 bp outside of its recognition sequence thereby capturing 19 bp of genomic DNA. Adapters (D7 and D5 are annealed to either end of the target fragment prior to amplification via PCR. Two size selection steps are used to remove unwanted genomic DNA.

Important Notes

Starting material

The starting material can either be cells collected from clearing plates that have grown in the presence of bacteria as a food source, or cells grown axenically in shaking suspension.

Nuclei must be collected from cells directly and cannot be frozen prior to nuclei collection. Once collected, nuclei can be stored at -80°C indefinitely.

Using these methods

To aid you in completing the protocol each section lists the materials (items that must be prepared), the equipment and the consumables (items purchased for this step). The whole process takes a minimum of 4 days, although 5-8 days is usual due to the number of long incubation steps.

Multichannel and repeat pipettes

If you are running a large number of samples in parallel it can be beneficial to make use of repeating pipettes and multichannel pipettes.

Bottlenecks

A population bottleneck occurs when there is a sharp reduction in the size of a population. Such events can reduce the variation of a population. When setting up your selection/screen it is important to use sufficient cells so as to not limit the abundance of each variant in your pool and generate a bottleneck. Variation will depend on the total number of unique mutants in your pool and the total number of cells in each of your starting cultures. A planning tool is available on our website (www.remi-seq.org) to assist you in experimental set up.

01 Preparation of genomic DNA

01a Preparing clearing plates (optional)

A. *Materials (**per sample**)*
   - *2.5 – 5.0 x10^5 Dicty cells*
   - *300 µl Kleb culture*
   - *1 SM agar plate (see Appendix A: how to make SM agar plates)*

B. *Equipment*
   - *Haemocytometer*
   - *Microscope*
   - *Bench top centrifuge*
   - *Flow cabinet*
   - *Liquid waste container*

C. *Consumables*
   - *1.5 ml microcentrifuge tubes, labelled*
   - *sterile L-shaped spreaders*
   - *paper towel*

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1. Record the cell density of your samples using a haemocytometer
2. For each sample calculate the volume containing a total of 2.5 – 5.0 x10^5 cells. This is the optimum number of cells per clearing.
3. Transfer the calculated volume of cells to 1.5 ml centrifuge tubes.
4. Centrifuge for 2 min at 540 *xg* to collect cells.
5. Discard the supernatant by decanting, dab away excess liquid with clean blue roll and replace with 400 µl *Ka* culture.
6. Resuspend the Dicty cells and spread on a SM agar plate
7. Allow plates to absorb, and incubate at 22°C for 2+ days.

01b Prepare nuclei

A. Materials (per sample)

- *1 cleared plate OR cells grown in liquid culture (start from step 9 and wash 3 times)*
- *400 ml KK2, stored at 4°C*
- *10 ml nuclei buffer, stored at 4°C* ****** add DTT just prior to use ******
    i. *add 500 ul of 1M DTT for 100 ml nuclei buffer*
    ii. *add 900 ul of 1M DTT for 180 ml nuclei buffer*

B. Equipment

- *Bench-top centrifuge with spin-out rotor for 50 ml tubes, cooled*
- *Racks for 50 ml tubes*
- *2 ice buckets*
- *Flat-ended spatula*
- *70% EtOH for cleaning spatulas*
- *5 L jug for collecting waste*
- *100 or 250 ml Duran bottle for nuclei buffer with DTT*

C. Consumables

- *50 ml centrifuge tubes, labelled (1 per sample)*
- *Blue roll*

Preparation (do this the day before):

- *Check the materials, equipment and consumables are available and booked.*
- *Label the 50 ml tubes (1 per sample)*
- *Add 35 ml KK2 to each of the 50 ml tubes, store at 4°C*

Do not process more than 24 samples at any one time.

8. Once the surface of the agar plates becomes clear after 2 days (wait for 3 days if less than 75% of the plate is translucent), scrap cells from the surface and transfer to a 50 ml falcon containing 35 ml cold KK2 using the flat end of a spatula – take care not to carry over any agar. Shake tube to resuspend cells
9. Collect cells by centrifugation at 540 *xg*, 2 minutes, 4°C
10. Decant supernatant carefully
11. Wash cells a total of ≥6 times with KK2 to remove *Ka.* Continue washing until the supernatant becomes clear
12. Resuspend the cells thoroughly in 10 ml nuclei buffer (**containing DTT**)
13. Centrifuge at 4,000 *xg* for 20 minutes at 4°C, or 2,500 *xg* for 45 minutes at 4°C.
14. Discard the supernatant and tap tubes upside down on clean blue roll to remove excess liquid. If pellets are not white repeat previous 2 steps
15. Continue with next stage or store pellets at -80°C.

