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RescueMu Protocols for Maize Functional Genomics

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Summary

RescueMu is a modified *Mu1* transposon transformed into maize to permit mutagenesis and subsequent recovery of mutant alleles by plasmid rescue. *RescueMu* elements insert late in the germline as well as in terminally dividing somatic (e.g., leaf) cells. Germinal insertions may result in a mutant phenotype, and *RescueMu* permits recovery of 5–25 kb of transposon-flanking genomic DNA without having to construct and screen genomic DNA libraries. Late somatic insertions of *RescueMu* do not result in a visible phenotype, but they are instead used to construct plasmid libraries of gene-enriched maize genomic DNA to facilitate the identification and sequencing of the euchromatic portion of the maize genome. This is because maize leaves contain abundant independent *RescueMu* somatic insertions, and 70–90% of these insertions occur preferentially into genes and not repetitive DNA. This chapter describes detailed protocols on how to obtain, generate, and use *RescueMu* for maize genomics, including resources developed by the Maize Gene Discovery Project (MGDP) consortium available online at ZmDB.

Key Words

Mutator, *RescueMu*, maize, genomics, transposon, genome survey sequence, plasmid rescue, techniques

1. Introduction

Mutator (*Mu*) is a large DNA transposon family in maize (see refs. 1,2 for reviews). Traditionally, *Mu* has been used to create novel mutants randomly in the search for new genes (forward mutagenesis) and to create saturating populations of transposon insertions useful for reverse-genetics screens. This is due to several factors: first, 70–90% of *Mu* elements insert into genes (3), not into the repetitive DNA fraction which constitutes >80% of the maize genome (4). Second, heritable *Mu* insertions occur late in germinal cells resulting in sibling progeny that carry independent insertions. *Mu* elements insert at a high fre-

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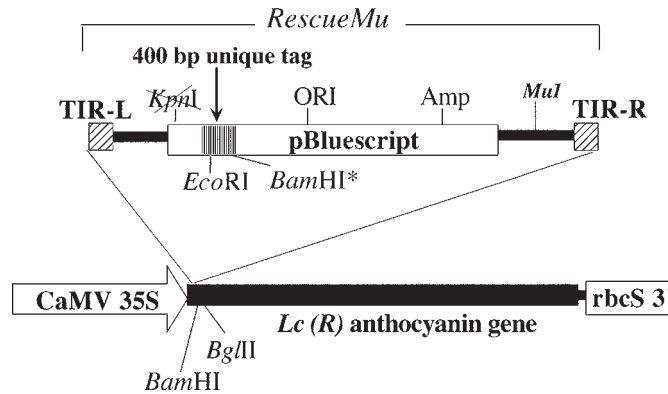


Fig. 1. Structure of the *RescueMu* vector. *RescueMu* consists of a plasmid inserted into an intact *Mu1* nonautonomous element. *RescueMu* is inserted downstream of a CaMV 35S promoter in the 5' untranslated leader of maize *Lc* (*Leaf Color*) a transcription factor of the *R* family required for anthocyanin production. Excision of *RescueMu* can restore tissue pigmentation. Two elements, *RescueMu2* and *RescueMu3*, differ by the presence of unique 400 bp heterologous tags of *Rhizobium* DNA, and both are present in the original *RescueMu* transgenic lines. The asterisk indicates that the internal *BamHI* site is present in *RescueMu3*, but absent in *RescueMu2*.

quency ($10^{-6} - 10^{-4}$ per locus per generation), to both linked and unlinked loci where they remain stable and transmissible through the germline. A mutant caused by a *Mu* element rarely ever reverts to wild-type. In contrast, maize *Ac/Ds* elements and *En/Spm* elements insert stochastically during maize development, preferentially insert within a 5 cM region of the donor site and may excise in subsequent generations (reviewed in ref. 1). Finally, because inherited *Mu* elements are not lost and continue to duplicate, they amplify over generations, up to hundreds of copies per plant, unlike *Ac/Ds* transposons that are inhibited by a negative feedback transposition control mechanism. Thus, random gene-targeted *Mu* amplification permits saturation mutagenesis.

Each member of the *Mu* element family is defined as sharing a common approx 215 bp terminal inverted repeat (TIR) to which the *Mu* transposase binds (reviewed in ref. 1). *MuDR* is a 4.9-kb *Mu* element that encodes two proteins required for transposition. The *Mutator* family was likely created by internal deletion and recombination of *MuDR* resulting in at least eight non-protein-coding subfamilies of smaller transposons (*Mu1-Mu8*), which are incapable of autonomous transposition, but may transpose in the presence of a functional *MuDR* element.

RescueMu2 and *RescueMu3* (Fig. 1) are modified *Mu1* elements into which high-copy number bacterial plasmids conferring ampicillin resistance were

inserted (3). They differ only by the presence of an internal 400-bp sequence tag derived from *Rhizobium*. These plasmids were stably co-transformed with the pAHC20 plasmid into maize by biolistic transformation. pAHC20 is a plasmid encoding *bar*, which is a selectable marker gene that confers resistance to the herbicide glufosinate/Basta (5). *RescueMu* transgenic lines must be crossed to an active *MuDR* line to transpose (3).

RescueMu was constructed to accelerate the discovery and characterization of *Mu*-mutagenized genes underlying mutant phenotypes of interest. Plasmid rescue can now be used to recover 5–20 kb of *Mu* element flanking DNA in plasmid form ready for DNA sequencing in only a few days (3), instead of having to construct a genomic library from a mutant plant.

In addition to germinal insertions, research using *RescueMu* uncovered that *Mu* elements also transpose at a very high frequency in terminally dividing somatic cells (e.g., leaf cells) (3). Late somatic *RescueMu/Mu* insertions are unlikely to cause a noticeable phenotype, and because they rarely occur in the shoot apical meristem, they are usually not transmitted to the next generation. However, the somatic behavior of *RescueMu* has created a novel resource for the construction of bacterial libraries of euchromatic-rich maize genomic DNA in plasmid form ready for DNA sequencing. This is because *RescueMu* somatic insertions also occur preferentially into genes (3). Read-out DNA sequencing from *RescueMu* elements recovered from a single leaf can rapidly identify significant numbers of independent genes and gene-rich DNA sequence (3). Because of the sensitivity of bacterial transformation and antibiotic selection, *RescueMu* insertions contained in single small leaf sectors can be recovered in *Escherichia coli* from a pool of plant material, filtering out all other maize genomic DNA. These features permit *RescueMu* sequencing to be an alternative to expressed sequence tag (EST) sequencing for gene discovery while offering several unique advantages: unlike EST sequencing, *RescueMu* may be used to find poorly transcribed genes. Second, *RescueMu* may lead to the discovery of large numbers of nontranscribed regulatory regions in maize located near *RescueMu* insertions (3), something not possible by EST sequencing. Finally, *RescueMu* sequencing from both the right and left borders allows more transcribed sequence to be obtained, including complete 5' and 3' untranslated regions. Whereas *RescueMu* plasmids can include up to 25 kb of genomic DNA (3), alternative methods to isolate genomic DNA flanking *Mu* insertions such as thermal asymmetric interlaced polymerase chain reaction (TAIL-PCR) using *Mu* read-out primers (6,7) typically result in <500 bp of readable DNA sequence.