01c Isolate DNA

A. Materials

- *Nuclei pellets (stored at -80°C)*
- *500 mM EDTA, pH 8*
- *10% SLS*
- *4M ammonium acetate*
- *10 mM Tris-Cl, pH 8.5 (a.k.a. E.B.) with RNase (Ambion® RNase Cocktail™)*

B. Equipment

- *Bench-top microfuge, cooled*
- *Heat block set to 55°C*
- *Beaker for liquid waste*
- *Incubator set to 37°C*

C. Consumables (for 48 preps)

- *Absolute ethanol*
- *70% ethanol*
- *Sterile 1.5 ml microcentrifuge tubes*

Preparation:

- *Check the materials, equipment and consumables are available and booked.*
- *Label 2 sets of 1.5 ml tubes*

Do not process more than 24 at any one time.

16. Thaw nuclei pellets on ice
17. Resuspend pellet to a final volume of 150 µl (X) and concentration of 100 mM EDTA, pH 8.0.
    a. For example, add 75 µl of 200 mM EDTA pH 8.0 if the pellet is approx. 75 µl
    b. Or, 30 ul of 500 mM EDTA pH 8.0 if the pellet is approx. 120 µl
18. Transfer samples to pre-labelled 1.5 ml tubes
19. Add an equal volume of 10% SLS (X: 150 µl), mix well, incubate at 55°C for 20 minutes
20. Add 375 µl 4M ammonium acetate, immediately mix well (volume is 2.5X)
21. Centrifuge at 16K *xg* for 15 min, 4°C
22. While the samples are in the centrifuge add 900 µl of abs. ethanol to the second set of 1.5 ml pre-labelled tubes
23. Transfer 450 µl of supernatant to the corresponding tube containing abs. ethanol taking care not to disturb the pellet
    - Any remaining supernatant can be stored at -20°C as backup
24. Mix well
25. Centrifuge at 16K *xg* for 15 min at 4°C
26. Discard supernatant
27. Wash pellet with 70% ethanol, bounce tube along rack to mobilise and break
28. Centrifuge at 16K *xg* for 10 min
29. Discard supernatant by decanting
30. Pulse briefly to collect remaining supernatant and remove with pipette tip
31. Air dry (37°C works well, check after 10 min, and every subsequent 5 min)
32. Resuspend in 49 µl E.B. and 1 µl RNase, incubate at r.t. to allow pellet to resuspend
33. Centrifuge at 16K x*g* for 1 minute to collect insoluble material
34. Transfer supernatant to fresh tubes
35. Store at -20°C.

01d Quantify DNA

A. *Materials*
   - *gDNA preps*

B. *Equipment*
   - *Gel tank*
   - *Power Pack*
   - *Gel tray and comb*

C. *Consumables*
   - *1x TAE*
   - *5x loading dye*
   - *NEB 1 kb ladder*
   - *Agarose*
   - *0.2 ml tubes*

Note:

- The purpose of this step is to determine the approx. concentration of the DNA preps prior to processing. There are 2 options: either quantify the samples using a Qubit or separate samples via agarose gel electrophoresis.
- Do not use spectrophotometry as degraded RNA interferes with this method
- To quantify via Qubit, follow the instructions in the HS dsDNA assay kit and remember to use the special tubes

36. Pour a 0.8% agarose gel
37. Make up the following master mix and add 8 µl to each tube

	1x	Master mix	Added?
Sample	2 µl	-	
5x loading dye	2 µl	___ µl	
H_2O	6 µl	___ µl	
TOTAL	10 µl	___ x 8 µl	

38. Add 2 µl gDNA to each tube
39. Load onto the agarose gel and add NEB 1 kb ladder (500 ng/lane)
40. Run at 100 V for 45 minutes
41. Photograph, save image to computer