The Maize Gene Discovery Project (MGDP) is a consortium of laboratories headed by Virginia Walbot (Stanford University) that is employing *RescueMu* on a large scale to accelerate the recovery of mutant-causing germinal

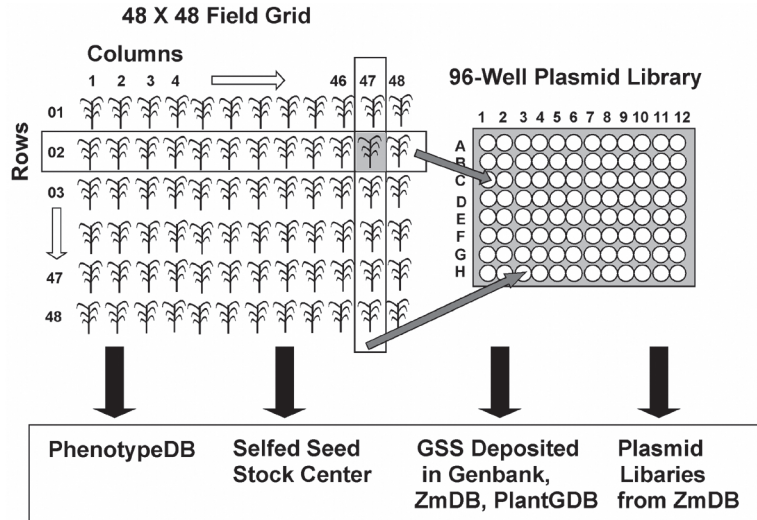


Fig. 2. Summary of *RescueMu* materials available from the MGDP.

RescueMu insertions and to construct libraries of *RescueMu*-mutagenized leaf DNA for maize euchromatic DNA sequencing. The MGDP makes available populations of *RescueMu* mutagenized seed, online descriptions of mutants, and 96-well microtiter plate libraries of recovered *RescueMu* plasmids representing somatic and germinal insertions. Each plate library represents plasmids recovered from a field grid consisting of 48 rows and 48 columns (2304 *RescueMu* plants) (Fig. 2). Each well contains *RescueMu* plasmids recovered from one row or one column (48 plants) in the grid. Each plant in the row or column is sampled by taking leaf punches from a single leaf. However, each plant is sampled twice, one leaf for the row sample and the second leaf for the column sample. If a *RescueMu*-flanking genomic DNA sequence is recovered in both a row and a column of a grid, the logical intersection identifies the single plant in the grid as the donor of the common *RescueMu* allele. Because each row and column are sampled from separate leaves, and because only a germinal insertion would be expected to extend beyond a single leaf, then double-sampling is used to distinguish between the more frequent late somatic insertions (leaf sector) and the rarer germinal insertions (whole plant). The MGDP makes available approx 100–500 bp read-out sequences from these libraries, known as genome sequence surveys (GSSs), which may be queried online at GenBank®, PlantGDB, or ZmDB. For online links, detailed information, or to order materials, the reader is encouraged to visit the Web site of the MGDP, known as ZmDB (www.zmdb.iastate.edu).

The first part of this chapter describes how to generate, recover, and analyze novel *RescueMu* insertions in-house, including: (i) how to obtain and choose *RescueMu* seed stocks; (ii) how to perform *RescueMu* plasmid rescues from maize; (iii) how to select against contaminating plasmids using restriction enzymes and filter hybridization techniques; and (iv) how to read-out and analyze sequence from recovered *RescueMu* elements. In **Subheadings 2.8.** and **3.8.**, I have included additional descriptions on how to request and use materials generated by the MGDG in combination with these basic protocols.

2. Materials

2.1. Selecting *RescueMu* Plant Material to Generate Novel Insertions

1. Glufosinate ammonium/phosphinothricin-tripeptide (PPT)/Basta (Liberty® Herbicide; Aventis Crop Science).
2. Tween® 20.

2.2. Genomic DNA Isolation (see Note 4)

1. Chloroform.
2. Isoamyl alcohol.
3. Isopropanol.
4. 70% (v/v) Ethanol.
5. Water.
6. TE buffer: 10 mM Tris-HCl, pH 8.0, 1 mM EDTA.
7. Prepare plasmid-free CTAB buffer: 100 mM Tris-HCl, pH 7.5/8, 2% (w/v) CTAB, 1.4 M NaCl, 20 mM ethylene diamine tetraacetic acid (EDTA), pH 7.5/8, 1% (v/v) β-mercaptoethanol, 1% (w/v) sodium bisulfite. For 100 mL of CTAB buffer, dissolve CTAB in 60 mL water by heating in a microwave for 20 s and then add other components. Add β-mercaptoethanol just before use. Store at room temperature or 4°C.

2.3. Plasmid Rescue

1. Enzymes needed: *Kpn*I, RNaseA, *Bgl*III, *Eco*RI, T4 DNA ligase (Invitrogen).
2. ElectroMAX DH10B competent cells (>10¹⁰ colony-forming units [cfu]/μg) (Invitrogen or LIFE Technologies).
3. 3 M Sodium acetate.
4. Buffer-saturated phenol, pH 8.0.
5. Chloroform.
6. Isoamyl alcohol.
7. 70% (v/v) Ethanol.
8. Water (plasmid-free).
9. SOC media (Invitrogen or LIFE Technologies).
10. DNA Electroporator and 0.1-cm cuvettes.
11. LB-carbenicillin (100 mg/L).
12. 100-mm Petri plates (see **Note 9**).

2.4. Isolating DNA Fragments for DNA Hybridization Probing of Rescued Colonies

1. Enzymes needed: *Pst*I, *Sac*I, *Xba*I, *Xho*I, *Bsp*HI.
2. Plasmids needed: pR, pBluescript[®] KS (Stratagene), pRescueMu2 and pRescueMu3, pMR15 and pMR17 (see **Note 13**).
3. *RescueMu* probe amplification primers: (i) primer p173+155F GCGAATTC GACAGCCGGCAGGGCATTTC; (ii) T7 primer CGCGTAATACGACTCACT ATAGGGC; and (iii) primer p192+130F TTCCTGCAGCGGCCGCGGATCAG.

2.5. Preparing Filters for Screening of Rescued Colonies

1. Whatman 3 MM filter paper (Whatman).
2. 0.5 M NaOH.
3. 1 M Tris-HCl (pH 7.5).
4. UV cross-linker (e.g., Stratalinker[®]; Stratagene).
5. India ink.
6. Nitrocellulose filters (e.g., NEN Colony/Plaque Screen).
7. 80°C Oven (if using nitrocellulose).

2.6. Confirming RescueMu Insertions Using Colony-Lift Hybridizations

1. Randomprime labeling kit (e.g., DecaPrimeII; Promega).
2. ³²P-λ [dCTP] (2000–3000 Ci/mmol) (Amersham Pharmacia Biotech).
3. NucTrap Push Columns (Stratagene).
4. 2× SSC, pH 7.0: 0.3 M sodium citrate, 0.3 M NaCl.
5. 10% (w/v) Sodium dodecyl sulfate (SDS).
6. 10 mg/mL Salmon sperm DNA.
7. Prehyb buffer: 1% (w/v) SDS, 2× SSC, 10% (w/v) dextran sulfate, 50% deionized formamide, 3× Denhardt's reagent (1% [w/v] Ficoll[®] 400, 1% [w/v] polyvinylpyrrolidone, 1% [w/v] bovine serum albumin [Fraction V; Sigma]).

2.7. Analyzing and Sequencing of RescueMu Plasmids

1. Enzymes needed: *Kpn*I, *Hind*III, *Eco*RI.
2. Sequencing primers: *Mu3*-R TGCTGTCTTGTGTCCGTTTAA and *Mu3*-L AGCTGTCTCGTATCCGTTTTG.

2.8. Requesting RescueMu MGDG Materials

1. 96-Well plates of *RescueMu* plasmids, each recovered from a field grid of 48 × 48 plants, may be purchased for \$150 US dollars at (www.zmdb.iastate.edu). Click on Order Materials, then follow the Library Plate link.
2. Pictures and descriptions of visible mutants in each MGDG *RescueMu* field grid may be found at the ZmDB Maize Phenotype Database (PhenotypeDB) at (<http://www.zmdb.iastate.edu/zmdb/phenotypeDB/index.htm>). Selfed seed from these grid plants are available from the Maize Genetics Cooperation Stock Center (<http://w3.ag.uiuc.edu/maize-coop/mgc-home.html>) (see **Note 1**). Send an e-mail

to maize@uiuc.edu indicating the *RescueMu* field grid letter, row, and column numbers.

3. To screen *RescueMu* 96-well plasmid libraries by PCR to search for an insertion into sequence of interest (reverse genetics), the right-side *RescueMu* read-out primer (*Mu1-R*) is 5'-TAT TTC GTC GAA TCC GCT TCT-3', and the left-side read-out primer (*Mu1-L*) is 5'-CAT TTC GTC GAA TCC CCT TCC-3'.

3. Methods

3.1. Selecting *RescueMu* Plant Material to Generate Novel Insertions

1. Request and select active *RescueMu* seeds (see **Notes 1, 2, and 3**).
2. Confirm the presence of the *RescueMu* transgene via its linkage to plasmid pAHC20 (5), which encodes resistance to the herbicide glufosinate/PPT/Basta. To test for herbicide resistance, a 5-cm-diameter circle is made using a black marker onto a leaf, which is then painted with 0.75% (v/v) glufosinate ammonium (Liberty Herbicide, 18% [v/v] solution) containing 0.1% (v/v) Tween 20 using a Q-tip. Only plants that are non-necrotic, 5–7 d after herbicide application, should be used.

3.2. Genomic DNA Isolation

1. Using plasmid-free solutions (see **Note 4**), isolate genomic DNA, preferably from young leaves 1–5, using the urea extraction method (8) or the CTAB method below (9). Both methods work well.
2. Grind 0.1–0.3 g of tissue to a fine powder in liquid nitrogen using a mortar and pestle.
3. Add tissue to a 2-mL Eppendorf® tube containing 0.9 mL of CTAB buffer.
4. Vortex mix sample briefly and keep on ice until all samples are ground.
5. Incubate the tubes at 60°C for 30 min, then cool at room temperature 10 min.
6. Add 1 vol chloroform:isoamyl alcohol (24:1) and invert tubes continuously for 5 min.
7. Centrifuge the tubes 5 min in a microcentrifuge at >14,000g, then remove the upper aqueous phase to a clean 2-mL Eppendorf tube.
8. Repeat **steps 6 and 7**. Transfer the upper, aqueous phase to a 1.5-mL Eppendorf tube.
9. Add 1 vol isopropanol and invert the tubes gently until the DNA precipitates.
10. Either spool the DNA with the curled-by-flaming tip of a sterile Pasteur pipet or minicentrifuge for 2 min at >14,000g.
11. Resuspend the DNA in 1 mL of 70% (v/v) ethanol. Incubate at room temperature 20 min.
12. Centrifuge the tube at >14,000g for 15 s, then air-dry the pellet.
13. Dissolve the DNA in 50–200 µL of TE. Incubate overnight at 4°C to dissolve.
14. Store at –20°C until next step.

3.3. Plasmid Rescue

1. Digest 10 μg of genomic DNA with 50 U of *Kpn*I in the presence of RNaseA in a vol of 150 μL , for 90 min at 37°C (see **Notes 5** and **11**).
2. Add 150 μL of phenol:chloroform:isoamyl alcohol (25:24:1), mix by inversion, microcentrifuge at >14,000g, remove the upper aqueous phase to a fresh tube. Repeat once (see **Note 6**).
3. Add 100 μL of chloroform, mix by inversion, microcentrifuge at >14,000g, and remove upper aqueous phase to a fresh tube.
4. To precipitate the DNA, add one-tenth vol of 3 M sodium acetate, mix by tapping, then add 2.5 vol of 95% ethanol.
5. Centrifuge for 20 min at >14,000g at 4°C.
6. Wash the pellet with 1 vol 70% (v/v) ethanol, then air-dry.
7. Dissolve in >20 μL water.
8. An optional *Bg*III selection step (see **Note 3**) is performed as follows: digest DNA with 30 U of *Bg*III in a final vol of 100 μL for 1 h at 37°C. Extract once with 1 vol of phenol:chloroform:isoamyl alcohol (25:24:1), then once with 1 vol chloroform as in **steps 2** and **3**. Ethanol precipitate and wash with 70% (v/v) ethanol as in **step 4**, but dissolve the final DNA pellet in >50 μL water.
9. Self-ligate at 14°C for 16 h with 10 U of T4 DNA ligase and 100 μL of fresh 5 \times ligation buffer (Invitrogen or LIFE Technologies) in a final vol of 500 μL (see **Notes 7** and **10**).
10. Extract the ligation mixture twice with 500 μL of phenol:chloroform:isoamyl alcohol (25:24:1) and once with 500 μL of chloroform as in **steps 2** and **3**.
11. Precipitate the DNA by adding one-tenth vol of 3 M sodium acetate, mix by tapping, then add 1 vol isopropanol. Invert.
12. Centrifuge 20 min, 14,000g, 4°C. Wash the pellet with 500 μL of 70% (v/v) ethanol and air-dry.
13. Dissolve the pellet in 10 μL water.
14. For each sample, aliquot 1 mL of SOC medium in a 3 to 10-mL tube.
15. For electroporation, thaw 30–50 μL of ElectroMAX DH10B cells (>10¹⁰ cfu/ μg DNA) in an ice slurry exactly according to the manufacturer's recommendations (see **Notes 8** and **10**).
16. As the cells are thawing, aliquot 2 μL of DNA (approx 1 μg) per sample in a separate Eppendorf tube and chill on ice (see **Note 10**).
17. When the cells are thawed, aliquot 30–50 μL of cells in each tube containing the DNA and incubate on ice >1 min.
18. Just prior to each electroporation, pipet up the SOC media in a Pasteur pipet, ready for pipeting into the cuvette immediately after electroporation. A delay of only 20–30 s in the addition of SOC causes a significant decrease in transformation efficiency.
19. Electroporate exactly according to the instructions accompanying the competent cells. For a Bio-Rad device, cells are placed in a 0.1-cm gap disposal cuvette (Bio-Rad) set at 100 ohms, 2.5 kV, 25 μF , then discharged (time constant approx 2.3).