02 Cleaving the insert flanking DNA and adding indexed adapters

02a Digest gDNA with MmeI followed by I-SceI

A. Materials
- *gDNA preps*

B. Equipment
- *Bench-top microfuge*
- *Heat block set to 65°C*
- *Incubator set to 37°C*
- *Rack for 1.5 ml tubes pre-warmed to 37°C*

C. Consumables
- *MmeI (NEB, 2 U/µl) – 5 µl per sample, stored at -20°C*
- *I-SceI (NEB, 5 U/µl) – 5 µl per sample, stored at -80°C*
- *10x CutSmart® Buffer*
- *S-adenosylmethionine (SAM)*
- *1.5 ml microcentrifuge tubes, sterile*

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42. Transfer gDNA to each labelled 1.5 ml tube. Use a total of 500 ng to 1 µg per sample.
43. Set up a master mix according to the following table:

	1x	Master mix	Added?
gDNA (0.5 to 1 µg)	___ µl	-	
10x CutSmart buffer	20 µl	___ µl	
SAM	0.5 µl	___ µl	
H_2O	___ µl	___ µl	
MmeI (25 U/rxn)	5 µl	___ µl	
TOTAL	200 µl	___ x ___ µl	

44. Add the master mix to each sample, and mix
45. Centrifuge tubes briefly to collect sample at base of tube
46. Incubate overnight at 37°C

47. Inactivate MmeI by incubating at 65°C for 20 min in a heat block
48. Pulse tubes briefly to collect sample at base of tube

49. Add 5 µl of I-SceI to each of the samples
50. Mix
51. Pulse tubes briefly to collect sample at base of tube
52. Incubate at 37°C for 6 hours

53. Incubate samples at 65°C for 20 minutes to heat inactivate I-SceI.
54. Samples can now be stored at -20°C, to save time it is useful to add the NaOAc and EtOH at this stage.

02b Ethanol Precipitation

A. Materials
- *Your digest reactions*
- *3 M NaOAc, pH 5.2*
- *Abs. ethanol*
- *70% ethanol*
- *10 mM Tris-Cl, pH 8.5 (a.k.a. E.B.)*

B. Equipment
- *Bench-top microfuge, cooled*
- *Incubator set to 37°C*
- *Ice box*
- *1.5 ml tube rack*

C. Consumables
- *Paper towel*

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55. Prepare a master mix according to the table below:

	1x	Master mix	Added?
Sample	200 µl	-	
3 M NaOAc pH 5.2	20 µl	___ µl	
Abs. ethanol	550 µl	___ ml	
TOTAL	770 µl	___ x 570 µl	

56. To each 200 µl sample add 570 µl of master mix
57. Mix and incubate on wet ice for 30 min (or at -20°C if a break in protocol is required)
58. Centrifuge at 16K *xg* for 30 min at 4°C
59. Decant supernatant and dap rim with clean paper towel to remove excess liquid
60. Add 300 µl 70% EtOH, resuspend pellet by bouncing sealed tubes along rack
61. Centrifuge at 16K *xg* for 15 min at 4°C
62. Decant supernatant, remove excess liquid with clean paper towel from the rim and reseal tubes
63. Pulse briefly to collect residual supernatant
64. Remove the residue supernatant with P200 pipette
65. Air dry for 10-20 min at 37°C until pellets become translucent
66. Resuspend in 50 µl of 10 mM Tris-Cl, pH 8.5 (a.k.a. E.B.)

02c Ligate adapters to digested gDNA

A. Materials
- *Digested samples*
- *Pre-annealed adapters; D701-712 (200 pg/µl) and D501-508 (20 ng/µl)*
  - *Refer to appendix A03 for adapter preparation protocol*
  - *Refer to appendix A04 for adapter sequences*

B. Equipment
- *Incubator, 16°C*
- *Ice box*
- *Bench-top microfuge*

C. Consumables
- *T4 DNA ligase (400 U/µl)*
- *10x T4 DNA ligase buffer*

IMPORTANT: Ensure samples are fully suspended following ethanol precipitation

67. Decide which adapters will be used to identify each sample and record below.
68. If a NextSeq System will be used for sequencing bear in mind the following:
    a. NextSeq Systems use a green laser to sequence G/T and a red laser to sequence A/C. At each cycle at least 1 of 2 nucleotides for each colour channel must be read to ensure proper registration.
    b. Refer to Illumina's Technical Note regarding Low-Plex Pooling Guidelines
    c. Refer to the TruSeq Sample Preparation Guide (Pooling Guidelines).
    d. And lastly, check the specific NextSeq System Guidelines as it uses the new RTA2.0