20. Immediately add 1 mL of SOC media into the cuvette, pipet up and down gently once, then remove into the 3 to 10-mL tube.
21. Shake at 37°C for 1 h at 225–300 rpm to allow expression of the antibiotic resistance gene.
22. To concentrate the cells, aliquot the SOC bacterial media into a 1.5-mL Eppendorf tube, and microcentrifuge for 5 s at 14,000g at room temperature.
23. Remove the SOC and gently resuspend in 200 μ L of fresh SOC.
24. Plate 20 and 180 μ L of cells onto ampicillin–carbenicillin-containing LB plates (see **Notes 9** and **11**).

3.4. Isolating DNA Fragments for DNA Hybridization Probing of Rescued Colonies (see Note 12)

1. The *RescueMu2*-specific probe is obtained as a 520-bp *XhoI*-*XbaI* fragment from pMR15 (see **Note 13**).
2. The *RescueMu3*-specific probe is obtained as a 478-bp *XhoI*-*SacI* fragment from pMR17.
3. Alternatively, PCR may be used to amplify *RescueMu2* and *RescueMu3* probes. To amplify *RescueMu2*, use 5' primer p173+155F and the 3' T7 primer. To amplify *RescueMu3*, use the 5' primer p192+130F and the 3' T7 primer 3. PCR cycle conditions are 94°C for 45 s, 50°C for 45 s, and 72°C for 60 s (30–35 cycles) in the presence of 2 mM MgCl₂. PCR products should be purified on an agarose gel.
4. Instead of using *RescueMu*-specific probes to detect new *RescueMu* insertions, an ampicillin probe may also be used. It is isolated as a 1-kb *BspHI* fragment from pBluescript KS+ and will detect both *RescueMu* plasmids.
5. Cauliflower mosaic virus (CaMV) 35S and maize *R(Lc)* probes should also be isolated to be used to screen against the recovery of the original *Lc::RescueMu* alleles after plasmid rescue (see **Note 12**). The CaMV 35S probe extends from +7072 to +7565 (**10**) and is isolated as a *XbaI*-*PstI* fragment from plasmid pR (**11**). The maize *R(Lc)* probe is isolated as an approx 800-bp *PstI* fragment from pR (see **Note 13**).

3.5. Preparing Filters for Screening of Rescued Colonies

1. This is the Grunstein-Hogness method (**12**).
2. Chill bacterial plates at 4°C for >1 h.
3. Lay out 4 pieces of cellophane (each >15 \times 15 cm). Label 1, 2, 3, and 4. Place a square of Whatmann 3 MM blotting paper (>10 \times 10 cm) beside, though not touching, each piece of cellophane. Have a timer ready for each of the four stations. Have a bottle of India ink with a gauge needle ready.
4. Pipet 1 mL of 0.5 M NaOH onto each of cellophane 1 and 2, and 1 mL of 1 M Tris-HCl buffer (pH 7.5) onto each of cellophane 3 and 4.
5. Use forceps to place a dry piece of nitrocellulose membrane onto each bacterial plate, one at a time. Wait 2 to 3 min. During this time, use a unique dot pattern and stab the membrane and LB with the India ink. This will be used to orient the

X-ray film after hybridization with the bacterial plates to pick positive *RescueMu* clones.

6. Transfer the filter onto cellophane 1 directly onto the pool of NaOH, colony-side facing up. Incubate 2 min to lyse the cells.
7. Transfer onto cellophane 2 and again incubate for 2 min as in **step 6**. Briefly blot onto Whatmann 3 MM paper to remove excess NaOH.
8. Transfer filter onto cellophane 3, directly onto solution of 1 M Tris-HCl, colony-side up. Incubate 2 min. Briefly blot onto Whatmann paper.
9. Transfer onto cellophane 4 and repeat as in **step 8**. Blot onto Whatmann 3 MM paper.
10. Immobilize DNA by UV cross-linking using manufacturer's recommendations, then place in an 80°C oven for 2 h. Store in a dry place until needed. Store the LB plates at 4°C.

3.6. Confirming *RescueMu* Insertions Using Colony-Lift Hybridizations

1. Prepare 10–50 ng of radioactive probe DNA using a random prime labeling kit (e.g., DecaPrimeII) and ^{32}P - γ [dCTP]. Incubate at 37°C for >3 h, and then purify on a NucTrap push column to remove unincorporated nucleotides.
2. In the first round of hybridization, to identify plasmid contamination (*see Note 4*), colonies should be hybridized to a mixture of the two *RescueMu*-specific probes to confirm colony identity as described below.
3. Filters should be wetted in 2× SSC for 1 min, then prehybridized in Prehyb buffer in the presence of 0.1 mg/mL single-stranded DNA (prepared by boiling a 10 mg/mL stock of salmon sperm DNA for 5 min, then quick-chilled on ice). The filters should be incubated for 30 min to 24 h at 42°C in a shaking tupperware container or hybridization oven.
4. Following prehybridization, radiolabeled probe should be denatured by boiling for 5 min with 50% (v/v) formamide, then quick-chilled on ice. The denatured probe should be added directly to the filters in Prehyb buffer, and hybridization carried out for 16–24 h at 42°C.
5. The hybridization solution should be removed and the filters washed in 0.2× SSC/0.1% (v/v) SDS at 65°C (100–500 mL/10 filters) for 15 min, with 2 changes of wash buffer. The filters should be wrapped in cellophane paper and exposed to X-ray film for 6–24 h.
6. Using the India ink markings on the filters, the X-rays should be marked, allowing them to be aligned with each original LB plate.
7. Positive colonies from the first hybridization screen should be picked with sterile toothpicks, arrayed on duplicate LB plates (50–100/100-mm-diameter LB plate) and numbered. The plates are then incubated overnight at 37°C.
8. This entire procedure (**steps 1–7**) should be repeated on the duplicate plates of selected positive colonies in order to screen out colonies that represent recovery of the original *RescueMu*/pAHC20 transgene array (*see Note 12*). Colonies from the first plate should be hybridized to a mixture of CaMV 35S- and maize *Lc(R)*-specific probes; colonies from the second plate should be hybridized again to the

mixture of *RescueMu*-specific probes. Colonies that are positive for the *RescueMu* probes but negative for CaMV 35S, and *Lc(R)* should then be chosen for DNA sequencing.