	Sample name	D5 adapter	D7 adapter
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69. Add 10 µl of the appropriate D7 pre-annealed adapter to each sample according to the record table above
70. Add 10 µl of the appropriate D50x pre-annealed adapter to each sample according to the record table above
71. Thaw reagents fully and prepare the following master mix:

	1x	Master mix	Added?
Sample	50 µl	-	✓
Adapter D7xx (200 pg/µl)	10 µl	-	
Adapter D50x (20 ng/µl)	10 µl	-	
10x ligase buffer	18 µl	___ µl	
H_2O	111 µl	___ µl	
T4 DNA ligase (400 U/µl)	1 µl	___ µl	
TOTAL	200 µl	___ x 130 µl	

72. Add 130 µl of master mix to the 70 µl of sample already present in the tubes
73. Mix well and spin cells briefly to collect sample at base of tube
74. Incubate at 16°C overnight.

03 Removal of competing products

03a PshAI digestion

A. Materials

- *Ligation reactions*
- *Sterile water*

B. Equipment

- *Bench-top microfuge*
- *Incubator set to 37°C*
- *Heat block set to 65°C*
- *Rack for 1.5 ml tubes pre-warmed to 37°C*

C. Consumables

- *PshAI (NEB, 10 U/µl) – 1 µl per sample, stored at -20°C*
- *10x CutSmart® Buffer*
- *1.5 ml microcentrifuge tubes, sterile*

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75. Transfer 40 µl of sample to each labelled 1.5 ml tube.
76. Set up a master mix according to the following table. Make a 10% excess of master mix.

	1x	Master mix	Added?
Sample	40 µl	-	
10x CutSmart buffer	10 µl	___ µl	
H_2O	49 µl	___ µl	
***Psh*AI (25 U/rxn)**	1 µl	___ µl	
TOTAL	100 µl	___ x 100 µl	

77. Add the master mix to each sample, and mix.
78. Centrifuge tubes briefly to collect sample at base of tube.
79. Incubate at 37°C for 3 or more hours.
80. Heat inactivate at 65°C for 20 minutes.

04 Target amplification

Note:

- The end point of this process is **1.3 ml** of **1.8 pM** target DNA that will be loaded onto the MiSeq/NextSeq System.
- Given the target is 183 bp this equates to 280 pg dsDNA, or 0.28 ng.
- Additional sample is required for QC.
- Since very little DNA is required for sequencing the limiting amount of DNA is the amount that can be visualized, excised from a gel and purified, twice.
- It is recommended that at least 500 µl of pooled PCR sample is loaded onto the first size selection gel (see 04b). Depending on the number of samples, the preceding steps should be scaled accordingly bearing in mind that 15 µl per sample will be used to check targets prior to pooling. Use larger reaction volumes for fewer samples.

04a Amplify fragment via PCR

A. Materials
- *Samples post-pshAI digestion (100 µl each)*
- *Primers P5_Illumina and D701_a to D712_a (10 µM)*
- *Sterile water*

B. Equipment
- *Thermocycler*
- *Ice box*
- *Mr Frosty 96-well cold block (stored in -20°C, purple: cold, pink: warm)*

C. Consumables
- *0.2 ml tubes – strips/plates*
- *Lids for 0.2 ml tubes*
- *GoTaq G2 flexi*

To monitor whether all reactions have amplified successfully PCR each sample individually.

81. Add 1 µl of each sample to the appropriate labelled 0.2 ml tubes
82. Add 2.5 µl of the appropriate D7xx_a oligo to each of the reaction tubes (not the pre-annealed double stranded adapter)
83. Set up a master mix as follows:

	1x	Master mix	Added?
Sample	1.0 µl	-	
D7xx_a (10 µM)	2.5 µl	-	
P5 (10 µM)	2.5 µl		
5x flexi buffer	10.0 µl		
$MgCl_2$ (25 mM)	2.0 µl		
dNTPs (40 mM)	1.0 µl		
H_2O	30.75 µl		
GoTaq G2	0.25 µl		
TOTAL	50 µl	___ x 45.5 µl	

84. Add 46.5 µl of master mix to each of the reaction tubes
85. Seal the tubes, mix, and collect sample at base of the tubes by briefly spinning
86. Amplify the product using the following cycling conditions.

Step	Temp.	Time	Note
1	95°C	2 min	
2	95°C	30 sec	Return to step 2 34 times
3	66°C	5 sec	
4	68°C	1 min	
5	68°C	10 min	
6	10°C	∞	

87. After cycling samples can be stored at -20°C.

04b Electrophoresis to check targets

A. Materials
- *PCR samples*
- *Sterile water*

B. Equipment
- *Gel tank*
- *Power pack*
- *Wide-toothed comb*
- *Gel doc*
- *USB stick*

C. Consumables
- *1x TAE*
- *SYBR Safe*
- *NEB low molecular weight ladder*
- *Agarose*

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88. Pour a 2.5% agarose gel
    a. The bands will appear more clear if a comb with wide teeth is used
89. Once set, load 15 µl of PCR into each well
90. Note which sample was added to each well
91. Add 6 µl of low molecular ladder (500 ng/lane)
92. Run for 45 minutes at 100 V, photograph, continue to separate samples for a total of 75 minutes
93. Photograph gel and save images
    a. The intensity of the bands will be used to combine the samples in equal amounts so it's worth getting a good photo which isn't overexposed
94. Transfer images to computer using a USB stick, annotate

95. If any reactions have not generated target then repeat those reactions only and check on gel before proceeding

Note:
The target is 183 bp. Any secondary bands will be removed via size selection

05 Combining samples and size selection

05a Pooling samples

A. *Materials*
   - *Image of the gel from previous step (un-modified)*
   - *PCR samples*

B. *Equipment*
   - *Multichannel pipette*
   - *ImageJ (download for free here)*

C. *Consumables*
   - *1.5 ml low retention microcentrifuge tube*

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96. Upload your gel image to ImageJ.
97. Measure the intensity of each band
98. Calculate the volume of each sample required according to band intensity (less sample is required for brighter PCR samples)
99. Combine the appropriate volume of each sample into a 1.5 ml microcentrifuge tube
    a. If particular samples are considerably less intense than the average the PCR should be repeated and checked via agarose gel electrophoresis
100. Mix and store at -20°C

05b First size selection

A. Materials
- *Pooled PCRs*

B. Equipment
- *Gel tank and comb*
- *Power pack*
- *Gel Doc or Safe Imager™ 2.0 Blue Light Transilluminator*

C. Consumables
- *NuSieve GTG Agarose*
- *SYBR® Safe DNA Gel Stain*
- *Cooled TAE*
- *NEB low molecular weight ladder (500 mg per well)*
- *Loading buffer*
- *Razor blade*
- *1.5 ml microcentrifuge tube*
- *Qiagen **QIAQuick** Gel Extraction Kit*

Preparation

- Cool the 1x TAE buffer to 4ºC
- Tape the teeth of the comb together to make an extra large well.
- Pour a 3.5 % NuSieve GTG agarose gel (50 ml) with SYBR Safe gel stain and store at 4ºC for 30 min or more.

101. Separate the pooled PCR product on a 3.5% NuSieve GTG low melting temperature agarose gel in fresh, cooled TAE buffer
    a. It may be necessary to adjust the volumes of samples. Larger volumes of sample can be separated by loading once as usual, running for 2 min at 80 V, then loading more sample into the same well a second time
    b. Load 6 µl NEB low molecular weight ladder (500 ng)
    c. Run for ~1 hour at 80 V
102. Photograph quickly gel and save image
103. Using the Blue Light Transilluminator excise the 183 bp band and transfer to a microcentrifuge tube
104. Purify the DNA using a Qiagen QIAQuick Gel Extraction Kit and elute in 30 µl E.B.
105. Quantify using a spectrophotometer.