3.7. Analyzing and Sequencing of RescueMu Plasmids

1. As a final check to confirm that the selected colonies represent true *RescueMu* insertions, plasmid DNA should be isolated and digested with *KpnI* and *HindIII*. If a plasmid corresponds to a new insertion, there should be at least one fragment >4.7 kb (*see Note 4*). A comparison of restriction patterns of plasmids recovered from the same plant may be useful in determining if the recovered plasmid represents a somatic or germinal insertion (*see Note 14*).
2. For cleaner sequencing of flanking genomic DNA, plasmids may first be linearized with *EcoRI*, then repurified by ethanol precipitation.
3. For sequencing, the primers are located –122 bp from the outside edge of *RescueMu*. The right TIR out primer is *Mu3-R* and the left border TIR out primer is *Mu3-L*.
4. The first several bases will correspond to *Mu1* TIR sequence, followed by novel sequence. The first 9 bp immediately flanking TIR sequence should be duplicated at both the left and right borders of *RescueMu*, which is a hallmark of *Mu/RescueMu* transposition.

3.8. Using Existing RescueMu MGDG Resources

3.8.1. How to Query MGDG RescueMu Plasmid Library GSS Databases

1. The *RescueMu* GSS collection consists of tens of thousands of partial read-out sequences from recovered *RescueMu* elements, representing both somatic and germinal insertions in pools of maize leaves (**Fig. 2**) (*see Note 15*).
2. Go to (www.zmdb.iastate.edu) and click on the Search ZmDB button.
3. To search for a sequence of interest in the GSS collection, use the ZmDB Basic Local Alignment Search Tool (BLAST). In the new page, specify GSS database, enter the sequence, and then Run BLAST.
4. A ZMDB BLAST Results page will open to indicate if a successful alignment was found.
5. In the Results Summary box, look for the word *RescueMu* under Description. Click on the corresponding sequence name; this will open up a new page.
6. At the bottom of the new page, there will be a box to indicate if the *RescueMu* GSS aligns with maize EST sequences. There will be a second box that indicates which field grid library the GSS was obtained from (e.g., Library 1006 Grid G) and the plant location within the grid (e.g., row 16).
7. Alternatively, *RescueMu* GSS sequences may also be accessed using GenBank® National Center for Biotechnology Information (NCBI) by delimiting the search to the dbGSS database or via the Plant Genome Database at (www.plantgdb.org). PlantGDB permits other useful search options such as searching using a text identifier:

- a. In PlantGDB, specify GSS or GSS contig under Sequence and *Zea mays*.
 - b. A query results page will open and list any *RescueMu* sequences that match the text.
 - c. Clicking on a sequence name will open up a new page that will indicate if the *RescueMu* GSS is part of a larger GSS contig and/or aligns with maize ESTs.
8. To identify upstream and downstream sequences to the original query sequence, look for overlapping ESTs or GSS contigs. For example, if RMTuc appears in the Results page, click on the corresponding link. This will open up a new page specifying that the GSS is part of a *RescueMu* tentative unique contig (RMTUC) assembled by aligning overlapping *RescueMu* GSSs and displaying overlapping EST sequences. For an example, go to (www.plantgdb.org), select Text Search, type in myb, specify GSS contig and *Zea mays*, then hit Search.

3.8.2. How to Retrieve a *RescueMu* Genomic Plasmid from a MGDG Grid Library for Further Sequencing

1. This section describes how to retrieve a plasmid encoding a GSS of interest from a MGDG 96-well grid library of *RescueMu* recovered plasmids in order to sequence further upstream or downstream (*see Subheading 3.8.1., step 6*). *RescueMu* GSS plasmids are not individually distributed by the MGDG.
2. Perform a sequence similarity search against the *RescueMu* GSS collection (*see Subheading 3.8.1.*).
3. In the Query Results page, note the grid origin of the GSS sequence (e.g., Grid G).
4. To identify the precise location of the GSS in a 96-well plate and the direction of the read-out sequence, locate the sequence identification (I.D.). Examples are 1006162C04.x2 1006 and 1008035A02.y1 1008 (*see Note 15*).
5. Purchase the correct 96-well *RescueMu* grid library plate online (*see Subheading 2.8.*).
6. After receiving the plasmid library, there are two methods to retrieve the GSS plasmid of interest from the correct well, PCR, or bacterial colony hybridization.
7. To PCR amplify the entire maize genomic DNA insert flanking *RescueMu* (up to 25 kb), design a PCR primer specific to the GSS to amplify in the direction away from *RescueMu*.
 - a. Synthesize a *RescueMu* read-out primer located on the opposite edge of the genomic insert. For example, if the GSS is from an “x” (right TIR) sequence, then the *RescueMu* read-out primer should correspond to the left TIR. The *RescueMu* left primer is 5'-CACCGCCGTGCTGCCGTAGAGCG-3' and the *RescueMu* right primer is 5'-CGCGTGACTGAGATGCGACGGAG-3'. These are located >220 bp internal to the left or right edge of the *RescueMu* element.
 - b. Use MasterAmp Extra Long DNA Polymerase with High Fidelity 2× Extra Long PCR Premix 9 (Epicentre), 5 ng of library plate DNA, the GSS primer, and the *RescueMu* primer.
 - c. Following an initial denaturation at 94°C for 1 min, perform 40 PCR cycles as

- follows: 94°C for 15 s, 60°C for 30 s, and 68°C for 25 min. The long extension time is to amplify inserts up to 25 kb in length.
- d. Additional details for PCR amplification may be found at (www.zmdb.iastate.edu); click on the Protocols button and follow the PCR link.
 8. For bacterial colony hybridization, transform the DNA from the correct well (e.g., C04 or A02) into *E. coli* strain DH10B and ensure that the colonies are well separated (*see Subheading 3.3., steps 15–24*).
 - a. To screen colonies containing the GSS plasmid of interest, generate a DNA probe corresponding to the GSS by PCR using the library well DNA or maize genomic DNA as the template or request an overlapping EST fragment (available online from ZmDB).
 - b. Follow **Subheadings 3.5.** and **3.6.** to immobilize the bacterial colonies onto nylon–nitrocellulose and to screen colonies using the radiolabeled probe.
 - c. Isolate plasmid DNA from positive colonies.
 9. Confirm the identity of the recovered clone by DNA sequencing, and then design specific DNA sequencing primers to sequence upstream and downstream of the GSS.
 10. For PCR cycle sequencing, consult (www.zmdb.iastate.edu); click on the Protocols button and follow the Cycle Sequencing link.

3.8.3. How to Use an EST or Heterologous Sequence to Screen RescueMu Libraries by Reverse Genetics

1. This section describes how to use a sequence (EST, heterologous sequence) with no similarity in the online ZmDB GSS collection to screen *RescueMu* plasmid libraries generated by the MGDP to identify a somatic or germinal insertion by reverse-genetics.
2. Purchase 96-well *RescueMu* grid library plates online.
3. Synthesize the *RescueMu* read-out primers (*Mu1-L* and *Mu1-R*) (*see Subheading 2.8.*).
 - a. Design and synthesize two or more PCR primers for the sequence of interest, both 5' to 3', one for the top strand and one for the bottom strand.
4. Perform a 96-sample PCR using the 4 PCR primers and use the following initial conditions: 0.5 mM dNTPs, 2.5 mM Mg⁺⁺, 0.8 μM of each specific primer, 4.0 μM of each *RescueMu* primer, 2 U *Taq* DNA polymerase and 5 ng library plate DNA. Denature 95°C for 5 min, then amplify for 40 cycles (95°C for 30 s, 55°C for 30 s, then 72°C for 2 min), followed by a single extension at 72°C for 5 min.
5. Consult (www.zmdb.iastate.edu) for a grid-specific list of positive control PCR primers and other recommendations. Click on RMu Libraries and then Screening.
6. Sequence the fragment to confirm its identity.
7. If the insertion of interest is found in both a row well and a column well, this indicates a likely germinal insertion event and pinpoints the exact plant. Note the grid letter, row and column number to request seed from the Maize Genetics Cooperative Stock Center (*see Note 1*).