05c Second size selection

A. Materials
- *30 µl sample (pooled PCRs, size-selected once)*

B. Equipment
- *Gel tank and comb with wide teeth*
- *Power pack*
- *Gel Doc*
- *Safe Imager™ 2.0 Blue Light Transilluminator*

C. Consumables
- *NuSieve GTG Agarose*
- *SYBR® Safe DNA Gel Stain*
- *Cooled TAE*
- *NEB low molecular weight ladder (500 ng per well)*
- *Loading buffer*
- *Razor blade (1 per sample)*
- *1.5 ml microcentrifuge tube (1 per sample)*
- *Qiagen Gel Extract **MinElute** Kit*

Preparation
- Cool the 1x TAE buffer to 4ºC
- Pour a 3.5 % NuSieve GTG agarose gel (50 ml) with SYBR Safe gel stain and store at 4ºC for 30 min or more.

106. Separate the pooled PCR product on a 3.5% NuSieve GTG low melting temperature agarose gel in fresh, cooled TAE buffer
    a. Add loading buffer to the
    b. Load 500 ng of NEB low molecular weight ladder
    c. Run for 1 hour at 80 V
107. Quickly photograph gel and save image
108. Using the Blue Light Transilluminator excise the 183 bp band and transfer to a microcentrifuge tube
109. Purify the DNA using a Qiagen MinElute Gel Extraction Kit
    - (https://www.qiagen.com/gb/products/catalog/sample-technologies/dna-sample-technologies/dna-cleanup/minelute-gel-extraction-kit/)

06 Sample QC and sequencing

06a Quantification

A. Materials
- *DNA sample (Pooled and size-selected twice)*

B. Equipment
- *Qubit*

C. Consumables
- *Qubit® dsDNA HS Assay Kit (Standards are stored at 4°C)*
- *0.5 ml Qubit tubes*

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110. Dilute samples and standards with reagent (r.t.) as described
    - Remember to use the relevant assay tubes
111. Read on a Qubit

06b Transferring samples to the sequencing facility

A. Materials
- *Sample (pooled, size-selected twice, quantified)*

B. Equipment
- *none*

C. Consumables
- *Paper towel*
- *Sealable bag*
- *Nescofilm*
- *Padded envelope (if posting)*
- *30 ml universal (if posting)*

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112. Transfer the sample to a fresh 1.5 low retention tube and label clearly
113. Seal with Nescofilm, and if posting place inside a 30 ml universal and to prevent it from being squashed
114. Put the sample into a re-sealable bag with blue roll to collect any spillage
115. Label with the following information:
    a. Your name and date
    b. Sample name
    c. Concentration determined by Qubit
    d. Your email address if this is your first sample
116. Deliver the sample, and if posting inform recipient of the expected arrival time.

Note

- The NextSeq system requires 1.3 ml of 1.8 pM sample
- Given the target is 183 bp this equates to ~280 pg dsDNA, or 0.28 ng
- To convert ng/ul and nM:
    - http://www.molbiol.edu.ru/eng/scripts/01_07.html
- Calculator for determining the number of copies of a template
    - http://cels.uri.edu/gsc/cndna.html

- The target length is most often 183 bp, however occasionally MmeI cuts one base closer or further from the recognition site which will generate products of 182 and 184 bp. NEB have observed that MmeI cuts one base further from the recognition site in up to 40% or cases. They suggest this is due to the structure of the DNA between the recognition site and the cut site.
- Prior to sequencing the sample will by analysed via Tapestation, dPCR and qRT-PCR
- Sequencing results can be accessed via Illumina's BaseSpace

Secondary bands

It is usual to see additional bands following amplification via PCR since the whole genomic DNA is used. The desired band is 183 bp. 2x size selection is sufficient to enrich your sample for sequencing

Is the insertion the only change in the genome?

The mutants have been grown for 4-8 weeks in the lab. Secondary mutants are possible. Any phenotype should be confirmed by a secondary allele or recapitulation.

Appendices

Appendix A: SM agar plates

A. Materials
- *SM agar powder (Formedium, Ref# SMA0102)*

B. Equipment
- *500 ml Duran bottles*
- *Autoclave*
- *Woolly gloves*
- *Lab gloves (1 size larger than usual to fit over woolly gloves)*

C. Consumables
- *9 cm petri dishes*

N.B. one bottle (400 ml) will make 8 SM agar plates

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1. Add 16.7 g pre-mix powder to a 500 ml Duran bottle.
2. Add dH_2O to 400 ml.
3. Label bottle. Repeat 1 to 3 if necessary.
4. Sterilise by autoclaving. Two bottles can be sterilized at a time using the bench top autoclave (121°C for 11 min).
5. Once sterilized transfer to 55°C water bath until media has become clear and ready to pour. Media can be poured while warmer if isolated gloves are used.
6. Arrange petri dishes (9 cm) in stacks of 4.
7. Starting from the bottom of each stack pour 50 ml agar into each petri dish; 8 petri dishes per 400 ml bottle. Each dish will be more than 2/3rd full.
8. Once set, plates can either be used immediately or stored at 4°C.

[Final]	Component	Amount	Added?
40 mM	Tris, pH 7.8	40 ml of 1M	
1.5%	Sucrose	15 g	
0.1 mM	EDTA	200 µl of 0.5 M EDTA	
6 mM	$MgCl_2$	1.22 g of $MgCl_2.6H_20$	
40 mM	KCl	2.98 g	
	H_2O	To 1 L	
Mix the above components then autoclave. Once cooled continue.			
0.4%	NP40 substitute	4 ml **(ADD AFTER AUTOCLAVING AND COOLING)**	
5 mM	DTT (Stored at minus 20°C)	Add 500 µl of 1 M per 100 ml directly prior to use.	***Add immediately prior to use***

- Combine the first 6 components in a 1 L Duran
- Label and autoclave
- Once cooled add the NP40 substitute, mix well and mark on label
- Store and use at 4°C
- Prior to use add DTT to the required amount of nuclei buffer. Discard any excess nuclei buffer following procedure.

Note

- 1 M DTT is stored at -20°C in 1 ml aliquots

Appendix C: Preparation of adapters

A. Materials
- *Complementary 100 µM oligos, desalted*

B. Equipment
- *Thermocycler or heat block (do not a thermocycler unless it is capable of a ramp rate of 0.02 °/sec)*

C. Consumables
- *0.2 ml tubes (if using a thermocycler)*
- *1.5 ml tubes (if using a heat block)*
- *10x T4 DNA ligase buffer (NEB)*

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1. Order oligos from IDT-DNA, desalted and 100 nM scale
   - The 5' ends of 'a' are outward facing
   - The 5' ends of 'b' are inward facing and ligate to the gDNA
2. Suspend oligos to 100 µM in dH_2O
3. Mix oligos 'a' and 'b' according to the tables below resulting in a concentration of 400 ng/µl.

For D7 adapters		1x	Added?
D7xx_a (100 µM)	20.7 µg	9.6 µl	
D7xx_b (100 µM)	19.3 µg	9.6 µl	
H_2O		80.8 µl	
TOTAL	**40 µg**	**100 µl**	

For D5 adapters		1x	Added?
D50x_a (100 µM)	20.0 µg	9.1 µl	
D50x_b (100 µM)	19.9 µg	9.1 µl	
H_2O		81.8 µl	
TOTAL	**40 µg**	**100 µl**	

4. Dilute 'a + b' according to the following table. This will give a final concentration of 20 ng/µl in 1x ligase buffer.

	1x	Master mix	Added?
'a + b'	5 µl	____ µl	
10x ligase buffer	10 µl	____ µl	
H_2O	85 µl	____ µl	
TOTAL	**100 µl**	**____ x 100 µl**	

5. Using a thermocycler run the samples on the following programme:
   - Lid temp: 104°C
   - 85°C, 10 min
   - 10°C, pause (ramp rate set to 0.02 °/sec)
6. Alternatively, using a heat block:
   - Incubate at 85°C for 10 min
   - Remove the block from the incubator and transfer to the bench
   - Incubate at r.t. for more than 1 hour