3.8.4. How to Screen the MGDG RescueMu PhenotypeDB to Obtain a Mutant of Interest

1. Grids of 48 × 48 *RescueMu* plants have been screened by the MGDG for visible mutant phenotypes and descriptions are available online (see **Subheading 2.8.**). Mutants may be caused by either *RescueMu*, but more likely by background *Mu/MuDR* elements. Go to the PhenotypeDB index page at (www.zmdb.iastate.edu/zmdb/phenotypeDB/index.htm).
2. For relative mutation frequencies in each grid, consult the Grid Summary Table.
3. Begin the search by taking the Interactive PhenotypeDB Tutorial.
4. Choose one of three search tools. To search using a specific phenotype, for example a Knotted adult leaf, then use the Phenotype Lists search engine. To search by general category, for example all adult leaf mutants, then use the Mutant Browser. To search by a specific location within a grid, use the Location Search engine.
5. Hit Start Search.
6. In the Query Results page, the column and row of each mutant is listed. Click on the corresponding Grid letter; this opens up the PhenotypeDB Search Details page, which is a summary card of the scoring details.
7. At the bottom of the PhenotypeDB Search Details page, there are links to all the *RescueMu* GSSs recovered from the row and column pool that contained the mutant plant.
8. Use the grid, row, and column information to request selfed seed from the Maize Genetics Cooperation Stock Center (see **Subheading 2.8.** and **Note 1**).
9. Once seed have been received, the user may wish to backcross to create an isogenic background. *RescueMu* seed populations are in a mixed genotype, typically A188 > W23 > Robertson > K55 > Freeling > B73. For more details, go to (www.zmdb.iastate.edu), open the *RescueMu* Index menu on the right side and choose *RescueMu* Tagging Populations.

3.8.5. How to Use a RescueMu GSS to Identify a Corresponding Mutant Phenotype

1. Perform a Blast search in ZmDB. Select the GSS database (see **Subheading 3.8.1.**).
2. In the Results Summary page, note whether the GSS appears as a single hit or multiple hits in the same library grid (indicated by the first 4 or last 4 letters under Description). Determine the row or column source of each GSS (see **Subheading 3.8.1., steps 3–6**).
3. If the GSS appears as only a single hit within any one grid, then proceed with **step 3**. For multiple hits, go to **step 4**.
 - a. There is a high probability that the *RescueMu* GSS corresponds to a somatic insertion, with no phenotype.
 - b. To determine if the GSS instead corresponds to a germinal insertion, purchase the corresponding 96-well *RescueMu* grid library plate (see **Subheading 2.8.**).

- c. To screen the 96-well plate for a *RescueMu* germinal insertion, use a *RescueMu* read-out primer and a primer to the GSS of interest to screen by PCR using **steps 3–8** in **Subheading 3.8.3**. If the GSS of interest is found in both a row and column sample, then proceed to **step 6**.
- d. If the GSS of interest is not found in both row and column samples, it is possible that a germinal insertion does exist, but was not retrieved during plasmid rescue in both row and column pools. To proceed, request *RescueMu* seed for all of the 48 plants in the row or column pool of the GSS. After growing these progeny, isolate leaf genomic DNA (**Subheading 3.2.**), then use PCR to screen leaves for the GSS-specific *RescueMu* insertion by following **Subheading 3.8.3., steps 3–7**. If an insertion is found, it is likely to be germinal, and thus, proceed to **step 7** of this section.
4. If multiple GSSs are retrieved, then click on the Sequence code of each GSS. At the bottom of each new page, note the Grid letter and Row/Column location.
5. If the multiple GSSs belong to only a row(s) or column(s) within a grid, but not both, then proceed with **step 5**. If the GSSs belong to both a row and column within a grid, then go to **step 6**.
 - a. As the number of duplicate GSSs in only a row or column sample increases, the probability that the GSSs correspond to a germinal insertion increases.
 - b. To determine if the GSS instead corresponds to a germinal insertion, purchase the corresponding 96-well *RescueMu* grid library plate (*see Subheading 2.8.*).
 - c. To screen the 96-well plate for a *RescueMu* germinal insertion, use a *RescueMu* read-out primer and a primer to the GSS of interest to screen by PCR using **steps 3–8** in **Subheading 3.8.3**. If the GSS of interest is found in both a row and column sample, then proceed to **step 6**.
6. If the GSSs correspond to a row and column within a grid, then request seed for the *RescueMu* plant at the field grid intersection (**Fig. 2**).
7. Search for a visible phenotype in PhenotypeDB using Location Search by entering the Grid letter, Row, and Column numbers (*see Subheading 3.8.4.*).
8. Isolate genomic DNA from the candidate plant(s) and confirm the presence of a *RescueMu* germinal insertion by PCR using the appropriate *RescueMu* read-out primer and a gene-specific primer (*see Subheading 3.8.3., steps 3–6*).
9. Perform a segregation analysis of the progeny by PCR to determine if the *RescueMu* allele cosegregates with the mutant phenotype.

3.8.6. How to Identify a *RescueMu* Insertion Responsible for a MGDP Mutant Phenotype

1. In the initial MGDP *RescueMu* grids, most mutants are caused by *MuDR/Mu* elements, not *RescueMu* (*see Note 2d*).
2. Use PhenotypeDB to locate the Grid letter, row and column numbers of the mutant (*see Subheading 3.8.4.*).
3. At the time of this protocol submission, the *RescueMu* GSS database could not be searched by row or column location. Instead, there is a link in PhenotypeDB

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from any plant grid location to all GSSs found in the corresponding row and column. These may be queried against one another, but they may total hundreds of sequences. Consult PhenotypeDB or ZmDB for future upgrades.

4. Alternatively, request seed from the mutant plant (*see Subheading 2.8.*).
5. Isolate genomic DNA from a mutant plant from two leaves not likely to share a clonal sector and perform separate plasmid rescues (*see Subheadings 3.2.–3.6.*).
6. Perform restriction digests on the plasmids recovered from both leaves and electrophorese the plasmids on an ethidium bromide-stained gel. If any plasmids appear to be identical between the two leaves, these may be germinal *RescueMu* insertions and should be sequenced (*see Subheading 3.7.*).
7. To determine if a candidate *RescueMu* insertion is responsible for the mutant phenotype, perform a segregation analysis of the progeny by PCR to determine if the *RescueMu* allele co-segregates with the mutant phenotype (*see Subheading 3.8.3., steps 3–6.*).