7. Dilute D7 pre-annealed adapters from 20 ng/µl to 200 pg/µl in 1x T4 DNA ligase buffer
8. Store at -20°C

D501_a	AATGATACGGCGACCACCGAGATCTACAC**TATAGCCT**ACACTCTTTCCCTACACGACGCTCTTCCGATCTN
D502_a	AATGATACGGCGACCACCGAGATCTACAC**ATAGAGGC**ACACTCTTTCCCTACACGACGCTCTTCCGATCTN
D503_a	AATGATACGGCGACCACCGAGATCTACAC**CCTATCCT**ACACTCTTTCCCTACACGACGCTCTTCCGATCTN
D504_a	AATGATACGGCGACCACCGAGATCTACAC**GGCTCTGA**ACACTCTTTCCCTACACGACGCTCTTCCGATCTN
D505_a	AATGATACGGCGACCACCGAGATCTACAC**AGGCGAAG**ACACTCTTTCCCTACACGACGCTCTTCCGATCTN
D506_a	AATGATACGGCGACCACCGAGATCTACAC**TAATCTTA**ACACTCTTTCCCTACACGACGCTCTTCCGATCTN
D507_a	AATGATACGGCGACCACCGAGATCTACAC**CAGGACGT**ACACTCTTTCCCTACACGACGCTCTTCCGATCTN
D508_a	AATGATACGGCGACCACCGAGATCTACAC**GTACTGAC**ACACTCTTTCCCTACACGACGCTCTTCCGATCTN
D501_b	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT**AGGCTATA**GTGTAGATCTCGGTGGTCGCCGTATCATT
D502_b	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT**GCCTCTAT**GTGTAGATCTCGGTGGTCGCCGTATCATT
D503_b	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT**AGGATAGG**GTGTAGATCTCGGTGGTCGCCGTATCATT
D504_b	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT**TCAGAGCC**GTGTAGATCTCGGTGGTCGCCGTATCATT
D505_b	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT**CTTCGCCT**GTGTAGATCTCGGTGGTCGCCGTATCATT
D506_b	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT**TAAGATTA**GTGTAGATCTCGGTGGTCGCCGTATCATT
D507_b	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT**ACGTCCTG**GTGTAGATCTCGGTGGTCGCCGTATCATT
D508_b	AGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT**GTCAGTAC**GTGTAGATCTCGGTGGTCGCCGTATCATT
D701_a	CAAGCAGAAGACGGCATACGAGAT**CGAGTAAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D702_a	CAAGCAGAAGACGGCATACGAGAT**TCTCCGGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D703_a	CAAGCAGAAGACGGCATACGAGAT**AATGAGCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D704_a	CAAGCAGAAGACGGCATACGAGAT**GGAATCTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D705_a	CAAGCAGAAGACGGCATACGAGAT**TTCTGAAT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D706_a	CAAGCAGAAGACGGCATACGAGAT**ACGAATTC**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D707_a	CAAGCAGAAGACGGCATACGAGAT**AGCTTCAG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D708_a	CAAGCAGAAGACGGCATACGAGAT**GCGCATTA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D709_a	CAAGCAGAAGACGGCATACGAGAT**CATAGCCG**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D710_a	CAAGCAGAAGACGGCATACGAGAT**TTCGCGGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D711_a	CAAGCAGAAGACGGCATACGAGAT**GCGCGAGA**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D712_a	CAAGCAGAAGACGGCATACGAGAT**CTATCGCT**GTGACTGGAGTTCAGACGTGTGCTCTTCCGATCTTTAT
D701_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ATTACTCG**ATCTCGTATGCCGTCTTCTGCTTG
D702_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TCCGGAGA**ATCTCGTATGCCGTCTTCTGCTTG
D703_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CGCTCATT**ATCTCGTATGCCGTCTTCTGCTTG
D704_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GAGATTCC**ATCTCGTATGCCGTCTTCTGCTTG
D705_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**ATTCAGAA**ATCTCGTATGCCGTCTTCTGCTTG
D706_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**GAATTCGT**ATCTCGTATGCCGTCTTCTGCTTG
D707_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CTGAAGCT**ATCTCGTATGCCGTCTTCTGCTTG
D708_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TAATGCGC**ATCTCGTATGCCGTCTTCTGCTTG
D709_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**CGGCTATG**ATCTCGTATGCCGTCTTCTGCTTG
D710_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TCCGCGAA**ATCTCGTATGCCGTCTTCTGCTTG
D711_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**TCTCGCGC**ATCTCGTATGCCGTCTTCTGCTTG
D712_b	AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**AGCGATAG**ATCTCGTATGCCGTCTTCTGCTTG
P5_Illumina	AATGATACGGCGACCACCGA

Notes

Further Information

You can find further information from the following sources:

- The publication
- Our website www.remi-seq.org
- DictyBase and the Dicty Stock Centre www.dictyBase.org
- The Dicty mailing list