4. Notes

1. Because *RescueMu* seed is transgenic, permission is required for interstate shipment in the U.S. or to other countries from the Maize Coop Stock Center in Illinois, U.S.A. In the U.S.A., a letter of notification to ship or grow transgenic seed must be submitted to APHIS, which is a branch of the U.S. Department of Agriculture, 10–30 d in advance (<http://www.aphis.usda.gov/ppq/biotech>). A detailed *RescueMu* APHIS notification template letter can be obtained from the Maize Coop Stock Center at (<http://w3.aces.uiuc.edu/maize-coop/Aphis-notification.html>). Combined with information found in ref. 3, these documents contain detailed information about the origin and construction of the *RescueMu* transgenes for permit documentation.
2. The utility of *RescueMu* is entirely determined by the choice of the starting plant material. The following parameters should be used when requesting or selecting *RescueMu* stocks:
 - a. Of the original 20 *RescueMu* lines constructed, the most active lines are designated R3-4, R3-8, R3-13, and R3-17 (3). Line R3-8 has a complex transgene array and is a rich source of somatic transposition events, but it has a very low frequency of transmissible *RescueMu* insertions.
 - b. From the original *RescueMu* lines, seed containing transposing *RescueMu* elements should be hand-selected based on the appearance of frequent single-cell red spotting on the kernel (aleurone) surface when viewed under a microscope (3); these represent *RescueMu* excisions from the anthocyanin *R* transcription factor. To observe such spots, seed lines should be in a *MuDR r CI* genetic background. *RescueMu* spotting is rarely observed on other tissues including leaves and anthers regardless of the anthocyanin genotype.
 - c. *MuDR* is prone to epigenetic inactivation (1,2). Poorly spotted kernels or ears with few spotted kernels should not be chosen for plasmid rescue. Unfortunately, even spotted kernels may give rise to seedlings with inactive *RescueMu* elements. There is a tendency for kernels, leaf sheaths and leaves to turn solid

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or patchy red when *MuDR/RescueMu* is in the process of epigenetic inactivation, though lack of a red color is not a reliable indicator of *RescueMu* activity. *Mu* epigenetic inactivation is lower when seed stocks are maintained by outcrossing; hence it is useful to have or request non-*Mutator r C1* seeds from the Maize Co-op Stock Center to serve as parents.

- d. *RescueMu* seed lines may be in a background with 1–3 copies of *MuDR* (known as the Minimal Line) or 10–50 copies of *MuDR* (known as the Standard Line) (reviewed in refs. 1,2). In the Minimal Line background, *RescueMu* has excellent kernel spotting, high somatic transposition, low epigenetic inactivation, but extremely weak germinal transposition. In the Standard Line, *RescueMu* may have excellent kernel spotting, high somatic transposition, high rates of epigenetic inactivation, and a >10% frequency of *RescueMu* transposition. The advantage of the Minimal Line backgrounds is that they contain few *Mu1–Mu8* elements, whereas Standard Lines may contain >100 *Mu1–Mu8* elements. Hence, mutants found in Standard Lines have a low probability of being caused by a *RescueMu* element.
 - e. Because developmentally older leaves (higher up the plant) have a greater probability of epigenetic activation, it may be best to collect DNA from lower on the plant if somatic insertion events are of interest. It has been observed that the first 4 leaves are a rich source of *RescueMu* somatic transposition events.
 - f. Though *RescueMu* elements at their original and complex transgene integration site have a low frequency of germinal transposition, this frequency may increase to 20–100% after the *RescueMu* element has transposed to a new location (3). These seed stocks are designated tr-*rMu* (transposed *RescueMu*), and are better stocks than the original *RescueMu* lines for germinal transposition, though they offer no advantages for late somatic transposition.
 - g. If tr-*rMu* seed stocks are requested in which *RescueMu* has transposed from the original *RescueMu/pAHC20* transgene array and the array has been outcrossed away, these seed lines should not be glufosinate herbicide-resistant. If using a tr-*rMu* line, a molecular strategy must be designed to prevent repeated recovery of the tr-*rMu* plasmid during plasmid-rescue experiments (see Note 3). The genomic DNA sequence flanking the tr-*rMu* must be known in order to identify a unique restriction site to restrict recovery of this locus, or the DNA can be used as a hybridization probe on a colony-lift.
 - h. In coming years, new *RescueMu* seed lines will be available from the Maize Gene Discovery Project which have smaller *RescueMu* elements, will use kanamycin instead of ampicillin to reduce general laboratory plasmid contamination, will have better restriction sites for plasmid rescue, will use *Bronze2* instead of *R* as the excision marker, and will likely have fewer background *MuDR/Mu* elements. These lines will be called *MiniMu* and *MidiMu*. See (www.zmdb.iastate.edu) for updates and details.
3. Ampicillin-encoding plasmids rescued from *RescueMu* stocks can include the original *RescueMu* transgene array in addition to the genetically linked plasmid

pAHC20 encoding the herbicide-resistance gene *bar*, which is also ampicillin-encoding. The original *RescueMu* donor element may be recovered, because new germinal *RescueMu* insertions are caused by duplication rather than “cut and paste” transposition (1,2). Fortunately, there is a unique *Bgl*II site 100 bp downstream of *RescueMu* in the original *RescueMu* transgene and an additional site in pAHC20. Therefore, to bias against the recovery of these plasmids, after they ligate to form circular DNA, the DNA is restricted with *Bgl*II in order to linearize them and prevent their replication in *E. coli*. Some percentage of true *RescueMu* insertions containing flanking *Bgl*II sites will also be selected against using this method.

4. Because *RescueMu* plasmid rescue involves the use of highly competent *E. coli* cells with selection on ampicillin media, contaminating laboratory plasmids found in laboratory solutions or aerosols are a severe problem unless actively prevented. For example, if 1 µg of contaminating plasmid DNA was diluted into 10,000 L of water, then even 1 µL of this solution would result in a transformed *E. coli* colony. The following measures should be taken to prevent plasmid contamination:
 - a. A separate plasmid-free bench area should be designated for the sole purpose of genomic DNA isolation and plasmid rescue. Other plasmid work should not be performed at the same time as this procedure.
 - b. Fresh reagents (enzymes, buffers, alcohol, water) and solutions should be purchased, aliquoted for single-use experiments in sterile plastic tubes if possible, labeled as plasmid-free, and segregated from general laboratory use. Alternatively, water and solutions may be treated with activated charcoal and then filtered.
 - c. Where possible, sterile plastic instead of laboratory glassware should be used. Mortars, pestles, spatulas, tube racks, and other reusable materials including pipetors should be treated with UV light for 1 min.
 - d. Pipetors and the bench area may be treated with a 0.2% (v/v) HCl solution or DNA-Zap spray (Ambion, Austin, TX, USA). The bench coat should be changed frequently.
 - e. Aerosol barrier pipet tips should be used throughout the procedure.
 - f. To detect external plasmid contamination, two plasmid rescue controls should always be performed. First, a control omitting genomic DNA will indicate if one of the plasmid rescue solutions or the handling itself is creating plasmid contamination, while a control omitting ligase will indicate if the genomic DNA has been contaminated.
 - g. To detect the extent of foreign plasmid contamination within a rescued DNA sample, random bacterial colonies should be restriction digested on an agarose gel. Because *RescueMu* is 4.7 kb, true rescued plasmids range in size from 5–25 kb. Small plasmids are an indication of external plasmid contamination. Alternatively, *RescueMu* plasmids may be positively identified using *RescueMu*-specific PCR amplification (see **Subheading 3.4.**).
5. Do not attempt to digest the genomic DNA with restriction digest for long peri-

ods of time or with excess enzyme (as when preparing a Southern blot), because the activity of contaminating nucleases will result in loss of the restriction fragment overhang and inhibit subsequent ligation.

6. I have found that genomic DNA purification using phenol–chloroform gives the highest transformation efficiency compared to sepharose and other minicolumn purification procedures.
7. For the ligation step, the concentration of genomic DNA is kept deliberately low (<20 µg/mL) to promote intramolecular ligation rather than intermolecular ligation (see ref. **13**). Unfortunately, early *Arabidopsis* T-DNA rescue protocols called for much higher concentrations of genomic DNA.
8. Until now, only Electromax DH10B cells from Life Technologies have worked efficiently for plasmid rescue for the following reasons: (i) the electroporation efficiency of these cells is 10¹⁰ colonies/µg plasmid DNA; (ii) these cells carry the *mcrA*, *mcrBC*, *mrr*, and *hsdRMS* mutations, preventing methylated cytosine and adenine residues from plant genomic DNA from being restricted; (iii) the cells carry the *deoR* mutation, allowing them to accept plasmids as large as 150 kb; (iv) While DH5-α cells also carry this mutation, although high efficiency transformation is limited to plasmids <30 kb in size; and (v) DH10B cells carry the *recA1* mutation, thus decreasing the frequency of recombination between the 215 bp terminal inverted repeats of *RescueMu*.
9. Carbenicillin (final 100 mg/L) should be substituted for ampicillin when preparing LB plates. Carbenicillin is more stable than ampicillin and will reduce satellite colony formation.
10. In addition to tissue containing inactive *RescueMu* elements, there are three major reasons for poor plasmid rescue efficiencies. First, the DNA pellet may be lost during the numerous genomic DNA precipitation steps. To help reduce loss of DNA, it may be useful to add 1 µL of glycogen as a carrier. Prior to transformation, an aliquot of the digested DNA should be measured to quantitatively assess the plasmid rescue efficiency. Second, the ATP in the ligase buffer may degrade due to numerous freeze–thaw cycles. The ligase buffer should be aliquoted into single-use tubes and frozen, and then thawed only once. Finally, even a slight loss in bacterial cell transformation competency will result in a significant decrease in the number of colonies recovered. For this procedure, cells should not be used if previously thawed, and the thawing and handling instructions of the manufacturer (Invitrogen or LIFE Technologies) should be followed exactly.
11. Up to 100–800 colonies are recovered on carbenicillin plates/µg of *KpnI*-digested genomic DNA electroporated into 50 µL of Electromax DH10B cells, when the genomic DNA is isolated from an active *RescueMu* seedling. If the DNA is also digested with *BglIII* to bias against recovery of plasmids from the *RescueMu* transgene array, the colony number drops to 20–300/µg of genomic DNA. Because there is no *KpnI* site internal to *RescueMu*, these plasmids contain maize genomic DNA flanking both sides of *RescueMu* and are large plasmids (mean = 12 kb). If genomic DNA is only needed from one flank, an internal *EcoRI* site may be used for plasmid rescue instead of *KpnI*, resulting in the recovery of smaller plasmids,

and therefore, a higher frequency may be expected (1500–4000 colonies/ μg using *EcoRI* alone, and 150–1000 colonies/ μg using both *EcoRI* and *BglII*). Alternatively, the MGDG uses a *BamHI/BglII* double digest (**Fig. 1**) to permit digestion and selection to be performed in a single step; *RescueMu2* does not have an internal *BamHI* site, though *RescueMu3* does. There is a flanking *BamHI* site near *BglII* at *Lc::RescueMu*, which further helps to prevent recovery of the original *RescueMu* transgene array. Regardless of the enzyme combination chosen, it is important to note that the plasmids recovered after this step may still be from the original *RescueMu* transgene array, and further colony-lift hybridizations are recommended.

12. Colony-lift hybridizations are performed to screen for the recovery of true transposition events. In the first screen, colony hybridization to *RescueMu2*- and *RescueMu3*-specific probes specifically detect *RescueMu* plasmids and thus screen against recovery of foreign plasmid contamination and the ampicillin-encoding plasmid pAHC20 (the *bar* transgene). The purpose of the second screen is to avoid the recovery of *RescueMu* plasmids from the original *RescueMu::Lc/pAHC20* transgene array. To do this, a set of filters is hybridized with the *RescueMu*-specific probes, while a duplicate set is hybridized to a combination of probes which flank *RescueMu* in the transgene array, namely the CaMV35S promoter and the maize *Lc(R)* cDNA. Colonies are then selected which hybridize to *RescueMu*, but do not hybridize to the CaMV35S/R probes. These colonies should represent new insertions of *RescueMu*.
13. Plasmids pRescueMu2, pRescueMu3, pMR15, and pMR17 and maps may be obtained from Virginia Walbot at Stanford University (walbot@stanford.edu) (**3**). The pR plasmid for the CaMV35S and maize *Lc(R)* probes may be obtained from Sue Wessler at the University of Georgia (sue@dogwood.botany.uga.edu) (**11**).
14. Recovered colonies may represent *RescueMu* somatic or germinal insertions. Typically, if 8–10 colonies are sampled by restriction digest on an agarose gel, unique plasmids represent late somatic insertions, whereas duplicated plasmids represent germinal or rare early somatic insertions (**3**). In the case of a germinal insertion, the same plasmid should be recovered from two separate leaves on the same plant.
15. Each GSS from the MGDG has a sequence I.D. code. Examples are 1006162C04.x2 1006 and 1008035A02.y1 1008. The 3 digits preceding the x or y digit or the period (e.g., C04 and A02) indicate the exact well location in the 96-well PCR library plate (e.g., C is a row and 04 is a column on the plate) (**Fig. 2**). The first 4 digits, sometimes repeated at the end (e.g. 1006 and 1008), also identify the field grid (e.g., G and I). X means that the read-out sequence was from the right *RescueMu* TIR (primer *Mu3-R*) while y refers to a read-out from the left TIR (primer *Mu3-L*) (see **Subheading 2.7.**). In addition, some sequence I.D.'s are longer, e.g., 1006162C04.1EL_x2 1006. The extra 3 digits (i.e., 1EL) indicate that the sequence from the recovered *RescueMu* plasmid came after one restriction site (*BamHI* or *BglII*) used during plasmid rescue, and hence this may not be a contiguous sequence. If the sequence came after a junction recognized as

the possible ligation between a *Bam*H1 overhang and a *Bgl*III overhang, then “2EL” is added to the GSS I.D. instead.

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